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The Evaluation on the Effectiveness as a Cosmetic Material of *Ascidian shell* Extract Using Zebrafish model

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Abstract : The extracts of AS contain in alloxanthin, halocynthiaxanthin, astaxanthin and 13 kinds of carotenoids. The aim of the study was to assess the anti-oxidant activity and cell viability of AS. The anti-oxidant activity was determined by using DPPH radical inhibition activity and superoxide dismutase (SOD)-like activity. The results of cell viability assay showed that the extracts from AS were cytotoxic at concentrations above 5.0 ㎎/㎖. This study was designed to examine inflammation induced by LPS, protection effect by UVB and the toxicity of *Ascidian shell* extract(ASE) as a functional cosmetic ingredient. Evaluation of embryo toxicity resulted in embryo coagulation and mortality when treated at 5.0, 10.0, 20.0 ㎎/㎖. At the lowest concentration of 1.0 ㎎/㎖, hatchability resulted in 100.0 % rate. The results of arrhythmia measurement in larvae showed similarity to the evaluation of embryo toxicity. This result demonstrated that toxicity is present at concentrations greater than 5.0 ㎎/㎖. The protective effect of ASE on LPS and UVB-induced in the zebrafish was investigated. Intracellular reactive oxygen species(ROS) generated by the exposure of zebrafish to LPS, UVB-radiation were significantly decreased after treatment with ASE at 0.1 ㎎/㎖. As a result, ASE similarly reduced UVB-induced ROS generation and cell death in live zebrafsih. Therefore, it is suggested that ASE has anti-Inflammatory effects and can possibly be used as a functional substance for skin protection in the future.

Keywords : Ascidian shell, Zebrafish, Anti-Inflammation, Ultraviolet B, Toxicity, Antioxidant

1. Introduction

The role of active oxygen and free radicals in various human diseases is becoming increasingly recognized. A radical is any molecule that contains one or more unpaired electrons [1]. Some of these radicals can exist in a free form and continuously interact with various tissue components resulting in disorder. When any living organism gets thrown off balance, its cells help it return to normal reduced environment. In other words, living organisms have the ability to keep a stable internal environment. When this condition is lost due to the production of reactive oxygen species (ROS) like peroxides and free radicals, there is considerable damage to the cell components such as proteins, lipids and DNA [2]. The potential role of oxygen or xenobiotic

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derived free radicals in the pathology of several human diseases has prompted extensive research linking the toxicity of numerous xenobiotics and disease processes to a free radical mechanism [3].

Oxidative stress plays important role in the pathophysiology of various diseases. Oxidative stress will be defined in the origin of reactive oxygen species and other free radicals. Complex antioxidant defense mechanisms have evolved to protect tissues because of the potential for catastrophic damage to free radical attack [4]. Antioxidants are the species that compensate the effect of oxidants or they are the compounds that act against the ROS and hence prevent the loss to be made of the body [5]. Antioxidants play an important role in maintaining a balance between antioxidants and free radicals produced by metabolites or derived from environmental sources, and exploring potential as UV protectors. There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body, and to prevent the deterioration of food ingredients. Furthermore, there is a preference for antioxidants from natural rather than from synthetic material [6].

There has been considerable interest in elucidating the mechanism by which carotenoids may exert a protective effect in biological systems. The role of carotenoids in human health is thought to provide health benefits of decreasing the risk disease particularly certain cancer, cardiovascular diseases, cataract and muscular degeneration [7-8]. In particular, it has been proposed that carotenoids activates reactive chemical species such as singlet oxygen, triplet photochemical sensitizers and free radicals which would otherwise induce potentially harmful processes [9]. The edible *Halocynthia roretzi* (*Ascidian*) is a sea squirt that is consumed primarily in Korea and northern Japan. The inedible outer shell part, tunic, also has been used as resources of natural bioactive compounds. The chondroitin found in the shell of *Ascidian* is used in cosmetics, while product development of its dietary fiber content is well under way [10]. *Halocynthia roretzi*, a byproduct of seafood industry in Korea, contains many high value substances one of which is carotenoids [11]. It has 13 types of carotenoids isomers containing the 6 most abundance of these such as alloxanthin, halocynthiaxanthin, diatoxanthin, diadinochrome, mytiloxanthinone, and astaxanthin, with minor concentrations of lutein, mytiloxanthin, and β-carotene [12]. In this study, we have experiment the antioxidants, toxicity and anti-inflammatory properties of *Ascidian shell* carotenoids using zebrafish.

2. Experiment

2.1. Experimental materials

The shell of *Ascidian* were collected in Daejeon, Korea in March 2017. B16F10 melanoma cell were provided by Keimyung University, and HaCaT cells were supplied by the Daegu Haany University. SOD Assay Kit-WST was purchased from Dojindo Laboratories, Inc (Kumamoto, Japan). 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma (St Louis, MO). DCF-DA (2',7'-Dichlorofluorescin diacetate), LPS (Lipopolysaccharides from Escherichia) and Acridine orange were supplied by Sigma. Adult zebrafish were purchased from a commercial dealer (Seoul Aquarium, Daejeon, Korea).

2.2. Cell Culture

B16F10 melanoma and HaCaT, a cell line selected *in vivo* for high pulmonary colonization were cultured in dulbecco modified eagle medium (DMEM) containing 10.0% fetal bovine serum (FBS), 1.0% penicillin, and passaged twice weekly by short exposure to 0.05% trypsin-EDTA (GIBCO). After 8 days incubation 5 % CO_2 at 37℃, the number of original cells forming colonies was

determined visually by counting random microscope fields.

2.3. Maintenance of zebrafish

Zebrafish were kept in zebrafish breeding system (S type) at 28℃ with a 16/15 h light/dark cycle. Zebrafish were fed 3 times a day, 7 days/week, with tetramin flake supplemented with live *Artemia*. Embryos were obtained from natural spawning, induced in the morning by turning on the light. Collection of embryos was completed within 1 hour.

2.4. WST-1 assay

Seed cells in a 96-well plate at a density of $5x10^3$ cells/well injunction. Culture the cells in CO_2 incubator at 37℃ for 24 hours. Remove the culture medium from wells of the 96-well plate in the tissue culture hood. Add 100.0㎕ (The ASE concentration is 0.1, 1.0, 5.0, 10.0, 20.0 ㎎/㎖) to each well of the plate, and place the plate back in to incubator. 24 h later, remove the plate from CO_2 incubator. Transfer 100㎖ of medium/WST-1 (Dilute WST-1 reagents 1:10 into culture growth medium) from each well into a culture plate. Incubate the cells for 2 hours at 37℃ CO_2 incubator. Measure the absorbance of each sample using a microplate reader (ELSA, Sunnyvale, USA) at a wavelength of 450 nm.

2.5. DPPH assay

The scavenging activity against DPPH radical was evaluated according to the method of Brand-Williams [13], with minor modifications. For the DPPH radical scavenging assay, 20.0 ㎕ of extract diluted appropriately in water was mixed with 180.0 ㎕ of DPPH in methanol (0.20 mM) in wells of a 96-well plate. The plate was kept in the dark for 30 min, after which the absorbance of the solution was measured at 492 nm in microplate reader.

2.6. SOD assay

The levels of SOD-like activity in the extracts were measured using the SOD Assay Kit-WST according to the technical manual (Dojindo). In a 96-well microplate, 20.0 ㎕ of sample solution (Sample(Extract) well and Blank2 well) or distilled water (Blank1 and Blank3) was mixed with 200.0 ㎕ of WST working solution. For Blank2 and Blank3, 20.0 ㎕ of dilution buffer was added. Then, 20.0 ㎕ of enzyme working solution was added to each Sample well and Blank1 well. The plate was incubated at 37℃ for 20 min and the absorbance was determined at 450 nm using a microplate reader.

2.7. Zebrafish embryo toxicity test

At 6 hour post-fertilization (hpf), embryos were examined under a dissecting microscope, and those embryos that had developed normally and reached the blastula stage were selected for subsequent experiments. Briefly, 30 normal embryos were randomly distributed into 24-well plate containing 2.0 ml of exposure extract solution (The ASE concentration is 0.1, 1.0, 5.0, 10.0, 20.0 ㎎/㎖). Mortality was identified by coagulation of the embryos, missing heartbeat. Sublethal endpoints included embryo malformation, heart rate, hatching success and mortality. During the 72 h exposure, embryos and larvae were examined under a microscope to screen for morphological abnormalities, and survival rates were recorded within each treatment. Mortality was recorded at 24, 48, 72 hpf.

2.8. Zebrafish Arrhythmia test

Zebrafish larvae were 72 hpf of age when assigned to experimental treatments, and were exposed to extract solution (The ASE concentration is 0.1, 1.0, 5.0, 10.0, 20.0 ㎎/㎖) or control (Egg water) treatments until 90 min. For all experiments, embryos and larvae were maintained at 28℃ and reared in sterile 24-well plates at a density of five fish per 2.0 ml well. Larvae arrhythmia test were examined under a microscope to screen for heart beat.

2.9. Estimation of intracellular ROS generation and generation and cell death in zebrafish embryos and image analysis for UV-B protected

Generation of ROS production zebrafish embryos was assessed using DCF-DA. Cell death was detected in live embryos using acridine orange. At 2 day post-fertilization (dpf), the embryos were treated with 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 ㎎/㎖ ASE, and 1 h later, the plate was irradiated with UV-B (50.0 ㎖/㎠). After irradiating embryos with UV-B, the embryos were transferred into a 24-well plate and treated with DCF-DA (20.0 ㎍/㎖) or acridine orange solution (7.0 ㎍/㎖), after which the plates were incubated for 1h in the dark at 28℃. After incubation, the embryos were rinsed in embryo medium and anesthetized with phenoxyethanol before visualization. embryos were observed under a fluorescence microscope (AM415T-GFBW, Dino-Lite, Taipei). The fluorescence intensity of individual zebrafish embryos was quantified using the image J program [14].

2.10. Estimation of LPS-stimulated intracellular ROS and cell death in zebrafish embryos and image analysis

At 6 h, the embryos were treated with 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 ㎎/㎖ ASE and 10.0 ㎍/㎖ LPS was added to the 24-well plate. At 3 dpf, the embryos were transferred into 24-well plate and treated with DCF-DA or acridine orange. Then, the plates were incubated for individual reaction times in the dark at 28℃. After incubation, the embryos were rinsed in fresh embryo media and anesthetized before visualization. The images of stained embryos were observed using a fluorescent microscope. The fluorescence intensity of individual zebrafish embryos was quantified using the image J program [15-16].

3. Results and Discussion

3.1. WST-1 cell viability assays

The effects of the ASC on cell viability were investigated. As shown in Fig. 1, all ASE reduced cell viability for at least 20.0 %. The HaCaT cell were confirmed by the WST-1 viability tests, which showed that, following the ASE treatment, cell viability decreased to 35.9, 24.8, 41.7, 84.0 % for 0.1, 1.0, 5.0, 10.0, 20.0 ㎎/㎖, respectively. B16F10 melanoma cell lines were treated with 0.1, 1.0, 5.0, 10.0, 20.0 ㎎/㎖ of ASE. ASE treatment significantly reduced cell viability in B16F10 melanoma cells, except at 0.1, 1.0 ㎎/㎖ and 5.0 ㎎/㎖ concentration(Fig. 2).

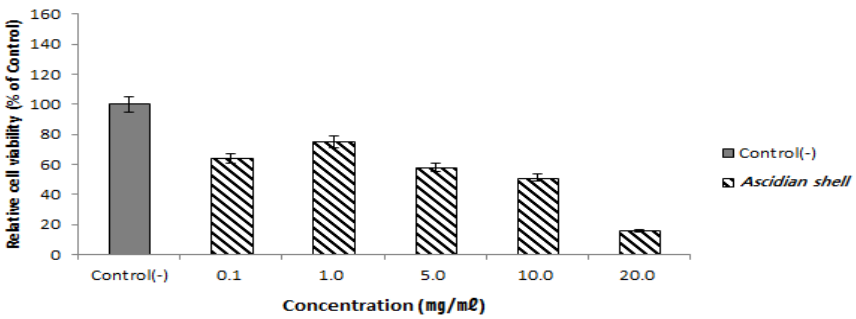

Fig. 1. The effects of *Ascidian shell* extract on the growth of HaCaT cell line. Serum-starved HaCaT cells were incubated with *Ascidian shell* extract for 24 hours at the indicated concentration of *Ascidian shell* extract. The cell viability was estimated using WST-1 assay.

3.2. Evaluation free radical DPPH assays

The DPPH radical scavenging test is one of the shortest available to investigate the overall hydrogen electron donating activity of single antioxidants and health promoting dietary antioxidant supplements. The scavenging ability of the ASE rose dose dependently in the range of 5.0-20.0 ㎎/㎖ of the final assay solution. Fig. 3 shows the scavenging ability expressed on the DPPH radical.

3.3. Evaluation free radical SOD assays

Fig. 4 shows the SOD-like activity in ASE. The SOD-like activities of ASE at 0.1, 1.0, 5.0, 10.0, 20.0 ㎎/㎖ were 27.4, 26.2, 42.1, 39.2 and 71.7 %, respectively. A significant difference was detected in which ASE showed higher SOD-like activities than ascorbic acid. These results were similar to the DPPH-scavenging ability data. The results show that ASE can promote increases in the activities of these antioxidant activity.

3.4. Zebrafish embryo toxicity assay

After 24 h, exposure to ASE at 0.1, 1.0, 5.0, 10.0, 20.0 ㎎/㎖, resulted in mortalities of 0.0, 0.0, 30.0, 30.0, 50.0% for embryos. The assay result at 0.1 and 1.0 ㎎/㎖ is within the range expected for the positive control in the standard fish embryo test. Typically, about 30~50% of the embryos exposed to 5.0, 10.0 and 20.0 ㎎/㎖ showed additional sublethal effects. Prolongation of the exposure to an age of 72 h (including hatching) resulted in 100% mortality at all concentrations except for the lowest with 0.1 ㎎/㎖ (Fig. 5). After 72 h of exposure, predominant

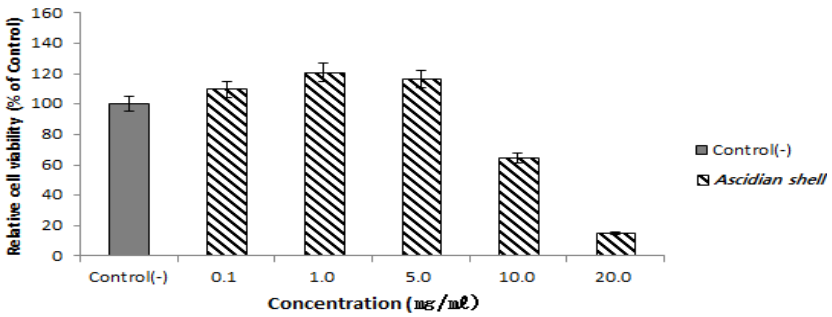

Fig. 2. The effects of *Ascidian shell* extract on the growth of B16F10 cell line. Serum-starved B16F10 cells were incubated with *Ascidian shell* extract for 24 hours at the indicated concentration of *Ascidian shell* extract. The cell viability was estimated using WST-1 assay.

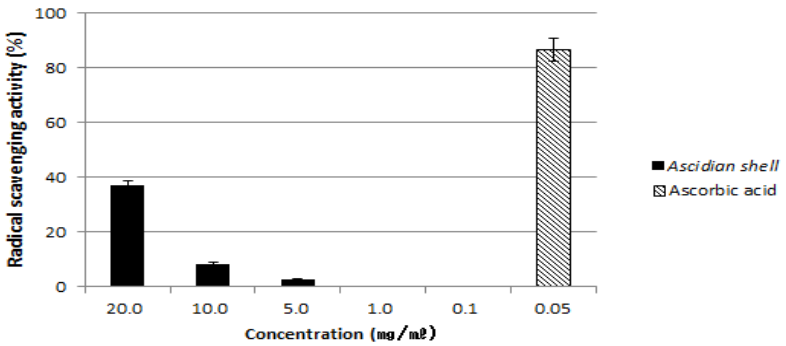

Fig. 3. DPPH radical scavenging activity(%) of *Ascidian shell* extract.

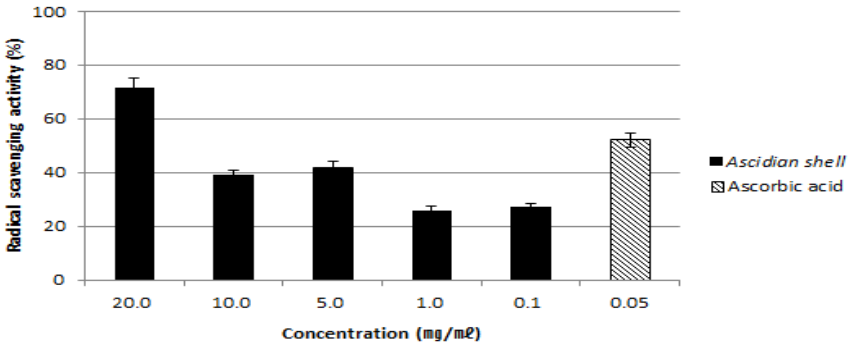

Fig. 4. SOD-like activity (%) of *Ascidian shell* extract.

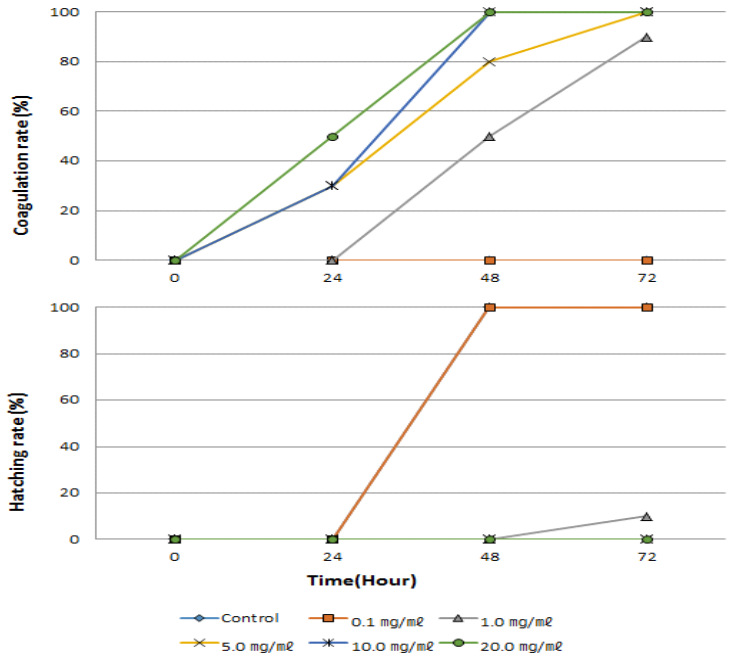

Fig. 5. Coagulation rate (%), and Hatching rate (%) of *Ascidian shell* extract.

sublethal effects were edema formation at the pericardium or the yolk sac, impairment of cardiovascular functions and development of general malformations.

3.5. Zebrafish Arrhythmia assay

The drop in heart rate continued to plunge at ASE compared to untreated embryos. No recovery was observed throughout the test period. ASE showed a gradual drop in heart rate from lowest concentration onwards, but at a much lower intensity than for untreated embryos. ASE exposed embryos developed a reduced heart beat from 5.0, 10.0 ㎎/㎖, which became more noticeable by 20.0 ㎎/㎖. Controls showed no adverse effects on the embryos. At

higher concentrations, the pumping efficiency was remarkably slow.

3.6. Effects of ASE on UVB-induced intracellular ROS generation and cell death in zebrafish

We have investigated the protective efficacy of ASE against UVB induced oxidative stress in zebrafish as an alternative animal model system. ROS scavenging effect of ASE in zebrafish is shown in Fig. 7(A). The level of ROS was 599.0 % in UVB irradiated zebrafish compared to non-irradiated control zebrafish. However, the addition of ASE to the zebrafish after exposure to UVB significantly reduced ROS level to 246.0, 169.0, 146.0, 100.0, 198.0 and 356.0 % at 5.0, 1.0, 0.5, 0.1, 0.05 and 0.01 ㎎/㎖. Fig. 7(B) is a typical fluorescence micrograph of ROS in the zebrafish. The negative control generated a clear image, whereas the positive control, wich was irradiated with UVB, showed a increase in the fluorescence. But, in zebrafish treated with ASE to UVB irradiation, a dramatic reduction in the amount of ROS was observed.

We determined UVB-induced cell death by measuring acridine orange fluorescence intensity in the body of the zebrafish (Fig. 8(C)). The UVB irradiation induced cell death in the zebrafish was increased 755.0% compared to the negative control. However, cell death was reduced 315.0, 307.0, 130.0, 115.0, 371.0, 502.0 % by the addition of ASE(5.0, 1.0, 0.5, 0.1, 0.05 and 0.01 ㎎/㎖) to UVB irradiated zebrafish. In agreement, images of UVB induced zebrafish showed a significant increase in fluorescence compared to non-irradiated zebrafish (Fig. 8(D)).

3.7. Effects of ASE on LPS-stimulated intracellular ROS generation and cell death in zebrafish

The production of ROS in the LPS induced inflammatory zebrafish model was analyzed using DCF-DA. Fig. 9(E) illustrates the ROS levels in zebrafish with or without LPS and/or ASC. These data show that, the ROS level in LPS stimulated zebrafish increased to 773.0 % compared with the negative control group. However, the ROS productions in zebrafish treated with different concentrations of ASC (5.0, 1.0, 0.5, 0.1, 0.05 and 0.01 ㎎/㎖) were reduced, and a significant reduction was observed at 0.1 ㎎/㎖. Fig. 9(F) is a typical fluorescence micrograph of ROS in the zebrafish. Fig. 10(G) shows that there is a high level of cell death in LPS treated zebrafish. These data show that, the cell death level in LPS stimulated zebrafish increased to 963.0 % compared with

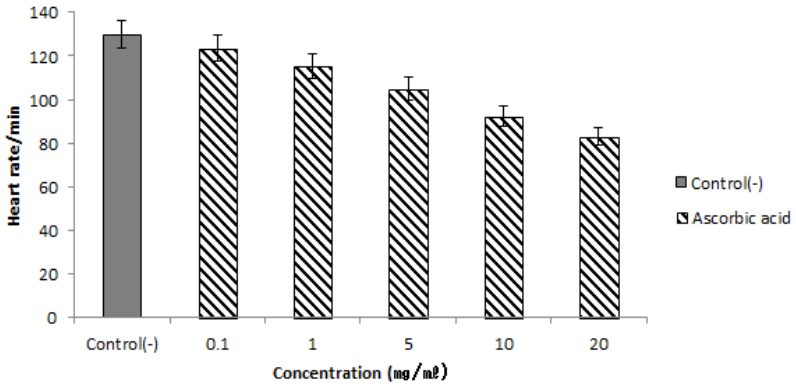

Fig. 6. Arrhythmia test of *Ascidian shell* extract on zebrafish larvae. The heartbeat of zebrafish larvae after having been treated with *Ascidian shell* extract were counted.

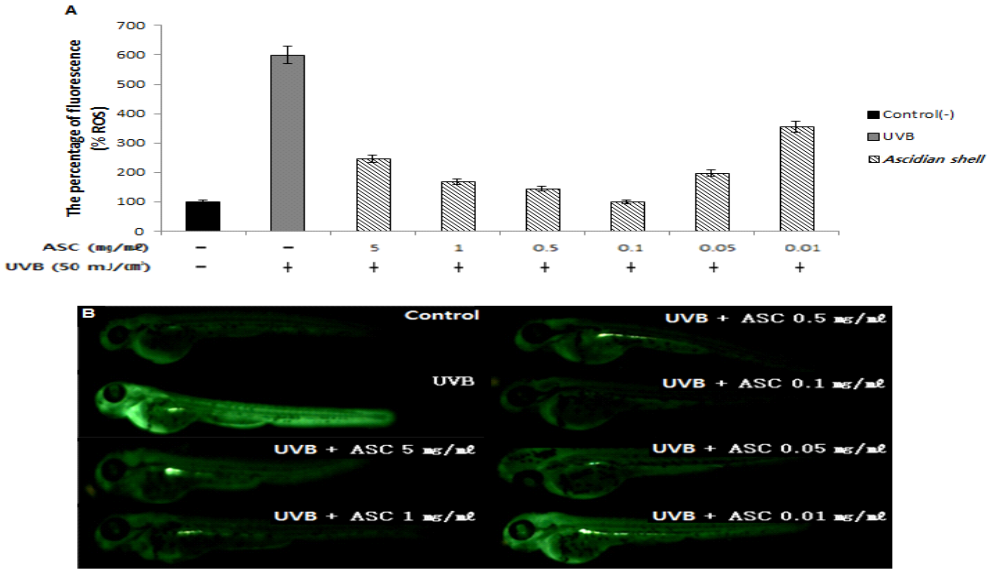

Fig. 7. Protective effect of ASE against UVB-induced ROS generation in zebrafish. The embryos were exposed to UVB (50 mJ/㎠) and treated with ASE. After incubation, the embryos were stained with DCF-DA and intracellular ROS were detected by spectrofluorometry (A) and fluorescencemicroscopy (B).

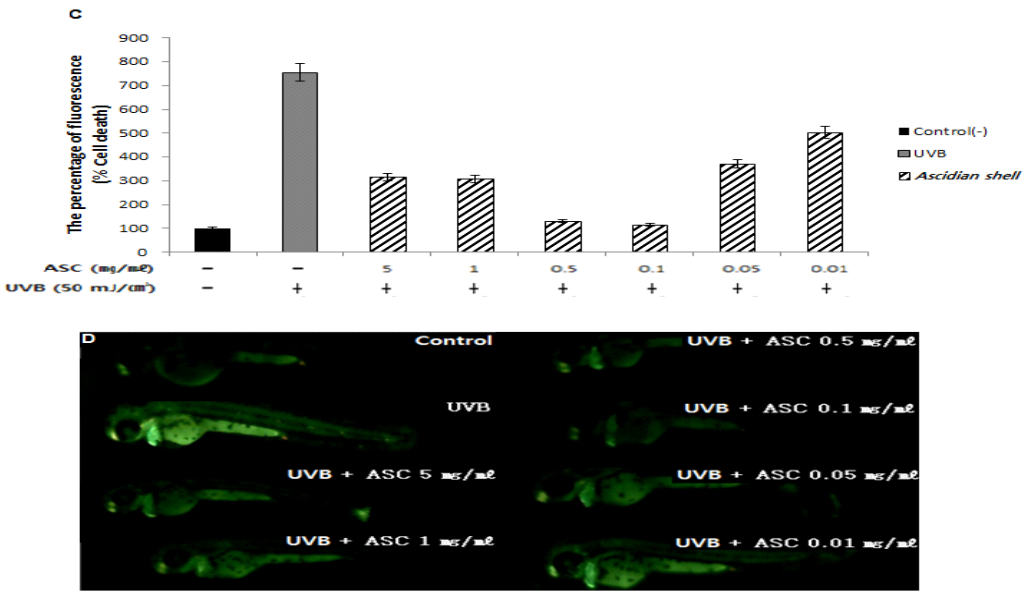

Fig. 8. Protective effect of ASE against UVB-induced cell death in zebrafish. The embryos were exposed to UVB (50 mJ/㎠) and treated with ASE. After incubation, the embryos were stained with acridine orange and cell death was detected by spectrofluorometry (C) and fluorescence microscopy (D).

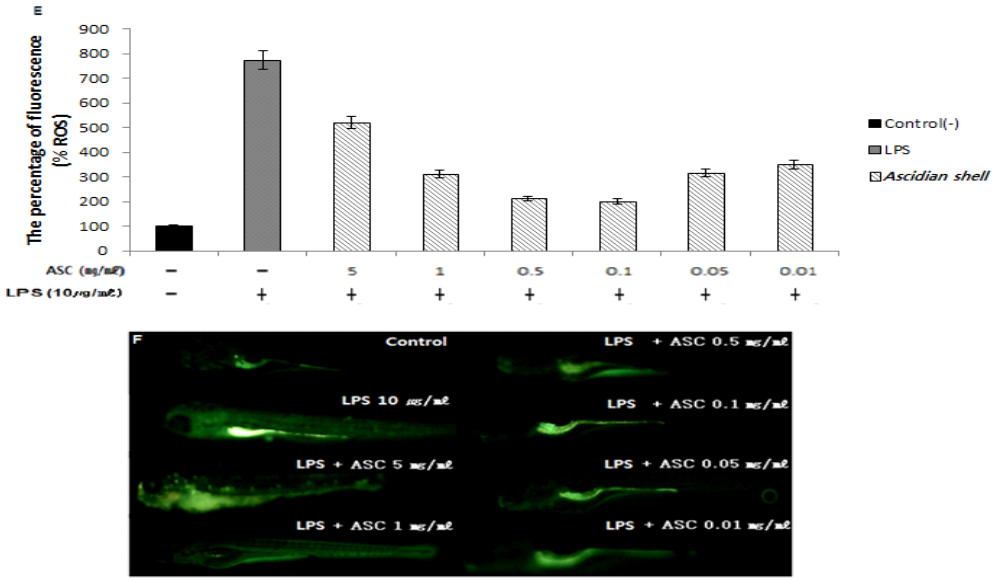

Fig. 9. Protective effect of ASE against LPS-induced ROS production in zebrafish. After incubation, the embryos were stained with DCF-DA and intracellular ROS were detected by spectrofluorometry (E) and fluorescence microscopy (F).

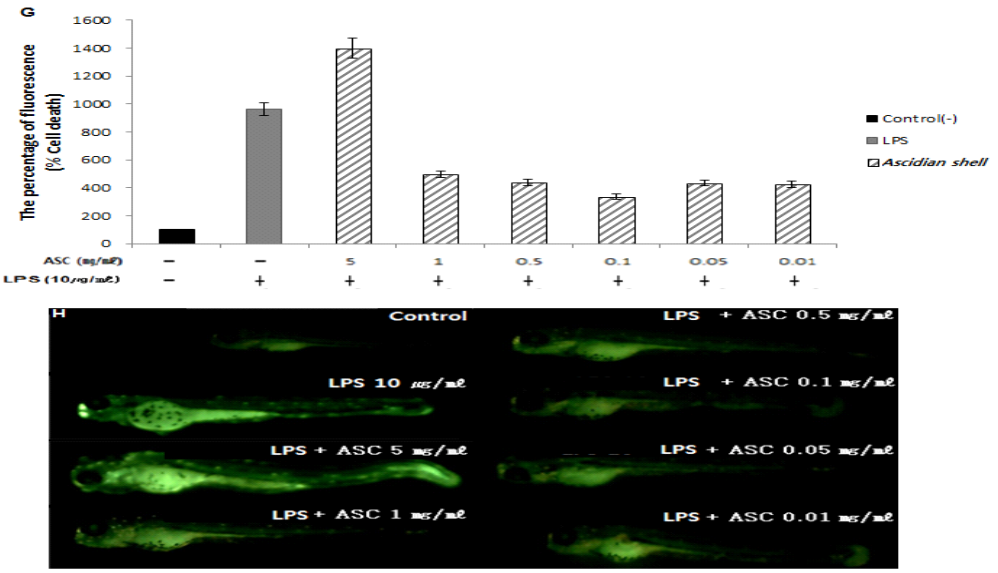

Fig. 10. Protective effect of ASE against LPS-induced cell death in zebrafish. After incubation, the embryos were stained with acridine orange and cell death were detected by spectrofluorometry (G) and fluorescence microscopy (H).

the negative control group. However, ASE significantly reduced the cell death in a concentration dependent manner, and a significant reduction was observed at 0.1 ㎎/㎖. Fig. 10(H) is a typical fluorescence micrograph of cell death in the zebrafish.

4. Conclusions

The results of evaluating the toxicity of *Ascidian shell* extract in HaCaT and B16F10 cells are as follows. As the concentration of *Ascidian shell* extract increased, cell viability decreased and toxicity was observed at higher concentrations. Evaluation of toxicity in the zebrafish model resulted in that the coagulation rate of embryo was found to be higher than 30.0 % beginning from the concentration samples of 5.0 ㎎/㎖ *Ascidian shell* extract, therefore proving that toxicity is apparent when in higher concentrations. The evaluation results of toxicity in zebrafish larvae heart was also similar to that of embryo.

In the present study, ASE provided protection in a UVB-induced zebrafish model through the inhibition of ROS generation. Furthermore, ASE inhibited ROS generation and cell death induced by UVB irradiation in a zebrafish. These results indicate that ASE could be used as a potent skin damage protective agent in cosmeceutical products after further study in zebrafish. ROS were assessed in LPS induced inflammation zebrafish model. In the present study, ROS and cell death in zebrafish were significantly increased with the LPS treatment. However, ASE treatment significantly decreased the LPS induced inflammation in the zebrafish. Cell death was also significantly reduced in ASE treated zebrafish.

References

1. G. C. Yen, H. Y. Chen, "Antioxidant Activity of Various Tea Extracts in Relation to Their Antimutagenicity", *J. Agric. Food Chem,* Vol.43, No.1 pp. 27-32, (1995).
2. M. Schieber, N. S. Chandel, "ROS Function in Redox Signaling and Oxidative Stress", *Current Biology*, Vol.24, No.10 pp. 453-462, (1995).
3. J. P. Kehrer, L. O. Klotz, "Free radicals and related reactive species as mediators of tissue injury and disease implications for Health", *Critical Reviews in Toxicology*, Vol.45, No.9 pp. 765-798, (2015).
4. V. Kumar, A. A. Khan, A. Tripathi, P. K. Dixit, U. K. Bajaj, "Role of oxidative stress in various diseases : Relevance of dietary antioxidants", *J. Phytopharmacology*, Vol.4, No.2 pp. 126-132, (2015).
5. P. Ahmad, C. A. Jaleel, M. M. Azooz, G. Nabi, "Generation of ROS and Non-Enzymatic Antioxidants During Abiotic Stress in Plants", *Botany Research International*, Vol.2, No.1 pp. 11-20, (2009).
6. P. Molyneux, "The use of the stable free radical diphenylpicryl-hydrazyl (DPPH) for estimating antioxidant activity", *J. Sci. Technol*, Vol.26, No.2 pp. 211-219, (2004).
7. A. Rodriguez-Bernaldo, H. S. Costa, "Analysis of carotenoids in vegetable and plasma samples: A review", *J. Food Composition and Analysis*, Vol.19. No.2 pp. 97-111, (2006)
8. Y. N. Kim, D. W. Giraud, J. A. Driskell, "Tocopherol and carotenoid contents of selected Korean fruits and vegetables", *J. Food Compos Anal.* Vol.20, No.6 pp. 458-465, (2007)
9. N. I. Krinsky, "Carotenoids as antioxidants", *Nutrition*, Vol17, No.10, pp. 815-817, (2001)
10. J. K. Song, D. W. Kime, J. B. Eun, B. D. Choi, M. J. Oh, S. J. Jung, "Identification of Cellulolytic Bacteria Associated with Tunic Softness Syndrome in the Sea Squirt, Halocynthia roretzi", *Food Sci. Biotechnol*,

Vol.21, No.5 pp. 1405-1411, (2012).
11. B. D. Choi, S. J. Kang, Y. J. Choi, M. G. Youm, K. H. Lee, "Utilization of *ascidian* (*Halocynthia roretzi*) tunic: Carotenoid composition of *ascidian* tunic. *Korean J Fish Aqua Sci*. Vol.27, No.4 pp. 344-350, (2014).
12. Z. Rohmah, L. Fitria, S. J. Kang, J. S. Kim, J. J. LEE, Y. L. Ha, B. D. Choi, "Anti-obesity effects of Lipid Extract from Sea-reared of Rainbow Trout (*Oncorhynchus mykiss*) Fed with Sea Squirt (*Halocynthia roretzi*) Tunic's Carotenoids and CLA, *J. Nutrition & Food Sciences*, Vol.6, No.4 pp. 1-7, (2014).
13. W. Brand-Williams, M. E. Cuvelier, C. Berset, "Use of a Free Radical Method to Evaluate Antioxidant Activity", *Lebensm. Weiss. U. Technol*, Vol.28, pp. 25-30, (1995).
14. A. Tingaud-Sequeira, N. Ouadah, P. J. Babin, "Zebrafish obesogenic test: a tool for screening molecules that target adiposity", *Published by the American Society for Biochemistry and Molecular Biology*, Vol.52, No.9 pp. 1765-1772, (2011).
15. A. Grarcia-Lafuente, E. Guillamon, A. Villares, M. A. Rostagno, J. A. Martinez, "Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease", *Inflammation Research*, Vol.58, No.9 pp. 537-552, (2009).
16. H. Wilms, J. Sievers, U. Rickert, M. Rostami-Yazdi, Ulrich Mrowietz, R. Lucius, "Dimethylfumarate inhibits microglial and astrocytic inflammation by suppressing the synthesis of nitric oxide, IL-1β, TNF-α and IL-6 in an in-vitro model of brain inflammation", *J. Neuroinflammation*, Vol.30, No.7 pp. 1-9, (2010).