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Comparison of the Antioxidant Activity of Maysin (C-Glycosylflavone) and Other Flavonoids

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The antioxidant activity of maysin (C-glycosylflavone), isolated from the silks of *Zea mays* L., was assessed relative to three other flavonoid compounds *i.e.*, rutin, quercetin and luteolin. Except for luteolin maysin was the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenger among the flavonoids tested at 0.5 mg/mL peroxidase activity assays revealed that maysin does not show any measurable difference in peroxidase activity with increasing concentrations. At the lowest concentration tested (0.1 mg/mL), the nitroblue tetrazolium (NBT) reduction activities of rutin, quercetin, luteolin and maysin were found to be 5.12, 1.98, 4.66 and 12.02 %, respectively. Finally, maysin exhibited higher lipid peroxidase inhibition by the thiobarbituric acid method at all concentrations tested.

Keywords: Maysin, *Zea mays* L., Antioxidant, Flavonoids.

INTRODUCTION

Maize (*Zea mays* L.) is the world 3 major crops together with rice and wheat and it annual production including the purposes of grain and silage was about 12×10^9 bushels in US in 2010-2012 year[1]. Maize is an important food crop, serving as a source of syrup, dextrose, dextrin, lactose and sucrose. Furthermore, maize fermented is used as the production of various staple medical products such as penicillin, bacitracin and neomycin[2].

The silk of maize has also been used in various applications in traditional medicine[3,4]. Several prior studies have reported that maize exhibits antioxidant and anticarcinogenic effects similar to those by white corn polyphenolics such as ferulic and *p*-coumaric acid and their respective derivatives[5-9]. In particular, Snook *et al.*[10,11]. and Kim *et al.*[12]. isolated and identified maysin [2'-O-a-L-rhamnosyl-6-C-(6-deoxy-xylose-hexose-4-ulosyl)luteolin] and related flavonoid analogs from maize silk.

To date, only the growth-inhibitory and antibiotic effects of maysin against corn earworm have received much attention[10,13]. However, a recent study showed that maysin also possesses significant antioxidant properties[14]. Kim *et al.*[15] reported that maysin showed 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, primarily through the glycosylation of two sugar moieties *i.e.*, ketofucose and rhamnose.

Although several studies have investigated the general properties of maysin. The specific antioxidant activities of maysin and its analogs remain poorly characterized. The aim of this study was to investigate the antioxidant activity of maysin isolated from the maize silk by using a variety of techniques and was to compare these to the activities of three other popular flavonoid compounds.

EXPERIMENTAL

Three flavonoids (rutin, quercetin, luteolin, Fig. 1) standards, nitro blue tetrazolium, DPPH, trichloroacetic acid and thiobarbituric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents including methanol, ethanol, ethylacetate and dichloromethane were also purchased from Sigma-Aldrich.

Extraction and isolation of maysin from silk of maize: Maysin (C-glycosylflavone) used in the present study was extracted, isolated and identified by using the previous study[15]. Corn silks were collected and soaked immediately in 100 % MeOH. Silks were then ground using a high-speed homogenizer and filtered. The filtrate was concentrated with a vacuum evaporator at 35-40 °C and then dissolved in distilled water. Subsequently, CH_2Cl_2 added to the concentrated aqueous solution to remove chlorophyll and lipids and the solution evaporated to remove remaining CH_2Cl_2. The final concentrated solution (50 mL) was submitted to preparative reversed-phase column chromatography (C18, 55-105 mm, 25 mm × 54 cm) and washed using distilled water (2 × 250 mL) under nitrogen to remove sugars and water-soluble pigments. Bound

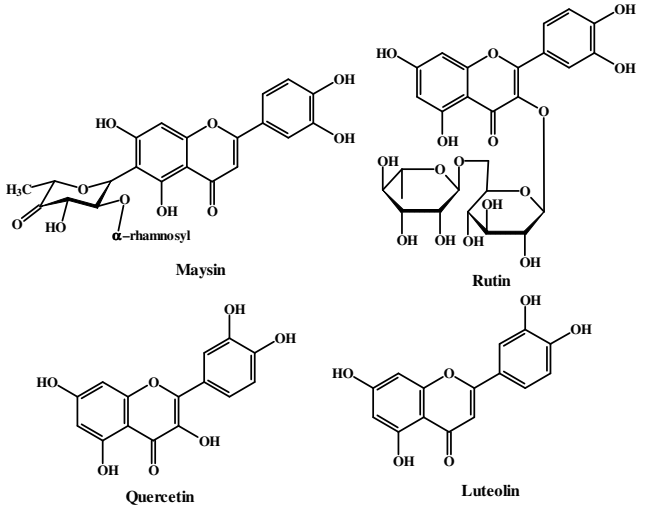

Fig. 1. Chemical structures of Maysin and 3 other flavonoids

materials were eluted from the column by using MeOH and the eluents were then pooled and evaporated to dryness. The residue was dissolved in MeOH and subjected to silicic acid column chromatography. Elution was performed using a mixture of CH_2Cl_2 and EtOAc (50:50, 3 × 250 mL) and the 100 % EtOAc eluents were collected as fractions. The 100 % EtOAc fractions were combined and evaporated to dryness. The final purification step was performed using C18 column (12.7 × 110 cm) chromatography with 50 % (v/v) MeOH as the eluting solvent to obtain pure maysin[15].

DPPH radical-scavenging assay: The free radical-scavenging activities of the maysin purified in the present study and three other flavonoids were measured using the DPPH assay. Reaction mixtures were prepared by mixing 2.5 mL of DPPH solution (0.35 mM DPPH dissolved in 50 % EtOH) and 0.2 mL of each sample. Reaction was incubated for 10 min at room temperature and the changes in DPPH absorbance at 517 nm were measured with a spectrophotometer. The anti-oxidant activity was calculated in terms of the per cent inhibition caused by the hydrogen donor activity of each sample.

Nitro blue tetrazolium (NBT) reduction assay: Borosilicate tubes containing the reaction solution (3 mL of assay buffer, 60 µL of enzyme, 30 µL of riboflavin) were illuminated using 20-W Sylvania Gro-lux fluorescent lamps at 25 °C. After 7 min, the absorbance at 560 nm of the blank and reaction solutions was measured in a UV/visible spectrophotometer. Activity was measured as "per cent NBT inhibition" and calculated as follows: Antioxidative activity (%) = (1-A/B) where A is the absorbance of the sample of interest and B is the absorbance of the control.

Peroxidase activity assay: Borosilicate tubes containing the samples and the reaction solution (0.5 mL of 0.3 % hydrogen peroxide, 0.5 mL of 1 % *o*-phenylenediamine and 7.9 mL of sodium phosphate buffer, pH 6.8) were incubated at room temperature (~25 °C) for 0.5 h and the absorbance of the solution was measured at 430 nm. The peroxidase activity was calculated in terms of percent inhibition as like the calculation of the NBT assay.

Lipid peroxidase assay (LPA): About 30 mM of linoleic acid solution was prepared with a mixture of 100 mM phosphate buffer and EtOH (v/v, 4:1) and it was used as the substrate solution. Mixture of 20 mL of substrate solution, 19.2 mL of 100 mM phosphate buffer and 0.8 mL of each sample solution was shaken at 100 rpm at 40 °C for 24 h. This solution was used as lipid peroxidase assay reaction mixture. Solutions containing 2.0 mL of lipid peroxidase assay reaction mixture, 1.0 mL of 35 % trichloroacetic acid (TCA) and 2.0 mL of 0.75 % thiobarbituric acid (TBA) were mixed by vortexing for 30s and incubated in a 95 °C water bath for 40 min. Then, 1.0 mL of acetic acid and 2.0 mL of chloroform were added to the cooled borosilicate tube. Following agitation, the solutions were then centrifuged at 3,000 rpm for 5 min. The absorbance of the supernatants was measured at 532 nm with a spectrophotometer and the inhibitory activity was calculated as same as the NBT assay.

Ferric-reducing antioxidant power (FRAP) assay: The reducing ability of each sample was determined using the FRAP assay[16]. Briefly, the FRAP reagent prepared by mixing 300 mM sodium acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl and 20 mM $FeCl_3$ solution at a ratio of 10:1:1 (v/v/v). Ferric-reducing antioxidant power reagent was prepared fresh daily and pre-warmed in a 37 °C water bath prior to use. Then, 0.1 mL of extract was mixed with 1.8 mL of FRAP reagent and 3.1 mL of ultrapure water (HPLC grade). The reaction mixture was incubated at 37 °C for 0.5 h and then, the absorption of the mixture was measured at 593 nm with a spectrophotometer. Ferric-reducing anti-oxidant power values were calculated on the basis of $FeSO_4$ standard curve (100-1000 µM) and reported as µmoles of Fe(II) per gram of dry weight.

Trolox equivalent antioxidant capacity (TEAC) assay: The TEAC assay which measures the reduction of the radical cation 2,2'-azino-*bis* (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by some antioxidants was conducted by using the prior study with some minor modifications[16,17]. Briefly, the $ABTS^+$ radical cation was generated by reacting 7 mmol/L ABTS with 2.45 mmol/L potassium persulfate. The reaction mixture allowed to stand at room temperature (keeping in the dark) for 16-24 h before use and used within 2 days. The $ABTS^+$ solution diluted with methanol to an absorbance of 0.700 ± 0.050 at 734 nm. All samples of interest were diluted appropriately to provide 20-80 % inhibition of the blank absorbance. About 50 µL of each of the diluted extracts was mixed with 1.9 mL of diluted $ABTS^+$ solution. Mixtures prepared in triplicate were incubated at room temperature for 6 min and the absorbance at 734 nm was measured with a spectrophotometer. Trolox concentration was calculated on the basis of trolox solution standard curve (100-1000 µM) and reported as µmoles of trolox equivalents (TE) per gram of dry weight.

Statistical analysis: Statistical analyses are performed with the general linear model of the statistical analysis program SAS (version 9.2; SAS Institute Inc., Cary, NC, USA). The experimental design is completely randomized with triplicates. The least significant difference (LSD) test is performed for a 0.05 probability level.

RESULTS AND DISCUSSION

The results of the experiments monitoring the free radical-scavenging abilities of the purified maysin and 3 flavonoid

standards (rutin, quercetin, luteolin) are summarized in Table- 1. The DPPH radical-scavenging assay was performed with the flavonoid compounds at the levels of 0.1, 0.5 and 1 mg/mL. Maysin exhibited free radical-scavenging activities of 5.53, 20.80 and 39.52 % at each of these respective concentrations. Maysin was the most effective DPPH radical scavenger compared to the 3 flavonoids at the level of 0.5 mg/mL and also was secondly effective at the level of 1.0 mg/mL except for only luteolin, which exhibited an inhibition activity of 48.96 %.

In the inhibition of reactive oxygen species (ROS), the purified maysin demonstrated no concentration-dependent peroxidase effects, maintaining a value of 10 % at all three concentrations tested. Overall, rutin and quercetin both displayed higher peroxidase activity than maysin or luteolin (Table-1). The NBT reduction assay revealed significant differences between maysin and the other three flavonoid compounds. The purified maysin usually had a higher NBT reduction activity at the all levels tested. Especially at the lowest concentration tested (0.1 mg/mL), the NBT reduction activity of maysin (12.02 %) was much higher than those of rutin, quercetin and luteolin and it was statistically significant ($P < 0.05$).

Maysin had the lipid peroxidase inhibition of 28.1-42.3 % at the all concentrations tested by the lipid peroxidase assay (LPA) method. Three flavonoids tested had also higher activity at the all concentrations, especially quercetin had a relatively higher inhibition activity of lipid peroxidase compared to the purified masin (Table-1). Therefore, we assume that all the four flavonoids including maysin had a significant inhibition activity of lipid peroxidase.

In the ferric-reducing antioxidant power (FRAP) assay, the antioxidant activity of the four flavonoids ranged from 133.3 µmol Fe(II)/g (luteolin at 0.1 mg/mL) to 1070.1 µmol Fe(II)/g (quercetin at 1.0 mg/mL). Antioxidant activity of maysin was not statistically differed with 3 other flavonoids at the all concentrations tested in the present study. Except for quercetin at 1 mg/mL, it had the significantly higher antioxidant activity compared to others (Table-1). Quercetin (1 mg/mL) showed the highest activity in the (trolox equivalent antioxidant capacity) TEAC assay (203.21 µmol TE/g). Maysin, luteolin and rutin at 1 mg/mL showed reducing activities of 177.99 µmol TE/g, 168.82 µmol TE/g and 135.43 µmol TE/g, respectively and it was statistically differed with one another ($P < 0.05$). Maysin had a relatively high TEAC at the lower level (41.22 µmol TE/g) than those of 3 other flavonoids.

Table-2 shows a regression analysis of flavonoid types and antioxidant activity types. The results from the DPPH, FRAP and TEAC assays showed high correlation values, while those from the peroxidase, NBT and LPA assays showed a relatively low correlation values. Inhibition of free radical-scavenging activity for four flavonoid compounds tested in the present study was usually concentration-dependent, except for the peroxidase assay of the four flavonoids. Furthermore, inhibition of free radical-scavenging activity was also flavonoid types-dependent (Table-2).

The antioxidant capacities of flavonoids including maysin varied with the used method. The prior study[18] performed a systematic assessment of the antioxidant activity of six flavonoid compounds by using several methods and reported

TABLE-1
COMPARISON OF FREE RADICAL SCAVENGING ACTIVITIES OF MAYSIN AND THREE FLAVONOIDS DEPENDING ON THE VARIOUS MEASUREMENT METHODS

Compounds	Concentration (mg/mL)	DPPH	POD	NBT	LPA	FRAP	TEAC
		Inhibition (%)				(µmol Fe(II)/g)	(µmol TE/g)
Maysin	1	39.5 ± 4.2	12.4 ± 0.4	17.5 ± 3.5	42.3 ± 16.1	642.4 ± 33.9	178.0 ± 5.1
	0.5	20.8 ± 0.7	12.0 ± 0.04	16.8 ± 0.9	27.1 ± 9.4	246.0 ± 34.2	76.5 ± 1.1
	0.1	5.5 ± 2.7	11.6 ± 0.2	12.0 ± 1.4	28.1 ± 4.4	144.1 ± 5.22	41.2 ± 4.2
$LSD_{(0.05)}$		5.73	0.53	4.49	22.15	62.90	7.74
Rutin	1	24.7 ± 1.0	24.6 ± 1.0	5.7 ± 0.7	31.5 ± 2.5	633.3 ± 19.0	135.4 ± 22.4
	0.5	13.4 ± 1.2	17.8 ± 7.2	5.6 ± 0.4	22.5 ± 12.2	241.8 ± 10.9	48.4 ± 3.0
	0.1	4.9 ± 1.1	22.7 ± 2.6	5.1 ± 1.6	27.7 ± 5.4	137.1 ± 2.7	32.9 ± 3.2
$LSD_{(0.05)}$		2.19	8.91	2.07	15.67	20.34	26.27
Quercetin	1	37.5 ± 0.2	22.2 ± 7.0	18.1 ± 2.9	58.9 ± 22.9	1070.1 ± 55.0	203.2 ± 6.1
	0.5	20.5 ± 1.0	22.3 ± 3.8	11.6 ± 0.5	55.2 ± 2.1	410.8 ± 24.6	99.3 ± 5.1
	0.1	8.8 ± 0.5	20.8 ± 5.2	2.0 ± 1.1	34.8 ± 6.0	172.1 ± 11.3	37.4 ± 0.9
$LSD_{(0.05)}$		1.35	10.98	3.59	27.39	45.28	9.21
Luteolin	1	49.0 ± 2.7	13.7 ± 5.3	12.4 ± 1.8	37.7 ± 16.7	525.7 ± 26.6	168.8 ± 17.6
	0.5	18.9 ± 0.6	12.7 ± 5.9	9.6 ± 1.6	42.3 ± 8.4	222.7 ± 16.2	69.2 ± 5.9
	0.1	7.5 ± 2.0	6.8 ± 5.6	4.7 ± 0.1	36.9 ± 15.3	133.3 ± 5.1	34.3 ± 1.0
$LSD_{(0.05)}$		3.85	11.12	2.74	27.87	30.58	21.46

TABLE-2
CORRELATION ANALYSIS OF THE DPPH, POD, NBT, TBA, FRAP, AND TEAC ASSAYS CARRIED OUT WITH VARIOUS CONCENTRATIONS OF EACH COMPOUND

Compound	DPPH	POD	NBT	LPA	FRAP	TEAC
Maysin	0.986^{**}	0.831^{**}	0.707^{*}	0.526^{NS}	0.969^{**}	0.976^{**}
Rutin	0.993^{**}	0.121^{NS}	0.251^{NS}	0.620^{NS}	0.973^{**}	0.995^{**}
Quercetin	0.998^{**}	0.21^{NS}	0.237^{NS}	0.237^{NS}	0.982^{**}	0.923^{**}
Luteolin	0.977^{**}	0.499^{NS}	0.920^{**}	0.016^{NS}	0.975^{**}	0.967^{**}

*Significant difference ($P < 0.05$), **Significant difference ($P < 0.01$), NS: Not Significant difference

that significant differences were observed in the values obtained by the same method in different solvents, as well as from different methods in the same solvent. The other previous study[19] identified three major types of antioxidant capacity assays *in vitro*: assays involving hydrogen atom transfer reaction (HAT), such as the oxygen radical-absorbance capacity (ORAC) assay; assays based on electron transfer (ET) reactions such as TEAC; and "other" assays such as chemiluminescence. Previous studies[19,20] have demonstrated that a single determinant of antioxidant activity is not sufficient and that a reliable and accurate assessment of antioxidant capacity can be feasible through the application of a variety of methods. The DPPH, peroxidase, NBT, LPA, FRAP and TEAC assays produced comparable results for the antioxidant capacity of the flavonoid compounds. Maysin, isolated and purified from corn, had high antioxidant activity when compared to the three other flavonoid compounds (rutin, quercetin, luteolin). With the prior study[15], maysin has a higher scavenging activity than other compounds, therefore it has the potential be a potent antioxidant compound. In conclusion, antioxidative activity of maysin varied with the antioxidative assays used, so further studies need to evaluate the biological activities of maysin *in situ* or/and *in vivo* level as well as its application as a nutraceutical.

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