Antioxidative Activity of Five Flavones Glycosides from Corn Silk (*Stigma maydis*)

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Abstract

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Five flavones glycosides (i.e., 2''-*O*-α-L-rhamnosyl-6-*C*-3''deoxyglucosyl-3'-methoxyluteolin, ax-5'-methane-3'-methoxymaysin, ax-4''-OH-3'-methoxymaysin, 6,4'-dihydroxy-3'-methoxyflavone-7-*O*-glucoside, and 7,4'-dihydroxy-3'-methoxyflavone-2''-*O*-α-L-rhamnosyl-6-*C*-fucoside) were successfully isolated from corn silk and identified. We evaluated their antioxidant activity among *in vitro* assay systems. Most of the flavones glycosides showed a high antioxidant activity in a lecithin liposome system, as well as a strong scavenging activity against radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide, and hydroxyl radicals. The study indicated that the corn silk flavonoids, especially 6, 4'-dihydroxy- 3'-methoxyflavone-7-*O*-glucoside, may be beneficial natural food antioxidants.

Keywords: flavonoids; antioxidant; radical scavenger

Antioxidant elements in food play an extremely important role in preventing flavour and nutrition losses. Consumers all over the world are becoming increasingly conscious of the nutritional value and safety of foods and their ingredients (Labuza 1971; Shahidi *et al*. 1992). At the same time, preference for natural foods and food ingredients has been increasing because they are generally believed to be safer, more healthy, and less subjected to hazardous elements than the foods containing artificial food additives (Yu & Cui 2001). Natural antioxidants, especially phenolics, are safe and bioactive. In recent years, considerable attention has been directed towards the identification of plants with antioxidant ability that may be used for human consumption.

Flavonoids occur ubiquitously in plant foods and are important constituents of the human diet, being a class of widely distributed phytochemicals with antioxidant and biological activities (Muthia & Paramasivam 2004). Flavonoids are phenolic substances consisting of two phenolic rings connected by a three-carbon unit, which, along with an oxygen atom, form the heterocyclic ring. Flavonoids are systematically classified into subgroups, including flavones, isoflavones, flavonols, flavanones, flavanonol, isoflavanone, chalcone, dihydrochalcone, aurone, flavanols, anthocyanidin, and other flavonoids, which are characterised by differences in the third ring structure (Li *et al*. 1990; Gao *et al*. 1998a,b; Kazuki *et al.* 1998; Liu *et al.* 2002).

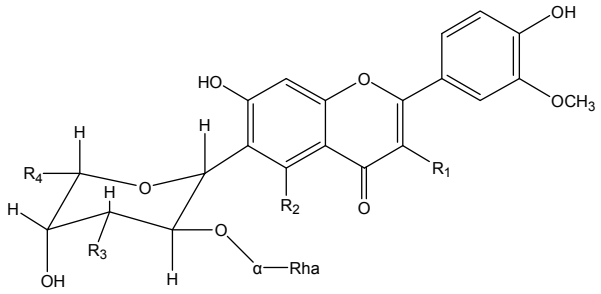
Corn silk is made up of the stigmas and styles of the maize plant from the grass (Gramineae) family. Corn silk has been used in traditional Chinese medicine for the treatment of hypertension, hepatitis, tumours, and hyperglycemia, among others (Li *et al*. 1995; Liu 1995; Ma & Gao 1998). The compounds present in corn silk exhibit immune-enhancing effects, as well as diuretic, cholagogic, and demulcent functions (Namba *et al.* 1993; Tang & Ding 1995). As a soothing diuretic, it is mainly used clinically for the treatment of urethritis, cystitis, nephritis, lithiasis (urinary stone), gonorrhea, and prostatitis in China (Wang & Guo 1991).

Corn silk is an excellent source of many bioactive compounds, including flavonoids, saponin, alkaloids, tannins, chlorogenic acid, phytosterols, allantoin, and so on (Bushman 2002). To date, several flavonoids, such as maysin, apigmaysin, and 3-methoxymaysine, ax-4-OH-maysin, among others, have been isolated and identified from corn silk (Waiss *et al.* 1979; Snook *et al.* 1995; Ren *et al.* 2009). Recently, the authors successfully isolated and identified five flavones glycosides from corn silk, including 2''-*O*-α-L-rhamnosyl-6-*C*-3''-deoxyglucosyl-3'-methoxyluteolin (Compound I), ax-5''-methane-3'-methoxymaysin (Compound II), ax-4''-OH-3'-methoxymaysin (Compound III), 6,4'-dihydroxy-3'-methoxyflavone-7-*O*-glucoside (Compound IV), and 7,4'-dihydroxy-3'-methoxyflavone-2"-*O*-α-L-rhamnosyl-6-*C*-fucoside (Compound V) (Figure 1) (Yi *et al.* 1997). Nevertheless, the capacity of the five flavones glycosides to act as antioxidants has not yet been reported. In this regard, the present study was carried out to determine their antioxidant activity in sin *in vitro* assay systems.

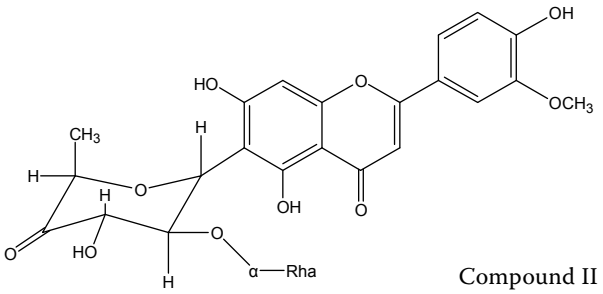
MATERIAL AND METHODS

General. UV spectra were measured on a Shimadzu UV 1601 (Shimadzu, Kyoto, Japan). Mass spectra were collected on a Micromass ZMD LC/MS spectrometer (Waters, Milford, USA) operated in ESI negative mode. The ^{1}H, ^{13}C, DEPT, and HMQC nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avanced 500 MHz NMR spectrometer, using standard pulse sequences. Chemical shifts were reported on the δ scale in parts per million downfield from TMS. TLC was carried out on a precoated silica gel 60 F_{254} plates (Merck, Darmstadt, Germany), developed with chloroform-methanol-water-acetic acid (70:30:10:1, v/v). Chromatography was performed on polyamide column (Marine Chemical Factory, Qingdao, China) and Toyopearl HW-40 (TOSOH, Yamaguchi Prefecture, Japan).

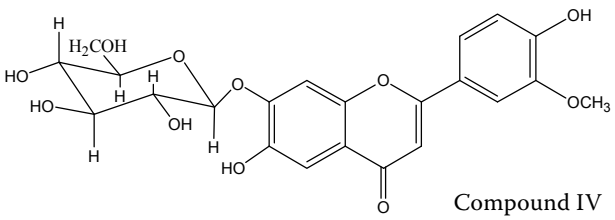
Materials and chemicals. Food-grade soya bean lecithin was purchased from Central Soya Co. (Cincinnati, USA). The five flavones glycosides from corn silk were self-provided in a laboratory (Henan University of Technology, Zhengzhou. China) (HPLC purity 95%). Butylated hydroxytoluene (BHT), dimethyl sulfoxide (DMSO), 1,1-diphenyl-2-picrylhydrazyl (DPPH), linoleic acid (99%), luminal, and phenanthroline were purchased from Sigma-Aldrich (St. Louis, USA). L-Ascorbic acid (vitamin C – Vc), ferrous sulphate, thiobarbituric acid (TBA), trichloroacetic acid (TCA), thiocyanate amine, copper sulphate, potassium phosphate, sodium carbonate, ethylenediamine tetracetic acid (EDTA), hydrochloric acid, hydrogen peroxide, boric acid, sodium borate, methanol, and ethanol were purchased from Huashuo Fine Chemicals Co., Ltd. (Shanghai, China). Butylated hydroxytoluene (BHT) was dissolved in methyl alcohol (0.5 mg/ml); luminol was dissolved in modicum 0.1 mol/l Na_2CO_3 and diluted with distilled water (1 mmol/l); 0.05 mol/l buffer bicarbonate (CB-L, pH 10.2) was prepared when needed using distilled water containing 0.1 mmol/l of EDTA; 1 mmol/l pyrogallol was dissolved in modicum 1 mmol/l HCl and diluted with distilled water containing 0.1 mmol/l of EDTA;



Compound I: R_1 = H, R_2 = OH, R_3 = H, R_4 = CH_2OH
Compound III: R_1 = H, R_2 = OH, R_3 = OH, R_4= CH_3
Compound V: R_1 = OH, R_2 = H, R_3 = OH, R_4 = CH_3



Compound II



Compound IV

Figure 1. Structures of Compounds I–V

0.1 mmol/l Vc and 33.3 mmol/l H_2O_2 were prepared using 0.05 mol/l phosphate buffer (pH 6.2); 1 mmol/l phenanthroline was prepared using distilled water in water bath; and 1 mmol/l $CuSO_4$ and 0.05 mol/l boric acid borax buffer solution (BB, pH 9.0) were prepared using distilled water.

Extraction and isolation. A total of 8 kg of corn silk powder was divided into 4 portions, then each portion (2 kg) was extracted 3 times at room temperature using 5 l of 80% v/v ethanol/water (3 × 5 l). The three extracts of each portion were combined and concentrated to 1.5 l (745 g dry weight) at 45°C under reduced pressure using rotary evaporator, then washed three times with 500 ml of petroleum ether and then extracted three times using 500 ml of ethyl acetate. These three extracts of each portion were then combined and concentrated to 160 ml (4.8 g dry weight) at 45°C under reduced pressure.

Aliquots of 100 ml of water were added to the above given solutions and the resulting mixtures were fractionated using polyamide column chromatography (100 mesh, 40 × 600 mm), eluted with ethanol-water (0–60%), and monitored by thin layer chromatography (TLC) performed on GF_{254} silica gel plates. Four fractions per portion were collected (1203, 505, 146, 34, 87 mg dry weight, respectively) and independently further fractionated on a Toyopearl HW-40 column (35 × 500 mm) with 10, 20, 30, and 40% ethanol, respectively, to give Compounds I–V (567, 232, 48, 19, 32 mg dry weight, respectively) in > 95% purity as analysed by HPLC.

Lipid peroxidation assay. Using ultrasound treatment to allow for fully homogeneous emulsions (surrounded by ice water protection), 8 g of soy lecithin was scattered in 1000 ml deionised water to prepare the liposomes. For the experimental group, the liposomes (10.0 ml) were placed into a 50 ml conical flask, with 0.05–0.20 ml of the tested samples already dissolved in methanol. Then, 2.0 µl of 0.05 mol/l $CuSO_4$ was added. For the control group, a generous amount of methanol was added to the tested samples, placed in water bath thermostatic oscillator at 100 times/min and 37°C, to determine the absorbance according to the procedure described in McDonald and Hultin (1991), Huang and Frankel (1997), Heinonen *et al*. (1998), and Conception *et al.* (1999) (UV-spectrophotometric method). In the test tubes, 0.1 ml of the each above mentioned antioxidant solution and 5 ml of absolute methanol were thoroughly mixed with absolute methanol at room temperature as the blank. The absorbance of the mixture was measured at 234 nm. The data determination was first performed before oscillation, after which the data determination was performed every 12 hours. Once the maximum value was reached in the control group, the determination was repeated onece or more times.

DPPH assay. To measure antioxidant activity, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging assay was carried out according to the procedure described by Hu (1997) and Gao *et al.* (2002), with slight modifications. Briefly, the DPPH radical-scavenging activity was measured in a reaction mixture containing 7.8 ml of 0.025 mg/ml DPPH radical solution and 0.2 ml of each one of the tested samples (1.25–40 µg/ml) dissolved in absolute methanol. The blank was measured using methanol to replace the tested samples in the reaction solution. The solution was rapidly mixed and incubated in a water bath at 25°C for 50 minutes. The scavenging capacity was then measured by monitoring the decrease in absorbance at 515 nm. The DPPH radical-scavenging activity was calculated according to the following formula:

$$[\text{DPPH}^{\cdot}]\text{scavenging ratio}(\%) = [1 - A_{\text{sample}}/A_{\text{control}}] \times 100\%$$

The scavenging capacity was expressed as IC_{50}, which was defined as the concentration of the tested sample required for the inhibition of the [DPPH•] radicals formation of by 50%.

Superoxide anion assay. The superoxide anion $[O_2^-]$-scavenging ability of five flavones glycosides was determined by the pyrogallol autoxidation system according to a method described previously (Wang *et al*. 1997; Fan *et al.* 1998; Heinonen *et al*. 1998). The reaction mixture contained 20 µl of pyrogallol (0.05 mmol/l), 970 µl of solution mixed with luminol (0.05 mmol/l), and 10 µl of different concentrations of the tested samples dissolved in bicarbonate buffer (pH 7.8) with 0.1 mmol of EDTA. The integral intensity of chemiluminescence (CP_{6s}), for every 6 s within 1 min was recorded using *R*-procedure (at high voltage of 800 V, discrimination voltage 0.2 V, 35°C) at the beginning of the luminous reaction. Maximum CP_{6s} was used to calculate the scavenging ratio. The blank substituting for methanol in the tested samples was measured in the same way. The final volume was always the same (1 ml) for all assays. The intensity of chemiluminescence (CL) was simultaneously recorded and processed with a SHG-D instrument. The $[O_2^-]$-scavenging activities of the

tested samples were calculated according to the following formula:

$$[O_2^-] \text{ scavenging ratio (\%)} = [(A_0 - A_S)/A_0] \times 100\%$$

where:

A_0 – chemiluminescence value of experimental group

A_S – chemiluminescence value of the blank group

The scavenging capacity was expressed as IC_{50}, which was defined as the concentration of the tested samples required for the inhibition of the $[O_2^{\bullet}]$ radicals formation by 50%.

Hydroxyl radical assay. The determination of hydroxyl radical [$^{\bullet}$OH] was exerted according to the same method used for $[O_2^-]$. Briefly, the assay solutions were added to a test tube in the following sequence: 20 µl of Vc (0.1 mmol/l), 50 µl of $CuSO_4$ (1 mmol/l), 50 µl of phenathroline solution (1 mmol/l), 830 µl bicarbonate buffer, and 50 µl of H_2O_2 (33.3 mmol), and finally the volume was made up to 1.06 ml exactly with methanol. The integral intensity of chemiluminescence every 6 s was recorded in succession using *R*-procedure with a biological chemiluminescence analyser (SHG-D; Shanghai Shangli Detecting Instrument Factory, Shanghai, China). The integral intensity CP_{6s} at the sixth 6 s was calculated. The blank was measured in the same way using methanol to replace the samples in the reaction solution. The IC_{50} determination was the same as that in the $[O_2^-]$ determination (Wang & Liu 1989; Lim *et al*. 1992; Frankel *et al.* 1998; Zhao 2002).

Statistical analysis. All experiments were conducted in three independent replicates at least. The data are expressed in terms of mean ± SD.

Table 1. ^{13}C NMR chemical shift assignments and DEPT of compounds

Carbon assignment	Compound I	Compound II	Compound III	Compound IV	Compound V
C-4	182.2(C)	182.3(C)	181.9(C)	177.2(C)	181.7(C)
C-2	164.0(C)	163.7(C)	163.6(C)	163.0(C)	163.3(C)
C-7	163.2(C)	162.6(C)	163.1(C)	161.2(C)	159.1(C)
C-5	157.9(C)	160.2(C)	159.3(C)	106.0(CH)	130.2(CH)
C-9	156.2(C)	156.8(C)	156.8(C)	158.7(C)	156.7(C)
C-4'	150.9(C)	148.2(C)	148.2(C)	148.1(C)	148.2(C)
C-3'	148.1(C)	151.9(C)	150.9(C)	150.4(C)	141.7(C)
C-1'	121.3(C)	121.5(C)	121.6(C)	121.7(C)	121.1(C)
C-6'	120.5(CH)	120.6(CH)	120.5(CH)	120.0(CH)	120.3(CH)
C-5'	115.8(CH)	116.0(CH)	116.0(CH)	115.8(CH)	115.9(CH)
C-2'	110.0(CH)	110.2(CH)	110.2(CH)	110.0(CH)	110.0(CH)
C-6	113.0(C)	107.6(C)	109.1(C)	158.5(C)	109.1(C)
C-10	105.1(C)	103.3(C)	103.3(C)	108.0(C)	102.9(C)
C-3	103.5(CH)	103.7(CH)	103.7(CH)	104.5(CH)	136.3(C)
C-1'''	102.2(CH)	99.3(CH)	100.7(CH)		100.6(CH)
C-8	95.1(CH)	93.5(CH)	94.1(CH)	98.5(CH)	94.8(CH)
C-3''	30.1 (CH)	78.3(CH)	76.0(CH)	75.7(CH)	76.0(CH)
C-2”	75.2 (CH)	78.2(CH)	73.9(CH)	73.7(CH)	74.0(CH)
C-5”	77.6(CH)	75.6(CH)	72.3(CH)	77.6(CH)	72.2(CH)
C-4'''	73.7 (CH)	71.5(CH)	71.6 (CH)		71.5(CH)
C-1'''	64.7(CH)	71.3(CH)	71.5(CH)	104.5(CH)	71.3(CH)
C-2'''	69.9(CH)	70.5(CH)	70.7(CH)		70.6(CH)
C-3'''	70.0(CH)	70.3(CH)	70.6(CH)		70.4(CH)
C-5'''	69.2 (CH)	69.0(CH)	68.3(CH)		68.2(CH)
C-4”	67.2(CH_2)	206.3(C)	74.1(CH)	69.7(CH)	73.6(CH)
C-6'''	17.2(CH_3)	17.4(CH_3)	17.8(CH3)		17.6(CH3)
C-6”	60.8(CH_2)	19.1(CH_3)	17.1(CH_3)	60.9(CH_2)	17.5(CH3)
OCH_3	55.9(CH_3)	56.1(CH_3)	56.1(CH_3)	56.0(CH_3)	56.0(CH3)

RESULTS AND DISCUSSION

Structure identification

Five flavonoid monomers (Compounds I–V) were isolated and identified as the major products of the column separation. Among which I (2''-*O*-α-L-rhamnosyl-6-*C*-3''-deoxyglucosyl-3'-methoxyluteolin), II (ax-5-methane-3-methoxymaysin), III (ax-4-OH-3-methoxymaysin), IV (6,4'-dihydroxy-3'-methoxyflavone 7-*O*-glucoside), and V (7,4-dihydroxy-3-methoxyflavone-2-*O*-α-L-rhamnosyl-6-*C*-fucoside) had been previously isolated and identified by Ren and Ding (2004, 2007) and Ren *et al.* (2009) (^{13}C NMR and DEPT of five flavones see Table 1, and their chemical structure see Figure 1). However, this is the first report on their antioxidant activity.

Effect of flavonoids on lipid peroxidation

Generally lipid peroxidation (LPX) is an oxidative process that occurs at low levels in all human cells and tissues. Membrane phospholipids are the target materials of lipid peroxidation. Lecithin liposomes, artificial fat double millirod 0.03–0.1 μm in diameter, are used to imitate the biomembrane (Zhao 2002). This method is extensively used in the lipid peroxidation in vitro test (Lim *et al.* 1992). Conjugated diene hydroperoxides (CD-POV), showing absorptions at 230–235 nm, were produced in the process of unsaturated fatty acids oxidation. Therefore, LPX can be estimated by the formation of the oxidative product using colorimetric methods. The content of CD-POV in the process depends on the rate of breakdown and formation. The speed of formation was greater than that of breakdown in the initial stage of oxidation; thus, the reverse occurred in the latter stage. Therefore, the extent of lipid peroxidation can be represented by the content of CD-POV, and the effect of antioxidants on the oxidation process can also be measured.

Different samples tested showed various antioxidant effects, and all samples tested were able to suppress the generation of CD-POV before 70 h (Figure 2). The peak in the blank appeared roughly at 84 h and Compounds I–III roughly at 96–100 h and their peaks were weaker than that of the blank. During the tested time, both compounds IV and V showed a remarkable ability to inhibit the formation of CD-POV. The results indicate that the five flavones glycosides from corn silk had a high antioxidant activity. Among these, Compounds II and IV exhibited the greatest antioxidant capability antecedent antecedent, with IC_{50} of 0.5 and 0.3 μg/ml, respectively, close to that of BHT (IC_{50} = 0.37 μg/ml). Their antioxidant activity also exhibited obvious dose-effect relations (Figure 3). The IC_{50} decreased in the following order: Compound I, Compound III, rutin, Compound V, Compound II, BHT, and Compound IV (Table 2).

[DPPH•]-scavenging activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH), in a stable radical-generating procedure, can respond purple with different intensity and light absorptions at 515 nm when dissolved in methanol at different concentrations. Absorbance decreases when radical scavengers are added into DPPH solutions. Thus, the scavenging effect on the DPPH free radical can be detected by determining the absorption at 515 nm.

The scavenging capacity increased with increasing concentrations of the tested samples, espe-

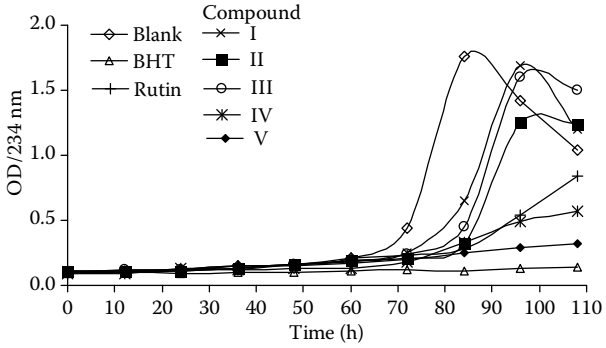


Figure 2. Effects of the tested samples (10 μg/ml) on lipid peroxidation measured at 234 nm

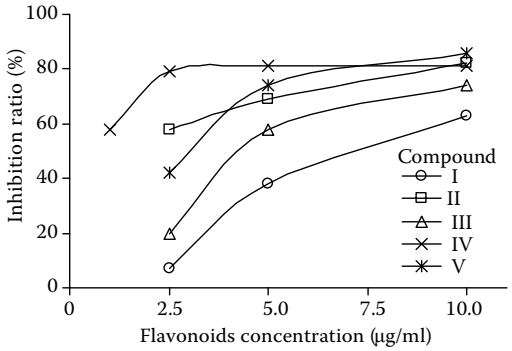


Figure 3. Effect of the tested samples concentration on lipid peroxidation

Table 2. IC_{50} of the tested samples inhibiting LPX and scavenging free radicals

	LPX	[DPPH•]	$[O_2^{-\bullet}]$	[•OH]
Rutin	3.8 ± 0.16	3.2 ± 0.12	10.6 ± 0.38	8.3 ± 0.25
BHT	0.4 ± 0.01	4.2 ± 0.17	ND	ND
Vc	ND	ND	14.8 ± 0.42	ND
Compound I	7.1 ± 0.31	83.8 ± 2.02	CD	CD
Compound II	0.5 ± 0.01	44.3 ± 1.58	10.5 ± 0.34	7.8 ± 0.28
Compound III	4.9 ± 0.21	50.5 ± 1.46	12.0 ± 0.41	10.5 ± 0.39
Compound IV	0.3 ± 0.01	0.3 ± 0.01	5.0 ± 0.21	1.9 ± 0.06
Compound V	2.9 ± 0.14	47.9 ± 1.37	3.4 ± 0.17	21.0 ± 0.85

Each value represents the mean ± SD of triplicate measurements; IC_{50} values expressed as μg/ml; ND – not detected; CD – considerable IC_{50}

cially Compound IV (Figure 4). Among the tested samples, Compound IV had the highest [DPPH•]-scavenging activity (IC_{50} = 2.7 μg/ml), which was much greater than that of BHT (IC_{50} = 4.2 μg/ml) or rutin (IC_{50} = 3.4 μg/ml), and also much greater than those of the other flavonoids (1.9 μg/ml IC_{50} of quercetin, luteolin, rutin, hydroxygenkwanin and kaempferol 7.0 μg/ml, while [DPPH•]-scavenging activity of the other 15 flavonoids can be neglected at the concentrations tested (Li *et al*. 2008). Compound I was the weakest, and no obvious differences were observed between Compounds II, III, and V (Table 2).

$[O_2^-]$-scavenging activity

The superoxide anion $[O_2^{-\bullet}]$ was produced by the autoxidation of pyrogallol in alkali solution, which stimulated luminol emission of λ = 430 nm chemiluminescence. A linear relation exists between the intensity of light emission and $[O_2^-]$ quantity in a certain concentration range. The effects of radical scavengers on superoxide anion radicals can be determined by the intensity of the light.

Great differences were observed in corn silk flavonoids scavenging $[O_2^-]$ activity. Compound I had a little effect on $[O_2^-]$ scavenging; Compounds IV and V had stronger effects, their IC_{50} values being 5.0 μg/ml and 3.4 μg/ml, respectively, far below those of Vc (IC_{50} = 14.8 μg/ml) and rutin (IC_{50} = 10.6 μg/ml), also much stronger than the other flavonoids (0.2 μg/ml IC_{50} of 10 flavonoids 23 μg/ml, while $[O_2^-]$-scavenging activity of the other 10 flavonoids could be neglected at the concentrations tested (Li *et al*. 2008); they also exhibited obvious dose-effect relations (Figure 5). $[O_2^-]$-free-radical-scavenging ability decreased in the following order: Compound V, Compound IV, rutin, Compound II, Compound III, Vc, and Compound I (Table 2).

[•OH]-scavenging activity

The phenanthroline-$CuSO_4$-Vc-H_2O_2 system is commonly applied to study the hydroxyl radical [•OH]. [•OH] causes phenanthroline to radiate stably for 1 min, and it exhibits a better relation between the luminous intensity and [•OH] concentration (Wang & Liu 1989; Heinonen *et al.* 1998).

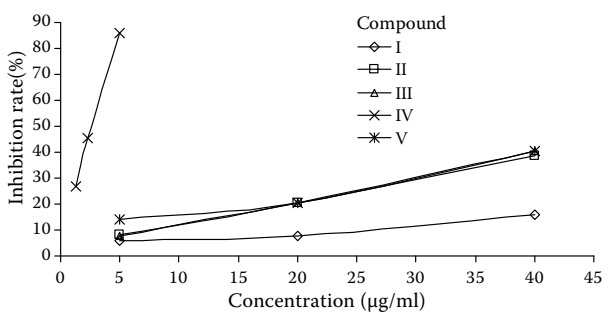


Figure 4. [DPPH•]-scavenging activity of the tested samples

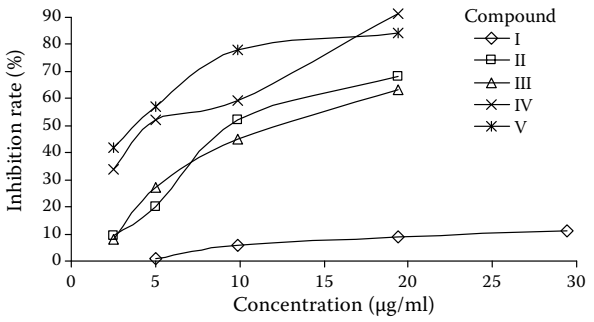


Figure 5. $[O_2^{\bullet}]$-scavenging activity of the tested samples

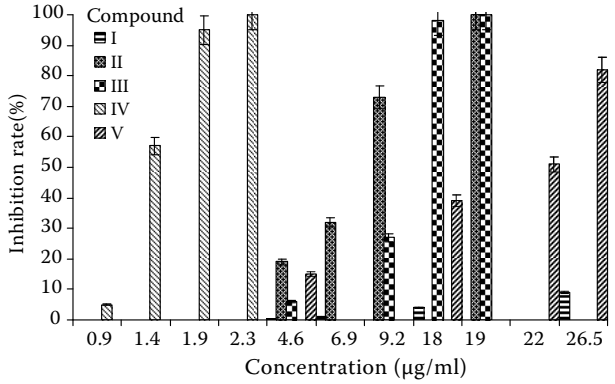


Figure 6. [•OH]-scavenging activity of the tested samples

Great differences could be observed between corn silk flavonoids scavenging [•OH] (Figure 6). Compound I had a little effect on [•OH]. Compound IV (IC_{50} = 1.9 μg/ml) had a stronger effect, but far lower than were those of rutin (IC_{50} = 8.3 μg/ml) and other tested samples. Compound II, Compound III, and rutin had similar effects on [•OH]. The [•OH]-scavenging ability decreased in the following order: Compound IV, Compound II, rutin, Compound III, Compound V, and Compound I (Table 2).

CONCLUSIONS

In recent years, many papers have been published on the antioxidant activity of flavonoids. However, the antioxidant efficacy of the five flavones glycosides from corn silk has not yet been reported, possibly due to the difficulty in obtaining their monomers. The five flavones glycosides exhibited different inhibiting capability toward LPX and different scavenging capability toward different free radicals. Among these, Compound I had the weakest effect, and Compound IV had the strongest effect, and in some aspects even much stronger than rutin, Vc, or BHT. Our results indicated that the five flavones glycosides, especially 6,4'-dihydroxy-3'-methoxyflavone-7-*O*-glucoside (Compound IV), could be a beneficial natural food antioxidant.

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