Preventive effect of *Angelica gigas* Nakai extract oral administration on dry eye syndrome

Younje Lee¹, Kang Min Kim², Jae Seon Kang¹

¹Department of Pharmacy, Kyungsung University, Busan, Republic of Korea
²Department of Pharmaceutical Science and Technology, Kyungsung University, Busan, Republic of Korea

**ARTICLE INFO**

*Article history:*
Received 19 December 2017
Revision 3 January 2018
Accepted 2 April 2018
Available online 20 June 2018

**Keywords:**
*Angelica gigas* Nakai
Benzalkonium chloride
Dry eye syndrome

**ABSTRACT**

**Objective:** To identify the preventive effect of *Angelica gigas* Nakai (*A. gigas* Nakai) extract in a benzalkonium chloride-induced dry eye model. **Methods:** A total of 28 mice were divided into 4 groups: 1) Normal group: mice received only saline; 2) positive control group: mice received an oral solution without *A. gigas* Nakai extract at 10:00 a.m. and 0.2% benzalkonium chloride eye drops at 2:00 p.m.; 3) *A. gigas* Nakai extract (5 mg); 4) *A. gigas* Nakai extract (10 mg). Both group 3) and group 4) received an oral solution with *A. gigas* Nakai extract (either 5 mg/kg or 10 mg/kg) at 10:00 a.m. and 0.2% benzalkonium chloride eye drops at 2:00 p.m. After 14 d of follow-up, tear volume measurement and fluorescein staining were evaluated for the recovery effects on ocular surface. Histologic analysis was conducted by hematoxylin and eosin staining. Apoptosis on ocular epithelium layer was examined using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining. Expression of TNF-α was also measured using western blot analysis. **Results:** An increase in both the tear volume and the sustained fluorescein staining scores was observed, demonstrating the preventive effects of *A. gigas* Nakai extract. Structure changes such as irregularity of the epithelial layer and corneal epithelial cell death were inhibited in the *A. gigas* Nakai extract groups. Expression of TNF-α moderately declined; however, its expression level was still higher, compared to the normal group. **Conclusions:** Results from the current study show the significant preventive effect of *A. gigas* Nakai extract in a mouse model of benzalkonium chloride-induced dry eye syndrome. Thus, *A. gigas* Nakai extract could be considered as an oral preventive agent for dry eye syndrome in the future.

1. Introduction

Dry eye syndrome is a symptom of dryness, visual disturbance, and burning sensation in the eyes caused by tear deficiency[1]. It increases the ocular surface inflammation by increasing tear osmolarity and tear film instability[2-4]. According to the Korea National Health and Nutrition Examination Survey of the 17 542 subjects between 2010 and 2012, about 10.4% of the subjects were found to have dry eye syndrome[5]. Cyclosporine and steroids (e.g. prednisolone, dexamethasone, fluorometholone, medrysone, rimexolone, and loteprednol) are frequently used to treat eye inflammation[6,7]. Despite the newly published evidence on the use of omega-3 and omega-6 for dry eye syndrome treatment, the evidence supporting their clinical significance is scarce[8].

National Health and Nutrition Examination Survey of the 17 542 subjects between 2010 and 2012, about 10.4% of the subjects were found to have dry eye syndrome[5]. Cyclosporine and steroids (e.g. prednisolone, dexamethasone, fluorometholone, medrysone, rimexolone, and loteprednol) are frequently used to treat eye inflammation[6,7]. Despite the newly published evidence on the use of omega-3 and omega-6 for dry eye syndrome treatment, the evidence supporting their clinical significance is scarce[8]. This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms. For reprints contact: reprints@medknow.com ©2018 Asian Pacific Journal of Tropical Medicine Produced by Wolters Kluwer Medknow

How to cite this article: Lee Y, Kim KM, Kang JS. Preventive effect of *Angelica gigas* Nakai extract oral administration on dry eye syndrome. Asian Pac J Trop Med 2018;11(6):369-375.
Angelica gigas Nakai (A. gigas Nakai) is a Korean traditional herbal medicine used in Asian countries such as Korea, China, and Japan. A. gigas Nakai of Korea has deep-purple flowers while Angelica sinesis and Angelica acutiloba of China and Japan have white flowers[9]. A. gigas Nakai is also used in food products in the Americans and different European countries[10]. Many substances including the coumarins such as decursin and decursinol angelate, which have many pharmacological activities, have been identified in A. gigas Nakai. The coumarins are used for applications of anemia, dysmenorrhea, amenorrhea, menopausal syndromes, arthritis, pain injuries, migraine headaches, and also as an anti-benzalkonium chloride terial, sedative, anodyne, and tonic agents[11-15]. Decursin and decursinol angelate and decursinol were isolated by recycling high-performance liquid chromatography (HPLC) conducted at a ratio of 54:44:2; these compounds upregulated the phosphorylation of AMP-activated protein kinase and the expression of glucose transporter type 2 and 4 in skeletal muscle and pancreas[16,17]. Decursin and decursinol angelate also regulates the expression of antioxidant enzymes as Nrf2 and suppresses the accumulation of amyloid β-protein in oxidative stress-related diseases[18].

Previously, we reported the genotoxicity, oral acute and subacute toxicity, pharmacokinetics, reproductive toxicity, and spermatogenesis of A. gigas Nakai extracts including 95% decursin and decursinol angelate[10,19-22]. In the present study, we aim to investigate the effects of A. gigas Nakai extract on dry eye syndrome in a benzalkonium chloride-induced mouse model. Benzalkonium chloride-induced mouse is characterized by low cytotoxicity in the cultured ocular cell lines caused by dry eye syndrome[23]. The primary objectives of the current study are: 1) to identify the decursin and decursinol angelate components in A. gigas Nakai extract; 2) to identify inhibition of TNF-α expression by A. gigas Nakai extract; and 3) to examine clinical signs using hematoxylin and eosin staining, tear volume, transferase-mediated dUTP nick-end labeling (TUNEL) assay, and fluorescein staining.

2. Materials and methods

2.1. Reagents and animals

A. gigas Nakai extract was isolated, purified, and extracted from A. gigas Nakai, with a purity of 70%, as revealed by HPLC analysis[16,22]. All chemicals and solvents were obtained from Sigma (St. Louis, MO, USA). Anti-TNF-α, goat anti-mouse conjugated to hors eradish peroxidase (HRP) (IgG H&L), mouse IgG kappa binding protein conjugated to HRP (m-IgG κ BP-HRP), and anti-β-actin were purchased from Abcam Inc. (Cambridge, MA, UK) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Male C57BL/6 ICR mice (7 weeks old) were obtained from Hyochang science (Daegu, Korea). The mice were housed under the following conditions for one week to adapt to the environmental changes: controlled temperature of (23±1)°C, relative humidity of 60%±5% and a 12 h light/12 h dark cycle. The study was carried out in accordance to the World Health Organization guideline and the Institutional Review Board of Kyungsung university for the evaluation of the safety and efficacy of herbal medicines (Confirmation number: research-16-006).

2.2. Preparation and analysis of A. gigas Nakai extract

The A. gigas Nakai was purchased from the Simmani Wild Ginseng Farm Association Corporation (Hamyang, Kyungnam, Korea) in 2017. A voucher specimen of A. gigas has been deposited at the herbarium located at the College of Pharmacy, Kyungsung University (No.17-01-AG). A. gigas Nakai was fully dried at room temperature condition followed by grinding a total of 10 kg. The ground A. gigas Nakai was extracted 2-3 times with equal volumes of 95% ethanol (50 L). The extract of A. gigas Nakai was consequently filtered to remove precipitates and evaporated using rotary evaporator. A. gigas Nakai was again dissolved and extracted using 95% ethanol (1 L) to remove extra impurities at 4°C for 16 h. Finally, 95% ethanol (50 L) was added to the extract and centrifuged at 5 000 rpm for 10 min to collect the supernatants. The dried supernatants (at 80°C) produced about 330 g of A. gigas Nakai extract. The identification of decursin and decursinol angelate from A. gigas Nakai extract was analyzed using an Agilent HPLC 1100 series (Agilent Technology, Santa Clara, CA, USA) with an Zorbax SB-C18 column (250.0 mm x 4.6 mm, 5.0 mm) and a UV monitor of 329 nm (Photo-Diode Array UV/Vis detector, Agilent Technology)[16].

2.3. Preparation of A. gigas Nakai extract solution

A solution of A. gigas Nakai extract was prepared to be used for oral, eye drop, and injection administration. The solution contained A. gigas Nakai extract (100.0 mg), ethanol (1.0 g), lysine (1.5 g), tween 80 (1.0 g), sodium hydroxide for pH (8.0) adjustment (Quantum satis, QS), and sterile water (QS). To reach the required composition, the A. gigas Nakai extract (100 mg) was solubilized first in ethanol (1.0 g), and then added to lysine (1.5 g) and tween 80 (1.0 g). The pH of dissolved A. gigas Nakai extract solution was adjusted to 5.6 using sodium hydroxide, and the extract was diluted up to 100 mL with sterile water. Finally, the solution was sterilized before using to prevent benzalkonium chloride terial and fungal growth.
2.4. Animal experimental procedures

The mice were separated into a normal group, a positive group, and two treatment groups (n=7 per group). The mice in the normal group received 200 μL saline orally at 10:00 a.m. for 2 weeks, followed by saline intracocularly (5 μL) in the eyes at 2:00 p.m. for 2 weeks (normal group). The placebo group (receiving a solution except for A. gigas Nakai extract), served as a positive control and received 200 μL saline orally once per day at 10:00 a.m. for 2 weeks, then 5 μL benzalkonium chloride eye drops at 2:00 p.m. for 2 weeks (resembling a positive control group). The mice from the two treatment groups received oral A. gigas Nakai extract solution (100 μL and 200 μL at 10:00 a.m.) at a daily dose of 5 mg/kg (i.e. A. gigas Nakai extract 5 mg group) and 10 mg/kg (i.e. A. gigas Nakai extract 10 mg group) of body weight for 2 weeks, andthen received 5 μL benzalkonium chloride eye drops at 2:00 p.m. for 2 weeks.

2.5. Tear secretion measurement

Tear volumes of A. gigas Nakai extract-treated animals were identified using an analysis recommended by Arakaki et al[24]. In order to establish pilocarpine-stimulated mice, anesthesia of animals from all groups were induced by ketamine (60.0 mg/kg body weight) and xylazine (6.0 mg/kg), and then intraperitoneally induced by pilocarpine (2.5 mg/kg) (Isopto carpine eye drops 2%, Alcon, Belgium). Tear volumes were measured by the length of the phenolred thread (Tianjin Jingming New Technological Development Co., Ltd., Tianjin, China) left in contact with the eye for 5 min. Tear volumes were determined by tear volume/body weight.

2.6. Fluorescein staining

The corneal epithelial surface was observed by the fluorescein staining method[25]. A total of 1 μL of 0.1% sodium fluorescein was adjusted to the right eye. After 1 min, the corneal epithelial surface was detected by the slit-lamp microscope with a cobalt blue filter (DM6C ophthalmoscope, Zumax Medical Co. Ltd., Germany). Fluorescein corneal staining scores (ranging between 1 and 4) from all groups were measured by 4 observers and the average was calculated. The scores observed were classified as follows: score 1 (no staining), score 2 (minimal staining), score 3 (mild/moderate staining) and score 4 (severe staining).

2.7. Histopathological study

The hematoxylin and eosin technique used in this study is modified with the previous method developed by Ok et al[26]. The eyeball was first fixed in 10% formalin solution. Next, the cornea was separated from the formalin fixed-eyeball and then dehydrated and embedded in paraffin. The cornea (4 μm) was consequently cut from the paraffin blocks, and the 4 μm sections were stained with 0.1% hematoxylin and 1.0% eosin. Each section was detected with a light microscope (DP-70, Olympus, Tokyo, Japan) for histopathology tests.

2.8. TUNEL assay

TUNEL assays were performed using a modified method of Ok et al[27]. In brief, to observe apoptotic cells in corneal epithelial layer, the eyeball was fixed and embedded in formalin and paraffin, and then deparaffinized and rehydrated sections (5 μm) were obtained. The sections were placed in 3% hydrogen peroxide for 10 min at room temperature and treated with 20 μg/mL proteinase K for 10 min at 37 °C. The treated sections were washed three times in 1xphosphate buffered saline and the apoptotic cells were stained using POD Kit (In situ cell death detection kit, Roche, Mannheim, Germany) according to the supplier’s instructions and observed with light microscopy (Panoramic Viewer, 3DHISTECH Ltd., Budapest, Hungary).

2.9. Western blot analysis

After TUNEL observation, cryosections were evaluated for TNF-α expression. Protein analysis was performed following the methods of Li et al[28]. Cells were lysed and extracted in RIPA buffer [50 mM Tris-Cl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1mM EDTA] and aliquots of supernatants were collected by 14 000 rpm centrifugation at 4 °C for 15 min. Protein concentrations were analyzed using a Bio-rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Proteins (30 μg) were resolved on 15% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE). Nonspecific binding was blocked by TBST (25 mM Tris–HCl, 50 mM NaCl, 0.05% Tween-20) containing 5% dry milk for 60 min at room temperature and incubated at 4 °C for 16 h with 1 : 400 and 1 : 10 000 diluted primary antibodies of anti-TNF-α and anti-β-actin. After 1 h of incubation at room temperature with IgG H&L and m-IgG κ BP-HRP secondary antibody, the signal was detected by the ChemiDoc-It Imaging system (UVP 97-0650-05) using enhanced Chemiluminescent solutions (enhanced Chemiluminescent Substrate, Thermo Scientific, Rockford, IL, USA). The relative densities were analyzed and presented using Image J software (version 1.51j8; public domain program created by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA).

2.10. Statistical analysis

The data were expressed as mean±SD values. Statistical analyses were performed and analyzed by one-way analysis of variance (n=7). P values<0.01, 0.05 and 0.001 were considered significant.

3. Results

3.1. The effect of A. gigas Nakai extract on tear secretion

Two treatment groups of mice were administered A. gigas Nakai extract twice daily for 2 weeks. The tear volume significantly decreased in the positive control group ([0.35±0.04] mm/μL body
weight] compared to that in the normal group [(0.44±0.01) mm/g body weight] (26.14% decrease, \(P<0.01\)). No significant differences were found in the A. gigas Nakai extract 5 mg group [(0.44±0.07) mm/g body weight] compared to the normal group (Table 1). However, the decreased tear volumes in the positive control group were restored by treatment with A. gigas Nakai extract 5 mg (approximately 26.14%, \(P<0.05\)) and 10 mg [(0.55±0.04) mm/g body weight] (approximately 57.15%, \(P<0.001\)) (Table 1). There were statistically significant differences between the positive control group and the 10 mg group with respect to the protective effects of A. gigas Nakai extract (Table 1).

3.2. Clinical evaluation of A. gigas Nakai extract in mouse

The positive control group showed a significant increase in fluorescein staining scores compared to the normal group (Figure 1). Moreover, a decrease in fluorescein staining scores in the A. gigas Nakai extract 5 mg and 10 mg groups was observed compared to the positive control group. However, no statistically significant differences were observed in the effects of different concentrations of A. gigas Nakai extract (Figure 1).

Figure 1. Clinical evaluations of dry eye including fluorescein staining scores (A) after 14 d and representative images of corneal fluorescein staining in the normal control (B), positive control group (C), A. gigas Nakai extract 5 mg group (D), and A. gigas Nakai extract 10 mg group (E) after 14 d. Results are expressed as means±SD for 7 mice per group. *\(P<0.01\).

3.3. Histopathological changes of corneal surface in A. gigas Nakai extract and benzalkonium chloride-induced mouse

Corneal epithelial cells were considerably damaged by the prolonged exposure to benzalkonium chloride (Figure 2B)[29]. However, when A. gigas Nakai extract 5 mg and 10 mg groups were administered benzalkonium chloride, the morphology of the superficial epithelium in the corneas significantly recovered, similar to the morphology of the normal group (Figure 2A, 2C and 2D).

Figure 2. Effect of A. gigas Nakai extract on corneal epithelial cells of benzalkonium chloride-induced mice. (A) Normal group, (B) Positive control group, (C) A. gigas Nakai extract 5 mg group, (D) A. gigas Nakai extract 10 mg group. Arrows are irregular and vacuolated corneal epithelial cells.

3.4. TUNEL staining

TUNEL staining results are shown in Figure 3. No apoptotic cells were found in the corneas of the normal control group (Figure 3A). However, TUNEL-positive cells were observed in A. gigas Nakai extract 5 mg and 10 mg, as well as the positive control group (Figure 3B, 3C and 3D). As seen in Figure 3E, the positive control group had mean apoptotic cells (punctate and round cells) of 72%. Means of apoptotic cells in the corneas were 41% and 25% in the A. gigas Nakai extract-treated groups 5 mg and 10 mg, respectively (Figures 3E). TUNEL-positive cells in corneal epithelial cells were also calculated per square 100 μm of tissue. The effect of exposure to A. gigas Nakai extract and benzalkonium chloride on the corneal epithelium cells in mice is presented in Figure 3E. Results showed a significant reduction (\(P<0.001\)) in the average number of apoptotic cells per tissue in mice treated with 5 mg and 10 mg of A. gigas Nakai extract compared to the positive control group (Figure 3E).

Figure 3. Detection of apoptosis via TUNEL assay on corneal sections. (A) Normal group, (B) Positive control group, (C) A. gigas Nakai extract...
5 mg group, (D) A. gigas Nakai extract 10 mg group. (E) The number of apoptotic (TUNEL-positive) cells per 100 μm tissue. Arrows are irregular and punctate apoptotic cells. Results are expressed as means±SD for 7 mice per group. Significantly different from positive control group, respectively (P<0.001).

3.5. Apoptotic enzyme expression in mice

The positive control group showed a significant increase in TNF-α / β-actin ratio compared to the normal control group (P<0.05, Figure 4). On the other hand, the expression of the apoptotic enzyme (TNF-α) was significantly reduced in the A. gigas Nakai extract 5 mg and 10 mg groups with a decrease in the TNF-α / β-actin ratio (P<0.05, Figure 4).

![Figure 4](image)

**Figure 4.** Effect of A. gigas Nakai extract on TNF-α levels in cornea tissue. The ratio of protein expression between the target protein and β-actin. Results are expressed as means±SD for 7 mice per group; AGNE: A. gigas Nakai extract; *Significantly different from the normal and positive control groups, respectively (P<0.05).

4. Discussion

Benzalkonium chloride as a preservative in eye drops is harmful to the ocular surface[30]. However, patients with prolonged exposure to benzalkonium chloride can experience eye discomfort, conjunctival inflammation, and corneal damage through cell suicide and oxidative stress caused by cytokine secretion[31,32]. A benzalkonium chloride-induced animal models (rabbit and mouse) has been recently studied using a benzalkonium chloride concentration of 0.2% for more than 1 week, administered as 5 μL twice daily, which causes dry eye syndrome by inflammation of the corneal surface[32,33]. Based on the evidence of dry eye syndrome induction of benzalkonium chloride, the aim of this study was to investigate the preventive effect of A. gigas Nakai extract in benzalkonium chloride-induced dry eye syndrome mice.

Tear volumes were measured to identify the preventive effects of A. gigas Nakai extract on tear fluid secretion. In this study, tear volumes of 0.2% benzalkonium chloride-induced dry eye syndrome mice significantly decreased, similar to the results of Lin et al[32]. In this result, tear volumes were recovered by treatment with A. gigas Nakai extract 5 mg and 10 mg. This result confirms the successful development of a mouse model of dry eye.

A total of 0.1% oral doxycycline was previously studied to identify its anti-inflammatory effect using fluorescein staining[25]. Nevertheless, the recovery effect on the corneal surface by A. gigas Nakai extract administration has not been confirmed using fluorescein staining methods. Our study investigated the protective effect of A. gigas Nakai extract administration on benzalkonium chloride-induced dry eye using fluorescein staining scores. Clinical evaluation of A. gigas Nakai extract for corneal surface inspection in mouse was performed after 14 d of oral administration. The fluorescein staining scores was also significantly decreased by A. gigas Nakai extract, compared to the positive control group (Figure 1). A. gigas Nakai extract may serve as an anti-inflammatory agent in the treatment of dry eye and could also maintain the normal corneal epithelium as results by Cho et al[34].

Prolonged exposure times and high concentrations of benzalkonium chloride significantly increase the damage to corneal epithelial cells[35]. Burstein et al[35] also showed that the corneal epithelial cell morphology underwent significant changes upon exposure to benzalkonium chloride as compared to controls. The corneal epithelial cells of 0.1% benzalkonium chloride-treated rats presented dilated rough endoplasmic reticulum, enlarged mitochondria, loss of microvilli, and disrupted cytoplasmic membrane[29,35]. Our results also showed that the positive control group (only benzalkonium chloride-treated rats) developed squamous metaplasia[36]. Damaged cells with irregular and vacuolated shapes were observed in the corneas of the positive control group. In this study, A. gigas Nakai extract improved the histopathological changes induced by benzalkonium chloride in the corneas. The morphology of the superficial epithelium was significantly recovered by A. gigas Nakai extract. Since decursinol and decursin and decursinol angelate from A. gigas Nakai have protective and therapeutic effects against oxidative stress-related diseases, it is reasonable to assume that the observed protective effect of A. gigas Nakai extract may be attributed to corneal epithelium protection from oxidative stress[18].

The rate of apoptotic cell death was measured and quantified with a TUNEL assay. TUNEL-positive cells include dark brown stained nuclei in a round form and show DNA breaks with apoptotic bodies[37,38]. Pre-treatment with A. gigas Nakai extract resulted in a significant decrease of apoptotic cells compared to treatment with benzalkonium chloride alone (positive control group). The number
of apoptotic cells also decreased with the increase in A. gigas Nakai extract concentration. These results represent evidence of a key therapeutic potential, shown as reductions in various inflammatory elements and an improvement in relevant clinical symptoms[39].

The expression levels of apoptotic enzymes in mice after exposure to A. gigas Nakai extract and benzalkonium chloride were observed by western blot analysis of the corneas. As shown in Figure 4, the apoptotic protein expression levels (TNF-α) after benzalkonium chloride treatment were only upregulated in the corneas in the positive control group, similar to results of Lin et al[32]. Prolonged topical treatment with benzalkonium chloride caused dry eye syndrome in both animal models and humans[32,40]. TNF-α, a major cause of the inflammation developed during dry eye syndrome, has a pathogenetic role[41]. We focused on the mechanism of apoptosis induced by A. gigas Nakai extract in a mice model. Accordingly, a decrease in TNF-α expression by A. gigas Nakai extract treatment may be a useful cellular defense strategy. A. gigas Nakai extract concluded positive effects on dry eye syndrome.

Oh et al[39] reported that A. gigas Nakai extract inhibits interleukin-6 and TNF-α, and suppresses cyclooxygenase-2, hypoxia inducible factor-1α, and prostaglandin-E2 in mice with dextran sulfate sodium-induced murine ulcerative colitis. Our findings further support existing data showing that benzalkonium chloride-induced dry eye syndrome in mice after exposure to A. gigas Nakai extract and benzalkonium chloride were observed by western blot analysis of the corneas. As shown in Figure 4, the apoptotic protein expression levels (TNF-α) after benzalkonium chloride treatment were only upregulated in the corneas in the positive control group, similar to results of Lin et al[32]. Prolonged topical treatment with benzalkonium chloride caused dry eye syndrome in both animal models and humans[32,40]. TNF-α, a major cause of the inflammation developed during dry eye syndrome, has a pathogenetic role[41]. We focused on the mechanism of apoptosis induced by A. gigas Nakai extract in a mice model. Accordingly, a decrease in TNF-α expression by A. gigas Nakai extract treatment may be a useful cellular defense strategy. A. gigas Nakai extract concluded positive effects on dry eye syndrome.

References

[1] Lemp MA, Foulks GN. The definition and classification of dry eye disease: report of the definition and classification subcommittee of the international dry eye workshop. Ocul Surf 2007; 5(2): 75-92.
[2] Javadi MA, Feizi S. Dry eye syndrome dry eye syndrome. J Ophthalmic Vis Res 2011; 6(3): 192-198.
[3] Messmer EM, Bulgen M, Kampik A. Hypersmolarity of the tear film in dry eye syndrome. Dev Ophthalmol 2010; 45: 129-138.
[4] Tsuota K, Yokoi N, Shimazaki J, Watanabe H, Dogru M, Yamada M, et al. New perspectives on dry eye definition and diagnosis: A consensus report by the Asia dry eye society. Ocul Surf 2017; 15(1): 65-76.
[5] Kim MK. The relation between dry eye syndrome and allergic conditions. Masters thesis. Ewha Womans University, School of Medicine; 2015.
[6] Donnenfeld E, Sheppard JD, Holland EJ. Prospective, multi-center, randomized controlled study on the effect of loteprednol etabonate on initiating therapy with cyclosporin A. In: Proceedings of the AAO Annual Meeting 2007. New Orleans: AAO; 2007.
[7] Brugger O, Chalouhi M, Yassin H, Sharaf S, Farouki A. The simultaneous determination of ciprofloxacin and ofloxacin in liver and lung tissues by HPLC. J Pharm Biomed Anal 2006; 40(3): 827-832.
[8] Kim KM, Kim TH, Park YJ, Kim IH, Kang JS. Evaluation of the genotoxicity of decursin and decursinol angelate produced by Angelica gigas Nakai. Mol Cell Toxicol 2009; 5(1): 83-87.
[9] Lee YY, Lee SH, Jin JL, Yun-Choi HS. Platelet anti-aggregatory effects of coumarins from the roots of Angelica genuflexa and A. gigas. Arch Pharm Res 2003; 26(9): 723-726.
[10] Konoshima M, Chi HJ, Hata K. Coumarins from the root of Angelica gigas Nakai. Chem Pharm Bull 1968; 16(6): 1139-1140.
[11] Ryu KS, Hong ND, Kim NJ, Kong YY. Studies on the coumarin constituents of the root of Angelica gigas Nakai. Isolation of decursinol angelate and assay of decursinol angelate and decursin. J Korean Soc Food Sci Nutr 2009; 38(5): 653-656.
and decursinol angelate against amyloid β-protein-induced oxidative stress in the PC12 cell line: The role of Nrf2 and antioxidant enzymes. *Biosci Biotechnol Biochem* 2011; 75(3): 434-442.

[19] Kim KM, Lee YJ, Hong YG, Kang JS. Oral acute and subacute toxicity studies of decursin and decursinol angelate of *Angelica gigas* Nakai. *Mol Cell Toxicol* 2009; 5(2): 153-159.

[20] Kim KM, Kim MJ, Kang JS. Absorption, distribution, metabolism, and excretion of decursin and decursinol angelate from *Angelica gigas* Nakai. *J Microbiol Biotechnol* 2009; 19(12): 1569-1572.

[21] Kim KM, Ok S, Go YS, Kang JS. Recovery from the two-generation reproductive toxicity in Sprague-Dawley rats by treatment with decursin and decursinol angelate. *J Life Sci* 2015; 25(7): 765-772.

[22] Kim KM, Seo JL, Kang JS. Decursin and decursinol angelate affect spermatogenesis in the adult rat at oral administration. *Mol Cell Toxicol* 2014; 10(1): 83-89.

[23] Iwasawa A, Ayaki M, Niwano Y. Cell viability score (CVS) as a good indicator of critical concentration of benzalkonium chloride for toxicity in cultured ocular surface cell lines. *Regul Toxicol Pharmacol* 2013; 66(2): 177-183.

[24] Arakaki R, Eguchi H, Yamada A, Kudo Y, Iwasa A, Enkhmaa T, et al. Anti-inflammatory effects of rebamipide eyedrop administration on ocular lesions in a murine model of primary Sjögren’s syndrome. *PLos One* 2014; 9(5): e98390.

[25] Zhang Z, Yang WZ, Zhu ZZ, Hu QQ, Chen YF, He H, et al. Therapeutic effects of topical doxycycline in a benzalkonium chloride–induced mouse dry eye model. *Invest Ophthalmol Vis Sci* 2014; 55(5): 2963-2974.

[26] Ok S, Kang JS, Kim KM. Testicular antioxidant mechanism of cultivated wild ginseng extracts. *Mol Cell Toxicol* 2016; 12(2): 149-158.

[27] Ok S, Kang JS, Kim KM. Cultivated wild ginseng extracts upregulate cell viability score (CVS) as a good indicator of critical concentration of benzalkonium chloride for toxicity in cultured ocular surface cell lines. *Regul Toxicol Pharmacol* 2013; 66(2): 177-183.

[28] Li B, Cong M, Zhu Y, Xiong Y, Jin W, Wan Y, et al. Indole-3-carbinol induces apoptosis of hepatic stellate cells through K63 de-ubiquitination of RIP1 in rats. *Cell Physiol Biochem* 2017; 41(4): 1481-1490.

[29] Cha SH, Lee JS, Oum BS, Kim CD. Corneal epithelial cellular dysfunction from benzalkonium chloride (BAC) in vitro. *Clin Exp Ophthalmol* 2004; 32(2): 180-184.