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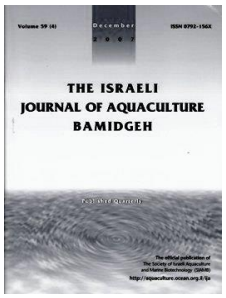

In Vitro Activation of *Epinephelus Malabaricus* Head-Kidney Leukocytes by *Angelica Sinensis* Polysaccharide

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Keywords: *Angelica sinensis* polysaccharide; bactericidal activity; *Epinephelus malabaricus*; leukocytes proliferation; phagocytic activity; respiratory burst

Abstract

Phagocytic, respiratory burst, bactericidal, and proliferative activity stimulation of *Epinephelus malabaricus* head-kidney leukocytes by *Angelica sinensis* polysaccharide (ASP) were evaluated *in vitro*. Leukocytes were incubated for 2, 6, 12 and 24 h in the presence of 0, 10, 100 and 1000 μg/mlASP or 100 μg/ml lipopolysaccharide (LPS) before phagocytic and respiratory burst activity assay, and for 24 h with 0, 100, 1000, 10000 μg ASP/ml before bactericidal activity detection. Meanwhile, leukocytes were incubated for 24 and 48 h followed by proliferative activity evaluation. The results showed that ASP boosted leukocytes phagocytic, respiratory burst, bactericidal, and proliferative activity significantly in a dose-dependent manner. Leukocytes incubated with 100 μg/ml ASP exhibited the highest phagocytic and respiratory burst activity. Leukocytes incubated with 1000 μg/ml ASP showed the highest bactericidal activity, which was followed by leukocytes incubated with 100μg/ml ASP. Leukocyte proliferation was significantly enhanced with increasing concentration of ASP, and reached the highest value with 10000 μg/ml ASP. Compared with LPS in the same concentration (100 μg/ml), ASP exerted a stronger stimulating effect on respiratory burst but was weaker on proliferation in *E. malabaricus* leukocytes. These findings indicate that ASP prototype enhanced cellular immunity of *E. malabaricus*, and potentially contributed to the pursuit of environmentally friendly prophylactic measures to enhance the immunity of *E. malabaricus*.

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Introduction

Epinephelus malabaricus is high quality seafood found in tropical waters throughout the Indo-West Pacific areas from East Africa to the Tonga Islands. In the Asia-Pacific region, it has become one of the most important cultured marine fishes, due to its tolerance to intensive culture conditions, rapid growth, large size, desirable taste, and high market demand. However, grouper culture has encountered outbreaks of various infectious diseases caused by virus, bacteria, and parasites. A review of grouper health, pointed out that the use of herbal medicine as a more environmentally friendly technique has achieved remarkable success in disease management (Harikrishnan et al. 2011). Herbal medicines and their extracts are considered promising immunostimulants, due to their easy availability, broad spectrum activity, high effectiveness, and eco-friendly prophylactic measures (Bhuvaneswari and Balasundaram, 2006). A wide variety of herbs and herbal extracts as immunostimulants have been used in aquaculture (Van Hai 2015).

Angelica sinensis polysaccharide (ASP) exists in the root of traditional Chinese herbal medicine *Angelica sinensis* (Oliv.) Diels. Biological activities, and correlation of structure and biological activities of ASP as well as extraction and purification techniques have been reviewed (Jin et al. 2012). They concluded that ASP exhibits various important bioactive properties including antioxidant, antitumor (Cao et al., 2010), hematopoietic, hepatoprotective (Ye et al., 2001), immunomodulatory (Yang et al., 2008), radioprotective activity as well as gastrointestinal protective effects on terrestrial animals. Several *in vitro* studies demonstrated that ASP prototype triggered immune responses in mice (Chen et al., 2010). However, there is limited data regarding the immunomodulatory function of ASP in fish. Dietary supplementation of ASP was found to enhance disease resistance in *E. malabaricus* against *Edwardsiella tarda* as well as phagocytosis, proliferation, and respiratory burst activity of head kidney leukocytes (Wang et al 2012).

The objective of the present study was to examine the *in vitro* effects of ASP prototype on *E. malabaricus* leukocyte phagocytic, respiratory burst, bactericidal, and proliferative activity.

Materials and methods

Fish and ASP. Thirty healthy groupers (initial mean weight 406 ± 12 g) were sampled from a culture tank (6.0 m × 6.0 m × 1.2 m) in a recirculating system (Haifa Seafood Industrial Development Co., Ltd) in Tianjin, China. Fish were fed a commercial feed (Haiqi® formulated feed for grouper, protein ≥ 52%, and lipid ≥ 12%, twice daily (7:30 am and 16:30 pm). The recirculating system consisted of a sieve bend screen, reservoir, biological filter, supplemental aeration, and circulation pump. The sieve bend screen was used to remove fish feces from seawater and maintain water quality. Water was renewed once a day at a rate of 2% of the system volume. Water temperature was maintained at 28.2 ± 0.4 ℃, salinity at 33.1 ± 1.2 ‰, dissolved oxygen at 8.5 ± 0.2 mg/L, pH 7.8 ± 0.2, ammonia below 0.12 mg/L, and nitrite below 0.07 mg/L. The light intensity was less than 500 lx and the photoperiod maintained at 12 h light/12 h dark cycle. The ASP was obtained according to the method described by Wang et al. (2011a). Total sugar, reducing sugar, uronic acid, and protein in the ASP were 76.72 ± 1.70%, 4.02 ± 0.12%, 13.82 ± 0.41%, and 4.87 ± 0.05%, respectively.

Preparation of glutaraldehyde-fixed rabbit red blood cells (GRRBCs). GRRBCs were prepared according to Wang et al. (2011a). Prior to use, GRRBCs were re-suspended in RPMI-1640 (Gibco, USA) maintenance medium (mRPMI) with 5% fetal bovine serum (FBS, Zhejiang Tianhang Biotechnology Co., Ltd., Zhejiang, China), 100 IU/mL ampicillin (Genview, Florida, USA), 0.1 mg/mL streptomycin sulfate (Genview, Florida, USA), counted in a Neubauer hemocytometer, and calibrated to 1.8×10^7 cells/ml.

Isolation of head-kidney leukocytes. Ten fish at a time were anesthetized with 100 mg/L MS-222 (Hangzhou Animal Pharmaceuticals Company, Zhejiang, China). Blood was drawn from the caudal vein with a 5 ml syringe, put into 1.5 ml Eppendorf tubes, left to clot overnight at 4^0C. Sera were pooled together and stored at -85^0C for later use. The anesthetized fish were sterilized with 75% ethanol (v/v). Head-kidney was aseptically

excised, rinsed with sterilized 0.85% sodium chloride solution, and cut into 5-10 mm^3 pieces with sterilized scissors. The head-kidney fragments were mixed with 5 ml of RPMI-1640 medium contained 20 IU/mL heparin sodium (Solarbio, Beijing, China), 100 U/mL ampicillin, 0.1 mg/mL streptomycin sulfate, transferred to a sterilized 150 μm stainless steel mesh. Cell suspension was obtained by forcing the fragments through the mesh with a syringe rod. The resulting cell suspension was centrifuged (400 *g*, 4^0C) for 10 min. The cell precipitation was washed (400 *g*, 4^0C, 10 min) three times with phosphate buffer solution (0.1 M PBS, pH 7.4). After three washes, the cells were resuspended in mRPMI, placed on a 1.020/1.059 Percoll (Sigma, Beijing, China) density gradient, and centrifuged at 400 *g* for 25 min at 4^0C. The isolated cells at the Percoll interface were collected and washed twice with mRPMI. Cell viability was examined by trypan blue (Sigma, Beijing, China) exclusion in a Neubauer hemocytometer. In all samples, cell viability was greater than 96%. The leukocytes were calibrated to 2×10^7 cells/mL of mRPMI for later use. The leukocytes of 10 fish were mixed together as one pooled sample. In total, three pooled samples were used to assay the phagocytic, respiratory burst, bactericidal, and proliferation activities.

Phagocytic activity. Phagocytic activity was measured according to the method described by Wang et al. (2011a) and Gebran et al. (1992) with some modifications. Cell suspension was calibrated to 5×10^7 cells/mL in RPMI-1640 solution. One hundred microliters of leukocytes suspension were placed in wells (n=6) of a flat-bottomed 96-well cell culture plate, and incubated for 3 h in a humidified incubator (Heracell™ 150i, Thermo Scientific) containing 5% CO_2. Non-adherent cells were removed by washing the cultures twice with phenol red-free Hank's balance salt solution (HBSS, Beijing Dingguo Changsheng Biotech. Co. Ltd., Beijing, China). The remaining monolayer was incubated for 2, 6, 12, or 24 h in mRPMI containing 0, 10, 100, 1000 μg/ml ASP in the humidified 5% CO_2 incubator. After incubation, half of the cells (three wells) from the same sample were fixed with 0.5% (v/v) paraformaldehyde (Beijing Dingguo Changsheng Biotech. Co. Ltd., Beijing, China) in PBS for 60 min and used as the control. All wells were washed once with mRPMI, and 100 μl GRRBCs (1.8×10^7cells/mL in mRPMI) was added to each well. Phagocytosis was allowed to proceed for 30 min at 27^0C in the humidified 5% CO_2 incubator. Then all wells were washed three times to remove non-ingested and non-attached GRRBCs. One hundred microliters of 0.2 M Tris-HCl (Roche, Sigma-aldrich, China, pH 7.4 in 6 M urea) were added to each well and left for 5 min at room temperature, then 100 μl 2,4-diaminofluorence (Sigma, Beijing, China) substrate solution containing 0.27% H_2O_2 was added to each well. The plate was incubated for 5 min at room temperature, and the O.D. was read at 608 nm using a microplate reader (VarioskanFlash, Thermo Scientific, China). Phagocytosis activity was calculated as $O.D._{test\ well} - O.D._{control\ well}$. Activation of phagocytosis was calculated as 100 × [(phagocytosis activity in wells with ASP – phagocytosis activity in wells without ASP)/phagocytosis activity in wells without ASP].

Respiratory burst. A nitroblue tetrazolium (NBT) reduction method was adopted to assay the respiratory burst, followed by Wang et al. (2011a) with some modifications. Briefly, a 100 μl aliquot of leukocyte suspension (5×10^6 cells/mL) was placed in a flat-bottomed 96-well cell culture plate (Greiner Bio-one, Germany) and incubated for 3 h at 27^0C in a humidified 5% CO_2 incubator. Non-adherent cells were removed by washing the cultures twice with phenol red-free HBSS. One hundred microliters of APS at 0, 10, 100, 1000 μg/ml in HBSS were added to three wells, and 100 μl of Lipopolysaccahride (LPS, Sigma, Beijing, China) at 100 μg/ml in HBSS were added to another three wells to serve as positive control. Then the leukocytes were incubated for 2, 6, 12, or 24 h in the humidified 5% CO_2 incubator. After incubation, a 100 μl aliquot of nitroblue tetrazolium (NBT, 2 mg/mL, Sigma, Beijing, China) was added to each well and incubated at 27^0C for 30 min. Then, the HBSS was discarded and the reaction was stopped by adding methanol. After washing twice with 70% (v/v) methanol, the formazan formed in each well was dissolved by adding 120 μl of 2 M KOH (Sigma-Aldrich, Beijing, China) and 140 μl of dimethyl sulphoxide (DMSO, Sigma, Beijing, China). The optical density (O.D.) was measured at 690 nm using the microplate reader mentioned above. Respiratory burst

activity was expressed as NBT reduction, expressed as $O.D._{690nm}$ per 5×10^6 cells. Activation of respiratory burst was calculated as 100 × [(O.D. in test cells – O.D. in control cells)/O.D. in control cells.

Bactericidal assay. The ability of head-kidney leukocytes to kill *Edwardsiella tarda* was assayed following Pohlenz et al. (2012) and Shoemaker et al. (1997) with some modifications. *E. tarda* was cultured in beef-extract peptone broth (BPB, Qingdao Hope Biol-Technology Co., Ltd., Qingdao, China) at 28^0C for 24 h, then centrifuged at 2000 *g* for 10 min, after which the pellets were washed twice in sterile 0.85% sodium chloride, and resuspended in 4 ℃ BPB. Bacteria suspension was calibrated to 1×10^8 cells/mL prior to use using a bacterial counter chamber, and was stored at 4^0C. One hundred microliters of leukocytes suspension (5×10^6 cells/mL) were added to each well in a sterile flat bottom 96-well microplate. This microplate was incubated at 27^0C for 2 h in a 5% CO_2 incubator for the attachment of macrophages, then washed twice with antibiotic free (af)-RPMI-1640. Then, 100 μl of af-RPMI-1640 solution containing ASP were added to each corresponding well. The final concentration of ASP in wells was 0, 100, 1000, 10000 μg/ml, respectively. Each ASP concentration was repeated in six wells. Twenty microliters of bacterial suspension in BPB was added to each well and the microplate was centrifuged at 150 *g* for 5 min to bring bacteria into contact with macrophages. The combined cell cultures (macrophages + bacteria) were incubated at 27^0C for 0 h or 2.5 h. After each incubation period, supernatants were removed and macrophages were lysed with 50 μl of 0.2% Tween 20 (Beijing Dingguo Changsheng Biotech. Co. Ltd., Beijing, China) to stop intracellular killing. Fresh BPB (100 μl per well) was added for *E. tarda* growth after macrophage lysis, and the plate was incubated for 18 h at 27^0C. After the incubation, 20 ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, 1 mg/mL, Sigma, Beijing, China) was added to each well and the plate was incubated for an additional 15 min. Reduction of MTT by *E. tarda* surviving in macrophages was used as a measure of intracellular killing. Absorbance was read at 620 nm using a microplate reader. Bacterial concentrations were calculated by comparing the absorbance obtained for each well to a standard curve previously constructed (data not shown). Bactericidal activity [% killing = (bacteria 0 h – bacteria 2.5 h) × 100/bacteria 0 h] was computed for each well and was presented as mean % killing per treatment.

Leukocyte proliferation. Leukocyte proliferation was measured according to Wang et al. (2011a) with minor modifications. The head-kidney leukocytes were resuspended in RPMI-1640 proliferation medium containing 10% FBS, 2% heat-inactivated grouper sera (45 ℃ for 30 min), 100 IU/mL ampicillin, and 0.1 mg/mL streptomycin sulfate. A 90 μl aliquot of proliferation medium containing 5×10^5 cells and 10 μl ASP or LPS (100 μg/mL mRPMI) was added to wells of a 96-well cell culture plate and incubated for 24 h or 48 h at 27^0C in a humidified 5% CO_2 incubator. The final concentration of ASP in the 96-well plate was 0, 100, 1000, 10000 μg/ml, respectively. Then, 20 μl of MTT (1 mg/mL) was added to each well and incubated for 4 h at 27^0C. The plate was then centrifuged at 500 *g* for 10 min at 4^0C. The supernatant was discarded, and the formazan crystals in each well were dissolved by adding 200 μl DMSO and 25 μl of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). The contents in the wells were thoroughly mixed on a micro-oscillator (ZW-A, Youlian Instrument Research Institute, Jiangsu, China), and the O.D. at 550 nm of the resulting suspension was measured in the microplate reader mentioned above.

Statistical analysis. Data were analyzed using one-way analysis of variance, followed by Tukey's Studentized Range (HSD) test to determine possible significant differences among treatments. Statistical analyses were conducted using SAS 9.4 (SAS Institute, Cary, NC, USA) software. Differences among treatments were considered significant at $P<0.05$. Data in figures were represented as means ± standard deviation (n=3, leukocytes from 10 fish were pooled as one replicate).

Results

Phagocytic activity. Phagocytic activity and activation of phagocytosis are presented in Fig. 1a, b. Leukocytes incubated in 10, 100 μg/mL ASP for 2, 6, 12, and 24 h exhibited significantly higher phagocytic activity than control. Phagocytic activity of leukocytes incubated in 1000 μg/mL ASP for 2, 6 h was not significantly higher, but for 12, 24 h was significantly higher than control. Leukocytes incubated in 100 μg/ml ASP exhibited the highest level of phagocytic activity among all the treatment groups at each incubation time. Activation of phagocytosis (Fig. 1b) of leukocytes incubated in 100 μg/ml ASP was highest among all the ASP incubated groups. Activation of phagocytosis in 1000 μg/L group was lower than the other two groups at 2, 6 h then increased to 10 μg/mL level at 12, 24h. Data in Fig. 1a, b indicate that 12 h incubation with ASP is suitable for the *in vitro* leukocytes phagocytic test.

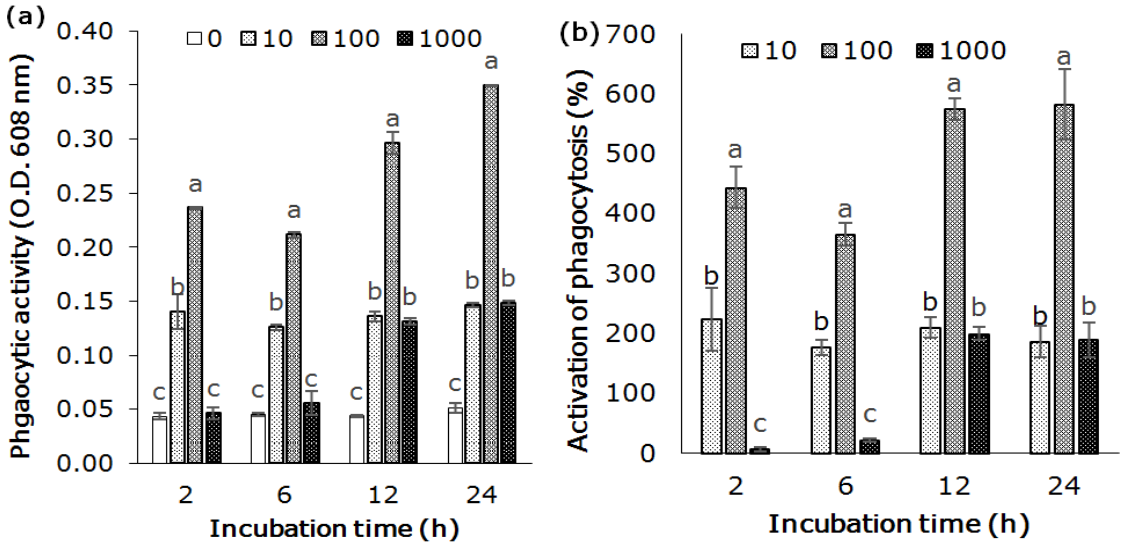

Fig. 1. Head kidney leukocytes phagocytic activity **(a)** and activation of phagocytosis **(b)** of *E. malabaricus* after 2, 6, 12, and 24 h incubation in 0, 10, 100, 1000 μg/ml ASP. Activation of phagocytosis (%) was calculated as [(phagocytosis activity in wells with ASP – phagocytosis activity in wells without ASP)/phagocytosis activity in wells without ASP] × 100. Each bar represents mean (± SD) of three replicates. Bars not sharing a common letter in the same incubation time are significantly different based on analysis of variance ($P < 0.05$) followed by Tukey's Studentized Range (HSD) test. ASP, *Angelica sinensis* polysaccharide.

Respiratory burst. The respiratory burst activity is presented in Fig. 2a. Compared with control group, LPS significantly stimulated respiratory burst activity, which indicates the vitality of leukocytes was normal *in vitro*. Both ASP and LPS significantly stimulated leukocyte respiratory burst at each incubation time. Leukocytes incubated in 100 μg/mL ASP exhibited highest respiratory burst activity among all groups at each incubation time. Data in Fig. 2b show that the activation of respiratory burst in 100 μg/mL group is the highest among all groups at each incubation time. In terms of leukocytes incubation time, two hours incubation with ASP is enough to test ASP's effect on leukocytes respiratory burst *in vitro*.

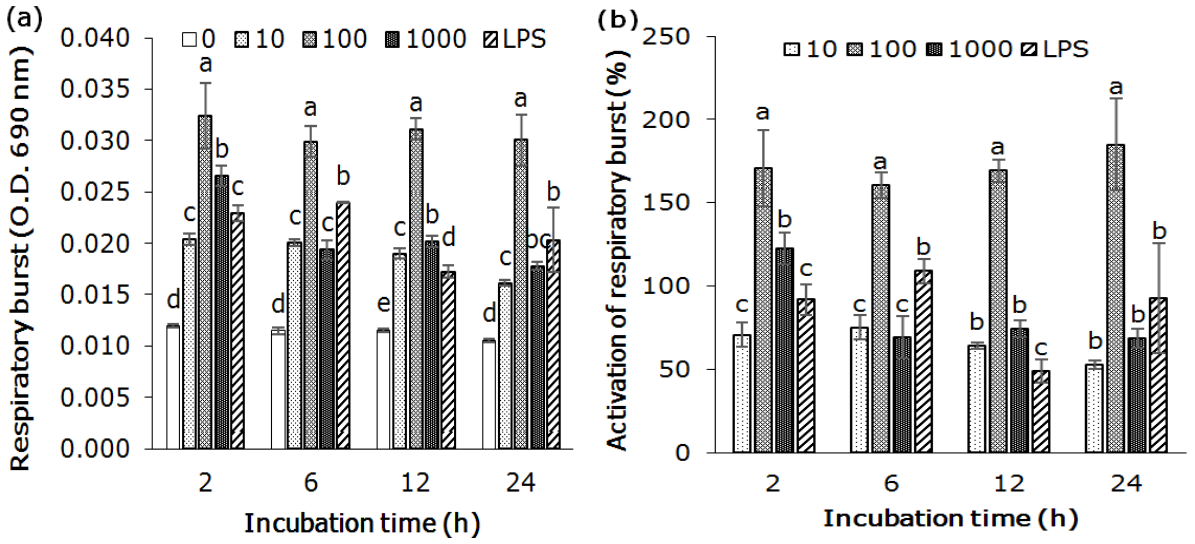

Fig. 2. Head kidney leukocytes respiratory burst activity **(a)** and activation of respiratory burst **(b)** of *E. malabaricus* after 2, 6, 12, and 24 h incubation in 0, 10, 100, 1000 μg/ml ASP or 100 μg/ml LPS. Activation of respiratory burst (%) was calculated as [(O.D. in test cells – O.D. in control cells) / O.D. in control cells] × 100. Each bar represents mean (± SD) of three replicates. Bars not sharing a common letter in the same incubation time are significantly different based on analysis of variance ($P < 0.05$) followed by Tukey's Studentized Range (HSD) test. ASP, *Angelica sinensis* polysaccharide. LPS, lipopolysaccharide.

Bactericidal assay. Bactericidal activity is presented in Fig. 3a. Compared with control, leukocytes bactericidal activity was significantly higher after 24 h incubation with ASP. Leukocytes incubated with ASP at 1000 μg/mL exhibited the highest bactericidal activity and activation of bactericidal activity (Fig. 3a, b). Similar bactericidal activity and activation of bactericidal activity were observed in leukocytes cultured in 100 and 10000 μg/ml ASP (Fig. 3a, b).

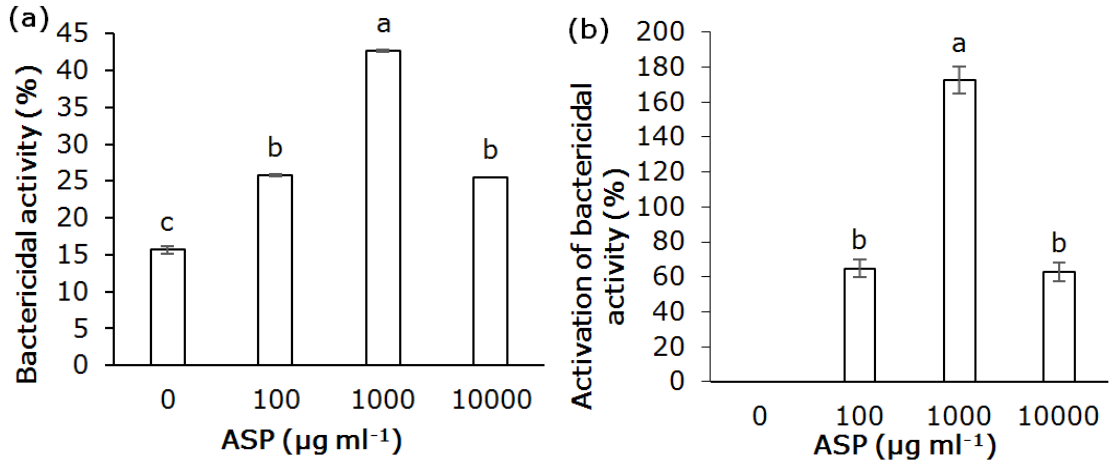

Fig. 3. Head kidney leukocytes bactericidal activity **(a)** and activation of bactericidal activity **(b)** of *E. malabaricus* after 24 h incubation in 0, 10, 100, 1000 μg/ml ASP. Bactericidal activity was calculated as [(bacteria 0 h – bacteria 2.5 h) / bacteria 0 h] × 100. Activation of bactericidal activity (%) was calculated as [(bactericidal activity in wells with ASP – bactericidal activity in wells without ASP) / bactericidal activity in wells without ASP] × 100. Each bar represents mean (± SD) of three replicates. Bars not sharing a common letter are significantly different based on analysis of variance ($P < 0.05$) followed by Tukey's Studentized Range (HSD) test. ASP, *Angelica sinensis* polysaccharide.

Leukocytes proliferation. The stimulated leukocytes proliferation cultured with ASP or LPS for 24 h and 48 h was observed in Fig. 4a. Leukocytes incubated in 1000, 10000 μg/ml ASP for 24, 48 h exhibited significantly higher proliferation than the control. Leukocytes incubated in 100 μg/ml ASP exhibited similar proliferation with control, but statistical differences between 100 μg/ml ASP and control were observed at 48 h. Activation of leukocytes proliferation varied significantly at 24 h, which was the highest in 10000 μg/mL, followed by 1000 μg/mL, and 100 μg/mL was the lowest (Fig. 4b). After 48 h incubation, activation of proliferation in 100 μg/ml ASP was the lowest but no statistically significant differences were observed among 1000, 10000 μg/ml ASP, and 100 μg/mL LPS groups (Fig. 4b). LPS exerted stronger stimulation on leukocyte proliferation than ASP in the same concentration (100 μg/ml) (Fig. 4a, b). Data in Fig. 4a, b indicate that 24 h incubation with ASP is apt for the *in vitro* leukocyte proliferation test.

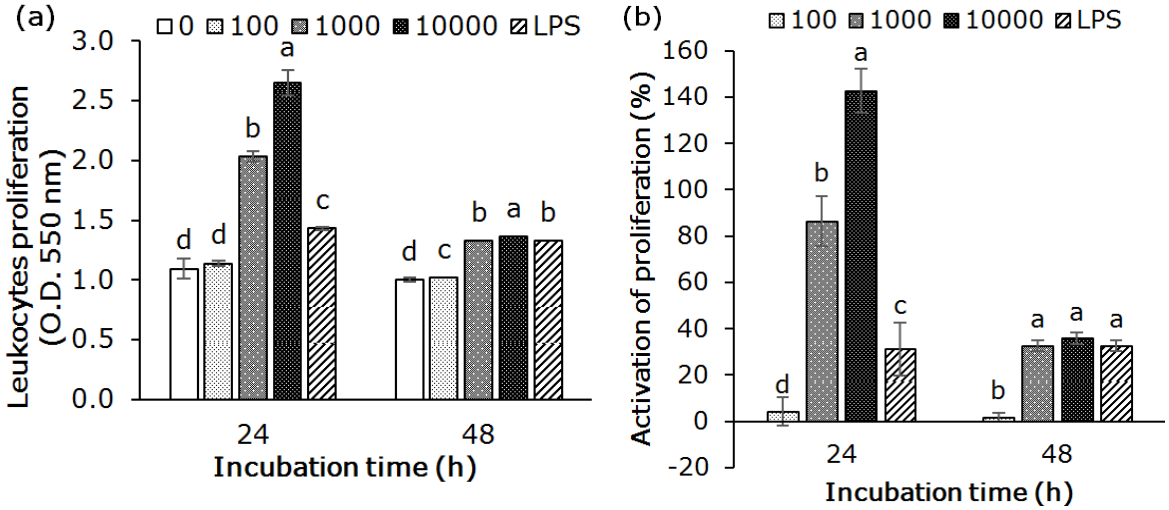

Fig. 4. Head kidney leukocytes proliferation **(a)** and activation of proliferation **(b)** of *E. malabaricus* after 24 and 48 h incubation in 0, 100, 1000, 10000 μg/ml ASP or 100 μg/ml LPS. Activation of proliferation (%) was calculated as [(proliferation in wells with ASP or LPS – proliferation in wells without ASP and LPS) / proliferation in wells without ASP and LPS] × 100. Each bar represents mean (± SD) of three replicates. Bars not sharing a common letter in the same incubation time are significantly different based on analysis of variance ($P < 0.05$) followed by Tukey's Studentized Range (HSD) test. ASP, *Angelica sinensis* polysaccharide. LPS, lipopolysaccharide.

Discussion

Botanical polysaccharides isolated from a wide range of flora species, including higher plants, mushrooms, lichens, and algae, exhibit robust immunostimulatory properties (Schepetkin et al., 2006). Most polysaccharides derived from higher plants are relatively nontoxic and exhibit limited side effects (Ovodov, 1998), that are considered a major issue deriving from immunomodulatory bacterial polysaccharides and synthetic compounds. To date, several studies have demonstrated immunity enhancement in aquatic animals with dietary supplemental higher plant-derived polysaccharide extracted from *Astragalus membranaceus* in sea cucumber *Apostichopus japonicas* (Wang et al., 2009), spotted maigre *Nibea albiflora* (Wang et al., 2012), yellow catfish *Pelteobagrus fulvidraco* (Bai et al., 2012), and tilapia *Oreochromis niloticus* (Zahran et al., 2014), *A. sinensis* in grouper *E. malabaricus* (Wang et al., 2012), *Ficus carica* in grass carp *Ctenopharyngodon idella* (Yang et al., 2015), *Druio zibethinus* in shrimp *Penaeus mondon* (Pholdaeng et al., 2010), and *Panax ginseng* in shrimp *Litopenaeus vannamei* (Liu et al., 2011). This indicates that plant-derived polysaccharides provide potential positive effects in aquaculture.

Plant-derived non-starch polysaccharide is not easily hydrolyzed by enzymatic systems secreted by digestive tract or gut microbiota of higher animals (Grosell et al., 2011). Consequently, such incompletely hydrolyzed polysaccharides serve as additional

immunostimulants (Westerhof et al., 2000). Although oral administration of aforementioned polysaccharide affects the immunity of aquatic animals, there is still no evidence that the prototype of these polysaccharides possess any immunostimulatory properties in aquatic animals. Our findings demonstrated that the ASP prototype can dose-dependently boost leukocyte phagocytic, respiratory burst, bactericidal activity and proliferation of *E. malabaricus*.

Macrophages play a vital role in host defenses against microorganisms by engulfing microbes and killing them by producing reactive oxygen species (ROS) during respiratory burst (Iwama et al., 1996).The phagocytic, respiratory burst, and bactericidal activity are useful indicators to assess the immune function of phagocytic cells, including circulating monocytes, neutrophils, and tissue macrophages. In the present study, the adherence method (Field et al., 1995) was used to harvest *E. malabaricus* head-kidney macrophages, which were further morphologically confirmed using an inverted microscope (Leica DMI4000B, Germany). Our results show that significantly high phagocytic and respiratory burst activity was observed in macrophages incubated with ASP, compared with control (Fig. 1, 2). Furthermore, ASP boosted macrophages phagocytic and respiratory burst activity in a dose-dependent manner: macrophages incubated in 100 μg/ml ASP exhibited the highest activity, while incubated in lower (10 μg/ml) and higher (1000 μg/ml) dosage of ASP exhibited relatively weaker activity. ASP exerted stronger activity to stimulate the respiratory burst of macrophages than LPS in the same concentration (Fig. 2a, b). Dietary ASP (500, 3000 mg/kg) enhanced both phagocytic and respiratory burst activity significantly in *E. malabaricus* head kidney leukocytes (Wang et al., 2011a). Dietary *Astragalus* polysaccharides significantly upregulated whole blood phagocytic activity and respiratory burst activity in Nile tilapia *O. niloticus (*Zahran et al. 2014). Dietary supplemental *A. membranaceus* polysaccharide significantly enhanced phagocytic activity and ROS production of coelomocytes in sea cucumber *A. japonicas* (Wang et al., 2009). Intraperitoneal injection of fucoidan, a polysaccharide mainly produced by brown algae, significantly enhanced phagocytic activity and respiratory burst activity in tilapia *O. niloticus* (Isnansetyo et al., 2015). These findings indicate that botanical polysaccharides upregulate phagocytic capacity of macrophages in aquatic animals.

However, the process of respiratory burst cannot kill all pathogens. For instance, *Renibacterium salmoninarum* can survive in mononuclear phagocytes depending on its ability to move from the phagosome into the cytoplasm (Gutenberger et al., 1997). Therefore, bactericidal assay is a further verification of phagocytized bacteria mortality in macrophages. Mosmann (1983) developed a rapid colorimetric assay based on MTT that measures only live cells utilizing spectrophotometry. This method was proved to be accurate to detect live *Escherichia coli* (Wang et al., 2011b). In the present study, ASP can significantly enhance the bactericidal activity of *E. malabaricus* head kidney phagocytes (Fig. 3a, b). Macrophages incubated in 1000 μg/ml ASP exhibited the highest bactericidal activity (Fig. 3a, b). So far, influence of botanical polysaccharides on macrophage bactericidal activity of other animals has not been found.

The ASP prototype is also a lymphocytes proliferation enhancer. The proliferation of T cells in BALB/c mice was directly activated by ASP, while B cells were not influenced, which indicated that ASP was T cell specific mitogenicity (Yang et al., 2006). Our results of leukocytes proliferation (Fig. 4a, b) indicated that *E. malabaricus* head kidney leukocytes were responsive to both LPS, which is B cell mitogenicity (Sizemore et al., 1984), and ASP, a T cell specific mitogenicity. These results reinforce the conclusion that teleost fish possess lymphocyte populations analogous in many respects to the T cells and B cells of mammals (Iwama et al., 1996).

In conclusion, ASP can enhance the *in vitro* phagocytic, respiratory burst, bactericidal, and proliferation responses of *E. malabricus* head kidney leukocytes, indicating that ASP prototype provides potential benefits on cellular immunity of *E. malabaricus*. The findings in this paper provide data showing the possibility that intraperitoneal injection of ASP could contribute to the immunity of *E. malabaricus*. Further intraperitoneal injection tests are recommended to verify the immune efficacy of ASP on *E. malabaricus*.

Acknowledgments

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