PERGAMON

Phytochemistry 62 (2003) 219–228

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Antioxidant flavonoids from leaves of *Polygonum hydropiper* L.

Zhao Feng Peng[a], Dieter Strack[b], Alfred Baumert[b], Ramanathan Subramaniam[a], Ngoh Khang Goh[a], Tet Fatt Chia[a], Swee Ngin Tan[a], Lian Sai Chia[a,*]

[a]*National Institute of Education, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616, Singapore*
[b]*Institute of Plant Biochemistry, Weinberg 3, D-06120, Halle, Germany*

Received 30 January 2002; received in revised form 8 October 2002

Abstract

Ten flavonoid compounds were isolated from the dried leaves of *Polygonum hydropiper* L. (Laksa leaves), and identified as 3-*O*-α-L-rhamnopyranosyloxy-3′,4′,5,7-tetrahydroxyflavone; 3-*O*-β-D-glucopyranosyloxy-4′,5,7-trihydroxyflavone; 6-hydroxyapigenin; 6″-*O*-(3,4,5-trihydroxybenzoyl) 3-*O*-β-D-glucopyranosyloxy-3′, 4′, 5, 7-tetrahydroxyflavone; scutillarein; 6-hydroxyluteolin; 3′,4′,5,6,7-pentahydroxyflavone; 6-hydroxyluteolin-7-*O*-β-D-glucopyranoside; quercetin 3-*O*-β-D-glucuronide; 2″-*O*-(3,4,5-trihydroxybenzoyl) quercitrin; quercetin. Evaluation of the antioxidative activity, conducted in vitro, by using electron spin resonance (ESR) and ultraviolet visible (UV–vis) spectrophotometric assays, showed that these isolated flavonoids possess strong antioxidative capabilities. Measurement of the Trolox equivalent antioxidant capacity (TEAC) values, against ABTS (2,2′-azinobis(3-ethyl-benzothiazoline-6-sulphonic acid) radicals and phenyl-*tert*-butyl nitrone (PBN) azo initiator (AI) also showed strong anti-oxidative activity. The most powerful of the antioxidants was 2″-*O*-(3,4,5-trihydroxybenzoyl) quercitrin (galloyl quercitrin). A combination of two flavonoid compounds was tested for synergistic anti-oxidative capacity, but no significant improvement was observed.

Keywords: *Polygonum hydropiper* L.; Flavonoid; Antioxidant; Trolox equivalent antioxidant capacity (TEAC); Galloyl quercitrin

1. Introduction

In recent years, flavonoids have attracted the interest of researchers because they show promise of being powerful antioxidants which can protect the human body from free radicals (Bors et al., 1996; Halliwell and Gutteridge, 1999). Flavonoids cannot be produced by the human body and have thus to be taken in mainly through the daily diet. The evidence reported in the chemistry, biochemistry and pharmacy literature supports the view that flavonoids play a vital biological role, including the function of scavenging reactive oxygen species (Bors et al., 1996; Pincemail, 1995; Pietta, 1998).

Chemically, there are three features that confer on flavonoids their remarkable antioxidant properties (Rice-Evans et al., 1997):

- the hydrogen donating substituents (hydroxyl groups), attached to the aromatic ring structures of flavonoids, which enable the flavonoids to undergo a redox reaction that helps them to scavenge free radicals more easily;
- a stable delocalization system, consisting of aromatic and heterocyclic rings as well as multiple unsaturated bonds, which helps to delocalize the resulting free radicals, and
- the presence of certain structural groups which are capable of forming transition metal-chelating complexes that can regulate the production of reactive oxygen species such as $OH^{\bullet}$ and $O_2^{2-\bullet}$.

Flavonoids are considered to be an active ingredient in a number of medicinal herbs (Okuda et al., 1992). In traditional Chinese herbal medical practice, raw plant materials are often used in dried form. As it always needs a rather long time to stew for preparation, we can infer that the components remaining in the dried material are those with good thermo-stable and water-soluble properties. Flavonoids, commonly occurring as their glycosides in plants, are some of the compounds which possess better chemical stability and hydrophilicity. This means that

* Corresponding author. Tel.: +65-67903885; fax: +65-68969414.
E-mail address: lschia@nie.edu.sg (L.S. Chia).

0031-9422/03/$ - see front matter
PII: S0031-9422(02)00504-6

the antioxidative activity of these plants can be attributed to flavonoids or other stable antioxidants.

This paper reports on a study of the flavonoids extracted from a common local herb, *Polygonum hydropiper* L. *(Polygonaceae)*. It is an erect herb with round stems (Fig. 1). The leaves are simple and dark green, with a sheathing stipule (or ochrea) at the petiole base. It is native to Southeast Asia and grows as a weed in some wet places (Kok et al., 1991). As this plant possesses a strong peppery taste, it is used as a kind of spice by the local people, especially the Chinese, to flavour Southeast Asia-style foods such as Laksa, and also by the Malays to flavor some of their traditional dishes.

It was reported by Yagi et al. (1994) that three sulfated flavonoids have been isolated and identified from the leaves of Polygonum hydropiper. Since then, no further work on the flavonoids in this plant has been published in the literature; there has, however, been some work done on the catechins and the terpenoids in this plant. Our preliminary screening of some tropical plants showed that this plant possesses very strong antioxidant capability. This led us to reinvestigate the leaves of this plant. The results are reported in this paper.

2. Results and discussion

2.1. The flavonoids from Laksa leaves

Ten main flavonoids were isolated and characterized. Six of them are flavone-3-ol derivatives and the other four are flavone derivatives. The total amount of flavonols, including the four quercetins and two kaempferols, that have high antioxidative potential (Table 1), constituted 3.2% by weight percentage, while the total amount of flavone made up 1.8%. 6″-*O*-(3,4,5-Trihydroxy-benzoyl) 3-*O*-β-D-glucopyranosyloxy-3′, 4′, 5, 7-tetra-hydroxyflavone (galloyl kaempferol 3-glucoside) gave the highest yield in terms of weight percentage (1%).

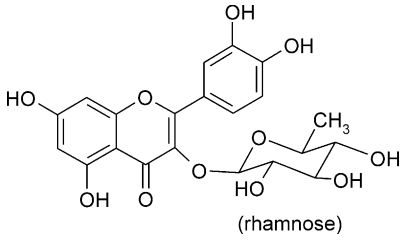
Fig. 1. Photograph of "Laksa" plant.

A summary of the isolated flavonoids is listed in Table 1, and the identification details are shown below.

2.1.1. Compound **1**: 3-*O*-α-L-rhamnopyranosyloxy-3′, 4′, 5, 7-tetrahydroxyflavone (quercitrin)

Formula: $C_{21}H_{20}O_{11}$

Molecular Mass: 448.4



NMR: ^{1}H (CD_3OD) $d = 7.38$ [*d*, H-2′, J(2′-6′) 2.1], 7.35 [*dd*, H-6′, J(6′-5′) 8.3], 6.95 [*d*, H-5′], 6.41 [*d*, H-8, J(8-6) 2.2], 6.24 [*d*, H-6], 5.40 [*d*, H-1″, J(1″-2″) 1.7], 4.26 [dd, H-2″, J(2″-3″) 3.4], 3.79 [*dd*, H-3″, J(3″-4″) 9.3], 3.46 [*dq*, H-5″, J(4″-5″) 9.6, J(5″-6″) 6.0], 3.37 [*dd*, H-4″], 0.98 [*d*, H-6″].

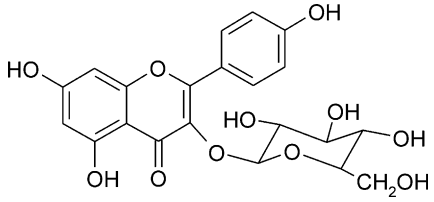
MS: Negative ES-MS, *m*/*z* (rel. int.): 447 ([M–H]$^-$, 100).

HPLC online UV–vis spectra: retention time at 16.7±0.1 min; absorption maxima at 256.3 and 345.9 nm.

2.1.2. Compound **2**: 3-*O*-β-D-glucopyranosyloxy-4′, 5, 7-trihydroxyflavone (kaempferol 3-glucoside)

Formula: $C_{21}H_{20}O_{11}$

Molecular Mass: 448.4



NMR: ^{1}H (CD_3OD) $d = 7.91$ [d(AA′), H-2′/H-6′, J(2′-3′) + J(2′-5′) 8.9], 6.96 [d(BB′), H-3′/H-5′], 6.43 [*d*, H-8, J(8-6) 2.1], 6.25 [*d*, H-6], 5.27 [*d*, H-1″, J(1″-2″) 7.6], 4.01 [*dd*, H-6″A, J(6″A-5″) 2.2, J(6″A-6″B 12.1], 3.77 [*dd*, H-6″B, J(6″B-5″) 6.1], 3.65–3.44 [*m*, H-2″/H-3″/H-5″], 3.39 [*dd*, H-4″, J(4″-3″) 9.5, J(4″-5″) 9.5].

MS: Negative ES-MS, *m*/*z* (rel. int.): 447 ([M–H]$^-$, 100).

Table 1
Flavonoids isolated from Laksa leaves

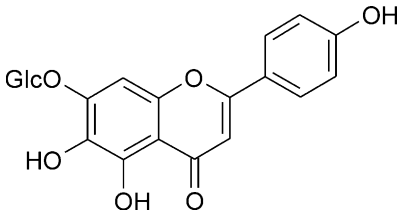
No.	Name (common name)	Abbreviation	Relative amounts (%)
1	3-*O*-α-L-Rhamnopyranosyloxy-3″, 4″, 5, 7-tetrahydroxyflavone (quercitrin)	L-QR	0.9
2	3-*O*-β-D-Glucopyranosyloxy-4′, 5, 7-trihydroxyflavone (kaempferol 3-glucoside)	L-KG	0.5
3	Scutillarein 7-*O*-β-D-glucopyranoside or 6-hydroxyapigenin	L-SG	0.3
4	6″-*O*-(3,4,5-Trihydroxybenzoyl) 3-*O*-β-D-glucopyranosyloxy-3′, 4′, 5, 7-tetra-hydroxyflavone (galloyl kaempferol 3-gluoside)	L-KGG	1.0
5	Scutillarein (aglycone)	L-SA	0.2
6	6-Hydroxyluteolin; 3′, 4′, 5, 6, 7-pentahydroxyflavone	L-LA	0.4
7	6-Hydroxyluteolin 7-*O*-β-D-glucopyranoside	L-LG	0.9
8	Quercetin 3-*O*-β-D-glucuronide	L-QG	0.4
9	2″-*O*-(3,4,5-Trihydroxybenzoyl) quercitrin; galloyl quercitrin	L-QRG	0.2
10	Quercetin	L-QA	0.2

HPLC online UV–vis spectra: retention time at 16.2±0.1 min absorption maxima at 267.0 and 356.2 nm.

*2.1.3. Compound **3**: Scutillarein 7-O-β-D-glucopyranoside or 6-hydroxyapigenin*

Formula: $C_{21}H_{20}O_{11}$

Molecular Mass: 448.4



NMR: ^{1}H (CD_3OD) d=7.92 [d(AA′), H-2′/H-6′, J(2′-3′)+J(2′-5′) 8.8], 7.06 [s, H-8], 6.97 [d(BB′), H-3′/H-5′], 6.68 [s, H-3], 5.50 [d, H-1″, J(1″-2″) 7.3], 4.01 [dd, H-6″A, J(6″A-5″) 2.1, J(6″A-6″B 12.0], 3.77 [dd, H-6″B, J(6″B-5″) 6.0], 3.66–3.55 [m, H-2″/H-3″/H-5″], 3.46 [dd, H-4″, J(4″-3″) 9.2, J(4″-5″) 9.2].^{13}C (DMSO) d=182.3(s, C-4), 164.1 (s, C-2), 161.2 (s, C-4′), 151.3 (s, C-9), 149.1 (s, C-7), 146.4 (s, C-5), 130.4 (s, C-6), 128.4 (d, C-2′/C-6′), 121.3 (s, C-1′), 115.9 (d, C-3′/C-5′), 105.9 (s, C-10), 102.5 (d, C-3), 101.1 (d, C-1″), 94.2 (d, C-8), 77.3 (d, C-5″), 75.7 (d, C-3″), 73.1 (d, C-2″), 69.6 (d, C-4″), 60.6 (t, C-6″). The assignment of the aglycone carbon signals, as well as the substituent position at C-7, follows from the correlations in the HMBC spectrum in CD_3OD.

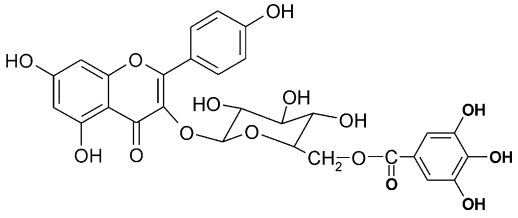
MS: Negative ES-MS, m/z (rel. int.): 447 ([M–H]$^-$, 100).

HPLC online UV–vis spectra: retention time at 15.8±0.1 minutes; absorption maxima at 267.0 and 338.7 nm.

*2.1.4. Compound **4**: 6″-O-(3,4,5-trihydroxybenzoyl)3-O-β-D-glucopyranosyloxy-3′, 4′, 5, 7-tetra-hydroxyflavone (galloyl kaempferol 3-glucoside)*

Formula: $C_{28}H_{24}O_{16}$

Molecular Mass: 616.5



NMR: ^{1}H (CD_3OD) d=7.57 [m, H-2′/H-6′ second order system], 6.98 [s, H-2‴/H-6‴], 6.76 [d (second order), H-5′, J(5′-6′) 8.3], 6.38 [d, H-8, J(8-6) 2.1], 6.22 [d, H-6], 5.24 [d, H-1″, J(1″-2″) 7.4], 4.38 [dd, H-6″A, J(6″A-5″) 5.0, J(6″A-6″B) 12.0], 4.31 [dd, H-6″B, J(6″B-5″) 2.0], 3.65–3.45 [m, H-2″-H-5″].

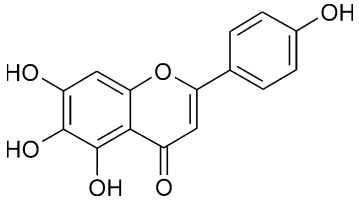
MS: Negative ES-MS, m/z (rel. int.): 615 ([M–H]$^-$, 100).

HPLC online UV–vis spectra: retention time at 15.6±0.1 minutes; absorption maxima at 263.4 and 353.1 nm.

*2.1.5. Compound **5**: Scutillarein (aglycone)*

Formula: $C_{15}H_{10}O_6$

Molecular Mass: 286.2



NMR: ^{1}H (CD_3OD) d=7.89 [d(AA′), H-2′/H-6′, J(2′-3′)+J(2′-5′) 8.9], 6.97 [d(BB′), H-3′/H-5′], 6.63, 6.62 [s ×2, H-3/H-8].

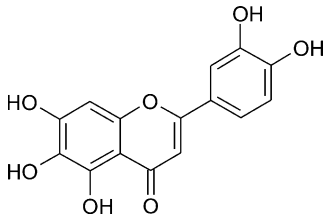
MS: Negative ES–MS, m/z (rel. int.): 285 ([M–H]$^-$, 100).

HPLC online UV–vis spectra: retention time at 19.0±0.1 min; absorption maxima at 282.6 and 335.8 nm.

*2.1.6. Compound **6**: 6-hydroxyluteolin; 3′, 4′, 5, 6, 7-pentahydroxyflavone*

Formula: $C_{15}H_{10}O_7$

Molecular Mass: 302.2



NMR: ^{1}H (CD_3OD) d = 7.42 [*dd*, H-6′, *J*(6′-2′) 2.2, *J*(6′-5′) 8.9], 7.41 [*d*, H-2′], 6.94 [*d*, H-5′], 6.61 [*s*, H-3], 6.58 [*s*, H-8]. ^{13}C (CD_3OD) d = 183.9 (*s*, C-4), 166.0 (*s*, C-2), 154.3, 151.7 (*s* ×2, C-9, C-7), 150.5 (*s*, C-4′), 147.5 (*s*, C-5), 146.7 (*s*, C-3′), 130.3 (*s*, C-6), 123.5 (*s*, C-1′), 119.9 (*d*, C-6′), 116.5 (*d*, C-5′), 113.8 (*d*, C-2′), 105.2 (*s*, C-10), 103.1 (*d*, C-3), 94.6 (*d*, C-8). The assignment of the carbon signals follows from the correlations in the HMBC spectrum. There are weak correlations between H-3/C-5 and H-8/C-4.

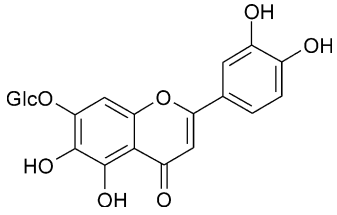
MS: Negative ES-MS, *m*/*z* (rel. int.): 301 ([M–H]$^-$, 100).

HPLC online UV–vis spectra: retention time at 16.8 ± 0.2 min; absorption maxima at 268.2 (double-hump) and 364.4 nm.

2.1.7. Compound 7: 6-hydroxyluteolin 7-O-β-D-glucopyranoside

Formula: $C_{21}H_{20}O_{12}$

Molecular Mass: 464.4



NMR: ^{1}H (CD_3OD) d = 7.45, 7.44 [*m* (second order system), H-2′/H-6′], 7.04 [*s*, H-8], 6.94 [*d*, H-5′, *J*(5′-6′) 8.2], 6.62 [*s*, H-3], 5.10 [*d*, H-1″, *J*(1″-2″) 7.4], 4.01 [*dd*, H-6″A, *J*(6″A-5″) 2.2, *J*(6″/A-6″B) 12.0], 3.63 [*dd*, H-6″B, *J*(6″B-5″) 6.0], 3.65–3.55 [*m*, H-2″/H-3″/H-5″], 3.47 [*dd*, H-4″, *J*(4″-3″) 9.1, *J*(4″-5″) 9.1].

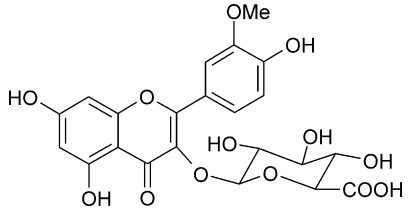
MS: Negative ES-MS, *m*/*z* (rel. int.): 463 ([M–H]$^-$, 100).

HPLC online UV–vis spectra: retention time at 14.8 ± 0.1 min; absorption maxima at 213.7, 281.3 and 342.3 nm.

2.1.8. Compound 8: quercetin 3-O-β-D-glucuronide

Formula: $C_{22}H_{20}O_{13}$

Molecular Mass: 492.4



NMR: ^{1}H (CD_3OD) d = 7.63, 7.64 [*m* (second order system), H-2′/H-6′], 6.89 [*m* (second order), H-5′], 6.43 [*d*, H-8, *J*(8-6) 2.0], 6.25 [*d*, H-6], 5.26 [*d*, H-1″, *J*(1″-2″) 7.6], 3.80 [*d*, H-5″, *J*(5″-4″) 9.7], 3.70 [s, 3′-OMe], 3.61 [*dd*, H-4″, *J*(4″-3″) 9.0], 3.56 [*dd*, H-2″, *J*(2″-3″) 9.1], 3.49 [*dd*, H-3″].

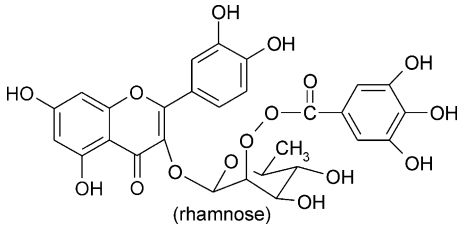
MS: Negative ES-MS, *m*/*z* (rel. int.): 491 ([M–H]$^-$, 100).

HPLC online UV–vis spectra: retention time at 16.1 ± 0.1 min; absorption maxima at 256.3 and 356.2 nm.

2.1.9. Compound 9: 2″-O-(3,4,5-trihydroxybenzoyl) quercitrin; galloyl quercitrin

Formula: $C_{28}H_{24}O_{15}$

Molecular Mass: 600.5



NMR: ^{1}H (CD_3OD) d = 7.40 [*bs*, H-2′], 7.39 [*dd*, H-6′, *J*(6′-2′) 2.2, *J*(6′-5′) 8.1], 7.11 [*s*, H-2‴/ H-6‴], 6.98 [*d*, H-5′], 6.42 [*d*, H-8, *J*(8-6) 2.1], 6.24 [*d*, H-6], 5.67 [*dd*, H-2″, *J*(2″-1″) 1.7, *J*(2″-3″) 3.4], 5.55 [*d*, H-1″], 4.05 [*m*, H-5″], 3.51 [*m*, H-3″/H-4″], 1.07 [*d*, H-6″, *J*(6″-5″) 5.7].

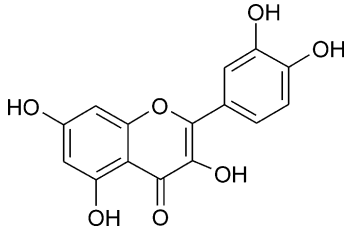
MS: Negative ES-MS, *m*/*z* (rel. int.): 599 ([M–H]$^-$, 100).

HPLC online UV–vis spectra: retention time at 18.8 ± 0.1 min; absorption maxima at 264.4 and 348.0 nm.

2.1.10. Compound 10: quercetin

Formula: $C_{15}H_{10}O_7$

Molecular Mass: 302.2



NMR: ^{1}H (CD_3OD) d = 7.77 [*d*, H-2′, *J*(2′-6′) 2.1], 7.67 [dd, H-6′, *J*(6′-5′) 8.5], 6.92 [*d*, H-5′], 6.43 [*d*, H-8, *J*(8-6) 2.0], 6.22 [*d*, H-6].

MS: Negative ES-MS, *m*/*z* (rel. int.): 301 ([M–H]$^-$, 100).

HPLC online UV–vis spectra: retention time at 20.2 ± 0.1 min; absorption maxima at 252.7 and 369.6 nm.

2.2. Antioxidative properties of the isolated flavonoids

Differences in antioxidative behavior of the isolated flavonoids was observed in their scavenging of two

types of free radicals. For instance, kaempferol glucoside (L-KGG), which possesses a more powerful antioxidative capability against $ABTS^{+\bullet}$ radicals than Trolox (antioxidant reference material), showed lower antioxidative activity than Trolox in PBN-AI system (see Table 2 and Fig. 2).

To resolve these differences, a semi-quantitative assessment by ESR, which measures the antioxidative capability of flavonoids in the PBN-AI systems, was conducted. In addition, a quantitative assay approach that measures the scavenging capability of the flavonoids against the $ABTS^{\bullet+}$ radical was used to determine the TEAC values of the flavonoids as well as to help elucidate the correlation between the structural features of the flavonoids and their antioxidative capabilities.

The results from the semi-quantitative study using the PBN-AI spin trapping technique are shown in Fig. 2. Significant antioxidative behavior for the flavonoids, as shown by the apparent suppression in the formation of the spin adduct (free radical, control in Fig. 2), was observed. Estimated semi-quantitatively, the antioxidative capabilities of the isolated flavonoids are similar to Trolox. In other words, it is very difficult to discriminate which flavonoid possesses a stronger antioxidative activity in the PBN-AI system.

The UV–vis measuring method was also used to determine the TEAC values of the flavonoids, and the results are presented in Table 2. The order of effectiveness in scavenging the $ABTS^{\bullet+}$, radicals, also a measure of their antioxidative potentials, is as follows:

$$\text{L-QRG} > \text{L-QG} > \text{L-QA} > \text{L-QR} > \text{L-KGG} > \text{L-LG} > \text{L-LA} > \text{L-SA} > \text{L-SG} > \text{L} - \text{KG}$$

Certain inferences can be drawn from these results.

1. The flavonoids belonging to the quercetin family generally appear in the first part of the TEAC order (their TEAC values are all beyond 3.4), which means that quercetin compounds possess stronger antioxidative activities than other flavonoids such as, for example, kaempferol, scutillarein and luteolin. This can be reasonably attributed to the structural features of quercetin, that is to the presence of the heterocyclic C-ring composed of a 4-keto group, 2,3-double bond and 3-hydroxyl group, which allows it to form a "huge" π-bond that links the A and B rings for electron delocalisation. This helps to stabilise the aryloxyl radical after hydrogen donation in the process of scavenging the free radicals (Rice-Evans et al., 1996).
2. Gallate esterification of the flavonoids, that have gallic acid attached to flavonoid nuclei at the 3-position by ester linkage via a sugar, can enhance its antioxidative activity. L-QRG (TEAC = 6.14, the most powerful antioxidant in our findings) and L-KGG (TEAC = 2.9) are more effective than their aglycones, quercetin (TEAC = 4.65) and kaempferol (TEAC = 1.39), respectively.
3. A sugar group attached to the flavonoid nuclei at the 7-position does not notably affect its antioxidative activity. For instance, the difference between the TEAC value of L-LG (TEAC = 2.87) and that of L-LA (TEAC = 2.33) is small. Similar results are also observed on L-SG (TEAC = 1.98) and L-SA (TEAC = 2.16). The attachment of water-soluble group(s) can thus improve the solubility of the flavonoid derivatives in hydrophilic media as compared with their aglycones.

It should be mentioned that the most interesting flavon-3-ols isolated are galloyl quercitin and galloyl kaempferol glucoside. They are noteworthy, both for their high-yield occurrences (rare in nature) in Laksa leaves and for their powerful antioxidative potentials (high TEAC values).

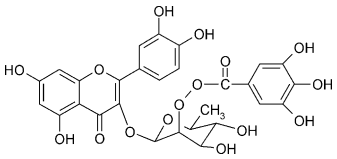
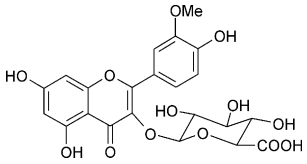
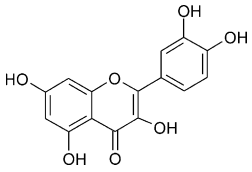
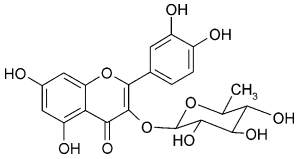
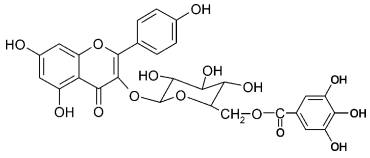
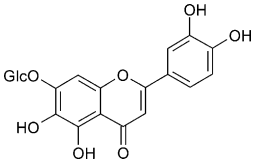
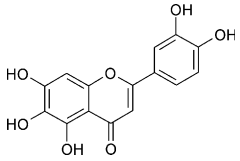
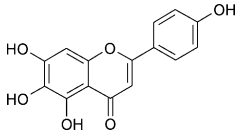
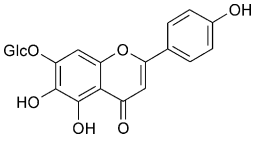
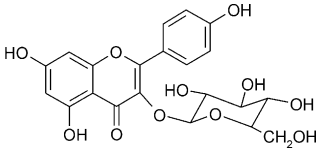
2.3. Possible synergy between flavonoids on antioxidative activity

It was of interest to examine whether there was the operation of a synergistic effect that contributes to the enhancement of antioxidative activity of a flavonoid in the presence of other flavonoids. Two experiments were conducted for this purpose.

The data from ESR assays showed that in the presence of flavonoids from different families, the antioxidant capability did not improve significantly in the PBN-AI measurement (Fig. 3). Mixing two types of flavonoids, e.g. quercetin (L-QA) and 6-hydroxyluteolin (L-LA), which have a pronounced difference in their structures, also did not bring about a noticeable change in their antioxidative capabilities.

The same conclusion was reached from the results of UV–vis measurements, when combinations of two flavonoids belonging to the same family were tested (Fig. 6) by monitoring the TEAC value changes when two different kaempferol compounds were mixed in various amounts. The results indicated that in the presence of a fixed amount of kaempferol aglycone(KA), the introduction of kaempferol 3-glucoside (L-KG) in various amounts did not cause significant fluctuations of the TEAC values (Fig. 4). This suggests that the capability of the scavenging $ABTS^{+\bullet}$ radical remained about the same in terms of the antioxidative potential per mole of antioxidant (solid line), although the total antioxidative capability was enhanced with the addition of another flavonoid, KGR (the white column and dotted line). As shown in Fig. 4, the TEAC values did not appear to significantly change in the presence of other flavonoids.

Table 2
TEAC values of the flavonoids isolated from Laksa leaves

Abbr.	Name (common name)	Structure	TEAC
L-QRG	2″-*O*-(3,4,5-Trihydroxybenzoyl) quercitrin; galloyl quercitrin		6.14±0.06
L-QG	Quercetin 3-*O*-β-D-glucuronide		5.08±0.09
L-QA	Quercetin		4.65±0.07
L-QR	3-*O*-α-L-Rhamnopyranosyloxy-3′, 4′, 5, 7-tetrahydroxyflavone (quercitrin)		3.46±0.11
L-KGG	6″-*O*-(3,4,5-Trihydroxybenzoyl) 3-O-β-D-glucopyranosyloxy-3′, 4′, 5, 7-tetra-hydroxyflavone (galloyl kaempferol 3-glucoside)		2.90±0.06
L-LG	6-Hydroxyluteolin 7-*O*-′-D-glucopyranoside		2.87±0.04
L-LA	6-Hydroxyluteolin; 3′, 4′, 5, 6, 7-pentahydroxyflavone		2.33±0.04
L-SA	Scutillarein (aglycone)		2.16±0.05
L-SG	Scutillarein 7-O-β-D-glucopyranoside or 6-hydroxyapigenin		1.98±0.08
L-KG	3-*O*-β-D-Glucopyranosyloxy-4′, 5, 7-trihydroxyflavone (kaempferol 3-glucoside)		1.39±0.07

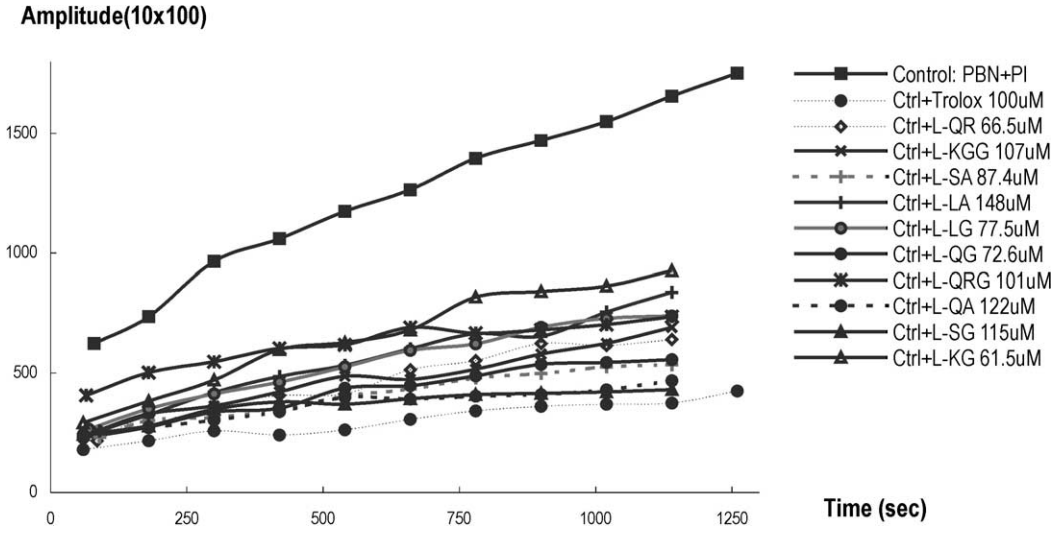


Fig. 2. Antioxidative capabilities of the flavonoids in PBN-Al system.

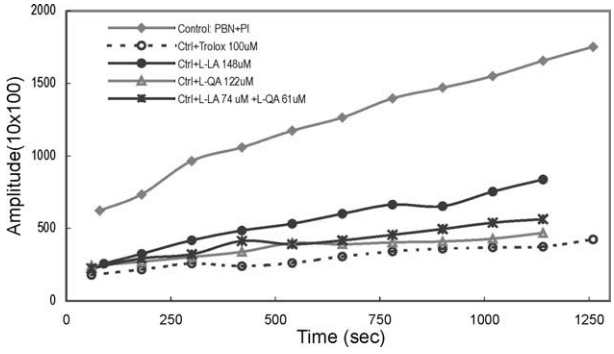


Fig. 3. Effect of combination of different flavonoids on the antioxidative activity in PBN-AI system.

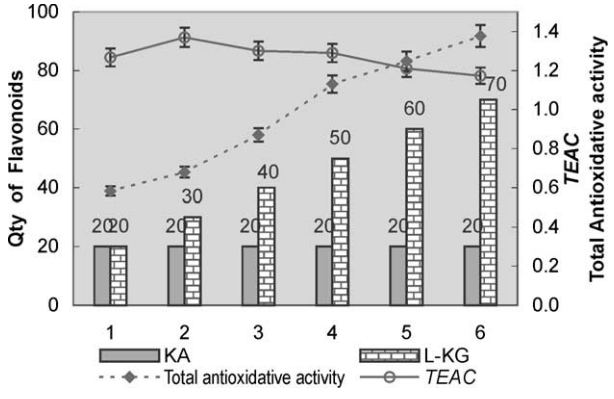


Fig. 4. Effect of the co-existing L-KG on the antioxidative potential of KA.

In summary, a combination of two flavonoid compounds did not significantly improve the overall antioxidative potential.

3. Experimental

3.1. Reagents and instrumentation

3.1.1. Chemicals

The following were purchased from Sigma-Aldrich, unless otherwise stated: phosphate buffer saline (PBS): pH = 7.4; myoglobin : 0.4 mg/ml; 2,2′-azino-bis(3-ethylbenzothiazolin-6-sulphonic acid) diammonium salt (ABTS): 0.1 mg/ml; hydrogen peroxide, 10 mM (Fisher, Fluka); horseradish peroxidase (HRP), 0.1 unit/ml; spin trapping agent: PBN, 100 mM; azo initiator: 2,2′-azobis (2-methyl propionamidine) dihydrochloride, 100 mM; reference material (Trolox): 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Tokyo Kasei).

3.1.2. HPLC system for analysis

Waters 600 HPLC system with 996 photo-diode array UV–vis detector.

Software: Millennium 2010; Column: ET 250/4 Nucleosil 120-5 C18 (Macherey-Nagel).

3.1.3. Prep-HPLC system for isolation

Beckman system gold equipped with a programmable solvent module-126NMP and a System Gold 168 dual-channel UV–vis detector.

Preparative column: VP250/40 Nucleoprep 100-10 (Macherey-Nagel), length: 250 mm, internal diameter 40 mm.

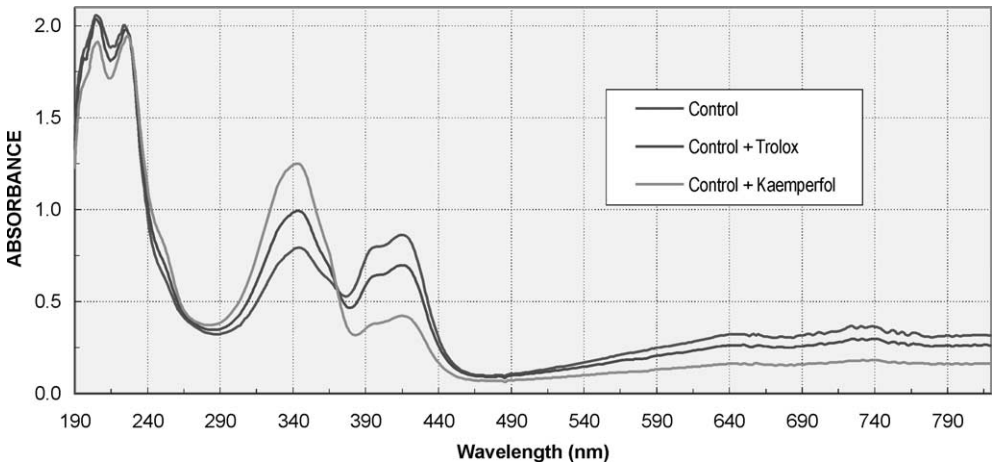


Fig. 5. UV–visible spectral change of ABTS/$ABTS^{+\bullet}$ in the presence of antioxidants.

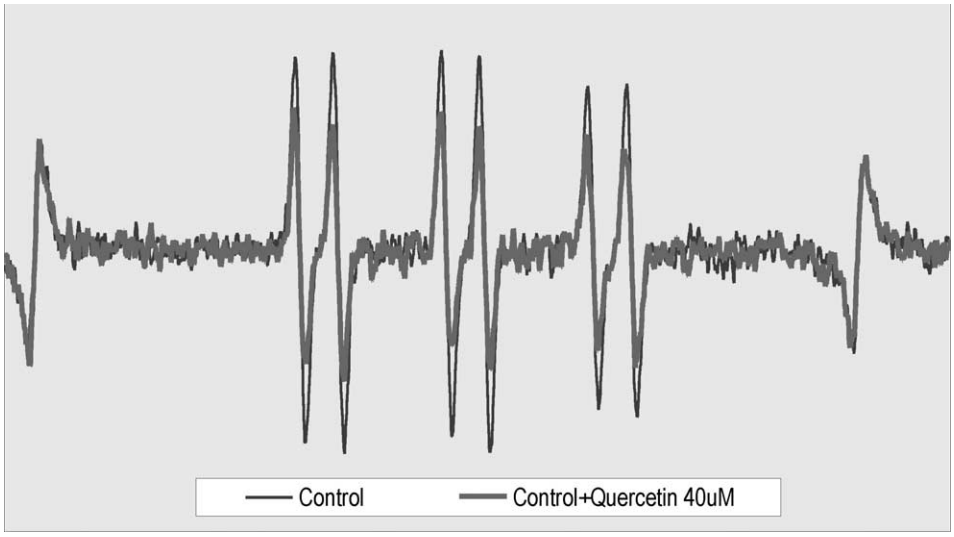


Fig. 6. ESR spectra of PBN radical and behavior of an antioxidant in PBN-AI assay.

3.1.4. UV–vis spectrophotometer

HP-8452A diode array spectrophotometer (Hewlett Packard).

3.1.5. ESR spectrometer

JES-TE100 ESR spectrometer (JEOL) inclusive of a microwave unit, cavity resonator, electromagnet, spectrometer as well as corresponding control and data processing software.

3.2. Procedures

3.2.1. Extraction and isolation

In order to obtain a pure flavonoid, a series of processing treatments comprising sample preparation, extraction, separation with column chromatography, prep-HPLC isolation and final purification, was used.

Fresh Laksa leaves were purchased from a wet market in Singapore. After the removal of any rotten leaves, the leaves were washed several times with tap water and then rinsed with distilled water. It was then dried in good air drift conditions, and kept in an oven at 100 °C for 2 h in order to deactivate the enzymes. The leaves were then chopped up roughly and sealed in plastic bags containing nitrogen for extended storage in freezer. Before extraction, it took about 30 min to grind the dried sample into fine powder in a ceramic mortar containing a fixed amount of dry ice.

The primary extraction was carried out as the first step since the complexity of the components in the sample was reflected in screening work with an HPLC assay. A sample was extracted using 4 aliquots of 80% MeOH solution, and the extracts then combined together, with the solvent being removed under reduced pressure. The chlorophyll was removed using hexane extraction; and the water phase was concentrated into a small volume. It was re-extracted using pure MeOH, 80% MeOH, 40% MeOH and water, in turn, to get crude extracts.

The polyamide-packed column chromatography proved to be the best way to achieve pre-isolation. The

above-mentioned crude extracts were loaded onto a MeOH-equilibrated chromatograph column packed with polyamide after removal of the organic solvent. Five fractions were collected by using a series of eluents in turn: water, 33% MeOH, 66% MeOH, 100% MeOH and pure MeOH containing a few drops of aqueous ammonia. These fractions were concentrated using a vacuum rotary evaporator for further prep-HPLC isolation.

A prep-HPLC isolation was conducted by gradient elution: the flowrate of the mobile phase was set at 10 ml/min; channel A was 0.04% aqueous formic acid and channel B was MeOH solution containing 0.04% formic acid. Fractions were collected by monitoring the change in the two UV–vis spectra (the ratio of pump A to pump B as well as the running time can be manipulate to permit better isolation, for various samples). The composition of the various fractions and the purity of the fractionated compounds were checked by running an analytical HPLC assay.

To produce a residue-free eluant, Sephadex gel LH-20 was used. For final "clear-up"(purification) of the isolated flavonoids, MeOH is used as the eluent.

3.2.2. Identification of isolated flavonoids

The structural elucidation of each isolated flavonoid was conducted by comprehensively analyzing the data from various assay techniques such as HPLC online-ultraviolet/visible spectrometry (UV/vis), mass spectrometry (MS) and nuclear magnetic resonance spectrometry (NMR) as well as by using hydrolysis techniques.

As a general rule, identification began with HPLC online UV–vis analysis under the following conditions. Time (min), A (%), B (%): 0.0, 100.0, 0.0; 30.0, 20.0, 80.0; 31.0, 0.0, 100.0; 35.0, 0.0, 100.0; 36.0, 100.0, 0.0; 46.0, 100.0, 0.0.

Column: ET 250/4 Nucleosil 120-5 C18, 250×4.6 mm; flow rate of gradient elution: 1 ml/min; mobile phase: (A) distilled water with 1.5% phosphoric acid, (B) acetonitrile.

The UV–vis spectral data of a flavonoid, obtained in each isolation process, was used to roughly classify the analyte through the monitoring of its characteristic maxima in the regions of 210–290 and 300–550 nm (Markham, 1982; Harborne, 1988; Mabry and Markham, 1975). Also, the HPLC behaviour (retention time) of the analyte indirectly reflected the bonding characterization of sugar(s) or other water-soluble group(s) in the analyte. MS was applied to determine the molecular mass of the analyte and confirm the position of various group(s) from a study of the fragmentation patterns. NMR spectrometry was used to re-confirm the positions of the various groups.

3.2.3. Evaluation of antioxidative activity of the isolated flavonoids

Two assay methods were used to evaluate the antioxidative activity of the isolated flavonoids.

3.2.3.1. Measurement of antioxidative potential (TEAC) of antioxidants.

A common method for testing the antioxidative potential of antioxidants as hydrogen-donating agents is to measure their ability to scavenge $ABTS^{+\bullet}$, the ABTS radical (Rice-Evans et al., 1996; Miller et al., 1993). This method is reliant on the generation of a long-lived specific $ABTS^{+\bullet}$ chromophore and its quenching (or suppression) by an antioxidant (Fig. 5). Trolox, the water-soluble vitamin E analog, is taken as a standard. Thus, Trolox Equivalent Antioxidant Capability (TEAC), which is defined as the concentration of the Trolox with the same antioxidant activity as a 1 mM concentration of the substance under investigation, is taken as an "index" to evaluate antioxidative activity of an antioxidant.

The procedure is as follows: mix 3.8 ml of PBS, 2 ml of ABTS and 200 μl of myoglobin in a measuring tube; add 80 μl of H_2O_2 (10 mM) and shake vigorously; incubate the resultant solution at 50 °C in a water-bath for 15 min to develop colour, then allow it to cool down to room temperature for 20 min. Add 50–200 μl of the sample, top up the mixture to 7 ml with PBS and mix thoroughly. Start a timer simultaneously. At exactly 2 min, measure the absorbance of the solution at 414 or 734 nm. Calculate the TEAC values of the analyte on the basis of the concentration change of $ABTS^{\bullet+}$ with a calibration curve plotted by using a series of Trolox standard solutions.

3.2.3.2. Estimation of antioxidative activity of antioxidants by electron spin resonance (ESR) spectrometry.

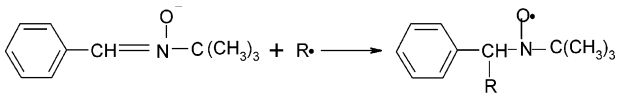
Using the ESR technique, the antioxidative activity of an antioxidant can be evaluated by monitoring its propensity to affect the generation of certain free radicals (Niki, 1990). The PBN-AI spin trapping method serves this purpose.

A water-soluble azo compound 2,2′-Azobis(2-methylpropion-amidine) dihydrochloride was taken as the initiator, AI, to produce the initial free radical.

$$R-N=N-R \rightarrow N_2 + 2R$$

(Azo Initiator, AI)

In the presence of the spin trapping reagent phenyl-*tert*-butyl nitrone (PBN), a nitroxide spin adduct is formed:



spin trap spin adduct

The spin trapping agent can trap free radicals rapidly to form more persistent radicals (spin adducts) which are detectable by ESR. As ESR signal strength is directly proportional to the number of radicals produced, spin

trapping can be used as a quantitative tool for counting radical events and thus to study antioxidants. Here, the evaluation of the antioxidative capability of an antioxidant is undertaken by monitoring the generation of the PBN spin adduct in the presence of the antioxidant (Fig. 6).

Experimentally, the ESR assay was conducted as follows.

Mix 20 μl of PBS, 40 μl of PBN and 20 μl of the sample or standard in a micro cell. Add 40 μl of AI and start the timer.

Transfer the resultant solution to a flat cell for ESR measurement under the following conditions.

Magnetic field: center field, 32.8.48 mT; sweep width, 5.0×1; sweep time, 30 s. Amplitude parameter: modulation frequency, 100 kHz; modulation width, 1.0×0.1 mT; amplification, 5.0×100; modulation, 1st; phase, 0; time constant, 0.03 s. Microwave status: frequency, 9.208 GHz; power, 10 mW; phase, 501; digital marker, 560. [Temperature: 25° (room temperature).]

Record, every minute, the peak heights that reflect the generation of PBN radicals. Plot the graph of time against amplitude.

4. Conclusions

Ten main flavonoids were isolated from Laksa leaves. Six of them belonged to flavone-3-ol and the other four belonged to flavone. 6″-*O*-(3,4,5-trihydroxybenzoyl) 3-*O*-β-D-glucopyranosyloxy- 3′, 4′, 5, 7- tetra-hydroxyflavone (galloylkaempferol 3-glucoside) gave the highest yield (1%) in weight percentage.

The isolated flavonoids showed strong antioxidative activity in vitro, in both the UV–visible and ESR assays. It should be noted that galloyl quercitrin, a flavon-3-ol compound, was found to have exceptional antioxidative activity—its TEAC value was 6.14.

A combination of two flavonoids did not significantly improve the antioxidative potential, that is, the synergistic antioxidative activity of two flavonoid compounds is not much enhanced in our experiments.

Acknowledgements

The research was supported by Nanyang Technological University Research Grant No. RP3/99 CLS. We thank the Nanyang Technological University and the Lee Foundation for their financial support.

References

Bors, W., Heller, W., Michel, C., Stettmaier, K., 1996. Flavonoids and polyphenols: chemistry and biology. In: Cadenas, E., Paker, L. (Eds.), Handbook of Antioxidants. Marcel Dekker Inc, New York, pp. 409–466.

Halliwell, B., Gutteridge, J.M.C., 1999. Free Radicals in Biology and Medicine. Oxford University Press Inc., New York. pp. 225.

Harborne, J.B., 1988. The Flavonoids: Advances in Research Since 1980. Chapman & Hall, London.

Kok, P.T., Hsaun, K., Avadhani, P.N., 1991. In: Foo, T.S. (Ed.), A Guide to Common Vegetables. Singapore Science Center, p. 124.

Mabry, T.J., Makham, K.R., 1975. In: Harborne, J.B., Mabry, T.J., Mabry, H. (Eds.), The Flavonoids. Chapman & Hall, London, p. 8.

Markham, K.R., 1982. Techniques of Flavonoid Identification. Academic Press, London and New York.

Miller, N.J., Rice-Evans, C.A., Davis, M.J., Gopinathan, V., Milner, A., 1993. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clinical Science 84, 407–412.

Niki Etsuo, 1990. Method in Enzymology. Vol. 186, p. 100.

Okuda, T., Yoshida, T., Hatano, T., 1992. Pharmacologically active tanins isolated from medicinal plants. Basic Life Science 59, 539–569.

Pietta, P., 1998. Flavonoids in medicinal plants. In: Rice-Evans, C.A., Packer, L. (Eds.), Flavonoids in Health and Disease. Macel Dekker Inc, New York, pp. 61–120.

Pincemail, J., 1995. Free radicals and antioxidants in human diseases. In: Faviler et al. (Eds.), Analysis of Free Radicals in Biological Systems. Birkhauser Verlay, Switzerland, p. 83.

Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine 20 (7), 933–956.

Rice-Evans, C.A., Miller, N.J., Paganga, G., 1997. Antioxidant properties of phenolic compounds. Trends in Plant Science 2 (4), 152–159.

Yagi, A., Uemura, T., Okamura, N., et al., 1994. Antioxidative sulphated flavonoids in leaves of *Polygonum hydropiper*. Phytochemistry 35 (4), 885–887.