Differential Toxicity of the Water and Ethanol Extracts of Chung-Sang, an Experimental Herbal Formula

Ran Won, Jun-Yong Choi[2], Chang Woo Han[2], Han-Sol Jeong[1], Sang Woo Shin[1], Hee Jae Jung[3], Myungsoo Joo[1]*

Department of Biomedical Laboratory Science, Division of Health Sciences, Dongseo University,
1 : Division of Applied Medicine, School of Korean Medicine, Pusan National University,
2 : Department of Internal Medicine, Korean Medicine Hospital, Pusan National University,
3 : Division of Allergy, Immune and Respiratory System, Department of Internal Medicine, College of Korean Medicine, Kyung Hee University

Chung-Sang (CS) is an experimental herbal remedy that is formulated to treat respiratory diseases implicated by inflammation. The herbs comprised of CS are frequently prescribed for treating various inflammatory symptoms: Menthae haplocalycis Herba, Magnoliae Flosis, Xanthii Fructus, Herba Asari, and Caryphylli Flos. Here, we prepared the extract of CS with boiling water (wCS) or with 50 % ethanol (eCS) and examined whether the two different extracts of CS exhibit a toxicity to cultured cells and mice. RAW 264.7 cells were treated with wCS or eCS, and the cytotoxicity of these extracts to RAW 264.7 cells was determined by an MTT assay. Although the production of intracellular reactive oxygen species that are detrimental to the cells was not increased by the extracts, the cytotoxicity to the cells was evident from 10 mg/ml of wCS and 100 mg/ml of eCS, suggesting that eCS is less cytotoxic. When mice (n = 10/group) received a single intratracheal wCS or eCS daily for 14 days, wCS yielded 40 % mortality, whereas eCS showed none. Both wCS and eCS did not significantly affect the weight of the body and of vital organs, except the lung. Biochemical analyses of mice blood indicated no damage to liver or kidney. However, unlike eCS, wCS significantly increased the level of IgE in serum. Collectively, our results show that eCS was less toxic than wCS, suggesting that CS prepared with 50 % ethanol is preferential over the conventional way of preparing CS.

keywords : Herbal remedy, Chung-Sang, New medicinal formula, Korean traditional medicine

Introduction

Herbal remedies are an essential component in Korean traditional medicine. According to the principles of the traditional Asian medicine, an herbal remedy consists of a key herb that executes a major pharmacologic effect towards a target symptom, and of other secondary herbs that help enhance the efficacy of the major herb[1)]. As a result, the traditional herbal remedies are mostly bulky. Although this traditional way of formulating herbal remedies has been practiced without significant adversary effects, it is possible that the bulkiness of the herbal remedies contributes to diluting the efficacy of the major herb, which could make the therapeutic effect of the remedy suboptimal.

Inflammatory diseases can be treated with various herbal remedies. The choice of a specific herbal remedy is dependent on the holistic diagnosis that considers various morbidities of a patient, the practice of which hinges on the principles of the traditional Korean medicine. Because diverse herbal remedies are available to regulate inflammation, it is possible that medicinal herbs can be collated based on an anti-inflammatory function[2)]. For example, *Mentha haplocalyx* Briq. has been traditionally used for decreasing the symptoms of common cold and sore throat[3)]. The dried flower bud of *Magnolia biondii* Pamp has been prescribed for relieving nasal congestion, nasosinusitis, and allergic rhinitis with purulent discharge[4)]. Along with anti-inflammatory effect, *Asarum sieboldii* Miq. has been used for treating cough and dyspnea and removing a nasal obstruction in sinusitis[4)]. *Magnolia biondii* Pamp showed some beneficial effect on asthma when co-administered with inhaled corticosteroid treatment in a clinical trial[5)]. *Xanthium sibiricum* Patr. is used for the treatment of chronic paranasal sinusitis, allergic and chronic rhinitis, and chronic bronchitis[4)]. Therefore, the effects of these herbs are likely similar and overlapping, which contributes to relieving respiratory symptoms.

We postulated that a concoction composed of herbs

* Corresponding author
Myungsoo Joo, Division of Applied Medicine, School of Korean Medicine, Pusan National University, Yangsan 50612, Republic of Korea
·E-mail : mjoo@pusan.ac.kr ·Tel : +82-51-510-8462

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that are used for a similar target disease can be more effective than a traditional herbal remedy for the same disease. To explore our hypothesis, we formulated Chung-Sang (CS), which comprises 5 different herbs. Among them, Menthae haplocalycis Herba, Magnoliae Flosis, Xanthii Fructus, and Herba Asari are traditionally used to relieve symptoms associated with inflammatory respiratory diseases, such as cold, allergic rhinitis, and asthma. Since many respiratory symptoms are often associated with bacterial infection, Caryphylli Flos was included as the fifth constituent of CS because *Caryophyllus aromaticus* L. has antimicrobial activity against various species of bacteria including *Staphylococcus ssp.*, *Streptococcus ssp.*, and *Pseudomonas aeruginosa*[4,6]. Since this is a new formula, it would be necessary to determine whether or not CS exhibits any toxicity. In this study, we prepared two different extracts of CS, aqueous extract (wCS) and 50 % ethanol extract (eCS) of CS, and determined whether they show any cytotoxicity and toxic effect in mice. Our results suggest that eCS is less toxic than wCS and CS needs to be prepared with 50 % ethanol. Our findings also suggest that the choice of solvent for extraction matters to achieve the desired effect of an herbal remedy.

Materials and Methods

1. Preparation of Chung-Sang

The herbal composition of Chung-Sang (CS) was shown in Table 1. CS is composed of *Caryophyllus aromaticus* L. *Mentha haplocalyx* Briq., *Magnolia biondii* Pamp., *Xanthium sibiricum* Patr., and *Asarum sieboldii* Miq.. The amount of each herb in CS was based on a daily dose typically prescribed to patients. The aqueous and 50% ethanol concoctions of CS (number: pnukh-CS01 and pnukh-CS02, respectively) were prepared in Kyung Hee Hanbang Clinic, Kyun Hee University, Seoul, Korea. The water extract of CS (wCS) started with 500 g of CS mixed with ddH_2O, which was heated at 80 ℃ for 2 h in a reflux apparatus (Global lab, Seoul, Korea). The primary extract was subject to the second round of heating in the reflux apparatus, concentrated to 50ml by a low-pressure evaporator (Bchi rotavapor R-220, Canada), and underwent freeze-drying processes to yield 7 g of powder. The ethanol extract of CS (eCS) was similarly prepared in 50 % ethanol. The yield of ethanol extraction of CS was 6 g in powder. The powder of wCS or eCS was dissolved in phosphate buffered saline (PBS), which was sterilized by being passed through a 0.45 mm filter prior to the experiment.

Table 1. Composition of Chung-Sang

Scientific name	Herbal name	Amount (g)
Mentha haplocalyx Briq.	Menthae haplocalycis Herba(薄荷)	10
Magnolia biondii Pamp	Magnoliae Flosis(辛夷)	5
Xanthium sibiricum Patr.	Xanthii Fructus(蒼耳子)	5
Asarum sieboldii Miq.	Herba Asari(細辛)	5
Caryophyllus aromaticus L.	Caryphylli Flos(丁香)	5
Total		30

2. Cell culture

RAW 264.7 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine (200 mg/L) (Thermo Fisher Scientific, Waltham, MA, USA) containing 10 % (v/v) heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin and 100 mg/ml streptomycin (Thermo Fisher Scientific). Cells were maintained in a humidified incubator at 37℃ and 5 % CO_2.

3. Measurement of Cytotoxicity

Cytotoxicity was determined by Vybrant MTT assay kit and the protocol of the manufacturer (Thermo Fisher Scientific). The percentage of metabolically active cells was calculated over untreated cells. The experiment was performed in triplicate three times independently.

4. Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS were measured by using 5-(and-6)-carboxy-2',7'-dichlorodihydro-fluorescein diacetate (carboxy-H2DCFDA; Molecular Probes, Eugene, OR, USA). Briefly, RAW 264.7 cells (1×10^6 cells/well) were incubated with 100 M carboxy-H2DCFDA for 30 min at 37℃ and analyzed by the BD FACS Canto II system (BD Biosciences, San Jose, CA, USA) at the excitation wavelength of 488 nm and the emission wavelength of 525 nm. Data were analyzed by FlowJo software (Tree Star, San Carlos, CA, USA).

5. Measurement of Toxicity in Animals

All experiments with mice were conducted per the NIH of Korea Guidelines for the Care and Use of Laboratory Animals. Experimental procedures with mice were approved by the Institutional Animal Care and Use Committee of Pusan National University (protocol number: PNU-2016-1139). C57BL/6 mice purchased from Samtaco Bio Korea, Ltd. (Osan, Korea) were housed in certified, standard laboratory cages. Mice (n = 10 per group) received 5, 10, or 20 mg/kg body weight of a single intratracheal (i.t.) injection of wCS or eCS every day for 14 days. Mortality during treatment was monitored. At day 15, the weights of the body were measured and then mice were sacrificed for

the measurement of the weights of vital organs, including lung, spleen, liver, and kidney. As for a control of damaged liver, mice received a single intraperitoneal (i.p.) injection of d-(+)-galactosamine hydrochloride (500 mg/kg body weight; Sigma-Aldrich, Seoul, Korea). Blood was drawn from the heart, from which plasma was prepared for the measurement of creatinine, GOP (Glutamate Oxaloacetate Transaminase), GPT (Glutamate Pyruvate Transaminase), and immunoglobulin E (IgE).

6. Measurement of GOP, GPT, and Serum IgE

Glutamate oxaloacetate transaminase (GOT), glutamate pyruvic transaminase (GPT), and creatinine in plasma were analyzed using a Fuji DRI-CHEM 3500i and the protocol provided the manufacturer (Fuji Photo Film, Ltd., Japan). Serum IgE was measured by an ELISA kit and the protocol of the manufacturer (Thermo Fisher Scientific, Waltham, MA, USA).

7. Statistical analysis

To compare the results of groups, one-way analysis of variance (ANOVA) tests with Tukey's post hoc test was used (with the assistance of InStat, Graphpad Software, Inc., San Diego, CA). P values less than 0.05 are considered significant. All experiments were performed at least three times independently.

Results

1. Differential cytotoxicity of the two different extracts of Chung-Sang

Prior to determining the effectiveness of CS in a clinical setting, it would be necessary to examine whether CS exhibits toxicity. Since CS was formulated to treat inflammatory diseases and macrophages play a critical role in regulating inflammation, we took RAW 264.7 cells and determined whether wCS or eCS exhibits a toxicity to the cell. RAW 264.7 cells were treated with various amounts of wCS or eCS, from 1 mg/ml to 500 mg/ml. At 16 h after treatment, an MTT assay was performed. As shown in Fig. 1 A, wCS showed a slight, but statistically significant, cytotoxicity at 10 mg/ml. The cytotoxicity of wCS became more severe as the amount of wCS increased. When treated with 500 mg/ml of wCS, about 40 % of RAW 264.7 cells were survived, showing approximately 60 % mortality. Similar experiments were performed with eCS (Fig. 2B). Unlike wCS, RAW 264.7 cells did not exhibit a cytotoxicity at 10 mg/ml of eCS. When treated with 100 mg/ml of eCS, RAW 264.7 cells exhibited a slight, yet statistically significant, cytotoxicity. Substantial cytotoxicity was observed when the cells were treated with 500 mg/ml of eCS.

Since inflammatory reaction accompanies the production of intracellular reactive oxygen species (ROS) that inflict cellular damage, we also examined whether wCS or eCS elicits the production of ROS, contributing to cytotoxicity. RAW 264.7 cells were similarly treated with 50 mg/ml of wCS or eCS for 16 h. The production of intracellular ROS was determined by a flow cytometer. As shown in Fig. 2, while LPS induced the production of intracellular ROS, neither wCS nor eCS significantly generated intracellular ROS (bottom two panels in Fig. 2C). Combined with the findings that intracellular ROS were near a basal level in RAW 264.7 cells treated with either wCS or eCS, these results suggest that eCS is less toxic than wCS, to which intracellular ROS contribute insignificantly.

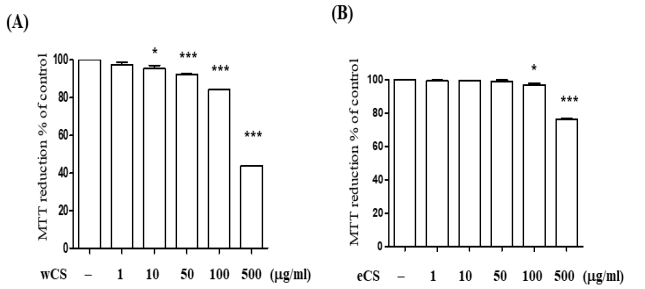


Fig. 1. Cytotoxicity of two different extracts of Chung-Sang. RAW 264.7 cells were treated with increasing amounts of wCS (A) or eCS (B) for 16 h. Cytotoxicity induced by wCS or eCS was determined by an MTT assay Data represent the mean SEM in triplicate. *P* and ***P* were less than 0.05 and 0.0001, respectively, compared to the untreated control.

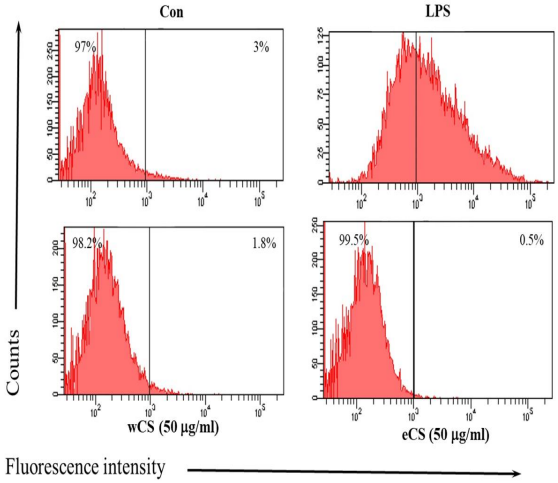
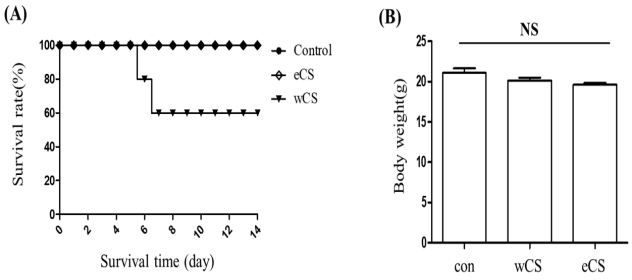


Fig. 2. Effects of the two extracts on the production of intracellular ROS. RAW 264.7 cells were treated with 50 mg/ml of wCS or eCS. At 16 h after treatment, intracellular ROS were measured by a flow cytometer. Treatment with LPS (100 ng/ml) for 16 h was included as a positive control for ROS.

2. The water extract of CS increased the mortality of mice

Given that herbal remedies are administered regularly and repetitively to patients during a morbid period, we determined whether there is any toxic effect because of a repetitive administration of CS. C57BL/6 mice (n = 10/group) were administrated with an intratracheal (i.t.) injection of 20 mg/kg body weight of wCS or eCS daily for 14 days. At day 15, mice survived were weighted and then sacrificed for further analyses of vital organs and blood. We found that during the experiment, mice that received wCS exhibited an increased mortality (40 %), as oppose to mice treated with eCS or PBS, in which no mortality was observed (Fig. 3A), suggesting that wCS has some adversary effect. This finding was somewhat consistent with the cytotoxicity of wCS to RAW 264.7 cells. However, when the body weights of survived mice were measured, there was no difference with a statistic significance between mice administered with wCS and eCS (Fig. 3B). Similarly, the weights of spleen, liver, and kidney of mice treated with wCS were not different from those of eCS or sham control group.

Although there were weight deviations between the groups, the differences fell within the standard deviations of the means of the sham controls (Fig. 3C). However, there was an exception; the weight of lung was significantly increased in mice treated with wCS.



(C)

(unit : g)

20mg/kg	control	wDS	eDS
Lung	0.1354±0.0178	0.2342±0.0385	0.1758±0.0111
Spleen	0.0758±0.0096	0.0734±0.0104	0.0642±0.0034
Liver	1.0162±0.0924	1.1652±0.1401	1.0238±0.0448
Kidney	0.2612±0.0243	0.2702±0.0128	0.2592±0.0101

Fig. 3. Effects of the two extracts on the mortality of mice. (A) C57BL/6 mice (n = 10/group) received daily a single i.t. PBS or 20 mg/kg body weight wCS or eCS for 14 days. During this experiment, mouse morbidity and mortality were closely monitored. At day 15, mice were weighed (B) and sacrificed for further analyses of key internal organs such as lung, spleen, liver, and kidney (C).

3. The water extract of CS increased the level of Immunoglobulin E (IgE) in mouse serum

Given the cytotoxicity and increased mortality of wCS, it is conceivable that without apparent changes in the weights of key organs, wCS inflicts damage to the tissues of kidney or liver. Therefore, we first examined whether i.t. wCS administration to mice can inflict damage to the kidney by measuring the level of creatinine in the blood. Blood was withdrawn from the mice treated with wCS or eCS, which were sacrificed at day 15, and the level of creatinine in the blood was determined. As shown in Fig. 4A, the level of creatinine in mice treated with wCS was similar to that of mice with eCS. Similarly, we measured GOP and GPT in blood to determine whether wCS causes any damage to the liver. As shown in Fig. 4B, the levels of both GOP and GPT in mice treated with wCS were similar to those of mice with eCS, while the levels of GOP and GPT in mice treated with d-(+)-galactosamine hydrochloride were increased. These results suggest that neither wCS nor eCS has no significant toxicity towards the kidney and the liver.

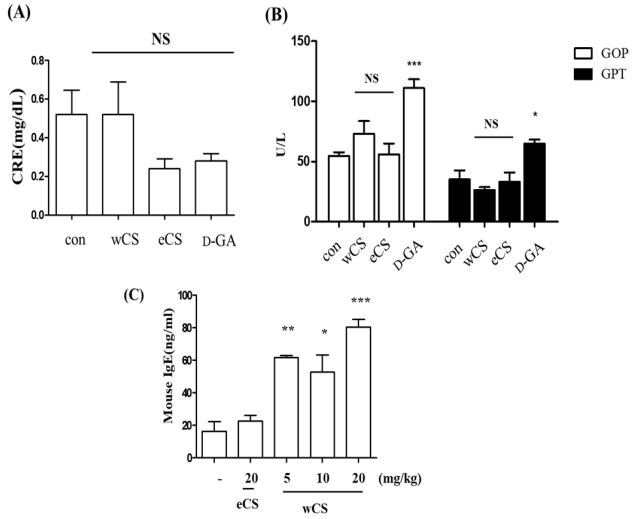


Fig. 4. Pathologic effects of the two extracts. Blood was withdrawn from the mice that were treated with 20 mg/kg of wCS or eCS as described above (n = 5/group), and creatinine (CRE) and GOT and GPT (B) in serum were measured. As controls for damaged liver, mice (n = 5) were treated with a single intraperitoneal D-(+)-galactosamine hydrochloride (500 mg/kg body weight) for 24h (D-GA). (C) Along with the mice treated with 20 mg/kg body weight of wCS or eCS, two different doses, 5 mg/kg and 10 mg/kg body weight, of wCS were administered to mice for 14 days (n = 5/group). At day 15, mice were sacrificed and blood was withdrawn. IgE in serum was measured. *P, **P, and ***P were less than 0.05, 0.001, and 0.0001, respectively, compared to control groups. NS means no statistical significance, compared to control groups.

Since wCS increased the weight of the lung, we postulated that wCS may increase allergic reaction in the lung. To test this possibility, we measured IgE in the blood of mice treated with wCS or eCS. As shown in Fig. 4C, while eCS did not change the level of blood IgE (the 2nd column from the left), wCS significantly increased the level of blood IgE (the 5th column from the left). To verify this observation,

we performed a similar experiment by administering lower amounts of wCS, 5 or 10 mg/kg body weight, to mice for 14 days and measuring blood IgE. Similar to the mice received 20 mg/kg body weight of wCS, mice received the low doses of wCS elicited the production of IgE (3rd and 4th columns from the left). Together, these results show that wCS is highly allergenic, suggesting that wCS can cause harm by eliciting an allergic reaction in the respiratory organ.

Discussion

Medicinal herbs are a key component in Korean traditional medicine and the herbal remedies concocted with the medicinal herbs have long been prescribed to patients as instructed by Korean traditional medicine (KTM). The herbal remedies in Korean medicine comprise one or two key herbs that exert a major therapeutic function and other peripheral herbs that support the function of the key herb. This way of formulating herbal remedies reflects the principles in practicing KTM, a holistic treatment of diseases by regaining homeostasis perturbed. Despite the herbal remedies that have been successful in treating patients, however, they have been challenged by the advent of modern pharmaceutics. Since pharmaceutical drugs consist of a single or few active chemicals that act on molecular targets, a small quantity of them is often sufficient to exert their therapeutic effect. One of the ways of coping with this challenge could be of reformulating traditional herbal medicine that is made of key herbs. We have studied experimental formulas[7]. In this study, we took and examined the toxicity of an experimental formula Chung-Sang (CS). CS is composed of 5 herbs that have been used as key herbs in various formulas that are used to treat inflammatory symptoms as a major target symptom 4. Although the herbs comprised of CS have been widely used for treating inflammatory symptoms in respiratory diseases, CS is a new herbal remedy. Therefore, we tested a possible toxicity of CS using cultured cells and C57BL/6 mice. The extract of CS was prepared by boiling CS with water (wCS) and by submerging CS in 50 % ethanol (eCS). The powder of each extract was prepared, sterilized, and used for the experiment. Results show that wCS was more toxic than eCS to RAW 264.7 cells and C57BL/6 mice. Compared to eCS, wCS was allergenic to mice. Therefore, our findings suggest that CS needs to be prepared with 50% ethanol, rather than water, a conventional media for preparing herbal remedies.

It appears that wCS was more cytotoxic than eCS because treatment of RAW 264.7 cells with 10 mg/ml of wCS exhibited cytotoxicity, while 100 mg/ml of eCS did. Cytotoxicity can be caused by various factors, including intracellular ROS because excessive ROS can damage cells. Our initial analyses indicated that 10 mg/ml of wCS or eCS did not induce the ROS (data not shown). Thus, we tested a higher amount of wCS or eCS, 50 mg/ml, for ROS production, showing no substantial production of the ROS by this amount of wCS or eCS. Given that 50 mg/ml of wCS induced a significant cytotoxicity, our results suggest that the intracellular ROS contributes marginally, if any, to the cytotoxicity of wCS.

Respiratory diseases ridden with inflammation are common. Since CS was formulated with the intent of treating inflammatory respiratory diseases, it would be necessary to determine whether a repetitive administration of CS to the lung exhibits any toxicity to mice. We administered mice (n = 10/group) with a single, 20 mg/kg of i.t. wCS or eCS daily for 14 days. This dose was calculated based on the minimum amount used for measuring cytotoxicity, 10 mg/ml of wCS, at which a marginal, yet statistically significant cytotoxicity was observed. For the sake of testing toxicity, however, we doubled the amount to 20 mg/kg body weight as a daily dose. We found that mice received repetitive wCS exhibited a high mortality (about 40 %), as opposed to eCS. The cause of mortality shown by wCS is unknown. Given that the kidney and liver of survived mice were normal, it is likely that the mortality by i.t. wCS may not be directly related to the damages in liver or kidney. Although damage in liver was detectable in mice died during the experiment (data not shown), we do not have convincing evidence that links the untimely death to a liver damage. Rather, since the weight of lung in mice received wCS appeared to be higher than mice received eCS, it is possible that the repetitive i.t. administration of wCS could induce edema or inflammation in the lung, increasing mortality. In support of this notion, we found increased inflammation in the lung of the mice died during the experiment (data not shown). In addition, i.t. wCS increased the levels of IgE in serum in mice. Even lower amounts of wCS consistently increased serum IgE in mice. A study shows that a high level of IgE makes mice susceptible to anaphylaxis, contributing to a high mortality of mice[8]. Therefore, it is conceivable that while the lethal level of IgE is likely varied among mice, an allergic reaction or anaphylaxis induced by wCS could contribute, at least in part, to the high mortality in mice received wCS.

We delivered wCS or eCS to the lung via an intratracheal route, in lieu of the traditional oral administration. Direct delivery of drugs to target lesions or organs is known to increase the efficacy of a drug[9]. In fact, nasal congestion, allergic rhinitis, and asthma can be treated with devices that enable a direct delivery of drugs to the target areas. Therefore, it is worth considering to develop an equivalent in herbal remedies in Korean medicine for treating inflammatory, respiratory diseases.

Conclusion

wCS showed higher toxic effects than eCS to RAW 264.7 cells and mice. It appeared that the toxicity found in mice was associated with serum IgE, the level of which was significantly increased by repetitive administration of wCS. Our results suggest that a solvent to prepare herbal medicine needs to be carefully chosen.

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