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Original Article

Effects of *Daegangwhal-Tang* Hot Aqueous Extract on Anti-inflammation and Anti-oxidation in RAW 264.7 Macrophage

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ABSTRACT

Background: The objective of this study was to determine the effects of *Daegangwhal-Tang* (DGHT) hot aqueous extract on production of inflammatory mediators and antioxidants in RAW 264.7 macrophage.
Methods: DGHT was extracted with water, filtered, concentrated and freeze-dried to perform. Cytotoxicity of DGHT extract was performed by MTT assay. Activated macrophages were treated with varying concentrations of DGHT extract (10, 50, 100 and 200 μg/mL), and nitric oxide (NO) and prostaglandin E2 (PGE_2) concentrations were measured to detect anti-oxidative effects. Interleukin-6 (IL-6), interleukin-1 beta (IL-1ß) and tumor necrosis factor-alpha(TNF-α) concentrations were also measured to detect inflammatory responses to DGHT
Results: Cytotoxicity of DGHT extract at concentrations of 10, 50, 100 and 200 μg/mL were not observed. NO production was significantly decreased in the DGHT hot aqueous extract 200 μg/mL concentration group. PGE_2, IL-6, IL-1ß and TNF-α production was significantly decreased in the DGHT hot aqueous extract 100 and 200 μg/mL concentration groups. DGHT hot aqueous extract appeared to have DPPH free radical scavenging capability at all of concentrations, but did not exceed 50%.
Conclusion: These results suggest that DGHT hot aqueous extract has concentration-dependent anti-inflammatory and anti-oxidative effect.

Introduction

In Korean Medicine, *Daegangwhal-Tang* (DGHT) is used to treat joint pain, swelling, and movement disorders caused by the invasion of wind-dampness pathogens [1,2]. This may result from various symptoms of arthritis, suggesting that DGHT can be used for treatment [3].

Inflammation is an immune reaction in the human body in response to tissue injury or infection. Arthritis is a disease characterized by joint pain and stiffness due to inflammatory reactions when the normal tissue of the joint is damaged [4]. Arthritis can occur in any joints depending on the initial cause. In addition, the progress is slow, and early diagnosis is difficult since the onset of symptoms appears diverse. Discovery of more effective drugs may help to prevent the decline in the quality of life in affected patients. [5,6].

There are several studies which have examined the anti-inflammatory and analgesic effects of DGHT in arthritis induced in rats [7-11], but not in lipopolysaccharide(LPS) activated macrophages. Therefore, the aim of this study was to determine the effects of DGHT hot aqueous extract on the production of inflammatory [interleukin-6 (IL-6), interleukin-1 beta (IL-1ß) and tumor necrosis factor-alpha (TNF-α)], and anti-oxidative [nitric oxide(NO), prostaglandin E2 (PGE_2)], mediators and DPPH free-radical scavenging rate in LPS activated macrophage.

Materials and Methods

Preparation of DGHT extracts

The prescription of DGHT was based on *Donguibogam* [2] and is shown in Table 1. The materials of the prescription used in this study were purchased from Omniherb Company (Gyeong-buk, Korea), and the hot aqueous extraction method is as follow. The materials of DGHT 192g ware boiled in 2L of distilled water for 4 hours. The extract was filtered and centrifuged (Hanil, Korea) at

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3,000 RPM for 3 minutes. The supernatant was secondary filtered with a 0.03 mm filter (Nalgene, New York, USA) and concentrated to 100mL using a rotary evaporator (Korprotech, Korea) and frozen at -80°C. The concentrated extract was lyophilized for 7 days using a freeze dryer system (Labconco, USA) to obtain 38.42g of powdered DGHT extract (Fig. 1).

Table 1 Prescription Contents of *Daegangwhal-Tang*.

Scientific name	Dose (g)
RhizomaNotopterygii	30
RhizomaCimicifugae	30
Radix AngelicaePubescentis	20
RhizomaAtractylodis	14
Radix StephaniaeTetrandrae	14
Radix Clematidis	14
RhizomaAtractylodisMacrocephalae	14
Radix AngelicaeGigantis	14
Poria	14
RhizomaAlismatis	14
Radix Glycyrrhizae	14
Total	192

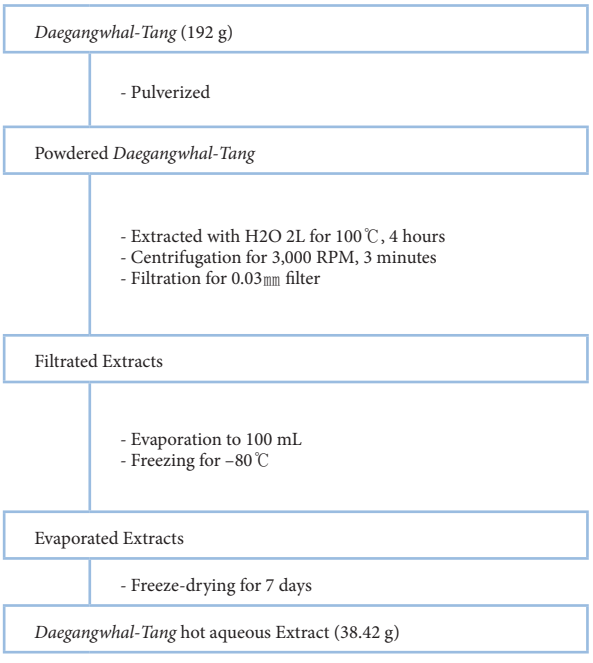

Fig. 1. Manufacturing procedure of *Daegangwhal-Tang* hot aqueous extract.

Cell culture and LPS treatment

RAW 264.7 macrophages were obtained from the Korean Cell Line Bank (Seoul, Korea) and used for experiments. The RAW 264.7 macrophage cell line was cultured in DMEM containing 10,000 units/mL penicillin and 10,000 μg/mL streptomycin in a 37°C incubator (Sanyo, Japan). 1μg of LPS was dissolved in 1mL of 1 x PBS 1mL and filtered for treatment. In each experiment, the cells were cultured for 24 hours, and incubated for 1 hour, and then treated with LPS at a concentration of 1μg/mL.

Evaluation of cytotoxicity

Cell viability was evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The stabilized cells were treated with 10, 50, 100, 200 and 400 μg/mL of DGHT extract, cultured for 24 hours, andthen MTT sample (Sigma, USA) was added and the cells were incubated for 4 hours. After removing the medium, 200 μg DMSO (Sigma, USA) was mixed and the absorbance was measured at 570 nm using a microplate reader (Labsystems, Finland).

NO measurement

The concentration of NO was measured using Griess reagent composed of 1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride and 2% phosphoric acid. The stabilized RAW 264.7 macrophages were treated with 1 μg/mL LPS and DGHT extracts at 10, 50 100 and 200 μg/mL, respectively, and cultured for 18 hours. Cultured supernatant 100 μg and Griess reagent 100 μg were mixed and measured at 540 nm using a microplate reader.

PGE_2 measurement

The concentration of PGE_2 was measured using a commercial competitive enzyme immunoassay kit (R&D, USA). RAW 264.7 macrophages were treated with 1 μg/mL LPS and DGHT extracts at 10, 50, 100 and 200 μg/mL, respectively, and cultured for 18 hours. Cultured supernatant 100 μg was loaded on a 96-well plate coated with goat anti-mouse, primary antibody solution and PGE_2 conjugate 50 μg were added and incubated overnight at 4°C. A 200 μg substrate solution was added and left to react for 15 minutes, treated with 50μg stop solution, and the absorbance was measured at 450nm using a microplate reader.

Cytokine measurement

ELISA kit (R&D, USA) was used to measure the amounts of IL-6, IL-1ß and TNF-α. RAW 264.7 macrophages were treated for 1 hour with 10, 50, 100 and 200 μg/mL DGHT, respectively, and then treated with 1 μg/mL of LPS for 18 hours. Treated solutions were loaded on a 96-well plate coated with capture antibody for IL-6, IL-1β, and TNF-α, and incubated overnight at 4°C. After washing 3 times with washing buffer, antibody reagent 100 μg was added to each well and reacted at room temperature for 1 hour. Then, it was washed 3 times again, tetramethylbenzidine (TMB) substrate 100 μg was added for 30 minutes, and stop solution 100 μg was added to stop the reaction. The absorbance was measured at 450 nm using a microplate reader.

DPPH radical scavenging measurement

DGHT extracts were diluted with methanol (Honeywell, USA) using 10, 50, 100 and 200 μg, mixed with 80 μg of 0.15mM DPPH

solution (Sigma, USA) and 96-well plate. After blocking the light at room temperature and reacting for 30 minutes, the absorbance was measured at 520 nm using a microplate reader. DPPH radical scavenging activity was calculated by subtracting the control group from the treated group, dividing by the control group, and then multiplying by 100.

Statistical analyses

Statistical analyses were performed with IBM SPSS program Ver. 22.0 (IBM Corp., USA). It was performed using Kolmogorov-Smirnov test, followed by Student's t-test. All measurements were expressed as mean ± SD, and statistical significance level was $p < 0.05$.

Result

Effect on cytotoxicity

Cell viability of the normal untreated group was 100 ± 1.52%, while cell viability of the LPS-activated group was 99.16 ± 0.52%, 98.76 ± 0.44%, 95.01 ± 0.51%, 86.60 ± 1.72%, and 65.63 ± 2.9% in the 10, 50, 100, 200, and 400 μg/mL DGHT groups, respectively. There was no significant cytotoxicity in the 10, 50, 100, and 200 μg/mL DGHT treated groups (Fig. 2).

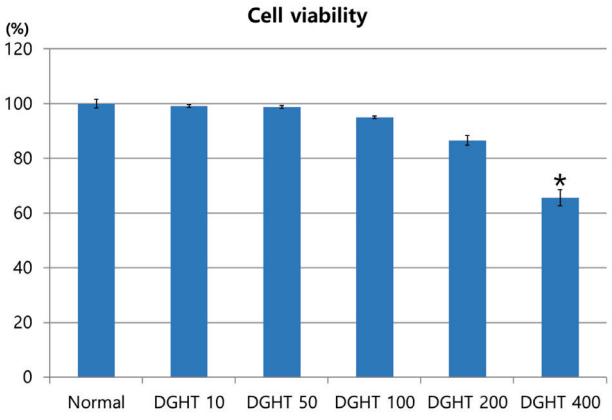

Fig.2. Effect of *Daegangwhal-Tang* hot aqueous extraction the viability of RAW 264.7 macrophage.
Normal: Non-treated.
DGHT 10: *Daegangwhal-Tang* Hot Aqueous Extract 10 μg/mL treated.
DGHT 50: *Daegangwhal-Tang* Hot Aqueous Extract 50 μg/mL treated.
DGHT 100: *Daegangwhal-Tang* Hot Aqueous Extract 100 μg/mL treated.
DGHT 200: *Daegangwhal-Tang* Hot Aqueous Extract 200 μg/mL treated.
DGHT 400: *Daegangwhal-Tang* Hot Aqueous Extract 400μg/mL treated.
* Statistically significant difference from the Control group, as determined by the Student's t-test $p < 0.05$.

Effect on NO and PGE_2 production

NO production was found to be 100 ± 0.68% in LPS-activated alone group (control group), compared with 98.07 ± 1.83%, 95.75 ± 1.41%, 84.56 ± 1.60%, and 73.86 ± 1.63% in the 10, 50, 100, and 200 μg/mL DGHT treated groups was observed. There was a significant decrease of NO production in the 200 μg/mL DGHT treated group (Fig. 3).

PGE_2 production was found to be 100 ± 1.63% in the control group, compared with 98.43 ± 0.53%, 94.14 ± 1.23%, 80.31 ± 0.71%, and 72.71 ± 0.71% in the 10, 50, 100, and 200 μg/mL DGHT treated groups. There was significant decrease of NO production in the 100 and 200 μg/mL DGHT treated groups (Fig. 3).

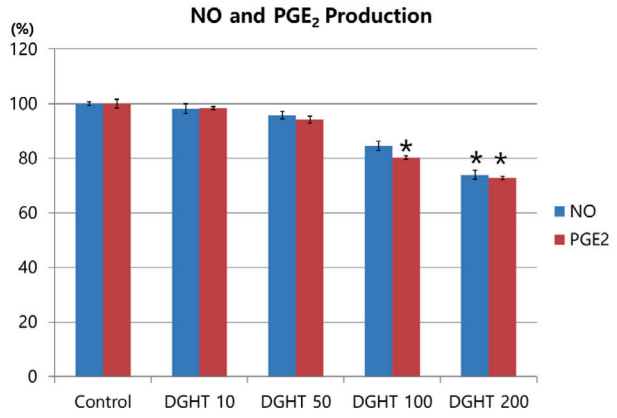

Fig. 3. Effect of *Daegangwhal-Tang* hot aqueous extracton the NO and PGE2 production in RAW 264.7 macrophage.
Control: 1 μg/mL LPS treated.
DGHT 10: 1 μg/mL LPS + *Daegangwhal-Tang* Hot Aqueous Extract 10 μg/mL treated.
DGHT 50: 1 μg/mL LPS + *Daegangwhal-Tang* Hot Aqueous Extract 50 μg/mL treated.
DGHT 100: 1 μg/mL LPS + *Daegangwhal-Tang* Hot Aqueous Extract 100 μg/mL treated.
DGHT 200: 1 μg/mL LPS + *Daegangwhal-Tang* Hot Aqueous Extract 200 μg/mL treated.
* Statistically significant difference from the Control group, as determined by the Student's t-test as $p < 0.05$.

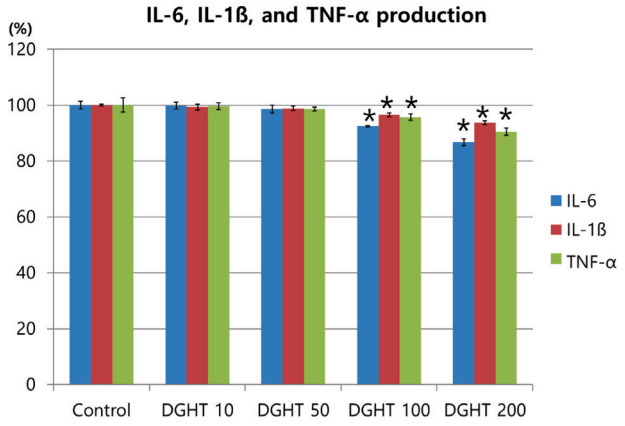

Fig. 4. Effect of *Daegangwhal-Tang* Hot Aqueous Extracton IL-6, IL-1ß,and TNF-α production in RAW 264.7 macrophage.
Control: 1 μg/mL LPS treated.
DGHT 10: 1 μg/mL LPS + *Daegangwhal-Tang* Hot Aqueous Extract 10 μg/mL treated.
DGHT 50: 1 μg/mL LPS + *Daegangwhal-Tang* Hot Aqueous Extract 50 μg/mL treated.
DGHT 100: 1 μg/mL LPS + *Daegangwhal-Tang* Hot Aqueous Extract 100 μg/mL treated.
DGHT 200: 1 μg/mL LPS + *Daegangwhal-Tang* Hot Aqueous Extract 200 μg/mL treated.
* Statistically significant difference from the Control group, as determined by the Student's t-test as $p < 0.05$.

Effect on IL-6, IL-1ß, and TNF-α production

IL-6 production was found to be 100 ± 1.33% in the LPS-activated control group, compared with 98.78 ± 1.26%, 98.59 ± 1.37%, 92.41 ± 0.20%, and 86.74 ± 1.25% in the 10, 50, 100, and 200 μg/mL DGHT treated groups. There was a significant decrease in NO production in the 100 and 200 μg/mL DGHT treated groups (Fig. 4).

IL-1ß production was found to be 100 ± 0.28% in the LPS-activated control group, compared with 99.25 ± 1.05%, 98.80 ± 0.81%, 96.48 ± 0.68%, and 93.74 ± 0.70% in the 10, 50, 100, and 200 μg/mL DGHT treated groups. There was a significant decrease of NO production in the 100 and 200 μg/mL DGHT treated groups (Fig. 4).

TNF-α production was found to be 100 ± 2.53% in the control group, compared with 99.60 ± 1.26%, 98.58 ± 0.76%, 95.69 ± 1.14%, and 90.42 ± 1.28% in the 10, 50, 100, and 200 μg/mL DGHT treated groups. There was a significant decrease in NO production in the 100 and 200 μg/mL DGHT treated groups (Fig. 4).

Effect on DPPH radical scavenging rate

The DPPH radical scavenging rate was found to be 3.96 ± 1.79%, 13.93 ± 0.74%, 31.46 ± 1.24%, and 49.26 ± 0.86% in the 10, 50, 100, and 200 μg/mL DGHT treated groups. There was DPPH free radical scavenging capability at all of concentrations, but this did not exceed 50% (Fig. 5).

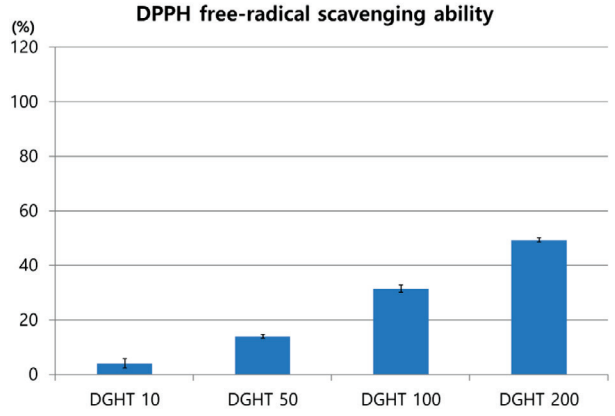

Fig. 5. Effect of *Daegangwhal-Tang* hot aqueous extracton DPPH radical scavenging rate in RAW 264.7 macrophage.
DGHT 10: 1 μg/mL LPS + *Daegangwhal-Tang* Hot Aqueous Extract 10 μg/mL treated.
DGHT 50: 1 μg/mL LPS + *Daegangwhal-Tang* Hot Aqueous Extract 50 μg/mL treated.
DGHT 100: 1 μg/mL LPS + *Daegangwhal-Tang* Hot Aqueous Extract 100 μg/mL treated.
DGHT 200: 1 μg/mL LPS + *Daegangwhal-Tang* Hot Aqueous Extract 200 μg/mL treated.

Discussion

Homeostasis is maintained within joints by chondrocytes which balance between damage and reconstruction by synthesizing and decomposing extracellular matrix. When this process becomes imbalanced, arthritis occurs, leading to an accumulation of inflammatory mediators, cytokines, growth factors, and enzymes.

NO and PGE_2 also play key roles in the damage and reconstruction of cartilage through interactions with inflammatory mediators. NO is released by chondrocytes, along with matrix metalloproteinase(MMP) and production of IL-1β. Furthermore, it discourages proteoglycan and collagen production, and acts as an inhibitor of recovery since proteoglycan and collagen are a key in the formation of cartilage [12]. PGE_2 is produced by cyclooxygenase-2(COX-2) and PGE_2 synthase enzyme [13].

IL-6, IL-1ß, and TNF-α are known to be cytokines that cause catabolism and are mainly expressed by chondrocytes in patients with degenerative osteoarthritis. Cytokines expressed through various pathways in activated chondrocytes, synovial cells, and monocytes induce matrix metalloproteinase, aggrecanase, inducible NOS, and cyclooxygenase-2, inhibit tissue inhibitor of metallo-proteinase. Consequently, it causes inflammation [14].

Damage of joins and persistence of inflammation are also associated with disorder of the anti-oxidative process. Anti-oxidative dysfunction is caused by oxygen free radical overproduction via macrophage, leukocyte, and prostaglandin pathways, and playing a major role in inflammatory reactions [15]. For that reason, many herbal medicine extracts have been studied using inflammatory mediators and cytokines to determine if the herbal medicine has anti-inflammatory and anti-oxidative properties [16-21].

There is a slight difference in composition and dosage of DGHT in the literature, with the prescription of DGHT based on *Donguibogam* [2] in this study. Composition of DGHT consists of *Rhizoma Notopterygii, Rhizoma Cimicifugae, Radix Angelicae Pubescentis, Rhizoma Atractylodis, Radix Stephaniae Tetrandrae, Radix Clematidis, Rhizoma Atractylodis Macrocephalae, Radix Angelicae Gigantix, Poria, Rhizoma Alismatis,* and *Radix Glycyrrhizae* [2,3]. It has been widely used for arthritis treatment in clinical practice due to the invasion of wind-dampness pathogens [3,22].

Previous studies about DGHT resulted in arthritis caused by collagen and carrageenan in rats and oral administration of DGHT to alleviate progression of arthritis [9,10]. Also, it has been reported that DGHT has an inhibitory effect on IgG anti-collagen antibody [7], analgesic effects in chronic knee arthritis models [8], and anti-oxidative and immunomodulatory effects [11]. Taken together, these results support the proposal.

DGHT has anti-inflammatory and anti-oxidative effects on damage and recovery mechanisms in inflammatory joints.

Conclusion

The results in this study showed that a significant decrease in cell viability was observed at 400 μg/mL, but not in the treatment groups of 10, 50, 100, and 200 μg/mL DGHT.

NO production was decreased in a concentration-dependent manner for all concentrations of DGHT extracts, and significantly at a concentration of 200 μg/mL.

PGE_2, IL-6, IL-1β, and TNF-α decreased in a concentration-dependent manner for all concentrations of DGHT extract, and significantly at concentrations of 100 and 200 μg/mL.

DGHT extract appeared to offer DPPH free radical scavenging capability at all of the concentrations analyzed, but not exceeding 50%.

These results suggest that DGHT hot aqueous extract has anti-inflammatory and anti-oxidative effects, by inhibiting inflammatory mediators and cytokine production. DGHT extracts decrease NO, PGE_2, IL-6, IL-1β, and TNF-α production in a concentration-dependent manner. However, in this study, only hot aqueous extraction was used. Further studies should be conducted using different extraction methods and various concentrations to accumulate further evidence to support the use of DGHT for inflammatory conditions.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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