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Korean Mistletoe *Viscum album coloratum* Induces Specific and Non-Specific Immune Responses in Japanese Eel *Anguilla japonica*

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Effects of Korean mistletoe extracts (KM-110), *Viscum album coloratum* on the specific and non-specific immune responses of Japanese eel *Anguilla japonica* were examined. The optimal concentration not showing toxicity of KM-110 was determined to 30-40 μg/ml in vitro and 100 μg/100 g of fish in vivo. Even 1000 μg of KM-110/100 g of fish did not show any clinical problem in fish though the levels of toxic parameters were slightly increased. In terms of antibody production, KM-110 significantly elicited more antibody production than FCA or β-glucan. β-glucan plus KM-110 group synergistically enhanced antibody production. There was no significant difference between KM-110 and KM-110 plus β-glucan group. The ROI production by head kidney (HK) leucocytes of eel injected with 500 or 1000 μg KM-110 was significantly ($P<0.05$) enhanced than the control and FCA-treated group. Maximum increase in the NBT reduction value was observed in 1000 μg KM-110 group but no significant difference was found between 500 and 1000 μg KM group. The level of serum lysozyme activity was significantly ($P<0.05$) higher in the 500 and 1000 μg KM-110- or FCA-treated group than in the control and 200 μg KM-110 group. The phagocytic activities of HK leucocytes isolated from eel injected with 500 and 1000 μg KM-110 were significantly ($P<0.05$) higher than 200 μg KM-110 and PBS-injected control group. Korean mistletoe appeared to be a good activator of the specific and non-specific immune responses of Japanese eel.

Keywords: Mistletoe, Japanese eel, Lysozyme, Non-specific immunity, Kidney phagocytes

Introduction

Traditional disease control strategies employ antibiotics and chemical disinfectants, but these are no longer recommended practices due to the emergence of bacterial resistance, and also due to concerns over environmental impacts and wildlife protection. Although vaccinations have been indicated as an effective prophylactic method for use in the disease control of fish (Midtlyng et al., 1996), there are some methodological problems insofar as they may be very expensive and stressful for fish (Ellis, 1999). Already, remarkable success has been achieved with immunostimulants as a more environmentally friendly approach to disease management (Raa, 1996; Sakai, 1999; Peddie et al., 2002)

Several compounds, including β-glucans, chitin, algal extracts and bacterial polysaccharides, have been used to enhance immunity and disease resistance in a variety of fish species (Raa, 1996; Anderson, 1996). β-glucan administration has been reported to augment antibody production, complement activity, lysozyme activity, phagocytic activity, and respiratory bursts of channel catfish *Ictalurus punctatus* (Chen and Ainsworth, 1992), Atlantic salmon *Salmo salar* (Engstad et al., 1992), rainbow trout *Oncorhynchus mykiss* (Verlhac et al., 1996), gilthead rainbow trout *Sparus auratus* (Castro et al., 1999), and sea bass *Dicentrarchus labrax* (Bagni et al., 2005). However, some of the immunostimulants could not be used due to various disadvantages, such as high cost or limited effectiveness.

Mistletoe is a semi-parasitic woody perennial commonly found growing on oak other deciduous trees. The extract of European mistletoe (*Viscum album* Loranthaceae, *V. album* L.; EM) shows various biological activities such as induction of cytokines (Mueller and Anderer, 1990; Mannel et al., 1991), enhancement of natural killer (NK) cell activity (Kuttan et al., 1992) and immunoadjuvant activities (Mertzer et al., 1985; Hajto, 1986)

Moreover, many investigators have demonstrated that the extract of *V. album* L. augmented anti-tumor effect by enhancing the cytotoxic activity of NK cells, lymphokine-activated killer (LAK) cells and macrophages. However, even if the characteristics and biological properties of EM have been stud-

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ied intensively, there is only little knowledge about the biological and physiological functions of Korean mistletoe (*Viscum album* Coloratum, *V. album* C.; KM), a different subspecies of *V. album* from EM. Some investigators have reported that KM is much more effective in inducing anti-tumor and immunoadjuvant activities (Lyu, et al., 2000; Yoon et al., 2001). Both *in vivo* and *in vitro* research have so far mainly been focused on the effect of the mistletoe on non-specific immune responses.

Since no studies have been reported on the use of KM in aquaculture, this preliminary study was performed to investigate the effect of KM on specific and non-specific immune responses in eel. To overcome the disease problem in fish culture systems, the present study was focused on the possibility of using this indigenous KM as an appropriate immnoadjuvant for fish to augment the immunity and disease resistance of the fish.

Materials and Methods

Fish

Eel *Anguilla japonica* weighing about 150-200 g were purchased from a commercial fish farm in Kunsan, Korea and acclimated for 1 week to laboratory conditions in 70 L glass aquaria with re-circulated and aerated water at 21-23°C, and fed daily with commercial diet during the adaptation and experimental period. They were acclimated to this environment for at least 2 weeks prior to use. The health status of the animals was daily checked by observing the fish behavior and no clinical symptoms such as abnormal swimming pattern and a change of body color were observed during that time.

Reagents

Nitroblue tetrazolium (NBT), Percoll, hemocyanin (HC), 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), phorbol myristate acetate (PMA) and Minimum essential medium (MEM) were purchased from Sigma Chemicals CO. Hanks balanced salt solution (HBSS), fetal bovine serum (FBS), antibiotic-antimycotic were obtained from Gibco BRL, Grand Island, NY. Sodium nitrite, sulfanylamide, phosphoric acid were purchased from ICN Biomedicals. Bakers' yeast, *Saccharomyces cerevisiae* purchased from Oriental Yeast Co. Ltd., Tokyo, and thioglycollate broth was obtained from Difco Laboratories, Detroit, U.S.A.

Extraction of mistletoe

Mistletoe growing at oak in January was harvested from Gangwondo, Korea. The mistletoe was 1 or 2 years old, and their leaves, trunks, and fruits were cut into 2 joints from the end of a branch followed by washing with distilled water (D.W.) and drying. The vacuum-packed mistletoe was stored at -80°C until extracted. The lyophilized leaves and trunks of mistletoe were mashed up and ground in approximately 10 vol. of D.W. for 30 s. After washing, they were pulverized in a mixer for 2 min and stirred for 16 h at 4°C. Mistletoe was centrifuged at 15,000×g for 30 min at 4°C and the suspension was filtered through different pore sizes, 7.2, 0.45, and 0.22 μm, successively. The mistletoe extract named KM-110 was lyophilized and re-suspended in D.W. at an appropriate dilution factor.

In vitro and in vivo toxicity of mistletoe

In vitro toxicity of mistletoe was tested against RTG-2 and CHSE-214 fish cell lines. The cells (1×10^6) from the respective cell lines were dispensed into each well of 24-well plate (Costar, USA) followed by administration of various concentration of KM-110 ranged from 10 ng to 100 μg/mL and incubated at 21°C for 3 days. Thereafter, MTT assay was done according to the method of Daly et al. (1995). Briefly, the tissue culture plates were centrifuged at 500×g for 10 min and then the supernatant fluids were carefully removed without disturbing the cell pellet or formazan precipitate. The formazan crystals were dissolved by the addition to each well of 200 μL of dimethyl sulphoxide (DMSO) (Sigma) followed by 25 μL of glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). The contents of the wells were then thoroughly mixed with a multichannel pipette. Ten minutes later, formazan development was read at 595 nm using ELISA reader (ASYS HITECH, Austria).

To study whether KM-110 has a serious toxic effect in vivo, 100 and 1000 μg of KM-110/100 g of fish were intraperitoneally (I.P.) injected to 5 fish per group, respectively. Four days after injection, fish blood was collected and the concentration of glutamic oxaloacetatic trams aminase (GOT), glutamic pyruvate transaminase (GPT) and c-creatin was determined on Fuji Dry Chem System (Fuji Photo Film Co. Ltd, Japan).

Administration of mistletoe for a non-specific immune response in eel.

The eels were divided into 4 or 5 groups of 5 eels per group. Fish in each group were I.P injected with 200, 500 and 1000 μg of KM-110/100 g of fish (200, 500 and 1000 μg

KM-110) in 0.5 ml of phosphate buffered saline (PBS), respectively. The remaining group of fish was injected with an equivalent volume of sterile PBS or 1:1 emulsified Freund's complete adjuvant (FCA) (Sigma) as a control. At day 4 post-injection, blood and head kidney leucocytes were obtained from each fish.

Antibody production by administration of mistletoe plus other immunostimulants

The immunostimulating effect of KM-110 was compared with FCA and β-glucan based on an elevated level of antibody production. HC was used as an antigen to evoke the specific antibody response. For the I.P. injection, 500 μg KM-110 and 100 μg of HC was mixed and administered in the volume of 200 μL. Each five fish was treated for a control (KM-110 non-treated) and an experimental group. The eel were divided into 6 groups (5 fish/group) and HC was only injected (control) or mixed and injected with other adjuvants of 100 μL and a total injection volume was adjusted to 200 μL through the all experiments. KM-110 was I.P. injected in the concentration of 500 μg suspended in 200 μL of phosphate buffered saline (PBS). In the group of FCA, 100 μL of FCA was 1:1 emulsified with HC suspended in 100 μL of PBS. In the FCA plus KM-110 treated fish group was added to FCA and mixed with HC. The optimized concentration (50 μg) of β-glucan was injected with HC in the volume of 200 μL. In the β-glucan plus KM-110 administered group, 50 μg of β-glucan and 500 μg KM-110 was mixed and I.P. injected in the volume of 200 μL. At day 30 post-injection, blood was harvested from fish per group followed by an assay of antibody titer in ELISA reader.

In our preliminary study, the induction of non-specific immune response by mistletoe was the highest at day 4 post-injection.

Isolation of head kidney (HK) leucocytes

The method described by Santarem et al (1997) was used with some modification. The eel HK was dissected out by a ventral incision, cut into small fragments and transferred to 5 ml HBSS, respectively. Cell suspensions from HK were obtained by teasing the HK tissues with 2 slide glasses in HBSS in a Petri dish (Coring, USA). After sedimentation of tissue debris at 4°C for 1 min, the supernatants were removed. HK cell suspensions were layered over a 34-51% Percoll gradient and centrifuged at 1000×g for 40 min at 14°C. After centrifugation, the bands of leucocytes above the 34-51% interfaces were collected with a Pasteur pipette and washed twice at 120×g for 8 min in HBSS. The concentration of viable cells was determined by trypan blue exclusion.

Serum

Blood was collected from the dorsal aorta of the eel. It was allowed to clot at 20°C for 30 min and cooled at 0°C for 1 h. Serum was obtained by centrifugation at 1000×g for 8 min. The sera were frozen at -20°C until used.

Reactive oxygen intermediates (ROI) production assay

ROI production from eel kidney cells after administration with KM-110 was assessed by monitoring their ability to reduce NBT (Secombes et al., 1988). The leucocytes (1×10^5 cells) were washed 1 time with HBSS at 60×g for 3 min at 4°C and incubated in 100 μL of complete media in the presence of PMA, 1 μg/mL and NBT (mg/mL). After 1 h at 25°C, excess amount of NBT was washed out with PBS and the leucocytes were fixed with 70% methanol. After discarding the methanol, the leucocytes were washed twice with PBS. The reduced formazan was solubilized with 120 μL of KOH and 140 μg of DMSO and optical density values were read at 620 nm in an ELISA reader.

Lysozyme activity

Serum lysozyme activity was measured using a modified turbidimetric microtitre plate technique according to Ellis (1999). Briefly, a standard suspension of 0.15 mg/mL *Micrococcus lysodeikticus* (Sigma) was prepared in 66 mm phosphate buffer (pH 6.0). Eel serum (50 μL) was added to 1 mL of bacterial suspension, and the decrease in absorbance was recorded at 0.5 and 4.5 min intervals at 450 nm in a spectrophotometer (SHIMADZU UV-1600PC). One unit of lysozyme activity was defined as reduction in absorbance of 0.001/min.

Phagocytic activity

Eel HK leucocytes were adjusted to 1×10^6 cells/200 μL/well in 5% FBS-MEM and dispensed in 8-well slide chamber (Nunc, Denmark) followed by overnight incubation at 25°C. Following incubation, 1×10^7 cells/mL of zymosan (Sigma) were added. The mixture was incubated at 25°C for 1 h with occasional shaking and 50 μL of the mixture was smeared on a glass slide, air-dried and stained with Wright's solution. Phagocytic index (PI) (Sahoo and Mukherjee, 2001) was calculated by enumerating 500 leucocytes per fish under a microscope. PI = number of cells ingesting zymosans/

number of cells observed × number of zymosans ingested/ number of cells observed × 100.

Statistical analysis

The statistical significance of differences between groups was calculated by applying Student's 2-tailed *t*-test.

Results

In vitro and in vivo toxicity of KM-110

The KM-110 concentrations of showing 50% killing CHSE-214 and RTG-2 were 30 μg /ml and 40 μg /ml, respectively (Fig. 1). The level of in vitro toxicity was similar to that reported in the previous study in which the KM-110 toxicity impacting the mammalian cell lines appeared to be negligible (Yoon et al., 2001). Table 1 shows the level of GOT, GPT and c-creatin in blood from the eel sensitized with KM-110. No significant difference between the fish group administered with 100 μg KM-110/100 g of fish and a KM-110 non-treated group was observed. However, when ten-fold more concentration of KM-110 was administered, the level of the three toxicity indicating materials was augmented with maximum in the normal range of indicating non-toxicity.

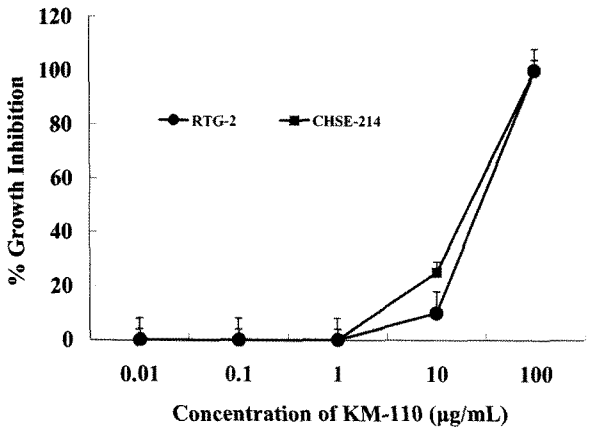

Fig. 1. In vitro cytotoxicity of mistletoes on fish cell lines. The cells (1×10^6) from the respective cell lines were incubated with various concentrations of KM-110 ranging from 10 ng to 100 μg/ml and incubated at 21°C for 3 days. Formazan development was read at 595 nm using ELISA reader (ASYS HITECH, Austria). Error bars represent SD from the mean of triplicate wells. The results are representative of three experiments.

Table 1. The levels of GOT, GPT and c-creatin in eel sera following injection of mistletoe

Standards for toxicity	Dose of mistletoes injected in fish		
	PBS	M100/100[1]	M1000/100
GOT	20±4 mg/mL	23±5 mg/mL	33±3 mg/mL
GPT	40±5 mg/mL	43±4 mg/mL	50±6 mg/mL
c-creatin	31±7 mg/mL	28±5 mg/mL	41±8 mg/mL

[1]μg of mistletoe/g of fish

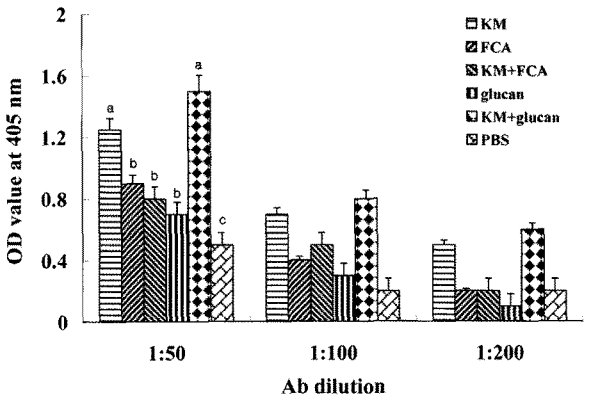

Fig. 2. The effect of mistletoe-supplemented conventional adjuvants on inducing antibody production in eel. Six groups (5 fish/group) of fish were i.p. immunized with hemocyanin supplemented with KM-110 (KM), FCA, KM-110+FCA (KM+FCA), β-glucan, KM-110+β-glucan (KM+β-glucan) and PBS, respectively. Statistical differences ($P<0.05$) between groups are indicated by different letters over the bar.

Humoral immune response elicited by KM-110

Fig. 2 shows a humoral effect of KM-110 compared with other immunostimulating molecules. As shown in Fig. 2, KM-110 significantly induced more antibody production than FCA or β-glucan. Although β-glucan failed to elicit an efficient antibody production, the β-glucan plus KM-110 group synergistically enhanced antibody production. There was, however, no significant difference between KM-110 and KM-110 plus β-glucan group.

ROI production

The results of the ROI production are shown in Fig. 3. The ROI production by the cells of fish injected with 500 and 1000 μg KM-110 was significantly ($P<0.05$) higher than the control and FCA-treated group. Though the ROI production in 200 μg KM-110 group was higher than the control group, the difference was not significant. Maximum increase in the NBT reduction value was observed in 1000 μg KM-110 group but no significant difference was found between 500 and 1000 μg KM group.

Lysozyme activity

Fig. 4 shows lysozyme activity in serum of eel sensitized with/without KM-110 administration. The level of serum

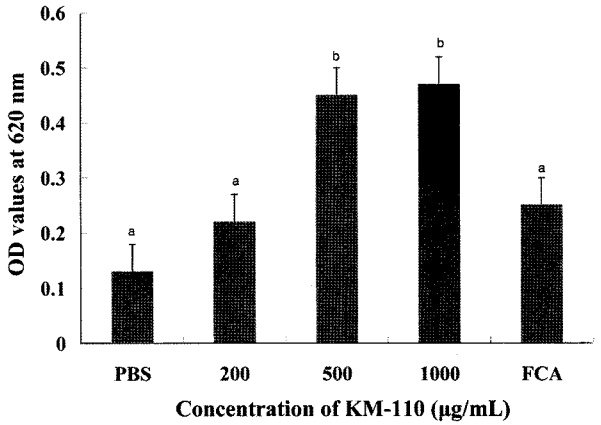

Fig. 3. The effect of mistletoe on the ROI production of head kidney leucocytes at 4 days post-injection. Five groups (5 fish/group) of fish were i.p injected with 4 different concentrations of KM-110 and FCA. Statistical differences ($P<0.05$) between groups are indicated by different letters over the bar.

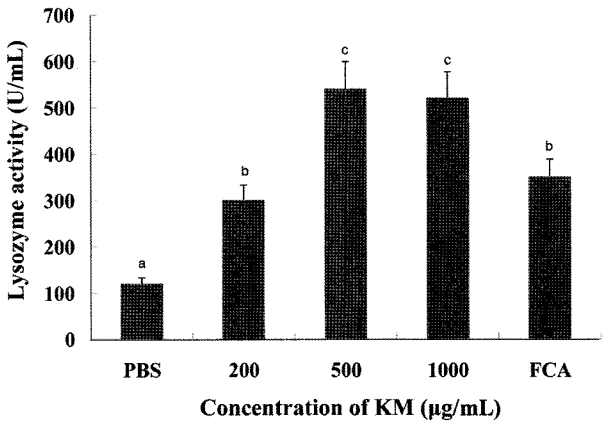

Fig. 4. Lysozyme activity of eel kidney leucocytes at 4 days post-injection of mistletoe. Data represent the mean+S.D. (n = 5). Five groups (5 fish/group) of fish were i.p. injected with 4 different concentrations of KM-110 and FCA. Statistical differences ($P<0.05$) between groups are indicated by different letters over the bar.

lysozyme activity was significantly ($P<0.05$) lower in the control and 200 μg KM-110 group than in the 500 and 1000 μg KM-110- or FCA-treated group. Maximum activity was found in the 500 μg KM-110 group. There was no significant difference between 500 and 1000 μg KM-110-treated group.

Phagocytic activity

To test whether mistletoe can have an influence on inducing phagocytic activity, eel kidney leucocytes previously sensitized with KM-110 (200, 500 and 1000 μg KM-110) were incubated overnight with zymosans. As shown in Fig. 5, the phagocytic activities of HK leucocytes isolated from eel injected with 500 and 1000 μg KM-110 were significantly

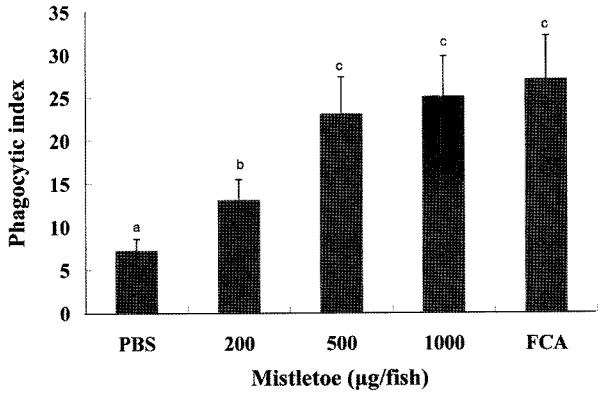

Fig. 5. Phagocytic activity of eel leucocytes injected with mistletoe. Fish were i.p. injected with 0, 200, 500, 1000 ug/100 g of fish, and FCA. Phagocytic activity was measured on day 4 after injection. Statistical differences ($P<0.05$) between groups are indicated by different letters over the bar.

higher than 200 μg KM-110 and PBS-injected control group. There was a significant difference ($P<0.05$) between 200 μg KM-110 group and 500 or 1000 μg KM-110 group, but no significant difference between 500 and 1000 μg KM-110 was observed.

Discussion

We have studied whether a specific and non-specific immune response was induced in eel by an administration of mistletoe. Mistletoe *V. album* is propagated by birds that eat berries and then excrete by wiping the sticky pulp off their beaks. Mistletoe contains lectins, protein toxins, and polysaccharides. Also, mistletoe's lectins are cytotoxic glycoproteins of approximately 10,000 molecular weight and activates macrophages and lymphocytes, leading to secretion of various kinds of cytokines (Mueller and Anderer, 1990; Mannel et al., 1991). Lectins are believed to mediate pathogen recognition, which can lead to neutralization of the invading organism (Vasta et al., 1994; Weis et al., 1998) during the early stages of an infection (Holmskov et al., 1994; Ni and Tizard, 1996; Lu, 1997).

Mistletoe, therefore, has been widely used for therapeutic purpose in Europe and in Korea as well. Furthermore, mistletoe preparations had increase in natural killer cells, T-helper cells, cytokine release, and peripheral blood mononuclear cells and lymphocytes. In mammalians, Korean mistletoe has shown 10 to 1000-fold higher anti-tumor effect than Europe mistletoe (Yoon et al., 2001). In our previous study, eel kidney leucocytes were not able to induce *in vivo* or *in*

vitro nitric oxide (NO) production when administrated with mistletoe (data not shown). In gilthead seabream head-kidney leucocytes, however, macrophage activating factor (MAF) could induce NO production by macrophages when used in conjunction with bacterial lipopolysaccharide (LPS) (Mulero and Meseguer, 1998). Furthermore, there is increasing evidence that fish are able to generate reactive nitrogen species such as NO (Cameron et al., 2003; Singru et. al., 2003). Therefore, it is necessary to further study whether eel phagocytes do not possess the mechanism to synthesize NO against foreign stimulators or mistletoe itself is not able to induce NO synthesis from kidney phagocytes.

On the other hand, ROI production from eel kidney leucocytes was readily elicited by the mistletoe administration, suggesting that ROI might be one of the indicators of mistletoe-induced non-specific immunity. In fact, ROIs such as the superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and singlet oxygen, play an important role in the antimicrobial activity of phagocytic cells (Babior, 1984). In *in vivo* experiments, the level of ROI production was highly augmented in kidney leucocytes from eel injected with 500, 1000 or 1500 μg of mistletoe /100 g of fish. At a higher concentration of mistletoe, ROI product was expressed at a similar level to 500 or 1000 μg However, mistletoe more than 1000 μg/100 g of fish failed to up-regulate the further induction of ROI production. Usually, immunostimulants do not show a linear reaction between doses and effect but a maximum effect at intermediate doses and no effect and even toxicity at high doses (Bliznakov and Adler, 1972; Gialdroni-Grassi and Grassi, 1985). The fact has been established in fish through *in vivo* (Kenyon et al., 1985; Anderson and Jeney, 1992) and *in vitro* studies (Siwicki et al., 1990). In *in vitro* experiment, 10 μg/mL of mistletoe was revealed to an optimal concentration to induce ROI production without any cellular cytotoxicity.

According to Fulton (1957) and Dianoux and Jolles (1969), 'true' lysozyme has to satisfy the following criteria: (1) the enzyme lyses *Micrococcus lysodeikticus* cells; (2) is readily adsorbed by chitin-coated cellulose; (3) is a low molecular weight protein; and (4) is stable at acidic pH at higher temperatures, but is inactivated under alkaline conditions. Considering the augmentation of lysozyme activity in serum from the mistletoe-injected fish, it is assumed that mistletoe can play a critical role in evoking lysozyme activity from eel kidney phagocytes. In the injection of 1500 μg/fish of mistletoe, lysozyme activity was down-regulated relatively to a low concentration of mistletoe (data not shown).

It is necessary to ascertain that phagocytes are working on phagocytosis in the presence of foreign pathogens. In our study, instead of some pathogens, zymosans were treated to either phagocytes from mistletoe-injected eel or those from mistletoe non-treated eel. Expectedly, phagocytes from mistletoe-treated eel were aggregated together and bunch of zymosans were engulfed by the phagocytes, indicating that mistletoe play an excellent role in activating the non-specific immune response in eel.

In conclusion, considering that most of all indicators for non-specific immune mechanism were revealed with positive results, Korean mistletoe could be a promising immunoadjuvant inducing the non-specific immune response in fish. Based on our preliminary results, the specific immune response by mistletoe needs to be further studied and further fish immunoadjuvant for a diet should be developed in future.

Acknowledgement

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