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COMPARATIVE FUNCTION-STRUCTURAL ANALYSIS OF ANTIPLATELET AND ANTIRADICAL ACTIVITIES OF FLAVONOID PHYTOCHEMICALS

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ABSTRACT

The antiplatelet and antiradical activities of 8 dietary flavonoid phytochemicals were investigated and compared. Quercetin, rutin, isoquercitrin, hesperetin, naringenin, hesperidin, naringin and icariin inhibited ADP-induced rat platelet aggregation by 68.33±2.43%, 55.34±2.09%, 52.27± 4.65%, 50.80±7.03%, 33.07±2.09%, 31.28±4.65%, 22.91±1.68% and 20.94±3.20%, respectively. Only quercetin, rutin and isoquercitrin showed clear scavenging HO· radical activity. Function-structure analysis indicated 1) flavonoids with more free phenolic hydroxyl groups showed relative higher antiplatelet and antiradical activities; 2) substitution of 7-OH of A ring affected antiplatelet capacities of flavanones apparently; 3) free 3-OH of C ring was important for both the antiplatelet and antiradical activities of flavonol (quercetin) and flavones (rutin, isoquercitrin); 4) C ring C_2-C_3 double bond and B ring 3'-4'orthodihydroxy might be important for its antiradical function. This study provides certain clues for evaluating a favorable dietary flavonoid drugs from our foods in prevention and treatment of related diseases associated with platelet aggregation and hyroxyl radical oxidation.

Keywords: Flavonoids, Antiplatelet, Antiradical, Function, Structure.

INTRODUCTION

Cardiovascular disease has become markedly prevalent by threatening human health seriously. Platelets play important roles in cardiovascular disease (Dohadwala and Vita 2009; Hodgson and Croft 2010). Platelets are anucleate discoid-shaped cell fragments derived from the cytoplasm of bone marrow megakaryocytes, playing a pivotal role in hemostasis and thrombosis (Guerrero *et al*., 2005; Hubbard *et al*., 2003; Wright *et al*., 2010). Upon vascular damage, subendothelium macromolecules such as collagen, thrombin, ADP, serotonin (5-HT) and thromboxane A_2 (TxA_2) are exposed or secreted at the site of damage, and platelets adhere to exposed subendothelium, which are activated and leads to hemostasis (Hubbard *et al*., 2004). Inefficient regulation of platelet activation leads to thrombosis, resulting in stroke, coronary artery and myocardial infarction associated diseases (Bucki *et al*., 2003). Anti-platelet therapy is an important aspect of cardiovascular disease prevention. However, few drugs currently available due to their high toxicity and apparent side effects. Extensive studies indicated that dietary flavonoids showed significant effects on platelet aggregation (Guerrero *et al*., 2005; Wright *et al*., 2010).

Free radicals are reactive oxygen species (ROS) with unpaired electron, including superoxide anion ($O\cdot_2^-$), hydroxyl radical (HO·), nitric oxide (NO·), hypochlorite ion (ClO^-) and peroxynitrite ($ONOO^-$) (Ak and Gulcin 2009; Li *et al*., 2011; Ningappa *et al*., 2008; Prakash *et al*., 2007; Renuka Devi and Arumughan 2007). ROS readily attack and induce oxidative damage to proteins, lipids, nucleic acids, DNA and carbohydrates (Gulcin *et al*., 2010; Kalaivani and Mathew 2009; Wang *et al*., 2007). The oxidative damages caused by free radicals leading to cancer, inflammation, coronary heart diseases, atherosclerosis, rheumatism, cataracts and neurodegenerative disorders (Kyung *et al*., 2008; Silici *et al*., 2010). Antioxidant can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidation chain reactions, counteracting the damaging effects of oxidation, and therefore, limiting the risk of various degenerative diseases associated with oxidative stress (Khoo *et al*., 2010).

Flavonoids are a class of polyphenol phytochemicals widely distributed in nature (especially in dietary foods) with antiplatelet, antioxidant, antiradical, anticarcinogenic, antiviral, antimicrobial, antithrombotic, antiinflammatory and antimutagenic activities (Cavia-Saiz *et al*., 2010; Macready *et al*., 2009). The intake of dietary flavonoid phytochemicals could decrease the incidence and mortality of cancer, coronary artery and myocardial infarction associated diseases (Khoo *et al*., 2010; Kalgaonkar *et al*., 2010; Mulvihill and Huff 2010). Flavonoids are divided into flavonol, flavone, isoflavone, flavanone, flavanol and anthocyanidin depending on their molecular substitutes. The polyphenolic flavonoids share a basic 15-carbon skeleton core structure (represented as C_6-C_3-C_6) consisting of two phenylbenzene (chromanol) rings linked through a pyran ring. Their functions are potentially affected by their individual structures, their

hydroxylation patterns as well as their functional groups glycosylated and/or alkylated (Khoo *et al*., 2010; Rao *et al*., 2010; Whitman *et al*., 2005). Flavonoids show antiplatelet effects attributable by the inhibition of platelet activation, 5-HT secretion (Guerrero *et al*., 2005; Wright *et al*., 2010), phospholipase C activation, Ca^{2+} influx, internal Ca^{2+} release (Pignatelli *et al*., 2000), protein kinase C (PKC) activation (Hodgson and Croft 2010), actin polymerization (Bucki *et al*., 2003), platelet-derived nitric oxide (NO) enhancement (Carusio *et al*., 2008), TxA_2 receptor antagonism (Hubbard *et al*., 2003; Navarro-Núñez *et al*., 2009) and blunting hydrogen peroxide (H_2O_2) or ROS production (Pignatelli *et al*., 2000). The free radicals scavenging activity of flavonoids is mainly attributed to their redox properties, which allow them to act as reducing agents, hydrogen donors and quenchers of singlet oxygen (Li *et al*., 2011; Gulcin2010).

In this study, we systemically investigated the antiplatelet and antiradical activities of 8 dietary flavonoid phytochemicals that are rich in our daily foods and fruits with similar structures on ADP-induced platelet aggregation and HO· radical scavenging and explored the association between flavonoids' structures and their antiplatelet/antiradical activities. The results indicated that their antiplatelet and antiradical activities were closely related to their intrinsic structures. Minor modifications and/or arrangements of the functional groups of flavonoids may apparently affect their antiplatelet and antiradical activities. Current work provided certain clues for evaluating the contribution impacts of functional groups of flavonoids on antiplatelet aggregation and free hydroxyl radical scavenging, which might also help in selecting effective and favorable dietary flavonoid drugs in the prevention and treatment of related diseases.

MATERIALS AND METHODS

Materials and instruments: Quercetin, isoquercitrin, rutin, hesperetin, hesperidin, naringenin, naringin and icariin were generous gifts from Prof. Hongshan Yu at the School of Bioengineering, Dalian Polytechnic University, Dalian, China. Adenosine diphosphate (ADP) with the purity over 98% was purchased from Sigma (USA). Adult female SD rats were purchased from SPF animal laboratory center, Dalian Medical University, Dalian, China. All other chemicals were analytical grade from commercial sources. LBY-NJ4 platelet aggregometer was from Beijing Precil Instru. Co. Ltd, China. UV-754 spectrophotometer was from Shanghai Jinghua Biotech. Co. Ltd, China.

Purity analyses of flavonoids: 0.55 mg of each of the 8 flavonoids was dissolved separately in 2 ml of methanol and filtered through a 0.2 μm filter. 10 μl of each flavonoid sample was analyzed by Waters 2690/996 high performance liquid chromatography (HPLC). An Intersil ODS-3 C18 column (4.6 × 250 mm) operated at 35 ^oC was used for HPLC analysis. The mobile phase was 60% (V/V) methanol supplemented with 40% (V/V) of 0.2% phosphoric acid. Elution was performed at 1.0 ml/min. The monitoring wavelengths were set at 254 nm for quercetin, isoquercitrin, rutin, hesperetin and hesperidin, 283 nm for naringenin and naringin, and 270 nm for icariin, respectively. Triplicates were performed for each sample. HPLC chromatograms were processed by using Millennium 32 software.

Platelet anti-aggregation assay of flavonoids: Blood was collected from adult female SD rats who had not been administrated with any drugs prior to sampling. Blood was diluted (1:9) with 3.8% sodium citrate. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared by centrifugation at 800 rpm for 20 min and 7000 rpm for 15 min at room temperature, respectively. PRP was diluted with PPP to make the final platelet count of 2.5×10^8/ml. Eight flavonoids were dissolved in dimethyl sulfoxide (DMSO) with the final concentration of 331 μmol/l, mixed well with 450 μl platelet plasma solution, and incubated at 37 ^oC for 3 min. Then 3 μl of 30 μmol/l ADP was added into the mixture to induce platelet aggregation. Aggregation was recorded for 5 min using an LBY-NJ4 platelet aggregometer. Platelet aggregation inhibitory percentage was calculated using following equation: Inhibiting percentage (%) = $(T_0\text{-}T)\ /T_0{}^*100\%$. T_0 is the transmittance of control sample, T is the transmittance of experimental sample. All results are the averages of triplicate measurements.

Radical scavenging activity of flavonoids: The evaluation of radical scavenging activity of flavonoids was based on HO· generated by Fenton reaction (Haber and Weiss, 1932): $Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+HO\cdot+OH^-$. 2,3-dihydroxy benzoic acid was produced through the oxidation of salicylic acid (SA) by HO·, which was used for analyzing the absorbance at 510 nm for activity assay. Briefly, the reaction mixtures (4 ml) containing 1ml of 1.5 μmol/l $FeSO_4$, 1ml of 9 μmol/l SA and 1ml of each flavonoid sample dissolved in DMSO or control with the same volume of DMSO or blank with the same volume of ddH_2O were mixed, then 1ml of 9 μmol/l H_2O_2 was added to initiate reaction, the mixture were incubated at 37 ^oC for 0.5 h. The scavenging percentage was calculated as following: Scavenging percentage (%)=$[A_0\text{-}(A_X\text{-}A_{X0})]/A_0{}^*100\%$. A_0 is the absorbance of control, A_X is the absorbance of sample and A_{X0} is the absorbance of blank. All results are the averages of triplicate measurements.

Data Statistical analysis: Data analysis was performed using SPSS 11.5 software. All results are presented as means ± standard deviations (S.D) when a minimal

number of two independent experiments were performed in triplicate. The differences between groups were evaluated using a one-way analysis of variance (ANOVA) with all pair wise multiple comparison procedures conducted using unpaired *t* test. Values with $P<0.05$ were considered statistically significant differences.

RESULTS AND DISCUSSION

In recent years, the intakes of dietary flavonoid phytochemicals was found to reduce and prevent human related diseases, primarily contributed to their antiplatelet and antiradical activities. However, the function-structural relevances of antiplatelet/ antiradical activities of flavonoids are still unclear. Comprehensive dissections of the correlations between discrete functional groups of flavonoids and their antiplatelet and antiradical activities are necessary to consider their modification into more potent and selective small-molecule inhibitors. In current work we investigated the antiplatelet against ADP-induced platelet aggregation and antiradical (HO·radical scavenging) activities of 8 flavonoid phytochemicals.

Structural comparisons of flavonoid phytochemicals: The 8 flavonoids were quercetin, isoquercitrin, rutin, hesperetin, hesperidin, naringenin, naringin and icariin, which can be cataloged as flavonol (quercetin), flavone (rutin, isoquercitrin and icariin) and flavanone (hesperetin hesperidin, naringenin and naringin). As schemed in Figure 1, quercetin, rutin, isoquercitrin and icariin are similar chemical derivatives originated from the core structure of flavone; while hesperetin, hesperidin, naringenin and naringin share the same core structure of flavanone. On the one hand, these flavonoid phytochemicals show similar structure, on the other hand, they also have individual intrinsic structures with the substitutions of functional groups (Figure 1). The structure differences among these flavonoids might result in and explain their functional differences.

Flavonoid phytochemicals used are in high purity (> 95%): The peak of individual flavonoid sample dominated overwhelmingly in its HPLC chromatogram. The purities of 8 flavonoid phytochemicals were greater than 95% calculated based on both the HPLC peak area and intensity height (Table 1), which testifies the high purities of flavonoids used for the antiplatelet and antiradical experiments and also ensures that all the results in current work to be obtained with high positivity, accuracy and credibility.

Comparative antiplatelet activities of flavonoid phytochemicals: The antiplatelet activity determinations for all 8 flavonoids were performed at the same concentration of 331 μmol/l, which made it possible to compare their activities at same level and to explore the structure-function relationship. All flavonoids showed apparent inhibitory effects on ADP-induced rat platelet aggregation (Figure 2). Their antiplatelet aggregation activities were in below order: quercetin (68.33± 2.43%) > rutin (55.34±2.09%) > isoquercitrin (52.27± 4.65%) > hesperetin (50.80± 7.03%) > hesperidin (33.07 ±2.09%) > naringenin (31.28±4.65%) > naringin (22.91± 1.68%) > icariin (20.94±3.20%). Rutin, isoquercitin and hesperetin showed similar inhibiting effects on ADP- induced platelet aggregation. The differences of antiplatelet aggregation activities for hesperidin and naringenin, for naringin and icariin, showed no statistical significance ($P>0.05$). There were significant difference among the rest groups ($P<0.05$).

Flavonoids with more phenolic hydroxyl groups show relative higher antiplatelet activity: The inhibitory percentages of rat platelet aggregation for quercetin, rutin, isoquercetin and icariin were 68.33±2.43%, 55.34±2.09%, 52.27±4.65% and 20.94±3.20%, respectively (Table 2). Quercetin, rutin, isoquercetin and icariin own same core structure from flavone, the numbers of free phenolic hydroxyl groups for them are 5, 4, 4 and 1. It seems that the more free phenolic hydroxyl groups a flavone has, the higher antiplatelet activity it will exhibit (Table 2), which is consistent with the results obtained for flavonoids on their antiplatelet activities in other systems (Wright *et al*., 2010; Cavia-Saiz *et al.*, 2010; Navarro-Núñez *et al*., 2009).

The 7-OH group of A-ring of flavanone is important for its antiplatelet activity: Hesperetin, hesperidin, naringenin and naringin can be regarded as the derivatives originated from same structure core of flavanone (Figure 1). The differences between hesperetin and hesperidin, between naringenin and naringin, are that the 7-OH of A-ring of flavanone is replaced by O-β-Glc-α-Rha. Obviously, O-β-Glc-α-Rha substituent of 7-OH decreased the antiplatelet capacities of hesperidin and naringin by ~26.76% and ~34.90% as their antiplatelet abilities were decreased from 31.28±4.65% and 50.80± 7.03% to 22.91±1.68% and 33.07±2.09% (Table 2). The above results indicated the 7-OH of A-ring of flavanone is critical for its antiplatelet function. Considering the only structural differences between hesperidin and hesperetin, naringin and naringenin were the free 7-OH group replaced by 7-O-β-Glc (6→1)-α-Rha and 7-O-β-Glc(2→1)-α-Rha, the presence of free 7-OH enhances the antiplatelet activities of flavanones. The free 7-OH group in the A ring might potentially affect flavonoids by binding to TxA_2 receptor (Cavia-Saiz *et al*., 2010; Navarro-Núñez *et al*., 2009) for exerting their activities.

The 3-OH group of C-ring of flavone is important for its antiplatelet activity: Quercetin, rutin and isoquercitrin share same core structure of flavone (Figure 1). Quercetin showed highest antiplatelet activity with

inhibitory percentage of 68.33±2.43%, followed by rutin and isoquercitrin (Table 2). Rutin and isoquercitrin have no free 3-OH group in the C ring, replaced by the disaccharide glucorhamnoside, which is also the only structural difference from quercetin. Compared with quercetin, following the replacements of O-Glc-α-Rha and O-β-Glc of 3-OH of C-ring (Figure 1), the antiplatelet activities of rutin and isoquercitrin decreased by ~19.01% and ~23.50%. It implicates that the 3-OH of C-ring of flavone is important for their ADP-induced antiplatelet activity. The above results are consistent with the antiplatelet activities induced by other reagent for flavonoids that 3-OH of C-ring increases antiplatelet activities (Wright *et al*., 2010; Navarro-Núñez *et al*., 2009; Bojic *et al*., 2011).

C_2-C_3 double bond and 3'4'-OH group might not be important for antiplatelet aggregation: Rutin, isoquercitrin and hesperetin showed similar inhibiting affinities on ADP-induced platelet aggregation with inhibitory rates of 55.34±2.09%, 52.27±4.65% and 50.80 ±7.03%, respectively. Rutin and isoquercitrin have C_2-C_3 double bond in C ring, while hesperetin has C_2-C_3 single bond and 4'-OH of B-ring was substituted by -O-CH_3. The above results indicated that the C ring C_2-C_3 double bond and the B ring 3'-4'orthodihydroxy group might not be important for its antiplatelet function. The structure differences of naringin from icariin were also the C ring C_2-C_3 double bond and B ring 4'-OH group, they showed similar antiplatelet aggregation activity. The C_2-C_3 double bond in the C ring as well as the 4'-OH in the B ring might not be important for its antiplatelet function. Controversially, previous experiments results indicated that the C_2-C_3 double bond and the 4'-OH played important roles in antiplatelet activity of structural similar flavonoids on collagen-stimulated platelet aggregation (Navarro-Núñez *et al*., 2009), this difference may be attributed to different mechanisms.

Radical scavenging activity of flavonoids

Quercetin shows hydroxyl radical scavenging activity dose-dependently: Among ROS, HO· exhibits the strongest oxidative activity, as it can nonspecifically oxidize all classes of biological biomolecules by causing lipid oxidation and enormous biological damage (Ozyurek *et al*., 2008; Ammar *et al*., 2009). Fig. 3 showed the HO· scavenging activity of quercetin at the concentrations of 19.89 μmol/L, 41.43 μmol/L, 82.86 μmol/L, 165.73 μmol/L, 331.46 μmol/L and 662.91 μmol/L. Following the concentration increase of quercetin, the absorbance at 510 nm decreased correspondingly, which indicated quercetin showed scavenging HO· radical activity dose-dependently.

We measured the antiradical activities of all 8 flavonoids at the same concentration of 260 μmol/L. Among them, only quercetin, rutin and isoquercitrin showed clear scavenging HO· radical activity (Figure 4). Quercetin showed the strongest HO· radical scavenging activity with the percentage of 30.69%, followed by rutin and isoquercitrin with the percentages of 13.70% and 10.23%, respectively. While hesperetin hesperidin, naringenin, naringin and icariin showed no scavenging HO· radical activity. The scavenging activity between rutin and isoquercitrin was not statically significant ($P > 0.05$). The differences of rutin and isoquercitrin compared to quercetin were with statical significances ($P<0.05$).

Flavonoids with more phenolic hydroxyl groups show relative higher antiradical activity: The spatial arrangement of substituents is probably a greater determinant of antiradical activity than the flavan backbone alone, both the configuration and total number of hydroxyl groups substantially influence several mechanisms of antiradical activity of flavonoids (Sekher Pannala *et al*., 2001; Burda and Oleszek 2001). Free radical scavenging capacity of flavonoids primarily attributes to high reactivations of hydroxyl substituents that participate in production process of free radicals (Heim *et al*., 2002). The B-ring hydroxyl configuration is generally the most significant determinant of scavenging ROS (Sekher Pannala *et al*., 2001; Burda and Oleszek 2001). Hydroxyl groups in the B-ring donate hydrogen and an electron to hydroxyl, peroxyl and peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoid radical. Among structurally homologous flavonoids, hydroxyl scavenging increases according to the total number of OH groups (Heim *et al*., 2002), which was consistent with the results obtained in current work. The antiradical activity experiment results showed that the scavenging percentage for quercetin, rutin and isoquercetin were 30.69%, 13.70% and 10.23%, respectively (Table 2). Quercetin, rutin and isoquercetin own the same core structure of flavone, overall, the numbers of free phenolic hydroxyl groups for them are 5, 4 and 4. It seems that the more free phenolic hydroxyl groups a flavone has, the higher antiradical activity it will exhibit (Table 2), which is consistent with previous results obtained for flavonoids in other reaction systems on their antiradical activities that the number of hydroxyl groups enhance antiradical activity of flavonoids (Sroka 2005).

The 3-OH group of C-ring of flavone is important for its antiradical activity: Free radical scavenging by flavonoids is highly dependent on the presence of a free 3-OH increasing the stability of the flavonoid radical, and the torsion angle of B-ring with respect to the rest of the molecules. Flavonol with a 3-OH is planar, while the flavones and flavanones lacking this feature are slightly twisted. The planarity of flavonoids was reported to permit conjugation and electron dislocation by increasing the flavonoid-phenoxyl radical stability (Sekher Pannala

et al., 2001; Burda and Oleszek 2001; Heim *et al*., 2002). Rutin and isoquercitrin have the same core structure of flavone as quercetin. Rutin and isoquercitrin have no free 3-OH group in the C ring, replaced by the disaccharide glucorhamnoside, which is also the only structural difference from quercetin. Quercetin showed the highest antiradical activity with scavenging percentage of 30.69% (Table 2). Compared with quercetin, following the replacements of O-Glc-α-Rha and O-β-Glc of OH at C_3 of the flavone C-ring (Figure 3), the hydroxyl radical scavenging activities of rutin and isoquercitrin decreased by ~55.36% and ~66.67%. Clearly, the 3-OH of C-ring of flavone is critical for its antiradical activity. The glycosylation blocks the 3-OH group in C-ring by decreasing the antiradical activities of flavonoids. Antiradical properties of flavonoids decreased as the number of glycosidic moieties increased, aside from mere presence and total number, the position and structure of sugar played an important role, as the *O*-glycosylation interferes with the co-planarity of B-ring with the rest of flavonoid and affects the ability to delocalize electrons (Plumb *et al*., 1999). The sugar moieties also prevent flavonoids access to the lipid membranes by affecting anti-radical activity (Saija *et al*., 1995). Above result was consistent with the results obtained on the antiradical activities of other series of flavonoids or flavonoids studied in other systems (Walker *et al*., 2000; Deliorman-Orhan *et al*., 2009; Mustafa *et al*., 2010; Wolfe and Liu 2008; van Acker *et al*., 1996A; Dugas *et al*., 2000; Bors *et al*., 1990). 3-OH is also a potential chelating and oxidation site (van Acker *et al*., 1996A). The substitution of 3-OH by a methyl or glycosyl group could completely abolish the activity of quercetin and kaempferol against β-carotene oxidation (Burda and Oleszek 2001). B-ring hydroxyl groups could form hydrogen bond with 3-OH by aligning the B-ring with the heterocycle and A-ring. The elimination of this hydrogen bond causes a minor twist of the B-ring by compromising the electron delocalization capacity (van Acker *et al*., 1996B). In addition, the influence of 3-OH on anti-radical activity might be potentiated by the of 3'-4'-catechol structure through this intramolecular hydrogen bond (Rice-Evans *et al*., 1996).

C_2-C_3 double bond and 3',4'-OH might be important for antiradical activity: As a salient feature of the most potent scavengers, the 3'-4'-catechol structure of B-ring for flavonoids strongly enhances the activities against peroxyl, superoxide and peroxynitrine radicals (Heim *et al*., 2002; Dugas *et al*., 2000). Rutin and isoquercitrin showed clear scavenging HO· radical activity, while hesperetin showed no HO· radical scavenging activity. Rutin and isoquercitrin have C_2-C_3 double bond in C ring and 3'-4'orthodihydroxy group of B ring, while hesperetin has single bond at C_2-C_3 and the 4'-OH of B-ring substituted by-O-CH_3. As the 3-OH of rutin and isoquercitrin was glycosylated, comparing to hesperetin, rutin and isoquercitrin have two ortho hydroxyl groups in the B ring, while there is only one hydroxyl group at 3' position of hesperetin, which implicated that the two adjacent hydroxyls improved the antiradical capacities of flavonoids. The above results fit well with previous experiment evidences obtained by using other reaction systems that a 2,3-double bond and orthodiphenolic structure enhanced the antiradical activities of flavonoids (Mustafa *et al*., 2010; van Acker *et al*., 1996A; Tsimogiannis an Oreopoulou 2004; Russo *et al*., 2000). C-ring C_2- C_3 double bond increases the antiradical activities of flavonoids by affording a more stable flavonoid radical through conjugation and electron delocalization. Oxidation of a flavonoid occurs on the B-ring when the catechol is present, yielding a fairly stable ortho-semiquinone radical through facilitating electron delocalization. Flavanone lacking catechol form relatively unstable radicals and are weak scavengers (Heim *et al*., 2002; Hu *et al*., 1995; Gao *et al*., 1999).

Taken together, for the flavonoids that share same structural core, their antiplatelet and antiradical activities were mainly attributed to the numbers and positions of hydrogen donating hydroxyl groups on the aromatic ring of the phenolic molecules and also affected by other factors such as glycosylation and other H-donating groups. The more free hydroxyl groups a flavonoid has, the higher antiplatelet and antiradical activities it exhibits among the same type flavonoids with same core structure. The 7-OH of A-ring and 3-OH of C-ring of flavonoids are important for the antiplatelet activity. The C_2-C_3 double bond of C ring and 4'-OH showed no effects on the antiplatelet activity for ADP-induced platelet aggregation of flavonoids. Principal functional groups attributed to potent antiplatelet activity were 7-OH of A-ring and 3-OH of C-ring. The two adjacent 3',4'-OH groups at B ring owing better electron donating properties as a radical target improve radical scavenging activity. The presence of 3-OH of C-ring, giving a catechol-like structure in ring C, which is also beneficial for free radical scavenging activity of flavonoids. The C_2-C_3 double bond was known to be responsible for electron delocalization from ring B and it increase the radical scavenging activity.

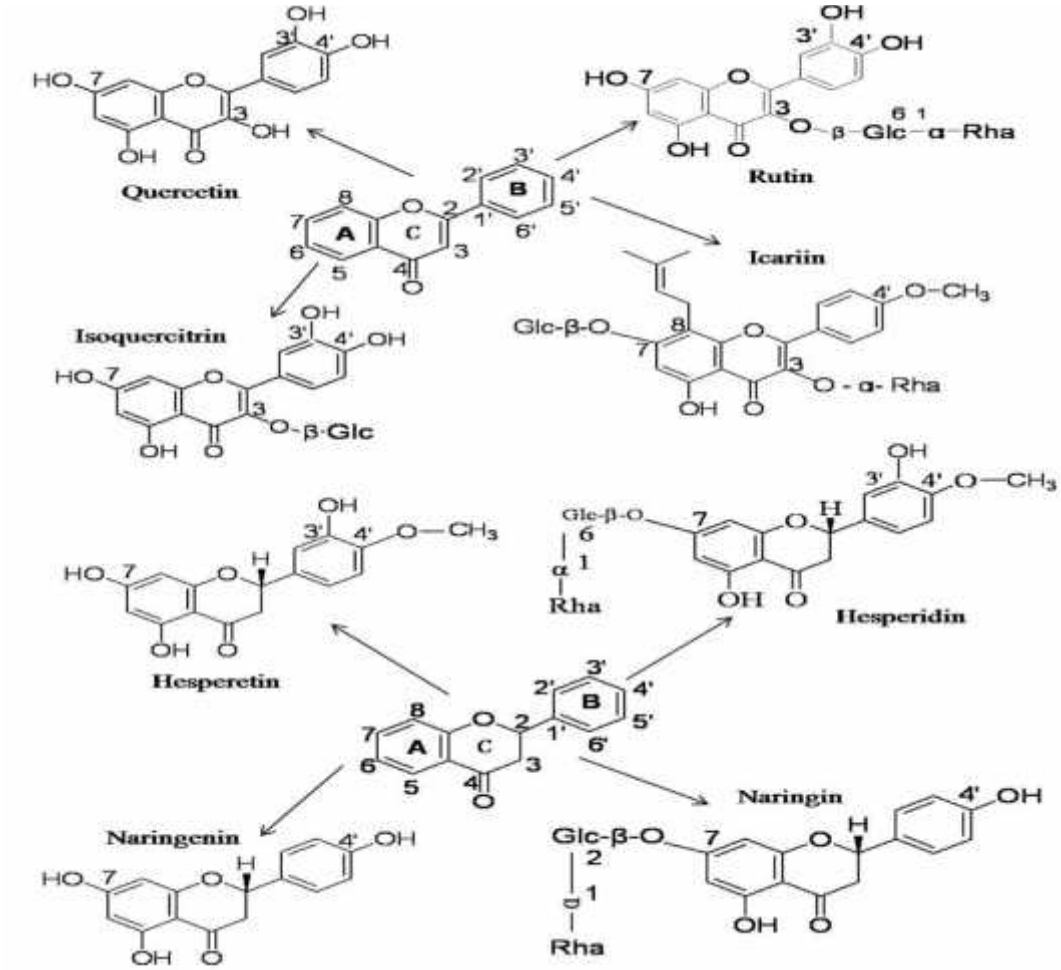


Figure 1.The chemical structures of quercetin, rutin, isoquercitrin, icariin, hesperetin, hesperidin, naringenin and naringin.

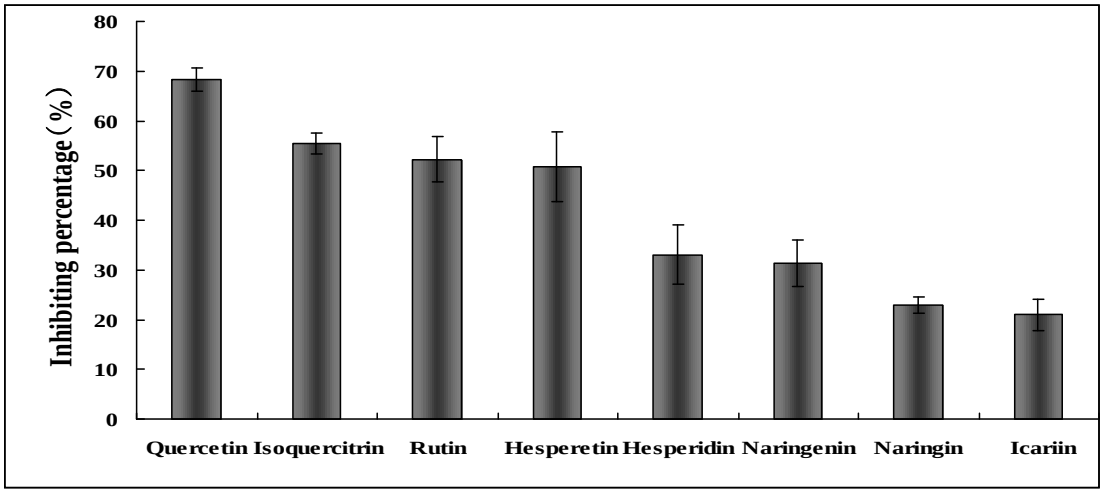


Figure 2. Inhibitory effect of 8 flavonoids on ADP-induced rat platelet aggregation. 331 «mol/l flavonoids incubated with PRP at 37 °C for 3 min, in prior to the addition of platelet aggregation inducer ADP (30 «mol/l). Inhibiting percentage (%)=(T_0-T)/ T_0*100%. T_0 is the inhibiting percentage of control, T is the inhibitiory percentage of flavonoids. Inhibiting percentages are presented as means ±S.D (*n*=3). There were no significant difference between rutin and isoquercitin (*P*=0.368), isoquercitin and hesperetin (*P*=0.636), rutin and hesperetin (*P*=0.207), hesperidin and naringenin (*P*=0.613), naringin and icariin (*P*=0.603) on anti-platelet activity. There were significant difference among the rest groups (*P*<0.05).

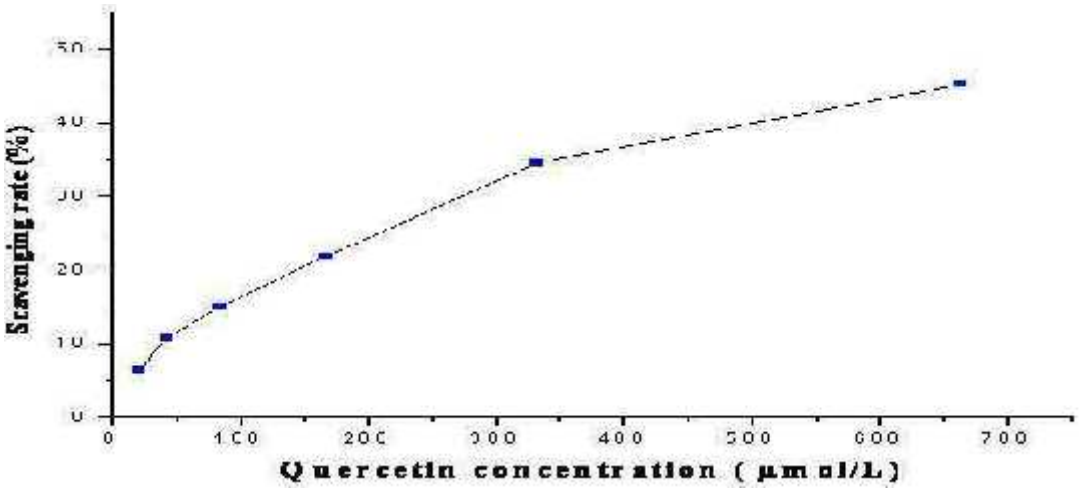


Figure 3. The hydroxyl radical scavenging activity of quercetin. The hydroxyl radical scavenging activity of different concentration of quercetin (19.89 «mol/l, 41.43 «mol/l, 82.86 «mol/l, 165.73 «mol/l, 331.46 «mol/l and 662.91 «mol/l) were determined by Fenton reaction, absorbances were measured at 510 nm. The scavenging rate was calculated by using the following formula: Scavenging rate (%)=[A_0-(A_X-A_{X0})]/ A_0*100%. A_0 is the absorbance of control, A_X is the absorbance of flavonoids and A_{X0} is the absorbance of blank.

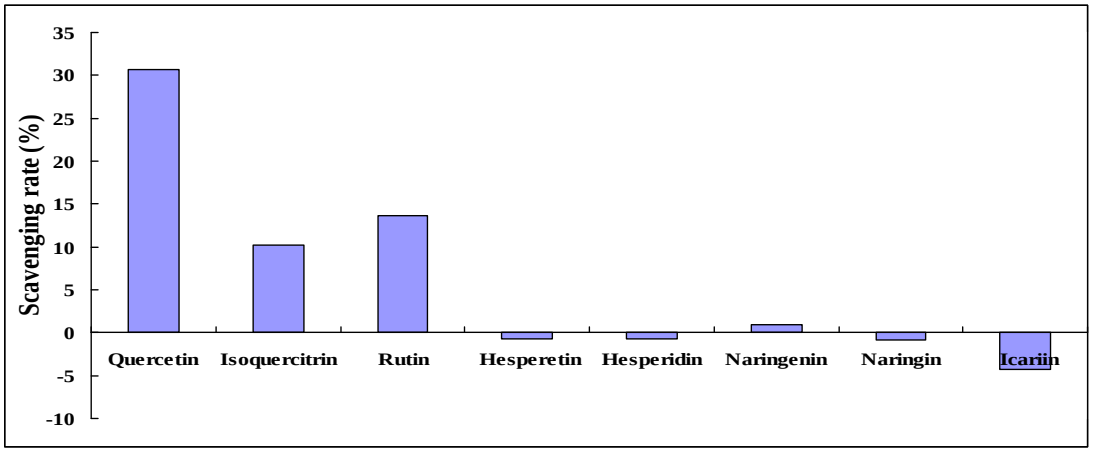


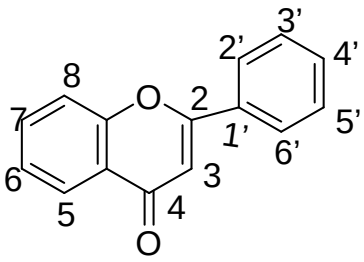
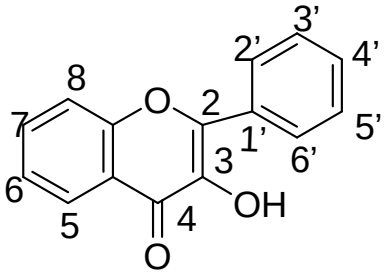
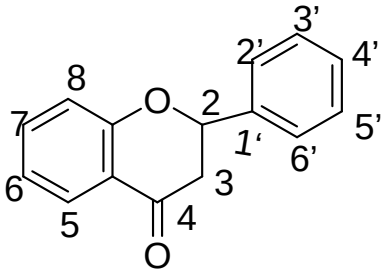
Figure 4. The hydroxyl radical scavenging activity of 8 flavonoid phytochemicals. The hydroxyl radical scavenging activity of 8 flavonoids at the same concentration of 260 «mol/l were determined by Fenton reaction, absorbances were measured at 510 nm. The scavenging rate was calculated by using the following formula: Scavenging percentage (%)=[A_0-(A_X-A_{X0})]/ A_0*100%. A_0 is the absorbance of control, A_X is the absorbance of flavonoids and A_{X0} is the absorbance of blank.

Table 2. The purity determinations of 8 flavonoid phytochemicals by HPLC.

Flavonoids	N[a]	Wavelength (nm)[b]	Retention time(min)	Purity (area) (%)[c]	Purity (height) (%)[d]
Quercetin	3	254	6.75±0.01	98.22±0.02	98.49±0.02
Isoquercitrin	3	254	4.51±0.01	97.12±0.02	97.20±0.02
Rutin	3	254	4.40±0.01	99.33±0.03	99.33±0.02
Naringenin	3	254	7.38±0.02	95.64±0.02	95.10±0.02
Naringin	3	254	4.26±0.01	95.06±0.04	95.31±0.03
Hesperetin	3	283	7.83±0.02	95.85±0.03	95.02±0.03
Hesperidin	3	283	4.35±0.02	96.53±0.02	96.78±0.03
Icariin	3	270	9.78±0.02	96.85±0.03	96.29±0.03

[a] Represents the number of measurement per experiment; [b] Indicates the wavelength used for monitoring the elution of flavonoid phytochemicals by HPLC; [c] Means the purities of 8 flavonoid phytochemicals were calculated based on their peak areas; [d] Represents the purities of 8 flavonoid phytochemicals were calculated based on their peak heights.

Table 2. antiplatelet and antiradical effects of 8 flavonoid phytochemicals.

Flavonoids subclass	Name of flavonoids	Mw	Inhibitory percentage (%)	Scavenging percentage (%)
Flavone 	Rutin (5,7,3',4'-OH)	610.52	55.34±2.09	13.70±0.002
	Isoquercitrin (5,7, 3',4'-OH)	464.38	52.27±4.65	10.23±0.002
	Icariin (5-OH, 4'-OMe)	676.66	20.94±3.20	-4.29±0.002
Flavonol 	Quercetin (3,5,7, 3',4'-OH)	302.20	68.33±2.43	30.69±0.003
Flavanone 	Hesperetin(5,7,3'-OH, 4'-OMe)	302.29	50.80±7.03	-0.66±0.007
	Naringenin (5,7,4'-OH)	272.26	31.28±4.65	-0.83±0.001
	Hesperidin (5,3'-OH, 4'-OMe)	610.58	33.07±2.09	-0.66±0.003
	Naringin (5,4'-OH)	580.55	22.91±1.68	0.99±0.001

Flavonoids are divided into classes based on the structure of ring C. Basic structure corresponds to flavan which are named flavanol if hydroxylated at C3. Flavanone have keto group on C4. If the double C2=C3 is present in flavanols are named Flavone.

Conclusion: In conclusion, the present study showed that the minor modification and arrangement of these functional groups could affect the antiplatelet and antiradical activities of dietary flavonoids apparently. The antiplatelet and antiradical activities of flavonoids were closely associated with their characterized intrinsic structures/conformations. The results from current work provide certain insights for estimating/evaluating the contribution impacts of different functional groups of flavonoids on the inhibition of platelet aggregation and scavenging HO· radical, which might also help in selecting and confirming the effective and favorable dietary flavonoids rich in our food and fruits in the prevention and treatment of related human diseases.

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