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Original Article

Anti-Inflammatory and Anti-Oxidative Effects of Danggwisusan on Macrophages

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ABSTRACT

Background: Danggwisusan is a herbal medicine which is used to treat bruises, static blood, external injuries, and somatalgia in Korean medicine. The objectives of this study were to investigate whether Danggwisusan hot aqueous extract had an inhibitory effect upon inflammatory cytokine production and oxidation.
Methods: Cytotoxic activity of Danggwisusan extract was examined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. The amount of nitric oxide produced was measured using Griess reagent. Prostaglandin E2 production was measured using an enzyme immunoassay. Inflammatory cytokines (IL-1β, IL-6 and TNF-α) were measured by an enzyme linked immunosorbent assay. The anti-oxidative effect of Danggwisusan was measured by the 1,1-Diphenyl-2-picryl hydrazyl method. The amount of polyphenol and flavonoid contents were measured by Folin and Ciocalteauea phenol reagent and aluminum nitrate.
Results: Danggwisusan hot aqueous extracts did not show significant toxicity at 10, 20, 50, and 100 μg/mL. At a dose of 100 μg/mL, Danggwisusan hot aqueous extract significantly inhibited nitric oxide and PGE_2 production, and significantly reduced IL-1β, IL-6 and TNF-α production. At a dose of 100 μg/mL, 1,1-Diphenyl-2-picryl hydrazyl free radical scavenging capability was over 50%.
Conclusion: This study showed that Danggwisusan hot aqueous extract may have anti-inflammatory and anti-oxidative effects on macrophages.

Introduction

Inflammation is the mechanism by which immune cells secrete inflammatory mediators to protect the body. This mechanism occurs when external substances such as bacteria or viruses are introduced. Inflammation is a very important immune reaction that occurs when tissues are damaged. Inflammation reactions are typically accompanied by fever, pain, and edema [1]. Inflammatory reactions are a common symptom of gout, tendinitis, sprained joints, aponeurositis, and osteoarthritis [2].

Danggwisusan is used to treat bruises, static blood, external injuries and somatalgia on Yixuerumen [3]. Most medicines that constitute Danggwisusan perform Qi moving, static blood breaking, heat-clearing, and blood-cooling activities [4].

Previous studies have reported headache relief [5], external injury amelioration [6], fracture healing [7], subcutaneous hematoma amelioration [8], antithrombotic [9], cerebral ischemic injury amelioration [10], allergic purpura amelioration [11], and pain control [12] effects of Danggwisusan.

Likewise, many studies have suggested that Danggwisusan might have anti-inflammatory and antioxidant effects. However, the properties of Danggwisusan as an anti-inflammatory or antioxidant has not been fully investigated. Therefore, the effects of Danggwisusan hot aqueous extract upon nitric oxide (NO) and prostaglandin E2 (PGE_2) production were studied. The amounts of interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) were also investigated, to understand the anti-inflammatory response more clearly. We investigated antioxidant efficacy using 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity in macrophages. The amount of polyphenol and flavonoid contained in Danggwisusan was also measured.

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Materials and Methods

Materials

For the composition of Danggwisusan, refer to the Donguibogam (Table 1) [13].

The herbs used in this experiment were purchased from Omniherb (Kyongbuk, Korea). Danggwisusan, 160 g was mixed with 1.6 L of water and boiled for 4 hours at 100°C. The extract was filtered and centrifuged at 3,000 g. It was then filtered again with 0.03 mm filter paper (Nalgene, New York, USA). The filtrate was concentrated to 100 mL and frozen at -80°C. The frozen solution was freeze-dried for 7 days using a freeze-dryer system (Labconco, Santa Clara, USA). The yield was 12.25%, indicating that 19.6 g of Danggwisusan extract was obtained.

Macrophage cell culture

The RAW 264.7 macrophage cell line was used in this experiment (ATCC, Manassas, USA). Macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) that included 10% fetal bovine serum (FBS) and were incubated at 37°C, 5% CO2. Cells cultured below 10 passages were used.

Cytotoxicity

Cytotoxicity was evaluated by performing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Macrophages were seeded at 1×10^5/well in a 96 well plate and were incubated for 18 hours. A control and the experimental groups of Danggwisusan 10, 20, 50, 100, 150, and 200 μg/mL were used. 20 μg of MTT reagent was added to each well after being left in a dark room for 4 hours. The supernatant was removed and 100 μg of DMSO reagent was added to each sample and shaken for 30 minutes. Absorbance was measured at 570 nm.

NO production

Macrophages were incubated for 18 hours at a concentration of 1×10^5/well in a 96 well plate. Griess reagent was prepared with 0.1% naphthylethylenediamine -dihydrochloride 50 μL, and 1% sulfanilamide 50 μL dissolved in 5% H_3PO_4. Control and experimental groups consisting of, Danggwisusan 10, 20, 50, and 100 μg/mL were used. Each group was treated with 1 μg/mL lipopolysaccharide (LPS). Cells were cultured for 24 hours. Then, 100 μL supernatant and 100 μL Griess reagent were mixed. A standard curve was produced based upon the concentration of $NaNO_2$. Absorbance was measured at 540 nm.

Table 1. The Prescription of Danggwisusan.

Pharmacognostic name	Weight (g)
Angelicae Radix	6
Caesalpiniae Lignum	4
Linderae Radix	4
Paeoniae Radix Rubra	4
Cyperi Rhizoma	4
Persicae Semen	3
Carthami Flos	3
Glycyrrhizae Radix et Rhizoma	2
Cinamomi Ramulus	2
Total	32

PGE_2 production

PGE_2 was measured using a commercial competitive enzyme immunoassay kit (R&D Systems, Minneapolis, USA). The control and the experimental groups were, Danggwisusan 10, 20, 50, and 100 μg/mL were treated with 1 μg/mL LPS. The treated macrophages were incubated for 18 hours. Each well was aspirated and washed 4 times with 400 μL wash buffer. 200 μL of substrate solution was added to each well. The treated cells were incubated at room temperature for 30 minutes then 100 μL of stop solution was added to each well. Absorbance was measured at 450 nm.

Cytokine

The amount of IL-1β, IL-6, and TNF-α produced was measured by an Elisa kit (R&D Systems, Minneapolis, USA). The control and the experimental groups were Danggwisusan 10, 20, 50, and 100 μg/mL and were treated with the 1 μg/mL LPS reagent. The treated macrophages were incubated for 18 hours. The culture was diluted to the appropriate concentration so that 50 μL of culture could be added to each cytokine-coated well which was then left at room temperature for 2 hours. The wells were then washed 3 times with wash buffer and treated with 100 μL of biotinylated antibody reagent. This was carried out at room temperature, for 1 hour, and then washed 3 times. 100 μL of streptavidin-HRP solution was added and left at room temperature for 1 hour. Each well was then washed 3 times with wash buffer. Then, 100 μL of di (2-ethylhexyl) -2,4,5-trimethoxy benzalmalonate (TMB) substrate was used for 5 to 30 minutes. After the reaction, 100 μL of stop solution was added to each sample. Absorbance was measured at 450 nm.

Measuring the anti-oxidative effect

To investigate the potential antioxidative capability of Danggwisusan, DPPH free radical scavenging capability was measured. Macrophages were seeded at a concentration of 1×10^5/well in a 96 well plate and incubated for 16 hours. The experimental groups were Danggwisusan 10, 20, 50, and 100 μg/mL) were used. Each group was diluted with 50μL of 100% methanol and mixed with 0.15 mM DPPH (Sigma, California, USA) 80 μL per well and left for 15 minutes at room temperature. Absorbance was measured at 520nm. A standard curve was made based upon the concentration of ascorbic acid. DPPH free-radical scavenging capability was evaluated using the equation below.

DPPH free-radical scavenging activity (%)

$$= \frac{Absorbance\ of\ Control - Experimental}{Absorbance\ of\ Control} \times 100$$

Polyphenol in Danggwisusan

Folin and Ciocalteau's phenol reagent method was used for polyphenol measurement. 0.5 mL of Folin and Ciocalteau's phenol reagent was added to 0.5 mL of Danggwisusan diluted in 50% methanol. The mixture was left at room temperature for 5 minutes, after which 0.5 mL of 10% sodium carbonate solution was added and left at room temperature for 1 hour. The supernatant was removed and the absorbance was measured at 725 nm. The polyphenol content was determined using a standard curve of tannic acid dissolved in 70% methanol.

Flavonoid in Danggwisusan

The aluminum chloride method was used to measure flavonoid concentration. The Danggwisusan hot aqueous extract was dissolved in methanol at a concentration of 1 mg/mL. To determine the total flavonoid content, 20 µL of 10% (w/v) aluminum, 20 µL 1M potassium acetate, and 860 µL methanol was added to 0.5 mL of Danggwisusan. Absorbance was measured at 415 nm. The total flavonoid content was determined using a standard curve of quercetin.

Statistical analysis

SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) was used to analyze results and statistical significance was confirmed using Student t test; the level of significance was $p<0.05$.

Results

The toxicity of Danggwisusan hot aqueous extract for the control group was 100 ± 0.86%, 97.62 ± 0.29% for the 10 µg/mL group, 96.47 ± 2.07% for the 20 µg/mL group, 91.84 ± 1.95% for the 50 µg/mL group, 88.03 ± 2.93% for the 100 µg/mL group, 82.21 ± 1.53% for the 150 µg/mL group, and 71.65 ± 1.68 % for the 200 µg/mL group. These results indicate that there was no significant toxicity up to 100 µg/mL (Fig. 1).

The NO production rate of the macrophages treated with Danggwisusan hot aqueous extract for the control group was 100.00 ± 0.37%, the 10 µg/mL group was 91.39 ± 2.94%, the 20 µg/mL group was 88.48 ± 3.24%, the 50 µg/mL group was 83.49 ± 3.72%, and that of the 100 µg/mL group was 76.19 ± 2.57%. The NO production rate was significantly decreased in the Danggwisusan hot aqueous extract 100 µg/mL group (Fig. 2).

The PGE_2 production rate of the macrophages treated with Danggwisusan hot aqueous extract for the control group was 100.00 ± 2.56%, the 10 µg/mL group was 98.67 ± 2.22%, the 20 µg/mL group was 94.38 ± 4.19%, the 50 µg/mL group was 91.63 ± 2.58%, and that of the 100 µg/mL group was 79.75 ± 3.85%. The PGE_2 production rate was significantly decreased in the Danggwisusan hot aqueous extract 100 µg/mL group (Fig. 2).

The IL-1β production rate of the macrophages treated with Danggwisusan hot aqueous extract for the control group was 100.00 ± 0.21%, the 10 µg/mL group was 96.91 ± 4.16%, the 20 µg/mL group was 94.36 ± 3.97%, the 50 µg/mL group was 88.14 ± 6.65%, and the 100 µg/mL group was 80.87 ± 3.14%. The IL-1β production rate was significantly decreased in the Danggwisusan hot aqueous extract 100 µg/mL group (Fig. 3).

The IL-6 production rate of the macrophages treated with Danggwisusan hot aqueous extract for the control group was 100.00 ± 0.42%, the 10 µg/mL group was 98.73 ± 6.95%, the 20 µg/mL group was 96.17 ± 2.84%, the 50 µg/mL group was 90.03 ± 6.19% and the 100 µg/mL group was 83.15 ± 2.11%. The IL-6 production rate was significantly decreased in the Danggwisusan hot aqueous extract 100 µg/mL group (Fig. 3).

The TNF-α production rate of the macrophages treated Danggwisusan with hot aqueous extract for the control group was 100.00 ± 1.38%, the 10 µg/mL group was 99.08 ± 1.07%, the 20 µg/mL group was 98.10 ± 2.62%, the 50 µg/mL group was 91.91 ± 3.41%, and the 100 µg/mL group was 79.54 ± 5.86%. The TNF-α production rate was significantly decreased in the Danggwisusan hot aqueous extract 100 µg/mL group (Fig. 3).

The DPPH free-radical scavenging ability of the macrophages treated with Danggwisusan hot aqueous extract for the 10 µg/mL group was 9.86 ± 2.51%, the 20 µg/mL group was 13.41 ± 3.86%, the 50 µg/mL group was 41.63 ± 3.59%, and the 100 µg/mL group was 51.31 ± 2.97%. The Danggwisusan hot aqueous extract provided 50% or more free-radical scavenging ability in the 100 µg/mL group (Fig. 4).

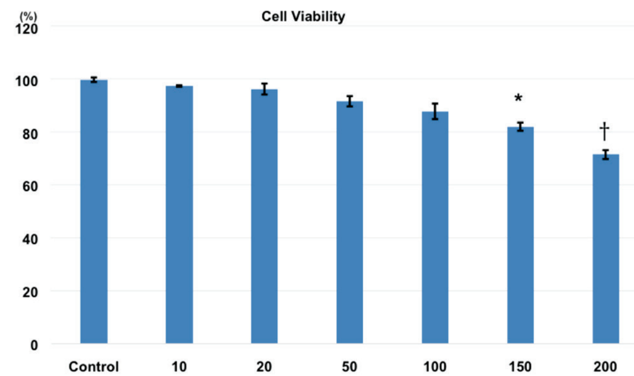

Fig. 1. Cytotoxic effects after treatment with Danggwisusan hot aqueous extract in macrophage.

10: 10 µg/mL Danggwisusan hot aqueous extract treated group
20: 20 µg/mL Danggwisusan hot aqueous extract treated group
50: 50 µg/mL Danggwisusan hot aqueous extract treated group
100: 100 µg/mL Danggwisusan hot aqueous extract treated group
150: 150 µg/mL Danggwisusan hot aqueous extract treated group
200: 200 µg/mL Danggwisusan hot aqueous extract treated group

Values are presented as mean ± SD.
*Statistically significant difference from the control group ($p<0.05$).
†Statistically significant difference from the control group ($p<0.01$).

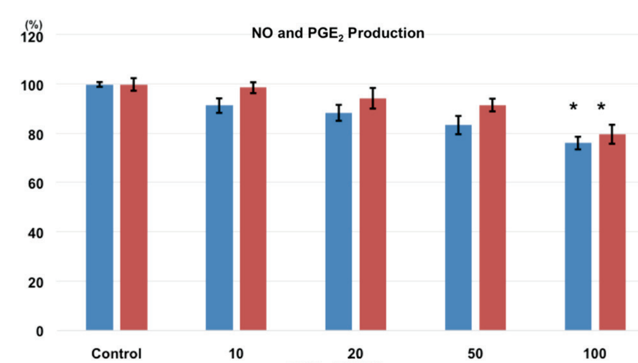

Fig. 2. NO and PGE_2 production after treatment with Danggwisusan hot aqueous extract in macrophage.

Values are presented as mean ± SD.
10: 1 µg/mL LPS and 10 µg/mL Danggwisusan hot aqueous extract treated group
20: 1 µg/mL LPS and 20 µg/mL Danggwisusan hot aqueous extract treated group
50: 1 µg/mL LPS and 50 µg/mL Danggwisusan hot aqueous extract treated group
100: 1 µg/mL LPS and 100 µg/mL Danggwisusan hot aqueous extract treated group
Control: 1 µg/mL LPS treated group

* Statistically significant difference from the control group ($p<0.05$).

LPS, lipopolysaccharide; NO, nitric oxide; PGE_2, prostaglandin E2

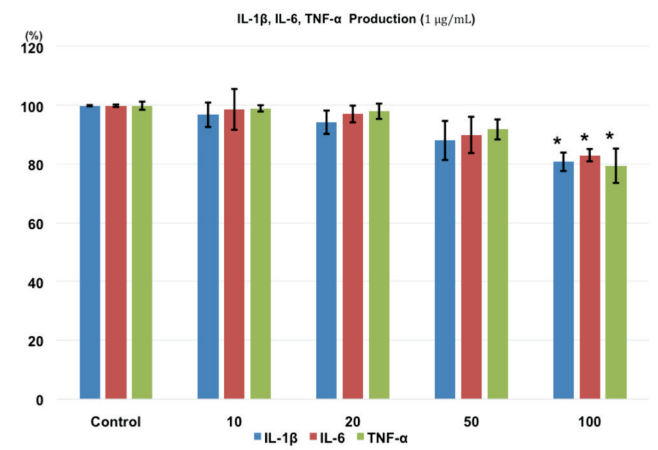

Fig. 3. IL-1β, IL-6, and TNF-α production after treatment with Danggwisusan hot aqueous extract in macrophage.

Values are presented as mean ± SD.
10: 1 μg/mL LPS and 10 μg/mL Danggwisusan hot aqueous extract treated group
20: 1 μg/mL LPS and 20 μg/mL Danggwisusan hot aqueous extract treated group
50: 1 μg/mL LPS and 50 μg/mL Danggwisusan hot aqueous extract treated group
100: 1 μg/mL LPS and 100 μg/mL Danggwisusan hot aqueous extract treated group
Control: 1 μg/mL LPS treated group
* Statistically significant difference from the control group ($p<0.05$)
IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

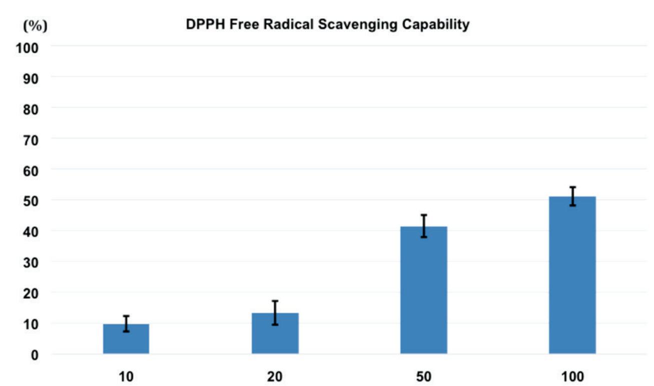

Fig. 4. DPPH Free radical scavenging after treatment with Danggwisusan hot aqueous extract.

Values are represented as mean ± SD.
10: 10 μg/mL Danggwisusan hot aqueous extract treated group
20: 20 μg/mL Danggwisusan hot aqueous extract treated group
50: 50 μg/mL Danggwisusan hot aqueous extract treated group
100: 100 μg/mL Danggwisusan hot aqueous extract treated group
DPPH, diphenyl-2-picryl hydrazyl.

Table 2. Total Polyphenoric Compounds of Danggwisusan Hot Aqueous Extract.

Compounds	Danggwisusan
TPC (mg tannic acid equivalents/ g)	26.41 ± 4.58

Danggwisusan, Danggwisusan hot aqueous extract; TPC, total polyphenoric compounds.

Table 3. Total Flavonoid Compounds of Danggwisusan Hot Aqueous Extract.

Compounds	Danggwisusan
TF (mg quercetin equivalents/ g)	9.49 ± 2.26

Danggwisusan, Danggwisusan hot aqueous extract; TF: total flavonoids.

The amount of polyphenol in Danggwisusan was 26.41 ± 4.58 mg/g (Table 2).

The amount of flavonoid in Danggwisusan was 9.49 ± 2.26 mg/g (Table 3).

Discussion

Danggwisusan on Donguibogam [13] is used when the patient complains of pain due to bruising. Thus, in Korean medicine, Danggwisusan has been used for sprain, strain, and bruises in patients. The components of Danggwisusan have been analyzed in previous studies showing that the standard substances of Danggwisusan are brazilin, safflomin, paeoniflorin, boldenone, amygdalin, coumarin, cinnamic acid, juglone and other compounds [14].

In Korean medicine, the inflammatory reaction may be considered as both competing positive and negative factors. Inflammation provides positive immunological resistance against disease. In contrast, it may also have a negative impact as immunity can cause disease [15]. In pathology, the inflammatory reaction is an important process of tissue repair and recovery of damaged tissue. This process can cause pain, edema, redness, heat, swelling and functional disability symptoms [2]. These symptoms are similar to the concepts of hemorrhage, bruising, and blood damage in Oriental medicine. Danggwisusan can be used for these symptoms.

The viability of macrophages did not significantly decrease in the 10, 20, 50, and 100 μg/mL Danggwisusan hot aqueous extracts compared with the control group.

LPS is produced by pathogenic fungus and consists of phospholipids, polysaccharides and small amounts of protein. LPS activates macrophages to produce TNF-α and plays a major role in inflammation [16]. It also promotes the production of reactive oxygen species and over-activates NO, causing inflammation and tissue damage [17]. Therefore, LPS is widely used in experimental models to study the inflammatory response [18].

NO acts as a signal transducer in the nervous system and protects against microorganisms invading from the outside [19]. However, excessive formation of NO not only promotes inflammatory reactions such as edema but also promotes inflammation by promoting the biosynthesis of inflammatory mediators [20]. In this study, NO production in LPS-stimulated macrophages was significantly inhibited in 100 μg/mL Danggwisusan hot aqueous extract compared with the control group.

Cyclooxygenase-2 (COX-2) is an enzyme that acts to speed up the production of prostaglandins that play a key role in promoting inflammation. Activation of PGE_2 is an important agonist in inflammatory responses. If PGE_2 accumulates, swelling and pain will persist [21,22].

PGE_2 production in LPS-stimulated macrophages was significantly inhibited in 100 μg/mL Danggwisusan hot aqueous extract compared with the control group.

IL-1β, IL-6, and TNF-α are proinflammatory cytokines involved in immunity. Cytokines are released from leukocytes and other cell types and are particularly important in both innate and adaptive immune responses, and play a central role in the immune system mediating and regulating immunity. Each cytokine binds to cytokine-specific cell surface receptors leading to enhanced intracellular signaling. Most immune functions can be effective when combined with various modulators. The combination of cytokines has an additive, inhibitory, or synergistic effect. In addition to the effects of other cytokines, the effects of cytokines can be suppressed or facilitated by hormone and cytokine receptor antagonism.

Cytokines play important roles in immunity, infectious diseases, hematopoietic function, tissue regeneration, and cell development and growth, induce the production of antibodies against antigens, and control and stimulate the body's defense against external invasion. A healthy immune system "remembers" external antigens and then reacts more quickly when exposed to the same antigen again. Cytokines such as interleukins (IL-1, -6, -12), IFNγ, and TNF, play a major role in regulating inflammatory responses [23]. IL-1β, IL-6, and TNF-α production in LPS-stimulated macrophages was significantly inhibited in 100 μg/mL Danggwisusan hot aqueous extract compared with the control group.

Polyphenol compounds are found widely in plants and occur in large amounts in fruits and leafy vegetables [24]. Phenolic antioxidants act by inhibiting oxidation by donating hydrogen to alkyl radicals, or alkylperoxy radicals in a chain reaction, and by removing the radicals [25]. DPPH (1,1-Diphenyl-2-picryl-hydrazyl) radical scavenging activity is a method of measuring antioxidative activity by reducing DPPH to DPPHH and reducing the absorbance by reacting with DPPH which is a free radical antioxidant [26]. The DPPH radical scavenging capability of Danggwisusan hot aqueous extract in macrophages was more than 50% at 100 μg/mL. The amount of polyphenol in Danggwisusan was 26.41 ± 4.58 mg/g. The amount of flavonoid in Danggwisusan was 9.49 ± 2.26 mg/g.

The results in this study demonstrate that Danggwisusan hot aqueous extract decreased NO and PGE_2, IL-1β, IL-6, and TNF-α production rate, and scavenging DPPH radicals at 10, 20, 50 and 100 μg/mL. Therefore, Danggwisusan hot aqueous extract is believed to have anti-inflammatory and antioxidant effects. Further studies may help to further understand safe and more effective use of Danggwisusan.

Conclusion

The results in this study suggest that Danggwisusan hot aqueous extract has the ability to suppress NO, PGE_2, and IL-1β, IL-6, and TNF-α production, and improve DPPH free-radical scavenging activity. Danggwisusan also contains polyphenol and flavonoid, which are antioxidants. Therefore, Danggwisusan hot aqueous extract may have anti-inflammatory and antioxidant activity.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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