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Anti-Stress Effects of *Glycine tomentella* Hayata in Tilapia: Inhibiting COX-2 Expression and Enhancing EPA Synthesis in Erythrocyte Membrane and Fish Growth

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ABSTRACT: The objective of this study was to elucidate the *in vivo* effects of the ethanol extract of wooly *Glycine tomentella* Hayata (GTE) root on tilapia to elucidate whether GTE has antistress activity. Tilapia as an animal model were fed with or without GTE, then injected with lipopolysaccharide (LPS) or ammonium chloride (NH_4Cl). The tilapia were exposed to 100 mg/L of aqueous NH_4Cl, and/or acute cold stress. Growth parameters of the tilapia were measured during the feeding trials. Tilapia injected with GTE (20 μg/g of fish), NH_4Cl (100 μg/g of fish) and/or LPS (1 μg/g of fish) were then sampled 2 h poststimulation. GTE significantly inhibited cyclooxygenase-2 expression and hemoglobin (Hb) dimer formation (36 kDa). GTE also improved growth and blood viscosity and upregulated eicosapentaenoic acid content of erythrocytes. The *in vivo* results indicated that GTE (20 μg/g of fish) can be applied as a stress-tolerance enhancing agent for the aquaculture industry.

KEYWORDS: *Glycine tomentella*, tilapia, cyclooxygenase, stress, eicosapentaenoic acid, EPA, erythrocyte, hemoglobin dimerization, tumor necrosis factor-alpha, TNF-α

INTRODUCTION

Herbs are used in fish health management.[1] Previous studies have shown that the ethanolic extract of *Glycine tomentella* Hayata (GTE) inhibits TNF-α expression by 67−90% during the early phase of lipopolysaccharide (LPS) stimulation.[2] The study on salmonid macrophages (TO) demonstrated that GTE induces TO proliferation and regulates eicosanoids and NADPH formations during LPS stimulation. Prostaglandin E_2 (PGE_2) (>100 nM) induces COX-2 and regulates TNF-α expression. Inhibition of TNF-α by GTE (<25 μg/mL) seems to reduce thromboxane 2 (TXA_2) and blocks PGE_2 9-reductase by arresting NADPH production, which is a cofactor regulating PGE_2 degradation.[3] In trout, PGE_2 affects cell permeability, erythrocyte deformability and flexibility.[4] Peroxides induce COX-2 activity,[5] alter the erythrocyte membrane lipids and cause structural changes in hemoglobin (Hb),[6,7] which mediated lipid peroxidation, Hb dimer formation, heme release from Hb and cross-linking of Hb fragments.

Erythrocytes are the dominant cells in blood. In mammals, the arachidonic acid (AA) level and the ratio of n-3 to n-6 highly unsaturated fatty acids (HUFA) in the erythrocyte membrane and Hb dimer have been used as biomarkers for monitoring inflammatory diseases.[8−12] The anti-inflammatory drugs generally inhibit COX-2 and lipoxygenase (LOX). Dietary fatty acids affect the tissues and plasma fatty acid composition of tilapia, trout and salmon.[13−16] Little is known about fatty acid metabolism in erythrocyte membrane of fish. Changes in a few fatty acids in erythrocyte membrane cause alterations in erythrocyte morphology and O_2 release rate of fish *in vivo*. Therefore, erythrocyte response may serve as an indicator for monitoring fish health during stress.[15]

GTE may be used to manage fish health and to reduce stress responses in fish. GTE strongly inhibits COX-2 activities and the iron-induced oxidation of linoleic acid *in vitro*.[17] GTE increases total antioxidative activities in plasma, prolongs the lag-phase of LDL oxidation and shows hypolipidemic and hypocholesterolemic effects in mammals and tilapia.[18−21] Dietary lipid intakes affect fatty acid contents and stability of trout erythrocyte membrane.[22] However, *in vivo* evidence of GTE treatment on teleosts regarding to erythrocyte responses and proinflammatory responses is unknown yet. Since stress stimuli induce a proinflammatory response such as tumor necrosis factor (TNF-α) and eicosanoid production in Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss*) and goldfish (*Carassius Auratus* L.),[23,24] the anti-inflammatory mechanism of herbs is worthy of investigation.

The aim of this study was to elucidate the effects of GTE on erythrocyte responses, proinflammatory gene expressions, hematological parameters, including plasma TNF-α, PGE_2, hemolysate electrophoresis profile and growth of tilapia. Whether TNF-α expression in plasma is strongly associated with AA or other HUFA metabolism in the erythrocyte membrane deserves investigation. The underlying mechanism and efficacy of GTE in tilapia may serve as a model to study natural anti-inflammatory products. In addition, GTE may be usable as a fish feed supplement or as an adjuvant to vaccine or antibiotics to improve the anti-inflammatory ability of cultured fish. The fish can then cope with stress from drastic temperature changes or chemical pollution or during live-fish transportation. The same efficacy may also be used to improve human stress tolerance.

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Table 1. Primers and Experimental Conditions Used in Real-Time PCR

gene	direction	primer sequence (5′−3′)	product size (bp)	annealing temp (°C)
TNF-α	forward	TAACACAAACAGCACACTCAA	78	55
	reverse	TAAAGAAATCCAGAAACCGAACA		
COX-2	forward	AGCAGCCAGAAGGAAGGCGG	130	62
	reverse	GACTGAGTTGCAGTTCTCTTAGTGTGC		
β-actin	forward	CCAGCCTTCCTTCCTTGGTAT	76	55
	reverse	GACGTCGCACTTCATGATGCT		

MATERIALS AND METHODS

GTE. The dried radix of *Glycine tomentella* Hayata was purchased from Kinmen, Taiwan. The root was powdered (<30 mesh) with a Chinese herbal grinder, then combined with 95% ethanol at a ratio of 1:10 (w/v) and refluxed for 2 h at 70 °C.[17] The solid residue was refluxed with 95% ethanol again. The solvent extracts were combined, and ethanol was removed under vacuum at 40 °C (Eyela digital water bath SB-1000, Tokyo Rikakikai, Japan) followed by freeze-drying to powder (100 mesh) designated as GTE, which was analyzed at 210 and 262 nm using RP-HPLC photodiode array detection as described.[17,25] GTE is composed of flavonoids that include vitexin (7.56 mg/g), daidzein (9.34 mg/g), daidzin (11.63 mg/g), malonyldaidzein (0.82 mg/g), genistein (0.16 mg/g), and organic acids including malic acid (511 mg/g), sugars (371 mg/g) including glucose and galactose (45.4% of total sugars), maltose (36.1%), fructose (11.5%), rhamnose (4.8%) and raffinose (2.2%). Other components of GTE are to be identified.

Feeding Trials. Tilapia (*Oreochromis mossambicus*) were transported to our laboratory from the Mariculture Research Center of the Fisheries Research Institute (Tainan, Taiwan). In trial 1, fish were acclimated and then separated into two groups: the control group weighed 101 ± 10 g/fish (n = 24), and the GTE group weighed 99 ± 17 g/fish (n = 24). In trial 2, both the control group (n = 8) and the GTE group weighing 59 ± 3 g/fish (n = 8) were designated as trial 2-1, while another group of smaller tilapia weighing 45 ± 12 g/fish (n = 8) in the control group and 41 ± 4 g/fish (n = 8) in the GTE group were designated trial 2-2. All fish were kept in glass tanks (60 × 58 × 42 cm, L × W × H) and reared under a 12:12 light−dark cycle.

Commercial feed powder (Dachan, Tainan, Taiwan) was mixed with 1% GTE as the test diet which was formulated according to our previous study.[18] Cellulose (1%) was used to substitute GTE in the control diet. The diets were isonitrogenic and isocaloric. Different feeds were made into pellets of 5 × 5 × 2 mm (L × W × H) with a pasta machine (Marcato S. p.A., Campodarsego, Italy) and fed to tilapia at 0.8% body weight per day.

Growth Analysis. The weight gain of each diet group of tilapia was calculated as the average of the total final body weight (FBW) divided by the total initial body weight (IBW) of each group. The specific growth rate (SGR, %/day) was determined as (ln FBW − ln IBW)/day × 100. The feed conversion ratio (FCR) was calculated as the mass of feed consumed (g) divided by the body mass gained (g). The protein efficiency ratio (PER) was determined as the weight gain (g) divided by the protein intake (g).

Injection Test. At the end of trial 1, the final weight of the control group averaged 124 ± 16 g/fish and that of the GTE group was 126 ± 24 g/fish. Fish fed a diet either with or without GTE were divided into three subgroups of treatments. Two of the three subgroups were exposed to aqueous NH_4Cl at 100 mg/L (NH_4Cl-w) for 2 h, denoted with (+), while one subgroup was not exposed to NH_4Cl-w, denoted with (−). A group of tilapia fed a GTE-free diet without injection nor exposure to aqueous NH_4Cl was considered as the blank. There were seven subgroups in total.

The four subgroups of fish exposed to NH_4Cl-w are denoted with (+). The test agent was dissolved in DMSO. The total volume of the test agent injected into the fish was 1 μL/g. For the GTE-free diet group, the test agents were DMSO (vehicle, as control) 1 μL/g of fish and 100 μg of NH_4Cl/g of fish (NH_4Cl-i). For the GTE-fed subgroups, the injected test agents were 20 μg of GTE (GTE) and a combination of 20 μg of GTE and 100 μg of NH_4Cl/g of fish (GTE/NH_4Cl). The one subgroup fed the GTE-free diet was injected with DMSO, and the GTE-fed subgroup was injected with GTE 20 μg/g of fish (GTE); neither was exposed to NH_4Cl-w, and they are denoted with (−).

The agents were injected into the caudal muscle of tilapia and then circulated into the blood. Each injection was completed within 16 min and was performed in triplicate. After exposure to 100 mg/L NH_4Cl for 2 h, fish blood samples were withdrawn from the caudal vein using a syringe with a needle (TOP Surgical, Kaohsiung, Taiwan) containing lithium heparin (357 unit/mL), an anticoagulant.

TNF-α and COX-2 Gene Expression Experiments. Nile tilapia (*Oreochromis niloticus* L.) were injected with test agents dissolved in DMSO. A total volume of 1 μL of each of the following test agents was injected into each fish. In the first experiment, the test agents injected included DMSO (as control), 20 μg of GTE, and a combination of 20 μg of GTE and 100 μg of NH_4Cl/g of fish (GTE + NH_4Cl). Following these injections, fish were exposed to aqueous 100 mg/L NH_4Cl at 14 °C for 2 h. In the second experiment, fish were injected with LPS (3 μg/g of fish) and a combination of 25 μg of GTE and 3 μg of LPS/g of fish (LPS + GTE) 2 h before blood was sampled. In the third experiment, each fish was injected with DMSO (as control), 25 μg of GTE/g of fish, 25 μg of isoflavone mixture (Seichen, Changhua, ROC)/g, 25 μg of daidzein/g and 1 μL of DMSO/g (as control) prior to injecting 1 μg of LPS/g of fish to determine the effects of the individual components of GTE on TNF-α and COX-2 gene expressions. Blood was sampled from tilapia at 2 h poststimulation. In all three experiments, each treatment was done in triplicate.

Total RNA from 200 μL of whole blood was isolated using TRIzol reagent (Invitrogen) and treated on-column with Total RNA Miniprep Purification (GeneMark) according to the protocol of the kit for tissue culture cell. The total RNA was used for the production of cDNA utilizing the iScript cDNA Synthesis Kit (Bio-Rad) according to the protocol for cDNA synthesis. Real-time PCR was run on the iQLight-Cycler 2.0 instrument (Bio-Rad) using iQ SYBRR Green Supermix (Bio-Rad). Each real-time PCR master mix contained 1 μL of each primer (10 μM each), 10 μL of enzyme Mastermix and 8 μL of diluted template. The primers for real-time PCR are listed in Table 1. PCR was performed with an initial preincubation step of 3 min at 95 °C, followed by 40 cycles of 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 55 °C for 10 s. Melting curve analysis was performed to confirm the formation of the expected PCR products. The iQLightCycler 2.0 software (Bio-Rad) was used to analyze the data obtained. The real time reaction products were resolved by 1% agarose gel electrophoresis in TAE (Tris-acetate-EDTA) buffer,[26] then stained with 0.1% ethidium bromide and photographed under 304 nm. β-Actin was used for normalization. Gene expressions was presented as expression changes relative to DMSO-treated control.

Measurement of Plasma TNF-α and PGE2. Blood samples were analyzed for erythrocyte count with a hematological analyzer

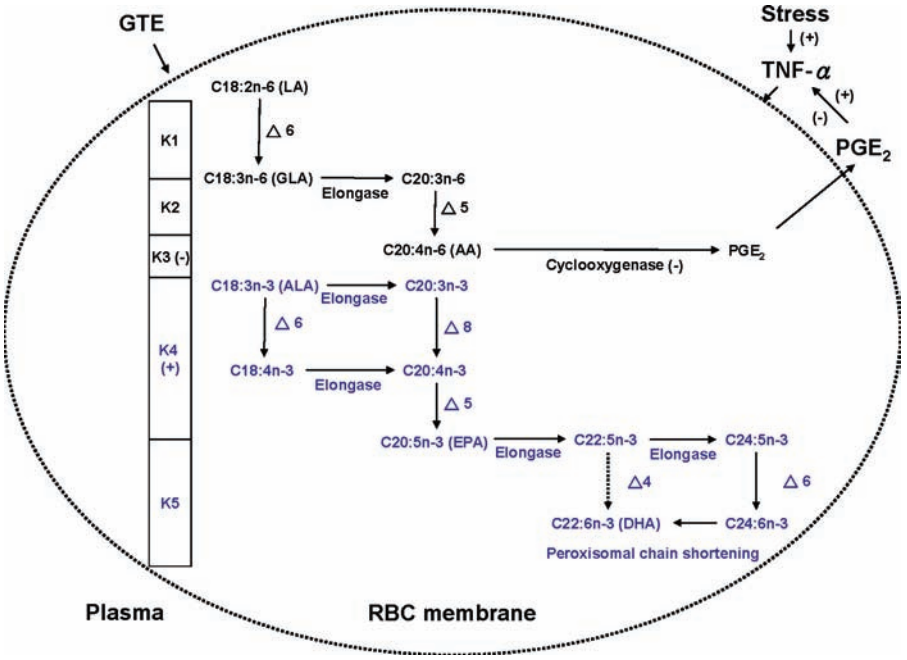

Figure 1. The proposed regulatory scheme of fatty acid synthesis and TNF-α expression induced by GTE and NH_4Cl in tilapia. RBC: red blood cell. K_1: Δ-6 desaturase of n-6 pathway, GLA/LA ratio. K_2: Δ-5 desaturase of n-6 pathway, AA/GLA ratio. K_3: PGE_2/AA ratio (COX-2 activity). K_4: Δ-5/6 desaturase of n-3 pathway, EPA/ALA ratio. K_5: Δ-4 desaturase of n-3 pathway, DHA/EPA ratio. (−): inhibition. (+): activation. The biosynthesis of highly unsaturated fatty acids (HUFA) in fish was adapted from Sargent et al. 2002[26] and Tocher et al. 2001.[30]

(Metertech Cell-Dye, Taiwan, ROC). The samples were centrifuged for 15 min at 700*g* to separate the erythrocytes and plasma. The plasma were transferred into 96-well plates. The content of intracellular TNF-α and PGE_2 in plasma was determined using the TiterZymeEIA kit (Stressgen 900-099, Ann Arbor, MI) and correlate-EIA (Stressgen 900-001) respectively. All treatments were done in triplicate, each of which was split into two parallel analyses.

Analyses of Erythrocyte Fatty Acids. Erythrocytes separated from 1 mL of blood were washed 3 times with phosphate-buffered saline (Bio-Rad, Hercules, CA). Tris-HCl buffer (10 mL of 1 mM) was added to the washed erythrocytes for lysis overnight at 4 °C. The samples were then centrifuged at 200*g* to collect the lipids from erythrocyte membrane. Total erythrocyte lipids were extracted with chloroform−methanol (2:1, v/v). The lipids extracted in the chloroform−methanol layer were concentrated under vacuum. The lipids were then mixed with 1 mL of hexane containing 0.2% BHT and spiked with C13:0 as an internal standard (Sigma). BF_3/MeOH reagent (1 mL of 14%, Sigma) was added to the mixture in a glass methylation tube. The mixture was heated to 100 °C for 10 min and then cooled to room temperature. The solution was allowed to settle until it formed two layers. The methyl esters were in the hexane phase. The solvent was removed under vacuum, and the lipids were concentrated in a glass bottle. The erythrocyte membrane fatty acids were weighed and then dissolved with 40 μL of hexane, 4 μL of which was analyzed using gas chromatography (Shimadzu GC-14A, Kyoto, Japan) with a 10% phenylcyanopropyl polysiloxane packed column (100 m × 0.25 mm i.d., Rt-2560, Restek, PA). The temperature of the injector and the FID detector was set at 250 °C. The oven temperature was programmed from 120 to 240 °C at a rate of 4 °C/min with a 10 min hold. The carrier gas was nitrogen at a flow rate of 3 mL/min. Fatty acids were identified by comparing their retention times with those of the standard fatty acids (Supelco37 Component FAME Mix, Bellefonte, PA). Quantification was based on C13:0, the internal standard.

Content of each fatty acid (pg) was divided by the number of erythrocytes per mL of blood sampled from each treatment. Fatty acid desaturation was determined by measuring the product-to-substrate ratio of the erythrocyte membrane.[10] The notations of the relative reaction rates in the fatty acid synthesis followed the proposed regulatory scheme (Figure 1). The Δ-6 and Δ-5 desaturases in the n-6 pathway are denoted K_1 and K_2, respectively. In addition, the production of PGE_2 by cyclooxygenase from the n-6 pathway was calculated as $K_3 = PGE_2$/AA, indicative of COX-2 activity. The ratio of AA to EPA also reflects the COX-2 activity.[26] In the n-3 pathway, the relative reaction rates of Δ-5/6 desaturase denoted K_4 for EPA to ALA and Δ-4 desaturase denoted K_5 for DHA to EPA were determined respectively. EPA synthesis was calculated as $K_4/(K_1 + K_2)$. Each K value was divided by that of the DMSO (control) treatment as fold of increase in reaction rate.

SDS−PAGE Electrophoresis of Hb. Blood samples were centrifuged at 700*g* at 4 °C for 10 min to separate cells from the plasma. The red blood cells were immediately washed 3 times, each time with 5 mL of ice-cold 100 mM Tris-HCl buffer (pH 7.6) containing 1.7% NaCl.[27] The washed erythrocytes were incubated in an ice bath for 1 h with a total of 3 volumes of 1 mM Tris-HCl buffer (pH 7.4) for lysis, which was confirmed by microscopic examination. One milliliter of lysed erythrocytes was mixed with 130 μL of 1 M NaCl to remove stroma by precipitation and centrifugation at 200*g* for 5 min at 4 °C. To the supernatant, another 130 μL of 1 M NaCl was added to remove stroma by precipitation, and the mixture was centrifuged again at 28000*g* for 15 min at 4 °C to collect the hemolysate. The protein content was determined by the Bradford method using commercial reagent (Bio-Rad).

A discontinuous 4−20% SDS−PAGE gel was used to analyze the hemolysate (Hbs) prepared from the control and the GTE groups of trial 1. The denatured hemolysates (10−20 μL) and 10 μL of the protein standards including lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), albumin (66 kDa) and phosphorylase b (97 kDa) (Amersham Bioscience, Piscataway, N.J.) were loaded separately onto the gel and run at 160 V for 1 h. The gel was stained with Coomassie Brilliant Blue R (Sigma) for 1 h and then was soaked at 25 °C in a solution containing 10% acetic acid and 20% methanol. Bands on the SDS−PAGE gel were analyzed with

the ImageMaster VDS software (version 2.0). The percentages of the Hb subunits or their components in the hemolysates were examined with the Image J program (National Institutes of Health, version 1.37v). The band or fraction (%) in the hemolysate was calculated as the density of each band divided by the sum of total band densities × 100%.

The tilapia hemolysate from the blank group of trial 1 was treated with 0, 12.5, 25, and 50 mg/mL NH_4Cl for 30 min before SDS−PAGE analysis of Hb dimer. In the second experiment, tilapia hemolysates (0.74 mg/mL) were treated with H_2O_2 (0, 50, 100, and 200 mM) for 1 h, while GTE (5 mg/mL) was mixed with hemolysate and treated with 100 mM H_2O_2 for 1 h. The hemolysate mixtures were denatured by heating at 95−100 °C for 5 min. The denatured hemolysates were mixed with tracking dye (1 mg of bromophenol blue dissolved in 5 mL of distilled water and 5 mL of glycerol), 2% SDS and 5% β-mercaptoethanol (v/v/v = 1/1/1). The mixed samples from triplicates were loaded onto 4−20% SDS−PAGE gel for Hb dimer analysis.

Erythrocyte Viscosity Measurement. At the end of trial 2 was an acute cold stress test. Ice was added into the aquaria to decrease the water temperature from 26 to 12.0 °C at a rate of 4.2 °C/h. Blood sampled from fish was transferred to 2.0 mL tubes. The erythrocytes were separated and immediately washed as described before. The washed erythrocytes were mixed with Tris-HCl buffer at a ratio of 1:2 (v/v) to a hematocrit (Hct) of 16%. The apparent viscosity was determined at a shear rate of 0−150/s using a viscometer with CP40 spindle (Brookfield, Middleboro, MA). Blood smears were stained with Liu stain prior to and after the rheological measurements.

Total Anti-H_2O_2-Induced Oxidation Capacity. The scavenging H_2O_2 effect was determined using a total antioxidant status (TAS) kit (Randox Laboratories, Antrim, U.K.) according to the manufacturer's instruction. The test agents included GTE, malic acid, vitexin, and daidzein, components of GTE; DMSO as solvent; NH_4Cl as chemical stress agent; Trolox, nordihydroguaiaretic acid (NDGA), and vitamin C as positive control, and an isoflavone mixture (Seichen, Changhua, ROC) including daidzein, glycitein, and genistin glycosides as reference. 25 μg of each test agent or combined agent was dissolved in 1 mL of DMSO, of which 1μL was mixed with 1 mL of chromogen for the TAS assay 10 μL of the test agents and their combinations were mixed with 1 mL of chromogen for the TAS assay. All treatments were assayed in triplicate.

Statistical Analysis. Data from each treatment were expressed as mean ± standard deviation (SD). Significance of difference between treatments in plasma TNF-α, PGE_2, fatty acids of tilapia erythrocyte membrane and gene expressions was determined using analysis of Kruskal−Wallis test followed by LSD (Analyze-it) post multiple comparison test. Correlations between TNF-α, PGE_2, K_3 and the ratio of AA to EPA indicative of COX-2 activity were analyzed by *t* test and Altman Bland test. The mean value of the GTE-fed group and the control group was evaluated using the Mann−Whitney *U* test. The level of significance was $P < 0.05$.

RESULTS

GTE Inhibited COX-2 and TNF-α Gene Expressions. The proinflammatory response and eicosanoid production of fish exposed to stressors were examined. Following injection of NH_4Cl (100 μg/g of fish) and exposure to cold temperature or LPS (3 μg/g of fish) stimulation at 2 h prior to sampling, TNF-α and COX-2 gene expressions were significantly induced (Figure 2A). There were significant differences ($P < 0.05$) between NH_4Cl or NH_4Cl/GTE treatments and the control and significant differences ($P < 0.01$) between the LPS or GTE + LPS treatments and the control. However, GTE + LPS combined did not inhibit TNF-α expression, but it significantly ($P < 0.01$) suppressed COX-2 expression (Figure 2A).

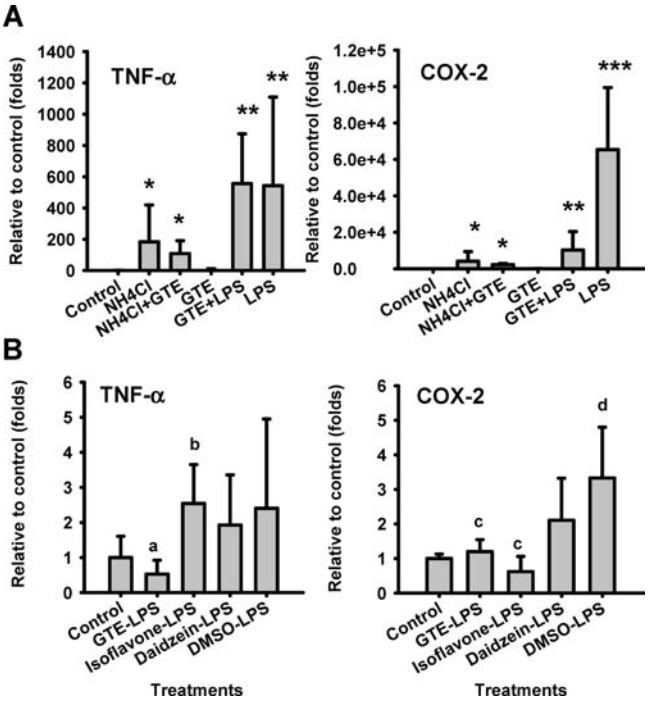

Figure 2. TNF-α and COX-2 genes expression in whole blood of Nile tilapia (*Oreochromis niloticus* L.). (A) Fish were injected with test agents, including GTE (25 μg/g of fish), NH_4Cl (100 μg/g of fish), combination of GTE and NH_4Cl (GTE + NH_4Cl), lipopolysaccharide (LPS, 3 μg/g of fish) and combination of LPS and GTE (LPS + GTE). Blood was sampled 2 h after injection. (B) Fish were injected with DMSO, GTE, isoflavones mixture, daidzein at a concentration of 25 μg/g of fish 15 min before injection with LPS (1 μg/g of fish). Blood was sampled 2 h after injection with LPS. All values were normalized to the housekeeping gene β-actin as fold induction relative to the control (DMSO injection group). Results reported as mean ± SD ($n = 3$) were analyzed by Kruskal−Wallis post LSD test. The asterisk (*) indicates significant differences ($P < 0.05$) between LPS or GTE + LPS and the control. (**) denotes significant differences ($P < 0.01$) between NH_4Cl or GTE + NH_4Cl treatments and the control. (***) denotes significant differences ($P < 0.01$) between GTE + LPS treatments and LPS. (a, b) TNF-α: significant difference ($P < 0.05$) between GTE−LPS and isoflavone−LPS treatments. (c,d) COX-2: significant difference ($P < 0.01$) between DMSO−LPS and control, GTE−LPS and isoflavone−LPS treatments.

When fish were preinjected with GTE or isoflavones and then stimulated 15 min later with a low level of LPS (1 μg/g of fish), DMSO−LPS significantly induced ($P < 0.05$) TNF-α and COX-2 expressions, while GTE−LPS treatment did not (Figure 2B). COX-2 expression was reduced by DMSO−LPS and showed significant ($P < 0.01$) difference from the control, GTE−LPS and isoflavone−LPS, indicative that GTE strongly inhibited inflammatory responses.

Plasma PGE_2 and TNF-α The trends of plasma TNF-α and PGE_2 in tilapia showed slightly reversed responses (correlation = 0.99, $P = 0.0143$) (Figure 3). The GTE-free group injected with DMSO (control), GTE or NH_4Cl showed an increase in TNF-α expression compared to blank. TNF-α was significantly different ($P < 0.05$) between control and blank; GTE and NH_4Cl; GTE/NH_4Cl; GTE, GTE/NH_4Cl and control. However, PGE_2 showed no significant ($P > 0.05$) differences between treatments, except GTE and blank ($P = 0.0057$). The GTE-fed subgroup injected with GTE + NH_4Cl significantly suppressed TNF-α

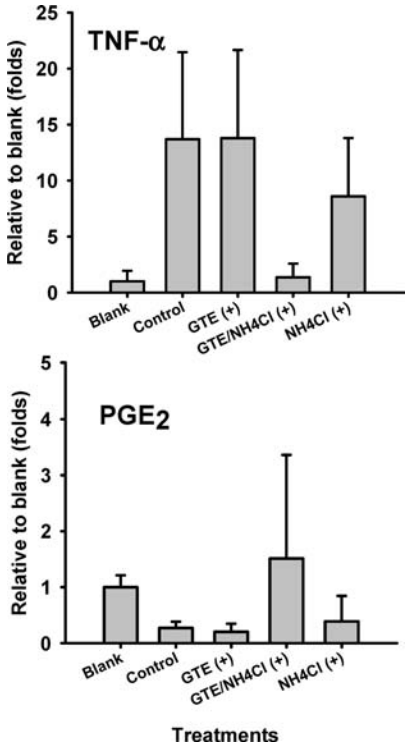

Figure 3. Effect of GTE and NH_4Cl on plasma TNF-α and PGE_2 production in tilapia. TNF-α and PGE_2 were evaluated by immunoassay and compared to the mean values of the untreated tilapia (no injection) as blank group. All fish with injection were exposed to aqueous NH_4Cl (100 mg/L). The treatments included fish being injected respectively with NH_4Cl (100 μg/g of fish), DMSO (1 μL/g of fish) as control, a combination of NH_4Cl (100 μg/g of fish) and GTE (20 μg/g of fish) as GTE + NH_4Cl, and GTE alone (20 μg/g of fish). Fish blood was sampled at 2 h after injection. Each treatment group was assayed in triplicate. All data are expressed as mean ± SD (n = 3). TNF-α: significant differences between blank and control (DMSO); GTE and blank; NH_4Cl and blank; GTE/NH_4Cl and control; GTE and GTE/NH_4Cl, P = 0.0322, by Kruskal−Wallis post LSD test. PGE_2: no significant differences between treatments, P = 0.2006, except blank vs GTE, P = 0.0057. Correlation between TNF-α vs PGE_2, correlation = −0.99 (t test), P = 0.0143, by Altman Bland test.

expression by 90% compared to the control (DMSO), indicative that GTE regulated plasma PGE_2 and TNF-α.

GTE Enhanced the Level of Highly Unsaturated Fatty Acids (HUFAs) in Erythrocyte Membrane. Whether fatty acids of erythrocyte membrane correlated with COX-2 activity and plasma PGE_2 level was determined. The GTE-fed tilapia (GTE) followed by injection with GTE showed higher level of n-3 HUFAs (P < 0.05) in the erythrocyte membrane than fish from other treatment groups (Figure 4). In the GTE treatment groups, the n-3 HUFAs increased including EPA (C20:5n-3) (1.22 pg/cell) and DHA (C22:6n-3) (0.64 pg/cell) followed by other HUFAs in minor quantities, i.e., alpha (α)-linolenic acid (ALA, C18:3n-3) (0.05 pg/cell) and gamma (γ)-linolenic acid (GLA, C18:3n-6) (0.004 pg/cell). In the n-6 pathway of the erythrocyte membrane, AA (C20:4n-6) was the major HUFA (0.12 pg/cell) (P < 0.05), followed by a minor quantity of C20:2 (0.01 pg/cell). The AA content was only 10% of that of EPA in the erythrocyte membrane of the GTE group. The amounts of EPA or EPA plus DHA in the GTE treatment group were significantly (P < 0.05) higher than those of the other HUFAs, and the ratio of n-3 to n-6 was significantly (P < 0.01) higher than that of the control.

In the GTE/NH_4Cl treatment groups (Figure 4), the n-6 HUFAs were all present in minor quantities, ranging from 0.001 pg/cell of C18:3 to a maximal quantity of 0.074 pg/cell of AA. The levels of the n-6 HUFAs in erythrocyte membrane were lower than those of the n-3 HUFAs, of which DHA content was the most abundant (0.28 pg/cell), followed by EPA (0.23 pg/cell), then a trace amount of C18:3 (0.041 pg/cell) and a negligible amount of C20:3. The total amount of the n-3 HUFAs in the GTE/NH_4Cl treatment group (0.55 pg/cell) was significantly (P < 0.05) lower than those of the GTE group (1.91 pg/cell). In addition, the ratio of AA to EPA (indicative of COX-2 activity) had a correlation with plasma PGE_2 level (correlation = 0.99, P = 0.0128).

The ratio of n-3 to n-6 in the GTE group was significantly (P < 0.01) higher than the control (Figure 4). Therefore, GTE upregulated the n-3 synthesis pathway in erythrocytes, while GTE/NH_4Cl downregulated the n-3 pathway. In the GTE group, the n-3 synthesis pathway remained the dominant pathway.

According to the reaction rates indicated by K (Figure 5), the Δ-5/6 and -4 desaturase activities did not increase in rate constant K_2 for AA formation by treatment with either GTE or NH_4Cl-i, while combined GTE/NH_4Cl treatment caused large variance in K_2 in comparison to that of the control.

The K_3 for formation of AA downstream products (PGE_2) was lowered by the GTE. The GTE/NH_4Cl treatment also yielded a lower K_3 than those of the NH_4Cl-i and the control (Figure 5). The K_3 showed positive correlations with the ratio of AA to EPA and plasma PGE_2 (correlation = 0.99, P = 0.0128). Therefore, GTE and GTE/NH_4Cl treatments inhibited COX-2 activity in erythrocyte membrane (P < 0.05).

The K_4 (the ratio of EPA to ALA) for the EPA synthesis showed an approximately 17-fold increase in the GTE-i treatment group comparing to the control and a 9-fold increase in the NH_4Cl-i treatment group comparing to the control (Figure 5). Treatment with GTE or NH_4Cl-i did not activate the elongase to catalyze the elongation from C18 to C20 or Δ-4 desaturase on EPA to form DHA. However, the K_6 ($K_4/K_1 + K_2$) in the erythrocyte membrane showed an increase of 6.3-fold of the control after the GTE treatment. No significant (P > 0.05) changes were found in the ratios of C18:1 to C18:0, C18:2 to C18:1, GLA to LA or ALA to LA, indicating no changes in the activities of Δ-9, -12 and -15 desaturases in all treatments (data not shown).

Therefore, we are proposing a metabolic pathway for the n-3 and n-6 HUFA syntheses in regulating TNF-α and PGE_2 in the erythrocyte membrane of tilapia treated with GTE (Figure 1). EPA plays a crucial role in the regulation of PGE_2 and the inhibition of TNF-α expression in fish treated with GTE.

GTE Reduced Hemoglobin (Hb) Dimerization. Because Hb functions as oxygen carrier, lower Hb concentration in erythrocytes may cause anemia. Tilapia exposed to NH_4Cl-w and injected with DMSO [DMSO (+)] as control had higher amounts of Hb dimers (around 36 kDa) and Hb tetramers (64 kDa) than the blank (1 in Figure 6). Injection with GTE and exposure to NH_4Cl-w treatment (GTE (+)) inhibited formation of Hb dimer (36 kDa) and Hb tetramer (64 kDa) (5 in Figure 6). The amounts of the Hb dimer in the hemolysate of the GTE/NH_4Cl (+), GTE (+) and GTE (−) treatments (2, 4, and 6 in Figure 6) were lower than those found in the NH_4Cl-i (+) (3 in Figure 6) and DMSO (−) treatments (7 in Figure 6).

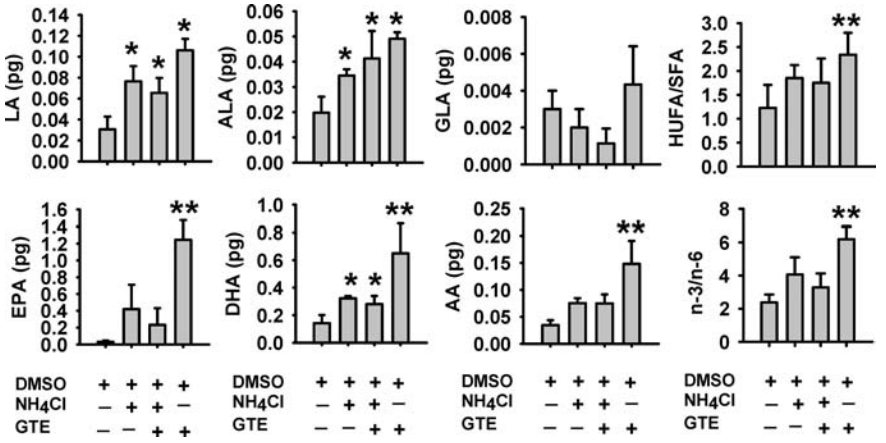

Figure 4. Highly unsaturated fatty acids (HUFAs) in erythrocyte membrane of tilapia exposed to aqueous NH_4Cl and injected with DMSO (control). Test agents dissolved in DMSO were NH_4Cl, a GTE/NH_4Cl combined or GTE alone. LA: linoleic acid, C18:2n-6. ALA: alpha (α)-linolenic acid, C18:3n-3. GLA: gamma (γ)-linolenic acid, C18:3n-6. AA: arachidonic acid, C20:4n-6. EPA: eicosapentaenoic acid, C20:5n-3. DHA: docosahexaenoic acid, C22:6n-3. HUFA/SFA: the ratio of HUFA to saturated fatty acid (SFA). n-3/n-6: the ratio of n-3 HUFA to n-6 HUFA in the erythrocyte membrane. Each treatment was assayed in triplicate. All data are expressed as mean $\pm$ SD ($n = 3$). The asterisk (*) indicates significant ($P < 0.05$) difference compared to the control. (**) denotes significant ($P < 0.01$) difference compared to the control. AA: significant differences between GTE and the control; NH_4Cl and the control; GTE and GTE + NH_4Cl, $P = 0.0234$, by Kruskal–Wallis post LSD test. EPA: GTE and control; GTE and NH_4Cl; GTE and GTE/NH_4Cl, $P = 0.0415$, by Kruskal–Wallis post LSD test. DHA: significant differences ($P = 0.0216$) between each treatment, except GTE and GTE/NH_4Cl, $P = 0.2029$, by Kruskal–Wallis post LSD test.

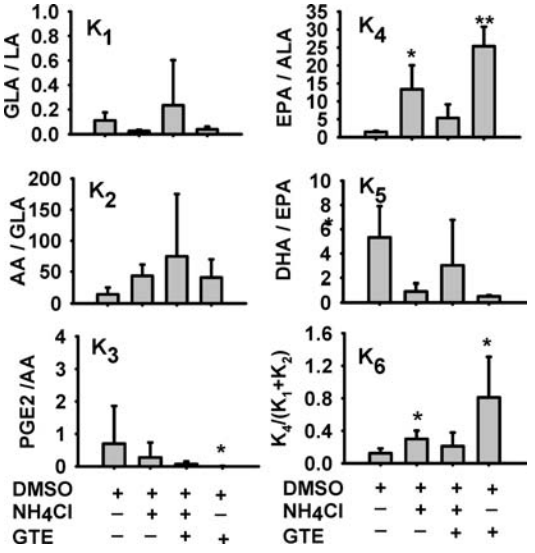

Figure 5. Effects of GTE and NH_4Cl on rate constants (K) of fatty acid syntheses in erythrocyte membrane of tilapia exposed to aqueous NH_4Cl. Each treatment group was assayed in triplicate. K_1, K_2 and K_5: no significant differences between treatments, $P > 0.05$, by Kruskal–Wallis post LSD test. K_3: significant differences between GTE vs control, $P = 0.0273$, by Kruskal–Wallis post LSD test. K_6 or $K_4/(K_1 + K_2)$: significant differences between GTE and the control; GTE and GTE/NH_4Cl; NH_4Cl and the control, NH_4Cl and GTE/NH_4Cl, $P = 0.0368$, by Kruskal–Wallis post LSD test. The ratio of AA to EPA as COX-2 activity and K_3: correlation = 0.89; t test, $P = 0.0102$, by Altman Bland test. Plasma PGE_2 vs K_3 or COX-2 activity of erythrocyte membrane: correlation = 0.99; t test, $P = 0.0128$, by Altman Bland test.

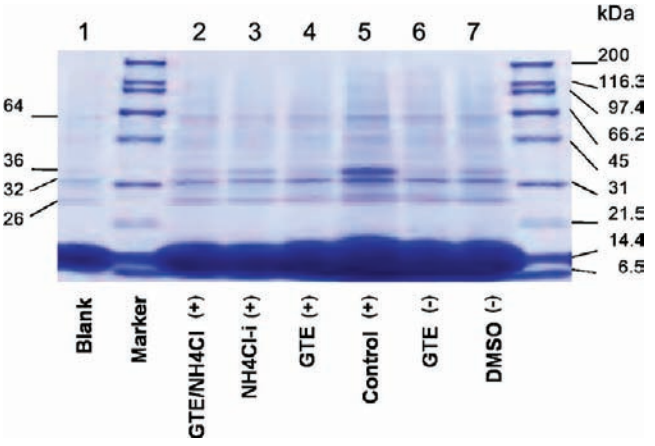

Figure 6. Hb dimer formation is inhibited by GTE. Effects of GTE and NH_4Cl on Hb dimer formation in erythrocytes of tilapia exposed to NH_4Cl. The 4–20% SDS–PAGE (pH 8.2) gel shows the Hb compounds in hemolysates from tilapia fed with GTE for a period of 3 months prior to injection and exposure to NH_4Cl stress for 2 h.

GTE Reduced Apparent Blood Viscosity. After feeding tilapia for 10 months, followed by exposure to cold temperature (14 °C) for 4 h, the GTE-fed fish had less blood aggregation in comparison to the control. The GTE-fed fish had significantly ($P < 0.01$) lower apparent blood viscosity (1.89 $\pm$ 0.02 cP) than the control group (2.14 $\pm$ 0.01 cP). In addition, the blood smear of the control group appeared darker in color than that of the GTE group (Figure 7), indicative of GTE protecting the tilapia from increases in erythrocyte aggregation and blood viscosity in addition to changes in erythrocyte morphology by regulating the syntheses of HUFAs.

GTE Did Not Inhibit Oxidation. GTE and its compounds in the H_2O_2-induced *in vitro* oxidation reactions are shown in Figure 8. The positive controls included ascorbic acid (vitamin C), Trolox and NDGA. For the positive controls, the total antioxidant status (TAS) is similar. TAS of the individual components of GTE on the same weight basis was in the order

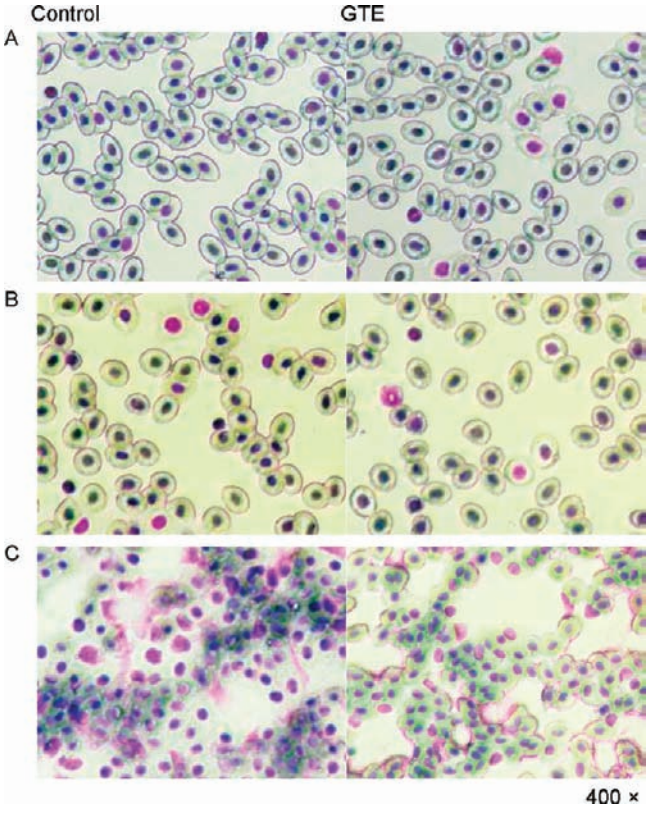

Figure 7. Effects of dietary GTE administered for 10 months on blood viscosity and aggregation in the erythrocyte membrane of tilapia exposed to acute cold stress. The acute cold stress test was performed on fish by adding ice into the aquaria to rapidly decrease the water temperature from 26 to 12.0 °C at a rate of 4.2 °C/h. Blood samples were collected from untreated fish (row A). Blood smears from cold-stressed fish (row B). Blood cells from cold-stressed fish washed 3 times with 0.1 M Tris-HCl buffer containing 1.7% sodium chloride and Hct adjusted to 16%, which was then sheared at 100/s (row C).

vitexin > isoflavone mix > daidzein > GTE > malic acid. DMSO and NH_4Cl significantly caused TAS to be lower than that of GTE and the combination of NH_4Cl, malic acid, vitexin, and isoflavone or daidzein. The results show that the anti-inflammatory responses of GTE did not correlate with the antioxidant capacity.

GTE Improved Tilapia Growth and Protein Utilization. The effects of dietary *Glycine tomentella* Hayata on growth of the tilapia are shown in Table 2. The GTE-fed groups were significantly ($P < 0.05$) higher in body weight and specific growth rate (SGR) than the control in both trials. The feed conversion ratios (FCRs) were lower in all of the GTE-fed groups than those of the control group, while the protein efficiency ratios (PERs) of GTE-fed were higher than those of the control groups, indicative of GTE inducing more efficient utilization of protein and feed conversion. Fish of smaller size (45 ± 12 g) had higher SGRs than the larger size fish (59 ± 3 g), indicative of SGR of fish decreasing with size and age of fish.

DISCUSSION

Stress Induced Proinflammatory Responses in Tilapia. *Glycine tomentella* Hayata has long been used to treat rheumatic diseases for humans.[18] The ethanolic extract was able to prolong

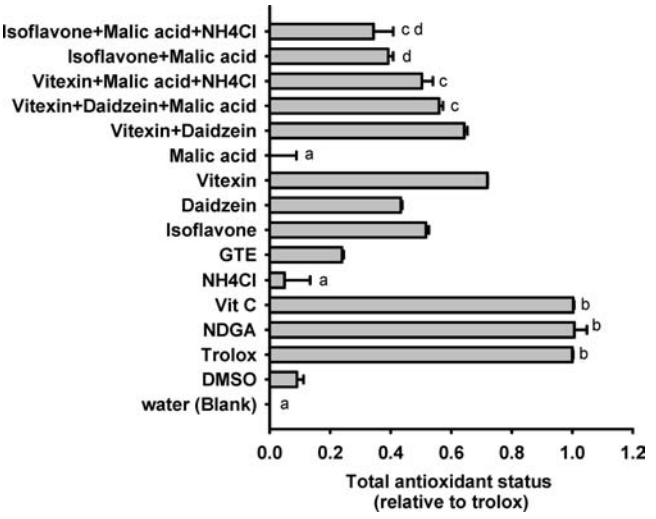

Figure 8. *In vitro* effects of GTE and NH_4Cl on hydrogen peroxide scavenging by the total antioxidant status. (a, b, c, d) No significant differences ($P > 0.05$) between the same letter denoted treatments.

the lag phase of LDL oxidation in tilapia.[21] Studies verified the anti-inflammation effect of GTE *in vivo* and *in vitro*, while daidzein was considered the major active flavonoid in GTE.[28] Tilapia was used as an animal model to study the anti-inflammation mechanism. GTE inhibited TNF-α *in vivo* similar to that in salmonid cell line.[2] When tilapia were exposed to NH_4Cl followed by injection with NH_4Cl or LPS, significantly induced proinflammatory gene expressions of TNF-α in blood and plasma occurred. The changes correlated with the HUFA syntheses in the erythrocyte membrane and the erythrocyte Hb dimerization. Changes in EPA, DHA and n-3/n-6 ratio in erythrocyte membrane corresponded to changes in erythrocyte morphology and O_2 release. The erythrocyte responses and proinflammatory gene expressions seem to be sensitive parameters for monitoring fish stress.[29]

Role of GTE on Anti-Inflammation and n-3 HUFA Syntheses. GTE accelerated the n-3 pathway from C18:3n-3 (ALA) to EPA and DHA most likely not via the intermediate of C20:3 which was detected at negligible amount (Figure 1). If it was the case, the C18:3n-3 should have higher affinity for Δ-6 desaturase than the elongase to form C18:4n-3 followed by catalysis of Δ-5 desaturase to form EPA. Therefore synthesis of EPA is achieved by Δ-6 desaturation of C18:3n-3 to produce C18:4n-3 that is elongated to C20:4n-3 followed by Δ5 desaturation. In addition, GTE increased the n-3/n-6 ratio of HUFA, which indicates that the n-3 synthesis pathway was activated to form higher levels of EPA and DHA than AA in the erythrocyte membrane. The n-3 fatty acids, primarily EPA and DHA, increased at the highest level in erythrocyte membrane of tilapia fed and injected with GTE compared to fish injected with DMSO (Figure 4). The ratio of AA to EPA to DHA was 1:10:5 similar to the AA to EPA ratio being 1:10 found in larva of cold-water marine fish.[29] The ratio of n-3/n-6 was the same in tissues of liver, muscle and brain from tilapia fed fish or vegetable oil diets.[30] The activities toward C24:6n-3 to form DHA and the high activities in both n-3 and n-6 pathway to form AA and EPA indicated tilapia treated with GTE might express bifunctional Δ-5/Δ-6 desaturase.

For proinflammatory factors in plasma, plasma PGE_2 was not significantly different between treatments (Figure 3), but the ratio of plasma PGE_2 to AA in erythrocyte membrane was significantly

Table 2. Effects of Dietary 95% Ethanol Extract from a Root of *Glycine tomentella* Hayata (GTE) on Growth Performance and Feed Utilization Efficiencies in Tilapia (*Oreochromis mossambicus*)[a]

	trial 1		trial 2-1		trial 2-2	
parameter	control	GTE	control	GTE	control	GTE
no. of fish	16	16	8	8	8	8
trial days	30	30	14	14	14	14
initial body wt (g)	101 ± 10	99 ± 17	59 ± 3	59 ± 3	45 ± 12	41 ± 4
final body wt (g)	113 ± 12	115 ± 22	64 ± 5	68 ± 4	50 ± 3	49 ± 6
wt gain (g)	13 ± 0	14 ± 4	5 ± 2	9 ± 1	5 ± 5	11 ± 5
Δ wt[b] (fold)	1.12	1.16	1.08	1.15	1.11	1.20
SGR (%/day)[c]	0.24 ± 0.01	0.43 ± 0.08*	0.62 ± 0.25	1.03 ± 0.15*	0.74 ± 0.3	2 ± 0.01*
FCR[d]	1.1 ± 1.1	1.0 ± 0.6	1.61 ± 0.97	0.89 ± 0.19	2.6 ± 1.3	0.71 ± 0.43
PER[e]	1.78 ± 0.11	2.12 ± 0.28	1.58 ± 0.65	2.68 ± 0.43**	1.54 ± 1.46	4.09 ± 2.03*

[a] * $p < 0.05$, ** $p < 0.01$ significantly different from the control. [b] Δ weight = final weight/initial weight. [c] Specific growth rate (SGR) = [(ln final body weight − ln initial body weight)/days] × 100. [d] Feed conversion ratio (FCR) = dry feed intake (g)/wet weight gain (g). [e] Protein efficiency ratio (PER) = body weight gain (g)/protein intake (g).

reduced by GTE and GTE/NH_4Cl (Figure 5). Changes in EPA to ALA (K_4) and PGE_2 to AA ratio (K_3) indicated that the fatty acids of erythrocyte membrane were sensitive to environmental stress. We propose a scheme of the GTE inhibition or regulation of plasma TNF-α expression as shown in Figure 1. Stressor-induced TNF-α expression was inhibited by GTE via the activation of Δ-5 desaturase. Both GTE and NH_4Cl activated Δ-5 but inhibited Δ-4 desaturase and enhanced the n-3 HUFA synthesis pathway in the tilapia erythrocyte membrane to increase the n-3/n-6 ratio. GTE is capable of regulating TNF-α, inhibiting COX-2 gene expressions and enhancing HUFA synthesis in the erythrocyte membrane as shown in this proposed pathway.

The Hb dimerization appeared when the fish were treated with H_2O_2 (data not shown), suggesting that Hb dimer formation is related to H_2O_2-induced oxidation similar to human Hb dimer formation, of which the molecular weight ranged from 25 to 30 kDa.[31] In this study, tilapia had molecular weights around 26 kDa, 32 kDa and 36 kDa slightly larger than the human Hb dimer. The tilapia Hb tetramers have molecular weight between 67 and 69 kDa which could split into monomers ranging from 16 kDa to 18 kDa.[32] GTE in the NH_4Cl-stimulated tilapia inhibited the formation of Hb dimer and tetramer (Figure 6). Formation of Hb dimers may result from changes in HUFAs of erythrocyte membrane leading to changes in erythrocyte deformability and increase in erythrocyte aggregation. GTE showed the opposite of H_2O_2-induced Hb dimerization in tilapia exposed to NH_4Cl stress. H_2O_2 is a strong reactive oxygen species that causes Hb aggregation and hemolysis and plays a role in innate immunity.[33] H_2O_2 induces TNF-α and changes of vasculature and causes cell and tissue injury.[34−36] Inhibition of Hb dimerization may be due to H_2O_2-induced oxidative stress (Figure 6) accompanied by reduction in TNF-α. It is consistent with the *in vitro* effects of GTE on proinflammatory responses, including inhibition of TNF-α via increasing IκB-α, inhibiting MAPK p38 and regulating arachidonate metabolites, e.g., PGE_2 and thromboxane B_2 (TXB_2).[2] In the previous study, GTE treatment resulted in a decrease in TXB_2, indicating that GTE has the ability to inhibit erythrocyte aggregation. Our *in vivo* findings showed that GTE reduced blood aggregation and improved blood characteristics. The intake of GTE lowers the level of TNF-α expression and increases unsaturated fatty acids in erythrocyte or increases HUFA level and the n-3/n-6 ratio resulting in increase of erythrocyte membrane fluidity,[37] and enhances blood circulation.[22], thus reducing blood viscosity (Figure 7), leading to increased survival rate in cultured fish especially during cold temperature.[38]

The survival and the SGR of the GTE-fed groups were higher than those fed the GTE-free diets. More fish body mass was produced at less feed consumption (FCR) and better protein utilization (PER) with GTE-fortified feed, indicating that GTE improved feed utilization in tilapia. The growth enhancing effect seemed to be more pronounced on smaller fish than larger fish.

Anti-Inflammation without Antioxidant Activity of GTE. According to the *in vitro* assay, we propose that the anti-H_2O_2-induced oxidative components in GTE are isoflavones, such as daidzein, and flavone glycosides such as vitexin, which were the active compounds. The efficacy of single compounds in comparison with the multiple compounds in the GTE showed that the composite had less synergistic effects on the H_2O_2-induced oxidative capacity (Figure 8). Regardless of the fact that the total antioxidative capacity of the GTE was much lower than that of the isoflavone mixture, *in vivo* study showed that GTE had strong antistress and anti-inflammatory effects. GTE also improved growth performance in smaller tilapia, which could be an overall effect derived from the antistress, anti-inflammatory and regulatory capacity of HUFA syntheses.

CONCLUSION

Based on herbal medicine, we verified the traditional concept that the root of *Glycine tomentella* Hayata (GTE) has functions of improving blood flow and anti-blood-coagulation. In addition, GTE has strong anti-inflammatory activities. We confirmed that the anti-inflammatory mechanism of GTE in NH_4Cl/LPS-induced tilapia was due to the inhibition of plasma TNF-α and COX-2 gene expression and regulation of EPA synthesis *in vivo*. Those proinflammatory factors can be good biomarkers to determine the animal responses to stress. The potential application of GTE as a fish feed supplement or a vaccine injection adjuvant may improve the ability of cultured fish to cope with stress, such as during live-fish transportation or sudden temperature changes, and the immunity needed to combat fish diseases without using antibiotics.

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