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ORIGINAL ARTICLE

Immunostimulatory Effects of Complex GPB through Increased TNF-α Expression and NK Cell Activation

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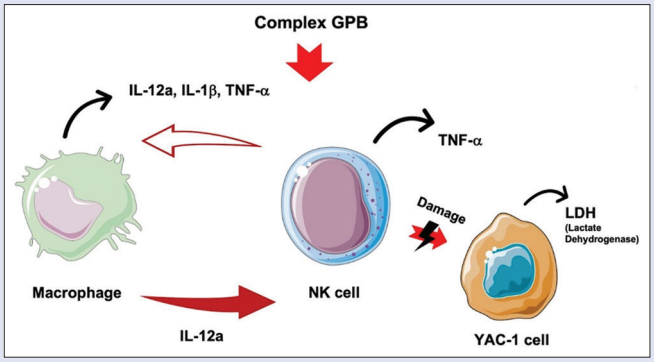
ABSTRACT

Background: Complex Great Powerful Blend (GPB) (a great powerful blend) is a hot water extract of three well-known dried fruits: goji berry, mulberry, and jujube. These fruits have been used as food for a long time, and this article sought to confirm the immunostimulatory effects of Complex GPB. **Materials and Methods:** To do this, *in vitro* study using RAW264.7 cells and *in vivo* using normal mice were performed. Cytokine production and NK cell activity levels were measured by enzyme-linked immunosorbent assay, real-time polymerase chain reaction (PCR), and the YAC-1 cell-killing assay. Transcription factor activation levels were also determined by western blotting analysis. **Results:** First, it was confirmed that Complex GPB had no cytotoxic effects on the cells and increased the production levels of tumor necrosis factor (TNF)-α in a concentration-dependent manner. Additionally, Complex GPB enhanced pro-inflammatory cytokine expressions such as TNF-α, IL-12a, and IL-1β. Western blotting analysis revealed that activation of the NF-κB and AP-1 pathways are involved. Furthermore, *in vivo* study confirmed that TNF-α was found to be elevated in the serum and splenocytes of mice-fed Complex GPB. Cytotoxic activation of NK cells was also increased in mice injected with Complex GPB. **Conclusion:** These results suggest that Complex GPB has an immune-enhancing effect, which can be used for immunomodulatory remedies.

Key words: Complex GPB, immune system, immunostimulatory effects, natural killer cells, tumor necrosis factor-α

SUMMARY

- Complex GPB increased the expression level of TNF-α *in vitro* by activating NF-κB and AP-1 pathways when administered to RAW264.7 cells, which are murine macrophages.
- Complex GPB plays a role in enhancing immune responses by releasing pro-inflammatory cytokines such as IL-12a, IL-1β, and TNF-α that act in activating macrophages and NK cells.
- Complex GPB did not show toxicity, and also increased the production of TNF-α and the level of activity of NK cells in mice.



Abbreviations used: TNF-α: Tumor necrosis factor-α; ; NK cells: Natural killer cells; MHC II: major histocompatibility complex II; IL: Interleukin; PBS: Phosphate buffered saline; FBS: Fetal bovine serum; ELISA: Enzyme-linked immunosorbent assay; HPLC: High-performance liquid chromatography.

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INTRODUCTION

The immune system defends the host against various pathogens and stressors. There are two types of immunological responses: innate and adaptive immune responses.[1] Innate immunity is the natural immunity the body has before being exposed to infectious agents and thus is always activated or can be activated immediately after infection.[2] However, since cells involved in this system exist naturally, they do not have antigen specificity or immune memory. Pattern recognition receptors can activate them by identifying pathogen-associated molecular patterns from pathogens.[3] Furthermore, they can rapidly respond to infection through phagocytosis, cytotoxicity, and inflammatory responses by dendritic cells, natural killer (NK) cells, neutrophils, and macrophages.[4]

Macrophages, a well-known cell type in innate immunity, are activated by a variety of infectious stimuli. Activated macrophages increase phagocytic activity to stimulate the uptake and degradation of infectious agents and secrete various inflammatory mediators.[5-7] In addition, they promote the expression of the major histocompatibility complex (MHC) II on the cell surface and induce the coordination of innate immunity and antigen-specific adaptive immunity by presenting antigens to T helper cells.[8]

When adaptive immunity is activated, NK cells recognize and lyse infected cells to effectively remove infectious agents at an early stage.[9] NK cells can differentiate between infected and uninfected host cells

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and induce apoptosis by targeting infected cells.[10] Additionally, NK cells have the major function of secreting pro-inflammatory cytokines. Representative examples include the cytokines interleukin (IL)-12 and tumor necrosis factor (TNF)-α, which trigger macrophage and NK cell activation.[11]

There is growing evidence that bioactive compounds obtained from natural products derived from edible plants have made a substantial contribution to pharmaceutical properties since they are generally harmless and have few adverse effects.[12,13] Therefore, providing scientific evidence for the activity of plants that are frequently utilized in traditional medicines could lead to the discovery of more effective and safer remedies.[14-17] In the last few years, customer demand for the beneficial properties of various kinds of berries, along with numerous scientific publications has rapidly increased reflecting the current interest in the qualities of berry-type fruits.[13,18] Its consumption has been reported to display a beneficial effect on human health including the immune system.

Complex Great Powerful Blend (GPB) is a hot water extract of three well-known dried fruits: goji berry, mulberry, and jujube. All three fruits are well-known foods, including the most widely consumed berry-type fruits, and each of them has medicinal properties.[19,20] Numerous reports suggest that consuming *Lycium barbarum* (goji berry), a traditional Chinese medicinal herb, that is consumed in various forms as popular nutraceutical meals or health dietary supplements, may exert essential biological activities such as prevention of cancer cell proliferation, improvement of metabolism, blood circulation, and immune response.[21-24] *Zizyphus jujuba* Mill., generally known as jujube, is a traditional and nutritious food consumed all over the world. Jujube also has antioxidant, anti-cancer, and immune-enhancing properties as a Chinese medicinal herb.[15,25] Meanwhile, mulberry (*Morus alba*) fruits, which are also widely consumed in various forms, also have been traditionally used in traditional medicine, consisting of several biologically active components that possess biological activities, including immunological effects.[26-28] The presence of bioactive components in these fruits, including alkaloids and flavonoids, is associated with bioactivities that are beneficial for health.[29,30] Several studies have revealed that mixing bioactive components may have a greater effect compared with compounds used individually.[15,25] However, there is little evidence on the efficacy of this mixture (Complex GPB), and in this paper, we aimed to confirm the immunity enhancement effects of Complex GPB. As mentioned above, these were measured based on the levels of TNF-α expression and NK cell activation *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials and reagents

RAW264.7 cells (ATCC number TIB-71) were received from the American Type Culture Collection (Rockville, MD, USA), and YAC-1 (KCLB number 40160) cells were received from the Korean Cell Line Bank (Seoul, Korea). Phosphate-buffered saline (PBS), Roswell Park Memorial Institute (RPMI) 1640 medium, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). A TNF-α enzyme-linked immunosorbent assay (ELISA) kit (Quantikine™ ELISA, Cat No.: MTA00B) was acquired from R&D Systems (Minneapolis, MN, USA). The NK Cell Isolation Kit (mouse), buffer (2 mM EDTA, 0.5% BSA in PBS, pH 7.2), and autoMACS® Rinsing Solution (2 mM EDTA in PBS, pH 7.2) were acquired from Miltenyi Biotec (Bergisch Gladbach, Germany). Antibodies for total forms and phospho-specific antibodies against p65 and c-Jun were acquired from Cell Signaling Technology (Beverly, MA, USA). β-actin antibody was acquired from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Preparation of complex GPB and high-performance liquid chromatography (HPLC) analysis

Complex GPB was provided by COSMAX NBT Inc. (Korea). Briefly, mixed dried fruits (goji berry, mulberry, and jujube) were extracted with water at 95°C for 4 hr. In order to standardize the extract, HPLC analysis was performed to assess the phytochemical profile of GPB, rutin.

Cell culture

RAW264.7 cells and YAC-1 cells were grown at 37°C under 5% CO_2 in RPMI 1640 media containing 10% inactivated FBS, glutamine, and 1% antibiotics.

Animals

Male C57BL/6 mice (18 – 20 g, 6-week-old,) were received from OrientBio (Sungnam, Korea). Fifteen mice per group were housed in cages of five and supplied with a pelleted meal and free access to tap water. For 30 days, mice were administered either vehicle (0.5% CMC) or Complex GPB (300 mg/kg or 600 mg/kg) orally once a day. All animal studies were carried out in accordance with the Institutional Animal Care and Use Committee of Sungkyunkwan University guidelines (Approval ID: SKKUIACUC2021-01-62-1).

Cell viability assay

RAW264.7 cells were plated in a 96-well plate with a concentration of 1×10^6 cells/mL per well and incubated overnight under 5% CO_2 at 37°C. Then, the cells were treated with Complex GPB (50 μg/mL or 200 μg/mL) and were additionally incubated for 24 hr. After incubation, 100 μL of the supernatant was withdrawn from each well, and 10 μL of MTT solution was given. After more than 3-hr incubation period at 37°C under 5% CO_2, 100 μL of MTT stopping solution (15% SDS) were applied, and the plate was incubated overnight. A multi-reader Spectramax 250 (BioTex, Bad Friedrichshall, Germany) was utilized to measure the absorbance at 570 nm, and the group values were normalized as reported previously.[31]

Enzyme-linked immunosorbent assay (ELISA)

For sample preparation, RAW264.7 cells were plated in a 96-well plate with a concentration of 1×10^6 cells/mL per well and Complex GPB (50 μg/mL or 200 μg/mL) was administered. The cells were cultured for 24 hr at 37°C with 5% CO_2, and 100 μL of the supernatants were transferred to an ELISA plate. The serum was extracted after centrifugation at 2,500 × g for 15 min. After that, further centrifugation at 15,000 × g for 1 min was performed to collect serum once again. TNF-α production levels were assessed according to the manufacturer's instructions using an ELISA kit (R&D Systems, MTA00B).

mRNA isolation and quantitative real-time PCR

RAW264.7 cells were treated with Complex GPB (0 – 200 μg/mL) and evaluated after 24 h. Mice were orally treated with Complex GPB (0 – 600 mg/kg), and spleens were isolated from the mice. Total RNA from cell lysates and splenocytes was extracted using TRIzol reagent, and cDNA was synthesized from 1 μg of mRNA as previously described.[32,33] The expression levels of TNF-α, IL-12a, and IL-1β were measured by quantitative real-time PCR on a real-time thermal cycler (Bio-Rad) using SYBR Premix Ex Taq (Takara, Japan) following the manufacturer's instructions. The primers used in this experiment are shown in Table 1.

Western blotting analysis

RAW264.7 cells were plated in 3-cm plates with a concentration of 2.5×10^6 cells/mL for overnight incubation at 37°C under 5% CO_2. Complex GPB (0 or 200 μg/mL) was administered at the indicated time. Cells were then collected using cold PBS and lysed with a western blotting analysis buffer. After quantifying the same amount of protein for each group using a Bradford protein assay (Bio-Rad), samples were loaded onto 10% sodium dodecyl sulfate (SDS)-PAGE gels. Electrophoresed gels were then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) which then were blocked in 3% BSA for 1 hr and incubated with primary antibodies diluted 1:2500 in 3% BSA overnight at 4°C or for 2 hr at room temperature. After that, the membranes were washed with 0.1% Tris-buffered saline with Tween®-20 buffer three times for 5 min each and then incubated with a secondary antibody diluted 1:2500 in 3% BSA for 2 hr at room temperature. After the washing process, the immunoblotted proteins were detected using an ECL reagent.

Table 1: Primer sequences for the analysis of mRNA prepared from RAW264.7 cells and splenocytes and used in quantitative real-time PCR

Gene	Direction	Sequences (5′ to 3′)
IL-12a	Forward	AAG ACC TGA AAA CCT ACA AGG C
	Reverse	GGC TTG CAT GTC ATC AA
TNF-a	Forward	TGC CTA TGT CTC AGC CTC TT
	Reverse	GAG GCC ATT TGG GAA CTT CT
IL-1b	Forward	GTG AAA TGC CAC CTT TTG ACA GTG
	Reverse	CCT GCC TGA AGC TCT TGT TG
GAPDH	Forward	CAC TCA CGG CAA ATT CAA CGG CAC
	Reverse	GAC TCC ACG ACA TAC TCA GCA C

NK cell isolation and LDH assay

Male C57BL/6 mice (15 mice per group) were orally treated for 30 days with Complex GPB (0-600 mg/kg) once a day. Spleens were collected and ground with 3 mL of RPMI 1640 media. The ground splenocytes were separated into groups of 3×10^7 cells; each group was placed in an e-tube, and the e-tubes were centrifuged for 10 min at 300 × g. The supernatants were then suctioned, and the remaining cells were suspended with 120 μL of NK cell isolation buffer. After that, 30 μL of NK cell biotin-antibody cocktail was added to each e-tube, and cells were pipetted to be resuspended. The mixture was put in an e-tube rotator and

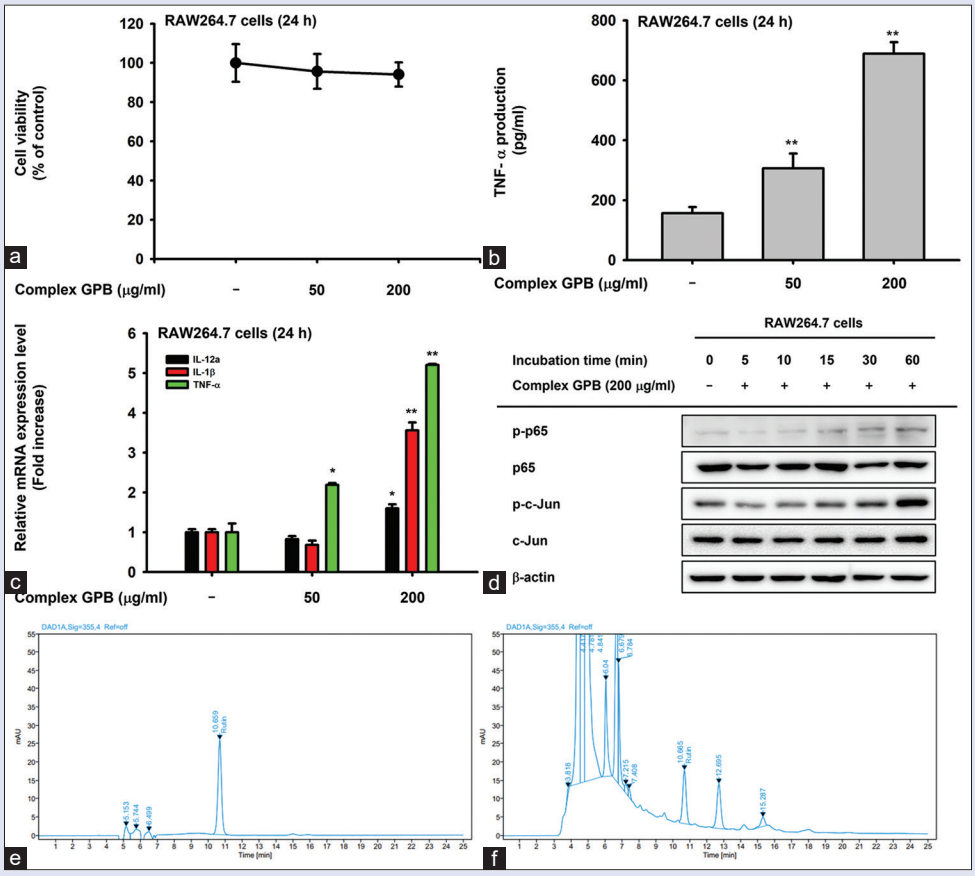

Figure 1: Immunostimulatory effects of Complex GPB *in vitro* in promoting the expression levels of TNF-a. (a) Cell viability was evaluated using the MTT assay. RAW264.7 cells were treated for 24 hr with different concentrations of Complex GPB (0-200 mg/mL) (b) Immunostimulatory effects of Complex GPB *in vitro* in promoting the expression levels of TNF-a. (b) The secretion levels of TNF-a from RAW264.7 cells which were treated with different concentrations of Complex GPB (0-200 mg/mL) for 24 hr, were measured by ELISA. **p < 0.01 compared to the induced group. (c) Immunostimulatory effects of Complex GPB *in vitro* in promoting the expression levels of TNF-a.(c) RAW264.7 cells were treated with Complex GPB (50 or 200 mg/mL) or remained untreated for 24 hr, and of the pro-inflammatory cytokine expression levels of IL-12a, IL-1b, and TNF-a were examined using quantitative real-time PCR. *p < 0.05 and **p < 0.01 compared to the induced group. (d) Immunostimulatory effects of Complex GPB *in vitro* in promoting the expression levels of TNF-a. (d) Complex GPB (200 mg/mL) was administered to RAW264.7 cells for the indicated times. The phosphorylated and total proteins of the NF-kB and AP-1 subunits (p65 and c-Jun) and b-actin were detected by western blotting analyses. (e) Immunostimulatory effects of Complex GPB *in vitro* in promoting the expression levels of TNF-a. (e) The contents of Complex GPB were detected by HPLC using rutin as a standard

incubated at 4°C for 5 min, and after another 1 ml of buffer was added, it was centrifuged at 300 × g for 10 min. After removing the supernatant, 240 µL of buffer and 60 µL of anti-biotin microbeads were given and incubated at 4°C using a rotator for 10 min.

LS columns were inserted into the MACS manual separator and washed with 2 mL of autoMACS® rinsing solution (2 mM EDTA in PBS, pH 7.2). Before passing the cells through the separator, the buffer was added so that the total volume of the cell suspension was at least 500 µL. NK cells flowed down the column; these cells were then centrifuged at 1000 × g for 5 min, and the supernatant was suctioned to obtain only NK cells. The NK cells thus obtained were suspended in RPMI 1640 media and plated at 5 × 10^4 cells/100 µL in round-bottom 96-well plates. YAC-1 cells were co-cultured with NK cells at a concentration of 10^4 cells/100 µL. The plates were incubated at 37°C under 5% CO_2 for 6 h and centrifuged at 250 × g at room temperature for 10 min. The supernatants were transferred to new flat 96-well plates, and 100 µL of reaction solution (catalyst: dye solution = 1:45) was added to each well. The plates were incubated for 5 to 15 min at room temperature with light shielding. Finally, 50 µL of 1 N HCl was added to stop the reaction, and absorbance was measured at 490 nm.

$$\text{Cytotoxicity (\%)} = \frac{\text{A - low control}}{\text{high control - low control}} \times 100$$

where A is [effector – target cell mix] – [effector cell control], high control is target cells + Triton X-100, and low control is only target cells.

RESULTS AND DISCUSSION

Complex GPB stimulated the expression of TNF-α *in vitro*

To investigate the immunostimulatory effects of Complex GPB *in vitro*, it was administered to RAW264.7 cells and it showed no cytotoxicity, as illustrated in Figure 1a. Next, we confirmed that the production levels of TNF-α in cell supernatants were increased by Complex GPB in a concentration-dependent manner [Figure 1b]. Furthermore, Complex GPB significantly increased the expression levels of cytokines, IL-12a and IL-1β, at a concentration of 200 µg/ml. Meanwhile, there was no significant difference in these mRNA at a concentration of 50 µg/ml, indicating that complex GPB is able to increase the expression of IL-12a and IL-1β at higher concentrations. Similar to the previous report study,[28,34] mRNA level of TNF-α found to be increased by ELISA was also enhanced by Complex GPB treatment at concentrations of 50 µg/ml and 200 µg/ml [Figure 1c]. Indeed, the foldchange difference was not very high. However, there was a significant difference ($p < 0.0001$), when we compared the foldchange at both concentrations (at 50 and 200 µg/ml), suggesting that the expression of TNF-α can be highly upregulated even by low concentration of Complex GPB. Why there is an activity discrepancy between TNF-α and the other two cytokines cannot be explained. However, activation of transcription factors such as NF-κB and AP-1 involved in induction of cytokine gene expression by ingredients of Complex GPB could be variable and sensitive to the level of active ingredients in this extract. Western blotting analysis confirmed that the NF-κB and AP-1 transcription factors, p65 and c-Jun, respectively, were activated by 200 µg/ml of Complex GPB in a time-dependent manner [Figure 1d]. This result implicates that the activation of these transcription factors requires a higher amount of active ingredients in Complex GPB. Also, it is suggested that strong induction of TNF-α could be due to the full activation of numerous transcription factors as assessed by the phosphorylation levels of transcription factors. Finally, HPLC analysis was conducted to obtain a phytochemical fingerprinting profile and to identify a representative indicator compound in Complex GPB. Three peaks were identified between 10 to 16 min [Figure 1e upper panel]. Of them, the first peak seen at 10.665 min was found to

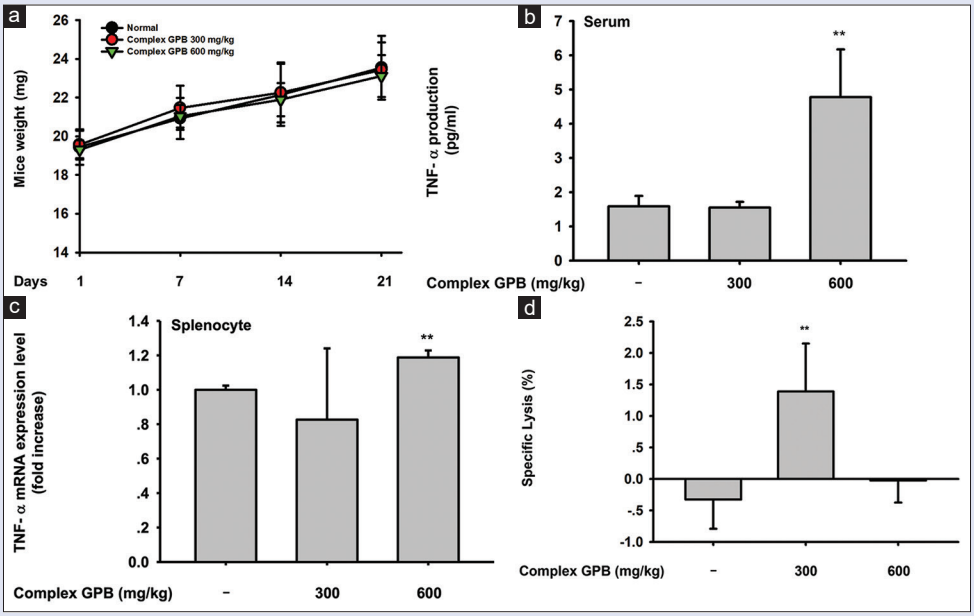

Figure 2: Immunostimulatory effect of Complex GPB *in vivo* by promoting the expression levels of TNF-a and NK cell activation. (a) The body weights of control mice and those orally injected with Complex GPB (300 mg/kg or 600 mg/kg) were evaluated weekly for four weeks. (b) Immunostimulatory effect of Complex GPB *in vivo* by promoting the expression levels of TNF-a and NK cell activation. (b) The TNF-a production in the serum of mice from each group was confirmed using ELISA. $^{**}P < 0.01$ compared to the induced group. (c) Immunostimulatory effect of Complex GPB *in vivo* by promoting the expression levels of TNF-a and NK cell activation. (c) TNF-a expression levels in splenocytes obtained from mice from each group were measured at the mRNA level by real-time PCR. $^{**}P < 0.01$ compared to the induced group. (d) Immunostimulatory effect of Complex GPB *in vivo* by promoting the expression levels of TNF-a and NK cell activation. (d) The degree of specific lysis of YAC-1 cells by activated NK cells was determined by LDH assay. $^{**}P < 0.01$ compared to the induced group

be rutin (quercetin-3-rhamnosyl glucoside), according to the profile of standard rutin [Figure 1e, lower panel]. This result is in line with previously published studies that used a variety of methods to investigate several phenolic acids and flavonoids that identify rutin as one of the phenolic compounds and the predominant flavonoid in goji berry.[20,29] Rutin is a type of citrus flavonoid glycoside that combines flavonol quercetin with the disaccharide rutinose.[35,36] It is a low molecular weight phenolic compound that is extensively found in fruits and vegetables. In addition, it is known to have potentially beneficial effects such as anti-inflammatory, antitumor, anti-allergic, cytoprotective, and antioxidant effects in preventing or treating diseases.[15,37-39] Therefore, as depicted in Figure 1, Complex GPB-enhanced expression levels of cytokines, including TNF-α, which induced immune enhancement *in vitro* by promoting NF-κB and AP-1 transcription factor activation, thereby inducing immune responses.

Complex GPB stimulated the expression of TNF-α and NK cell activation *in vivo*

An animal experiment was conducted to confirm the immunostimulatory effect *in vivo* confirmed at the cellular level in Figure 1. When Complex GPB (300 mg/kg or 600 mg/kg) was given to mice by oral injection for a month, toxicity was evaluated through changes in body weight. No change in body weight was evident among the three groups, confirming that Complex GPB was not toxic [Figure 2a]. Next, to check the expression level of TNF-α in serum isolated from blood, it was measured using ELISA, which showed that the serum TNF-α of mice fed Complex GPB (600 mg/kg) was significantly increased [Figure 2b]. Splenocyte TNF-α expression level was confirmed at the mRNA level, and the increase was dependent on the intake concentration of Complex GPB [Figure 2c]. Finally, the degree of cytotoxic activity of NK cells activated by Complex GPB was checked, and only the Complex GPB 300 mg/kg group showed a significant increase [Figure 2d].

CONCLUSION

We verified that Complex GPB increased the expression level of TNF-α *in vitro* by activating NF-κB and AP-1 pathways when administered to RAW264.7 cells, which are murine macrophages. Complex GPB plays a role in enhancing immune responses by releasing pro-inflammatory cytokines including TNF-α, IL-12a, and IL-1β which activate macrophages and NK cells. In addition, through *in vivo* experiments based on the results of the *in vitro* experiments, it was confirmed that Complex GPB did not show toxicity, and also that it increased the production of TNF-α and the level of activity of NK cells which may induce the activation of immune function, as summarized in Figure 3.

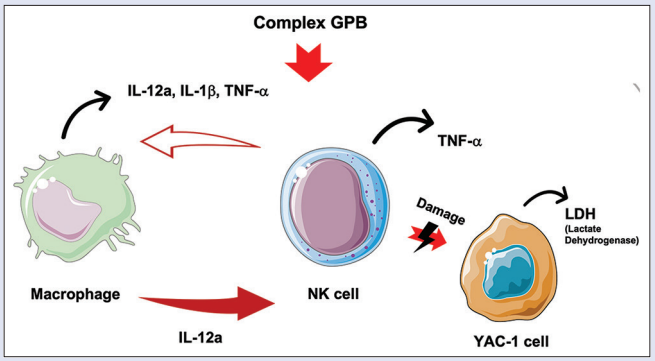

Figure 3: Scheme for immunostimulatory effect of Complex GPB

Overall, our findings provide experimental evidence that Complex GPB could be promising agents to enhance immune response, efficiently. Further investigations are needed to identify active ingredients by LC/GC-MS spectra analysis, validate the Complex GPB effect using various *in vitro* and *in vivo* models (eg., cyclophosphamide-induced immunosuppressive conditions), and perform clinical trials.

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Conflicts of interest

There are no conflicts of interest.

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