Original Research

Antioxidant Actions of Phenolic Compounds Found in Dietary Plants on Low-Density Lipoprotein and Erythrocytes in Vitro

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Key words: gingerol, aloe-emodin, barbaloin, rhapontin, anthraquinone derivatives, stilbenes, low-density lipoprotein, erythrocytes, erythrocyte membranes, oxidative stress, peroxyl radicals, lipid peroxidation, Na^+/K^+-ATPase, Ca^{2+}-ATPase, protein sulfhydryl groups

Objective: There is increasing interest in the study of the antioxidant actions of plant phenolic compounds as evidence shows that consumption of plant products rich in these compounds contributes to protection from a number of ailments including cardiovascular diseases. In the present study, the antioxidant effects of selected phenolic compounds from dietary sources, namely barbaloin, 6-gingerol and rhapontin, were investigated.

Methods: Low-density lipoprotein (LDL), erythrocytes and erythrocyte membranes were subjected to several *in vitro* oxidative systems. The antioxidant effects of the phenolic compounds were assessed by their abilities in inhibiting hemolysis and lipid peroxidation of LDL and erythrocyte membranes, and in protecting ATPase activities and protein sulfhydryl groups of erythrocyte membranes.

Results: 6-Gingerol and rhapontin were found to exhibit strong inhibition against lipid peroxidation in LDL induced by 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) and hemin while barbaloin possessed weaker effects. A similar order of antioxidant potencies among the three compounds was observed on the lipid peroxidation of erythrocyte membranes in a *tert*-butylhydroperoxide (*t*BHP)/hemin oxidation system. On the other hand, barbaloin and rhapontin were comparatively stronger antioxidants than 6-gingerol in preventing AAPH-induced hemolysis of erythrocytes. Among the three compounds, only barbaloin protected Ca^{2+}-ATPase and protein sulfhydryl groups on erythrocyte membranes against oxidative attack by *t*BHP/hemin. Interestingly, rhapontin demonstrated protective actions on Na^+/K^+-ATPase in a sulfhydryl group-independent manner under the same experimental conditions.

Conclusions: In view of their protective effects on LDL and erythrocytes against oxidative damage, these phenolic compounds might have potential applications in prooxidant state-related cardiovascular disorders.

INTRODUCTION

Reactive oxygen species (ROS) and other free radicals are characterized by their ability in causing oxidative damage to the body. They contribute to the etiology of a number of pathological conditions including cardiovascular diseases. Epidemiological studies suggest that dietary intake of antioxidants from fruits and vegetables is associated with a reduced risk for cardiovascular diseases [1,2]. The phenolic constituents in plants have been suggested to play a role [3]. Phenolic compounds are widely distributed in plants. They are comprised of several classes of compounds such as flavonoids, anthraquinones, stilbenoids, and their derivatives. The antioxidant properties of these phenolic compounds are related to their abilities to

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Abbreviations: AAPH = 2,2′-azobis(2-amidinopropane) hydrochloride, DMSO=dimethylsulfoxide, DW = distilled water, Hepes = 4-(2-hydroxyethyl)piperazine-1-ethanesulfonate, LDL = low-density lipoprotein, PBS = phosphate-buffered saline, Pi = inorganic phosphate, ROS = reactive oxygen species, TBARS = thiobarbituric acid-reactive substance, *t*BHP = *tert*-butylhydroperoxide.

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donate electrons and to act as free radical scavengers by the formation of stable phenoxyl radicals [4]. Many structure-activity relationship studies of the antioxidant mechanisms of phenolic compounds have been conducted using purely chemical model systems [5,6]. These include assays to assess the scavenging effects of the phenolic compounds on superoxide radicals, hydroxyl radicals, 2,2′-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) radicals 2,2-diphenyl-1-picrylhydrazyl radicals, and other free radicals. Simple biological models such as the lipid peroxidation systems of unsaturated fatty acids [7] and liver microsomes [8] are also commonly applied. The simplicity of these systems allows a large number of agents to be tested conveniently. However, these models also have the drawback of having low physiological relevance. Although the copper-catalysed low-density lipoprotein (LDL) oxidation system [9] is considered more physiological in this respect and has recently gained wide acceptance, care should be taken when applying this system to study the antioxidant mechanisms of phenolic compounds. It is because the antioxidant effects are often complicated by the metal-ion chelating effects of the test compounds [10,11]. In this regard, the development of robust *in vitro* model systems with high physiological relevance is still in need for the study of the antioxidant mechanisms of phenolic compounds.

In the present study, the antioxidant actions of a selected number of natural phenolics were studied in several carefully designed model systems of LDL and erythrocytes which allow the antioxidant mechanisms to be elucidated. The compounds studied originated from three common dietary plants, viz. ginger (*Zingiber officinale* Roscoe), aloe (*Aloe vera* (L.) Burm f.) and rhubarb (*Rheum rhabarbarum* L. or *R. undulatum* L.). They include 6-gingerol (5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-3-decanone) from ginger, rhapontin (rhaponticin or 3,3′,5-trihydroxy-4′-methoxystilbene 3-β-D-glucoside) from rhubarb, aloe-emodin (1,8-dihydroxy-3-(hydroxymethyl)anthraquinone) from rhubarb or aloe, and barbaloin (aloin or 1,8-dihydroxy-10-(β-D-glucopyranosyl)-3-(hydroxymethyl)-9-anthracenone) from aloe. Their chemical structures are shown in Fig. 1. The content of rhapontin in rhubarb root varies between 3.1% to 4.3% in different seasons [12]. Ginger was found to contain 11% of gingerols including 5% 6-gingerol [13]. Barbaloin and its aglycone aloe-emodin are the major ingredients of aloe leaves. The content of barbaloin in the juice of aloe leaves was reported to be 15–40% [14].

LDL and erythrocytes are important blood components that are exposed to increased oxidative stress under certain pathophysiological conditions. In familial hypercholesterolemia, a defect characterized by reduced cellular uptake of LDL, LDL stays in the circulation for an extended period of time. This will result in increased LDL oxidation [8] and a higher incidence of atherosclerosis as considerable evidence has implicated the role of oxidized LDL in atherogenesis [15]. The mechanism of LDL oxidation *in vivo* is not fully understood. Both cell-mediated and cell-independent mechanisms have been proposed [16–18]. In sickle cell anemia, studies have shown that the sickled erythrocytes produce more ROS [19]. In addition, serum antioxidants such as ascorbate and β-carotene are found to be lower in sickle cell anemic subjects [20,21]. Exposure of erythrocytes to this and other prooxidant conditions can lead to a number of membrane changes including lipid peroxidation [22,23], protein crosslinking [23] and sulfhydryl group oxidation [24], resulting subsequently in membrane damage and hemolysis [22,25]. Membrane enzymes such as ATPases are also targets of free radical attack [23,26]. Decrease in erythrocyte ATPase activities has been found to coincide with pathological changes of other clinical parameters in coronary heart disease [27]. Dietary antioxidants, which can reduce the oxidative damage on LDL and erythrocytes, might thus confer protective effects in prooxidant state-related cardiovascular disorders.

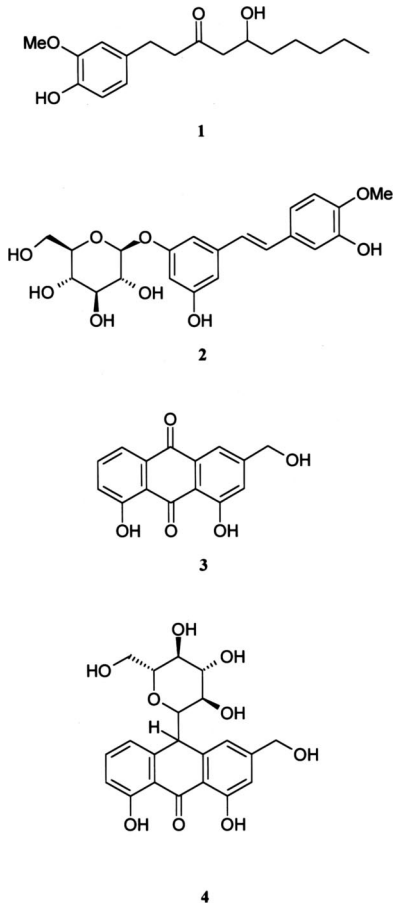

Fig. 1. Chemical structures of 6-gingerol (1), rhapontin (2), aloe-emodin (3), and barbaloin (4).

In the present investigation, the protective actions of the test compounds on LDL was studied by determining the extent of lipid peroxidation in LDL induced by 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) or hemin. Moreover, AAPH-induced hemolysis was used to assess the gross damaging effects of free radicals on erythrocyte membranes and the

protective actions of the test compounds. In addition, lipid peroxidation inhibition, ATPase protection and sulfhydryl group protection were also performed on isolated erythrocyte membranes exposed to *tert*-butylhydroperoxide (*t*BHP)/hemin oxidative damage to further characterize the different aspects of the protective actions of these compounds on the cell membranes. Hemin was used to catalyze the generation of peroxyl radicals and alkoxyl radicals by *t*BHP (reactions 1 and 2 below) [23], subsequently producing the oxidative damage on the erythrocyte membranes.

$$\text{HX-Fe}^{\text{III}} + t\text{Bu-OOH} \rightarrow \text{HX-Fe}^{\text{II}} + t\text{Bu-OO}^{\cdot} + \text{H}^{+} \quad (1)$$

$$\text{HX-Fe}^{\text{II}} + t\text{Bu-OOH} \rightarrow \text{HX-Fe}^{\text{III}} + t\text{Bu-O}^{\cdot} + \text{OH}^{-} \quad (2)$$

MATERIALS AND METHODS

Reagents

Human LDL suspended in phosphate-buffered saline (PBS) with EDTA was purchased from Calbiochem (La Jolla, CA). Trolox, 6-gingerol (>95%) and AAPH were supplied by Wako Chemical Company (Japan). Aloe-emodin (>95%), barbaloin (>97%), rhapontin (>99%), *t*BHP and hemin were purchased from Sigma-Aldrich (St. Louis, MO). Dimethylsulfoxide (DMSO) was purchased from Merck (Germany). All other reagents used were of analytical grade.

Preparation of Rat Erythrocytes

About 10 mL of blood from an adult male Sprague-Dawley rat was obtained from the Laboratory Animal Service Center of The Chinese University of Hong Kong. The blood sample, collected in heparinized tubes, was centrifuged at 1500g for 10 min at 4°C. The erythrocytes collected were resuspended gently with 5 parts of physiological saline and centrifuged again at 1500g for 10 min at 4°C. The washing procedure was repeated two more times. In the last wash, the centrifugal force was lowered to 1000g. The erythrocytes were finally suspended to a hematocrit of 0.20 in PBS.

Preparation of Erythrocyte Membranes

Approximately 100 mL of blood was obtained from a rabbit provided by the Laboratory Animal Service Center of The Chinese University of Hong Kong. The blood was collected in tubes with 60 mM EDTA in saline as anticoagulant (blood: anticoagulant = 9:1 v/v). After washing the erythrocytes, hemoglobin-free erythrocyte membranes were prepared according to a hypotonic lysis procedure [28]. Great care was taken to avoid contamination by potassium and phosphate ions which could affect the determination of ATPase activities in the subsequent assays.

Lipid Peroxidation of LDL

In the present investigation, oxidation of LDL was induced by AAPH, a water-soluble free radical generator [25]; or by hemin, a trivalent ferric oxidant which also exists *in vivo* [17]. AAPH undergoes thermal decomposition to produce carbon-centered radicals which are converted into peroxyl radicals in the presence of oxygen. The peroxyl radicals formed could then initiate lipid peroxidation chain reactions [25]. AAPH was used in the present study such that the antioxidant effects of free radical scavengers in aqueous phase could be assessed. In the absence of exogenous hydroperoxides, hemin ($\text{HX-Fe}^{\text{III}}$) propagates lipid peroxidation chain reactions by catalyzing the formation of lipid peroxyl ($\text{LOO}^{\cdot}$) or alkoxyl ($\text{LO}^{\cdot}$) radicals from endogenous lipid hydroperoxides (LOOH) in LDL (reactions 3–5 below) [29,30].

$$\text{HX-Fe}^{\text{III}} + \text{LOOH} \rightarrow \text{HX-Fe}^{\text{II}} + \text{LOO}^{\cdot} + \text{H}^{+} \quad (3)$$

$$\text{HX-Fe}^{\text{III}} + \text{LOOH} \rightarrow \text{HX-}[\text{Fe}^{\text{IV}} = \text{O}]^{2+} + \text{LO}^{\cdot} + \text{H}^{+} \quad (4)$$

$$\text{HX-Fe}^{\text{II}} + \text{LOOH} \rightarrow \text{HX-Fe}^{\text{III}} + \text{LO}^{\cdot} + \text{OH}^{-} \quad (5)$$

Therefore a compound that could inhibit lipid peroxidation induced by hemin is likely to be a chain-breaking antioxidant in lipid. By employing both oxidation systems, the relative potency of a test compound in resembling a free radical scavenger and/or a chain-breaking antioxidant could be evaluated.

The condition of the AAPH-induced lipid peroxidation of LDL was 37°C for 2 h in a final volume of 1 mL containing PBS (pH 7.4), 0.5 mM EDTA, 50 μL of the test compound dissolved in DMSO, 0.1 mg/mL LDL and 20 mM AAPH. The condition of the hemin-induced lipid peroxidation of LDL was 37°C for 2 h in a final volume of 1 mL containing PBS (pH 7.4), 0.5 mM EDTA, 50 μL of the test compound dissolved in DMSO, 0.1 mg/mL LDL and 1 μM hemin. Trolox was used as the reference antioxidant compound in the assays.

*t*BHP/hemin-Induced Lipid Peroxidation of Erythrocyte Membranes

The reaction was performed at 37°C for 4 h in a final volume of 1 mL containing PBS (pH 7.4), 50 μL of the test compound dissolved in DMSO, 0.05 mg/mL erythrocyte membranes, 0.5 mM *t*BHP and 1.5 μM hemin. Trolox was used as the reference antioxidant compound in the assay.

Determination of Lipid Peroxidation by Fluorescence Measurement of TBARS

Lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substance (TBARS) formed [31]. The

sample mixture containing oxidized LDL or erythrocyte membranes was mixed with one part of 20% trichloroacetic acid and one part of 0.8% thiobarbituric acid. The mixture was heated for 1 h at 95°C. After cooling, it was centrifuged at 3000g for 5 min. The supernatant was extracted with butan-1-ol and the organic layer was saved for subsequent TBARS determination. TBARS was measured fluorometrically at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. TBARS formation in the presence of the test compound was expressed as a percentage of the control to indicate the extent of inhibition of lipid peroxidation.

AAPH-Induced Hemolysis Assay

The assay was performed as described by Jimenez *et al.* [32] with minor modifications. The reaction mixture was made up by combining 500 μL of rat erythrocyte suspension, 250 μL of the test compound dissolved initially in DMSO and diluted with PBS (pH 7.4), and 250 μL of 400 mM AAPH in a 1.5 mL microfuge tube. Trolox was used as the reference antioxidant compound in the assay. The final concentration of DMSO was 2.5%. Preliminary experiments had shown that this concentration of DMSO had minimal effects on the assay. All assay mixtures were incubated at 37°C for 3 h with gentle rotation. After the incubation period, aliquoted samples of each reaction mixture were mixed with 9 parts of PBS or distilled water (DW), respectively. All samples were mixed well and centrifuged at 1500g for 10 min. Absorbance readings of the supernatants at 540 nm were measured. The percentage of erythrocyte lysed in each sample was calculated according to the following equation:

$$\text{Lysis } \% = A_{540} \text{ in PBS}/A_{540} \text{ in DW} \times 100\%$$

ATPase Protection Assay

The oxidation reaction was performed in a final volume of 800 μL containing 50 mM sodium 4-(2-hydroxyethyl)piperazine-1-ethanesulfonate (Hepes) buffer (pH 7.4), the test compound, erythrocyte membranes (0.3 mg/mL for Ca^{2+}-ATPase, and 1 mg/mL for Na^+/K^+-ATPase), 0.5 mM *t*BHP, and hemin (2 μM for Ca^{2+}-ATPase, and 1.5 μM for Na^+/K^+-ATPase). The mixture was incubated for 4 h at 37°C with gentle agitation. It was then centrifuged at 20,000g for 5 min at 4°C. The supernatant was discarded and the membrane pellet was kept on ice. The erythrocyte membranes were resuspended in 450 μL of assay buffer according to Reinila *et al.* [33] except that imidazole buffer was used instead of Tris buffer, with 0.01% Triton X-100 (to release the ATPase from the membranes) followed by incubation at 37°C for 10 min before commencement of the ATPase assay. At suitable time intervals after the addition of ATP (1 mM final concentration), aliquots (50 μL) of samples were drawn for inorganic phosphate (Pi) determination. The amount of Pi in each sample was determined by absorbance measurement at 650 nm after incubation for 30 min with 1 mL of malachite green reagent [34] and 450 μL of 1% polyvinyl alcohol. Absorbance readings were compared against a preconstructed calibration curve using potassium dihydrogen phosphate as standard. The amount of Pi released within a defined period (10 min) was calculated by subtracting the reading of an aliquoted sample at zero-time from the one after 10 min incubation. The enzyme activity of Ca^{2+}-ATPase or Na^+/K^+-ATPase in the membranes expressed as nmole Pi/mg/min was calculated by the difference in the activities determined in assay buffers with and without the activation ions (calcium ions for Ca^{2+}-ATPase, and potassium ions for Na^+/K^+-ATPase).

Sufhydryl Group Protection Assay

The oxidation reaction was performed in a final volume of 800 μL containing 50 mM Hepes buffer (pH 7.4), the test compound, 0.3 mg/mL erythrocyte membrane, 0.5 mM *t*BHP and 10 μM hemin. The mixture was incubated for 4 h at 37°C with gentle agitation. It was then centrifuged at 20,000g for 5 min at 4°C. The supernatant was discarded and the membrane pellet was kept on ice. The erythrocyte membranes were resuspended in 400 μL of 75 mM phosphate buffer (pH 7.4) with 2.7 mM EDTA. Afterwards, 500 μL of 10% sodium dodecyl sulfate was added to denature the proteins and 100 μL of 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) was also added for protein thiol determination [35]. Color was allowed to develop for 15 min before absorbance measurement at 412 nm. Net absorbance for each sample was obtained by subtracting the blank (containing an equivalent amount of membrane proteins). The amount of free sulfhydryl groups in the membrane proteins of the sample was determined from a standard curve constructed with a series of known amounts of reduced glutathione.

Protein Assay

Protein assay was conducted according to the method of Lowry *et al.* [36] using bovine serum albumin as standard.

Statistical Analyses

Statistical analyses of the results were performed using one-way ANOVA followed by Dunnett's test. A P value of < 0.05 was considered statistically significant.

RESULTS

The antioxidant actions of the phenolic compounds were studied in a multitude of experimental systems. Results of the initial screening are shown in Table 1. For those compounds with positive results, dose-dependent studies were subsequently performed to quantitate their potencies. The results are shown in Fig. 2, Fig. 3, Fig. 4, Fig. 5, Fig. 6, Fig. 7, Fig. 8. It was found that aloe-emodin is inactive in six out of the seven

Table 1. Results of Initial Screening of Compounds on All Assays

Compound	Effectiveness, maximum concentration tested (μM)						
	A	B	C	D	E	F	G
6-Gingerol	+, 1000	+, 50	+, 500	+, 70	−, 1000	−, 1000	−, 1000
Rhapontin	+, 1000	+, 50	+, 500	+, 70	−, 1000	+, 2000	−, 1000
Aloe-emodin	−, 20	+, 20	−, 5	−, 20	−, 15	−, 15	−, 15
Barbaloin	+, 1000	+, 100	+, 500	+, 1000	+, 2000	−, 2000	+, 2000

Column A: inhibition of AAPH-induced lipid peroxidation in LDL; Column B: inhibition of hemin-induced lipid peroxidation in LDL; Column C: inhibition of AAPH-induced hemolysis; Column D: inhibition of lipid peroxidation of erythrocyte membranes; Column E: protection of Ca^{2+}-ATPase activity in erythrocyte membranes; Column F: protection of Na^{+}/K^{+}-ATPase activity in erythrocyte membranes; Column G: protection of protein sulfhydryl groups in erythrocyte membranes. The effectiveness of the test compounds is indicated by the following symbols: +, active; −, inactive.

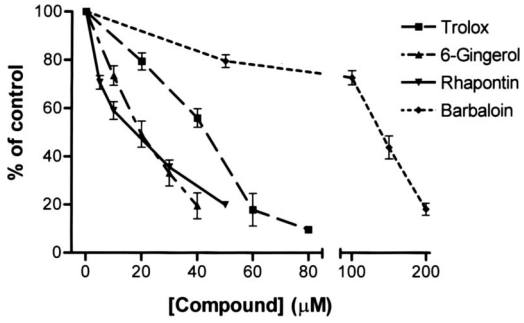

Fig. 2. The inhibitory actions of Trolox, 6-gingerol, rhapontin and barbaloin on AAPH-induced lipid peroxidation in LDL. Results are expressed as a percentage of the control. Data represent mean values ± S.D. from four independent experiments.

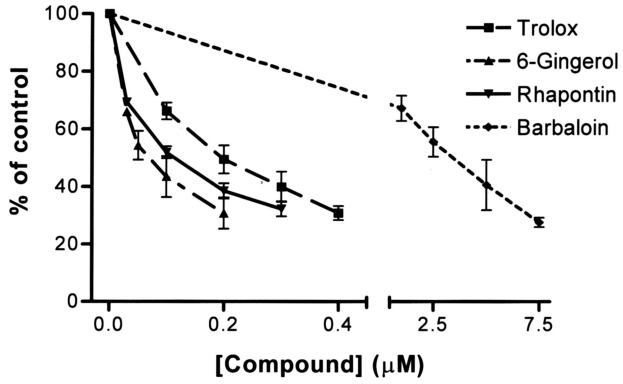

Fig. 3. The inhibitory actions of Trolox, 6-gingerol, rhapontin and barbaloin on hemin-induced lipid peroxidation in LDL. Results are expressed as a percentage of the control. Data represent mean values ± S.D. from four independent experiments.

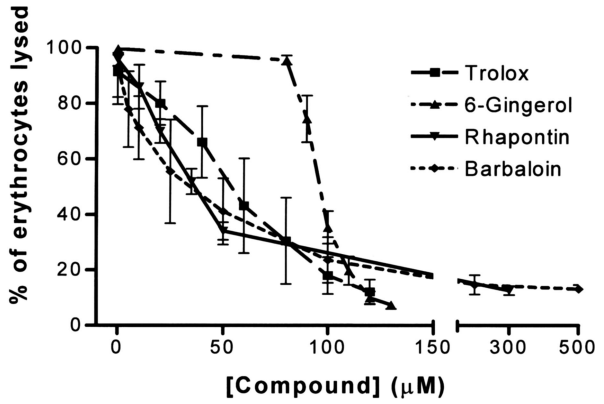

Fig. 4. The inhibitory actions of Trolox, 6-gingerol, rhapontin and barbaloin on AAPH-induced hemolysis. Results are expressed as a percentage of erythrocytes lysed. Data represent mean values ± S.D. from four independent experiments.

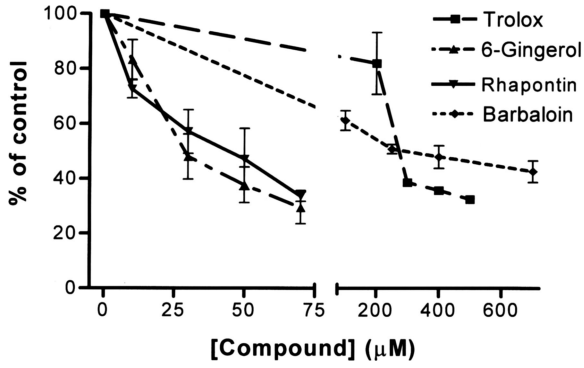

Fig. 5. The inhibitory actions of Trolox, 6-gingerol, rhapontin and barbaloin on lipid peroxidation of erythrocyte membranes. Results are expressed as a percentage of the control. Data represent mean values ± S.D. from four independent experiments.

assays (Table 1). In the hemin-induced LDL lipid peroxidation system (Column B in Table 1), the only assay showing some activity for aloe-emodin, the effectiveness of this compound was found to be much lower than those of the other compounds tested, being more than 100 times less potent than Trolox (data not shown).

The inhibitory actions of the active phenolic compounds against LDL oxidation were studied. Fig. 2 shows the antioxidant actions of 6-gingerol, rhapontin and barbaloin in the AAPH-induced LDL lipid peroxidation assay, compared against Trolox as the positive control. Fig. 3 shows the results in which LDL oxidation was induced by an alternative method, the hemin system. It was observed that the effective concentrations of all the test compounds in the hemin system were about two orders of magnitude lower than those in the AAPH system. This is probably due to the low level of endogenous

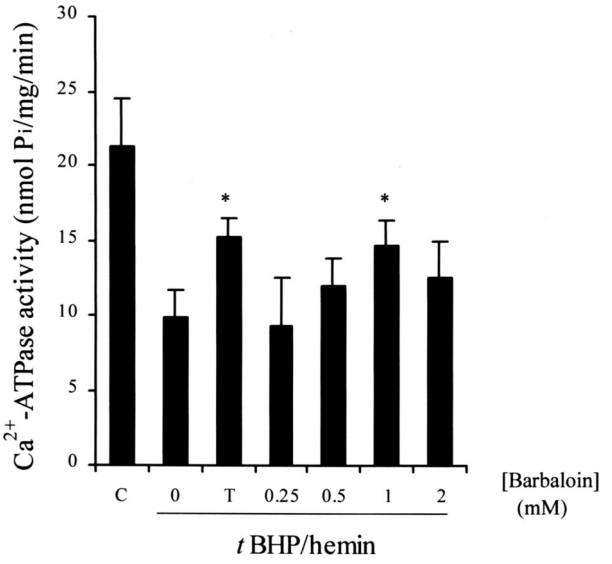

Fig. 6. The protective actions of barbaloin on Ca^{2+}-ATPase activity against oxidative damage in erythrocyte membranes. Results are expressed as enzyme unit in nmol Pi/mg/min. Data represent mean values ± S.D. from four independent experiments. C: control without the test compound, T: 1 mM Trolox. Results are compared against the control with oxidative damage by one-way ANOVA followed by Dunnett's test. *, $P < 0.05$.

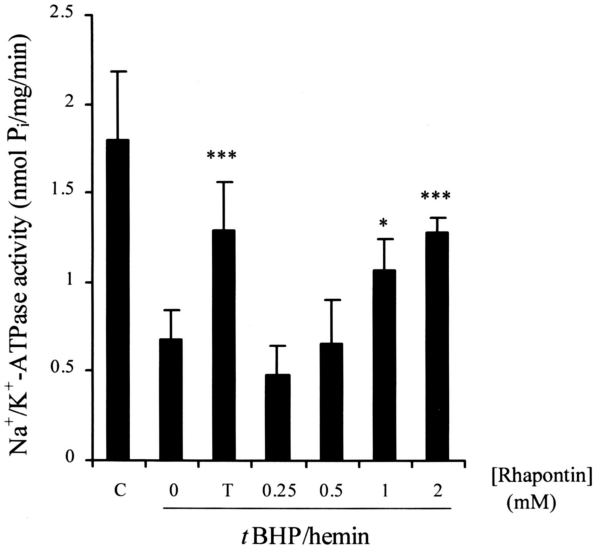

Fig. 7. The protective actions of rhapontin on Na^+/K^+-ATPase activity against oxidative damage in erythrocyte membranes. Results are expressed as enzyme unit in nmol Pi/mg/min. Data represent mean values ± S.D. from four independent experiments. C: control without the test compound, T: 1 mM Trolox. Results are compared against the control with oxidative damage by one-way ANOVA followed by Dunnett's test. *, $P < 0.05$; ***, $P < 0.001$.

lipid peroxides in LDL, and therefore a small amount of antioxidant is sufficient to inhibit lipid peroxidation. The antioxidant actions of the test compounds on AAPH-induced hemolysis are shown in Fig. 4. The lipid peroxidation inhibitory

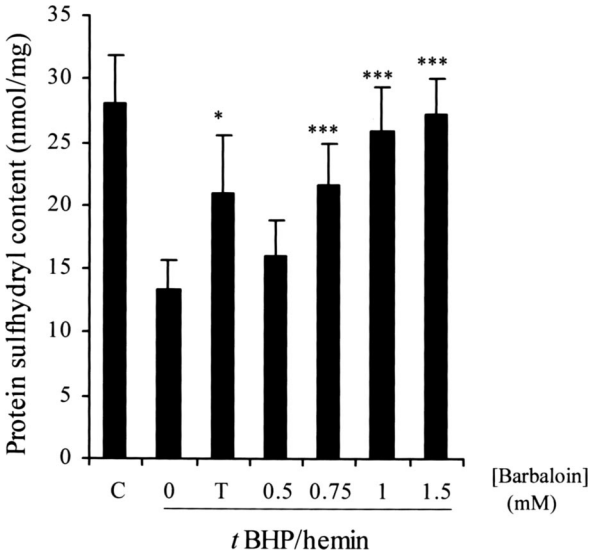

Fig. 8. The protective actions of barbaloin on sulfhydryl groups against oxidative damage in erythrocyte membranes. Results are expressed as nmol SH/mg protein. Data represent mean values ± S.D. from four independent experiments. C: control without the test compound, T: 1 mM Trolox. Results are compared against the control with oxidative damage by one-way ANOVA followed by Dunnett's test. *, $P < 0.05$; ***, $P < 0.001$.

actions of the phenolic compounds on erythrocyte membranes were also investigated (Fig. 5). In order to compare the antioxidant potencies of the phenolic compounds in these four assays, the IC_{50} value of each compound was determined and further transformed into the relative potency as compared with Trolox, and the results are listed in Table 2. These results show that 6-gingerol and rhapontin exhibit stronger antioxidant actions than Trolox in inhibiting lipid peroxidation in all the systems studied while barbaloin possesses a much weaker effect. 6-Gingerol is about two times stronger than rhapontin in inhibiting hemin-induced LDL lipid peroxidation and the hemin/*t*BHP-induced erythrocyte membrane lipid peroxidation. On the other hand however, 6-gingerol exhibits a weaker effect than rhapontin in inhibiting AAPH-induced LDL lipid peroxidation. Rhapontin and barbaloin are comparable in protecting erythrocytes from AAPH-induced hemolysis. They are almost two times more potent than Trolox while 6-gingerol was only about half as potent as Trolox in this assay. These results suggest that 6-gingerol exhibits a strong antioxidant action against lipid peroxidation but this compound possesses little activity in scavenging AAPH-derived radicals. The fact that rhapontin exhibits stronger antioxidant actions than 6-gingerol in all the AAPH-induced oxidation systems suggests that it is a stronger AAPH-derived radical scavenger than 6-gingerol.

Comparing the IC_{50} values of the phenolic compounds in assays A, C and D in Table 2, it is interesting to note that in the AAPH-induced hemolysis inhibition assay (Column C in Table 2), the IC_{50} value of 6-gingerol is about 5 times higher than that

Table 2. Relative Effectiveness of 6-Gingerol, Rhapontin and Barbaloin in Inhibiting Lipid Peroxidation and Hemolysis as Compared with Trolox

Compound	IC_{50} (μM) (Relative potency)			
	A	B	C	D
Trolox	40.9 (1.00)	0.195 (1.00)	59.2 (1.00)	224.4 (1.00)
6-Gingerol	19.1 (2.14)	0.054 (3.61)	95.3 (0.62)	20.7 (10.84)
Rhapontin	14.1 (2.90)	0.104 (1.87)	31.9 (1.86)	38.3 (5.86)
Barbaloin	142.4 (0.29)	2.845 (0.07)	31.7 (1.87)	71.0 (3.16)

The IC_{50} values of the phenolic compounds and Trolox in different assays (Column A: inhibition of AAPH-induced lipid peroxidation in LDL; Column B: inhibition of hemin-induced lipid peroxidation in LDL; Column C: inhibition of AAPH-induced hemolysis; Column D: inhibition of lipid peroxidation of erythrocyte membranes) were calculated from the dose-dependence curves in Fig. 2 to Fig. 5 by Sigmaplot. The relative potency (values shown in parentheses) for each compound was calculated by the IC_{50} of Trolox over the IC_{50} of the test compound.

in assays A and D. In the case of barbaloin, however, the reverse is true in that assay C exhibits the lowest IC_{50} value. A closer look at Fig. 4 shows that there is a dramatic decrease in the percentage of hemolysis from 80 μM of 6-gingerol onwards but there is virtually no protection below 80 μM. This result suggests that 6-gingerol does not directly eliminate AAPH-derived radicals in aqueous solution nor does it prevent the initial attack of AAPH on the erythrocyte membranes. It probably acts by protecting the erythrocytes from lysis by terminating the lipid peroxidation chain reactions. For barbaloin, the lower IC_{50} value in the hemolysis inhibition assay C than the lipid peroxidation inhibition assays A and D (Table 2) suggests that antioxidant mechanisms other than inhibition of lipid peroxidation might contribute to the protection of the erythrocytes from lysis. Considering the lower potency of barbaloin in inhibiting lipid peroxidation, it is possible that protection of membrane proteins is an important mechanism for the action of barbaloin in inhibiting hemolysis.

The protective actions of these phenolic compounds on membrane proteins were thus studied by the ATPase protection assays and sulfhydryl group protection assay. The results show that barbaloin is effective in protecting Ca^{2+}-ATPase (Fig. 6) and protein sulfhydryl groups (Fig. 8) against oxidative attack by *t*BHP/hemin while other phenolic compounds tested have no effects (Columns E and G in Table 1). Moreover, rhapontin protects Na^+/K^+-ATPase from oxidative attack (Fig. 7) in the absence of sulfhydryl group protection (Column F in Table 1). The protective potencies of barbaloin towards Ca^{2+}-ATPase and protein sulfhydryl groups, and of rhapontin towards Na^+/K^+-ATPase, were found to be comparable to that of Trolox (Table 3). The percentage recovery at 1 mM instead of the IC_{50} value was used to calculate the relative potency because the maximal protective effects afforded by Trolox in these assays was observed at 1 mM in the initial studies and the percentage recoveries afforded by the test compounds in some of these assays could not reach 50%. From the results, it could be concluded that protection of protein sulfhydryl groups may be related to the protective actions of barbaloin on Ca^{2+}-ATPase but not the protective actions of rhapontin on Na^+/K^+-ATPase. The ineffectiveness of 6-gingerol in these three assays also suggests that it is a poor *t*BHP-derived radical scavenger.

DISCUSSION

The *ortho*-hydroxyl-methoxyl structure in the phenyl rings of rhapontin and 6-gingerol probably contributes to the high antioxidant activities of the two phenolic compounds, due to the increase in stability of the phenoxyl radical formed by electron delocalization to the adjacent methoxyl group. 6-Gingerol is a hydrophobic molecule due to the presence of a long alkyl chain. This increases its solubility in lipid and the chance of encountering lipid peroxyl radicals in LDL or erythrocyte membranes, thus explaining its strong chain-breaking action in lipid. However, its lipophilicity may also be the cause of its

Table 3. Relative Effectiveness of Rhapontin and Barbaloin in Protecting ATPases and Protein Sulfhydryl Groups in Erythrocyte Membranes as Compared with Trolox

Compound	% Recovery at 1 mM of compound (Relative potency)		
	E	F	G
Trolox	46.5% (1.00)	55.1% (1.00)	52.1% (1.00)
Rhapontin	N.A.	35.4% (0.64)	N.A.
Barbaloin	42.3% (0.91)	N.A.	86.1% (1.65)

The percentage recovery in Ca^{2+}-ATPase activity (Column E), Na^+/K^+-ATPase activity (Column F) or free sulfhydryl group content (Column G) by 1 mM of the phenolic compounds or Trolox was calculated by regarding the difference in the determinants of the control with and without oxidant treatment to be equivalent to 100% recovery. The relative potency (values shown in parentheses) for each compound was calculated by the percentage recovery of the test compound over the percentage recovery of Trolox. N.A. stands for not applicable as the compound is inactive in that assay.

poor scavenging effect on the highly water-soluble AAPH-derived peroxyl radical (Fig. 9) as this radical contains two amine groups. On the other hand the glucosyl moiety increases the hydrophilicity of rhapontin, thus explaining the stronger scavenging activity of rhapontin on the AAPH-derived peroxyl radicals in solution than 6-gingerol.

Lipid peroxidation could be initiated by free radical generating compounds/systems such as AAPH, *t*BHP, Fe^{2+}/H_2O_2 and Fe^{3+}/ascorbate. At the AAPH concentration used in the hemolysis assay, there would be an abundant supply of lipid peroxidation initiators in the system. As a result, the lipid peroxidation chain reactions can still propagate if chain-terminators such as 6-gingerol are present below a certain threshold in the erythrocyte membranes. This is reflected in the requirement of a critical concentration of 6-gingerol to produce an inhibitory action on hemolysis (Fig. 4). On the other hand, antioxidants that could scavenge AAPH-derived radicals in aqueous phase such as rhapontin could reduce the degree of lipid peroxidation by reducing the number of chain initiators in the first place. Thus, a dose-dependent inhibition of hemolysis was evident starting at a low concentration (Fig. 4).

The only structural difference between aloe-emodin and barbaloin is the presence of an extra glucosyl moiety in barbaloin. The huge difference in their antioxidant activities suggests that substitution of this glucosyl group is important for the observed antioxidant activities of barbaloin. Solubility may be one of the factors. The absence of a glucosyl group in aloe-emodin increases the difficulty of dissolving this compound in an aqueous medium. It is possible that the antioxidant actions of aloe-emodin were not fully revealed because of the limitation imposed by its low water solubility. Of particular interest is the identification of 10-hydroxyaloins A and B as the main *in vitro* oxidation products of barbaloin diastereomers [37]. This suggests that the C-10-glucosylation in barbaloin renders the molecule a distinct electron-donating site as compared with its parent molecule aloe-emodin which has a C-10-carbonyl group instead. This may be the reason for a different and, perhaps, an enhanced free radical scavenging property for barbaloin as compared with aloe-emodin as indicated in the present study.

Barbaloin is shown in the present study to inhibit lipid peroxidation (Fig. 5) and to afford protection of protein sulfhydryl groups (Fig. 8) and Ca^{2+}-ATPase (Fig. 6) in erythrocyte membranes challenged with *t*BHP-derived free radicals. This wide spectrum of antioxidant actions may provide the basis for its high potency in inhibiting AAPH-induced hemolysis (assay C in Table 2). It is possible that the scavenging of *t*BHP- or AAPH-derived radicals in the aqueous phase may be involved in the protective mechanisms of barbaloin. An efficient free radical scavenger in aqueous solution is particularly effective in the protection of membranes since its presence could restrict free radical damage on lipids and proteins. The resultant preservation of membrane structure and function is essential for maintaining membrane fluidity and flexibility as well as ionic balance between the intracellular and extracellular compartments. These processes are crucial for the survival of a cell.

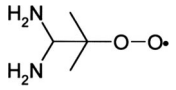

Fig. 9. Chemical structure of peroxyl radical derived from AAPH.

ATPase inhibition by oxidative attack has been suggested to involve mechanisms including protein crosslinking [23], sulfhydryl group oxidation [38,39] and lipid peroxidation [38,40]. From our data, it is conceivable that protection of protein sulfhydryl groups could explain, in part at least, the protective actions of barbaloin on Ca^{2+}-ATPase. However, our data also show that while barbaloin at 1 mM recovers 86% of the sulfhydryl groups in erythrocyte membranes oxidized by 0.5 mM *t*BHP and 10 μM hemin (Fig. 8), the same concentration of barbaloin could only recover 42% of the Ca^{2+}-ATPase activity (Fig. 6) and is completely ineffective in recovering Na^+/K^+-ATPase activity (Column F in Table 1) in the presence of 0.5 mM *t*BHP and 1.5–2 μM hemin. These results suggest that mechanisms other than sulfhydryl group oxidation are involved in the *t*BHP/hemin-induced inactivation of ATPases as this part of inactivation is irrecoverable by barbaloin. Of particular interest is the finding that rhapontin protects Na^+/K^+-ATPase against free radical attack independent of sulfhydryl group protection (Table 3). In addition, although rhapontin inhibits TBARS formation in erythrocyte membranes at micromolar concentrations (Fig. 5), its protective effect on Na^+/K^+-ATPase against the same *t*BHP/hemin system could only be observed at the millimolar range. While we cannot rule out the possibility that the enzyme could be inhibited by lipid-derived oxidants in the absence of TBARS formation, these results suggest that mechanisms other than sulfhydryl group protection and lipid peroxidation inhibition might be involved in the protective effect of rhapontin on Na^+/K^+-ATPase.

Oral administration of ginger, aloe and rhubarb have been reported to produce beneficial effects on experimental animals *in vivo.* Ginger has been demonstrated to decrease levels of lipid peroxidation in rabbits with experimental atherosclerosis [41], in Apo E-deficient mice [42], and in hyperlipidemic rats [43]. *Aloe vera* gel extract has been found to exhibit various antioxidant effects in streptozotocin-induced diabetic animals [44–46] and kainic acid-induced neurotoxicity in mice [47]. Oral treatment with *Aloe vera* leaf juice also significantly decreased malondialdehyde formation, improved superoxide dismutase and catalase activities, and reduced glutathione levels in various tissues of gamma-irradiated rats [48]. *Aloe vera* leaf pulp extract could also increase the hepatic enzyme activities of glutathione S-transferase, DT-diaphorase, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, and decrease hepatic malondialdehyde formation and lactate dehydrogenase activity in normal mice [49]. A rhubarb preparation has been shown to increase the enzyme activities of superoxide dismutase, catalase and glutathione peroxidase in

erythrocytes and to reduce lipid peroxidation in the brain, liver and whole blood of aging mice [50]. Results of the present investigation and other *in vitro* studies [51–57] have attributed these antioxidant activities to the phenolic compounds from these plants. Ginger, aloe and rhubarb are regarded as functional foods in China as well as in some other countries. Their consumption on a long-term basis is considered beneficial to human health. The present study illuminates a practical guide in nutritional medicine that by exploiting the different antioxidant mechanisms of different compounds found in dietary plants, a more complete spectrum of antioxidant protection could be achieved.

CONCLUSION

In summary, the phenolic compounds investigated in the present study exert their antioxidant actions through different mechanisms. 6-Gingerol possesses a strong antioxidant activity in lipid due to its strong chain-breaking activity. Barbaloin is a weak chain-breaking antioxidant against lipid peroxidation but it possesses multiple protective actions on membrane proteins. It is likely a good scavenger of free radicals in aqueous solution. Rhapontin is a good scavenger of AAPH-derived radicals and a good inhibitor of lipid peroxidation. It also possesses a specific protective effect on erythrocyte Na^+/K^+-ATPase independent of sulfhydryl group protection. Their combined use would produce a synergistic effect, suggestive of the therapeutic or prophylactic value of these compounds against ROS-related disorders such as atherosclerosis.

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REFERENCES

1. Gey KF: Prospects for the prevention of free radical disease, regarding cancer and cardiovascular disease. Br Med Bull 49:679–699, 1993.
2. Mayne ST: Antioxidant nutrients and cancer incident and mortality: an epidemiologic perspective. Adv Pharmacol 38:657–675, 1997.
3. Kris-Etherton PM, Hecker KD, Bonanome A, Caval SM, Binkosk AE, Hilpert KF, Griel AE, Etherton TD: Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. Am J Med 113(Suppl 9B):71S–88S, 2002.
4. Croft KD: Antioxidant effects of plant phenolic compounds. In Basu TK, Temple NJ, Garg ML (eds): "Antioxidants in Human Health and Diseases," New York: CABI Publishing, pp 109–121, 1999.
5. Heim KE, Tagliaferro AR, Bobilya DJ: Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J Nutr Biochem 13:572–584, 2002.
6. Gao Z, Huang K, Yang X, Xu H: Free radical scavenging and antioxidant activities of flavonoids extracted from the radix of *Scutellaria baicalensis* Georgi. Biochim Biophys Acta 1472:643–650, 1999.
7. Cheng Z, Ren J, Li Y, Chang W, Chen Z: Establishment of a quantitative structure-activity relationship model for evaluating and predicting the protective potentials of phenolic antioxidants on lipid peroxidation. J Pharm Sci 92:475–484, 2003.
8. Cholbi MR, Paya M, Alcaraz MJ: Inhibitory effects of phenolic compounds on CCl_4-induced microsomal lipid peroxidation. Experientia 47:195–199, 1991.
9. Yokode M, Kita T: LDL isolation and copper-catalysed oxidation. In Taniguchi N, Gutteridge MC (eds): "Experimental Protocols for Reactive Oxygen and Nitrogen Species." New York: Oxford University Press, pp149–151, 2000.
10. Lodge JK, Traber MG, Packer L: Thiol chelation of Cu^{2+} by dihydrolipoic acid prevents human low density lipoprotein peroxidation. Free Radic Biol Med 25:287–297, 1998.
11. Yokozawa T, Dong E: Influence of green tea and its three major components upon low-density lipoprotein oxidation. Exp Toxicol Pathol 49:329–335, 1997.
12. Aaviksaar A, Haga M, Kuzina K, Pussa T, Raal A, Tsoupras G: Hydroxystilbenes in the roots of *Rheum rhaponticum.* Proc Estonian Acad Sci Chem 52:99–107, 2003.
13. Nazemiyeh H, Delazar A, Afshar J, Eskandari B: Isolation, identification and quantitative determination of gingerols in ginger roots. Ulum-i Daroei 2002(2):61–68, 2002.
14. WHO: "WHO monographs on selected medicinal plants. Volume I." Geneva: World Health Organization, p 37, 1999.
15. Parthasarathy S, Rankin SM: Role of oxidized low-density lipoprotein in atherogenesis. Prog Lipid Res 31:127–143, 1992.
16. Hiramatsu K, Rosen H, Heinecke JW, Wolfbauer G., Chait A: Superoxide initiates oxidation of low density lipoprotein by human monocytes. Arteriosclerosis 7:55–60, 1987.
17. Miller YI, Felikman Y, Shaklai N: Hemoglobin induced apolipoprotein B crosslinking in low-density lipoprotein peroxidation. Arch Biochem Biophys 326:252–260, 1996.
18. Grinshtein N, Bamm VV, Tsemakhovich VA, Shaklai N: Mechanism of low-density lipoprotein oxidation by hemoglobin-derived iron. Biochemistry 42:6977–6985, 2003.
19. Hebbel RP, Eaton JW, Balasingam M, Steinberg MH: Spontaneous oxygen radical generation by sickle erythrocytes. J Clin Invest 70:1253–1259, 1982.
20. Jain SK, Williams DM: Reduced levels of plasma ascorbic acid (vitamin C) in sickle cell disease patients: its possible role in the oxidant damage to sickle cells *in vivo.* Clin Chim Acta 149: 257–261, 1985.
21. Natta C, Stacewicz-Sapuntzakis M, Bhagavan H, Bowen P: Low serum levels of carotenoids in sickle cell anemia. Eur J Haematol 41:131–135, 1988.
22. Sato Y, Kamo S, Takahashi T, Suzuki Y: Mechanism of free

radical-induced hemolysis of human erythrocytes: hemolysis by water-soluble radical initiator. Biochemistry 34:8940–8949, 1995.
23. Moore RB, Bamberg AD, Wilson LC, Jenkins LD, Mankad VN: Ascorbate protects against *tert*-butyl hydroperoxide inhibition of erythrocyte membrane $Ca^{2+}+Mg^{2+}$-ATPase. Arch Biochem Biophys 278:416–424, 1990.
24. Soszynski M, Bartosz G: Decrease in accessible thiols as an index of oxidative damage to membrane proteins. Free Radic Biol Med 23:463–469, 1997.
25. Niki E, Komuro E, Takahashi M, Urano S, Ito E, Terao K: Oxidative hemolysis of erythrocytes and its inhibition by free radical scavengers. J Biol Chem 263:19809–19814, 988.
26. Dwight JF, Hendry BM: The effects of tert-butyl hydroperoxide on human erythrocyte membrane ion transport and the protective actions of antioxidants. Clin Chim Acta 249:167–181, 1996.
27. Zhou X, Lin M, Guo Y: Change of activities of adenosine triphosphatase and superoxide dismutase, erythrocyte Na^+, K^+, Ca^{2+} and Mg^{2+}, lipid peroxide in coronary heart disease. Chin J Hypertension 7:24–26, 1999.
28. Matteucci E, Cocci F, Pellegrim L, Gregori G., Giampietro O: Measurement of ATPase in red cells: setting up and validation of a highly reproducible method. Enzyme Protein 48:105–119, 1995.
29. Miller YI, Shaklai N: Oxidative crosslinking of LDL protein induced by hemin: involvement of tyrosines. Biochem Mol Biol Int 34:1121–1129, 1994.
30. Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP, Rice-Evans C: Polyphenolic flavonols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. Arch Biochem Biophys 322:339–346, 1995.
31. Rice-Evans CA, Diplock AT, Symons MC: "Techniques in Free Radical Research." Amsterdam: Elsevier, pp 146–149, 1991.
32. Jimenez I, Garrido A, Bannach R, Gotteland M, Speisky H: Protective effects of boldine against free radical-induced erythrocyte lysis. Phytother Res 14:339–343, 2000.
33. Reinila M, MacDonald E, Salem N Jr, Linnoila M, Trams EG: Standardized method for the determination of human erythrocyte membrane adenosine triphosphatases. Anal Biochem 124:19–26, 1982.
34. Woo AYH, Waye MMY, Kwan HS, Chan MCY, Chau CF, Cheng CHK: Inhibition of ATPases by *Cleistocalyx operculatus.* A possible mechanism for the cardiotonic actions of the herb. Vasc Pharmacol 38:163–168, 2002.
35. Haest CW, Plasa G, Kamp D, Deuticke B: Spectrin as a stabilizer of the phospholipid asymmetry in the human erythrocyte membrane. Biochim Biophys Acta 509:21–32, 1978.
36. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275, 1951.
37. Rauwald HW, Lohse K: Structure revision of 4-hydroxyaloin: 10-Hydroxyaloins A and B as main *in vitro*-oxidation products of the diastereomeric aloins. Planta Med 58:259–262, 1992.
38. Hebbel RP, Shalev O, Foker W, Rank BH: Inhibition of erythrocyte Ca^{2+}-ATPase by activated oxygen through thiol- and lipid-dependent mechanisms. Biochim Biophys Acta 862:8–16, 1986.
39. Shao Q, Matsubara T, Bhatt SK, Dhalla NS: Inhibition of cardiac sarcolemma Na^+/K^+-ATPase by oxyradical generating systems. Mol Cell Biochem 147:139–144, 1995.
40. Thomas CE, Reed DJ: Radical-induced inactivation of kidney Na^+,K^+-ATPase: Sensitivity to membrane lipid peroxidation and the protective effect of vitamin E. Arch Biochem Biophys 281:96–105, 1990.
41. Verma SK, Singh M, Jain P, Bordia A: Protective effect of ginger, *Zingiber officinale* Rosc on experimental atherosclerosis in rabbits. Indian J Exp Biol 42:736–738, 2004.
42. Fuhrman B, Rosenblat M, Hayek T, Coleman R, Aviram M: Ginger extract consumption reduces plasma cholesterol, inhibits LDL oxidation and attenuates development of atherosclerosis in atherosclerotic, apolipoprotein E-deficient mice. J Nutr 130:1124–1131, 2000.
43. Liu N, Huo G, Zhang L, Zhang X: Effect of *Zingiber officinale* Rosc on lipid peroxidation in hyperlipidemia rats. Wei Sheng Yan Jiu 32:22–23, 2003.
44. Rajasekaran S, Sivagnanam K, Subramanian S: Modulatory effects of *Aloe vera* leaf gel extract on oxidative stress in rats treated with streptozotocin. J Pharm Pharmacol 57:241–246, 2005.
45. Rajasekaran S, Sivagnanam K, Subramanian S: Antioxidant effect of *Aloe vera* leaf gel extract in streptozotocin-induced diabetes in rats. Pharmacol Rep 57:90–96, 2005.
46. Parihar MS, Chaudhary M, Shetty R, Hemnani T: Susceptibility of hippocampus and cerebral cortex to oxidative damage in streptozotocin treated mice: prevention by extracts of *Withania somnifera* and *Aloe vera.* J Clin Neurosci 11:397–402, 2004.
47. Parihar MS, Hemnani T: Phenolic antioxidants attenuate hippocampal neuronal cell damage against kainic acid induced excitotoxicity. J Biosci 28:121–128, 2003.
48. Saada HN, Ussama ZS, Mahdy AM: Effectiveness of *Aloe vera* on the antioxidant status of different tissues in irradiated rats. Pharmazie 58:929–931, 2003.
49. Singh RP, Dhanalakshmi S, Rao AR: Chemomodulatory action of *Aloe vera* on the profiles of enzymes associated with carcinogen metabolism and antioxidant status regulation in mice. Phytomedicine 7:209–219, 2000.
50. Yao G, Zhang B, He L, Hao Y: Antioxidant effects of *Rheum palmatum* polysaccharides. Chinese Archives of Traditional Chinese Medicine 22:1295–1311, 2004.
51. Kikuzaki H, Nakayani N: Antioxidant effects of some ginger constituents. J Food Sci 58:1407–1410, 1993.
52. Aeschbach R, Loliger J, Scott BC, Murcia A, Butler J, Halliwell B, Aruoma OI: Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. Food Chem Toxicol 32:31–36, 1994.
53. Malterud KE, Farbrot TL, Huse AE, Sund RB: Antioxidant and radical scavenging effects of anthraquinones and anthrones. Pharmacology 47:S1:77–85, 1993.
54. Yen GC, Duh PD, Chuang DY: Antioxidant activity of anthraquinones and anthrone. Food Chem 70:437–441, 2000.
55. Yuan Z, Gao R: Antioxidant actions of anthraquinolines contained in *Rheum.* Pharm Pharmacol Lett 7:9–12, 1997.
56. Matsuda H, Morikawa T, Toguchida I, Park JY, Harima S, Yoshikawa M: Antioxidant constituents from rhubarb: structural requirements of stilbenes for the activity and structures of two new anthraquinone glucosides. Bioorg Med Chem 9:41–50, 2001.
57. He BF, Chen QH: Biochemical study of Chinese rhubarb. XXIV. Inhibitory effects of anthraquinonen derivatives on some flavin-linked enzymes. Acta Biochim Biophys Sin 21:72–77, 1989.

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