## Original Article

# Dunaliella salina alga extract inhibits the production of interleukin-6, nitric oxide, and reactive oxygen species by regulating nuclear factor-κB/Janus kinase/signal transducer and activator of transcription in virus-infected RAW264.7 cells

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## ARTICLE INFO

## ABSTRACT

Recent investigations have demonstrated that carotenoid extract of Dunaliella salina alga (Alga) contains abundant β-carotene and has good anti-inflammatory activities. Murine macrophage (RAW264.7 cells) was used to establish as an in vitro model of pseudorabies virus-induced reactive oxygen species (ROS) response. In this study, antioxidant activities of Alga were measured based on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, trolox equivalent antioxidant capacity assays, reducing power, and virus-induced ROS formation in RAW264.7 cells. Anti-inflammatory activities of Alga were assessed by its ability to inhibit the production of interleukin-6 and nitric oxide (NO) using enzyme-linked immunosorbent assay, then the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway was investigated by measuring the inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear factor-κB (p50 and p65), JAK, STAT-1/3, and suppressor of cytokine signaling 3 (SOCS3) by Western blotting. In addition, Alga inhibited virus replication by plaque assay. Our results showed that the Alga had high antioxidant activity, significantly reduced the virus-induced accumulation of ROS, and

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inhibited the levels of nitric oxide and interleukin-6. Further studies revealed that Alga also downregulated the gene and protein expressions of iNOS, COX-2, nuclear factor-κB (p50 and p65), and the JAK/STAT pathway. The inhibitory effects of Alga were similar to pre-treatment with specific inhibitors of JAK and STAT-3 in pseudorabies virus -infected RAW264.7 cells. Alga enhanced the expression of SOCS3 to suppress the activity of the JAK/STAT signaling pathway in pseudorabies virus-infected RAW264.7 cells. In addition, Alga has decreased viral replication ($p < 0.005$) at an early stage. Therefore, our results demonstrate that Alga inhibits ROS, interleukin6, and nitric oxide production via suppression of the JAK/STAT pathways and enhanced the expression of SOCS3 in virus-infected RAW264.7 cells.

# 1.    Introduction

Carotenoids are effective antioxidants that can quench singlet oxygen ($^1O_2$), suppress lipid peroxidation, and prevent oxidative damage. Recent investigations have also demonstrated that various carotenoids, including all-*trans* forms of ß-carotene, ß-cryptoxanthin, lycopene, crocin, and astaxanthin, have good anti-inflammatory activities [1–5]. *Dunaliella salina* (Chlorophyceae) is a halophilic unicellular microalga with a mucus surface coat (i.e., with no cell wall). *D. salina* alga contains abundant carotenoids (especially all-*trans*-ß-carotene and 9- or 9′-*cis*-ß-carotene) [6,7].

Virus infection of the human central nervous system is a significant source of morbidity and mortality worldwide. Infection of herpes simplex virus (HSV) is always observed in humans with hypoimmunity. HSV is the infectious agent most commonly responsible for sporadic acute encephalitis, and when left untreated, acute encephalitis has a mortality rate of 70% [8]. Pseudorabies virus (PRV) is a neurotropic alphaherpes virus that, after intranasal infection in adult mice, enters peripheral neurons and propagates to the central nervous system. Owing to its pathogenesis and immunobiology being similar to those of HSV, many studies have used PRV to mimic an HSV infection [8,9]. Viral infections often cause increased formation of reactive oxygen species (ROS) and reactive nitrogen species, either due to the direct effects of the virus on the cells or as a consequence of host inflammatory responses to the infections [10,11]. As important factors of innate immunity, ROS are also beneficial for a host in terms of limiting viral replication. It is intriguing to note that the induction of ROS production has been recognized as a common mechanism in counteracting viral infections [12]. A recent study indicated that HSV initially promotes the production of ROS, which in turn elicits a wide spectrum of responses by activating the transcription factor nuclear factor-κB (NF-κB) through mitogen-activated protein kinase pathways [13]. However, no reports have yet confirmed that infection of RAW264.7 cells with PRV induces increased ROS production.

Several hypothesis-based transcriptional analyses have been conducted that have provided considerable information. PRV infections activate signal transduction cascades, including the NF-κB and mitogen-activated protein kinase cascades [5,9,13]. These signaling events most likely result in the release of inflammatory mediators. However, cellular inflammatory responses differ, depending on the type of host cells and species. It is known, for example, that the interleukin-6 (IL-6) family of cytokines mediates its action primarily through activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway along with other intracellular signaling pathways [14,15]. However, this molecular mechanism has not been clearly defined in macrophages (such as RAW264.7 cells) by PRV infection. The data of the present study describe the critical roles of IL-6 in the JAK/STAT cascade-related effects induced by PRV infection.

Cytokines signal through JAK and STAT transcription factors. IL-6, which activates STAT-1 and STAT-3, has been hypothesized to contribute to the progression of many inflammatory diseases such as antineuroinflammation and Crohn disease [14–17]. In general, IL-6 is one of the major physiological mediators of acute-phase reactions, and is associated with or activates many inflammatory cytokines. As IL-6 can orchestrate or modulate the inflammatory response of macrophages, we evaluated the inflammatory cytokines that are dependent on IL-6.

JAK is responsible for the tyrosine phosphorylation of STAT-3 in response to extracellular signals and oncogenes. The newly described JAK inhibitor AG490 blocks the constitutive activation of STAT-1 and STAT-3 [16]. The pleiotropic cytokine IL-6 is a major activator of STAT-3; IL-6 stimulates the formation of tyrosine-phosphorylated STAT-1 (pSTAT-1) and STAT-3 (pSTAT-3) in cancer cells [18,19]. Through the JAK/STAT signaling pathway, IL-6 plays an important role in cell proliferation, apoptosis, metastasis, inflammation, and other biological activities [18–20]. Our aim in this study was to demonstrate that the JAK/STAT signaling pathway may be critical for the inflammatory responses to a viral infection. At the same time, inhibition of this pathway may offer a novel strategy for antiviral inflammatory treatment.

In the present study, we investigated the activity and action mechanism of *D. salina* alga extract on the production of IL-6, a pivotal cytokine of the ROS and inflammatory process in PRV-infected RAW264.7 cells.

## 2. Methods

### 2.1. D. salina sample

The *D. salina* sample used in this study consisted of spray-dried powder of *D. salina* algae cultivated in Taiwan in 2011 and was purchased from Gong Bih Enterprise Co., Ltd (Wunlin, Taiwan).

### 2.2. Preparation of D. salina extract

*D. salina* extract (Alga) was prepared according to the method of Hu et al [6]. The algal sample (10 g) was extracted for 2 hours at room temperature in 250 mL of hexane/acetone/ethanol (2:1:1, v/v/v) with a shaker. Saponification was then performed through the addition of 10 mL of 40% methanolic KOH at $25°C$ for 16 hours. After filtering, the extract was transferred to a separatory funnel and washed three times with 250 mL of distilled water. The solvent was evaporated to dryness in a rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan) to yield carotenoid extract. The composition of carotenoids in the extract was measured by high-performance liquid chromatography (HPLC) under the conditions established in the previous report [6]: column, YMC C30 ($250 \times 4.6$ mm, $5 \mu m$) (Waters Co., Milford, MA, USA); mobile phase, methanol (MeOH)–acetonitrile–$H_2O$ (84/14/2, v/v/v)/$CH_2Cl_2$ = 75/25 (v/v); flow rate, 1 mL/min; detection, 210–650 nm at a rate of 1.00 spectrum/s. The equipment consisted of a PrimeLine Gradient Model 500G HPLC pump system (Analytical Scientific Instruments, Inc., El Sobrante, CA, U.SA) and an S-3210 photodiode-array detector (Schambeck SFD GmbH, Bad Honnef, Germany). The Alga was then dissolved in dimethyl sulfoxide (DMSO) for the experiment.

### 2.3. Materials and reagents

The solvents used for the preparation of the algal carotenoid extract, including acetonitrile, methanol (MeOH), methylene chloride ($CH_2Cl_2$), ethanol, and n-hexane, were purchased from Merck Co. (Darmstadt, Germany). Distilled deionized water ($H_2O$) was prepared with the Ultrapure water purification system (Lotun Co., Ltd, Taipei, Taiwan). Potassium hydroxide (KOH) was purchased from Merck Co. All-*trans* forms of lutein (MW 569) and zeaxanthin (MW 569), glutamine, penicillin, streptomycin, DMSO, Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, and 2.5% phosphoric acid), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, EDTA, leupeptin, phenylmethanesulfonyl fluoride, phosphate-buffered saline (PBS), sodium chloride (NaCl), sodium nitrite ($NaNO_2$), Triton X-100, Tris, AG490, and STAT-3 inhibitor were purchased from Beyotime Inc. (Shanghai, China). RPMI1640, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hyclone (Logan, UT, USA). Enzyme-linked immunosorbent assay (ELISA) kits for the measurement of IL-6 were purchased from eBioscience (San Diego, CA, USA). The inhibitors of JAK2 (AG490) and STAT-3 were obtained from Cayman Chemical (Michigan, USA).

### 2.4. DPPH radical scavenging activity

The method used was the same as that previously described by Epsín et al [21]. An aliquot of each sample (30 μL, 0.5–40 mg/mL) in acetone/MeOH = 1/1 (v/v) was mixed with 200 μL of 100 μM DPPH (prepared with methanol). The mixture was shaken vigorously and then left to stand at room temperature for 60 minutes in the dark. The absorbance was measured spectrophotometrically at 520 nm. The scavenging activity was expressed as the percentage of DPPH radical scavenged. The $EC_{50}$ value (mg sample/mL) refers to the effective concentration at which 50% of the DPPH radicals were scavenged. The α-tocopherol was used as a positive control.

### 2.5. Trolox equivalent antioxidant capacity assay

Trolox equivalent antioxidant capacity (TEAC) assay was performed in the same manner as previously reported by Scalzo et al [22]. The absorbance was measured at 734 nm as a function of concentration, and the scavenging percentage of 2,2′-azinobis(3-ethylbenzthiazoline-6- sulfonic acid) ($ABTS^+$) was calculated relative to trolox, a water-soluble analog of vitamin E used as an antioxidant standard. Antioxidant activity was presented as micromoles of Trolox equivalent per gram of sample weight. The $ABTS^+$ solution ($OD_{734} = 0.7 \pm 0.02$) was prepared by mixing ABTS, peroxidase, and $H_2O_2$ with the final concentrations of 100 μM, 4.4 units/mL, and 50 μM, respectively, followed by incubation at $30°C$ for 6 minutes. The compound α-tocopherol was used as a positive control.

### 2.6. Reducing power

The reducing power was measured under the conditions previously described by Oyaizu [23]. An aliquot of each sample (0.5 mL, 0.5–10 mg/mL) in acetone/MeOH = 1/1 (v/v) was mixed with 0.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of 1% $K_3Fe(CN)_6$, and the mixture was then allowed to react at $50°C$ for 20 minutes. After adding 0.5 m of 10% trichloroacetic acid, the mixture was centrifuged at 8000 rpm for 10 minutes (Hermle Z300 K centrifuge; Hermle Labortechnik GmbH, Wehingen Württ, Germany). The supernatant (0.1 mL) was then taken out, and immediately mixed with 0.1 mL of methanol and 20 μL of 0.1% ferric chloride. After the reaction was allowed to proceed for 10 minutes, the absorbance was determined at 700 nm, with higher absorbance levels indicating stronger reducing power. The compound α-tocopherol was used as a positive control.

### 2.7. Cell culture and virus

Porcine kidney 15 (PK15) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Hyclone). The RAW264.7 cell line was cultured in RPMI1640 supplemented with 10% FBS. The stock of wild-type PRV (strain TNL) used in this study was amplified from PK15 cells, and the titer was determined by standard plaque assay in PK15 cells.

### 2.8. Nitrite assay

Nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction [24].

RAW264.7 cells were plated at a density of $1 \times 10^5$ cells/well into 24-well plates. After 4 hours of incubation, the cells were pretreated with DMSO (0.1%) or different concentrations (25 μM, 50 μM, or 100 μM) of Alga (DMSO concentration in each well was 0.1%) for 1.5 hours and then incubated for 3 hours, 6 hours, 9 hours, 12 hours, and 24 hours with PRV [0.1 multiplicity of infection (MOI)]. The supernatant (100 μL) was mixed with 100 μL of Griess reagent (1% sulfanilamide, 0.1% N-1-napthylethylenediamine dihydrochloride (NED), 2.0% phosphoric acid) and incubated for 10 minutes at room temperature, and the absorbance of the mixture at 595 nm was then determined with an ELISA plate reader (Multiskan Spectrum, Thermo Co., Vantaa, Finland). All measurements were performed in triplicate. The concentration of $NO_2^-$ was determined using a standard curve generated with $NaNO_2$.

### 2.9. Evaluation of ROS production

ROS production was detected by measuring intracellular ROS formation using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe. Briefly, RAW264.7 cells were pretreated with DMSO (0.1%) or different concentrations (25 μM, 50 μM, or 100 μM) of Alga (DMSO concentration in each well was 0.1%) for 1.5 hours and then incubated for 24 hours with PRV (0.1 MOI) to induce ROS production, followed by incubation with 20 μM DCFH-DA for 30 minutes in the dark. At the end of the DCFH-DA incubation, the cells were washed with PBS. After washing, the formation of fluorescence dichlorofluoroscein, which is the oxidized product of DCFH-DA in the presence of several ROS, primarily hydroperoxide, was measured using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA) at excitation/emission wavelengths of 485/535 nm. The ROS level was expressed as a percentage over basal levels.

### 2.10. Determination of IL-6 levels secreted from RAW264.7 cells

RAW264.7 cells were pretreated with DMSO (0.1%) or various concentrations of Alga (DMSO concentration in each well was 0.1%) for 1.5 hours and then infected with 0.1 MOI of PRV for 6 hours, 9 hours, 12 hours, and 24 hours, after which the absorbance of the given mixture at 450 nm was determined with an ELISA plate reader (Multiskan Spectrum, Thermo Co.). The amounts of IL-6 in the supernatant were detected by ELISA kits (eBioscience). All the experiments were performed in triplicate.

### 2.11. Western blot analysis

RAW264.7 cells were untreated or pretreated with DMSO or 100 μM of Alga for 1.5 hours before an infection with 0.1 MOI of PRV. After 6 hours, 9 hours, 12 hours, and 24 hours, the cells were washed with PBS and lysed in a lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/mL leupeptin] and kept on ice for 30 minutes. Cell lysates were centrifuged at 12,000$g$ at 4°C for 15 minutes, and the supernatants were stored at −70°C until analyses. Protein concentrations were measured using a protein assay kit (Bio-Rad, Laboratories, Inc., Hercules, CA, USA). Whole-cell lysate proteins or nuclear lysate proteins (20 μg) were resolved in loading buffer,

electrophoresed on sodium dodecyl sulfate (SDS)/polyacrylamide gels, and electroblotted onto polyvinylidene difluoride membranes. The membranes were then blocked in Tris-buffered saline–Tween 20 solution containing 5% nonfat dry milk and incubated overnight at 4°C with specific antibodies against iNOS, COX-2, NF-κB p50 and NF-κB p65 by Spring Bioscience (LabVision, Runcorn, England), JAK (Santa Cruz, Heidelberg, Germany), pJAK (Abcam, Cambridge, Cambridgeshire, United Kingdom), STAT-1 , STAT-3, pSTAT-3, SOCS3 by Cell Signaling (New England Biolabs, Hitchin, UK), pSTAT-1 (Epitomics, Burlingame, San Mateo, USA), and GAPDH (glyceraldehyde 3-phosphate dehydrogenase) (Novus, Littleton, Colorado, USA). Proteins were visualized using goat antirabbit or mouse antibody conjugated to horseradish peroxidase and a chemiluminescence Western blotting detection system (ECL Plus Western Blotting Reagents; Amersham Biosciences, Boston, MA, USA). Protein band densities were quantified using AlphaImager 2200 software.

### 2.12. Effects of JAK and STAT-3 inhibitors on PRV-infected RAW264.7 cells

The effects of the JAK–STAT pathway in PRV-infected RAW264.7 cells were examined via culturing of the cells with JAK inhibitor (AG490) or STAT-3 inhibitor. RAW264.7 cells were untreated (DMSO) or pretreated with AG490 or STAT-3 inhibitors 1.5 hours before an infection with 0.1 MOI of PRV. After 24 hours, the cells were washed with PBS, lysed, and then assayed via a Western blot analysis.

### 2.13. Plaque reduction assay

Viral titers of the PK15 cells were determined using a plaque reduction assay. RAW264.7 cells were pretreated with 100 μM of Alga or DMSO (0.1%) for 1.5 hours, and then incubated for 24 hours with PRV (0.1 MOI). Briefly, the supernatant (100 μL) was $10^{-1}$–$10^{-3}$-fold dilutions and then added to each well for 1.5 hour. Cells were washed twice with PBS and covered with an overlay of the maintenance medium (Dulbecco's modified Eagle's medium with 2% FBS and 1% methylcellulose), and the plate was further incubated for 2–3 days to allow for plaque formation. The cells were stained with crystal violet, and the number of plaques was counted.

### 2.14. Statistical analysis

The values are expressed as mean ± standard deviation. The significance of any difference from the respective controls for each experimental test condition was assayed using Student $t$ test for each paired experiment. A $p$ value of <0.01 was regarded as indicative of a significant difference.

## 3. Results and discussion

### 3.1. Antioxidant activities of Alga

Our previous reports demonstrated that Alga contained abundant β-carotene and had good anti-inflammatory activities [5]. In order to understand the quality of Alga stored under −20°C for 1 year, the total β-carotene contents in Alga

were measured in this study. Our results showed that *D. salina* contained abundant β-carotene (contains of all-*trans*-β-carotene, 9- or 9′-*cis*-β-carotene, and 13- or 13′-*cis*-β-carotene) about was 91.8%. Antioxidant activities of the Alga were determined using DPPH radical scavenging and TEAC assay. As shown in Table 1, the Alga showed a high DPPH radical scavenging activity. With regard to the TEAC assay, the TEAC values of the algal extract, all-*trans*-β-carotene, and α-tocopherol were 0.76 mmol trolox equivalent/g, 0.57 mmol trolox equivalent/g, and 1.38 mmol trolox equivalent/g, respectively (Table 1). In addition, the reducing power was increased with increasing concentrations of each experimental sample (Figure 1). Thus, Alga had high levels of antioxidant activities. Jimenéz and Pick [25] demonstrated that 9-*cis*-β-carotene was more effective than its all-*trans* isomer for scavenging of ROS. Alga was rich in *cis* forms and all-*trans* forms of carotenoids. Therefore, these *cis* forms of carotenoids, especially 9- or 9′-*cis*-β-carotene, should also contribute significantly to the antioxidant activities of Alga.

### 3.2. Effects of Alga on ROS generation

One important function of activated macrophages is to kill foreign invasive bacteria through the secretion of ROS and NO [26]. However, overexpression of ROS and NO can cause various illnesses by injuring surrounding cells and resulting, in turn, in acute or chronic inflammatory diseases [27]. In this study, we first performed experiments to determine the effects of Alga on intracellular ROS accumulation in cells infected with PRV. PRV-infected RAW264.7 cells exhibited a rapid increase in intracellular ROS levels at 24 hours, as determined using the $H_2O_2$-sensitive probe CM-$H_2$ DCFDA, which was effectively attenuated by pretreatment with Alga in a dose-dependent manner (Figure 2). As expected, Alga (at concentrations of 50 μM and100 μM) blocked ROS accumulation almost completely. Hence, our study indicated a remarkably higher antioxidant activity of Alga.

### 3.3. Alga inhibits the production of NO and IL-6 in PRV-infected RAW264.7 cells

IL-6 and NO are representative proinflammatory mediators generated by external stimuli in macrophages. To determine
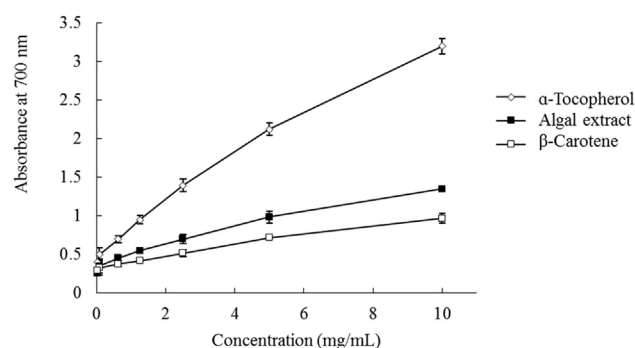


Figure 1 – Reducing power of Alga, β-carotene and α-tocopherol. Each value is expressed as mean ± SD (*n* = 3). Alga = *D. salina* carotenoid extract; SD = standard deviation.
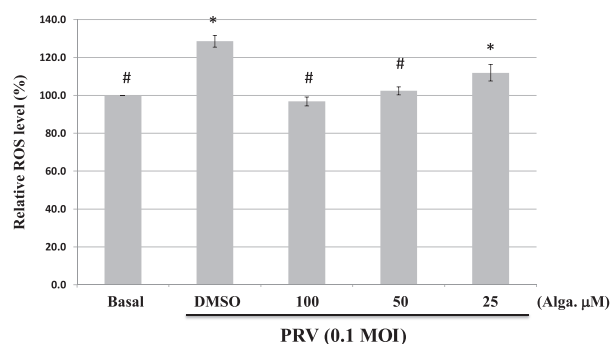


Figure 2 – Alga inhibits the production of ROS in PRV-infected RAW264.7 cells. RAW264.7 cells were untreated (DMSO and basal) or pretreated with 100 μM, 50 μM, and 25 μM of Alga for 1.5 hours, and then infected with PRV or left uninfected (basal). After 24 hours, the cells were stained with 10 μM of DCFH-DA for 30 minutes, and ROS production was determined using a fluorescence microplate reader. All measurements were performed in triplicate. Data are presented as mean ± SD (*n* = 3). #*p* < 0.01, significant compared with PRV-infected RAW264.7 cells (DMSO). *p* < 0.01, significant compared with basal. Alga = *D. salina* carotenoid extract; DMSO = dimethyl sulfoxide; DCFH-DA = dichloro-dihydro-fluorescein diacetate; MOI = multiplicity of infection; PRV = pseudorabies virus; ROS = reactive oxygen species; SD = standard deviation.

| Table 1 – $EC_{50}$ of DPPH radical-quenching activity and antioxidant activity (TEAC assay) values of the Alga, β-carotene, and α-tocopherol. | | |
| --- | --- | --- |
| Sample | $EC_{50}$ (mg sample/mL) | TEAC (mmol TE/g sample) |
| α-Tocopherol | 2.72 ± 0.09 c | 1.38 ± 0.07 a |
| β-Carotene | 13.23 ± 0.05 a | 0.57 ± 0.03 c |
| Alga | 8.15 ± 0.42 b | 0.76 ± 0.05 b |

Values (mean ± SD, *n* = 3) in the same column that are followed by a different letter are significantly different (*p* < 0.05), as determined by Duncan's multiple range tests.
Alga = *D. salina* carotenoid extract; $EC_{50}$ = effective concentration of sample that can decrease DPPH concentration by 50%; SD = standard deviation; TE = trolox equivalent; TEAC = trolox equivalent antioxidant capacity; DPPH = 1,1-Diphenyl-2-picrylhydrazyl.

the inhibitory effects of Alga on IL-6 and NO production, we investigated the inhibitory activity of Alga against NO and IL-6 production in PRV-infected RAW264.7 cells. In order to determine the time profile of NO and IL-6 production, we measured the amount of NO and IL-6 in the cultured medium at the indicated time points after PRV infection. NO production was induced after 9 hours and continuously increased up to 24 hours (Figure 3B), whereas IL-6 production was continuously induced from 6 hours up to 24 hours (Figure 3A), and IL-6 was produced earlier than NO. Next, we examined the effect of Alga on the production of NO and IL-6 induced by PRV

**(A)**



**(B)**
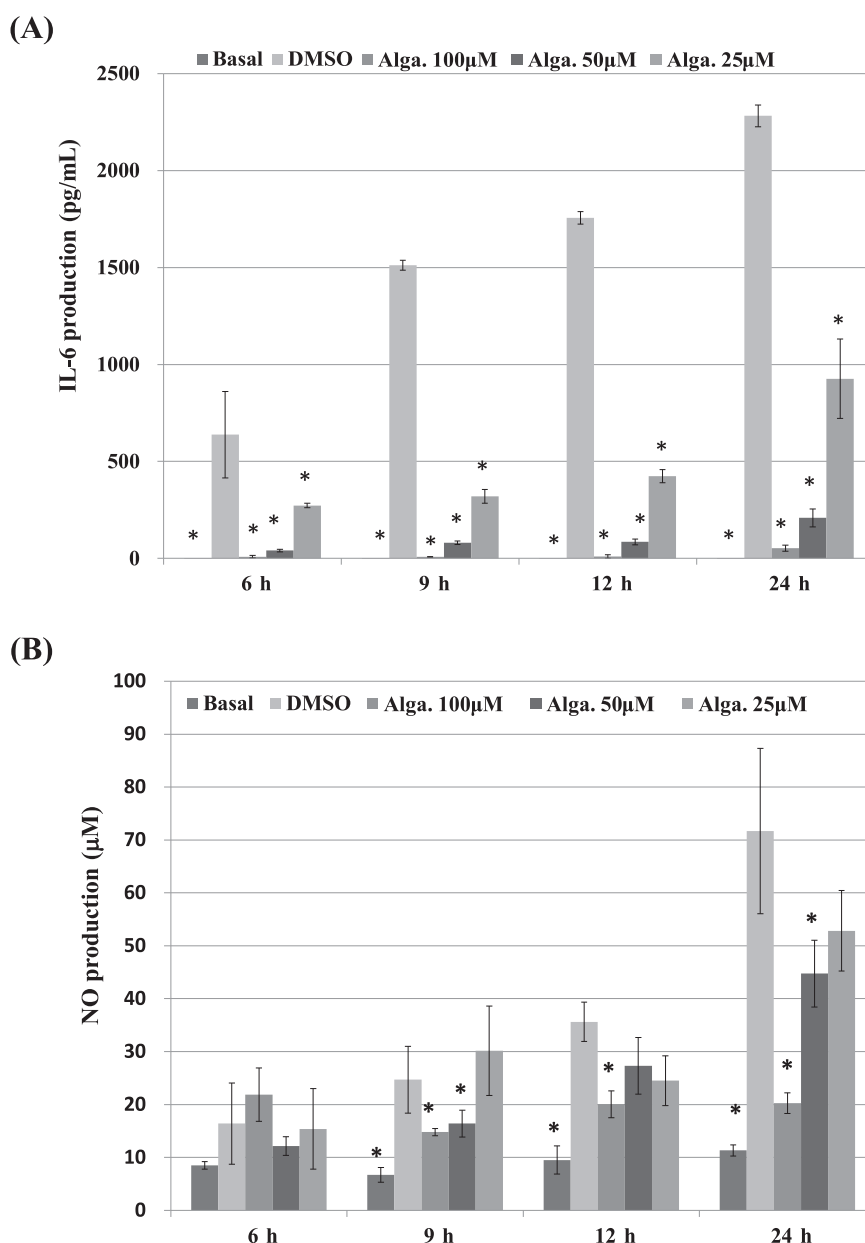


**Figure 3 — Alga inhibits the production of (A) IL-6 and (B) NO in PRV-infected RAW264.7 cells. RAW264.7 cells were untreated (DMSO and basal) or pretreated with 100 μM, 50 μM, and 25 μM of Alga for 1.5 hours, and then infected with PRV or left uninfected (basal). After 6–24 hours, IL-6 and NO production was measured by ELISA. All measurements were performed in triplicate. Data are presented as mean ± SD (_n_ = 3). \*_p_ < 0.01, significant compared with PRV-infected RAW264.7 cells (DMSO). Alga = _D. salina_ carotenoid extract; DMSO = dimethyl sulfoxide; ELISA = enzyme-linked immunosorbent assay; IL = interleukin; PRV = pseudorabies virus; SD = standard deviation.**

infection. Our results showed that Alga (100 μM) strongly inhibited the production of NO and IL-6 in a concentration-dependent manner. Therefore, we next sought to determine the mechanisms of IL-6 and NO production in PRV-infected RAW264.7 cells.

### 3.4. JAK/STAT pathway is involved in virus-mediated ROS, IL-6, and NO production

STATs are a family of nuclear proteins mediating the action of a number of cytokines, such as ILs, interferons, and others. In

particular, the JAK/STAT pathway is known to be involved in LPS-induced IL-6 production. Previous studies have reported that STAT-1 and STAT-3 play a role in IL-1β and IL-6 production in response to diverse stimuli [15,28]. To elucidate this, we tested the role of the JAK/STAT pathway, which is involved in PRV-mediated iNOS and COX-2 gene expressions. To our knowledge, this study is the first to investigate the effects of Alga on virus-induced IL-6 and NO production via the JAK/STAT pathway in PRV-infected cells.

To confirm the involvement of the JAK pathway, we pretreated cells with AG490, a specific inhibitor of JAK (10 μM and

20 μM), and STAT-3 inhibitor (20 μM and 50 μM). The results showed that the AG490 (20 μM) and STAT-3 inhibitor (50 μM) almost completely abrogated both virus-mediated IL-6 (Figure 4A) and NO (Figure 4B) production induced by PRV infection at 24 hours. We next tested which signaling pathway leads to the differential effects of AG490 and STAT-3 inhibitor on the regulation of iNOS, COX-2, NF-κB p50, and NF-κB p65 expressions in virus-infected cells (Figure 4C). The results showed that the inhibitory effect of AG490 and STAT-3 inhibitor on PRV-induced NO production was mediated by the inhibition of iNOS and COX-2 expressions via the NF-κB pathway.

We thus further investigated whether the suppressive effect of Alga on JAK–STATs is correlated to its antioxidant effects on ROS. We further examined the effect of Alga, AG490, and STAT-3 specific inhibitor on virus-induced ROS production. The results indicated that pretreatment with Alga, AG490, and STAT-3-specific inhibitor produced similar effects on ROS production in PRV-infected RAW264.7 cells (Figure 4D). The findings indicate that JAK–STAT signaling is required for ROS, IL-6, and NO release and provide evidence of the critical involvement of JAK/STAT signaling in PRV-infected RAW264.7 cells.

### 3.5. Alga inhibits virus-induced activation of NF-κB, iNOS, and COX-2 in RAW264.7 cells

NF-κB is a well-known transcription factor leading to the induction of proinflammatory genes, such as iNOS and
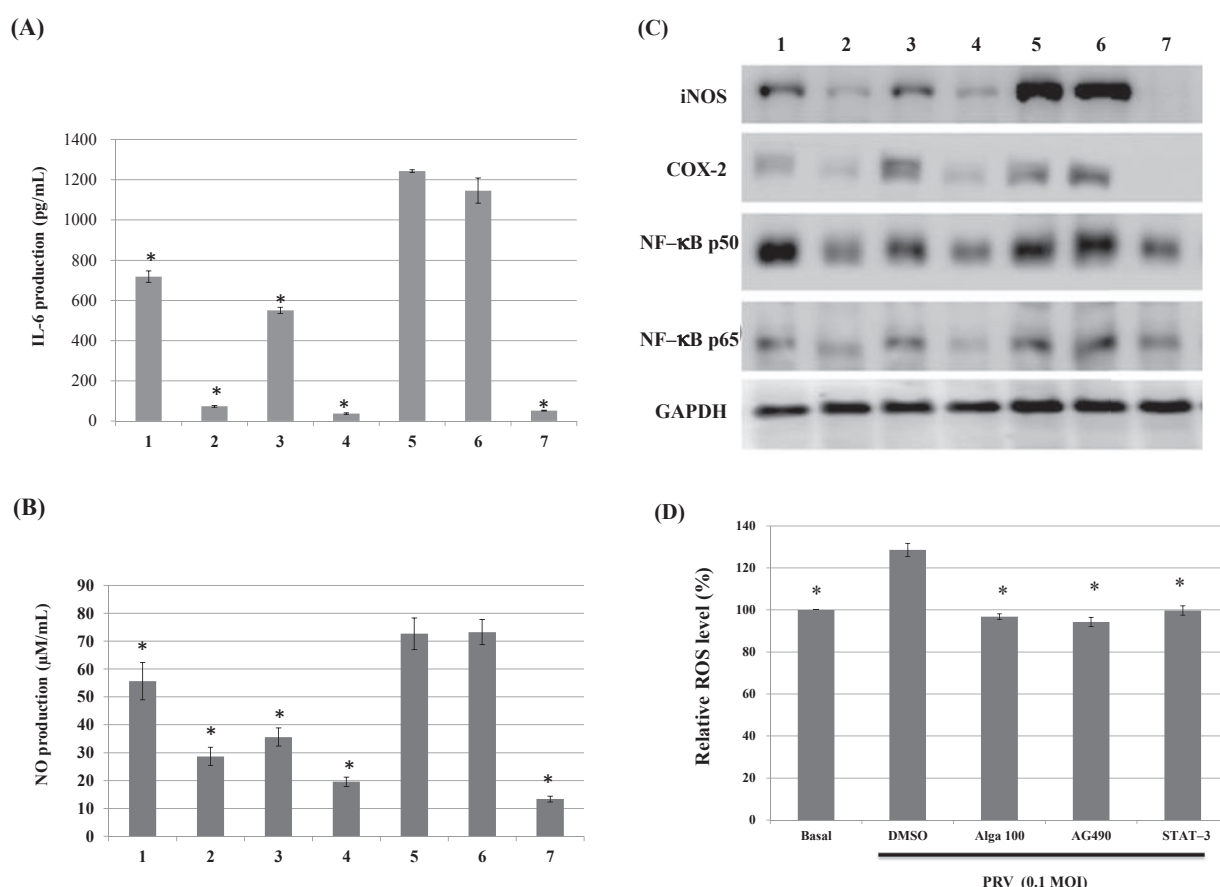


Figure 4 – Effect of JAK/STAT inhibitors on PRV-induced ROS and inflammatory response in RAW264.7 cells. (A) IL-6 and (B) NO production was determined by ELISA and Griess reagent. RAW264.7 cells were pretreated with different concentrations of STAT-3 inhibitor (Line 1: 25 μM; Line 3: 50 μM) or JAK2 inhibitor AG490 (Line 2: 10 μM, Line 4: 20 μM), and DMSO (Line 5), or received no treatment (mock, Line 6) for 1.5 hours; the cells were then infected with PRV or left uninfected (basal, Line 7) for 24 hours. (C) Cell lysates were prepared and subjected to Western blot analysis using antibodies against iNOS, COX-2, and NF-κB (p50 and p65); the signals were normalized against the GAPDH. (D) RAW264.7 cells were untreated (DMSO) or pretreated with Alga (100 μM), AG490 (20 μM), and STAT-3 inhibitor (50 μM) for 1.5 hours, and then infected with PRV or left uninfected (basal). After 24 hours, the cells were stained with 10 μM of DCFH-DA for 30 minutes, and the ROS production was determined using a fluorescence microplate reader. All measurements were performed in triplicate. The results are presented as means ± SD of three independent experiments. *p < 0.01, significant compared with PRV-infected RAW264.7 cells (DMSO). Alga = D. salina carotenoid extract; DMSO = dimethyl sulfoxide; ELISA = enzyme-linked immunosorbent assay; IL = interleukin; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; iNOS = inducible nitric oxide synthase; COX-2 = cyclooxygenase; JAK = Janus kinase; NF-κB = nuclear factor-κB; PRV = pseudorabies virus; SD = standard deviation; ROS = reactive oxygen species; STAT = signal transducer and activator of transcription.

COX-2 genes [12,15,16]. Our previous study showed that β-carotene has an anti-inflammatory effect on PRV-infected cells [9]. The results of the present study showed that all-*trans*-β-carotene and all-*trans*-α-carotene are components of commercial carotenoid products and are also the major components of *D. salina* algal extract [5,9,10].

We first tested whether the suppressive effects that Alga had on anti-inflammatory responses via the inhibition of iNOS, COX-2, and NF-κB expressions were similar to the effects of α- and β-carotene. In order to elucidate the mechanism by which Alga inhibits PRV-stimulated NO production, we investigated its effect on iNOS and COX-2 protein expressions. This result demonstrates that the Alga could inhibit NO production through downregulation of PRV-induced iNOS expression in RAW264.7 cells. In addition, Alga could also inhibit COX-2 expression in RAW264.7 cells infected by PRV (Figure 5A). The experimental data further revealed that NF-κB p50 and p65 expressions were upregulated in RAW264.7 cells infected with PRV when compared with the uninfected ones; however, Alga significantly inhibited ($p < 0.01$) the protein expressions of NF-κB p50 and p65 in PRV-infected RAW264.7 cells (Figure 5B). These results suggest that the inhibitory effect of Alga on PRV-induced NO production was mediated by the inhibition of iNOS and COX-2 expressions via the NF-κB pathway. These findings also indicated that the Alga had an anti-inflammatory effect via the inhibition of iNOS, COX-2, and NF-κB expressions.

### 3.6. Alga inhibits virus-mediated activation of the JAK/STAT pathway

Activation of the NF-κB pathway plays an essential role in mediating the response to proinflammatory stimuli. Therefore, to investigate the involvement of these signals in IL-6 regulation by Alga, we compared the phosphorylation levels of various signaling molecules in RAW264.7 cells during virus infection. The image in Figure 6 shows that virus-activated JAK2, STAT-1, and STAT-3 phosphorylation was completely blocked by Alga, supporting the hypothesis that Alga exerts its differential effects on the inhibition of mediated iNOS and COX-2 induction through regulation of the JAK/STAT pathway.

STAT-1 has been reported to be an important transcription factor for iNOS [14,19,21]. STAT-3 is a key signaling molecule for many cytokines and growth factor receptors. Therefore, to understand the Alga-induced downregulation of iNOS, we tested the hypothesis that Alga downregulates iNOS protein expression by suppressing the JAK/STAT signaling cascade. Phosphorylation of STAT-1 and STAT-3 peaked after 9 hours of virus infection, and this activation was reduced by pretreatment with 100 μM of Alga. As expected, Alga inhibited the phosphorylation of STAT-1 and STAT-3 in a time-dependent manner (Figure 6).

These data demonstrated that Alga caused decreased activation of STAT-1 and STAT-3 in the PRV-infected cells, implying that STAT-dependent inflammatory signaling may be related to the anti-inflammatory activity of Alga. To verify the hypothesis that Alga is a JAK/STAT pathway inhibitor, we first determined the time-course activation of JAK/STATs



Figure 5 — Alga inhibits virus-induced activation of iNOS, COX-2, and NF-κB in RAW264.7 cells. RAW264.7 cells were untreated (DMSO) or pretreated with 100 μM of Alga for 1.5 hours, and then infected with PRV (DMSO) or left uninfected (basal). After 6–24 hours, protein levels of (A) iNOS and COX-2, and (B) NF-κB p50 and p65 in the RAW264.7 cells were assayed via Western blot analysis. All measurements were performed in triplicate. Alga = *D. salina* carotenoid extract; DMSO = dimethyl sulfoxide; iNOS = inducible nitric oxide synthase; COX-2 = cyclooxygenase; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; NF-κB = nuclear factor-κB; PRV = pseudorabies virus.
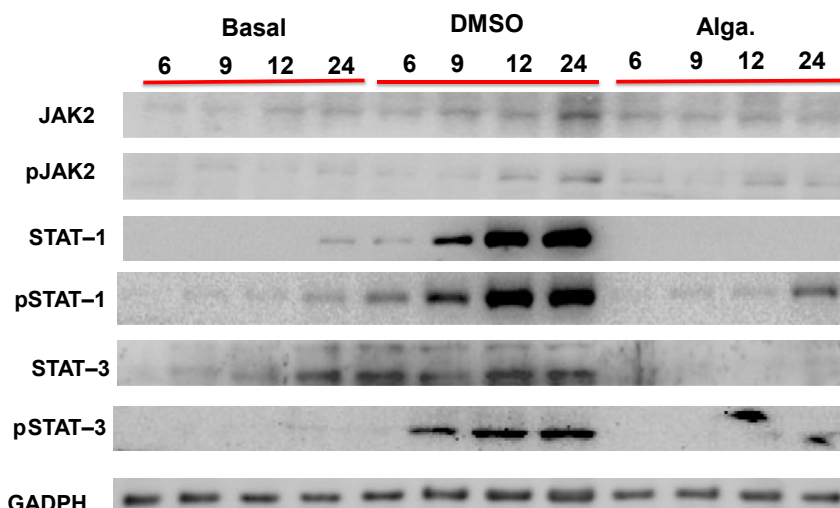
**Figure 6** — Alga inhibits the expressions of JAK, pJAK, STAT-1, STAT-3, pSTAT-1, and pSTAT-3 in PRV-infected RAW264.7 cells. RAW264.7 cells were untreated (DMSO) or pretreated with 100 μM of Alga for 1.5 hours, and then infected with PRV or left uninfected (basal) for 6—24 hours. Total protein was subjected to 10% SDS—PAGE followed by Western blotting using JAK, pJAK, STAT-1, pSTAT-1, STAT-3, pSTAT-3, and GAPDH antibodies. All measurements were performed in triplicate. Alga = *D. salina* carotenoid extract; DMSO = dimethyl sulfoxide; JAK = Janus kinase; PRV = pseudorabies virus; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; pSTAT = tyrosine-phosphorylated signal transducer and activator of transcription; STAT = signal transducer and activator of transcription.

induced by viral infection in RAW264.7 cells. In this study, we first examined whether Alga affects STAT-1 and STAT-3 activation in virus-infected RAW264.7 cells.

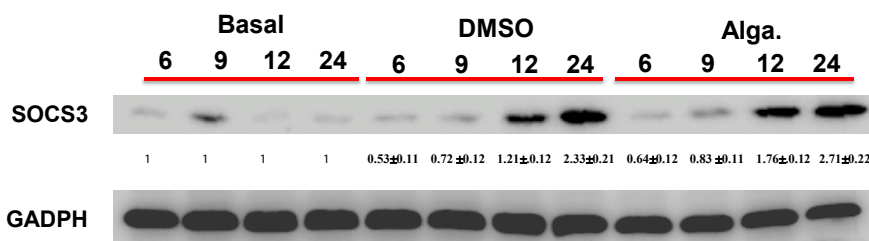### 3.7. Alga regulates the expression of SOCS3 in PRV-infected RAW264.7 cells

Experimental results of this study support the conclusion that Alga strongly inhibits IL-6 production (Figure 1). However, it is known that the IL-6 family of cytokines mediates its action primarily through activation of the JAK/STAT pathway along with other intracellular signaling pathways. A recent study reported that SOCS1 and SOCS3, intracellular negative-feedback molecules, selectively inhibit LPS-induced IL-6 production [17,19,29,30]. Therefore, we investigated whether

the JAK/STAT pathway and SOCS3 levels correlated with the inhibition of IL-6 production induced by Alga pretreatment. As expected, it was found that the SOCS3 level was increased at 12 hours and 24 hours after pretreatment with Alga (compared with DMSO) in PRV-infected RAW264.7 cells (Figure 7).

Our results suggest that Alga inhibits ROS, IL-6, and NO by suppressing the activation of STAT-1 and STAT-3 (Figure 6) and through the accumulation of SOCS3 (Figure 7).

### 3.8. Alga affects viral replication

On the basis of our findings, Alga decreases the expressions of iNOS, COX-2, ROS, and inflammatory cytokines by inhibiting the NF-κB pathway. This raises the concern that viral



**Figure 7** — Alga regulates the expression of SOCS3 in PRV-infected RAW264.7 cells. RAW264.7 cells were untreated (DMSO) or pretreated with 100 μM of Alga for 1.5 hours, and then infected with PRV or left uninfected (basal) for 6—24 hours. Total protein was subjected to 10% SDS—PAGE followed by Western blotting using SOCS3 and GAPDH antibodies. All measurements were performed in triplicate. Data are presented as mean ± SD ($n = 3$). Alga = *D. salina* carotenoid extract; DMSO = dimethyl sulfoxide; PRV = pseudorabies virus; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; SD = standard deviation; SOCS3 = suppressor of cytokine signaling 3.
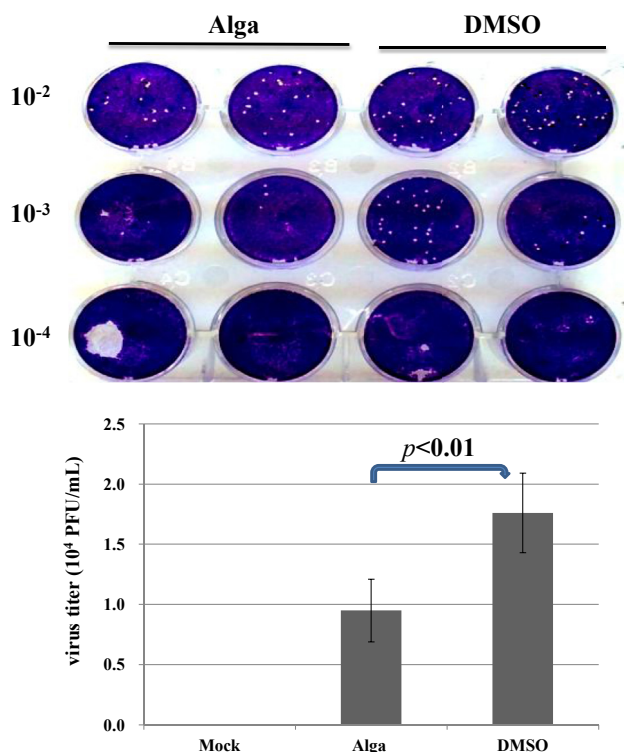
**Figure 8 – Antiviral activity of RAW264.7 cells treated with Alga. RAW264.7 cells were pretreated with the Alga (100 μM) or DMSO for 1.5 hours. The cells were infected with the PRV of 0.1 MOI for 12 hours. Supernatants were collected and frozen at −70°C. Afterward, the supernatants were used to determine the level of virus titer by plaque assay. The data represent the average number of PFU/mL. The results are presented as means ± SD (n = 3); p < 0.01, significant compared with PRV-infected RAW264.7 cells (DMSO). Alga = D. salina carotenoid extract; DMSO = dimethyl sulfoxide; MOI = multiplicity of infection; PFU = plaque forming unit; PRV = pseudorabies virus; SD = standard deviation.**

replication may be increased in the presence of Alga. Consequently, we infected RAW264.7 cells with PRV in the presence or absence of Alga and measured the viral titers by standard plaque assays. The results demonstrated that Alga decreased viral replication ($p < 0.005$) at 12 hours (Figure 8), but not at 24 hours (data not shown). Hence, this study indicated that Alga treatment interfered with PRV replication in RAW264.7 cells inhibiting the extracellular virus release.

These data showed that despite inhibition of inflammation mediator and viral replication in the presence of Alga.

## 4.  Conclusions

Experimental investigations in this study demonstrated that Alga shows good antioxidant and anti-inflammatory effects via its inhibition of ROS, IL-6, and NO release in virus-infected RAW264.7 cells (Figures 1–3). In addition, the inhibitory effects of Alga were similar to those caused by pretreatment with the specific inhibitors of JAK and STAT-3 in PRV-infected

RAW264.7 cells (Figures 4–6). Furthermore, Alga enhanced the expression of SOCS3 to suppress the activity of the JAK/STAT signaling pathway in PRV-infected RAW264.7 cells (Figure 7). At the same time, Alga treatment interfered with PRV replication in RAW264.7 cells inhibiting the extracellular virus release at early infection (Figure 8).

Therefore, our results demonstrate that Alga inhibits ROS, IL-6, and NO production via suppression of the NF-κB and JAK/STAT pathways and through regulation of SOCS3 in virus-infected RAW264.7 cells. In summary, the results of this study indicate that Alga *has moderate antioxidant and anti-inflammatory activities—findings that suggest potential new applications for Alga in the future.*

## Conflicts of interest

All authors declare no conflicts of interest.

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