A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Involvement of HPI Axis in the anti-stress activities of Hydrangeae Dulcis Folium in zebrafish.

Junyoung Oh

Department of Marine Life Science

GRADURATE SCHOOL

JEJU NATIONAL UNIVERSITY

FEBRUARY, 2016

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Junyoung Oh

(Supervised by ProfessorSeungheon Lee)

A thesis submitted in partial fulfillment of the requirement for the degree of

Master of Science

2016.02.

This thesis has been examined and approved.

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Thesis director, You-Jin Jeon, Prof. of marine life science

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Gi-Young Kim, Prof. of marine life science

_____

Seungheon Lee, Prof. of marine life science

2016.02

Date

Department of Marine Life Science

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

Abstract

In this study, the anti-stress effects of ethanolic extract of Hydrangeae Dulcis Folium (EHDF) were investigated. To determine the effects of EHDF on physical stress, changes in whole-body cortisol level or behavior were monitored in zebrafish. To induce physical stress, we used net handling stress (NHS). Fish were treated with EHDF for 6 min before exposing the animals to stress. And then, fish were used for evaluation through behavioral test including novel tank test or open field test, or sacrificed for collecting body fluid from whole-body. In result, increased anxiety-like behaviors in novel tank test and open field test under stress were recovered by treatment with EHDF at 5, 10 and 20 mg/L ($P< 0.05$). Moreover, compared with normal group which were not treated by NHS, whole-body cortisol level were significantly increased by treatment of NHS in control group. As compared with control group, pretreatment with EHDF at concentrations of 5, 10 and 20 mg/L for 6 min significantly prevented the increase of whole-body cortisol levels induced by NHS ($P< 0.05$). In addition, adrenocorticotropin hormone(ACTH) challenge studies showed that the EHDF completely blocked the effects of ACTH (0.2 IU/g, IP) on cortisol secretion. These results suggest that EHDF may be a good anti-stress candidate, and that its mechanism of action may be related to its positive effects on cortisol release.

Keyword: Net handling stress; Zebrafish; HydrangeaeDulcis Folium; Whole-body cortisol; Novel tank test; Open field test

Contents

Abstract i

Contents ii

List of Figures iv

I. Introduction 1

II. Materials and Methods 4

1. Materials 4

2. Preparation of extract 4

3. Animals 5

4. Drug administration and induction of stress 5

5. UPLC quantitative analysis 5

6. Novel tank test 7

7. Open field test 7

8. Measurement of whole-body cortisol level 7

9. ACTH challenge test 8

10. Statistics 9

III. Result 10

1. Anti-stress effect of EHDF on NTT 10

1.1. Duration in top 10

1.2. Frequency in top 10

1.3. Turn angle 11

1.4. Distance moved 11

1.5. Not moving duration 11

1.6. Velocity 12

2. Anti-stress effect of EHDF on OFT 14

2.1. Distance moved 14

2.2. Meandering movement 14

2.3. Turn angle 15

2.4. Not moving duration 15

2.5. Frequency of highly mobile movement 15

3. Effect of EHDF on whole-body cortisol level 18

4. ACTH challenge test 20

IV. Discussion 21

References 25

Abstract in Korean 30

감사의글 32

List of Figures

Figure 1. Ultra-high-performance liquid chromatography (UPLC) chromatogram of phyllodulcin and hydrangenol from ethanolic extract of Hydrangeae Dulcis Folium. 6

Figure 2. Behavioral effect of the 70% ethanolic extract of Hydrangeae Dulcis Folium (EHDF; 5-20 mg/L) and L-theanine (40 mg/L) on the novel tank test in adult zebrafish. The graph shows duration in top (A), Frequency in top (B), Turn angle (C), Distance moved (D), Not moving duration (E) and Velocity (F). Each bar represents the mean ± S.E.M. of 10-12 zebrafish. *P* values for the group comparisons were obtained by one way ANOVA followed by Student-Newman-Keuls test ($^{\#}P< 0.05$ compared with the normal group, $^{*}P< 0.05$ compared with stressed control group)..... 13

Figure 3. Behavioral effect of the 70% ethanolic extract of Hydrangeae Dulcis Folium (EHDF; 5-20 mg/L) and L-theanine (40 mg/L) on the open field test in adult zebrafish. The graph shows distance moved (A), Meandering movement (B), Turn angle (C), Not moving duration (D) and Highly mobile movement (E). Each bar represents the mean ± S.E.M. of 10-12 zebrafish. *P* values for the group comparisons were obtained by one way ANOVA followed by Student-Newman-Keuls test ($^{\#}P< 0.05$ compared with the normal group, $^{*}P< 0.05$ compared with the vehicle-treated control group)..... 17

Figure 4. Effect of the 70% ethanolic extract of Hydrangeae Dulcis Folium (EHDF; 5-20 mg/L) and L-theanine (40 mg/L) on whole-body cortisol in adult zebrafish. The data are expressed as the mean (± S.E.M.) of the whole-body cortisol level. Each bar represents the mean ± S.E.M. of 4 zebrafish. P values for the group comparisons were obtained by one-way ANOVA followed by Student-Newman-Keuls test (# P < 0.05 compared with the normal group, * P < 0.05 compared with the vehicle-treated control group). 19

Figure 5. Increase of cortisol induced by adrenocorticotropic hormone (ACTH) was blocked by 70% ethanolic extract of Hydrangeae Dulcis Folium (EHDF; 10 mg/L) in ACTH challenge test. The data are expressed as the mean (± S.E.M.) of the whole-body cortisol level (N = 4 zebrafish per group). P values for the group comparisons were obtained by two-way ANOVA followed by Student Newman-Keuls test (* P < 0.05 versus the vehicle-treated control # P < 0.05 compared with the ACTH-treated

group). 21

I. Introduction

Stress works as the cause of many diseases by being the origin of all diseases and causing circulation disorder and disharmonyin human body [1]. Stress response is classified into short term responses and long term responses. Short term responses include increase of blood pressure, increase of heart rate, and change of behavior and blood movement from gastrointestinal tract to skeletal muscle while long term responses involve decrease of spleen weight and immune suppression caused by the decrease in the number of leukocyte. If the intensity of stress is strong, if it continues for a long time or if human body has a defect in stress response system, the stress may cause a disease [2]. When human experience stress, the hypothalamic-pituitary-adrenal (HPA) axis is activated and secretion cortisol from the adrenal glands. Exactly, the activation of the HPA axis induce the release of corticotropin-releasing hormone (CRH) from the hypothalamic. CRH acts on the pituitary to stimulate ACTH synthesis. ACTH, in turn, stimulates cortisol secretion from the adrenal cortex. And then adrenal cortex secretes cortisol, glucocorticoid into the blood. And then adrenal cortex secretes cortisol, glucocorticoid into the blood. Also,increased glucocorticoids regulate the action of the HPA axis by negative feedback[3].

Cortisol has several significant functions and life sustaining adrenal hormone essential to the maintenance of homeostasis such as increasing access to energy stores, fat mobilization and increasing protein, as well as regulating the level and duration of inflammatory responses[4]. Cortisol has advantageous effects such as energy supply and immunity improvement at normal level[5]. However, when blood cortisol would be maintained at high level, it causes mental disease including depression panic disorder and hippocampal damage in animals, and the memory loss and Cushing's syndrome [6, 7].

The stress response transmitted by the hypothalamic-pituitary-adrenal (HPA) axis in mammals is similar to the situation of fish.The stress response is characterized by the activation of the hypothalamus-pituitary-interrenal (HPI) axis in fish[8].Structures in fishes homologous to the cortical tissue of the mammalian adrenal gland, they are in close proximity to or imbedded in the kidney.

The zebrafish is a good model for the study of development genetics, biology human disease, human disease and behavioral pharmacology. Zebrafish have the advantages of easy handling as an experiment organism, cheap price short generation length of about 3 months, and there is a high homogeny of genetic overlap between zebrafish and humans, with approximately 90%[9]. Moreover, zebrafish are ideal for developing valid experimental models of stress, anxiety, major depression and discovering new therapeutics[10].

Hydrangea macrophylla is a species of flowering plant in the family Hydrangeaceae,and widely cultivated in Asia, and mainly grows insouthern coast including Jeju Island and Geoje Islandin Korea. The total size is approximately 2 ~ 3m, in colors ranging from white through pinks and violet.Hydrangeae Dulcis Folium is the dry and fermented leaf of *Hydrangea macrophylla*.It has antimicrobial and anti-allergic effects [11]. Leaf extracts of *Hydrangea macrophylla* were examined as a possible anti-malarial activity and anti-diabetic[12, 13]. Chemical principal constituents of Hydrangeae Dulcis Folium and their derivatives is hydrangenol, phyllodulcin, thunberginols andhalofuginones[14, 15]. Hydrangenol, phyllodulcin and thunberginol A significantly lowers blood glucose and free fatty acid level[13].Newly demonstrated that halofuginone, inhibits the development of autoimmunity in a mouse model of activating the amino acid response pathway [16].

Recently, people have been exposed to various kinds of stress, which may causes mental diseases such as depression and panic disorder. Therefore, a study, which provides with theoretical background required for the development of new medicine and treatment

method by verifying the mechanism of endocrine system hormone and stress, is urgently required. However, until now, no attempts have been made to investigate the anti-stress activity of Hydrangeae Dulcis Folium. This study investigated the anti-stress activity of Hydrangeae Dulcis Folium in zebrafish using method of behavioral pharmacology and molecular biology.

II. Materials and Methods

1. Materials

ACTH and tricainewere purchased from Sigma-Aldrich Co.(St. Louis, MO). Hydrangeae Dulcis Folium was purchased at the Han-Nong-Won Co. (Daegu, Korea), and a voucher specimen (JJNUOPS 2014-01) was deposited at the Marine Biomedical Science of the College of Ocean Sciences, Jeju National University. L-theanine were purchased from Santa Cruz Biotech (Santa Cruz, CA). The material was authenticated by Prof. Yong Han Kim of Department of Herbal Medicinal Pharmacology, College of Herbal Bio-industry, Daegu Haany University. All other materials were of the highest grade and were obtained from standard commercial sources.

2. Preparation of extract

The pulverized Hydrangeae Dulcis Folium (63.3 g) were extracted with 70% ethalonic solution (about 1 L) at 60 °C for 2 h twice. The ethalonic extract of Hydrangeae Dulcis Folium (EHDF) was filtered, concentrated on a water bath under vacuum, frozen and lyophilized (Eyela, model FDU-1200, Japan) (yield: 29.18%).

3. Animals

Zebrafish of 5-6 months of age were purchased from the World fish aquarium (Jeju, Korea). All fish were acclimated for at least two weeks in the experimental room and maintained under constant temperature (26 ± 1 ℃) tanks with aerated water. Fish were kept on a 14–10 h light/dark cycle (lights on from 07:00-21:00 h) and fed two times a day with commercial flakes, TetraMin (Tetra, Germany). All protocols were approved by the Institutional Animal Care Committee [2].

4. Drug administration and induction of stress

Net handling stress (NHS) was induced using method of Ramsay, Feist [17]. **S**ubjected to NHS, zebrafish were netted suspended in the air for 3 min. And then, fish were returned to water for 3 min. Afterward fish were suspended in the air for an additional 3 min again. Fish were induced to NHS after treatment with EHDF for 6 min. The fish were randomly divided in unstressed normal group, stressed control group, stressed EHDF-treated groups, and stressed positive control group. EHDF or L-theanine were dissolved in 0.9% NaCl solution. And zebrafish was put into a medicated bath of 0.9% NaCl solution, EHDF (5-20 mg/L), or L-theanine (40 mg/L) for 6 min just before test.

5. UPLC quantitative analysis

Reverse-phase UPLC was performed on the Waters Acquity UPLC H class (Milford,

Massachusetts, USA), consisting of a Quarternary Solvent Manager pump, Sample Manager - TN and PDA detector. Empower 3 pro software (Milford, Massachusetts, USA) was used for UPLC data analysis. Chromatographic separation was accomplished on a Waters C18 reverse phase column (Acquity BEH 50 x 1.0 mm I.D., 1.7 μm) at 30 °C and monitored at 313 nm. A gradient solvent system consisted of methanol (solvent A) and water (solvent B) was used from 10% (solvent A): 90% (solvent B) to 100% (solvent A): 0% (solvent B) for 40 min at a flow rate of 0.5 mL/min. The standard stock solutions were prepared by dissolving standards in 100% methanol to set a final concentration of 10 mg/mL. After ultracentrifugation at 9000 rpm and filtration through a syringe filter (0.45 μm, Pall Co.), 5 μL of sample was injected 3 times. The average retention time of hydrangenol andphyllodulcin were22.94 min and 24.10 min. The calibration curve of hydrangenol and phyllodulcinwere drawn with (R^2) of hydrangenol and phyllodulcin were as follows: y = 9750x – 19,400; R^2 = 0.999759 in standard concentrations ranging from 1 to 500 μg/mL. Using these equations, the quantity of hydrangenol and phyllodulcin in the EHDF was calculated and determined as 18,580.5 ±30.48 μg/g and 7672.2 ± 10.06 μg/g, respectively (Fig.1).

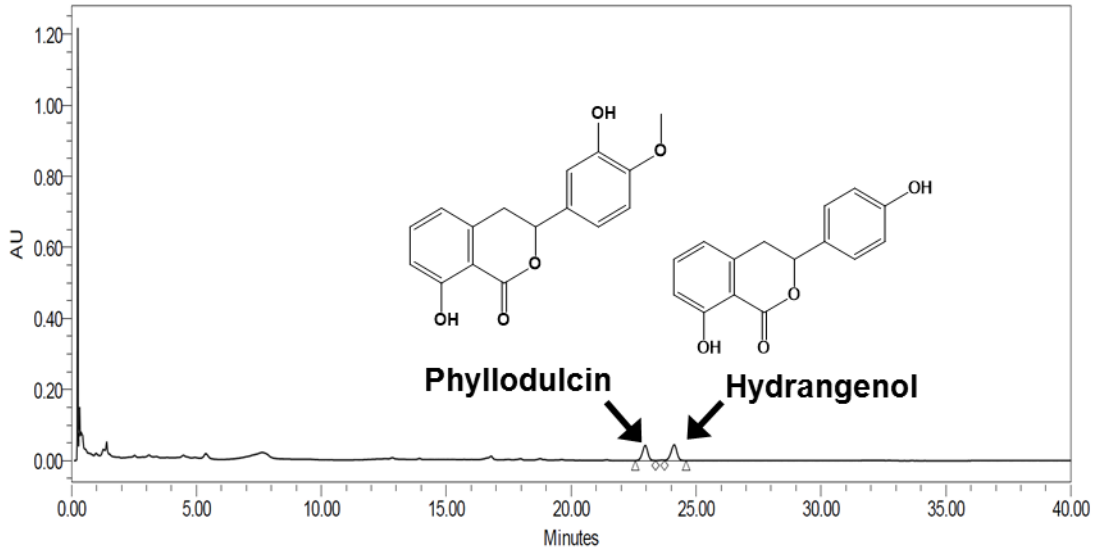


Figure 1. Ultra-high-performance liquid chromatography (UPLC) chromatogram of phyllodulcin and hydrangenol from ethanolic extract of Hydrangeae Dulcis Folium.

6. Novel tank test

For the novel tank test to assess zebrafish anxiety and locomotion [18-20], a 1.5-L trapezoidal tank (15 cm height×28 cm top×23 cm bottom×7 cm width) was maximally filled with water and divided into two equal virtual horizontal portions. In this experiment, fish (N = 10-12 zebrafish per group) were pre-treated with EHDF for 6 min. Zebrafish behaviors were recorded with subsequent automated analysis of generated traces by Ethovision XT 8.5 software (Noldus IT, Wageningen, Netherlands) from the top view for 6 min to calculate duration in top, frequency in top, turn angle, distance moved, not moving duration and velocity.

7. Open field test

Open field test (OFT) was performed to observe the anti-stress effect of EHDF on the swimming pattern and locomotor activity[21]. A white plastic cylinder (21 cm diameter, 24 cm height) filled with water to a height of 10 cm was implemented for this test. Following drug pretreatment for 6 min, the fish (N = 10-12 zebrafish per group) were individually placed in the center of the tank, and recorded with subsequent automated analysis of generated traces by Ethovision XT 8.5 software from the top view for 6 min, to distance moved, meandering movement, turn angle, not moving duration and highly mobile movement.

8. Measurement of whole-body cortisol level

The level of whole-body cortisol was measured using the method [22]. Zebrafish was put into a medicated bath of EHDF for 6 min. Zebrafish were sacrificed by tricaine (Sigma-Aldrich, Mo) at the concentration of 150 mg/L to obtain body fluid. After skin moistness of zebrafish was dried, it was put into a prepared cryo tube with 2 ml of 0.1M phosphate buffer saline (PBS) for homogenization. The ground mixture was added of 5 ml of diethyl ether and vortexed for 1 min. Then it was centrifuged at 3000 g for 10 min and then, frozen for 30 seconds in liquid nitrogen. The supernatant was moved into a new test tube and diethyl ether was evaporated by a vacuum centrifugal concentrator (CVE-2000, Eyela, Japan). After the evaporation of diethyl ether, 1 ml of 0.1M PBS was added to the test tube and the content was moved to a 1.7 mL tube. The tube was then stored at -20℃ until it was submitted for cortisol measurement. The whole-body cortisol level was measured by a cortisol assay kit (R&D system, USA). Absorbance was measured at a wavelength of 450 nm by a microplate reader (Molecular devices, USA) to analyze ELISA plate. Absorbance value was converted into the cortisol concentration value based on the 4-parameter sigmoid minus curve. The whole-body cortisol levels were expressed as the ratio of concentration to weight of each fish.

9. ACTH challenge test

Four treatment groups (N = 4 zebrafish per group) were used in order to determine whether an ACTH challenge could short-circuit possible inhibitory effects at the level of the brain and pituitary exerted by the 10 mg/L concentration of EHDF. The challenge dose of ACTH (Sigma-Aldrich Co., MO; freshly dissolved in sterile 0.1 MPBS) was paired with EHDF or home tank water pretreatment. The challenge dose of ACTHwas determined from a pilot dose-response study which examined four doses of ACTH in the range of 0.05–0.4 IU/g.

We used the lowest dose examined since it produced a maximal stimulatory effect on cortisolsecretion (data not shown). ACTH (0.2IU/40 μl/g) or vehicle (0.1 M PBS, pH 7.4) wereintraperitoneally injected after pretreatment with EHDF for 6 min or 0.9% NaCl solution. Fish were scarified15 min post ACTH injections for measurement of whole-body cortisol level[23].

10. Statistics

Values are expressed as means ± S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Student-Newman-keuls test for multiple comparisons. Statistical significance was set at p <0.05.For challenge study, the interactions between the agonist and EHDF were analyzed separately with a two-way ANOVA [factors: ACTH versus drug]; pairwise comparisons for the assessment of the drug influence on the ACTH effects were conducted by using Student-Newman-Keuls test. Statistical significance was set at p <0.05.

III. Result

1. Anti-stress effect of EHDF on NTT

1.1. Duration in top

Duration in top was measured for 6 min in NTT. The duration in top was 171.1 ± 13.7 sec in the normal group while it was 73.0 ± 18.0 sec in the control group which was about 58% decreased by NHS induction (p <0.05). Interestingly, the duration in top was 128.4 ± 12.6 sec at 5 mg/L of EHDF (p <0.05), 166.6 ± 20.3 sec at 20 mg/L of EHDF (p <0.05) and 204.8 ± 23.4 sec at concentration of 20 mg/L of EHDF (p <0.05). In the EHDF-treated groups at 5-20 mg/L concentrations, duration in top significantly increased compared to the control group. In the L-theanine-treated group, which was a positive control, duration in top was significantly decreased compared with the control group (152.2 ± 12.1 sec, p <0.05; Fig. 2A).

1.2.Frequency in top

Frequency in top was measured for 6 min in NTT. As shown in Fig. 1B, the frequency in top was 34.0 ± 2.0 times in the normal group while it was 10.4 ± 3.0 times in the control group which was about 71% decreased by NHS induction (p <0.05). Interestingly, the frequency in top were 28.5 ± 3.3 times at concentration of 5 mg/L of EHDF (p <0.05), 23.0 ± 2.3 times at concentration of 10 mg/L of EHDF (p <0.05) and 19.6 ± 2.7 times at concentration of 20 mg/L of EHDF (p <0.05). In the EHDF-treated groups at 5-20mg/L concentrations, frequency in top significantly increased compared to the control group. In the L-theanine-treated group, frequency in top was significantly increased compared with the

control group (21.6 ± 1.5 times, p <0.05; Fig. 2B).

1.3. Turn angle

The turn angle was 25.9 ± 2.1 deg in the normal group however, it increased by 88% to 47.1 ± 7.7 deg in the control group (p <0.05). It was 25.2 ± 3.4 deg in EHDF 5 mg/L (p <0.05), 26.8 ± 2.4 deg in EHDF 10 mg/L (p <0.05) and 25.1 ± 1.4 deg in EHDF 20 mg/L (p <0.05). Significant decrease was observed in the EHDF-treated groups at all concentrations compared to the control group. In the L-theanine-treated group, turn angle was significantly decreased compared with the control group (29.4 ± 3.4 deg, p <0.05;Fig. 2C).

1.4. Distance moved

Distance moved was measured for 6 min in NTT. The distance moved was 1921.0 ± 132.1 cm in the normal group while it was 1303.0 ± 143.8 cm in the control group which was about 33% decreased by NHS induction (p <0.05). Interestingly, the distance moved were 1977.0 ± 109.8 cm at concentration of 5 mg/L of EHDF (p <0.05), 2182.3 ± 125.6 cm at concentration of 10 mg/L of EHDF (p <0.05) and 2031.0 ± 100.5 cm at concentration of 20 mg/L of EHDF (p <0.05;Fig. 2D).

1.5. Not moving duration

Not moving duration was measured for 6 min in NTT. The not moving duration was 41.3 ± 3.3 sec in the normal group while it was 115.4 ± 23.8 sec in the control group which was about 180% increased by NHS induction (p <0.05). Interestingly, the not moving duration were 32.0 ± 4.2 sec at concentration of 5 mg/L of EHDF (p <0.05), 26.3 ± 4.2 sec at concentration of 10 mg/L of EHDF (p <0.05) and 27.0 ± 3.8 sec at concentration of 20 mg/L

of EHDF (p <0.05). In the EHDF-treated groups at 5-20 mg/L concentrations, not moving durations were significantly decreased, compared to the control group. In the L-theanine-treated group, not moving duration was significantly decreased compared with the control group (44.9 ± 2.3 sec, p <0.05; Fig. 2E).

1.6.Velocity

Velocity was measured for 6 min in NTT. The velocity was 5.1 ± 0.3 cm/s in the normal group while it was 3.8 ± 0.4 cm/s in the control group which was about 26% decreased by NHS induction (p <0.05). Interestingly, the velocity were 5.4 ± 0.3 cm/s at concentration of 5 mg/L of EHDF (p <0.05), 6.0 ± 0.3 cm/s at concentration of 10 mg/L of EHDF (p <0.05) and 5.6 ± 0.2 cm/s at concentration of 20 mg/L of EHDF (p <0.05). In the EHDF-treated groups at 5-20 mg/L concentrations, velocity significantly increased compared to the control group. In the L-theanine-treated group, velocity was significantly increased compared with the control group (29.4 ± 3.4 cm/s, p <0.05; Fig. 2F).

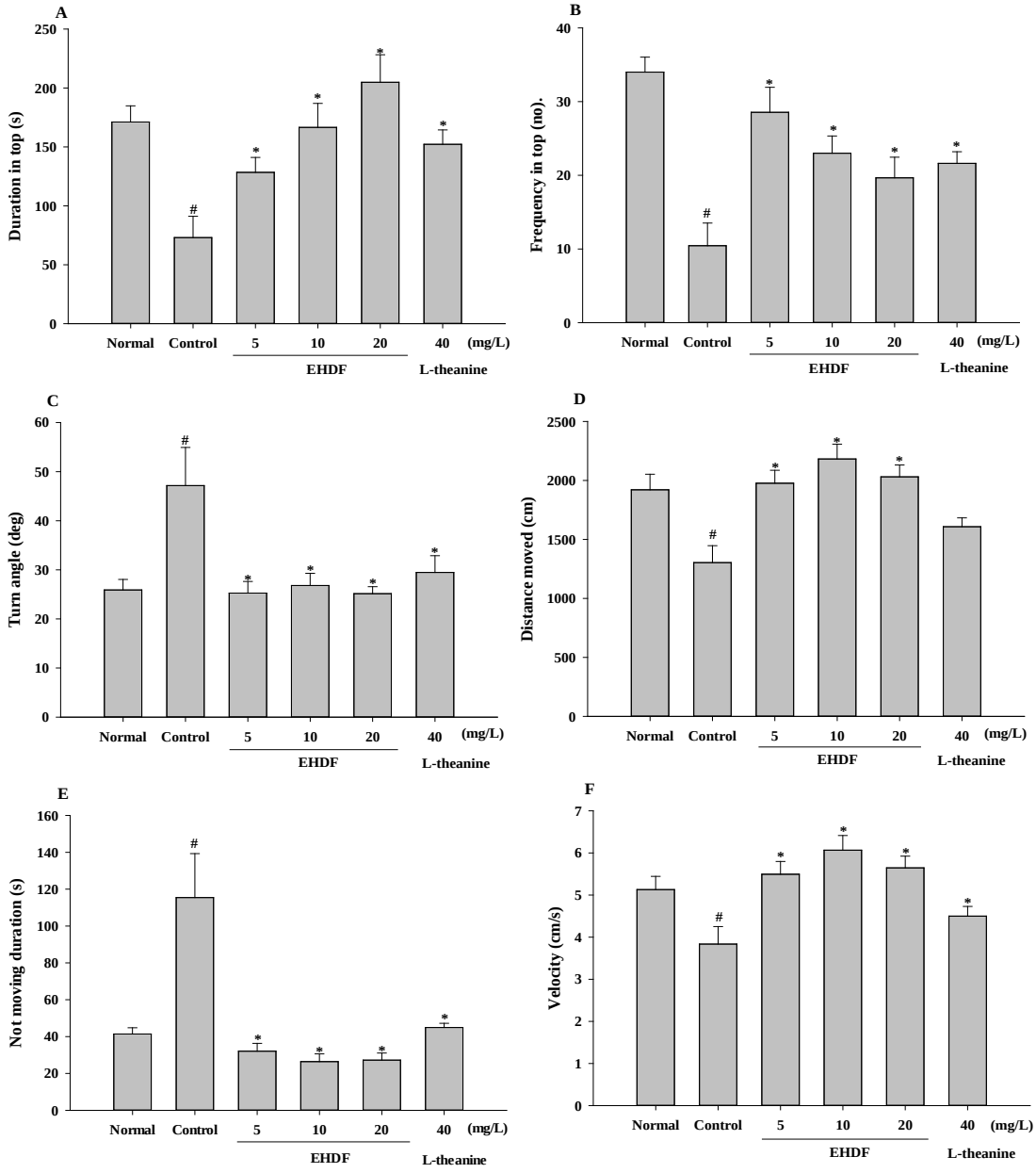


Figure 2. Behavioral effect of the 70% ethanolic extract of Hydrangeae Dulcis Folium (EHDF; 5-20 mg/L) and L-theanine (40 mg/L) on the novel tank test in adult zebrafish. The graph shows duration in top (A), Frequency in top (B), Turn angle (C), Distance moved (D), Not moving duration (E) and Velocity (F). Each bar represents the mean ± S.E.M. of 10-12 zebrafish. *P* values for the group comparisons were obtained by one way ANOVA followed by Student-Newman-Keuls test ($^{\#}P< 0.05$ compared with the normal group, $^{*}P< 0.05$ compared with stressed control group).

2. *Anti-stress effect of EHDF on OFT*

2.1. Distance moved

Distance moved was measured for 6 min in OFT. The distance moved was 2705.1 ± 217.5 cm in the normal group while it was 1964.3 ± 82.3 cm in the control group which was about 27% decreased by NHS induction (p <0.05). Interestingly, the distance moved were 2179.5 ± 187.0 cm at concentration of 5 mg/L of EHDF (p <0.05), 2391.1 ± 58.0 cm at concentration of 10 mg/L of EHDF (p <0.05) and 2543.7 ± 49.9 cm at concentration of 20 mg/L of EHDF (p <0.05). In the EHDF-treated groups at 5-20 mg/L concentrations, distance moved significantly increased compared to the control group.In the L-theanine-treated group, distance moved was significantly increased compared with the control group (2722.6 ± 168.2 cm, p <0.05; Fig. 3A).

2.2. Meandering movement

Meandering movement was measured for 6 min in OFT. The meandering movement was 416.0 ± 46.4 deg/s in the normal group while it was 752.5 ± 171.3 deg/s in the control group which was about 80% increased by NHS induction (p <0.05). Interestingly, the meandering movements were 469.3 ± 93.3 deg/s at concentration of 5 mg/L of EHDF (p <0.05), 372.2 ± 55.3 deg/s at concentration of 10 mg/L of EHDF (p <0.05) and 399.6 ± 62.7 deg/s at concentration of 20 mg/L of EHDF (p <0.05). In the EHDF-treated groups at all concentrations, meandering movement significantly decreased compared to the control group. In the L-theanine-treated group, meandering movement was significantly decreased compared with the control group (342.0 ± 43.3 deg/s, p <0.05; Fig. 3B).

2.3. Turn angle

The turn angle was 23.9± 1.3 deg in the normal group however, it increased by 30% to 30.5 ± 1.7 deg in the control group (p <0.05). It was 21.9 ± 1.2 deg in EHDF 10 mg/L (p <0.05) and22.8 ± 2.3 deg in EHDF 20 mg/L (p <0.05). Significant decrease was observed in the EHDF-treated groups at 10-20 mg/L concentrations compared to the control group. In the L-theanine-treated group, Turn angle was significantly decreased compared with the control group (21.9 ± 1.0 deg, p <0.05; Fig. 3C).

2.4. Not moving duration

Not moving duration was measured for 6 min in OFT. The not moving duration was 32.0 ± 5.4 sec in the normal group while it was 59.2 ± 6.7 sec in the control group which was about 84% increased by NHS induction (p <0.05). Interestingly, the not moving duration were 26.2 ± 6.3 sec at concentration of 5 mg/L of EHDF (p <0.05), 17.8 ± 2.6 sec at concentration of 10 mg/L of EHDF (p <0.05) and 25.6 ± 4.6 sec at concentration of 20 mg/L of EHDF (p <0.05). In the EHDF-treated groups at 5-20 mg/L concentrations, not moving durations were significantly decreased, compared to the control group. In the L-theanine-treated group, not moving duration was significantly decreased compared with the control group (19.7 ± 3.5 sec, p <0.05; Fig. 3D).

2.5. Frequency of highly mobile movement

Frequency of highly mobile movement was measured for 6 min in OFT. As shown in Fig. 1B, the frequency of highly mobile movement was 58.8 ± 3.7 times in the normal group while it was 245.1 ± 27.1 times in the control group which was about 322% increased by NHS induction (p <0.05). Interestingly, the frequency of highly mobile movement were 55.2

± 12.9times at concentration of 5 mg/L of EHDF (p <0.05), 61.8 ± 7.6 times at concentration of 10 mg/L of EHDF (p <0.05) and 130.0 ± 8.5 times at concentration of 20 mg/L of EHDF (p <0.05). In the EHDF-treated groups at 5-20mg/L concentrations, frequency of highly mobile movement significantly decreased compared to the control group. In the L-theanine-treated group,frequency ofhighly mobile movement was significantlydecreased compared with the control group (44.5 ± 7.0 times, p <0.05; Fig. 3E).

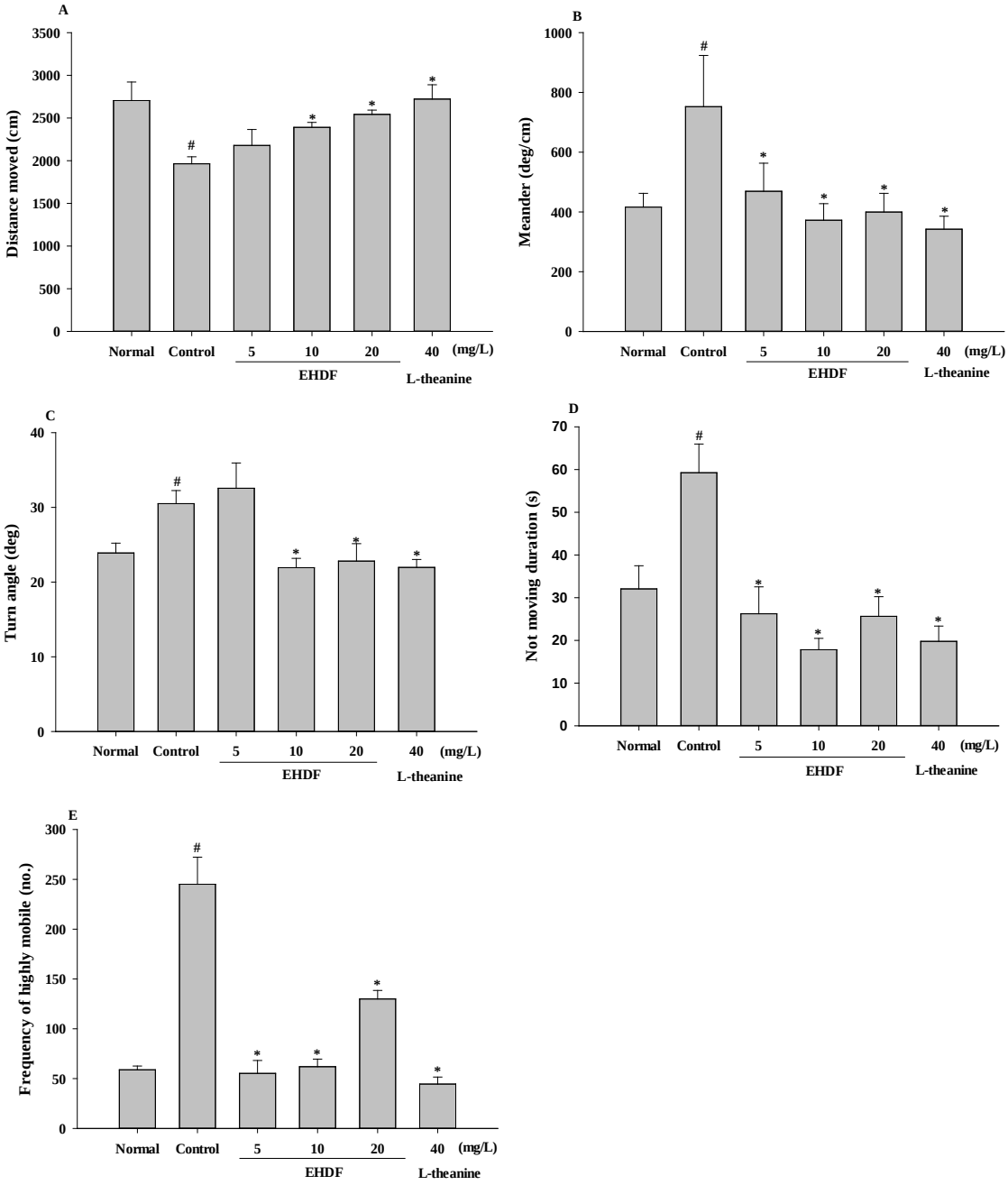


Figure 3. Behavioral effect of the 70% ethanolic extract of Hydrangeae Dulcis Folium (EHDF; 5-20 mg/L) and L-theanine (40 mg/L) on the open field test in adult zebrafish. The graph shows distance moved (A), Meandering movement (B), Turn angle (C), Not moving duration (D) and Highly mobile movement (E). Each bar represents the mean ± S.E.M. of 10-12 zebrafish. *P* values for the group comparisons were obtained by one way ANOVA followed by Student-Newman-Keuls test ($^{\#}P< 0.05$ compared with the normal group, $^{*}P< 0.05$ compared with the vehicle-treated control group).

3. Effect of EHDF on whole-body cortisol level

To investigate whether changes in biochemical parameters such as whole-body cortisol after stress are normalized or prevented by EHDF treatment, we measured whole-body cortisol level in zebrafish pretreated with EHDF (Fig. 4). In the unstressed normal group, the whole-body cortisol level was 3.73 ± 0.55 ng/g, and it was significantly increased in stressed control group(7.22 ± 0.78 ng/g, p <0.05). Increases of whole-body cortisol levels after stress were significantly reduced by EHDF at concentrationsof 5, 10, and 20 mg/L (4.58 ± 0.98, 3.65 ± 0.852, 3.23 ± 0.63 ng/g, respectively) compared with the stressed control group. Also, in the L-theanine-treated group, whole-body cortisol levels was significantly decreased compared with the control group (3.23 ± 0.45 ng/g, p <0.05).

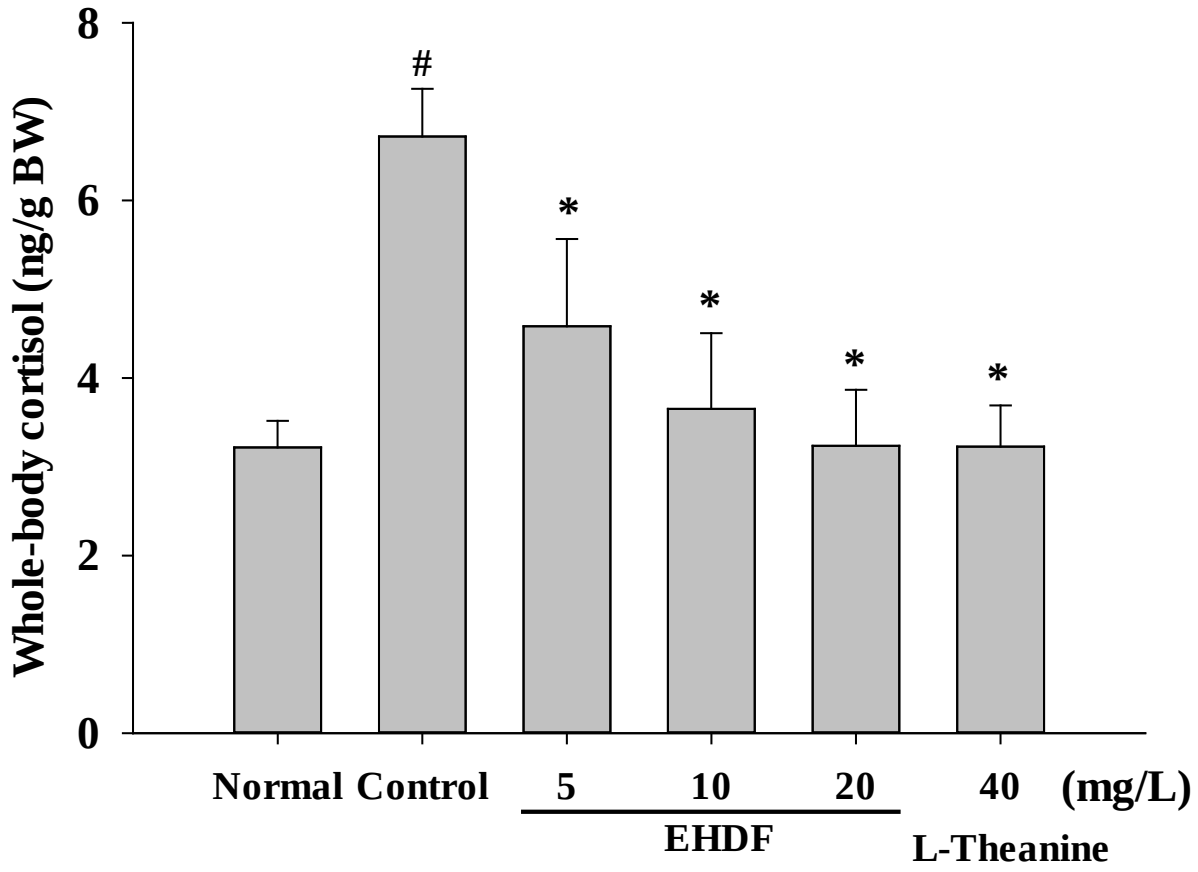
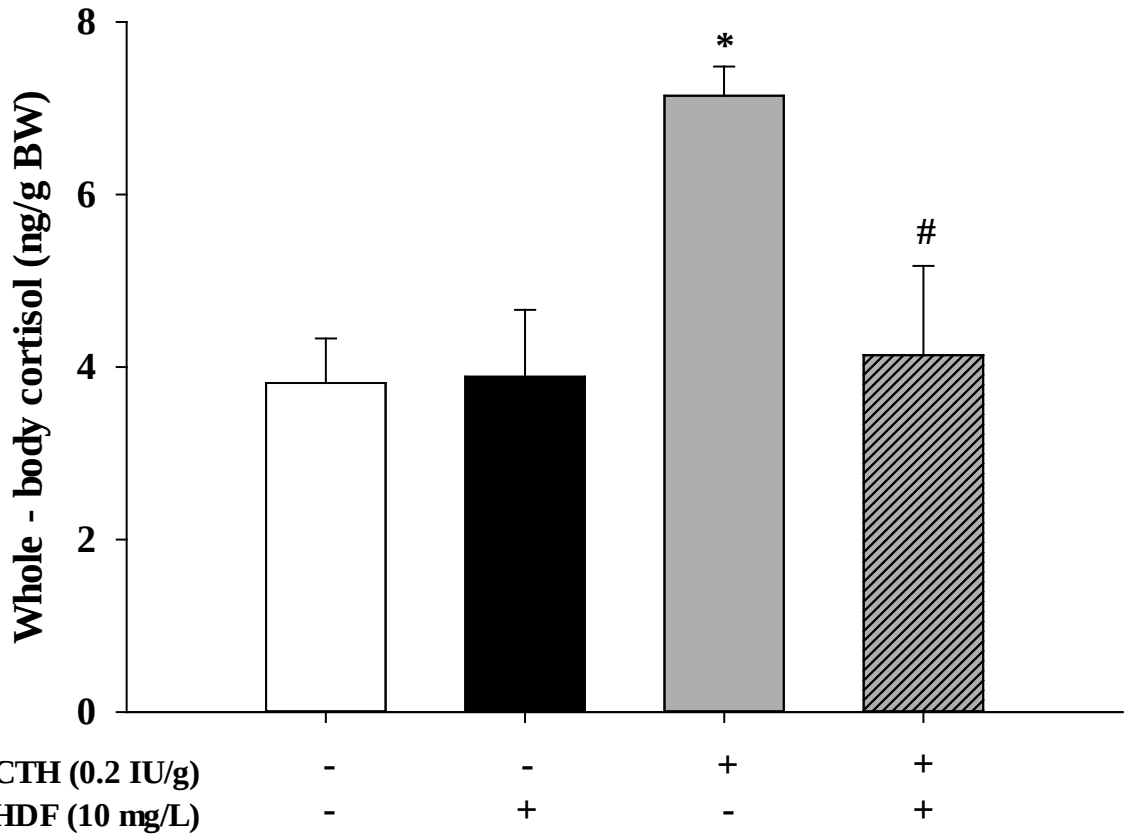


Figure 4. Effect of the 70% ethanolic extract of Hydrangeae Dulcis Folium (EHDF; 5-20 mg/L) and L-theanine (40 mg/L) on whole-body cortisol in adult zebrafish. The data are expressed as the mean (± S.E.M.) of the whole-body cortisol level. Each bar represents the mean ± S.E.M. of 4 zebrafish. P values for the group comparisons were obtained by one-way ANOVA followed by Student-Newman-Keuls test (#P< 0.05 compared with the normal group, * P< 0.05 compared with the vehicle-treated control group).

4. ACTH challenge test

An ACTH challenge was administered after pretreatment with either vehicle or EHDF (10 mg/L) to determine if EHDF exerted an inhibitory effect on cortisol secretion at the level of the interrenal glands (Fig. 5). The 0.2 IU/g dose of ACTH stimulated cortisol release comparable to typical stress response levels. EHDF completely blocked the ACTH induced



cortisol secretion.

Figure 5. Increase of cortisol induced by adrenocorticotropic hormone (ACTH) was blocked by 70% ethanolic extract of Hydrangeae Dulcis Folium (EHDF; 10 mg/L) in ACTH challenge test. The data are expressed as the mean (± S.E.M.) of the whole-body cortisol level (N = 4 zebrafish per group). P values for the group comparisons were obtained by two-way ANOVA followed by Student Newman-Keuls test (* $P < 0.05$ versus the vehicle-treated control # $P < 0.05$ compared with the ACTH-treated group).

IV. Discussion

Stress has provided a major information in explaining the behavioral change and the physiological factors that affect physical health[24]. Stress is all the responses, which a living organism shows to protect itself when it receives harmful stimulations from external environment[25]. Activation of the HPA by stressors, resulting in increasing plasma levels of ACTH and cortisol, is importantly involved in the responses that advance the protection of mammals [26].The cortisol-related endocrine system of zebrafish acts similarly to the one of endocrine system of mammals [27]. The stress response is characterized by the activation of the HPI axis in zebrafish[8].In other words, stress-induced synthesis and secretion of cortisol involves the activation of the hypothalamus, pituitary and interrenal gland in fish[28].

Exposuresto sudden excessive stress induce the secretion of stress hormone which works on central nervous system (CNS)orendocrine system. It results in the changesof behavior, immunity function or body weight[29, 30]. The previous studies reported that zebrafish tend to show not moving,sinking down to bottom or being observed that are overexcited and perform abnormal swimming patterns, erratic movement or jumps out of water under the anxiety, stress or panic state[31, 32]. Thereby, in this study, we observedbehavioral parameters including total distance moved, not moving duration, duration

in top, turn angle and meandering movement utilizing NTT or OFT. In the results of NTT,because of exploration response by stimuli of NHS, duration in top, frequency in top, distance moved and velocity were decreased.Andturn angle and not moving duration were significantly increased (Fig.2).In addition, in the results of OFT,distance moved was decreased. On the other hand,meandering movement, turn angle, not moving duration and frequency of highly mobile movement were increased (Fig.3).Accordingly,we verified that anxiety-like behaviors were increased and locomotor activity was reduced in zebrafish under stress.In the present study, we observed that EHDF recovered the anxiety-like behaviors or reduction of locomotor activity by NHS in the NTT and OFTin a dose-dependent manner with a profile comparable to that observed forL-theanine, an anti-stress functional supplement. The results of the present study provide evidence for the potential role of EHDF as an anti-stress material with some significant findings.

Exposure to stress also causes behavioral changes including increase of anxiety-like behaviors or decrease of locomotor activity as well as anxiety or depression [33]. Besides anxiety-like behaviors are relative with the increase of cortisol levels[34].also demonstrated that plasma corticosterone levels were elevated by obestatin, a chemical stressor, induced anxiety-like effect in mice in the elevated plus-maze and OFT test as the correlation between the activation of the stress axis and anxiety[35].Changes in whole-body cortisol level are useful biomarkers of the primary stress responses [36]

In our results, whole-body cortisol was increase by NHS in the same manner as the previous studies (Fig.4). Therefore, we speculate that behavioral changes induced by NHSresult from increase of whole-body cortisol. Interestingly, increases of whole-body cortisol levels induced by NHS were blocked by pretreatment of EHDF in a dose-dependent manner. This results suggest that anti-stress effect of pretreatment of EHDF, which prevent behavioral changes in NTT and OFT could be dependent upon blockade of increases of

whole-body cortisol.

HPA axis is well-known for many mammals, with CRF and ACTH being the most important hormones and corticosterone or cortisol the main end products of the HPA axis [37]. This general mammalian mode also applies to teleost[38]. However, secretion of corticosteroid occurs from the interrenal cells in fish. as ACTH is the main secretagogue responsible for cortisol secretion in mammals and fish[39].

Our results suggest that EHDF may effectively block NHS-induced cortisol secretion. Thereby, we hypothesized that EHDF might exhibit its effects through affecting through altering HPI axis because it is major part of the endocrine system that controls the secretion of cortisol. We investigated this hypothesis whether the secretion of cortisol by ACTH would be antagonized by anti-stress properties of EHDF [23]. In the result, EHDF completely blocked the induction of cortisol secretion by ACTH (Fig.5).In the present study, these results suggest that the anti-stress effects of EHDF are mediated through the melanocortin receptor.

In previous research,phyllodulcin, a constituent of Hydramgea macrophylla. is a potential phosphodiesterase (PDE) inhibitor.It is well-known that cyclic AMP is biotransformed to 5'-AMP, biologically inactive compound by intracellular PDE[40].Kawamura, Kagata [41] reported that phyllodulcin exhibited a PDE inhibitory effect due to enhance cyclic AMP-induced steroidogenesis in a concentration-dependent manner in bovine adrenocortical cells.Although authors did not perform a measurement of concentration of cortisolin bovine adrenocortical cells after treatment of phyllodulcin, for reasons mentioned above, phyllodulcin might potentiate production or secretion of cortisol.Nevertheless, in this study, the pretreatment of EHDF reduced the whole-body cortisol level. Unfortunately, we did not know any active compound of EHDF yet. However, we could speculate possible mechanism of EHDF on cortisol secretion through researches using bovine adrenal fasciculata cells. ACTH stimulates cytosolic calcium movement, which

was biphasically enhanced by its concentrations, and also induces cortisol production mainly via phospholipase A2-dependent processes. In addition, guanosine-5'-triphosphate (GTP) may physiologically play an important role in the regulation of cortisol synthesis [42]. Simultaneous activation of Ca^{2+} messenger systems by GTP could co-operatively enhance cortisol production [28]In this way, cortisol secretion is closely related with Ca^{2+} signaling.Thunberginols A, B, and F were isolated from Hydrangeae Dulcis Folium, inhibited rise in intracellular Ca^{2+} concentrations in RBL-2H3 cells induced by antigen [42].Our present results and these previous reports suggest that the anti-stress mechanism of EHDF could be dependent upon inhibition of Ca^{2+} signaling in interrenal gland of zebrafish.

The results showed that anti-stress effects of EHDF mediated the secretion of cortisol and may be partly attributable to endocrine. The present findings may also provide important scientific evidence for the application and development of relaxation functional food to conquer the stress and EHDF can be a potential candidate for treating stress-related disorders such as anxiety and depression.As diseases caused by stress rapidly increase recently, current study trend is the finding and studying natural substance materials such as *Schizandra chinensis* and*Scutellaria baicalensis*, which have less adverse effect in the efficient treatment of stress-related diseases [21]. The result of this study suggests that EHDF can be developed to a medicine, which restricts stress, as material for functional food. In further study, we need to clarify mechanism and active components in Hydrangeae Dulcis Foliumwhich inhibits stress response. We are currently working on the isolation of active components in EHDF using an activity-guided fractionation approach.

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Abstract in Korean

본연구에서는 ethalonic extract of Hydrangeae Dulcis Folium (EHDF)의스트레스예방효과를관찰하였다. 물리적스트레스유도방법인 Net handling stress (NHS)로스트레스를유도하여 EHDF 가동물모델인제브라피쉬의행동변화와 cortisol 조절효능을확인하고자하였다. 6 분간 EHDF 에약욕후 NHS 를유도하여 novel tank test, open field test 및 whole-body cortisol 의수준을측정하였다.NHS 를처리하지않은정상군과비교하여 NHS 를유도한대조군에서 NTT 와 OFT 결과, 대조군에서 meandering movement 및회전각, 부동시간의증가하고,총이동거리의감소하는행동변화가관찰되었다. 그러나 EHDF 를 5-20 mg/L 의농도로전처리한실험군에서 NHS 유도에의해나타나는행동변화가억제되었다(P<0.05).

또한 NHS 를처리하지않은정상군과비교하여 NHS 를유도한대조군에서유의성있는 whole-body cortisol 수준의증가가관찰되었다(P<0.05).흥미롭게도 EHDF 를 5-20 mg/L 를 6 분간전처리후 whole-body cortisol 의수준을측정하였을때,대조군과비교하여유의성있게감소하는결과가관찰되었다(P<0.05). ACTHchallenge test 결과, ACTH 처리에의한전신코티졸(whole-body cortisol) 분비증가가 EHDF 에의해현저하게억제됨을확인할수있었다(*P*<0.05). 따라서 EHDF 는 cortisol 분비량을억제하고, 스트레스를저하하는작용효과를나타내므로, 우울증, 불안장애, 피로증후군, 수면장애, 신경변성질병,

스트레스성정신병에피소드와같은다양한스트레스성질환의치료제또는개선제등의개발에유용하게이용될수있다

감사의글

오직열정과패기로시작했던대학원생활이어느새2년의석사과정을마치고학위논문을제출하게되었습니다.짧지도그렇다고길지도않았던시간동안저에게도움을주신분들이많습니다.미흡하지만학위논문을마치면서그분들께감사의말씀을전합니다.

학문적인가르침과동시에많은기회와지원을해주셨고못난제자를앞으로나아가게이끌어주신지도교수님인이승헌교수님께무엇보다더깊은감사의마음을전합니다.그리고논문심사를맡아주시고언제나열정적인강의와올바른연구자의자세를알려주신전유진교수님,김기영교수님께감사를드립니다.또한부족한점을언제나세심하게봐주시고자상함으로대해주신여인규교수님,

수산질병관리사면허증합격에많은도움을주신이제희교수님,정준범교수님께감사의말을드립니다.

제가연구실에그리고대학원을시작할수있게길을열어준근수형과은아누나그리고재영이형,

학부시절과연구실초창기부터함께했던은성이형,원보형,그리고내밑에서너무고생이많았던성은이와다예에게감사의말을전합니다.또한방황하고힘들때옆에서많은조언과이정표가되어준성도,

태수,민기,유정이,혜나,진이누나,윤택이,현경이,은란이,그리고그외석사동기들과현수형에게덕분에졸업을하게되어진심으로감사의마음을전합니다.가끔씩찾아와저에게힘이되어준완택이,앞으로대학원에진학하는천만이,가재를좋아하는현석이,

국립수산물품질관리원에서근무중인빛나에게그동안너무고마웠다고앞으로도가끔씩오래보자고감사의말을전합니다.

4년째유지하고있는 A5 – Synergy

모임회장김동광,부회장한정진,총무조승용과유치원부터대학교까지함께한조진수,고영일,그리고먼곳에서항상응원을해준이석진,많은정보를주고있는신훈,쉐보래에서근무하는박지훈,뒤늦게대학을다니며고생하는고경표,전화한통이면달려와주는용희,내부탁은다들어주는경윤이와용현이,함께동거동락하며그누구보다가까이에있던이제는내동생같은영석이에게고맙다는말전합니다.또한말썽꾸러기지만언제나내편이되어주는나은이,기쁠때나슬플때나함께술을나누며공감해주는민승이와용완이,민주,그리고하영드림에게도결코가볍지않은마음을전합니다.

마지막으로한없는사랑과믿음으로저에게자신감을심어주신아버지,철없는아들때문에마음고생이심하셨던어머니,저에게등대같은누나에게도고맙습니다.

앞으로는가족의은혜에조금이나마보답할수있도록노력하겠습니다.일일이언급을하지못했지만그동안저를아끼고도와주신모든분들께다시한번진심으로감사를드립니다.

2016年 2月

오준영