Immune enhancement effects of neutral lipids, glycolipids, phospholipids from Halocynthia aurantium tunic on RAW264.7 macrophages

A-yeong Jang
Gangneung-Wonju National University

Weerawan Rod-in
Gangneung-Wonju National University

Woo Jung Park (✉ pwj0505@gwnu.ac.kr)
Gangneung-Wonju National University

Research Article

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Abstract

The fractionated lipids of *Halocynthia aurantium* (Pyuridae) have been demonstrated to possess anti-inflammatory properties, but their modulatory properties have not been studied. This study aimed at estimating the immune enhancing effects of fractionated lipids from *H. aurantium* tunic on macrophage cells. The tunic of *H. aurantium* was used to isolate total lipids, which were then subsequently separated into neutral lipids, glycolipids, and phospholipids. RAW264.7 cells were stimulated with different concentrations (0.5, 1.0, 2.0, and 4.0%) of each fractionated lipid. The cytotoxicity, the production of NO, the expression of immune-associated genes, and signaling pathways were performed. The neutral lipids and glycolipids significantly stimulated the production of NO and the expression of *IL-1β*, *IL-6*, *TNF-α* and *COX-2* in a dose-dependent manner, while phospholipids ineffectively induced NO production and mRNA expression. Furthermore, it was found that both the neutral and glycolipids increased NF-κB p-65, p38, ERK1/2 and JNK phosphorylation, suggesting that these lipids may enhance immunity by activating the NF-κB and MAPK signaling pathways. In addition, *H. aurantium* lipids-induced *TNF-α* expression was also decreased by blocking the MAPK or NF-κB signaling pathways. These results suggest that the neutral lipids and glycolipids from *H. aurantium* tunic have potential as a source of immune-enhancement materials.

1. Introduction

*Halocynthia aurantium* (Pyuridae) is a marine ascidian that is primarily consumed in Korea and Japan. It can be found in the Eastern Sea of Korea, the Sea of Japan, and northern Russia (Fomenko et al., 2013; Kim et al., 2014; Lambert et al., 2016). Most of these species can be eaten raw, cooked, dried, or pickled and are available in seafood markets (Lambert et al., 2016). In the genus *Halocynthia*, the morphology and composition of the tunic in different species like *H. aurantium*, *H. papillosa*, and *H. roretzi* have been thoroughly investigated (Fomenko et al., 2013; Hirose et al., 2009; Song et al., 2020; Van Daele et al., 1992). However, *H. aurantium* has received little research, despite the fact that tunicates and ascidians are known to contain biologically active compounds (Arumugam et al., 2018; Palanisamy et al., 2017; Sawada, 1992).

Lipids are one of the active constituents in marine ascidians and play an essential role in modulating their composition for health benefits (Palanisamy et al., 2017; Tabakaeva and Tabakaev, 2017). They are classified into two major classes based on their chemical characteristics, namely polar lipids (phospholipids, glycolipids, sphingolipids, etc.) and non-polar lipids (as called neutral lipids, which consist of triacylglycerol, cholesterol, wax, free fatty acids, etc.) (Lordan et al., 2017). Polyunsaturated fatty acids (PUFAs) were found in marine lipids, particularly eicosapentaenoic (EPA; 20:5 n-3) and docosahexaenoic (DHA; 22:6 n-3), have been related to improve immune function (Calder, 2015). Some studies have reported that the total lipids, neutral lipids, and polar lipids from ascidian species showed a high amount of EPA and DHA (Ai-li and Chang-hai, 2006; Lee et al., 1993; Viracaoundin et al., 2003; Xu et al., 2003). Ascidian lipids possess improved health benefits such as anti-diabetic and antioxidant effects (Jang et al., 2021; Mikami et al., 2010).
H. aurantium has been reported to provide biological effects such as antimicrobial peptides (Jang et al., 2003; Jang et al., 2002; Lee et al., 2001), antioxidants (Jo et al., 2010), gastroprotective (Chiji et al., 2001), immune-enhancement (Monmai et al., 2018) and anti-inflammatory activities (Monmai et al., 2018). Fatty acids extracted from H. aurantium tunic were found to have immune-enhancing and anti-inflammatory properties (Monmai et al., 2018). In other studies, lipids and fatty acids derived from marine sources promoted several immune function effects in macrophages, resulting in immunological enhancement (Han et al., 2018; Lim et al., 2021). Our previous study reported that total lipids isolated from H. aurantium tunic had immune regulatory effects and the neutral lipids, glycolipids, and phospholipids exhibit anti-inflammatory effects on RAW264.7 cells (Jang et al., 2021; Jang et al., 2022). They have high concentrations of PUFAs and other fatty acids (Jang et al., 2022). Therefore, the purpose of this study was to evaluate the immune-enhancing effects of neutral lipids, glycolipids, and phospholipids, which were isolated from H. aurantium tunic, and their mechanisms were investigated on RAW264.7 cells.

2. Materials and Methods

2.1. Lipid extraction and fraction from H. aurantium tunic

Total lipids were extracted from the H. aurantium tunic using a modified method of Bligh and Dyer (Bligh and Dyer, 1959). Briefly, the lyophilized powder (4.5 g) of dried samples was mixed to the mixture solution of chloroform and methanol (1:2, v/v) containing 0.01% of butylated hydroxytoluene (BHT) to the solvent as an antioxidant (Christie, 1982), and centrifuged at 3000 rpm for 10 min. The homogenate was filtered and evaporated. After being dried, the samples were resuspended in hexane. To separate fractionated lipids, the total lipids were added and separated by silica gel column chromatography. The column was eluted with chloroform, acetone, and methyl alcohol, that produced the neutral lipids, glycolipids, and phospholipids, in order. The solvents were evaporated and dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA) and stored at -20°C until analysis.

2.2. Sample treatments

RAW264.7 cells were purchased from from Koran Cell Line Bank (KCLB). The cells were grown at 37°C in RPMI-1640 medium (GibcoTM, Waltham, USA) supplemented with 10% fetal bovine serum (FBS, Welgene, Korea) and 1% penicillin/streptomycin (Welgene, Korea) in a humidified atmosphere of 5% CO₂. In all experiments, each lipid was diluted in RPMI-1640 medium (no phenol red) supplemented with 1% FBS and 1% antibiotics before any treatments. Cells were pre-treated with various concentrations of three lipids (0.5, 1.0, 2.0, and 4.0%), or 1% DMSO as a model control for 1 h. Following the addition of RPMI to the wells were further incubated for 24 h. The effects of the H. aurantium lipids were evaluated.

2.3. Cell viability analysis

RAW264.7 cells were tested for cytotoxicity using the EZ-Cytox Cell Viability Assay kit (DaeilLab Service, Seoul, Korea). After removing the supernatant, the treated cells were reacted with the WST-solution for 1 h, and the absorbance at 450 nm was measured.
2.4. Nitric oxide (NO) assay

After incubation for 24 h, the nitric concentration of treated-lipid cells was measured using the Griess reagent (Promega, WI, USA). The culture supernatants were incubated with Greiss reagent A (1% sulfanilamide in 5% phosphoric acid) and Greiss reagent B (0.1% N-1-naphtylethylenediamine dihydrochloride in water), and the absorbance at 540 nm was determined.

2.5. Real-time PCR analysis

To analyze the relative expression of interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α), cyclooxygenases-2 (COX-2) and β-actin in the immune system qPCR was used as described previously (Jang et al., 2022). After extracting total RNA from the cells with TRI reagent, cDNA was synthesized from total RNA with high-capacity cDNA reverse transcription kit. cDNA was added into a mixture of TB Green® Premix Ex Taq™ II (Takara Bio Inc., Shiga, Japan, Cat#RR820A) and oligonucleotide primers. The reaction was operated by QuantStudio™ 7 FlexReal-Time PCR System (Applied Biosystems, Foster City, CA, USA).

2.6. Western blotting analysis

Cells were lysed in RIPA buffer (Tech & Innovation, Hebei, China) containing 0.5mM EDTA solution, and a protease & phosphatase inhibitor cocktail (Thermo Fisher Scientific, USA) to release the protein. The protein was loaded and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membranes. Immunoblots were probed with primary antibodies against p-NF-κB p65, p-p38, p-ERK1/2, p-JNK (Cell Signaling Technology, Danvers, MA, USA) and α-tubulin (Abcam, UK), followed by secondary antibodies against goat anti-rabbit IgG (H + L)-HRP (GenDEPOT, TX, USA). The Pierce® ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the protein bands, and the signal intensity was determined by the ChemiDoc XRS + imaging system (Bio-Rad, Hercules, CA, USA).

2.7. Pathway inhibition assay

RAW264.7 cells were treated for 3 h with 100 nM of NF-κB activation inhibitor (Merck, Rahway, NJ, USA) and for 1 h with 20 µM of ERK, JNK, and p38 activation inhibitors (Merck, Rahway, NJ, USA). The supernatant was removed, and the cells were treated with 4.0% of neutral lipids and glycolipids or 1 µg/mL of LPS. After 24 h, the cells were extracted for total RNA and the gene expression was evaluated by real-time PCR. The results of TNF-α expression quantification were compared to β-actin expression.

2.8. Statistical analysis

Statistix 8.1 Statistics Software (Tallahassee, FL, USA) was using to evaluate the Statistical differences. One-way analysis of variance (ANOVA) was used to analyze the data, followed by Duncan's multiple range test, at \( p < 0.05 \). Results are expressed as mean \pm standard deviation (SD).

3. Results
3.1. Effect of neutral lipids, glycolipids, and phospholipids from *H. aurantium* tunic on cell viability

The cytotoxicity of three lipids on RAW264.7 macrophages was evaluated. Cell viability was not affected by neutral lipids and glycolipids at concentrations up to 2.0%. However, the highest dose (4.0% of lipids) reduced cell viability by approximately 84% in neutral lipids (Fig. 1A) and by 91% in glycolipids (Fig. 1B).

3.2. Effect of neutral lipids, glycolipids, and phospholipids from *H. aurantium* tunic on NO production

As displayed in Fig. 1C, phospholipids had no effect on the RAW264.7 cells. In addition, the results showed that treatment with three lipids provided effects on NO production. The production of NO was significantly increased by neutral lipids and glycolipids (0.5-4.0%) in a dose-dependent manner (Fig. 2A and 2B). In contrast, the phospholipids did not show a significant increase in the production of NO compared to control (RPMI), although NO production was still dose-dependently (Fig. 2C).

3.3. Effect of neutral lipids, glycolipids, and phospholipids from *H. aurantium* tunic on gene expression

In order to determine whether *H. aurantium* lipids enhance immunity in RAW264.7 cells, the expression of immune-associated genes was measured. Except phospholipids, neutral lipids and glycolipids increased the expression of cytokine genes such as *IL-1β*, *IL-6*, and *TNF-α* in a dose-dependent manner (Fig. 3A and 3B). In particular, *COX-2* expression was highly increased by neutral lipids and glycolipids. However, phospholipid does not show any effects (Fig. 3C). These data suggested that neutral lipids and glycolipids of *H. aurantium* may act as signaling molecules, causing the increase of pro-inflammatory cytokine secretion, which means immunological enhancement. Therefore, the neutral lipids and glycolipids were selected for further experiments.

3.3. Effect of neutral lipids and glycolipids from *H. aurantium* tunic on MAPK and NF-κB signaling pathway

To determine the phosphorylation of NF-κB and MAPK of *H. aurantium* lipids, western blot analysis was performed. As shown in Fig. 4, neutral lipids (Fig. 4A) and glycolipids (Fig. 4B) dose-dependently increased the phosphorylation of NF-κB and MAPK signaling pathways. These results showed that neutral lipids and glycolipids have immune-enhancing activity through the NF-κB and MAPK signaling pathways in RAW264.7 macrophages.

3.4. Effect of neutral lipids and glycolipids from *H. aurantium* tunic on MAPK and NF-κB inhibited RAW264.7 cells

To evaluate the effects of neutral lipids and glycolipids on the mechanism of immune-regulation in NF-κB and MAPK activation, *TNF-α* expression was measured. The cells were pre-treated with different inhibitors of NF-κB, ERK, JNK, and p38, and then the cells were incubated with 4.0% *H. aurantium* lipids for 24 h before being tested for *TNF-α* expression by real-time PCR. As shown in Fig. 5, *TNF-α* expression was
increased in RAW264.7 cells treated with LPS as a positive control and the *H. aurantium* lipids without inhibitors, compared to RPMI even though *H. aurantium* lipids showed lower TNF-α expression than LPS. When compared to *H. aurantium* lipids without inhibitors, neutral lipids with specific NF-κB, ERK and p38 inhibitors suppressed TNF-α expression, but JNK inhibitors had no effect on TNF-α expression. Furthermore, TNF-α expression was comparable in glycolipids treated with JNK and p38 inhibitors to specific NF-κB, and ERK inhibitors.

4. Discussion

Three lipids, including neutral lipids, glycolipids, and phospholipids, were isolated from the total lipids of *H. aurantium* tunic. Our previous studies demonstrated that these lipids showed anti-inflammatory properties and contained diverse essential fatty acids (Jang et al., 2022). In the present study, these lipids were evaluated for their immune-enhancing activity on macrophage cells.

Several studies have demonstrated the immunomodulatory activities of bioactive compounds that enhance the production of reactive oxygen species (ROS) and NO, as well as the production of cytokines and chemokines such as IL-1β, IL-6, IL-12, IL-10, TNF-α, and TGF-β in RAW264.7 macrophages (Deng et al., 2020; Han et al., 2018; Kim et al., 2017; Xie et al., 2019). The fatty acids from *H. aurantium* tunic were reported to possess the immunomodulatory properties and reported that these compounds could regulate the production of NO and prostaglandin E2 (PGE₂) and the expression of iNOS, IL-1β, IL-6, COX-2, and TNF-α via MAPK and NF-κB signaling (Monmai et al., 2018). Han et al. (2018) demonstrated that DHA stimulated the GPR120, C-Raf, and MAPKs to activate the NF-κB p65 pathway, which increased mRNA and protein expression of iNOS as well as cytokine production of IL-1β, IL-6, IL-12, TNF-α, IFN-γ, and TGF-β (Han et al., 2018). In the present study, our results showed that neutral lipids and glycolipids significantly increased the production of NO in RAW264.7 macrophages compared to control groups. In addition, the expression of immune-associated genes such as IL-1β, IL-6, and TNF-α as well as COX-2 was also stimulated by neutral lipids and glycolipids in a dose-dependent manner (Fig. 2). Cyclooxygenases are metabolic enzymes that are involved in the production of prostaglandins, which are important components of the inflammatory response (Lee et al., 2008). Lipids derived from *Ammodytes personatus* (Ammodytidae) and *H. aurantium* increased the expression of COX-2 which stimulated the production of PGE₂ (Lim et al., 2021). Especially, our study showed that phospholipids did not stimulate NO production and gene expression, but neutral lipids and glycolipids increased NO production and immune gene expression, suggesting that these two lipids are involved in immunostimulatory activity.

Additionally, the effect of neutral lipids and glycolipids isolated from *H. aurantium* on NF-κB and MAPK signaling pathways were evaluated. Our results showed that those two lipids enhanced expression levels, leading to further activation of phosphorylated NF-κB p-65 and phosphorylated MAPK molecules, such as ERK1/2, JNK, and p38 (Fig. 3). Moreover, TNF-α expression by NF-κB and MAPK inhibition showed differently regulated when NF-κB and MAPK signaling were inhibited, meaning that the neutral lipids and glycolipids enhanced immunity with different signaling pathways in macrophage, respectively (Fig. 4).
Alfalfa (Fabaceae) polysaccharides reduced TNF-α production by blocking NF-κB or MAPK inhibitor in RAW 264.7 macrophages, which was similar to our results (Xie et al., 2019).

5. Conclusions

The current results demonstrated that the neutral lipids and glycolipids isolated from *H. aurantium* tunic stimulated NO production and immune-associated gene expression in RAW264.7 cells. The expression of phosphorylated NF-κB p-65, ERK1/2, JNK, and p38, was enhanced by neutral lipids and glycolipids. On the other hand, phospholipids do not stimulate macrophage function. Therefore, these results indicated that neutral lipids and glycolipids in *H. aurantium* tunic exhibit potential immune-enhancing activity.

Declarations

CRediT authorship contribution statement

A-yeong Jang: Methodology, Software, Validation, Formal analysis, Investigation, Writing—original draft preparation, Visualization. Weerawan Rod-in: Methodology, Software, Validation, Formal analysis, Data curation, writing—review and editing, Visualization. Woo Jung Park: Conceptualization, Resources, Data curation, Writing—review and editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References


**Figures**

![Figure 1](image)

**Figure 1**

Effects of *H. aurantium* lipids on cell viability. (A) Neutral lipids, (B) glycolipids, and (C) phospholipids. Values are expressed as mean ± SD (*n* = 3). Difference is declared significant when *p* < 0.05.
Figure 2

Effects of *H. aurantium* lipids on NO release. (A) Neutral lipids, (B) glycolipids, and (C) phospholipids. Values are expressed as mean ± SD (*n* = 3). Difference is declared significant when *p* < 0.05.
Figure 3

Effects of *H. aurantium* lipids on mRNA expression of immune genes. (A) Neutral lipids, (B) glycolipids, and (C) phospholipids. Values are expressed as mean ± SD (*n* = 3). Difference is declared significant when *p* < 0.05.
Figure 4

Effects of neutral lipids and glycolipids from *H. aurantium* tunic on the protein expression associated with NF-κB and MAPK pathways. (A) Neutral lipids and (B) glycolipids. Values are expressed as mean ± SD (*n* = 3). Difference is declared significant when *p* < 0.05.
**Figure 5**

Effect of neutral lipids and glycolipids from *H. aurantium* tunic on TNF-α expression with specific NF-κB and MAPK inhibitors. Values are expressed as mean ± SD (*n* = 3). Difference is declared significant when *p* < 0.05.