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Inhibition of Fish Gill Lipoxygenase and Blood Thinning Effects of Green Tea Extract

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The objective of the present study was to determine whether green tea extracts are inhibitory to lipid oxidations catalyzed by lipoxygenase (LOX) and hemoglobin (Hb) using fish as an animal model. Green tea was extracted with water. LOX was extracted from the gills of grey mullet and tilapia, respectively. The LOX activity was determined using chemiluminescence and high-pressure liquid chromatography. The green tea extract showed inhibitory effects on both LOX-catalyzed and Hb-catalyzed oxidation of arachidonic acid and linoleic acid. Blood thinning effects were observed ex vivo by mixing the green tea extract with fish red blood cells and showed that the flow behavior of fish blood becomes closer to the Newtonian type with a thinner consistency. Similar effects were found on tilapia and grey mullet.

KEYWORDS: Lipoxygenase; blood thinning; green tea; hemoglobin; antioxidant; fish gill

INTRODUCTION

Green tea is known to have an antioxidant activity that prevents coronary heart disease (*1*−*5*). Catechin mixtures prepared from tea effectively prevented the prooxidant activity of lipoxygenase (LOX) in fish skin extract (*6*). The topical application of green tea polyphenols (GTPs) significantly inhibited epidermal cyclooxygenase (COX) and LOX (E.C. 1.13.11.12), which are used as markers of skin tumor promotion (*7*, *8*). The metabolites from arachidonic acid (AA) catalyzed by LOX were bioactive compounds involved in inflammation (*9*−*11*). The LOX activity in the same tissue of diseased animals was higher than that in normal animals (*12*−*14*). The antiinflammatory activities of flavonoids have been evaluated by their modulation on COX and LOX (*15*). A synergistic interaction was observed between 15-LOX-catalyzed derivatives and heme protein-catalyzed oxidation that reduced the potency of the endogenous antioxidants and enhanced lipid or low-density lipoprotein (LDL) oxidation (*16*−*19*). A novel drug and a 15-LOX inhibitor, PD 146476, had a dramatic effect in reducing atherogenesis (*20*). Chinese green tea inhibited LDL oxidation induced by human endothelial cells (*5*), while pu-erh tea showed strong antioxidant inhibitory activities to human LDL oxidation ex vivo (*21*).

Both in vivo and in vitro results indicated the inhibitory effect of vitamin E on LOX-catalyzed oxidation of highly unsaturated fatty acids. The apparent viscosity of blood was reduced, indicative of the higher deformability of the erythrocytes in mullet fed with vitamin E-fortified diets (*22*). The treatment with vitamin E nicotinate on insulin-dependent diabetes mellitus improved the rheological properties of red cell membranes and retinal capillary blood flow (*23*). Tea polyphenols were able to reduce the swelling and perforation phenomena of red blood cells (RBCs) induced by peroxides in a dose-dependent manner (*24*).

The purpose of the present study was to determine whether green tea extracts (GTEs) are inhibitory to both the LOX-catalyzed and the heme protein-catalyzed oxidation of lipids. If so, the membrane of RBCs can be protected from oxidative damages, and then, the rheological properties of blood fluid should be able to reflect the differences.

Because fish are poikilothermic, their body temperature and blood viscosity are sensitive to changes in environmental temperatures. Therefore, fish were used as an animal model in this study to test the effects on fish blood for prescreening antioxidant and blood thinning activities (*25*) of green tea.

MATERIALS AND METHODS

Materials. Linoleic acid ($C_{18:2}$), AA ($C_{20:4}$), α-tocopherol acetate, sodium heparin, and 5-hydroperoxyeicosatetraenoic acid (HpETE) were from Sigma Chemical Co. (St. Louis, MO); ±5-hydroxyeicosatetraenoic acid (HETE), ±12-HETE, and 15-HETE were purchased from Cayman Chemical Co. (Ann Arbor, MI); hydroxyapatite was from Macro-Preceramic, BioRad (Hercules, CA); ammonium thiocyanate (NH_4-SCN), ferrous chloride ($FeCl_2$), L-(+)-ascorbic acid were from Riedel-deHaën Chemical Co. (RedH Laborchemilalien GmbH & Co. K. G., Seelze, Germany); and green tea was from a supermarket in Keelung, Taiwan.

Preparation of GTE and Green Tea Infusion (GTI). The green tea was ground to a powder (30 mesh, 0.59 mm diameter), mixed with 10 volumes of deionized water, and refluxed at 100 °C for 30, 60, and 90 min, respectively. The extracts were freeze-dried, respectively, as GTE and stored in a refrigerator until use. Then, the GTE was dissolved in deionized water. The aqueous extract was mixed with 0.5 g of activated charcoal for 1 h and centrifuged at 1400*g* for 10 min. The supernatant was used as the decolored GTE, which was used in the spectrophotometric measurements of antioxidant activity to reduce the interference in absorbance at 510 or 234 nm. The GTI was obtained

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by soaking the crushed green tea powder in boiling water for 5 min and filtering it, thus imitating the way people drink tea.

Preparation and Assay of Fish Gill LOX. LOX was extracted from the gills of instantaneously killed grey mullet (*Mugil cephalus*) and tilapia hybrid (*Oreochromis niloticus* × *Oreochromis aureus*), respectively. The crude extract and hydroxyapatite partially purified LOX were prepared, and the activities were assayed according to the method of Hsu and Pan (*26*). Soybean LOX (Sigma) was assayed as a reference.

Rapid Photometric Assay of Hemoglobin (Hb)-Catalyzed Oxidation. GTE (15 μL) at a concentration range of 47−57 mg/L was added with 0.3 mL of 0.05 M phosphate buffer (pH 7.0) containing 0.025% Tween 20 and 200 μL of 0.01 mM linoleic acid in ethanol. The mixture was incubated at 37 °C for 3 min followed by the addition of freshly prepared 0.003% Hb (50 μL) to catalyze the oxidation of linoleic acid. The assay mixture was incubated at 37 °C for 10 min and then terminated by the addition of 0.6% HCl−ethanol (5 mL), followed by 0.02 M $FeCl_2$ (0.1 mL) and 30% ammonium thiocyanate (0.1 mL), shaken for 10 s, and then set for 5 min. The absorbance of the assay mixture was measured at 510 nm (*27*). The peroxidation (%) is calculated as follows:

$$\text{peroxidation (\%)} = \frac{A_s - A_0}{A_h} \times 100\%$$

where A_s is the absorance of the sample containing antioxidant and Hb, A_0 is the absorbance of the antioxidant, and A_h is the absorbance of the control containing Hb but no antioxidant.

Chemiluminometric Assay. Hydroperoxide produced by LOX catalysis was detected using a chemiluminescence (CL) method (*28*). The assay mixture contained 0.78 mL of 0.1 M Na-borate buffer (pH 10.5) consisting of 0.04% Tween-20, 200 μM AA in 10 μL of ethanol as the substrate, GTE, 0.1 mL of gill LOX extract (protein content 1 mg/mL), and 0.1 mL of luminol (dissolved as 1 mM solution in 0.1 M Na-borate buffer, pH 10.5) in 3.5 mL disposable measuring cuvettes. The reaction temperature was 25 °C. The reaction rate of light emission was determined using a luminometer (model 1251, Bio-Orbit Oy, Turku, Finland).

Assay of Gill LOX Activity. The LOX activity was assayed spectrophotometrically by measuring the conjugated dienes indicated by the absorbance at 235 nm according to the method of Surrey (*29*). The gill LOX extract (protein content 1 mg/mL), 0.1 mL, was diluted with 0.9 mL of 0.05 M phosphate buffer, pH 7.0, containing 1 mM glutathione and 0.04% Tween-20 and was incubated at 25 °C with 50 μL of GTE containing 0.1−100 μg of solid for 5 min. The assay mixture was then incubated with 10 μL of 200 μM AA at 25 °C for 3 min. The LOX activity was determined by the increase in absorbance at 234 nm using a molar absorptivity of 27 000 M^{-1} to estimate the conjugated derivatives.

Identification of Isozymes. One milliliter of crude gill LOX extract and 3.95 mL of 0.05 mM phosphate buffer, pH 7, were mixed with AA in ethanol (0.05 mL) and incubated at 25 °C for 30 min. The reaction was terminated by adjusting it to pH 3 with 6 N HCl. The reaction products, HETE, were extracted with 2 volumes of ethyl acetate and methylated (*26*). The methylated compounds were absorbed on a solid phase extraction column (J&W Scientific, Folsom, CA) and then eluted with a hexane/ether mixture (75:25 v/v) to be analyzed by high-pressure liquid chromatography (HPLC, Waters, model 510, Milford, MA) equipped with a LiChrospher 100 RP-18 column (25 cm × 4 mm, 5 μL) (E. Merck Kga, Darmstadt, Germany) and a UV detector set at 235 nm (Waters, model 490E, Programable Multiwavelength Detector). The compounds were eluted with methanol/water (75:25 v/v) containing 1 mM ethylenediaminetetraacetic acid and 50 mM ammonium acetate at a flow rate of 1.0 mL/min. The presence of 5-, 12-, and 15-HETE was confirmed by comparison with authentic standards (Cayman) of their retention times. The protein content of the crude LOX extract was determined with the method of Lowry et al. (*30*) using bovine serum albumin (Sigma Chemical Co.) as the standard.

Collection of Fish Blood Samples. Live fish were put on a bed of crushed ice under dimmed light (*31*). A damp cloth was used to cover the fish head. A sample of 1 mL of whole blood was drawn from the caudal vein into 0.1 mL of physiological saline containing 143 USP sodium heparin (Sigma Chemical Co.), and it was mixed with 5 volumes of 0.05 M KH_2PO_4, pH 7.0.

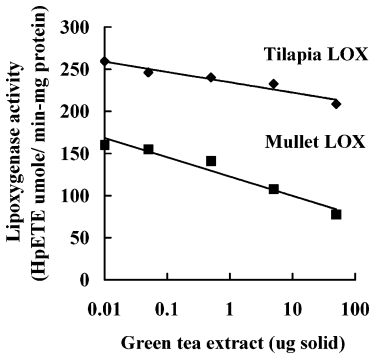

Figure 1. Effect of GTE on HpETE formation catalyzed by gill LOX (1 mg/mL); assayed in 0.05 M phosphate buffer, pH 7.0, using 100 μM AA as the substrate; incubated at 25 °C for 3 min; and measured with the spectrophotometric method.

Measurement of Rheological Properties of Blood. A sample of 1.5 mL of whole blood was measured using a Cone and Plate Microviscometer (Haake model PK45, Karlsruhe, Germany) at cone 4°, and the shear rate ranged from 80 to 300 s^{-1} for 10 min at 25 °C (*32*). Data were collected to fit a power law model $\sigma = K\gamma^n$, where σ = shear stress, γ = shear rate, K = fluid consistency, and n = flow behavior index, to determine the flow behavior indicated by K and n.

The hematocrit of the blood was measured before and after the shear by using NH_4-heparinized capillary tubes (75 ± 1 mm × 1.1−1.2 mm, height × diameter) to draw blood up to 2/3 of the height and at 15 000 cfw for 5 min. The red layer of the capillary tube was measured using a hematocrit ruler (Kubota, Tokyo, Japan).

RESULTS AND DISCUSSION

Dose-Dependent Inhibitory Effect on LOX. The effect of GTE on fish gill LOX is shown in **Figure 1**. The GTE was mixed with partially purified gill LOX of tilapia or grey mullet, assayed on AA ($C_{20:4}$), and determined using a spectrophotometric method. At an increased GTE concentration (x) from 0.01 to 100 μg dry weight, the antioxidant effects (y) increased linearly according to a regression equation of $y = -5.31 \ln(x) + 234.53$ ($R^2 = 0.93$) for tilapia gill LOX and $y = -9.94 \ln(x) + 122.65$ ($R^2 = 0.95$) for grey mullet gill LOX. The LOX inhibitory activity of GTE was dose-dependent.

Because either inhibition of LOX enzyme or suppression of the autoxidation could lead to reduction of the HpETE formation, LOX and arachidonate were pretreated separately with GTE prior to the determination of the HpETE produced. The CL intensity of the hydroperoxy derivatives was measured with a luminol-mediated CL assay. The gill LOX pretreated with GTE showed a 90.2% inhibition of tilipia gill LOX and a 76.7% inhibition of grey mullet gill LOX (**Figure 2**). AA was pretreated with GTE, and the autoxidation was inhibited by 98.5 or 95.7%, respectively, for the two fish (**Figure 2**), indicating that GTE can inhibit both the LOX-catalyzed dioxygenation and the autoxidation of the polyunsaturated fatty acids (PUFA).

According to HPLC analysis, the products resulting from the catalysis of gill LOX appeared in three isoforms, 5-, 12-, and 15-HETE, indicative of the presence of three LOX isozymes in fish gill (**Table 1**). By addition of GTE at 34 mg/L to tilapia gill LOX extract, the activities of 15-, 12-, and 5-LOX were inhibited by 69.7, 28.7, and 55.1%, respectively. Because antioxidants such as vitamin E regulated the acceleration of AA metabolism and reduced the activity of LOX and COX (*33*), vitamin E (83 mg/L) was used as a positive control for measuring the inhibitory effects of GTE on the three LOXs of

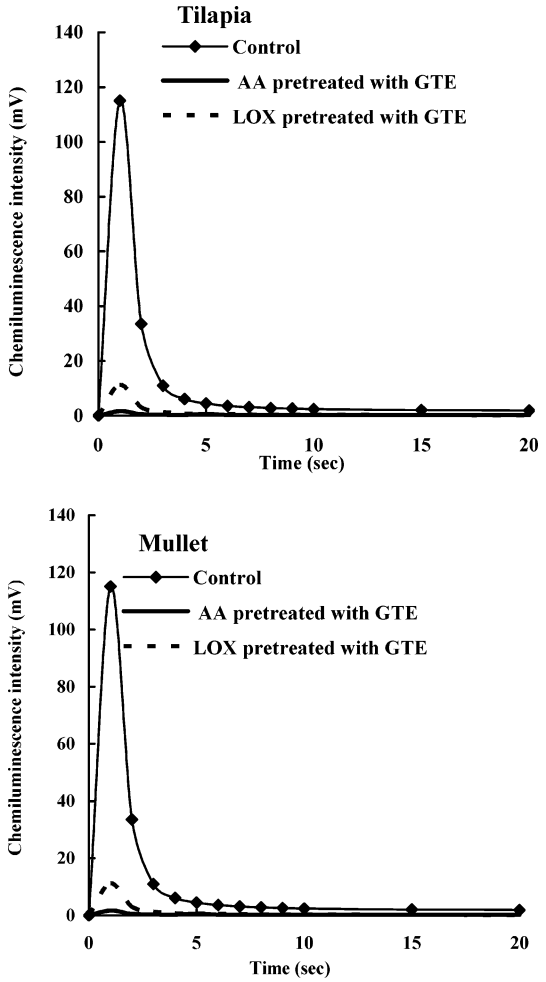

Figure 2. Inhibitory effect of GTE on AA and fish gill LOX indicated by chemiluminesence intensity. AA and LOX were pretreated with GTE, respectively, followed by the addition of 0.1 mL of gill LOX and 0.1 mL of 0.1 mM luminol to 0.05 M Na-borate buffer, pH 10.5, at 25 °C for 1 min using 200 μM AA in ethanol preincubated with GTE for 2 min.

Table 1. Effect of GTE[a] on HETE Formation Catalyzed by Tilapia Gill LOX[b]

	HETE (μmol/min mg protein)			
treatment	15	12	5	total
control	23.8	124.2	6.15	154.2
GTE	7.2	88.5	2.76	98.5
(34 mg/L)	(69.7%)[c]	(28.7%)[c]	(55.1%)[c]	(36.1%)[c]
vitamin E	6.9	76.2	2.71	85.8
(83 mg/L)	(71.0%)[c]	(38.6%)[c]	(55.9%)[c]	(44.3%)[c]

[a] Obtained by reflux for 90 min. [b] Assayed in 0.05 M phosphate buffer, pH 7.0, containing 0.04% Tween 20 and incubated at 25 °C for 30 min. [c] % inhibition = 100% − ($HETE_{GTE}/HETE_{control}$) × 100%.

tilapia gill. The different LOX isozyme showed a different susceptibility to GTE inhibition.

GTI at 57 mg/L inhibited 15-, 12-, and 5-LOX of grey mullet gill by 52.8, 49.2, and 55.6%, respectively (**Table 2**). The purified GTPs were also found to inhibit 5-, 12-, and 15-LOX and were considered to alter the risk for cancer (*34*). In our results, the GTE was inhibitory to 15-LOX and 5-LOX more than to 12-LOX (**Table 1**). The 15-LOX is a cause of LDL oxidation leading to atherogenesis (*35*). The activities of phospholipase A_2, COX, and 5-LOX increased significantly in the platelet of chronically cadmium-poisoned rats. However,

Table 2. Effect of GTI[a] on HETE Formation Catalyzed by Mullet Gill LOX[b]

	HETE (μmol/min mg protein)			
treatment	15	12	5	total
control	54.5	89.0	15.1	158.6
GTI	25.7	45.2	6.7	77.
(57 mg/L)	(52.8%)[c]	(49.2%)[c]	(55.6%)[c]	(51.1%)[c]

[a] Obtained by soaking in 100 °C hot water for 5 min. [b] Assayed in 0.05 M phosphate buffer, pH 7.0, containing 0.04% Tween 20 and incubated at 25 °C for 30 min. [c] % inhibition.

Table 3. Comparison on the Antioxidant Effects of Vitamin E and Freshly Prepared GTEs

	concentration[a] (mg/L)	antioxidant activity[b] (%)
GTE	47	49.7
	51	59.8
	55	79.7
vitamin E	83	20.5

[a] The three concentrations of GTE were obtained by reflux for 30, 60, and 90 min, respectively, to the described concentrations of GTE solid. [b] The antioxidant activity (%) was measured on Hb-catalyzed oxidation of linoleic acid (*27*).

Table 4. Comparison on the Antioxidant Effects of Vitamin E and Freeze-Dried GTEs and GTIs Refrigerated for 2 Months

treatment	antioxidant activity[c] (%)
blank	0
GTE[a]	38.4
GTI[b]	40.0
vitamin E (83 mg/L)	21.2

[a] Refluxed for 90 min, freeze-dried, and stored in a refrigerator for 2 months and then dissolved in deionized water to 57 mg/L. [b] Soaked in 100 °C hot water for 5 min, freeze-dried, and refrigerated for 2 months and then dissolved in deionized water to 57 mg/L. [c] The antioxidant activity (%) was measured on Hb-catalyzed oxidation of linoleic acid (*27*).

the activities of these enzymes were substantially decreased when these rats were administered with catechin, the antioxidant abundant in tea. The activity of 5-LOX is involved in normal leukotriene B_4 synthesis; thus, catechin showed an antiinflammmatory function (*36*).

Antioxidant Effect on Hb-Catalyzed Oxidation. The effect of GTE on Hb-catalyzed oxidation of linoleic acid was evaluated in reference to that of vitamin E (**Table 3**). The GTE was obtained by reflux for 30, 60, and 90 min up to concentrations of 47, 51, and 55 mg/L, respectively, and the corresponding antioxidant effects were 49.7, 59.7, and 79.7%, respectively, all of which were higher than that of vitamin E at 83 mg/L. Because the GTE obtained by reflux for 90 min showed the highest antioxidant effect, it was compared with the GTI imitating the way that people make tea. Both GTE and GTI at 57 mg/L (prepared after storage of the tea in a refrigerator for 2 months) had similar inhibitory effects on Hb-catalyzed oxidation of linoleic acid, either 38.4 or 40.0% (**Table 4**), being 48% less than the newly prepared GTE (**Table 3**). Nevertheless, both GTE and GTI of 57 mg/L were still 81−89% higher in LOX inhibitory effect than that of vitamin E at 83 mg/L.

LOX is a dioxygenase containing a nonheme single iron. It catalyzes the peroxidation of *cis*,*cis*-1,4-pentadiene structures. In its oxidized state, LOX (Fe^{3+}) can abstract a proton from a

Table 5. Effect of Decolored GTE on the Flow Behavior Index (n) and Fluid Consistency (K) of Fish RBCs Using the Power Law $\sigma = K\gamma^n + C$

treatment[a]	hematocrit (%)	K	n	R^2
	Tilapia RBCs			
plasma (control)	26	0.027	0.68	0.98
decolored GTE	26	0.015	0.76	0.98
ascorbic acid	26	0.008	0.87	0.99
	Mullet RBCs			
plasma (control)	27	0.028	0.65	0.98
decolored GTE	27	0.016	0.73	0.99
ascorbic acid	27	0.014	0.79	0.99

[a] The total volume (V) was 1.5 mL including 1/3 volume of RBCs, 1/3 volume of plasma, or isotonic saline containing 2.5 mg of decolored GTE or ascorbic acid as a positive control.

PUFA and reduce itself to LOX (Fe^{2+}), producing a fatty acid free radical. The derivatives of these free radicals are bioactive metabolites involved in inflammation (*9*−*11*) that can reduce the potency of the endogenous antioxidants and enhance lipid or LDL oxidation (*16*−*19*). GTPs can scavenge free radicals and active oxygen, thus inhibiting autoxidation and LOX-catalyzed oxidation of AA; GTPs are also capable of reducing Fe^{3+} to Fe^{2+} or chelating iron ions, which inactivate LOX and inhibit Hb-catalyzed oxidation (*34*, *37*−*39*).

Blood Thinning Effect. Because the GTE was a stronger antioxidant than vitamin E to the Hb-catalyzed oxidation of linoleic acid (**Tables 3** and **4**) and because blood is rich in Hb, the effect of GTE on hemorheology was studied. Mixing GTE with tilapia or grey mullet RBCs ex vivo resulted in a blood thinning effect shown by reduced a fluid consistency (K) in **Table 5**. The rheological data fit the power law model, $\sigma = K\gamma^n + C$. When tilapia or grey mullet RBCs were adjusted to a hematocrit of 26 or 27% and then mixed with GTE in comparison to those mixed with blood plasma to the same hematocrit, the fluid behavior index (n) of tilapia or mullet blood increased from 0.68 to 0.76 or 0.65 to 0.73, indicating that the presence of GTE enhanced the RBC to flow more like a Newtonian fluid ($n = 1$). The fluid consistency (K) of tilapia or mullet blood decreased from 0.027 to 0.015 or 0.028 to 0.016 in the presence of GTE. In a prolonged treatment time of GTE with RBCs up to 7 h (**Figure 3**), the fluid consistency (K) decreased linearly according to the regression equation of $y = 0.027 - 0.0024x$ ($R^2 = 0.99$) for tilpia LOX and $y = 0.023 - 0.0013x$ ($R^2 = 0.89$) and declined rapidly after 7 h of exposure time for grey mullet LOX. All indicated that the GTE exerted a thinning effect on fish RBC fluid. The use of ascorbic acid of equal dry weight resulted in a thinner consistency (lower K value) and was closer to a Newtonian type flow (larger n).

In conclusion, the results observed in this study favor the hypothesis that green tea was able to inhibit the oxidations catalyzed by LOX and Hb on PUFA such as those present in red cell membranes (*4*) to reduce the blood consistency and thus to enhance the blood fluidity. Our previous in vivo and ex vivo studies also showed that plant extracts that caused a reduction in LOX-catalyzed hydroperoxides present in fish gill also inhibited hemolysis, blood clotting, and reduced blood consistency (*25*).

The lipid peroxidation or damages on sulfhydryl (SH) groups of protein in erythrocyte membranes decreased the membrane fluidity and deformability of erythocytes (*40*). GTPs could protect SH groups of membrane proteins from Cr(VI)-induced oxidative damage (*4*) and inhibit H_2O_2-induced lipid peroxidation (*24*, *41*). The lipid oxidation of rat erythrocyte membranes

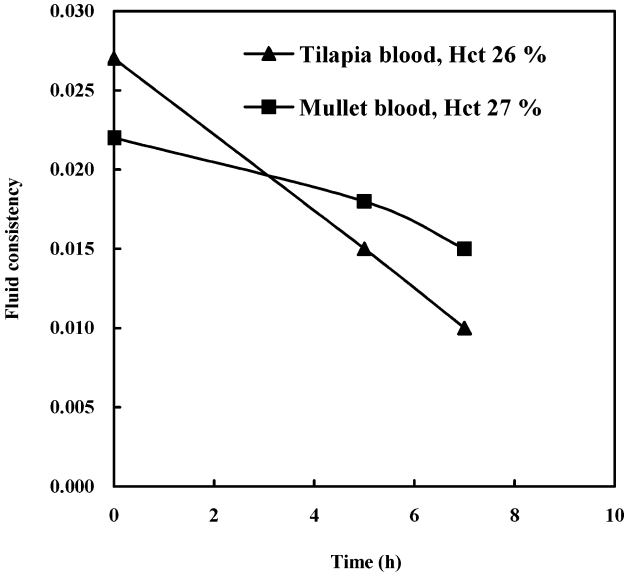

Figure 3. Effect of treatment time of GTE on fluid consistency (K) of tilapia or mullet RBCs at 25 °C using the power law $\sigma = K\gamma^n + C$ (σ, shear stress; r, shear rate). Blood plasma was used to adjust the hematocrit to 26–27%.

mediated by an azo-free radical initiator, 2,2′-azo-bis(2-amidinopropane)dihydrochloride (AAPH), induced membrane damage and hemolysis subsequently (*42*, *43*). GTPs exhibited a strong protection for RBC membranes to hemolysis induced by AAPH (*41*, *44*).

The findings that tea flavonoids decreased platelet aggregation (*45*), while tea catechin is able to improve the thrombogenesis index and has an antithrombotic function (*36*), support our observations of the blood thinning effect. Our current preliminary results showed that several plant extracts that had antioxidant activities also inhibited LOX and COX activities. Feeding fish with these plant extracts prolonged the lag phase and reduced the oxidation rate of LDL in fish blood.

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