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Identification of Antioxidants in Young Mango Leaves by LC-ABTS and LC-MS

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ABSTRACT

Thai eat the young leaves of mango as vegetables. Antioxidants in young leaves of mango cultivars 'Talapnak', 'Chok Anan' and 'Nam Dok Mai' were identified by high-performance liquid chromatography coupled with an online ABTS assay (HPLC-ABTS) and electrospray ionization mass spectrometer (HPLC-ESI-MS). Young leaves of mango cv. 'Nam Dok Mai' had the highest antioxidant capacity. Major antioxidants in young mango leaves were mangiferin and benzophenones (maclurin and iriflophenone derivatives). Mangiferin presented in higher quantities than other compounds in each cultivar, with cv. 'Talapnak' containing the most (37.92±0.98 mg/g DW). The compound with the highest antioxidant capacity in all cultivars was mangiferin pentoside (from 1.19±0.25 mmol TE/g DW in cv. 'Chok Anan' to 2.13±0.04 mmol TE/g DW in cv. 'Talapnak'). The compound with the highest Trolox equivalent antioxidant capacity was maclurin galloyl glucoside (1.75±0.62 mol TE/mol).

Keywords: Young mango leaf, Antioxidant, HPLC-ABTS, Xanthone, Benzophenone

INTRODUCTION

Mango (*Mangifera indica* L.) is a native plant in South Asia, and distributed across tropical and subtropical regions of the world. It is usually grown for its fruit, which is recognized as the 'king of fruits' or 'superfruit' (Mukherjee and Litz, 2009; Noratto et al., 2010). Besides the fruit, other parts of the mango tree provide value-added products. Mango peel and kernel are by-products of mango processing. Mango peel can be used as a dietary fiber, and mango kernel is used to manufacture mango kernel oil or mango kernel flour (Masibo and He, 2009). In

Thailand, the young leaves of mango are consumed as vegetables. Mango leaves have traditionally been used to treat diseases, including dysentery and flatulence. In Indonesia, reddish-colored young leaves are also eaten as a salad or side dish (Flach et al., 1993). In India, young mango leaves are used as ethnomedicine for treatment of diabetes (Sarmah and Hazarika, 2012). Mangiferin is the most important phenolic compound in mango leaves, bark, peels and kernels, and is present in particularly high quantities in young leaves (Barreto et al., 2008). Mangiferin has many biological activities, including anticancer, antimicrobial, anti-allergenic, anti-inflammatory, analgesic, immunomodulatory and hypolipidemia, as well as antioxidant activity (Masibo and He, 2008).

HPLC-ABTS is an analysis system developed for simultaneous purification, identification and capacity determination of the antioxidant compounds. After separating by HPLC column, the compounds were reacted with ABTS radical, which had an absorption maximum at 734 nm. In the presence of an antioxidant, ABTS radical loses its color and causes a negative peak in the chromatogram. Trolox equivalent antioxidant capacity (TEAC) value, a specific antioxidant capacity of individual compounds, can be calculated by this method (Koleva et al., 2001). This technique has been used to determine antioxidants in many plants, including gardenia fruit, pomegranate seed and green and black tea (Stewart et al., 2005; He et al., 2010; He et al., 2011).

Antioxidants in the young leaves of Thai mango have not been reported. In this study, HPLC-ABTS, in combination with HPLC-ESI-MS, was used to analyze the antioxidants in the young leaves of three different mango cultivars. The results provide qualitative and quantitative information about the antioxidants present, as well as the specific antioxidant activities of young mango leaves.

MATERIALS AND METHODS

Plant Materials and Chemicals

Young leaves of three different mango (*Mangifera indica* L.) cultivars, ‘Talapnak’, ‘Chok Anan’ and ‘Nam Dok Mai’, were obtained from a local farm in Chiang Mai, Thailand in November 2012. Young mango leaves were reddish-brown in color, while mature leaves were dark green.

2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and potassium persulfate were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of vegetable extracts

Fresh young mango leaves were selected and washed in tap water for 1 min. The leaves were extracted with 60% ethanol at the ratio of 1 g per 5 ml using a blender (Model MR 4050 CA, Braun, Spain) for 30 s. The extracts were then filtered through a double layer of muslin cloth and centrifuged at 2,500g for 20 min. The supernatant was kept at -80°C before analysis. Extraction was performed in duplicates.

Identification and quantification of antioxidants

HPLC analysis was performed with Agilent 1200 (Agilent Technologies, Santa Clara, CA, USA) according to the method of He et al. (2010) with slight modification. The sample (10 μl) was separated by Prevail C18 (250×46 mm) column (Alltech Associates, Lokeren, Belgium) with gradient elution (A: 100% methanol, B: methanol:water:formic acid at the ratio of 20:80:0.25 by volume) at a flow rate of 0.7 ml/min and 30°C. Gradient elution began with 100% B, then changed to 90% B at 6.25 min, 80% B at 12.5 min, 55% B at 50 min, 20% B at 68.75 min and returned to 100% B at 75 min. The compounds were detected at 280, 330 and 520 nm.

The compounds escaping from the UV-Vis detector were subjected to on-line-ABTS analysis. ABTS reagent (2 mM ABTS and 3.5 mM potassium persulphate) was prepared and kept in the dark at room temperature for 16 h to stabilize the radical before use. The reagent, at a flow rate of 0.5 ml/min, reacted with the eluted compounds and was detected at 734 nm. In the presence of antioxidants, it produced a negative peak in the chromatogram. Antioxidant capacity was calculated from the area of the negative peak and expressed as Trolox equivalent (TE). Specific antioxidant capacity of the compounds, or Trolox equivalent antioxidant capacity (TEAC), was obtained from the molar ratio between antioxidant capacity (TE) and the quantity of the compounds.

Identification of the compounds was performed by HPLC coupled with electrospray ionization mass spectrometer (HPLC-ESI-MS) under the same elution condition as described above. Quadrupole mass spectrometer was operated in both positive and negative mode. Mass spectra were recorded in the range of *m/z* 100-1500.

Statistical analysis

Multiple linear regression of TEAC value was performed by R version 2.15.2 (http://cran.r-project.org/) to study the effects of the number and position of the hydroxyl group on TEAC values of the compounds identified from young mango leaves. The number of OH groups at different positions was used as explanatory variables, while the TEAC value of the compound was used as the response variable. Predicted TEAC values from regression analysis were plotted against the actual TEAC value. Regression coefficients were used to determine the effect of the chemical structure of the compounds on antioxidant capacity.

RESULTS

Identification and quantification of antioxidants

HPLC-ABTS chromatograms of young leaves extracts of three mango cultivars are shown in Figure 1. Positive peaks were used for detection of phenolic compounds, while negative peaks were used for detection of antioxidant capacity. Identification of antioxidants in young leaves of three mango cultivars are shown in Table 1. The major antioxidants in young mango leaves were derivatives of xanthone and benzophenones. Xanthones including mangiferin (compound 11)

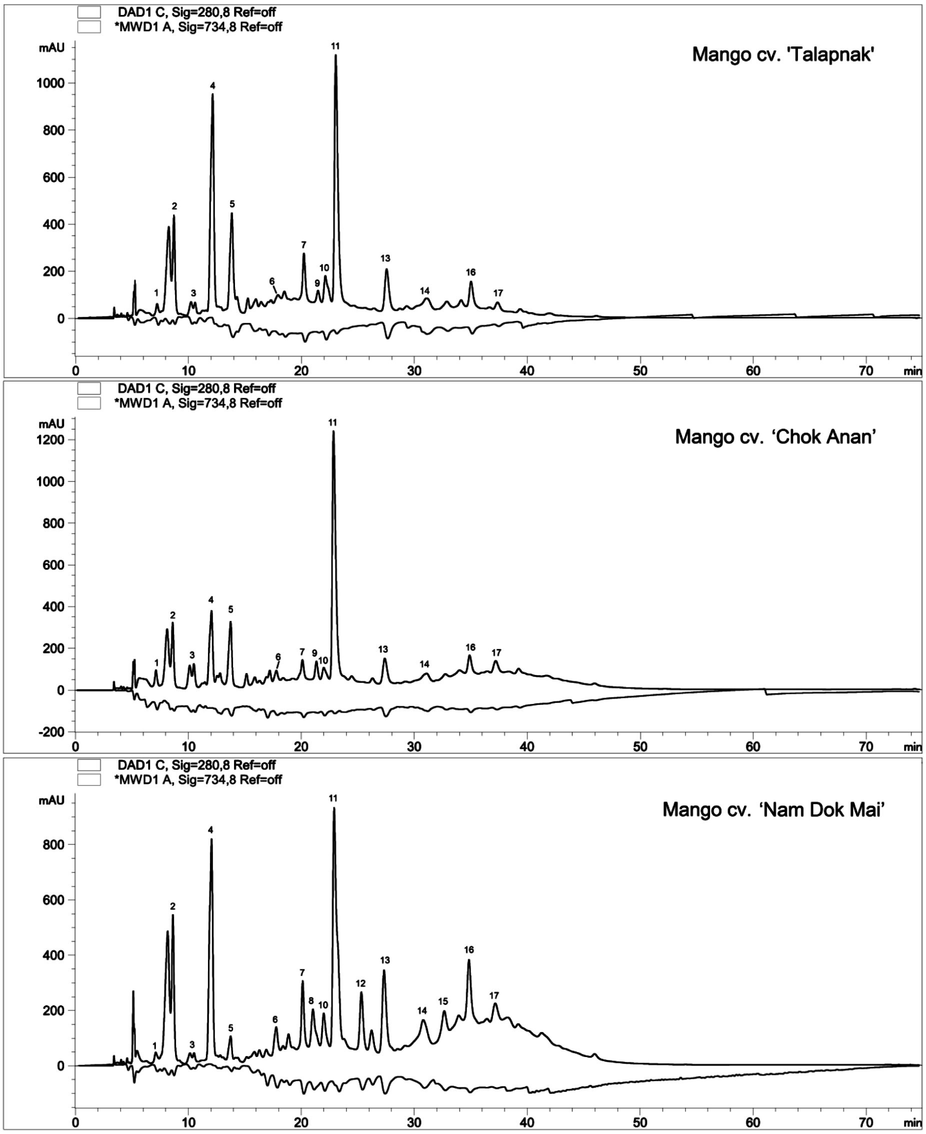


Figure 1. HPLC-ABTS chromatograms of young mango leaves extracts. Positive peaks were UV signal at 280 nm for the detection of phenolic compounds. Negative peaks were Vis signal at 734 nm for the detection of ABTS radical scavenging activity.

Table 1. Identification of antioxidants in young mango leaves.

Peak no.	$_{max}$ (nm)			MS negative ions (m/z)			MS positive ions (m/z)				MW	Proposed name	References
				$[M–H]^-$	$[2M-H]^-$	Other	$[M+H]^+$	$[M+Na]^+$	$[2M+Na]^+$	Other			
1	210	278		343	687	169 [galloyl]	345	367	711	169 [galloyl]	344	Galloylquinic acid	Stewart et al. (2005)
2	214sh	234sh	318	423	847		425	447	871	407 [M-OH] 287 [M-di-OH-benzoyl]	424	Maclurin glucoside	Barreto et al. (2008)
3	214sh	280sh	340sh	575			577	599	1175	467 [M-catechol] 261 [maclurin] 169 [galloyl]	576	Maclurin galloyl glucoside	Barreto et al. (2008)
4	202	214	296	407	815		409	431	839	246 [iriflophenone] 181 [glucose+H]	408	Iriflophenone glucoside	Barreto et al. (2008)
5	222sh	282	340sh	559		315 [galloyl-glucoside]	561	583	1143	169 [galloyl]	560	Iriflophenone galloyl glucoside	Zhang et al. (2013)
6	224	278		635				659	1295	467 [M-galloyl] 169 [galloyl]	636	Triglloylglucose	Vallejo et al. (2004)
7	196	220	274	197	395		199	221	419		198	Syringic acid	Sun et al. (2007)
8	208	280	342sh	727		363	729	751		169 [galloyl]	728	Maclurin digalloyl glucoside	Berardini et al. (2004)
9	232	272	340sh	527			529	551	1079	246 [iriflophenone] 169 [galloyl]	528	Iriflophenone benzoyl glucoside	Barreto et al. (2008)
10	204	258sh	276	441	883		443	465	907	273 [M-galloyl]	442	epicatechin gallate	Sun et al. (2007)
11	240	258	318	421			423	445	867		422	Mangiferin	Barreto et al. (2008)
12	214	280	342sh	711			713	735	1447	402 [M-2galloyl+H]	712	Iriflophenone digalloyl glucoside	Berardini et al. (2004)
13	194	220	282	553		421[mangiferin-H]$^-$	555	577			554	Mangiferin pentoside	Ajila et al. (2010)
14	222	278		545	1091			1115			546	NI[a]	
15	222	278		679			681	703		246 [iriflophenone] 122 [galloyl+H]	680	Iriflophenone galloyl hydroxybenzoyl glucoside	Zhang et al. (2013)
16	196	220	276	349	699		351	373	723		350	NI	
17	222	266	356	463	927		465	487	951	303 [quercetin] 181 $[hexose+H]^+$	464	Quercetin hexoside	de Brito et al. (2007)

Note: aNI, not identified.

Table 2. Quantity of antioxidants in young leaves of three mango cultivars.

Peak no.	Compounds	Antioxidant quantity[a] (mg/g DW)		
		cv. 'Talapnak'	cv. 'Chok Anan'	cv. 'Nam Dok Mai'
1	Galloylquinic acid	1.53±0.11	2.53±0.03	1.07±0.03
2	Maclurin glucoside	10.13±0.13	17.81±2.28	28.73±1.67
3	Maclurin galloyl glucoside	1.09±0.68	5.38±0.48	1.50±0.02
4	Iriflophenone glucoside	31.23±2.26	10.31±0.82	23.34±0.35
5	Iriflophenone galloyl glucoside	12.43±0.53	8.40±0.76	2.03±0.09
6	Triglloylglucose	ND[b]	1.34±0.04	2.88±0.07
7	Syringic= acid	4.16±1.73	1.71±0.32	6.13±0.87
8	Maclurin digalloyl glucoside	ND	ND	4.12±0.26
9	Iriflophenone benzoyl glucoside	0.57±0.80	1.84±0.13	ND
10	Epicatechin gallate	2.43±1.21	1.32±0.06	2.46±0.03
11	Mangiferin	37.92±0.98	29.11±11.42	33.27±2.65
12	Iriflophenone digalloyl glucoside	ND	ND	5.38±0.22
13	Mangiferin pentoside	6.96±3.77	6.50±0.73	14.20±1.17
15	Iriflophenone galloyl hydroxybenzoyl glucoside	ND	ND	3.09±0.27
17	Quercetin hexoside	3.97±1.56	4.97±0.29	6.41±1.13

Note: [a]Mean±SD of duplicate analyses; flavonoids were quantified as molar equivalent of quercetin; other phenolic compounds were quantified as molar equivalent of caffeic acid.
[b]ND, not detected.

and mangiferin pentoside (compound 13) were present at the quantities of 29.11-37.92 mg/g DW and 6.50-14.20 mg/g DW, respectively (Table 2). Compounds 2, 3 and 8 were maclurin derivatives, while compounds 4, 5, 9, 12 and 15 were iriflophenone derivatives.

Galloylquinic acid (compound 1), trigalloylglucose (compound 6) and syringic acid (compound 7) are hydroxybenzoic acids with antioxidant activity found in young mango leaves. Flavonoids, including epicatechin gallate (compound 10) and quercetin hexoside (compound 17), antioxidants as well, were also found in young mango leaves.

Antioxidant capacity of phenolic compounds

The compound with the highest antioxidant capacity in the young leaves of three different mango cultivars was mangiferin pentoside, with the highest value in cv. 'Talapnak' (2.13 mmol TE/g DW) and the lowest value in cv. 'Chok Anan' (1.19 mmol TE/g DW). However, the antioxidant activities of all of the benzophenone derivatives were higher than those of mangiferin and mangiferin pentoside in all cultivars (Table 3).

The compound with the highest TEAC value in young mango leaves was maclurin galloylglucoside (1.75 mol TE/mol), followed by trigalloylglucose (1.47 mol TE/mol) and iriflophenone galloyl hydroxybenzoyl glucoside (1.17 mol TE/mol) (Table 4).

Table 3. Antioxidant capacity of phenolic compounds in young leaves of 3 mango cultivars.

Peak no.	Compounds	Antioxidant capacitya (mmol TE/g DW)		
		cv. 'Talapnak'	cv. 'Chok Anan'	cv. 'Nam Dok Mai'
1	Galloylquinic acid	0.54±0.03	0.42±0.10	0.22±0.01
2	Maclurin glucoside	0.55±0.13	0.64±0.03	0.80±0.34
3	Maclurin galloyl glucoside	0.44±0.18	1.02±0.00	0.47±0.10
4	Iriflophenone glucoside	0.08±0.00	0.06±0.06	0.03±0.03
5	Iriflophenone galloyl glucoside	1.48±0.02	0.78±0.13	0.24±0.00
6	Triglloylglucose	NDb	0.30±0.03	0.69±0.06
7	Syringic acid	0.83±0.09	0.32±0.02	0.82±0.10
8	Maclurin digalloyl glucoside	ND	ND	0.61±0.05
9	Iriflophenone benzoyl glucoside	0.04±0.06	0.07±0.00	ND
10	Epicatechin gallate	0.68±0.25	0.31±0.05	0.54±0.08
11	Mangiferin	0.35±0.00	0.18±0.06	0.73±0.09
12	Iriflophenone digalloyl glucoside	ND	ND	0.78±0.18
13	Mangiferin pentoside	2.13±0.04	1.19±0.25	1.38±0.25
15	Iriflophenone galloyl hydroxybenzoyl glucoside	ND	ND	0.53±0.04
17	Quercetin hexoside	0.31±0.07	0.24±0.03	0.24±0.00
	Mangiferin and its derivativec	**2.48**	**1.37**	**2.11**
	Benzophenones	**2.59**	**2.57**	**3.46**
	Maclurin derivativesd	0.99	1.66	1.88
	Iriflophenone derivativese	1.6	0.91	1.58
	Total	**7.43**	**5.53**	**8.08**

Note: a Mean±SD of duplicate analyses.
b ND, not detected.
c Compounds 11 and 13.
d Compounds 2, 3 and 8.
e Compounds 4, 5, 9, 12 and 15.

DISCUSSION

HPLC-ABTS chromatograms of the ethanol extracts from young leaves of three different mango cultivars showed a similar pattern, indicating that the antioxidant activity of young mango leaves derived from either similar or the same compounds. Most of the antioxidants were hydrophilic compounds, which were eluted from the column within 40 min.

Benzophenones, including maclurin and iriflophenone, are also important antioxidants in young mango leaves. The highest quantity of mangiferin was found in the young leaves of mango cv. 'Talapnak' (37.92 mg/g DW), while the lowest quantity appeared in cv. 'Chok Anan' (29.11 mg/g DW). However, the quantities were lower than those found in the young leaves of mango cv. 'Van Dyke' (58.12 mg/g DW) and 'Embrapa-141-Roxa' (67.20 mg/g DW) from Brazil (Barreto et al., 2008). Mangiferin is a compound found in many parts of mango, including peels, kernel, bark and pulp. The quantity in mango pulp was lower than in other

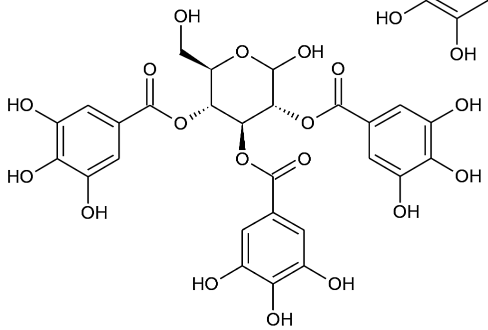
Table 4. Trolox equivalent antioxidant capacity (TEAC) of phenolic compounds in young leaves of 3 mango cultivars.

Peak no.	Compounds	TEAC (mol TE/mol)[a]			
		cv. 'Talapnak'	cv. 'Chok Anan'	cv. 'Nam Dok Mai'	cv. Mean±SD
1	Galloylquinic acid	1.22	0.57	0.69	0.83±0.35
2	Maclurin glucoside	0.23	0.15	0.12	0.17±0.06
3	Maclurin galloyl glucoside	2.34	1.10	1.81	1.75±0.62
4	Iriflophenone glucoside	0.01	0.02	0.00	0.01±0.01
5	Iriflophenone galloyl glucoside	0.67	0.52	0.66	0.62±0.08
6	Triglloylglucose	NDb	1.42	1.52	1.47±0.07
7	Syringic acid	0.40	0.37	0.27	0.35±0.07
8	Maclurin digalloyl glucoside	ND	ND	1.09	1.090.0
9	Iriflophenone benzoyl glucoside	0.37	0.21	ND	0.29±0.11
10	Epicatechin gallate	1.25	1.04	0.97	1.09±0.15
11	Mangiferin	0.04	0.03	0.09	0.05±0.03
12	Iriflophenone digalloyl glucoside	ND	ND	1.03	1.030.0
13	Mangiferin pentoside	1.70	1.01	0.54	1.08±0.58
15	Iriflophenone galloyl hydroxybenzoyl glucoside	ND	ND	1.17	1.17±0.00
17	Quercetin hexoside	0.36	0.23	0.17	0.25±0.10

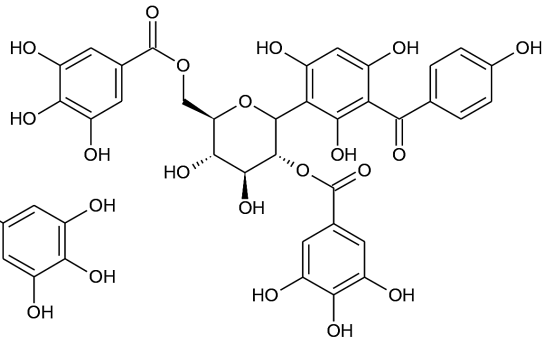
Note: [a]Molar of Trolox with equivalent antioxidant capacity of a 1 molar substance.
[b]ND, not detected.

parts (Ribeiro et al., 2008). Mangiferin content varied among mango cultivars (Berardini et al., 2005). Although mango is a primary source of mangiferin, it has also been identified from other plant families, including honeybush (*Clyclopia* spp.) and African mango (*Irvingia gabonensis*) (Sun and Chen, 2012; Matkowski et al., 2013).

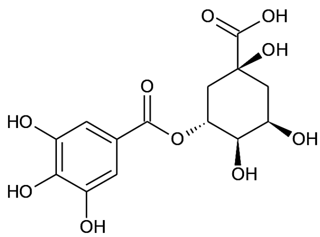
These benzophenones are attached to glucose, galloyl or benzoyl moieties. The quantity of monoglucoside derivative was higher than that of more complex derivatives for both maclurin and iriflophenone. A similar trend was also found in the young leaves of mango cv. 'Van Dyke' and 'Embrapa-141-Roxa'. However, the ratio between maclurin glucoside and iriflophenone glucoside varied among cultivars. The ratio of maclurin glucoside per iriflophenone glucoside was higher than found in the 'Chok Anan' and 'Nam Dok Mai' cultivars, while less than found in the 'Talapnak' cultivar. The quantity of iriflophenone glucoside was much higher than that of maclurin glucoside in the 'Van Dyke' and 'Embrapa-141-Roxa' cultivars (Barreto et al., 2008). Benzophenone glycosides with double substitution (from galloyl or benzoyl moieties) were found only in cv. 'Nam Dok Mai'. Maclurin is an intermediate in biosynthesis of mangiferin and isomangiferin (Berardini et al., 2004). In some literature, benzophenones from mango were named as folia-mangiferosides (Zhang et al., 2011). The chemical structures of some antioxidants found in young mango leaves are shown in Figure 2.



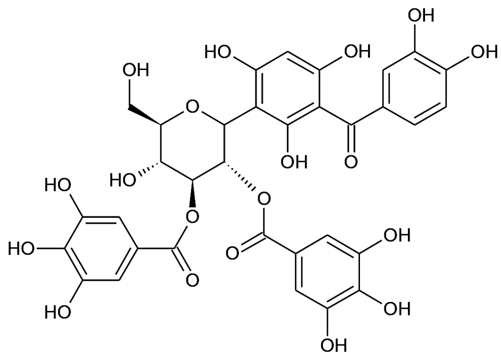
Triglloylglucose



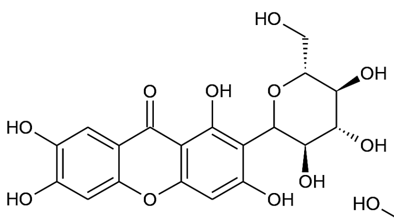
Iriflophenone digalloyl glucoside



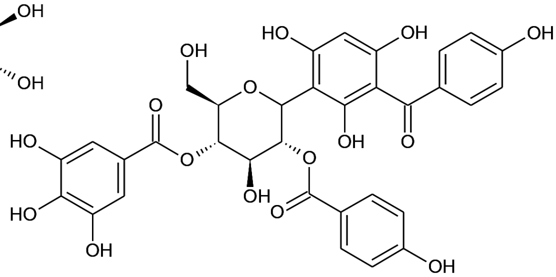
Galloylquinic acid



Maclurin digalloyl glucoside



Mangiferin



Iriflophenone hydroxybenzoyl galloyl glucoside

Figure 2. Some antioxidants found in young mango leaves.

Mono- and di-galloyl glucosides of maclurin were found in young Thai mango leaves. However, these compounds were not detected in both old and young Brazillian mango leaves. In Brazillian mango cultivars, gallic acid and gallate esters of methanol, glucose, mangiferin, maclurin and iriflophenone were identified. The number of galloyl moieties could be up to five in esterification with glucose, while benzophenones estirified with up to two galloyl moieties. Mangiferin was found in most parts of the mango tree. Benzophenones were absent in mango kernel, while isomangiferin was found only in mango bark (Barreto et al., 2008; Ribeiro et al., 2008). In this study, isomangiferin was also not detected in young Thai mango leaves.

Galloylquinic acid, trigalloylglucose, syringic acid, epicatechin gallate and quercetin hexoside were previously reported in many parts of mango (Gu et al., 2003; Elzaawely and Tawata, 2010; Ramirez et al., 2013). Kaempferol glucoside was identified from the peel of mango cv. ‘Tommy Atkins’ (Berardini et al., 2004). However, this compound was not detected in this study.

The ratio of total antioxidant capacity of maclurin derivatives to iriflophenone derivatives varied among cultivars, and was dependent on their contents. Antioxidant activity of maclurin derivatives was higher than those of iriflophenones in the ‘Chok Anan’ and ‘Nam Dok Mai’ cultivars, but was lower in the ‘Talapnak’ cultivar.

TEAC value measured the specific antioxidant capacity of the compounds. TEAC value of mangiferin was 0.05 mol TE/mol (Table 4), which was similar to the 133.07 mol TE/g (equivalent to 0.056 mol TE/mol) reported previously (Petrova et al., 2011).

Antioxidant activity of phenolic compounds was influenced by the number and position of hydroxyl (OH) groups (Arts et al., 2003). Multiple linear regression was performed on the antioxidants identified from young mango leaves. The number of OH groups at different position was used as explanatory variables, while the TEAC value of the compound was used as the response variable. Correlation between actual and predicted TEAC (Multiple R^2 = 0.7465) confirmed the structure-activity relationship of the OH groups (Figure 3). From the regression coefficient, OH groups substituted at the *para* position of aromatic compound influenced the antioxidant capacity of phenolic compounds the most (Figure 4). OH group of maclurin also had a positive effect on antioxidant activity, comparable to *meta*-substituted OH of phenolic compounds. On the other hand, OH attached to alkane had a negative effect on antioxidant activity. In most cases, the alkane OH was located in glucose moiety. Glycosides of phenolic compounds had lower antioxidant activity than their corresponding aglycone. Sugar moiety reduced antioxidant activity of phenolic compound by substituting free OH group, which was responsible for radical scavenging. Glycosylation also decreased coplanarity of the B-ring in flavonoids and increased hydrophilicity of the compounds (Heim et al., 2002).

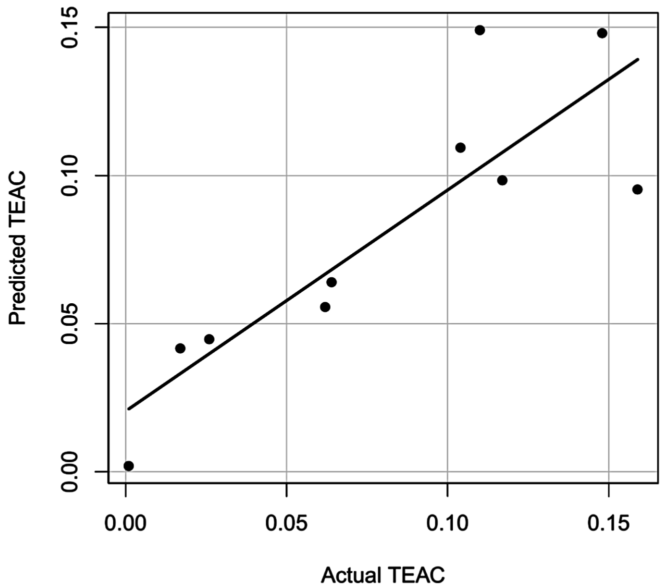


Figure 3. Actual and predicted TEAC values of antioxidants from young mango leaves.

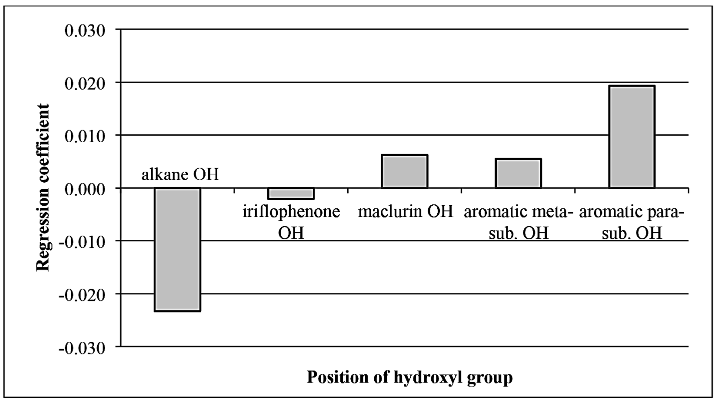


Figure 4. Regression coefficient of multiple linear regression of TEAC value as influenced by number and position of OH group in antioxidants from mango leaves.

CONCLUSIONS

Major antioxidants in young leaves of mango cv. 'Talapnak', 'Chok Anan' and 'Nam Dok Mai' were the derivatives of xanthone mangiferin and benzophenones (maclurin and iroflophenone). The content of mangiferin was higher than that of other compounds in each cultivar. The compound with the highest antioxidant activity was mangiferin pentoside, while the compound with the highest TEAC value was maclurin galloylglucoside. Further work should investigate antioxidants in the young leaves of other mango varieties and the effect of the growth period on the antioxidative compounds.

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