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Effect of *Punica granatum* solvent extracts on immune system and disease resistance in *Paralichthys olivaceus* against lymphocystis disease virus (LDV)

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ABSTRACT

We report the effect of aqueous, ethanol, and methanol solvent leaf extracts of *Punica granatum* on innate immune mechanisms, such as phagocytosis activity, respiratory burst activity, alternative complement activity, lysozyme activity and functional immunity in terms of percentage cumulative mortality and Relative Percent Survival (RPS) in olive flounder *Paralichthys olivaceus* naturally infected with lymphocystis disease virus (LDV) after 8 weeks. Infected fish were intraperitoneally administered with 0, 5, 50, and 100 mg kg^{-1} body weight of solvent extracts. In groups treated with 50 and 100 mg kg^{-1} body weight, the chosen innate immune parameters significantly increased after 8 weeks when compared to 0 mg kg^{-1} dose, but not with 5 mg kg^{-1}. Administration of *P. granatum* solvent extracts for 8 weeks significantly reduced the percentage mortality with the consequent increase in RPS. The results suggest that intraperitoneal administration of the leaf extracts of *P. granatum* at 50 or 100 mg kg^{-1} dose clearly enhance the innate immune responses and disease resistance after 8 weeks in *P. olivaceus* against natural LDV infection.

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1. Introduction

Olive flounder, *Paralichthys olivaceus* is one of the most important commercial food fish in Korea. Olive flounder production has increased through improved culture techniques [1]. Aquaculture production increased in South Korean from 147,000 metric ton (mt) in 1971 to over 1.2 million mt in 2006, of which olive flounder accounted for the higher production of about 48.1% [2].

Recently, mass mortality of adult flounder showing ascites occurred at many aquaculture farms in southern Korea due to diseases associated with viruses, bacteria, and parasites [3–5]. Of these, viral diseases such as Lymphocystis disease virus (LDV), viral haemorrhagic septicaemia virus (VHSV), and aquabirnavirus (ABV) are the most common pathogens in cultured fish causing serious economic loss in Asian countries such as Korea, Japan, and China [6–11].

Lymphocystis disease virus (LDV) belonging to the Iridovirus family is widely distributed over many countries; recently it has caused serious damage to olive flounder farms in South Korea [11–14]. Olive flounder infected with LDV develop characteristic hypertrophied lymphocystis cells that appear as nodules on skin, fins and/or mouth. The infected fish lose commercial value due to the appearance of tumor-like nodules; besides the nodules in mouth interfere with feeding, and sometimes lead to starvation. At present there are no medicines or commercially available vaccines to treat LDV infection [15]. Hence there is an urgent need to look for eco-friendly disease preventative measures to promote sustainable olive flounder production.

In aquaculture one of the most promising methods strengthening of the defense mechanism disease management is through prophylactic administration of immunostimulants [16]. In fish, the immunostimulants are known to increase certain aspects of innate immunity [17–20]. *Punica granatum* commonly known as the pomegranate, is a member of the Punicaceae (Pomegranate family) [21]. The primary bioactive constituents of pomegranate juice are the polyphenols and their potent antioxidant characteristics [22]. Punicalagin is a major antioxidant polyphenol in pomegranate juice [23]. The antibacterial and antimicrobial properties of *P. granatum* have been studied extensively. Extracts from the plant have been found to work against Methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant (MRSA) *S. aureus*, *Escherichia coli* O157:H7, *Salmoneela typhi*, and some streptococci strains [24–28]. The better

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antimicrobial activity of *P. granatum in vitro* against *Tenacibaculum maritimum* has been reported [29]. However, there is no report on the application of the herbal solvent extracts on the innate immune mechanism in olive flounder. This study aims to assess the effect of aqueous, ethanol, and methanol solvent extracts of *P. granatum* on innate immune mechanisms and disease resistance of *P. olivaceus* against natural lymphocystis disease virus infection.

2. Materials and methods

2.1. Fish

Lymphocystis disease virus (LDV) infected olive flounder *P. olivaceus* (total length 24 ± 3.5 cm, weight 547 ± 14.5 g) were transported from a local flounder farm of Jeju Island, South Korea, in January 2010. Fish were kept in 1000 l flow-through tanks at a rearing density of 16.5 kg/m^3under natural conditions. Temperature, salinity, and dissolved oxygen of the seawater during the experimental period were 18–20 °C, 32–35‰, and 5.7–6.3 ppm, respectively. The fish were fed with standardized pelletized feed *ad libitum* twice a day at 10:00 and 17:00 h at 3% of the body weight. Food was withheld from the fish for 24 h before sample collection.

2.2. Detection of LDV virus

To detect LDV in the collected olive flounder, the pooled skin and gills of each fish were homogenized in 10 volumes of HBSS. The homogenates were centrifuged at 2000 g at 4 °C to remove cell debris. The supernatant was collected and stored at −80 °C until used. For PCR amplification, total nucleic acids were extracted from 45 μl of homogenate supernatant using proteinase K (TaKaRa, Japan) and phenol–chloroform solution; the nucleic acid extracts were then subjected to PCR. The primer sequences and target regions for the fish viruses were followed as described in previous studies [10]. PCR amplification was performed using an AccuPower™ PCR premix kit (Bioneer, Korea), according to the manufacturer's instructions. PCR amplicons were subjected to electrophoresis in agarose gels containing ethidium bromide, and visualized under UV light.

2.3. Herbal solvent extracts

P. granatum was collected from Jeju Island, South Korea. The leaves were washed in sterile distilled water, shade dried, powdered, and stored at −20 °C until further use. Hundred grams each of the leaf powder was taken in three sterilized conical flasks (2 l) separately and added with 1 l of 94% solvents (distilled water, ethanol, or methanol) added and mixed well. Conical flasks tightly covered with aluminum foil were kept for 7 d at room temperature and agitated daily to ensure complete digestion; the extracts were then filtered through sterile muslin cloth. The solvent extracts were evaporated using rotary vacuum evaporator (Buchi SMP, Switzerland) and the obtained residue mixed with sterile distilled water, ethanol, and methanol were stored separately in sterilized screw cap glass container at −20 °C until use [30].

2.4. Experimental design

To study the innate immune mechanism four groups (n = 40 each) LDV positive olive flounders (n = 320) were injected (50 μl) intraperitoneally with 0, 5, 50 or 100 mg kg^{-1} body weight of aqueous, ethanol or methanol solvent extracts using 1-ml tuberculin syringe with 24-gauge needle. Each group was maintained in replicate. The cumulative mortality was recorded for 8 weeks. Relative percent survival (RPS) was calculated by the following formula of Amend [31].

$$\mathrm{RPS} = 1 - \left\{\frac{\text{Percent mortality in treated group}}{\text{Percent mortality in control group}}\right\} \times 100$$

2.5. Separation of leucocytes

Blood samples (6 fish at a time in each group) from the caudal vein were collected from one of the replicate tanks in each group on completion of 8th week of post-treatment. Heparin was used as an anticoagulant. Individual fish was sampled only once to avoid the influence on the assays due to multiple bleeding and handling stress on the fish. For assay leucocytes were separated from each blood sample by density-gradient centrifugation [32]. One milliliter of histopaque 1.119 (Sigma) containing 100 μl of bacto hemagglutination buffer, pH 7.3 (Difco, USA) was dispensed into siliconised tubes and added to 1 ml of a mixture of 1.077 density histopaque hemagglutination buffer; one ml of blood was carefully layered on the top and the tubes were centrifuged at 700 g for 15 min at 4 °C. After centrifugation, plasma was collected and stored at −20 °C until used for analysis. Separated leucocytes were gently removed and dispensed into siliconised tubes, containing phenol red free HBSS (Sigma). Cells were then washed twice in HBSS and adjusted to 1 × 10^7 viable cells ml^{-1}.

2.6. Phagocytosis activity

Phagocytic activity of blood leucocytes was determined spectrophotometrically; 1 ml of the leucocyte suspension was mixed with 2 ml of the congo red-stain and mixed with autoclaved yeast cell suspension so as to contain yeast cell:leucocyte ratio of 20:1 [33]. The mixtures were incubated at room temperature for 60 min. Following incubation, 1 ml ice-cold HBSS was added and 1 ml of histopaque (1.077) was injected into the bottom of each sample tube. Leucocytes were harvested by centrifugation at 850 g for 5 min to separate leucocytes from free yeast cells and washed twice in HBSS. The separated leucocytes were re-suspended in 1 ml trypsin–EDTA solution (5.0 g l^{-1} trypsin and 2.0 g l^{-1} EDTA, Sigma), and incubated at 37 °C overnight. The absorbance of the samples was measured at 510 nm using trypsin–EDTA as blank.

2.7. Respiratory burst activity

Respiratory burst activity of the isolated leucocytes was quantified by reduction of ferricytochrome C [34]. A volume of 100 μl of leucocyte suspension and an equal volume of cytochrome C (2 mg l^{-1} in phenol red free HBSS) containing phorbol 12-myristate 13-acetate (PMA, Cayman) at 1 mg ml^{-1} were placed in triplicate in microtitre plates. Correspondingly another 100 μl of leucocyte suspensions and solutions of cytochrome C containing PMA and superoxide dismutase (SOD, Cayman) at 300 U ml^{-1} were prepared in duplicate in microtitre plates. The plates were incubated at room temperature for 15 min and measured at 550 nm against a cytochrome C blank in a multiscan spectrophotometer. The obtained readings were converted to nmoles O_2^- by subtracting the O.D. of the PMA/SOD treated supernatant from that treated with PMA given alone for each fish, and converting O.D. to nmoles O_2^- by multiplying by 15.87. Final results were expressed as nmoles O_2^- produced per 10^5 blood leucocytes.

2.8. Natural haemolytic complement activity

Natural haemolytic complement of plasma was assayed using sheep red blood cells (SRBC, Biomedics) as targets [35]. Equal volumes of SRBC suspension (6%) in phenol red free HBSS containing Mg^{2+} and EGTA were mixed with serially diluted plasma to

give final plasma concentrations ranging from 10% to 0.078%. The samples were incubation at 22 °C for 90 min then centrifuged at 400 g for 5 min at 4 °C to avoid unlysed erythrocytes. The hemoglobin content of the supernatants was measuring at 550 nm in a microplate reader. The values of maximum (100%) and minimum (spontaneous) haemolysis were obtained by adding 100 µl of distilled water or HBSS to 100 µl samples of SRBC, respectively. The degree of haemolysis (Y) was estimated and the lysis curve for each specimen plotted Y/(1−Y) against the volume of plasma added (ml) on a $\log_{10}$–$\log_{10}$ scaled graph. The volume of plasma producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units ml^{-1} obtained for each experimental group.

2.9. Lysozyme activity

Plasma lysozyme activity was measured spectrophotometrically according to the method of Ellis [36]. A volume of 0.02% (w/v) suspension of *Micrococcus lysodeikticus* made up in 0.05 M phosphate buffer (pH 6.2) was used as substrate. Lyophilised hen egg white lysozyme was used as a standard. A new standard curve was prepared for each assay. Standard solutions as well as samples were added to the substrate at 25 °C. The results were expressed as mg ml^{-1} equivalent of hen egg white lysozyme activity.

2.10. Statistics

The data (mean ± standard error, SE) were analyzed by one way analysis of variance (ANOVA) followed by Tukey's test to compare the means between individual treatments using SPSS at $P < 0.05$ levels.

3. Results

3.1. Phagocytosis activity

The phagocytosis activity of LDV infected fish significantly increased after the administration of each solvent extract at 50 and 100 mg kg^{-1} dose after 8 week. However, at 5 mg kg^{-1} dose the solvent extracts did not enhance the phagocytic activity any time when compared to 0 mg kg^{-1} dose (Fig. 1).

3.2. Respiratory burst activity

The effect of different doses of solvent extracts on respiratory burst activity of isolated phagocytic cells is shown in Fig. 2. At 100 mg kg^{-1} the respiratory burst activity significantly increased with each solvent extracts after 8 weeks while at 5 and 50 mg kg^{-1} the extracts did not enhance the respiratory burst activity when compared to 0 mg kg^{-1} dose.

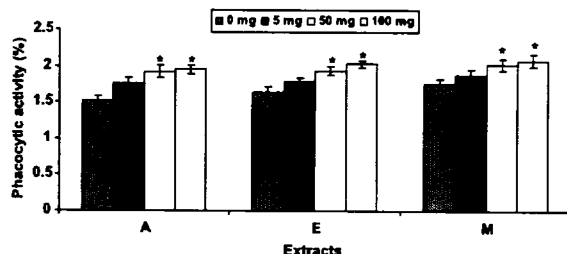

Fig. 1. Phagocytic activity of lymphocystis disease virus (LDV) infected fish after administration with aqueous (A), ethanol (E), and methanol (M) solvent leaf extracts and doses (0, 5, 50, and 100 mg kg^{-1}) in olive flounder (mean ± SEM, $n = 6$). Statistical differences ($P < 0.05$) compared with 0 mg kg^{-1} of each solvent extracts was indicated by asterisks.

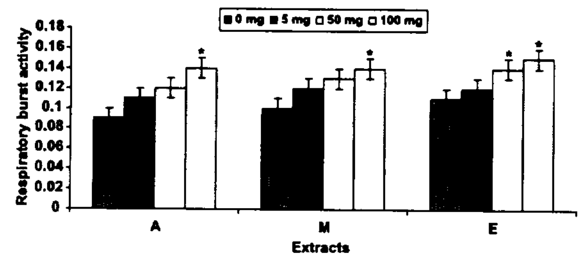

Fig. 2. Respiratory burst activity of lymphocystis disease virus (LDV) infected fish after administration with aqueous (A), ethanol (E), and methanol (M) solvent leaf extracts and doses (0, 5, 50, and 100 mg kg^{-1}) in olive flounder (mean ± SEM, $n = 6$). Statistical differences ($P < 0.05$) compared with 0 mg kg^{-1} of each solvent extracts was indicated by asterisks.

3.3. Natural haemolytic complement activity

The plasma natural haemolytic complement activities (ACH_{50}) of the extracts are shown in Fig. 3. Serum complement activity significantly increased with all the extracts at 50 and 100 mg kg^{-1} dose after 8 weeks when compared to 0 mg kg^{-1} dose. Infected fish injected with 5 mg kg^{-1} dose of all solvent extracts did not show an increase in the complement activity.

3.4. Plasma lysozyme activity

The lysozyme activity was significantly enhanced at concentration of 100 mg kg^{-1} dose in all solvent extracts after 8 weeks. However administration of 5 and 50 mg kg^{-1} dose of ethanol and methanol solvent extracts did not enhance the lysozyme activity except with aqueous extract (Fig. 4).

3.5. Disease resistance

The cumulative mortality of LDV positive group administered with 0 mg kg^{-1} dose was 90%. Infected fish treated with 5 mg kg^{-1} dose of aqueous extract had 75% mortality. However, fish injected with 50 and 100 mg kg^{-1} of aqueous extract had a lower mortality of 45% and 35%, respectively. Infected fish treated with 50 mg kg^{-1} dose of ethanol solvent extract suffered 30% mortality during 8 weeks. The high dose of 100 mg kg^{-1} dose of ethanol extract produced 20% mortality. The methanol solvent extract at concentration of 50 and 100 mg kg^{-1} doses produced the maximum

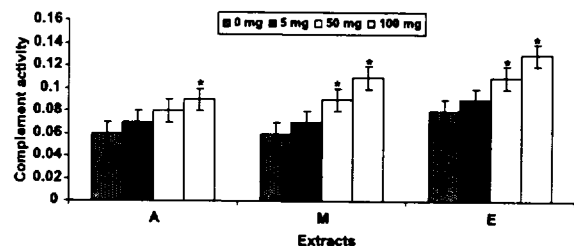

Fig. 3. Plasma natural haemolytic complement activity of lymphocystis disease virus (LDV) infected fish after administration with aqueous (A), ethanol (E), and methanol (M) solvent leaf extracts and doses (0, 5, 50, and 100 mg kg^{-1}) in olive flounder (mean ± SEM, $n = 6$). Statistical differences ($P < 0.05$) compared with 0 mg kg^{-1} of each solvent extracts was indicated by asterisks.

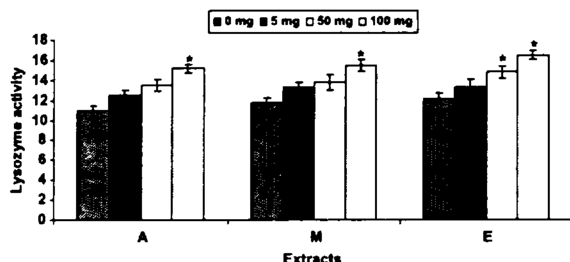

Fig. 4. Plasma lysozyme activity of lymphocystis disease virus (LDV) infected fish after administration with aqueous (A), ethanol (E), and methanol (M) solvent leaf extracts and doses (0, 5, 50, and 100 mg kg^{-1}) in olive flounder (mean ± SEM, n = 6). Statistical differences ($P < 0.05$) compared with 0 mg kg^{-1} of each solvent extracts was indicated by asterisks.

protection with 35% and 25% mortality, followed by 70% mortality when treated with 5 mg kg^{-1} dose (Fig. 5).

4. Discussion

The major components of the innate immune system are macrophages, monocytes, granulocytes, and humoral elements, like lysozyme or complement system [37,38] which constitute the first line of defense against invading pathogens in fish [39]. Medicinal herbs as immunostimulants of the medicinal herbs increase resistance to disease by enhancing nonspecific and specific defense mechanisms [18–20,40]. The active compounds of herbs activate several components of the immune system, such as phagocytes, natural killer cells, T-lymphocytes, B-lymphocytes, complement, and lysozyme [41]. Thus immunostimulants offer a promising alternative to antibiotics, chemicals, and vaccines.

In fish phagocytosis has been recognized as one of the important characteristics of the innate immune system in the host's defense against invading micro-organisms [34,42]. In LDV positive *P. olivaceus*, administration of 50 and 100 mg kg^{-1} doses of the extracts significantly enhanced the phagocytic activity of leukocytes after 8 weeks. Application of immunostimulants including plant extracts significantly enhance the phagocytic activity in various fish [43–45]. However, all the extracts at 5 mg kg^{-1} dose did not enhance the phagocytic activity. The present result suggests that all the solvent extracts at high concentration (50 and 100 mg kg^{-1} doses) promote better phagocytic activity after 8 weeks. The present results agree with the previous observations on triherbal extracts of *Azadirachta indica*, *Ocimum sanctum*, and *Curcuma longa* [45].

Phagocytes produce toxic oxygen forms during respiratory burst [46]. Since superoxide anion is the first product to be released from the respiratory burst, measurement of O_2^- has been accepted as a precise way to measure respiratory burst [47]. Infected fish treated with 100 mg kg^{-1} doses of all the solvent extracts significantly enhanced respiratory burst activity after 8 weeks. The present result is in agreement with the previous report in goldfish intraperitoneal administration with triherbal extracts [45] and in *Labeo rohita* fed with *Achyranthes aspera* [48] in large yellow croaker and common carp fed with a diet containing a mixture of *A. embranaceus* and *A. sinensis* extracts [49,50]. However, no significant respiratory burst activity was found in fish injected with all the solvent extracts at 5 and 50 mg kg^{-1}.

The present study all the solvent extracts at 50 and 100 mg kg^{-1} doses significantly enhanced the alternate complement activity. The bactericidal activity of serum complement activity has been well recognized as one of the key killing mechanisms of clearing bacteria in fish [51]. In marine teleost gilthead goldfish seabream the serum complement activity increased after administration with triherbal extracts and feeding levamisole (500 mg kg^{-1}) containing diet for 10 weeks [52,53]. However, in this study no significant activity was found with any extract at 5 mg kg^{-1} dose. Route of administration appears to be important in evidence positive response. Oral application of EcoActiva™ and MacroGard™ in snapper and turbot did not influence alternate complement activity [53,54].

Lysozymes of fish possess a high potential with bacteriocidal, bacteriolytic, anti-viral and anti-inflammatory properties. It plays an important role in the bio-defense system against Gram-positive and Gram-negative bacteria [55]. In the present study, all the extracts at 100 mg kg^{-1} dose significantly enhanced the lysozyme

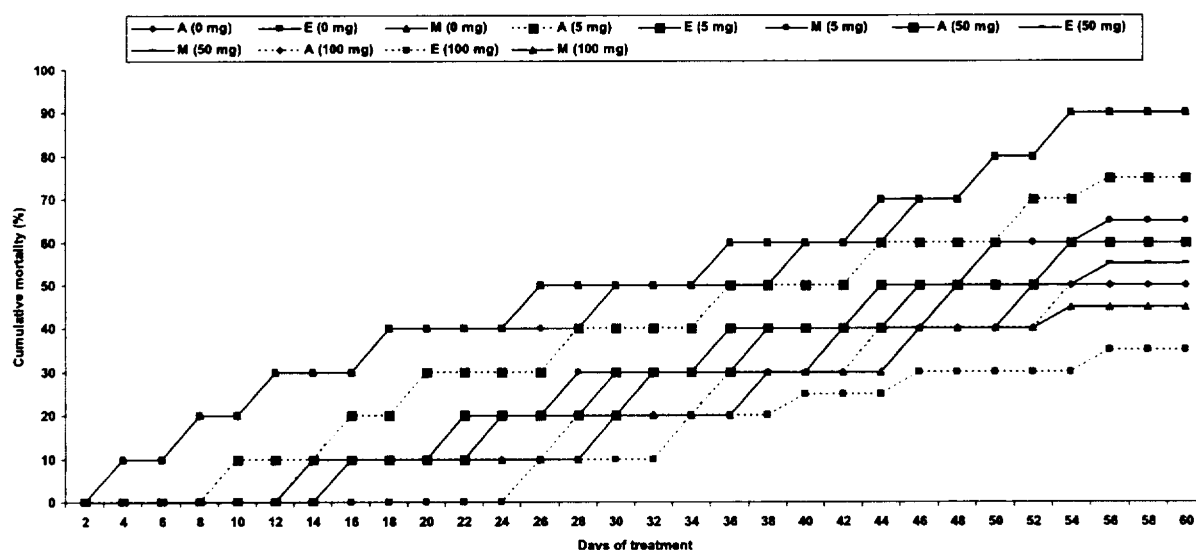

Fig. 5. Cumulative mortality of lymphocystis disease virus (LDV) infected fish after administration with aqueous (A), ethanol (E), and methanol (M) solvent leaf extracts and doses (0, 5, 50, and 100 mg kg^{-1}) in olive flounder.

activity. In Jian carp, the lysozyme activity was observed on 20th, 25th, and 30th day [50] and in large yellow croaker [49] fed with Traditional Chinese medicine (TCM) from *Astragalus radix*. *Oreochromis niloticus* fed with Astragalus root [56] and *L. rohita* fed with *A. aspera* also exhibited enhanced the lysozyme activity [48]. However, in the present study lysozyme activity did not increase at 5 and 50 mg kg^{-1} doses.

All the solvent extracts at 50 and 100 mg kg^{-1} doses increased the survival rate of infected fish. Earlier studies in this line also revealed that dietary supplementation of *O. sanctum* [18] and tri-herbal leaf extract through intraperitoneal injection enhanced the innate immunity and disease resistance against *Aeromonas hydrophila* in goldfish [45]. In conclusion this study showed that all the solvent extracts of *P. granatum* significantly enhance the innate immune response and disease resistance at 50 and 100 mg kg^{-1} doses. Further studies are needed to find out the effective use of this solvent extracts with special reference to the timing, dosage, and method of administration.

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