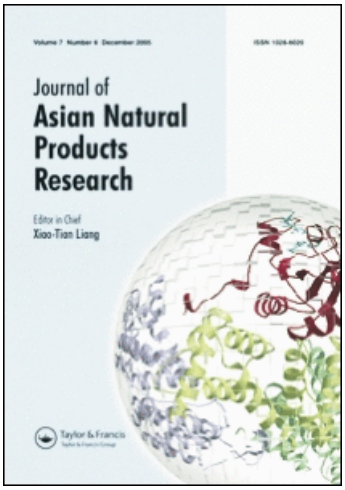

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Effect of 3,4,5,6-tetrahydroxyxanthone on erythrocyte deformability in apolipoprotein E-deficient mice

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Previous investigations have indicated that reduced erythrocyte deformability may be an important factor contributing to the development of atherosclerosis, and endogenous asymmetric dimethylarginine (ADMA) might be an important contributor to reduction of erythrocyte deformability in atherosclerosis. In this study, the effect of 3,4,5,6-tetrahydroxyxanthone (**1**), a kind of polyphenolic compound, on erythrocyte deformability in apolipoprotein E-deficient (apoE −/−) mice was evaluated. After treatment with compound **1** (10 or 20 mg/kg per day) for 4 weeks, erythrocyte deformability, antioxidant enzymes activity, erythrocyte dimethylarginine dimethylaminohydrolase (DDAH) activity, the plasma level of ADMA and malondialdehyde (MDA) level were determined. Treatment with compound **1** (10 or 20 mg/kg) increased erythrocyte deformability, antioxidant enzymes activity concomitantly, a decrease in the plasma levels of MDA and ADMA, and an increase in erythrocyte DDAH activity. The present result suggests that the beneficial effect of **1** on the erythrocyte deformability, besides inhibiting lipid peroxidation, may be related to reduction of ADMA concentration via an increase in DDAH activity.

Keywords: 3,4,5,6-tetrahydroxyxanthone; erythrocyte deformability; antioxidant enzymes; asymmetric dimethylarginine; dimethylarginine dimethylaminohydrolase; apolipoprotein E-deficient mice

1. Introduction

Erythrocyte deformability is a crucial determinant of microvascular perfusion. Impairment in erythrocyte deformability has been reported to be involved in the pathogenesis of many circulatory disorders including atherosclerosis [1]. Decreased erythrocyte deformability was seen in some pre-atherosclerotic conditions such as hypercholesterolemia, diabetes mellitus, lipid peroxidation, and hypertension [2,3]. There is evidence that nitric oxide (NO) participates in the regulation of erythrocyte deformability [3]. Previous studies have shown that erythrocyte deformability was decreased by treatment with NO synthase (NOS) inhibitors NG-nitro-L-arginine methyl ester (L-NAME) [2]. Asymmetric dimethylarginine (ADMA) is also an endogenous inhibitor of NOS, an enzyme catalyzing NO production from arginine. A great deal of information has been

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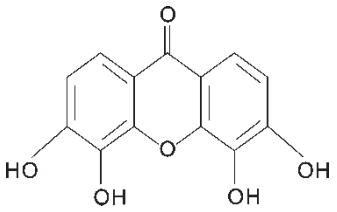

Figure 1. Chemical structure of compound **1**.

indicated that ADMA, besides inhibiting NO synthesis, plays a role as a novel cardiovascular risk factor [4]. It has been reported that decreased erythrocyte deformability may be related to the elevation of ADMA level in diabetic rats [5]. Our recent studies reported that erythrocyte deformability was significantly decreased concomitantly with an elevation of endogenous ADMA level in apolipoprotein E-deficient (apoE −/−) mice [6]. These findings suggest that decreased erythrocyte deformability is related to the reduction of NO production by elevation of ADMA level in some cardiovascular diseases.

Xanthone, as a kind of polyphenolic compound, has extensive pharmacological actions. Previous investigations have shown that some xanthones have a potent antioxidant activity and inhibit lipid peroxidation [7,8]. Previous studies have demonstrated that the protective effect of some xanthone compounds is related to the reduction of ADMA concentration in hypercholesterolemic animals and endothelial cells treated with low-density lipoprotein (LDL) [9,10]. As mentioned above, impaired erythrocyte deformability is related to the elevation of ADMA level. Therefore, we hypothesized that the beneficial effects of 3,4,5,6-tetrahydroxyxanthone (**1**) on erythrocyte deformability are related to the inhibition of lipid peroxidation, which in turn reduce the ADMA level. In the present study, we tested the effect of **1**, a xanthone derivative (Figure 1), on erythrocyte deformability in apoE −/− mice.

2. Results and discussion

2.1 Erythrocyte deformability

Erythrocyte deformability was expressed as the elongation index (EI) and integrated EI (IEI). Erythrocyte deformability (expressed as the EI and IEI) in the model group was markedly lowered compared with that in the control group ($P < 0.01$). After treatment with **1** (10 or 20 mg/kg) for 4 weeks, values of the EI and IEI were significantly increased in apoE −/− mice ($P < 0.05$ or $P < 0.01$; Figure 2).

2.2 Erythrocyte antioxidant enzymes activity

Catalase (CAT) and superoxide dismutase (SOD) activities in the model group were markedly lowered compared with that in the control group ($P < 0.01$). After treatment with **1** (10 or 20 mg/kg) for 4 weeks, activities of erythrocyte CAT and SOD were significantly increased in apoE −/− mice ($P < 0.05$ or $P < 0.01$; Table 1).

2.3 Erythrocyte dimethylarginine dimethylaminohydrolase activity

Dimethylarginine dimethylaminohydrolase (DDAH) activity in the model group was decreased significantly compared with that in the control group. Treatment with **1** (10 or 20 mg/kg) for 4 weeks significantly increased the DDAH activity in apoE −/− mice ($P < 0.01$; Figure 3).

2.4 Plasma malondialdehyde and ADMA concentrations

Plasma levels of malondialdehyde (MDA) and ADMA in the model group were increased significantly compared with that in the control group ($P < 0.01$). Treatment with **1** (10 or 20 mg/kg) for 4 weeks markedly decreased the plasma levels of MDA and ADMA in apoE −/− mice ($P < 0.01$; Figures 4 and 5).

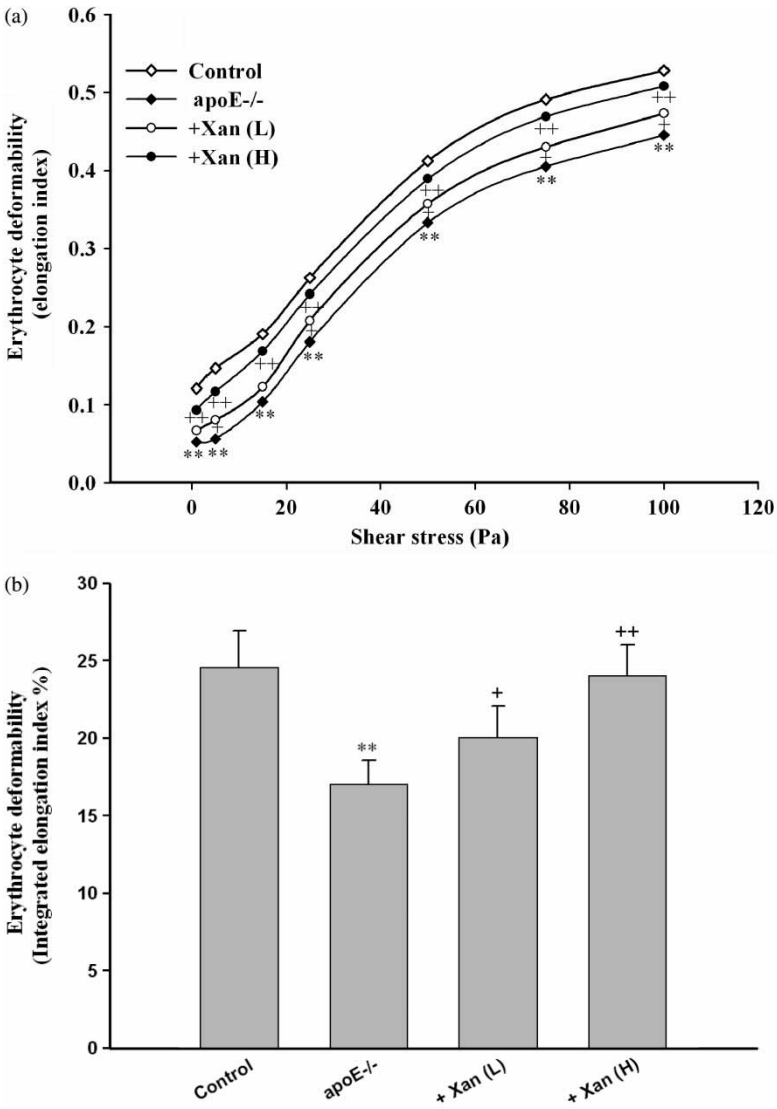

Figure 2. Effect of **1** on erythrocyte deformability. (a) The effect of **1** on EI (elongation index). (b) The effect of **1** on IEI% (integrated elongation index %). + Xan (L): **1**, 10 mg/kg; + Xan (H): **1**, 20 mg/kg. Values are mean ± SD, $n = 10$. $^{**}P < 0.01$, compared with control; $^{+}P < 0.05$, $^{++}P < 0.01$, compared with apoE −/− mice.

Table 1. Effects of **1** on activities of erythrocyte antioxidant enzymes activity.

	Control	apoE −/− mice	+Xan (L)	+Xan (H)
CAT (k/mg Hb)	0.065 ± 0.004	$0.052 \pm 0.002^{**}$	$0.058 \pm 0.003^{+}$	$0.061 \pm 0.003^{++}$
SOD (U/mg Hb)	3.64 ± 0.35	$2.02 \pm 0.11^{**}$	$2.86 \pm 0.18^{+}$	$3.38 \pm 0.22^{++}$

Values are mean ± SD, $n = 10$. + Xan (L): **1**, 10 mg/kg; + Xan (H): **1**, 20 mg/kg; **1**, Xan; CAT, catalase; SOD, superoxide dismutase. $^{**}P < 0.01$, compared with control; $^{+}P < 0.05$, $^{++}P < 0.01$, compared with apoE −/− mice.

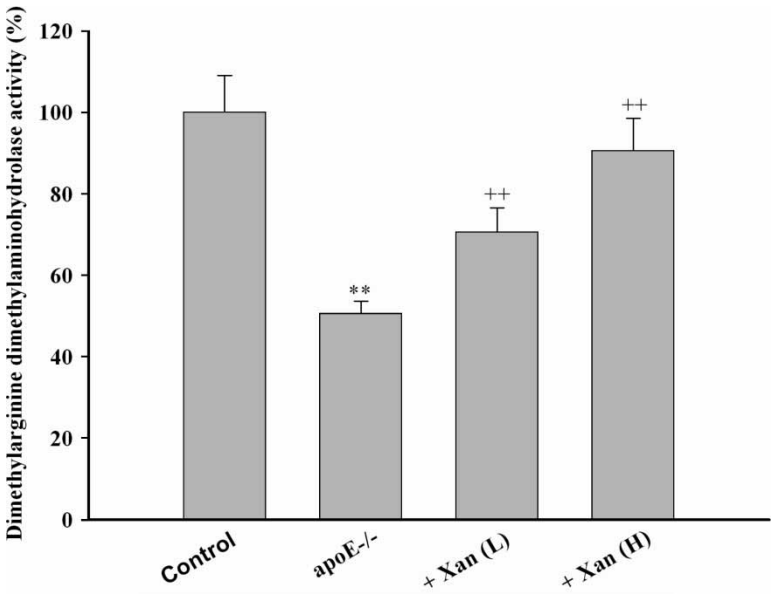

Figure 3. Effect of **1** on erythrocyte dimethylarginine dimethylaminohydrolase activity. + Xan (L): **1**, 10 mg/kg; + Xan (H): **1**, 20 mg/kg. Values are mean ± SD, $n = 10$. $^{**}P < 0.01$, compared with control; $^{++}P < 0.01$, compared with apoE −/− mice.

2.5 Discussion

Decreased erythrocyte deformability may be a contributor to damages of target organs in atherosclerosis. Previous investigations have shown that erythrocyte deformability in atherosclerotic animal

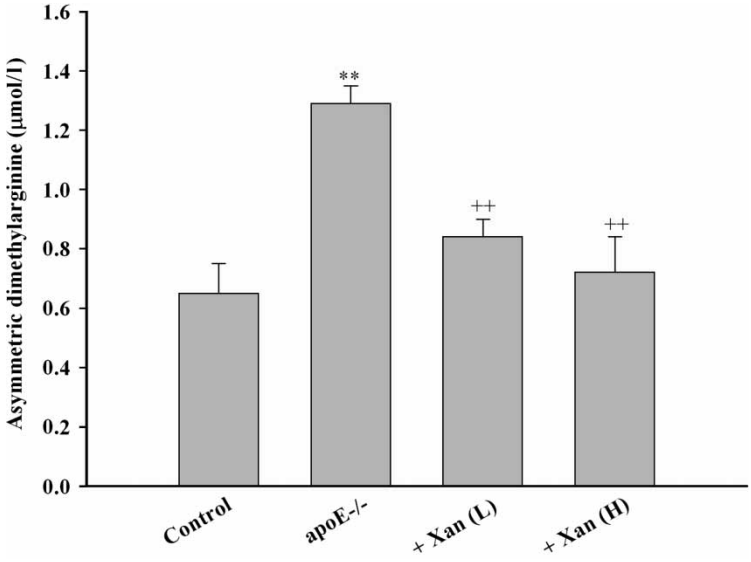

Figure 4. Effect of **1** on plasma asymmetric dimethylarginine level. + Xan (L): **1**, 10 mg/kg; + Xan (H): **1**, 20 mg/kg. Values are mean ± SD, $n = 10$. $^{**}P < 0.01$, compared with control; $^{++}P < 0.01$, compared with apoE −/− mice.

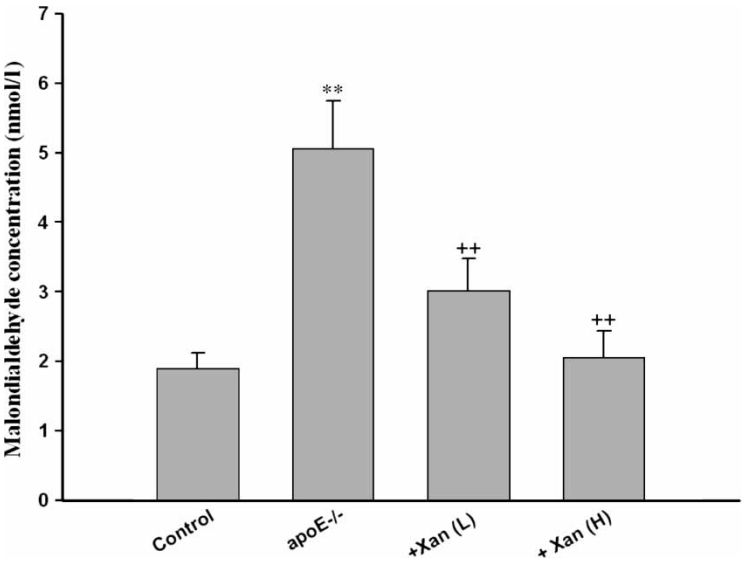

Figure 5. Effect of **1** on plasma malondialdehyde level. + Xan (L): **1**, 10 mg/kg; + Xan (H): **1**, 20 mg/kg. Values are mean ± SD, $n = 10$. $^{**}P < 0.01$, compared with control; $^{++}P < 0.01$, compared with apoE −/− mice.

model or patients with hypercholesterolemia was significantly decreased [11,12]. The present results confirmed our previous observations that erythrocyte deformability was significantly decreased in apoE −/− mice, a commonly used model for experimental atherosclerosis research [6]. The mechanism responsible for impaired erythrocyte deformability in atherosclerosis has not been fully elucidated. It has been reported that NO participates in the modulation of erythrocyte deformability. There is evidence that treatment with the exogenous NOS inhibitor L-NAME decreased erythrocyte deformability *in vitro* and *in vivo* [13]. Our recent observations showed that exogenous ADMA caused a decrease of erythrocyte deformability in normal mice or aggravated impairment of erythrocyte deformability in apoE −/− mice [6]. *In vitro*, exogenous ADMA caused a decrease of erythrocyte deformability in a concentration-dependent manner, and the effect of ADMA was reversed by L-arginine [6]. These results suggest that endogenous ADMA might be an important contributor to the reduction of erythrocyte deformability in atherosclerosis.

Nowadays, ADMA is regarded as a novel risk marker in cardiovascular medicine and beyond [4]. It is well known that ADMA is synthesized by the protein arginine methyltransferase (PRMT), which utilizes *S*-adenosylmethionine methyl group donor, and degraded by DDAH [4]. The increased level of ADMA may be related to the reduction of DDAH activity and/or upregulation of PRMT expression [14]. DDAH is widely distributed in nervous, cardiovascular, renal, and hepatic tissues. DDAH is also present in monocytic, polynuclear, and red blood cells (RBC) [15]. RBC could be used to assess the status of DDAH in various disease states. In a pilot study of end-stage renal disease patients, RBC DDAH activity with ADMA as the substrate correlated inversely with age, and enzyme activities were higher in patients with greater diastolic blood pressure drops during hemodialysis [15]. *In vitro*, the increased level of ADMA was accompanied by a reduction

in DDAH activity and/or upregulation of the protein expression of PRMT I [14]. Recent work showed that a single injection of native LDL caused a significant decrease in the activity of DDAH in the RBC of rats [16]. The results of the present study also showed that plasma levels of ADMA were significantly increased concomitantly with the reduction of erythrocyte DDAH activity in apoE −/− mice.

Xanthones, as a kind of polyphenolic compounds, have extensive pharmacological actions. Previous investigations have shown that some xanthones have a potent antioxidant activity and inhibit lipid peroxidation stimulated by CCl_4–NADPH or $FeCl_2$–ADP mixture in the rat liver homogenate and block the oxidation of LDL *in vitro* [8]. It has been documented that DDAH has a critical sulfhydryl group of its cysteine 249 residue which participates in the reaction-intermediate formation by its nucleophilic attack of guanidinium carbon of ADMA [17]. A sulfhydryl group in the catalytic region of the active site confers on DDAH its exquisite sensitivity to oxidative stress. Sulfhydryl blocking agents such as *p*-chloromercuribenzoate and $HgCl_2$ have been known to inhibit the activity of the enzyme [4]. Rats treated with native LDL markedly reduced DDAH activity and increased the plasma concentration of ADMA [9]; *in vitro*, ox-LDL or lysophosphatidylcholine (LPC), the major component of ox-LDL, increased the level of ADMA accompanied by a reduction in DDAH activity and/or upregulation of the protein expression of PRMT I [14]. The results of the present study revealed that plasma levels of MDA and erythrocyte DDAH activity were significantly increased concomitantly with the reduction of erythrocyte antioxidant enzymes activity in apoE − /− mice. It is probable that lipid peroxidation reduces DDAH activity. The present results showed that **1** also increased erythrocyte deformability, antioxidant enzymes activity concomitantly, a decrease in the plasma levels of MDA and ADMA, and an increase in erythrocyte DDAH activity. Erythrocytes are highly sensitive to oxidative stress and oxidative stress could impair erythrocyte deformability. Other polyphenol derivatives such as (−)-epigallocatechin gallate upregulated SOD and CAT levels [18]. Previous investigations have demonstrated that xanthones attenuated a decrease in the activity of DDAH induced by LPC [10]. These findings support the hypothesis that **1** can improve erythrocyte deformability, and the beneficial effects of **1** on erythrocyte deformability may be related to the reduction of ADMA concentration via an increase in DDAH activity by the inhibition of lipid peroxidation. However, further work is needed before drawing a definitive conclusion on this matter.

In summary, the present study suggests that the beneficial effect of **1** on the erythrocyte deformability, besides inhibiting lipid peroxidation, may be related to the reduction of ADMA concentration via an increase in DDAH activity.

3. Experimental

3.1 Reagents

Compound **1** (purity: 99.0%) was obtained from the School of Pharmaceutical Sciences, Central South University, China. Internal standard ADMA was purchased from Sigma Chemical Co. (St Louis, MO, USA). Polyvinylpyrrolidone (PVP-K30) and other reagents were obtained from Shuang-He Medical Corporation (Beijing, China).

3.2 Experimental animals

Male C57BL/6J and apoE−/− mice (on the C57BL/6 background) were obtained from the Department of Laboratory Animal Science, Beijing University (Beijing, China). Animals received humane care in compliance with the

'Guide for the Care and Use of Laboratory Animals' published by the National Institutes of Health (NIH publication 85-23, revised 1985). Animals were housed in cages under controlled temperature (23 ± 1°C) with a humidity level of 50–60% and a 12 h light–dark cycle with free access to water and normal mouse chow.

3.3 Experimental protocols

Mice aged 13 weeks were divided randomly into four groups ($n = 20$ per group): control group (wild-type C57BL/6J mice), model group (apoE − /− mice), and xan-treated groups (apoE − /− mice were intragastrically administered with **1**, 10 or 20 mg/kg per day for 4 weeks, respectively). Compound **1** was dissolved in a vehicle containing 10% gum acacia and 0.5% saline carboxymethyl cellulose. In the control group or model group (apoE − /− mice), the same volume of vehicle was given for 4 weeks. At the end of the experiment, the mice were sacrificed under anesthesia, and blood was collected from the carotid artery. Some anticoagulated blood samples were immediately collected for analysis of erythrocyte deformability. Plasma was prepared from other blood samples via centrifugation at 2500*g* for 20 min at 4°C and stored at −70°C for biochemical analysis.

3.4 Assay of erythrocyte deformability

Blood samples (anticoagulated with heparin 0.15 g/ml) were placed in tubes containing 15% polyvinylpyrrolidone (PVP-K30) solution (PVP 150 g/l, Na_2HPO_4 2.84 g/l, KH_2PO_4 0.68 g/l, NaCl 3.8 g/l, pH 7.4). Erythrocyte deformability was determined by laser diffraction analysis using an ektacytometer (LBY-BX3; Pu-Lisheng Corporation, Beijing, China). In brief, a low-hematocrit (2.5%) suspension of erythrocytes in 15% PVP solution was added to the sample cup, then laser beam was directed through, and erythrocytes were sheared at a series of shear stress. During the passage of the laser beam, the laser light was diffracted, forming an image correlating to the shape of all erythrocytes that pass the laser beam. Thus, the diffraction image represented the mean deformability of all these erythrocytes and was analyzed by a microcomputer. On the basis of the geometry of diffraction pattern, the EI was calculated: $EI = (L - W)/(L + W)$, where L and W are the length and width of the diffraction pattern, respectively. The IEI is an overall measurement of erythrocyte deformability when shearing erythrocytes at a series of shearing stress; all measurements were carried out at 37°C and within 4 h after collection of blood samples. Each sample was examined for three times to correct the results, an increased EI or IEI at a given shear stress indicates greater cell deformation and hence greater RBC deformability, as reported previously [19,20].

3.5 Assay of erythrocyte antioxidant enzymes activity and plasma lipids

CAT (EC.1.11.1.6) and SOD (EC.1.15.1.1) activities of RBC were determined as previously described [21–23].

3.6 Assay of erythrocyte DDAH activity

DDAH was estimated by measuring L-citrulline formation in RBC lysates. Briefly, in an ice bath, cell lysates were divided into two groups, and was added to the ADMA-treated group (final concentration 0.4 mmol/l). To inactivate DDAH, 10% trichloroacetic acid was immediately added to one experimental group. This group provided a baseline of 0% DDAH activity. The other lysate was incubated at 37°C for 4 min before the addition of 10% trichloroacetic acid. The amounts of L-citrulline formed were measured by the method of Precott and Jones. The difference in L-citrulline concentrations

between two groups reflected the DDAH activity. For every experiment, DDAH activity of RBC in the control group was defined as 100%, and DDAH activity in the other group was expressed as the percentage of L-citrulline from ADMA compared with the control group, as described previously [15].

3.7 Determination of plasma ADMA concentration

The protein in the plasma was removed using 5-sulfosalicylic acid. The content of ADMA was determined by high-performance liquid chromatography (HPLC), as described previously [24]. HPLC was carried out using a Shimadzu LC-6A liquid chromatograph with a Shimadzu SCL-6A system controller and Shimadzu SIC-6A autosampler. *O*-Phthaldialdehyde adducts of methylated amino acids and internal standard ADMA produced by precolumn mixing were monitored using a model RF 530 fluorescence detector set at $\lambda^{ex} = 338$ and $\lambda^{em} = 425$ nm on a Resolve C18 column. Samples were eluted from the column using a linear gradient containing the mobile phase. Phase A was composed of 0.05 mol/l (pH 6.8) sodium acetate–methanol–tetrahydrofuran (81:18:1, v/v/v) and mobile phase B was composed of 0.05 mmol sodium acetate–methanol–tetrahydrofuran (22:77:1, v/v/v) at a flow rate of 1 ml/min.

3.8 Determination of plasma MDA concentration

The content of thiobarbituric acid reactive substances, reflecting the level of lipid peroxide, was measured spectrophotometrically, as reported previously, and expressed as the amount of MDA [25].

3.9 Statistical analysis

Initial analyses were performed by Student's *t*-test. If the data did not fit the constraints of this parametric test, the data were analyzed using the one-way ANOVA test. $P < 0.05$ was considered significant. SPSS 10.0 software was used for all calculations. All data were presented as mean ± SD.

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