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Comparison of quercetin and dihydroquercetin: Antioxidant-independent actions on erythrocyte and platelet membrane

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ABSTRACT

We investigated the effects of two flavonoids quercetin and dihydroquercetin (DHQ), which have different solubilities and antioxidant capacities, on hemolysis and platelet aggregation in human blood. Exposure of human red blood cells (RBCs) to free radicals generated by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) for 2 h resulted in 63.5 ± 3.9% hemolysis (vehicle: 0.3 ± 0.4%). Pre-incubation of RBCs with lipid-soluble quercetin and water-soluble DHQ for 30 min significantly reduced the AAPH-induced hemolysis to 3.6 ± 1.5% and 32.5 ± 5.6% respectively. In contrast, quercetin and DHQ were similarly effective in reducing phospholipase C-induced hemolysis (37.2 ± 9.1% and 45.4 ± 10.0% versus vehicle 75.7 ± 5.2%, $P < 0.001$). Pre-incubation with quercetin, but not DHQ, inhibited the aggregation of platelets by adenosine diphosphate. DHQ was more potent than quercetin in inhibiting superoxide produced by xanthine oxidase. These results suggest that the antihemolytic effects of flavonoids may not be directly mediated by removal of free radicals and may likely be due to their interaction with cell membrane.

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1. Introduction

Flavonoids, compounds found in most plant species, are known to have a variety of beneficial biological effects [1], including protection of cells against oxidative stress, an action attributed to their antioxidant properties [2–4]. One of the potential hematologic benefits of flavonoids is to stabilize cell membranes by reducing lipid peroxidation and scavenging free radicals [2,5]. Erythrocytes are especially susceptible to oxidation due to their high lipid content and their rich oxygen supply and transition metals such as iron and copper [6,7]. Reactive oxygen species generated in plasma, cell membrane, or intracellularly can attack erythrocyte membranes, compromise cell integrity, and induce oxidation of lipids and protein, which results in hemolysis [6,8–10]. Increasing evidence suggests that flavonoids protect cell not only by buffering free radicals but also by altering cell membrane properties [2,11]. Certain flavonoid compounds may incorporate into the hydrophobic core of the membrane bilayer, causing a reduction in membrane fluidity and membrane stability [2]. This reduced membrane fluidity may limit diffusion of free radicals and improve the antioxidative effectiveness of flavonoids. Thus, direct interactions of flavonoids with cell membranes may alter their antioxidative and membrane stabilizing effectiveness. How flavonoid solubility affects cell membrane stability has yet to be tested. Also, the role of flavonoid antioxidant activity in cell membrane protection remains unclear.

The objective of the present study was to investigate whether the cell membrane action of selected flavonoids requires a lipophilic property and is independent on antioxidant capacity. We compared two flavonoid compounds, lipid-soluble quercetin and water-soluble dihydroquercetin (DHQ), for their effects on hemolysis of human red blood cells (RBCs). Hemolysis was induced by 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), a free radical generator, as well as phospholipase C (PLC), a hemolytic enzyme, to determine if antihemolytic action of flavonoids is a specific activity against free radicals. To further investigate potential antioxidant-independent membrane effect of flavonoids, we tested the effects of quercetin and DHQ on platelet aggregation induced by adenosine diphosphate (ADP).

2. Materials and methods

2.1. Chemicals and solutions

All chemicals and phosphate buffered saline (PBS, pH 7.4) were purchased from Sigma–Aldrich Corp. (St. Louis, MO). Stock solutions of quercetin and DHQ were prepared weekly in mixed dimethyl sulfoxide (DMSO) and PBS and kept at 4 °C. All other chemicals were prepared fresh in PBS. Quercetin has low water solubility, 50 μM in PBS [12], whereas the solubility of DHQ is ~8 mM in PBS. In contrast, the solubility of quercetin in DMSO is high: ~550 mM.

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2.2. Preparation of RBCs and platelets

All the procedures were approved by the Institutional Review Board of Uniformed Services University of the Health Sciences. Blood samples were obtained from adult volunteers who had consented to provide blood samples. Blood was collected in EDTA tubes and centrifuged for 15 min at 3000 rpm and 4 °C (Thermo IEC Centra CL3R). Plasma was removed and the buffy coat was discarded carefully by aspiration. RBCs were washed three times with a cold PBS and were finally suspended in PBS to obtain a hematocrit of approximately 20%. The samples were stored at 4 °C and used within 3 h.

Platelet-rich plasma (PRP) was prepared by a two-syringe technique described by Jaffe and Deykin [13]. Whole blood was transferred to plastic centrifuge tubes containing 0.5 ml of 3.8% trisodium citrate. The anti-coagulated blood was centrifuged at room temperature at 180 × *g* for 10 min. Plasma was carefully transferred to a plastic tube, stored at 4 °C and tested within 3 h.

Quercetin and DHQ were added to RBCs or PRP at a final concentration of 10 μM containing 0.05% DMSO. RBCs and PRP were incubated at 37 °C for 30 min. The flavonoids or vehicle-treated RBCs and PRP were centrifuged at 1000 rpm for 5 min and at 3000 rpm for 15 min (Eppendorf Centrifuge 5418). The supernatants were removed and discarded. The final pellets were resuspended with PBS to their original volumes for immediate measurements.

2.3. Measurement of hemolysis

Hemolysis was induced by AAPH and PLC separately. AAPH (50 mM) and PLC (10 mU/ml) were added to sample solutions immediately before measurement. AAPH decomposes in aqueous solution to generate free radicals at a steady rate for an extended period (half life 175 h) [3]. The samples ($n = 6$ for each of AAPH and PLC tests) were continuously assessed in triplicate using a Tecan Sunrise microplate absorbance reader (baseline optical density, OD, was adjusted to ~0.8). Hemolysis was measured as turbidity reduction (OD at 620 nm) at 37 °C [14,15]. In addition, a stop time assay was used to determine the amount of hemoglobin released from the lysed RBCs ($n = 5$ per group). As described above, RBCs samples were processed through incubation, centrifugation, supernatant removal and resuspension. Subsequently, AAPH was added to RBCs incubated at 37 °C. The sample solutions were taken and centrifuged immediately and at 2 h after addition of AAPH. The supernatants ($n = 5$) were measured at 405 nm [16]. The hemolytic values were calculated using that of RBCs resuspended with distilled water and AAPH as 100%. Similarly, PLC was added to the RBCs following resuspension and the samples were continuously monitored in a microplate at 620 nm and 37 °C.

2.4. Measurement of platelet aggregation

Platelet aggregation, carried out at 37 °C with 200 μl of PRP added to each well of a 96-well microplate [17], was initiated by adding ADP (final concentration: 10 μM). Transmittance was read at 609 nm [13] each minute for at least 15 min. The aggregation in the microplate was facilitated by shaking agitation for 45 s between transmittance readings. Some samples from one subject were also compared using a platelet aggregometer (Chrono-Log Corp.). All the experiments were carried out in duplicate. The rates of platelet aggregation (slopes) were compared.

2.5. Assessment of antioxidant activity of flavonoids

Quercetin and DHQ were evaluated for their efficacy in scavenging superoxide generated by xanthine plus xanthine oxidase in PBS [18]. Lucigenin (final concentration 5 μM)-enhanced chemiluminescence was used to determine the generation of superoxide anion. The oxidase reaction was initiated by adding 100 μM (final concentration) of xanthine to the sample solutions, which contained xanthine oxidase (350 mU/ml) and various concentrations of quercetin or DHQ in a tube luminometer with a built-in injector (Berthold AutoLumat Plus LB 953). The background-adjusted chemiluminescence signal was continuously recorded at 2 Hz and 21 °C for 3 min, and area under the curve (AUC) was used for comparisons.

Furthermore, quercetin and DHQ were tested for their effects on AAPH-generated radicals in the presence of plasma. The reaction solutions contained 10% plasma in PBS, 50 mM AAPH, 50 μM pyranine (a radical scavenger) and 10 μM quercetin or DHQ. AAPH-derived free radicals were measured as consumption of pyranine, which was followed at 454 nm and 37 °C [19]. The reaction delay (lag) was calculated for comparison.

2.6. Data processing and statistical analysis

Data are reported as mean ± SEM. The half effective concentrations (EC50) of quercetin and DHQ inhibiting superoxide chemiluminescence were obtained in each of the six samples using non-linear regression. Data were analyzed by using paired *t*-test or one-way ANOVA with Bonferroni post hoc test. The results were considered significant at $P \leq 0.05$.

3. Results

3.1. Effects of quercetin and DHQ on hemolysis induced by AAPH or PLC

The hemolysis induced by AAPH was tested by measuring hemoglobin at 405 nm. In the absence of AAPH, RBCs were stable and no significant hemolysis was detected after 2 h incubation (Table 1). When incubated with AAPH, about 63% of hemolysis was observed at 2 h (less than 1% at 0 h). Both quercetin and DHQ significantly inhibited hemolysis induced by AAPH. However, quercetin was more effective than DHQ. Quercetin reduced more than 90% of the hemolysis induced by AAPH, while DHQ reduced hemolysis by only ~50%.

The hemolytic activity of AAPH was also followed continuously at 620 nm to verify changes in the turbidity of RBCs. Changes in RBCs occurred immediately following addition of AAPH. As shown in Fig. 1, RBCs underwent an initial increase followed by irreversible loss of cloudiness of suspended RBCs. This biphasic response was not observed in the vehicle-treated RBCs. Reductions in AUC (dashed rectangular area in Fig. 1) were 19.1 ± 1.0%, 11.9 ± 1.1% and 21.3 ± 1.2% for vehicle, quercetin and DHQ respectively ($P < 0.01$, quercetin versus vehicle or DHQ, $n = 6$). Thus, the hemolytic process was delayed significantly in RBCs that were pre-incubated with quercetin for 30 min. Interestingly, unlike the results of the hemoglobin tests (Table 1), pre-incubation with DHQ was ineffective for the action of AAPH on RBCs. This discrepancy indicates that despite similar degrees of turbidity reduction, the

Table 1
Percent hemolysis within 2 h of incubation with AAPH.

	0 h	2 h	*P*-Value
Control	0.19 ± 0.25	0.32 ± 0.41	>0.05
AAPH	0.45 ± 0.32	63.47 ± 3.85*	<0.0001
AAPH + quercetin	0.39 ± 0.29	3.61 ± 1.49*,#,†	<0.001
AAPH + DHQ	0.33 ± 0.29	32.5 ± 5.62*,#	<0.0001

* $P < 0.001$, compared to control ($n = 5$).
\# $P < 0.01$, compared to AAPH ($n = 5$).
† $P < 0.01$, compared to AAPH + DHQ ($n = 5$).

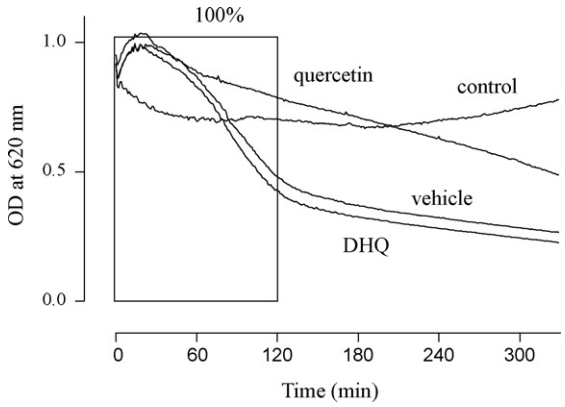

Fig. 1. Representative tracings showing the effects of quercetin and DHQ on lysis of RBCs by AAPH. Data were collected in a single experiment at 37 °C with intermittent shacking. After 30 min pre-incubation, quercetin or DHQ was removed before measurements. Recoding started immediately following addition of AAPH.

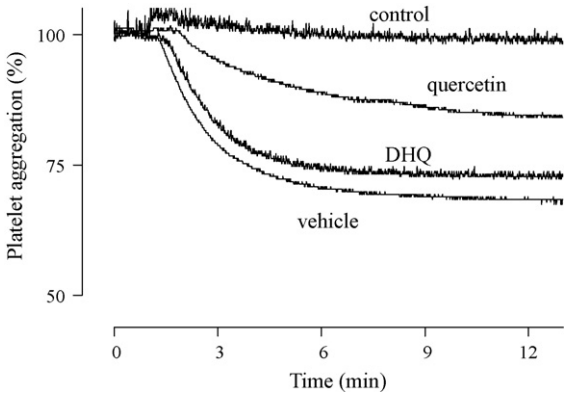

Fig. 3. Representative tracings showing the effects of quercetin and DHQ on platelet aggregation induced by ADP. Samples were obtained from a single subject and data were collected at 37 °C using a platelet aggregometer. After 30 min pre-incubation, quercetin or DHQ was removed before measurements.

DHQ-treated RBCs lost less hemoglobin content than the vehicle-treated ones during AAPH exposure.

To determine if the antihemolytic action was reflective of free radicals quenchings, quercetin and DHQ were further tested by examining hemolysis induced by PLC. Two hours of incubation with PLC caused 75.7 ± 5.2% (n = 6) hemolysis determined by changes in transmittance at 620 nm (Fig. 2). Pre-incubation with quercetin and DHQ significantly reduced hemolysis to 37.2 ± 9.1% and 45.4 ± 10.0% respectively (n = 6 per group, P < 0.01). No significant difference between quercetin and DHQ was found.

3.2. Effects of quercetin and DHQ on activation of platelets induced by ADP

Platelets were washed with PBS before the platelet aggregation tests. Thus, activation of platelets by ADP was performed in a plasma-free media, a non-optimal environment. At 10 μM, ADP caused slightly more than 20% platelet aggregation within 15 min when a microplate reader was used. Similar results were obtained using a platelet aggregometer (Fig. 3). When rates of platelet aggregation (reduction of OD at 609 nm) were compared, quercetin, but not DHQ, showed a significant inhibitory effect on platelet activation by ADP (−0.0036 ± 0.0011 versus vehicle −0.0061 ± 0.0013 and DHQ −0.0051 ± 0.0019, P < 0.05, n = 7). Ten minutes after addition of ADP, platelet aggregation reached 18.6 ± 2.1% in the vehicle-treated samples. The percentage of platelet aggregation was reduced in the quercetin-treated (9.5 ± 2.3%, P < 0.001), but not the DHQ-treated samples (16.6 ± 2.9%, P > 0.05).

3.3. Antioxidant capacities of quercetin and DHQ in PBS and plasma

When tested in PBS, both quercetin and DHQ produced a concentration-dependent inhibition on superoxide generated by xanthine oxidase, measured by lucigenin chemiluminescence (Fig. 4). However, DHQ was more potent than quercetin (EC50: 17.4 ± 3.6 μM versus 70.8 ± 19.3 μM, P < 0.0001). These two compounds were further compared for their effectiveness as an antioxidant against free radicals generated by AAPH. Pyranine was used as a probe and followed at 454 nm. In PBS, pyranine was consumed at a constant rate without any lag phase (Fig. 5A). As a result, a greater increase in AUC was noted for DHQ than quercetin in PBS (DHQ 17.3 ± 2.7% versus quercetin 11.6 ± 2.5%, P < 0.001) as compared to vehicle. The addition of human plasma produced a long lag phase, apparently because the endogenous antioxidants contained in plasma scavenged free radicals (Fig. 5B). Both quercetin and DHQ caused a significant delay in consumption of pyranine in PBS (Table 2). This inhibition of pyranine consumption on AAPH-generated free radicals was greater with DHQ than with quercetin. However, neither quercetin nor DHQ produced any further delay in consumption of pyranine in the presence of 10% plasma.

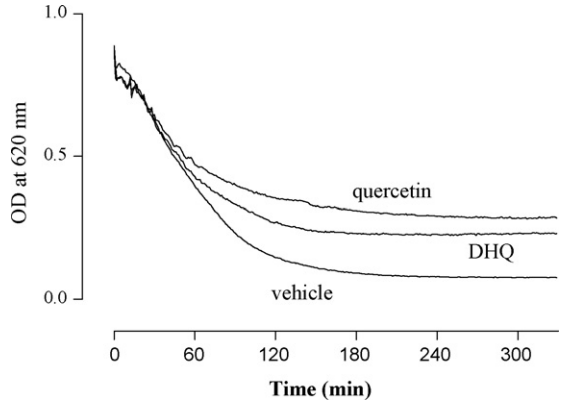

Fig. 2. Representative tracings showing the effects of quercetin and DHQ on lysis of RBCs by PLC. Data were collected in a single experiment under same conditions in Fig. 1. Recoding started immediately following addition of PLC.

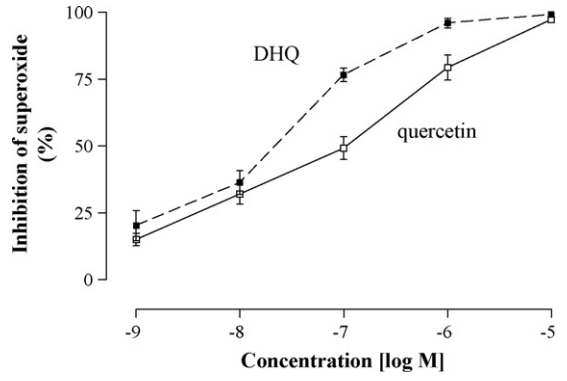

Fig. 4. Concentration-dependent inhibition of quercetin and DHQ on superoxide generation by xanthine oxidase. The experiments were conducted in PBS at 21 °C. Percent inhibition was calculated based on reductions in lucigenin chemiluminescnce relative to vehicle control.

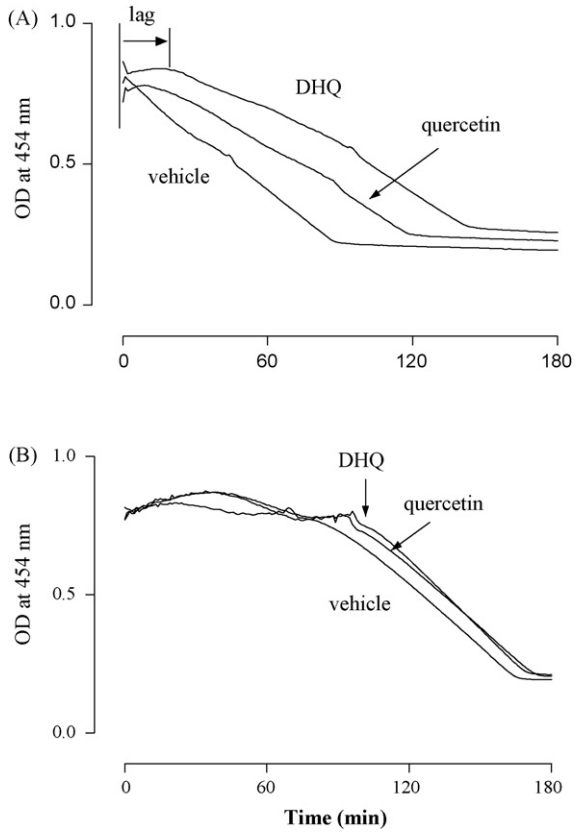

Fig. 5. Representative tracings showing the effects of quercetin and DHQ on consumption of pyranine by AAPH-generated free radicals in PBS (A) or in plasma (B). Data were collected in a single experiment under same conditions in Fig. 1. Recoding started immediately following addition of AAPH.

4. Discussion

The data from the present study extend previous studies in flavonoid research. We confirmed that flavonoid protection of RBCs may not be directly associated with antioxidant action. This is supported by several findings. First, quercetin, the less potent antioxidant, provided better protection against hemolysis induced by free radicals than DHQ, the more potent antioxidant. Second, quercetin and DHQ reduced lysis of RBCs caused by PLC, a nonoxidative hemolytic enzyme. Third, quercetin and DHQ inhibited ADP-induced platelet aggregation, which did not involve free radicals.

The findings from the present study also provide information to help potential clinical applications of flavonoids. Our results indicate the antioxidant capacity of blood is high in healthy adults. A solution containing only 10% plasma shows sufficient antioxidant capacity that could not be further enhanced by adding quercetin or DHQ (Fig. 5). These findings suggest that an antioxidant supplement may be needed or effective only when endogenous antioxidants become insufficient, such as under extreme conditions such as oxidative stress [4]. Also we found that the hematological effects can obtained via brief exposure to relatively low concentrations of flavonoids. The effective dose (10 μM) tested in the present study is comparable to what might be expected and achievable *in vivo*, as plasma concentration of quercetin can be maintained >10 mM through oral administration [20].

Table 2
Lag time (min) in consumption of pyranine.

	Vehicle	Quercetin	DHQ
PBS	1.7 ± 0.4	11.5 ± 1.4*	21.8 ± 2.6*,#
10% plasma	71.6 ± 5.2	78.2 ± 11.6	79.9 ± 10.1

* $P < 0.01$, compared to vehicle ($n = 6$).
\# $P < 0.01$, compared to quercetin ($n = 6$).

At a physiological temperature in aqueous solutions, AAPH decomposes steadily to generate peroxyl radicals. These peroxyl radicals induce oxidation of polyunsaturated lipids in RBC membranes to cause lipid peroxidation. As a result of lipid peroxidation, the RBC membrane undergoes damage quickly and losses its integrity, which leads to the release of hemoglobin or hemolysis [3]. Plant antioxidants have been shown to protect RBCs against hemolysis induced by AAPH. Banerjee et al. reported that treating RBCs with curcumin inhibits AAPH-induced hemolysis, as evidenced by reduced release of hemoglobin and formation of thiobarbituric acid reactive substances [3]. Similarly, Zhu et al. reported that treating rat erythrocytes with a cocoa extract containing flavanoid inhibits hemolysis induced by AAPH *in vitro* [21]. Furthermore, these investigators showed that hemolysis resistance to AAPH is enhanced in RBCs following feeding (gastric infusion) rats with the cocoa extract. The investigators attributed the antihemolytic actions of these plant-derived compounds to their antioxidant property. Our findings on an antihemolytic effect are consistent with these studies. But, our results suggest that flavonoids may reduce hemolytic damage by altering the cell membrane of RBCs, rather than by directly scavenging peroxyl radicals.

This antioxidant-independent antihemolytic action of flavonoids has also been supported by other studies. For example, flavonoids are reported to stabilize RBC membranes and inhibit hemolysis of RBCs in hypotonic solutions [4,11]. Most recently, flavonoids have been shown to reduce hemolysis of RBCs induced by a combination of heat and hypotonic exposure [22]. In the present study, we extended these findings by showing that quercetin and DHQ inhibited lysis of RBCs caused by PLC, which is known to hydrolyze phospholipids at the cell membrane.

We further verified the antioxidant-independent action of flavonoids by monitoring platelet aggregation. Previous studies have shown that consumption of flavonoid-rich drinks such as purple grape juice [23] and red wine [24] prolongs bleeding times and decreases platelet aggregation in humans and animals. Quercetin-rich compounds have been shown to inhibit platelet aggregation induced by collagen [25] and ADP [26]. We demonstrated that the lipid-soluble quercetin, but not water-soluble DHQ, indeed inhibits activation of platelet aggregation by ADP. These results suggest that quercetin may affect platelet function by altering the ADP-binding site.

It remains unclear why the lipid-soluble quercetin is more effective on RBCs and platelets but less potent in PBS, compared to the water-soluble DHQ. The antioxidant activity of the two flavonoids is associated with two aromatic rings (Fig. 6) [27], which actively scavenge free radicals. Adjacent hydroxyl groups in positions 3′ and 4′ are believed to be more effective for antioxidant activity in the flavonol class than groups in the 2′ and 4′ positions [28]. Quercetin and DHQ share these groups in the same positions. However, between C2 and C3, quercetin has the double bond, while DHQ has the single bond. Thus, quercetin and DHQ are formed in planar and nonplanar structures respectively. The unique formations of these two flavonoids may contribute to differences in antioxidant activity and solubility. Quercetin, which has poor solubility in water and high lipophilicity, encourages selective partition into hydrophobic regions [4] or alters lipid–protein components [11] of the RBC membrane. In contrast, the water soluble, lipophobic DHQ can easily move through the cell membrane. Thus, the presence of DHQ in the membrane region is likely limited after incubation and resuspension. The difference in membrane distribution between these two compounds may explain the antihemolytic and antiaggregatory effectiveness seen *in vitro*.

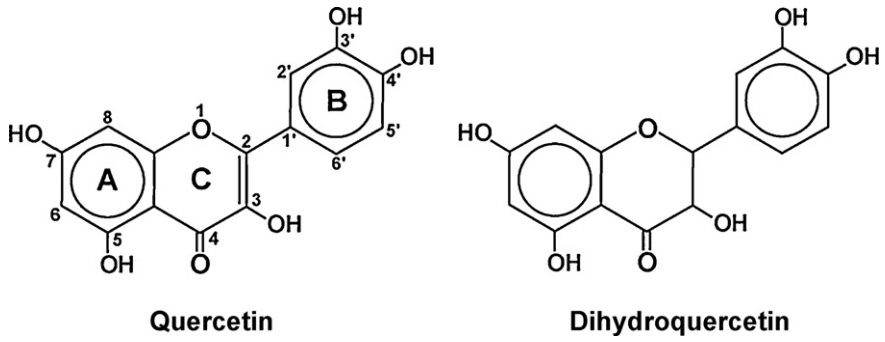

Fig. 6. Structures of quercetin and dihydroquercetin.

It is interesting to note that pre-incubation with DHQ reduced hemolysis when measured by the hemoglobin content in the solution (Table 1), but not when measured by the turbidity reduction (Fig. 1). One potential explanation offered for this inconsistency may be that during exposure to AAPH, the DHQ-treated RBCs lost their cellular fluid, but not hemoglobin content. That is, DHQ was able to maintain partial membrane integrity by allowing loss of fluid, but not hemoglobin from RBCs, whereas quercetin was able to keep both contents in RBCs. Technically, it is difficult to verify DHQ-induced semi-permeability in cell membrane, but we are confident of our major finding that the lipid-soluble quercetin confers better hematological benefits.

In summary, the present study demonstrates that the lipid-soluble quercetin is more effective for protecting RBCs against oxidative and enzymatic damages than the water-soluble DHQ. Also quercetin, but not DHQ, inhibits ADP-induced platelet activation. Our results suggest these actions are likely mediated through flavonoids interacting with cell membranes by, not through scavenging free radicals. These membrane effects may persist following removal of flavonoids. The membrane interactions may make lipid-soluble flavonoids a more favorable choice than water-soluble ones. Further studies will be required to determine whether administration of quercetin may be a potential preventive or therapeutic treatment for various disease states associated with hemolysis and platelet activation or thrombosis.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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