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Research Article

Anti-inflammatory property of hydrogen-rich *Gynostemma pentaphyllum* Makino distillate

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Abstract
Hydrogen-rich *Gynostemma pentaphyllum* Makino distillate (HRGD) is produced by distilling *G. pentaphyllum* Makino, with the addition of hydrogen gas. This study sought to explore the pharmacological and biological impacts of HRGD on the generation of pro-inflammatory cytokines and mediators in macrophages. The cells were treated with various concentrations of HRGD (2, 4 and 8 µg/mL) in the absence or presence of lipopolysaccharide (LPS) (1 µg/mL) for 24 h. The results indicated that HRGD is an effective inhibitor of LPS-induced nitric oxide ($NO^{\bullet}$) production in RAW 264.7 cells: The $NO^{\bullet}$ concentration was reduced dramatically after treatment with 8 µg/m98L HRGD (11.8 µM) compared to the LPS-induced group (28.4 µM). These inhibitory effects of HRGD included a dose-dependent decrease in the expression of cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) proteins. To evaluate the inhibitory effects of HRGD on other cytokines, we also measured cytokines tumor necrosis factor-α (TNF-α) level in the cell supernatants of LPS-stimulated RAW 264.7 macrophages by enzyme-linked immunosorbent assay. In this assay, HRGD significantly decreased the expression of TNF-α in a dose-dependent manner ($p < 0.05$). The study also performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on macrophages. HRGD did not display any cytotoxicity in this assay. The findings demonstrated that HRGD could modulate macrophage-mediated inflammatory functions such as the overproduction of cytokines, $NO^{\bullet}$ and PGE_2 without any cytotoxic.

Keywords: Inflammation, Hydrogen-rich *Gynostemma pentaphyllum* Makino distillate, Cytokines, RAW 264.7 macrophage cells

INTRODUCTION

Inflammation plays a key role in producing and regulating pro-inflammatory cytokines like nitric oxide, prostaglandins, and TNF-α in macrophages. Nitric oxide ($NO^{\bullet}$), an inflammatory mediator, is produced by nitric oxide synthases (NOS), with inducible NOS (iNOS) promoting inflammation by generating $NO^{\bullet}$ in macrophages, which can lead to tissue injury, genetic alterations, and nerve damage (Pradhan *et al*., 2024; Gupta *et al*., 2024). Cyclooxygenase-2 (COX-2) further promotes prostaglandin synthesis and $NO^{\bullet}$ production. While inflammation is a natural defense mechanism, persistent or chronic inflammation can contribute to diseases such as rheumatoid arthritis, arteriosclerosis, gastritis, and asthma (Gupta *et al*., 2024; Hou *et al*., 2016; Baito *et al*., 2023). Drugs such as non-steroidal *anti-inflammatory drugs* (NSAIDs) and corticosteroids are commonly employed to manage inflammation, while NSAIDs alleviate pain, fever, and inflammation, making them useful for treating various inflammatory conditions (Kaur *et al*., 2022). NSAIDs are a class of drugs that inhibit COX enzymes, which typically activate several processes that lead to pain, fever, and inflammation. By blocking these enzymes, NSAIDs reduce pain and inflammation (Kaur *et al*., 2022). However, these drugs have side effects and in some cases, they are irreversible. Plants can be used in their raw extract form to address inflammatory diseases or specific anti-inflammatory compounds can be extracted and used to treat inflammation (Kaur *et al*., 2022).

Gynostemma pentaphyllum (Thunb.) Makino has been widely used in traditional herbal medicine in Asian countries and exhibits a variety of pharmacological properties, including anti-inflammatory, antioxidative, antidiabetic, lipid metabolism regulatory and neuroprotective activities (Huang *et al*., 2021; Nguyen *et al*., 2021; Liu *et al*., 2021; Wang *et al*., 2019; Weng *et al*., 2021). This herb is also widely used as a health supplement in beverages, biscuits, noodles, face washes and bath oils (Huang *et al*., 2021; Nguyen *et al*., 2021; Liu *et al*., 2021; Wang *et al*., 2019; Weng *et al*., 2021).

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Extracting medicinal plants involves isolating bioactive compounds or secondary metabolites. The method chosen for extraction depends on factors such as the type of plant material, solvent properties, pH, temperature, and solvent-to-sample ratio, as well as the intended use of the final extract (Liu *et al*., 2021). Steam distillation is a commonly employed method for extracting natural products from plant materials (Gil *et al*., 2023). It is an effective technique that consumes less energy, preserves the quantity and quality of bioactive compounds, and allows for easy absorption by the skin and mucous membranes of the body (Gil *et al*., 2023; Russo *et al*., 2023).

Hydrogen has emerged as a novel antioxidant material that can mitigate cellular damage by selectively scavenging strong oxidants such as hydroxyl radicals. Drinking hydrogen-rich water has been shown to have positive effects in the treatment of several conditions, including metabolic syndrome, rheumatoid arthritis, chronic hepatitis B, Parkinson's disease, and cancer (Asgharzadeh *et al*., 2022; Ichihara *et al*., 2021; Song *et al*., 2022). The US Food and Drug Administration has recently recognized hydrogen gas as a safe food additive when used in drinking water or beverages (bulut *et al*., 2023). Despite the increasing evidence supporting the benefits of hydrogen, limited studies have been conducted on its effects. Furthermore, the systemic effect of *Gynostemma pentaphyllum* (Thunb.) Makino manufactured through steam distillation remains unclear. Therefore, this study aimed to examine the anti-inflammatory capacity of hydrogen-rich *G. pentaphyllum* (Thunb.) Makino distillate (HRGD).

MATERIALS AND METHODS

Preparation of hydrogen-rich *G. pentaphyllum* (Thunb.) Makino distillate (HRGD)

The HRGD sample was obtained from Youngmul Company (Jeju, Korea), washed thoroughly with water, and then dried overnight. The dried *G. pentaphyllum* (Thunb.) Makino was immersed in water and subjected to low-temperature vacuum extraction during distillation (Altieri *et al*., 2022). The first distillate was filtered to eliminate precipitates, infused with hydrogen gas (400 ppb), and then concentrated using a rotary evaporator (Buchi Rotavapor R-200, New Castle, DE, US). Afterwards, the extracts were freeze-dried by lyophilization and dissolved in dimethylsulfoxide (DMSO) at a concentration of 500 mg/mL until they were used for experimentation (Kim and Kim, 2023).

Cell culture

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing L-glutamine, supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% heat-inactivated fetal calf serum (Cambrex, Walkersville, MD). Cell cultures were maintained in a 5% CO_2 incubator with 70% humidity at 37 °C. Upon confluence, the medium and nonadherent cells were discarded and replaced with fresh culture medium. After another 24 hours, cells were harvested by gently scraping with a rubber scraper, washed three times, counted for viability, and reseeded into culture plates. They incubated for at least 2 h to allow them to adhere to the plates. After washing three times with medium, LPS (Sigma) at a concentration of 1 µg/mL was added and the cells were cultured for the indicated times (Mo *et al*., 2022).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay

Cytotoxicity in RAW 264.7 murine macrophages was measured using MTT assay in 96-well plates. Cellular toxicity of various inhibitors was evaluated using the MTT assay, based on the conversion of MTT to formazan by mitochondrial dehydrogenases (Mosmann *et al*., 1983). RAW 264.7 cells were seeded at 5×10^3 cells per well in 100 µL of medium in 96-well plates. After 24 hours, the cells were treated with HRGD at concentrations ranging from 0 to 8 µg/mL. The cells were then incubated with 10 µL of MTT (5 mg/mL) at 37 ºC in the dark for 4 hours. The resulting formazan crystals were dissolved by adding 100 µL of DMSO to each well, and the absorbance was measured at 540 nm after overnight incubation at 37 ºC using a Spectra MR microplate reader. Cell viability was expressed as a percentage of the control value. Non-toxic HRGD concentrations, which did not significantly affect the viability of RAW 264.7 cells (greater than 90% viability), were selected to assess anti-inflammatory effects (Lee and Park, 2016).

Measurement of nitric oxide ($NO^{\bullet}$) production

The accumulation of nitrite, a stable byproduct of $NO^{\bullet}$, in the culture medium was used as a marker of LPS-induced $NO^{\bullet}$ production in RAW 264.7 cells (Shih *et al*., 2010). $NO^{\bullet}$ production was assessed by measuring nitrite in media fractions by the Griess reaction. Subcultured cells were seeded at a density of 15×10^4 cells per well in a 48-well plate with DMEM containing 10% fetal bovine serum (FBS), then incubated for 24 hours in a 5% CO□, humidified atmosphere at 37 ºC. After 24 hours, cells were treated with HRGD, with or without LPS, at non-toxic concentrations (determined from the MTT assay) in phenol red-free DMEM with 10% FBS and further incubated. The optical density at 540 nm was measured using a Spectra Max 250 ELISA Reader (Molecular Devices, USA), and nitrite levels were determined from a standard curve derived from serial dilutions of $NaNO_2$ (Sigma) in the culture medium. An LPS-

untreated control was included to verify macrophage activation by LPS, while the LPS-treated control was compared to the effects of HRGD.

Determination of cyclooxygenase-2 (COX-2), prostaglandin E_2 (PGE_2) and tumor necrosis factor-α (TNF-α)

RAW 264.7 macrophages were seeded into 6-well plates and stimulated with lipopolysaccharide (LPS) (1 µg/mL) in the presence or absence of various concentrations of HRGD at concentrations ranging from 2 to 8 µg/mL for 24 h. The levels of COX-2, PGE_2 and TNF-α in cell culture supernatant were assayed by a commercially available ELISA kit (Mouse COX-2 ELISA kit, abcam; Mouse PGE_2 ELISA kit, Abcam; Mouse TNF-α ELISA kit, R&D systems, respectively); as per the manufacturer's guidelines. All controls and standards were performed in triplicate, with optical density readings taken using an ELISA reader (Molecular Devices, USA). The results were calculated using standard curves generated by the standards provided by the manufacturer (Mohammadi *et al*., 2024).

Statistics analysis

Results are shown as the mean of three independent experiments ± standard deviation (SD). A Mann-Whitney U test was used to assess statistical significance between groups in SPSS version 12.0. A p-value of less than 0.05 was considered statistically significant, representing 95% confidence.

RESULTS AND DISCUSSION

Cell viability

A murine RAW264.7 macrophage in vitro model was used to evaluate the anti-inflammatory effects of HRGD. The MTT assay is commonly employed to study cell proliferation and viability *in vitro*. RAW 264.7 macrophages treated with HRGD at different concentrations (0, 2, 4 and 8 µg/mL) in the absence or presence of LPS for 24 h. as shown in Fig. 1, did not show toxicity to the cells.

Additionally, alterations in the morphology of RAW 264.7 cells were noted in the control, LPS-treated, and HRGD-treated groups at various concentrations. LPS treatment induced morphological changes, confirming the establishment of the inflammation model. In contrast, co-treatment with HRGD reduced the irregular morphological changes, suggesting its potential to modulate cell inflammation. Therefore, HRGD at 2-8 µg/mL was used in subsequent experiments.

Effects of HRGD on LPS-stimulated $NO^{\bullet}$ production in RAW 264.7 macrophages

Next, the present study measured LPS-induced $NO^{\bullet}$ release by employing a Griess reagent. LPS increases the levels of pro-inflammatory cytokines such as $NO^{\bullet}$ and TNF-α in macrophages (Willeaume *et al*., 1995; Khan *et al*., 2022). It also induces diverse disease-related inflammatory responses. LPS-stimulated RAW 264.7 cells were treated with HRGD, and then $NO^{\bullet}$ production was measured in LPS-stimulated RAW 264.7 macrophages. LPS-induced $NO^{\bullet}$ production was 28.4 ± 0.14 µM in the LPS-treated group compared to the untreated group (Fig. 2).

However, the amount of $NO^{\bullet}$ after treatment with HRGD reduced dramatically in a dose-dependent way compared to the LPS-induced group (28.4 ± 0.14 µM). The $NO^{\bullet}$ concentrations were 22.7, 19.9 and 11.8 µM.

$NO^{\bullet}$ is an essential mediator in inflammation, with excessive $NO^{\bullet}$ production occurring during both acute and chronic inflammatory responses. $NO^{\bullet}$ is generated from L-arginine by NOS isoenzymes, particularly iNOS, which is predominantly expressed in activated macrophages (Janeway *et al*., 2002).

Suppression of HRGD on LPS-activated COX-2, PGE_2 and TNF-α activation and $NO^{\bullet}$ production in RAW 264.7 macrophages

On the inflammatory mediators COX-2, PGE_2 and TNF-α, the anti-inflammatory activities of HRGD were tested. COX-2 is a well-known enzyme that can catalyze the generation of prostanoids such as PGE_2. PGE_2 is involved in many diseases, particularly inflammation-related (Yang *et al*., 2021). $NO^{\bullet}$ plays a role in inflammatory diseases. Specifically, PGE2 production is initiated during the pre-inflammatory phase, contributing to pain, fever, and vasodilation (Yang *et al*., 2021). Since COX-2 and PGE_2 are the important inflammatory mediators (Yan *et al*., 2021), the effects of HRGD on the LPS-induced release of COX-2 and PGE_2 from RAW264.7 cells were measured by the ELISA kit (Table 1).

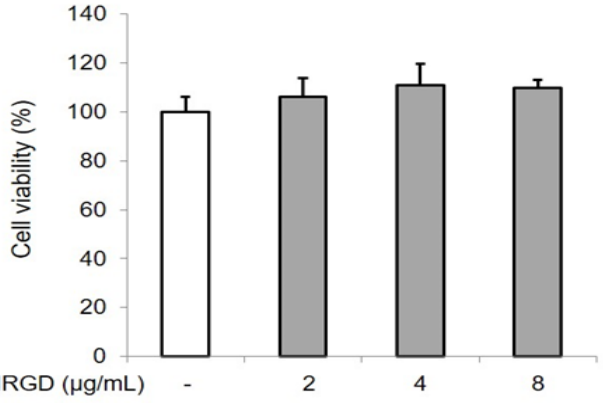

Fig. 1. *Effect of HRGD on cell viability of RAW 264.7 macrophages. RAW 264.7 macrophages treated with HRGD (2, 4 and 8 µg/mL) for 24 h, and then the cell viability was measured by MTT assay. Data are expressed as the Mean ± SD of three independent experiments*

Table 1. Inhibitory effects of HRGD on production of COX-2, PGE_2 and TNF-α in the LPS-stimulated RAW 264.7 macropages

	COX-2 (ng/mL)	PGE_2 (ng/mL)	TNF-α (ng/mL)
Untreated control	1.44 ± 0.088	0.56 ± 0.038	8.6 ± 0.14
LPS (1 μg/mL)	3.72 ± 0.174*	3.60 ± 0.008*	35.5 ± 2.28*
+2 μg/mL HRGD	3.73 ± 0.087*	0.98 ± 0.047$^{*,\#}$	34.6 ± 1.01*
+4 μg/mL HRGD	3.18 ± 0.156*	0.67 ± 0.020$^{\#}$	31.7 ± 0.74*
+8 μg/mL HRGD	1.68 ± 0.099$^{\#}$	0.56 ± 0.026$^{\#}$	26.6 ± 0.24$^{*,\#}$

Value means ± SD (n = 3); $^{*}p < 0.05$ is considered statistically significant compared to untreated control. $^{\#}p < 0.05$ is considered statistically significant compared to LPS. HRGD: Hydrogen-rich *Gynostemma pentaphyllum* Makino distillate; LPS: lipopolysaccharide

Treatment with LPS (1 μg/mL) enhanced COX-2 and PGE_2 secretion levels, and was notably reduced by HRGD (2, 4 and 8 μg/mL) in a dose-dependent manner in LPS-induced RAW 264.7 macrophages ($p < 0.05$) (Table 1). We treated LPS-stimulated RAW 264.7 macrophage cells with HRGD and observed its inhibitory activity on TNF-α production. When LPS-stimulated RAW 264.7 cells were treated with HRGD at a concentration of 1 μg/mL, LPS-induced TNF-α production was attenuated in a dose-dependent way when cells were co-treated with HRGD ($p < 0.05$) (Table 1). TNF-α is a cytokine with diverse effects on different cell types. It is a key regulator of inflammatory responses and plays a significant role in developing certain inflammatory and autoimmune disorders (Jang *et al*., 2021).

Gynostemma pentaphyllum (Thunb.) Makino has been widely used as an herbal medicine in Asian countries. Previous studies have demonstrated that long-term treatment with *G. pentaphyllum* Makino does not induce *in vivo* toxicity (Wong *et al*., 2017), and accumulating evidence has suggested the beneficial effects of *G. pentaphyllum* Makino in a wide range of chronic diseases (Attawish *et al*., 2004; Li *et al*., 2016). In particular, the strong reactive oxidative species (ROS) scavenging activity of *G. pentaphyllum* Makino is thought to be important for its activity (Megalli *et al*., 2021). This may potentially exhibit anti-inflammatory effects through the suppression of inflammatory mediators. The above results implied that HRGD can suppress the inflammatory stimulus response by reducing COX-2, PGE_2 and TNF-α levels.

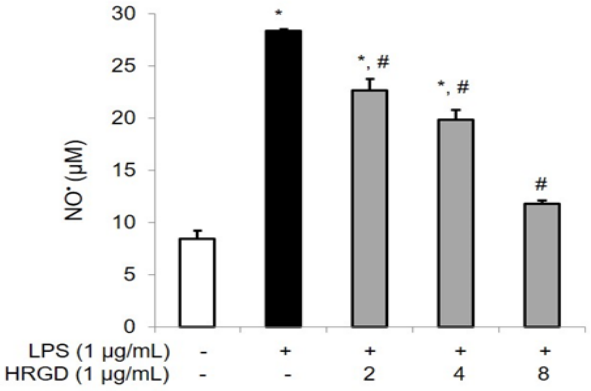

Fig. 2. *Effect of HRGD on LPS-induced NO• levels in RAW 264.7 macrophages. RAW 264.7 cells were treated with HRGD (2, 4 and 8 μg/mL) and LPS (1 μg/mL) for 24 h. production, determined in the culture supernatant by using the Griess reagent. Results are expressed as the Mean ± SD of three independent experiments. $^{*}p < 0.05$ is considered statistically significant compared to untreated control; $^{\#}p < 0.05$ is considered statistically significant compared to LPS. HRGD: Hydrogen-rich Gynostemma pentaphyllum Makino distillate. LPS: lipopolysaccharide*

Conclusion

The primary objective of this study was to explore the mechanisms responsible for the anti-inflammatory effects of HRGD in LPS-induced RAW 264.7 cells. The results indicated that HRGD effectively reduced the production of NO•, PGE2, and the pro-inflammatory cytokine TNF-α, along with the expression of COX-2 protein in LPS-activated RAW264.7 macrophages. These results imply that HRGD should be considered a candidate potential for anti-inflammatory agents for treating inflammation-related diseases. Further molecular studies are underway to elucidate the mechanisms underlying the anti-inflammatory properties of HRGD.

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Conflict of interests

The authors declare that he has no conflict of interest.

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