# Free Radical Scavenging of Flavonoids and Their Effects on Erythrocyte Na Leak, Platelet Aggregation and TBARS Production

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## ABSTRACT

We compared radical scavenging activity of flavonoids and their antioxidant effects on erythrocyte Na leak, platelet aggregation and TBARS production using Sprague Dawley rats. The concentrations of flavonoids scavenging radical by 50%($SC_{50}$) for 0.1mM DPPH were Quercetin; 7.4 $\mu$M, Catechin; 10.6 $\mu$M, Morin; 22 $\mu$M, Hesperidin; 400 $\mu$M, Naringin; 3.95mM respectively. Morin completed its antioxidant activity in 2minutes while catechin, hesperidin and naringin have slow but long lasting antioxidant activity. Whole blood platelet aggregation when incubated with quercetin or catechin was significantly decreased ($P<0.05$) compared with the control. Na-leak in intact erythrocyte was significantly lower when incubated with quercetin compared with other flavonoids ($P<0.05$). Morin, hesperidin and naringin rather increased Na leak in intact erythrocyte. Na-leak in erythrocyte treated with phenazine methosulfate (PMS) was overall increased, but was not affected by flavonoids. Intracelluar Na and K were not affected by treatment of PMS. TBARS production in platelet rich plasma (PRP) was significantly lower ($P<0.05$) when incubated with quercetin or hesperidin compared with the control. PMS treatment caused an increase in TBARS production regardless of flavonoids. Physiological functions of flavonoids are known to be associated with their radical scavenging activities, possibly preventing oxidative modification in many phases. In the present study, Antioxidant effects of flavonoids were not well correlated with their radical scavenging activities although quercetin which showed the strongest radical scavenging activity had the most antioxidant effects.

**KEY WORDS** : flavonoids, DPPH test, erythrocyte Na-leak, platelet aggregation, TBARS

## INTRODUCTION

Flavonoids are phenolic compounds widely distributed in plants with over 4000 variet ies. Recently, interest in food phenolics has greatly increased because their antioxidant ability might have beneficial effects on human health. High intake of foods rich in flavonoids such as wine, grape fruit juice and green tea might reduce the risk of cardiovascular diseases. Epidemiological studies indicated that consumption of flavonoid rich foods was associated with the lowered incidence of cardiovascular diseases (Hertog MG et al, 1993 ; Imai K et al, 1995 ; Arai Y et al, 2000). Dietary red wine had favorable effects on human plasma high-density lipoprotein and blood chemistry(Lavy et al, 1994), and consumption of catechin and quercetin or drinking grape juice inhibited platelet aggregation in human(Pignatelli P et al, 2000 ; Keevil JG et al, 2000). There are also reports that tea derived catechin had hypocholesterolemic and antihypertensive effects(Atensvold I et al, 1992) or tea flavonoids retarded LDL oxidation(Ishigawa T et al, 1997). On the other hand, consumption of green or black tea did not affect LDL oxidation in human(Van Het Hof KH et al, 1997) or high intake of black current and apple juices had a prooxidant activity on plasma protein(Young JF et al, 1999). These contradictions may result from the differences in the

* Running head : Radical scavenging of flavonoids and biological effects

dose or the time period of consumption and individual variations. It has been suggested that significant amounts of flavonoids can be absorbed in human subject and be bioavailable to act as antioxidant in vivo(Young JF et al, 1999 ; Donovan JL et al, 1999 ; Olthof MR et al, 2000 ; Scalbert A et al, 2000). However, it is not sure that the habitual diet for general population can provide sufficient flavonoids enough to the concentration at which they can exert the protective effect against free radicals. Physiological functions of flavonoid are based on its radical scavenging activity preventing oxidative modification on biomembrane, lipoprotein, platelet and prostanoid (Terao J et al, 1994 ; Salah N et al, 1995), but it is currently unclear whether flavonoids really can act as an antioxidant in vivo. Since beneficial effects of dietary flavonoids in human primarily are accounted for antioxidant ability, studies are priorly necessary to determine whether flavonoid can act as antioxidant in invitro or ex vivo system. Overall, more invitro and invivo studies are needed on exactly which flavonoid(s) is beneficial or what doses of flavonoids is effective with no complication before recommendation for therapeutic use.

We conducted an experiment to compare DPPH (2,2 Diphenyl 1-picryl hydrazyl) free radical scavenging activity of flavonoids and their antioxidant effects by examining erythrocyte Na leak, platelet aggregation and TBARS production in exvivo system after incubation of flavonoids. The scavenging activities of flavonoids can be evaluated in vitro by DPPH free radical assay(Brand-Williams W et al, 1995). DPPH is a stable nitrogen radical with purple color of absorbance at 517nm, and the absorbance decreases upon reaction with antioxidant. We chose five major dietary flavonoids such as flavonols; quercetin and morin (onion, red grape), flavanol; catechin (green and black tea) and flavanones; hesperidin and naringin (citrus fruits) and examined their antioxidant effects to give supportive data before clinical trial for therapeutic use.

# METHODS AND MATERIALS

## 1. Flavonoids and animal sample

DPPH(2,2-diphenyl-1-picrylhydrazyl) and test flavonoids; quercetin, catechin, morin, hesperidin, naringin were purchased from Sigma-Aldrich (St. Louis, MO). Male Sprague Dawley rats were ad libitum fed a commercial pellet and housed in a group of four in a plastic cage in a room maintained 20-25℃ with a 12 dark-light cycle. At age of 12-14 weeks, blood samples were obtained by cardiac puncture into vacuum tubes containing heparin, and platelet aggregation and erythrocyte Na leak were freshly performed.

## 2. DPPH radical scavenging test

Radical scavenging activity of flavonoids were evaluated by DPPH method of Brand-Williams.[16] Radical scavenging activity is defined as the amount of antioxidant necessary to decrease the DPPH concentration by 50%. Flavonoids were first dissolved in DMSO (Dimethyl sulfoxide, Sigma-Aldrich) to 50mM stock solution, then rediluted with methanol (Merck) to each working concentration. The DPPH concentration was measured by its absorbance of 517nm at 0 min through 10 min after addition of flavonoid in methanol to 0.1mM DPPH in methanol to be 1.5㎖ of total volume and $SC_{50}$ (50% scavenging activity) is the concentration of flavonoid at which decrease in absorbance by 50% at 10 minutes after addition of flavonoid.

## 3. Platelet aggregation

Platelet aggregation was measured using a Chronolog Whole Blood Aggregometor (model 500-Ca, Havertown, Pennsylvania, USA). The whole blood incubated with 100㎛M flavonoid for 10 minutes was then diluted with isotonic saline (1:2) to give platelet concentration 400,000/㎕. Adenosine diphosphate (ADP, 2㎛M) was added to initiate aggregation, and three

readings of impedance changes were averaged for each rat. Platelet aggregation caused an increase in impedance across two platinum electrodes in the whole blood and the record response was set 5Ω impedance. The impedance method using the fresh whole blood has the advantage of measuring platelet aggregation under nearly physiological conditions in the presence of other blood components.

### 4. Erythrocyte Na-leak

Sodium leak is defined as the sodium efflux through passive sodium channel occuring under inhibition of ouabain sensitive Na-pump and furosemide sensitive Na+-K+ cotransport and can be increased upon membrane damage of erythrocyte after exposure to free radical generating system such as phenazine methosulfate (PMS)(Maridonneau I et al, 1983). Four mℓ of blood preincubated with 100μM flavonoid for 10minutes was then aliquoted 2mℓ each with and without 0.5mM PMS and incubated in shaking water bath for 5 minutes. Blood was then centrifuged at 1,000×g for 10 minutes, and the plasma and buffy coat were removed. Red blood cells were washed 5 times with a cold isotonic washing solution [150mM choline chloride, 10mM Tris-4 morpholinopropane sulfonic acid (MOPS), pH 7.4 at 4℃], centrifuging at 1,000×g for 5 minutes after each wash. The final erythrocyte pellet was resuspended in the choline chloride washing solution to give 40-50% hematocrit. The hematocrit was measured. A 50μℓ aliquot of the suspended erythrocyte was added to 5mℓ of 0.025% acationox (a metal free detergent, Scientific Products, McGaw Park Illinois, USA) to be used for determination of intracellualr K and Na concentrations. Two mℓ of PMS treated and untreated erythrocyte suspensions were added to each 40mℓ of medium (150mM choline chloride, 10mM glucose, 1mM ouabain, 1mM furosemide, 10mM Tris -MOPS pH7.4 at 37℃), then mixed gently and aliquoted to 12 tubes. Tubes in duplicate were transferred to an ice bath after incubation in 37℃ shaking water bath for 0, 10, 20, 30, 40 and 50min. Tubes were centrifuged at 1,000×g for 5minutes, then the supernatants were removed and measured Na concentration.

Calculations :

Na μg/(ml×min)×60min×mmole/23mg×[44ml-(4ml×hct)/(0.0041×hct)]×(mg/1000μg)=mmole/ℓ rbc/hour (Na leak)

Na μg/(ml×min)×mmole/23mg×101/hct×(mg/1000μg)=mmole/ℓ rbc (Intracelluar Na)

### 5. TBARS production

Platelet rich plasma (PRP) were obtained after centrifuging PMS treated and untreated whole blood at 300 ×g for 10 minutes. After incubation of each PRP with 100μM flavonoids for 24 hours at 37℃ incubator, thiobarbituric acid reactive substance (TBARS) was determined according to a modified Yagi's method(Yagi K. et al, 1976). Plasma lipids were precipitated with phosphotungstic acid and TBARS in the pellet were measured by a fluometer (Kontron model SFM 25) using 1,1,3,3- tetraethoxypropane (Sigma-Aldrich) as a standard.

### 6. Statastical analysis

Values were analyzed using the SAS pakage (SAS, 1994). Analysis of varience were conducted in a completely randomized block design. Duncan's multiple test was applied to compare individual means when F-value was significant ($P<0.05$).

## RESULTS AND DISCUSSION

### 1. DPPH assay of flavonoids

Radical scavenging activity are shown in Table 1. $SC_{50}$ is the flavonoid concentration that can scavenge 0.1mM DPPH (2,2 Diphenyl 1-picryl hydrazyl) radical by 50% at 10 minutes after addition of flavonoids. Quercetin among 5 flavonoids had the lowest $SC_{50}$ of

7.4 $\mu$M meaning that quercetin had the strongest antioxidant activity. Catechin and morin also have comparatively low $SC_{50}$, while hesperidin and naringin have as high $SC_{50}$ concentration as 0.4mM and 3.95mM respectively. Morin had the same antioxidant activity as vitamin C. $SC_{50}$ can be useful to decide the optimum concentration of flavonoids for invitro experiment. Our result agreed that concentration of 5-10 $\mu$M for quercetin or catechin and 800-1200 $\mu$M for hesperidin or naringin are usually used in invitro experiment(Pignatelli P et al, 2000 ; Bok SH et al, 1999)

The pattern of decrease in absorbance after addition of flavonoid are shown in Figure 1. The time period lasting antioxidant activity when interpreted with the pattern of decrease in absorbance was different between flavonoids. Like vitamin C which completed its antioxidant activity in 10 seconds, morin showed 90% of its antioxidant activity in 10-15 seconds and completed in 2 minutes. Catechin, hesperidin and naringin had slow but lasting activity, decreasing absorbance until 10 minutes and longer. This suggests that the activities scavenging 50% DPPH radical by morin and vitamin C are the total antioxidant activity they have, while catechin, hesperidin and naringin have the remaining activity above scavenging 50% DPPH radical. The time period lasting antioxidant activity of flavonoid is important for the concentration and incubation time in invitro experiment. Potency and acting period of flavonoid may be associated with its chemical structure, and further study is needed in relation of the chemical characteristics of DPPH and its concentration.

## 2. Platelet aggregation

Whole blood platelet aggregation are shown in Table 2. All of test flavonoids decreased the maximum platelet aggregation and quercetin and catechin were

**Table 1. $SC_{50}$ concentrations of flavonoid for 0.1mM DPPH**

| | Quercetin | Catechin | Morin | Hesperidin | Naringin | Vit. C[2] |
|---|---|---|---|---|---|---|
| $SC_{50}$ ($\mu$M)[1] | 7.4 | 10.6 | 22 | 400 | 3950 | 22 |

[1] Flavonoid concentrations at which the decrease in absorbance at 517nm by 50% for 0.1mM DPPH in methanol at 10minutes after addition of flavonoid in methanol

[2] Vitamin C was tested to compare with flavonoids in its radical scavenging activity

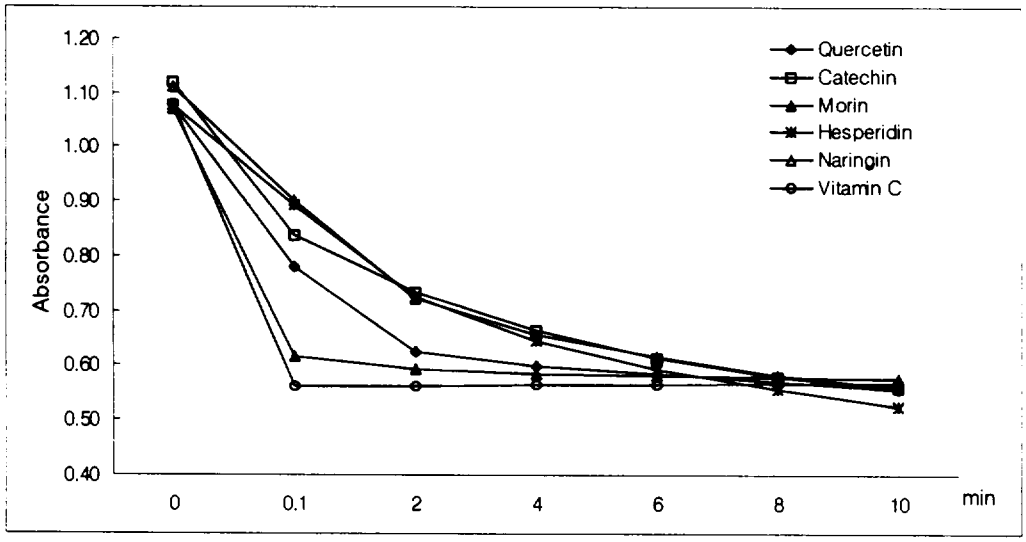


**Fig. 1 Patterns of decrease in absorbance after addition of $SC_{50}$ concentration of flavonoid to 0.1mM DPPH.**

**Table 2. Whole blood platelet aggregation**

| | Control | Quercetin | Catechin | Morin | Hesperidin | Naringin |
|---|---|---|---|---|---|---|
| Maximum($\Omega$)[1] | 12.10±2.05[a] | 9.59±1.07[b] | 10.28±0.87[b] | 10.67±0.77[ab] | 11.64±2.16[ab] | 10.60±1.02[ab] |
| Initial slope[2] ($\Omega$/min) | 7.69±0.90[a] | 6.58±0.70[b] | 7.34±0.83[ab] | 7.73±0.95[a] | 7.84±0.83[a] | 7.73±0.95[a] |

[1] Maximum aggregation in ohm at the point where aggregate dissociated.

[2] Initial slope is ohm change for the first one minute. Values in the same row not sharing the same superscript differ. ($P<0.05$) Values are means±SD of ten samples.

significantly different compared with the control ($P<0.05$). Quercetin only significantly decreased the initial slope of platelet aggregation ($P<0.05$), and hesperidin tended to rather increase the initial slope.

Inhibitory effect of flavonoid on platelet aggregation may be involved in oxidative events such as arachidonic acid metabolism and thromboxane production in platelet(Landolfi R et al, 1984). Flavonids may interact with oxidant species formed on platelet activation or flavonoid may decrease in platelet release of superoxide which is a platelet stimulator. Havsteen(Laughton MJ et al, 1991) reported that flavonoid inhibited cyclooxygenase. Grape juice incubated with platelet enhanced platelet release of nitric oxide which is platelet inhibitor(Sauter R et al, 1998). At level of 10-20 $\mu$M quercetin or 50-100 $\mu$M catechin inhibit platelet aggregation in vitro system using human PRP.[5] Considering their scavenging activities in DPPH assay, flavonoids of 100 $\mu$M used in this experiment may be enough for quercetin and catechin, but not sufficient enough for naringin and hesperidin to act as antioxidant.

### 3. Erythrocyte Na Leak

Intracellular Na and K and Na leak are shown in Table 3. Intracelluar Na and K were not different between intact and PMS treated red blood cells. Na leak in intact red blood cell was significantly decreased when treated with quercetin compared to the control or treated with other flavonoids ($P<0.05$). Although there is no statistical difference between groups when treated with PMS, Na leak after treatment of PMS was increased with quercetin, catechin or morin which is strong antioxidant when compared with their intacts, while Na leak was decreased with hesperidin and naringin. There might be some interactions between agents of radical generating and scavenging.

PMS which is used for radical production can readily cross the cell membrane and generate free radical in the presence of NADH causing damage inside of cell.[18] Unlike vitamin E that is localized in the membrane, flavonoid present in the aqueous phase can scavenge intracellular and extracellular free radicals. Some flavonoids such as flavanol appear to pass through biological membrane(Scarbert A et al, 2000). In the present study, PMS did not cause loss of intracellular Na and K compared with those of intact cells. Cells may close channels preventing toxic substance such as PMS to enter cell by the defence mechanism. All of test flavonoid except quercetin caused an increase in Na leak in intact cell, which can not be interpreted as the consequence of oxidative damage. Ouercetin and catechin with strong antioxidant activity potentiated the increase in Na leak by PMS, while Naringing and hesperidin inhibit the increase in Na leak by PMS. Maridonneau et al(1986) reported flavonoids have heterogenous effects such as protective, toxic, biphasic effective or inactive on K loss and lipid peroxidation induced by oxygen free

**Table 3. Intracellular Na and K concentrations and passive Na leak in intact or PMS treated red blood cell**

| | Intracellular (mmole/ ℓ rbc) | | Passive Na leak (mmole/ ℓ rbc/hr) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Na | K | Control | Quercetin | Catechin | Morin | Hesperidin | Naringin |
| Intact | 2.53±0.26 | 87.9±3.27 | 2.71±0.6$^{ab}$ | 2.21±0.4$^{b}$ | 2.89±0.1$^{ab}$ | 3.06±0.9$^{a}$ | 3.20±0.8$^{a}$ | 3.21±0.9$^{a}$ |
| PMS treated | 2.68±0.39 | 90.30±9.20 | 2.95±0.9$^{a}$ | 2.69±0.3$^{ab}$ | 3.09±0.5$^{a}$ | 3.08±0.1$^{a}$ | 3.06±0.2$^{a}$ | 2.81±0.4$^{ab}$ |

Values in the same row not sharing the same superscript differ ($P<0.05$)
Values are means±SD of ten samples

radical. Hydroxyl groups in the flavonoid and polarity of flavonoid led a significant impact on the flavonoid-biomembrane interaction, causing membrane leakage in immobilized artificial membrane(Ollila F et al, 2002). Radical scavenging may not be the sole mechanism of flavonoid in controlling Na efflux(Umarova FT et al, 1998). Further studies are needed to clarify that flavonoid can act as antioxidant in chain breaking on membrane lipid peroxidation and prevent oxidative damage.

### 4. TBARS production

TBARS production in platelet rich plasma (PRP) from PMS treated and untreated blood are shown in Table 4 and the comparison of TBARS production and the influence of PMS treatment on the effect of flavonoids are shown in figure 2. All of test flavonoids decreased TBARS production in untreated PRP compared with the control, showing a significant difference in quercetin and hesperidin ($P<0.05$). TBARS production in PMS treated PRP was overall increased compared with the untreated PRP, and the increase in quercetin and hesperidin was two times so that there was no difference between groups.

Diet with catechin and quercetin decreased vitamin E consumption and reduced TBARS production in vitamin E deficient rats(Fremont L et al, 1998). Flavonoids can replace vitamin E as antioxidant to the some extent. Flavonoid rich extract in invitro test reduced TBARS production and LDL oxidation and retarded the lag time of conjugate diene production(Viana M et al, 1996). In the present study, quercetin and hesperidin by themselves reduced TBARS production, but with PMS they potentiated it in producing TBARS. Compared with the effects of PMS on TBARS production, PMS on Na leak was comparatively minor, which may imply that functioning cells such as erythrocyte and platelet have significant defence mechanisms against toxic substance or free radical.

**Table 4. TBARS production in platelet rich plasma**

| | Control | Quercetin | Catechin | Morin | Hesperidin | Naringin |
|---|---|---|---|---|---|---|
| Untreated [1)] | 0.97±0.31$^{a}$ | 0.55±0.05$^{b}$ | 0.68±0.21$^{ab}$ | 0.70±0.06$^{ab}$ | 0.56±0.11$^{b}$ | 0.64±0.05$^{ab}$ |
| PMS treated[1)] | 1.15±0.38$^{a}$ | 1.04±0.60$^{ab}$ | 1.09±0.6$^{a}$ | 1.13±0.71$^{a}$ | 1.24±0.47$^{a}$ | 0.98±0.26$^{ab}$ |

[1)]TBARS concentration expressed in *nmole/ml* platelet rich plasma from intact or PMS treted and untreated blood Values in the same row not sharing the same superscript differ ($P<0.05$) Values are means±SD of ten samples

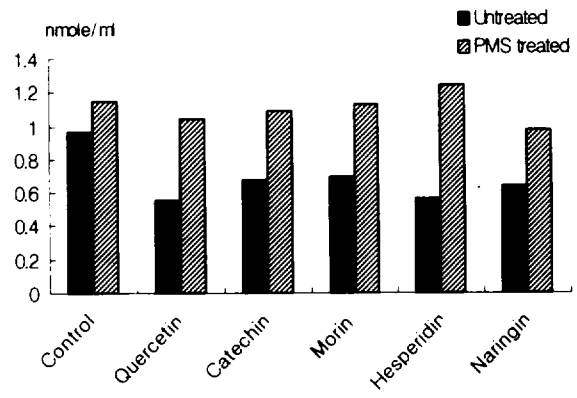


**Fig. 2** Comparison of TBARS production in PRPs from untreated and PMS treated blood

## CONCLUSIONS

The purpose of the present study was to correlate the antioxidant capacity of flavonoids and their effects on biological system. Quercetin and catechin have antioxidant activity of 40-400 times of hesperidin and naringin. Quercetin only have consistent antioxidant effect on platelet aggregation, Na leak and TBARS production. All of flavonoids except quercetin caused increase in Na leak, which can not be explained in the antioxidant view point. Recent interests focus on that structural feature of flavonoids such as hydroxyl group and the polarity of flavonoids is associated with its physiological function on biomembrane.

It is yet unclear whether flavonoid really can act as antioxidant in vivo system or whether the dose of flavonoid effective in vitro are achievable in plasma in vivo. More invitro and invo study are needed to determine conclusively which flavonoids are bioavailable and what dose of flavonoids are beneficial for human health. Overall, further studies are necessary to elucidate the favorable mechanism of flavonoids involved in degenerative diseases before recommending therapeutic use.

Acknowledgements:

This work was supported by grant No R02-2001-00718 from the Basic Research Program of the Korea Science & Engineering Foundation

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