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Effect of Browning on Total Phenolic, Flavonoid Content and Antioxidant Activity in Indian Gooseberry (*Phyllanthus emblica* Linn.)

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ABSTRACT

Browning of Indian Gooseberry (*Phyllanthus emblica* Linn.) for 4 hr caused significant ($p<0.05$) increase in total phenolic from 1,790.4 to 1,923.5 mg gallic acid equivalence/ 100 g fresh weight (FW) and in flavonoid content from 143.1 to 148.2 mg catechin equivalence/ 100 g FW. HPLC analysis indicated that quercetin also increased significantly ($p<0.05$) from 2.8 to 3.4 mg/ 100 g FW after 4 hr of browning. While kaempferol was only found in the 6 hr browning sample (0.2 mg/ 100 g FW). Total antioxidant activity (TAC) as determined by ORAC method increased to 98.3 μmoles Trolox equivalence/ 100 g FW in 6 hr browning sample. It was shown that TAC had a good correlation with quercetin ($r=0.711$). In addition, TAC and quercetin exhibited high correlation with browning index of the Indian Gooseberry ($r=0.720$ and 0.977, respectively). Therefore quercetin, as flavonol compound, could represent changes in flavonoids as well as a good quality indicator for antioxidant activity (ORAC) in Indian Gooseberry.

Key words: Indian Gooseberry, flavonoid, total phenolic, quercetin

INTRODUCTION

Indian Gooseberry (*Phyllanthus emblica* Linn.) is a local Thai fruit with 8-12 m tall, smooth, greenish grey, exfoliating bark tree. The fruits are globose, fleshy, greenish yellow and obscurely 6-lobed, containing 6 seeds. Its clinical properties include reducing LDL-cholesterol and blood-sugar, anti-inflammatory and anticarcinogen. It is rich in vitamin C, inositol, tannin, gallic acid, quercetin and kaempferol (Anonymous, 2006). Flavonoids are polyphenolic which have the diphenylpropane ($C_6C_3C_6$) skeleton (Heim *et al.*, 2002). They are usually found in plants as glycosides (Fuhrman and Aviram, 2002). Flavonoids family includes flavonols, flavones, flavanols, flavanones, anthocyanidins and isoflavones. Flavonols, such as quercetin, kaempferol and myricetin, are the most widely distributed flavonoids. Most fruits contain quercetin glycosides while kaempferol and myricetin glycosides are found only in trace quantities (Hertog *et al.*, 1992). The important health benefits of flavonols and flavonoids are their antioxidative activity which received much attention recently.

Browning reaction occurs in many fruits during processing. The reaction involves phenolics as substrates. Not only enzymatic but also non-enzymatic browning is responsible for

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browning reaction in fruit. Non-enzymatic browning includes metal ion interaction with phenolics, ascorbic acid degradation and Maillard reaction. Oxidation of phenolics in the first enzymatic browning phase leads to the formation of unstable quinine products while in a second non-enzymatic phase, these quinones react with other compounds to form browning pigment (Robards *et al*., 1999). Quinones formed by oxidation in the first phase, however, can react with ascorbic acid or other reducing compounds and regenerate back the phenol form. Browning is therefore inhibited until ascorbic acid is depleted, after that the formation of brown pigments occurs. It should be noted that not all phenolics presented in fruits are polyphenol oxidase (PPO) substrate, e.g. flavonol glycosides, but they can degrade when browning reaction take place (ToMas-Barberan and Robins, 1997).

The objective of this study was to determine the effect of browning in Indian Gooseberry on total phenolic and flavonoid content related to the changes in antioxidant activity of the fruit. In addition, quantitative analysis by HPLC of gallic acid (phenolic), quercetin and kaempferol (flavonol) were determined related to their occurrence with total phenolic and flavonoid content, as well as changes in antioxidative activity of the browning Indian Gooseberry. Changes in antioxidant activity were quantified by the Oxygen Radical Absorbance Capacity (ORAC) assay and 2,2–diphenly–1-picrylhydrazyl radical scavenging capacity (DPPH) assay.

MATERIALS AND METHODS

Browning treatment Fresh Indian Gooseberries were purchased from Nonthaburi Market, Nonthaburi province, Thailand. The fruits were washed, chopped and divided into 4 parts. Controls were immediately dipped in liquid nitrogen to stop browning reaction. The others were placed on petri dish in air conditioning room (25 °C) for browning reaction to take place for 4, 6 and 8 hr then dipped in liquid nitrogen. All of samples were freeze-dried and stored in -40 °C until analysis. Color changes due to browning reaction were measured by Hunter Lab colorimeter (Minolta, Japan) as L, a, b. Browning index (BI) was calculated as $BI = [100 (x - 0.31)] / 0.172$, where $x = (a + 1.75L) / (5.645L + a - 0.3012b)$ (Palou *et al*., 1999).

Total phenolic content Freeze-dried sample (2.00 g) was homogenized and extracted with 20 ml of 80% methanol for 1 min and sonicated for 20 min, followed by centrifugation at 4,598 ¥g for 20 min (RC 5C Plus, Rotor: F 28/36, Sorval Ins., USA.). The process was repeated twice. The methanol fractions were combined and adjusted to 50 ml in volumetric flask. All procedures were done at 4 °C. Total phenolic content was measured by modified colorimetric Folin-Ciocalteu method (Wolfe *et al*., 2003) and expressed as mg gallic acid equivalence/ 100 g fresh weight (FW) for the triplicate extracts.

Flavonoid content Samples were extracted in the same manner as for phenolic content. The flavonoid content was measured by a modified colorimetric method according to Wolfe *et al*. (2003) and expressed as mg catechin equivalence/ 100 g FW for the triplicate extracts.

Analysis by HPLC The extraction for HPLC analysis was modified from Hertog *et al*. (1992). Briefly, freeze-dried sample (0.500 g) was extracted with methanol and HCl. After refluxing for 2 hr, the extract was made up to 100 ml and filtered though a 0.45 μm filter (Regenerated cellulose, Agilent Technologies. USA) prior to injection. The HPLC method was modified from Huber (1998) and Breitfellner *et al*. (2003). Ten μl was injected onto Hypersil ODS (Agilent, USA) column (4×125 mm, 4 μm) protected by guard column at 25 °C oven temperature. Two mobile phases (A: 0.5% H_3PO_4 in water, B: methanol) were used. Gradient program was 95% A isocratic

for 5 min, from 95% to 85% A in 3 min, from 85% to 75% A in 4 min, from 75% to 62% A in 2 min, from 62% to 52% A in 12 min, from 52% to 0% A in 4 min and from 0% to 95% A in 5 min. Flow rate was 1 ml/min for 7 min and changed to 2 ml/min, hold for 28 min before returned to 1 ml/ min. UV detector was used with 280 nm for 7 min and changed to 370 nm, hold for 28 min before returned to 280 nm. Quercetin, kaempferol, catechin and gallic acid (Sigma-aldrich, Inc.) were used as external standard.

Oxygen radical absorbance capacity (ORAC) assay The extraction and measurement method were modified from Prior *et al*. (2003). The freeze-dried sample (0.500 g) was used in extraction step. Two hundred µl fluorescine sodium salt (Sigma-aldrich, Inc.) was used for fluorescence intensity reading by FLUOstar OPTIMA microplate reader for 1 hr or for until the intensity was less than 5% of the initial. The oxygen radical absorbance capacity (ORAC) values were calculated by regression equation between Trolox concentration and net area under the fluorescene curve. Data were expressed as total antioxidant capacity (TAC, µmoles of Trolox equivalents (TE)/ 100 g FW).

2,2–diphenly–1-picrylhydrazyl radical scavenging capacity (DPPH) assay The extraction was the same as for total phenolic assay. The 2,2-diphenyl-1-picryhydrazyl DPPH was determined according to the method of Singh *et al*. (2002). The radical scavenging activity was expressed as the inhibition percentage and was calculated as % radical scavenging activity = (control OD - sample OD/control OD) × 100. The DPPH values were expressed in antiradical efficiency (AE) which calculated as $AE = 1/ IC_{50}$ where IC_{50} was a concentration of samples required to scavenge 50% of free radicals generated by DPPH.

Statistic analysis All data were reported as mean of three samples with two replications each. Statistical analysis was performed using SPSS software (SPSS for WINDOWS 10.0). Differences between variables were tested for significance by using the ANOVA procedure (Duncan's Multiple Range Test, DMRT), using a level of significance of $p < 0.05$.

RESULTS AND DISCUSSION

Browning index, total phenolic and flavonoid content Browning Index (BI) of the browning samples was determined as shown in Table 1. All values were significantly different ($p<0.05$). The total phenolic of the control was 1,790.4 mg gallic acid equivalence/ 100 g FW. An increase of 7.4% in total phenolic was found in 4 hr browning sample. Higher total phenolic, 8.6 and 17.8% increase was found as browning time incresed to 6 and 8 hr, respectively. Increase in total phenolic content could be due to an increase in the complex polyphenol from later phase of browning reaction as suggested by Robards *et al*. (1999).

Table 1 Browning index, total phenolic and flavonoid contents in chopped Indian Gooseberry placed at 25 °C for 0, 4, 6 and 8 hours.

Time (hr)	Browning Index (BI)	Total phenolic (mg gallic acid/ 100 g FW)	Flavonoid content (mg catechin/ 100 g FW)
0	-	1,790.4^{c}	143.1^{c}
4	1.00^{c}	1,923.5^{b}	148.2^{b}
6	2.87^{b}	1,943.9^{b}	142.1^{c}
8	4.11^{a}	2,108.6^{a}	157.8^{a}

Mean values in the same column followed by different superscript are significantly different ($p<0.05$).

The flavonoid content of control sample was 143.1 mg catechin equivalence/ 100 g FW. An increase of flavonoid content 3.6 and 10.3% was found in 4 and 8 hr browning samples, respectively. The 6 hr sample had similar flavonoid content as control ($p \geq 0.05$). Durmaz and Alpaslan (2007) found an increase in total phenolic of roasting apricot when browning increased. Total phenolic and flavonoid of Shiitake mushroom were also increased during heating as browning increased (Choi *et al*., 2006).

Analysis by HPLC Table 2 shows flavonoid (quercetin, kaempferol and catechin) and phenolic (gallic acid) quantity as measured by HPLC. Figure 1 shows chromatogram of quercetin and keampferol in 4 and 6 hr browning sample as determined by HPLC. Quercetin increased as browning reaction progressed. The 4 hr browning sample had 3.4 mg quercetin/ 100 g FW which was 21.4% higher than control sample ($p<0.05$). Kaempferol was only found in trace amount (0.2 mg / 100 g FW) in 6 hr browning sample but not in the 8 hr sample. Kaempferol was increased during browning from 0 to 6 hr and then decreased in 8 hr sample. Kaempferol is normally found in low quantity compared to gallic acid and quercetin for most kind of fruits and vegtables (Hertog *et al*., 1992; Häkkinen *et al*., 1999). Noteworthy, catechin could not be found in any sample. On the other hand, gallic acid decreased significantly ($p<0.05$) from 931.1 to 642.8 mg /100 g FW during the browning period. The 31.0% decrease in gallic acid suggested an enzymatic reaction, such as polyphenol oxidase to occur utilizing gallic acid

Table 2 Quercetin, kaempferol, catechin and gallic acid contents in chopped Indian Gooseberry placed at 25 °C for 0, 4, 6 and 8 hours.

Time (hr)	Quercetin (mg/100 g FW)	Kaempferol (mg/100 g FW)	Catechin (mg/100 g FW)	Gallic acid (mg/100 g FW)
0	2.8^{d}	nd*	nd	931.1^{a}
4	3.4^{c}	nd	nd	642.8^{b}
6	4.2^{b}	0.2	nd	524.9^{d}
8	4.5^{a}	nd	nd	619.1^{c}

Mean values in the same column followed by different superscript letters are significantly different ($p<0.05$).
* nd = not detectable

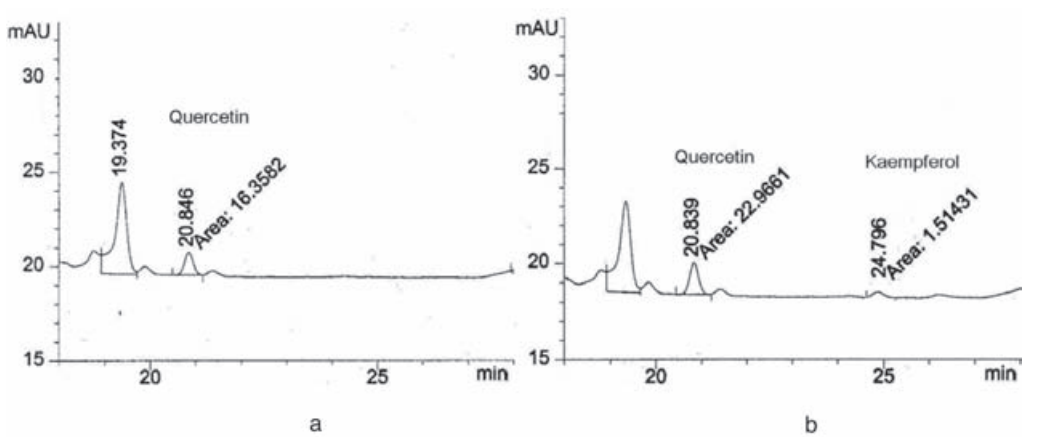

Figure 1 Chromatogram of (a) 4 hours browning Indian Gooseberry and (b) 6 hours browning Indian Gooseberry determined by HPLC.

at the early phase of browning reaction (Robards *et al*., 1999). The decrease in gallic acid might be due to transformation of gallic acid into other phenolic or flavonoid compounds (KulKarni and Aradhya, 2005) which could then convert to other complex polyphenol forms. Phenolic compounds could also degrade during browning reaction (ToMas-Barberan and Robins, 1997).

Antioxidant activity. Total antioxidant activity (TAC) from ORAC method and antiradical efficiency (AE) from DPPH method of browning Indian Gooseberry were shown in Table 3. TAC values increased significantly ($p<0.05$) in all browning samples. The 6 hr sample had the highest value at 98.3 μmoles TE/ 100 g FW, which signified a 25.7% increase in antioxidant activity in brown fruit. The TAC value showed high correlation (r = 0.711) with quercetin content (Table 4). Javier Moreno *et al*. (2006) also found that dehydrated onion, where Maillard reaction occurred, has positive correlation with TAC during storage.

The AE of four samples had similar values as those of control. This result from DPPH method was in agreement with changes in gallic acid content found in Table 3. Therefore, the AE showed positive correlation (r = 0.763) with gallic acid content. Many antioxidants that react quickly with peroxyl radicals (ORAC method) might react slowly or even be inert to DPPH as suggested by Huang *et al*. (2005). Li *et al*. (2005) also found the positive correlation between gallic acid and DPPH in Chinese black-grained wheat. The difference in antioxidant activity measured by ORAC and DPPH was inherited by their differences in mechanism of free radical scavenging and redox potential transfer of the two methods (Huang *et al*., 2005).

Pearson's correlation coefficients of browning index, total phenolic, flavonoid content, gallic acid content, quercetin content and antioxidant activity (ORAC and DPPH) were shown in Table 4. Browning index had high correlation with quercetin (r = 0.977), total phenolic (r = 0.858) and TAC from ORAC (r = 0.720). Quercetin also had positive correlation

Table 3 Total antioxidant capacity (TAC) from ORAC and antiradical efficiency (AE) from DPPH method in chopped Indian Gooseberry placed at 25 °C for 0, 4, 6 and 8 hours.

Time (hr)	TAC (μmoles TE / 100 g FW)	AE (1/ IC_{50})
0	78.2^{c}	0.094^{a}
4	80.4^{c}	0.092^{a}
6	98.3^{a}	0.080^{b}
8	90.3^{b}	0.090^{a}

Mean values in the same column followed by different superscript are significantly different ($p<0.05$).

Table 4 Pearson's correlation coefficients of browning index, total phenolic, flavonoid content, gallic acid content, quercetin content and antioxidant activity (ORAC and DPPH).

	Browning	Phenolic	Flavonoid	Gallic acid	Quercetin	ORAC
Phenolic	0.858**					
Flavonoid	0.596*	0.748**				
Gallic acid	-0.748**	-0.639**	-0.209			
Quercetin	0.977**	0.818**	0.522*	-0.820**		
ORAC	0.720**	0.392	0.094	-0.648**	0.711**	
DPPH	-0.571*	-0.239	0.233	0.763**	-0.655**	-0.799**

*,** = significant at $p<0.05$ and $p<0.01$, respectively

with antioxidant activity (TAC) as determined by ORAC method (r = 0.711) and with total phenolic (r = 0.818). Whereas gallic acid had positive correlation with antiradical efficiency (AE) as determined by DPPH (r = 0.763). These positive correlations suggested browning of the Indian Gooseberry resulted in more flavonoids and phenolics as well as antioxidant property (ORAC) pertaining to increase health benefit while DPPH assay does not represent antioxidative mechanisms in biological system.

CONCLUSIONS

Total phenolic and flavonoid content increased ($p<0.05$) as a result of browning in Indian Gooseberry. Total phenoic increased from 1,790.4 to 2,108.6 mg gallic acid equivalence/ 100 g FW, and flavonoid increased from 143.1 to 157.8 mg catechin equivalence/ 100 g FW, at 8 hr of browning. Browning reaction also resulted in an increase of quercetin and antioxidant activity evaluated by ORAC method. Kaempferol was found in trace amount only in 6 hr sample while other treatments have too low kaempferol to be detected by this method. Browning index showed good correlation with quercetin (r = 0.977), total phenolic (r = 0.858), flavonoid content (r = 0.596), and TAC from ORAC method (r = 0.720). In addition, quercetin exhibited good correlation with TAC from ORAC method (r = 0.711), and with total phenolic (r = 0.818). Therefore, quercetin, as flavonol compound, could represent changes in flavonoids as well as a good indicator for antioxidant activity (ORAC) in Indian Gooseberry.

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