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Total Peroxyl Radical-Scavenging Capacity of the Chemical Components from the Stems of *Acer tegmentosum* Maxim

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Acer tegmentosum is a type of deciduous tree that grows in Korea. It has been used in traditional medicine for the treatment of hepatic disorders. In this study, chromatography fractionation and isolation have been successfully used to yield 15 compounds, including 10 flavonoids, 4 phenylethyl glycosides, and 1 other glycoside. Their structures were determined on the basis of their physical and spectral properties [^{1}H and ^{13}C nuclear magnetic resonance (NMR) and mass spectrometry (MS)] and by comparison of these results to similar data in the literature. The total peroxyl radical-scavenging capacity of each isolated compound was evaluated. Among them, the most active components belong to the flavonoids. Among these, quercitrin (**1**), 6-hydroxy-quercetin-3-*O*-galactose (**6**), and (+)-catechin (**8**) showed stronger activity than the positive control Trolox.

KEYWORDS: ***Acer tegmentosum*; Aceraceae; antioxidant; peroxyl radical; TOSC; flavonoid**

INTRODUCTION

Oxidative stress generated by reactive oxygen species (ROS), such as hydroxyl radicals and hydrogen peroxide, has been linked to several cellular toxicity processes, including damage to proteins, membrane lipid peroxidation, DNA alteration, and enzyme inactivation (*1, 2*). These may be attributed to chemical carcinogenesis, heart diseases, reperfusion injury, rheumatoid arthiritis, inflammation, and aging (*3*). Therefore, studies on antioxidants, especially those thought to prevent the presumed deleterious effects of ROS in the human body, are becoming more widespread and are receiving much attention from biologists, medicinal chemists, etc. Until now, phytochemicals (especially secondary metabolites) have been recognized as antioxidant sources in crude extracts and dietary foods. Similarly, there is also an increase in the use of methods for estimating the efficiency of antioxidants.

The total oxidant scavenging capacity (TOSC) assay, developed by Regoli and Winston, is a method for evaluating antioxidant activity based on the inhibition of oxy-radical-induced ethylene gas production from α-keto-γ-methiolbutyric acid (KMBA) (*4*). The advantages of this method are that it is simple, rapid, and applicable for either pure antioxidants or biological tissues. It is worth noting that the number of publications focusing on TOSC has increased in recent years.

Acer tegmentosum (Aceraceae) is a type of deciduous tree that grows in Korea, Russia, and northern areas of China. In Korea, *A. tegmentosum* has been used in traditional medicine for the treatment of hepatic disorders (*5*). Its phytochemical constituents have been investigated in the past but not extensively; only some of the isoprenoids, flavonoids, and other phenolic compounds of the plant were reported (*6*−*8*). As part of our ongoing investigations of bioactive compounds from Korean medicinal plants, we have successfully isolated 15 compounds, including 10 flavonoids and 5 others, from a MeOH extract of the stems of *A. tegmentosum*. Their antioxidant activities against peroxyl radicals were evaluated using the TOSC assay.

MATERIALS AND METHODS

General Procedures. Melting points were measured using a Fisher−Johns melting point apparatus and are uncorrected. Optical rotations were measured using a JASCO DIP-360 (Tokyo, Japan) automatic digital polarimeter. The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker DRX NMR spectrometer (Germany) using Bruker's standard pulse program. Chemical shifts were reported in ppm downfield from tetramethylsilane (TMS), with *J* in Hz. The electronspray ionization (ESI) mass spectra were recorded on an AGILENT 1100 LC-MSD trap spectrometer. Silica gel (70−230 and 230−400 mesh, Merck), YMC RP-18 resins (30−50 μm, Fuji Silysia Chemical Ltd.), and Dianion HP-20 (Mitshubishi Chemical Corporation) were used as absorbents in the column chromatography performed in this research. Thin-layer chromatography (TLC) plates (Silica gel 60 F_{254} and RP-18 F_{254}, 0.25 mm, Merck) were purchased from Merck KGaA (Darmstadt, Germany). Spots were detected under UV radiation (254 and 365 nm) and by spraying the plates with 10% H_2SO_4, followed by heating with a heat gun.

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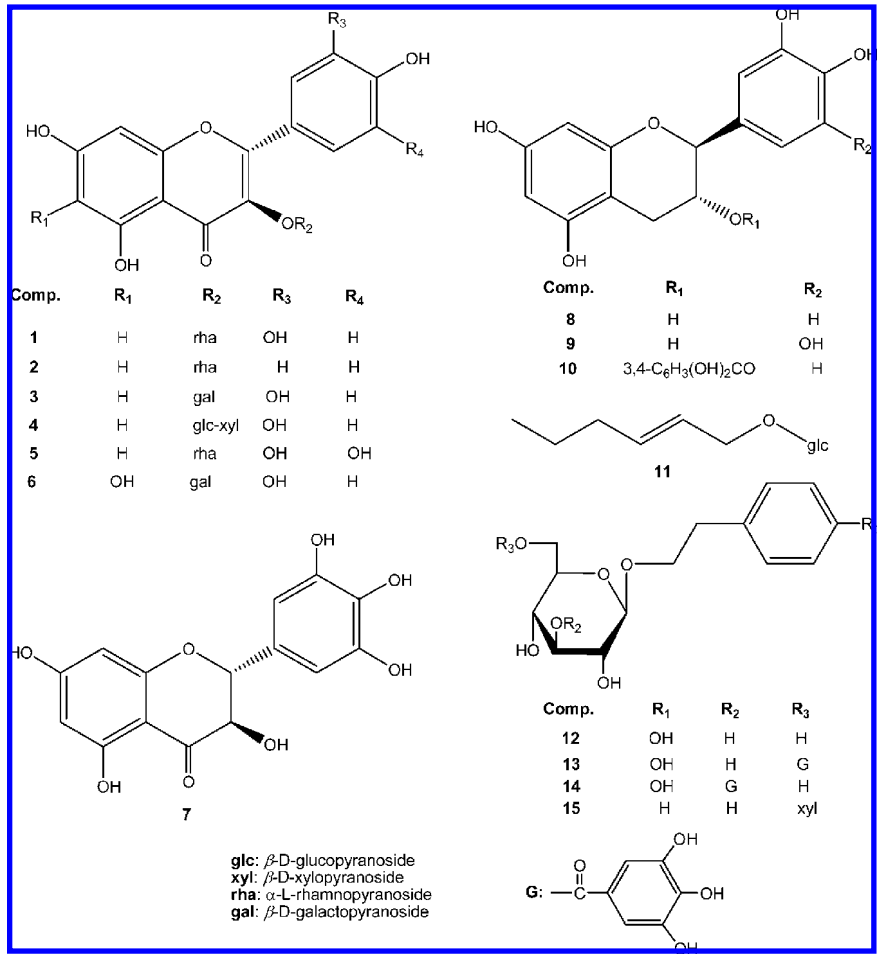

Figure 1. Structures of the isolated compounds (**1**−**15**).

Plant Materials. The stems of *A. tegmentosum* were collected at Duk Yu Mt., Cheon Buk, Korea, in August 2005 and taxonomically identified by one of us (K. Bae). A voucher specimen (CNU 05012) has been deposited at the College of Pharmacy, Chungnam National University, Daejeon, Korea.

Chemicals. Ultra-high quality (UHQ) water was used to make all of the solutions. α-Keto-γ-methiolbutyric acid (KMBA) and 2,2′-azobis-amidinopropane (ABAP) were purchased from Sigma-Aldrich Corp.

Extraction and Isolation. The dried stems of *A. tegmentosum* (2.5 kg) were extracted with MeOH (6 L) 3 times at room temperature. The combined MeOH extract (210 g) was suspended in water (1.5 L) and then partitioned with *n*-hexane, CH_2Cl_2, and EtOAc (each 1.5 L × 3), successively. The water layer was subjected to a Dianion HP-20 column eluted with a gradient of MeOH in H_2O (25, 50, 75, and 100% MeOH) to give four fractions (**1a**−**d**). The EtOAc extract (30 g) was chromatographed on a silica gel column using a gradient of CH_2Cl_2/MeOH (30:1−0:1) as the eluent to afford seven fractions (**2a**−**g**). Repeated silica gel column chromatography of fraction **2c** with $CHCl_3$/MeOH (6:1) gave six subfractions (**3a**−**f**). Fraction **3b** was further chromatographed using RP column chromatography with MeOH/H_2O (2:3) to give dihydromyricetin [**7**, 5.0 mg, yellow powder, mp 245−246 °C, $[\alpha]_D^{20}$ +41 (*c* 0.5, MeOH), ESI−MS *m*/*z* 321 $[M + H]^+$] (*9*); (+)-catechin-3-*O*-(3,4-dihydroxybenzoyl) [**10**, 4.0 mg, yellow powder, $[\alpha]_D^{20}$ +35 (*c* 0.4, MeOH), ESI−MS *m*/*z* 427 $[M + H]^+$] (*10*); and erigeside B [**11**, 4.3 mg, colorless syrup, $[\alpha]_D^{20}$ +15 (*c* 0.6, MeOH), ESI−MS *m*/*z* 263 $[M + H]^+$] (*11*). Next, kaempferol-3-rhamnoside [**2**, 16.4 mg, yellow powder, mp 172−174 °C, $[\alpha]_D^{20}$ −60 (*c* 0.61, MeOH), ESI−MS *m*/*z* 433 $[M + H]^+$] (*12*) and (+)-catechin [**8**, 25 mg, colorless needle, mp 240−241 °C, $[\alpha]_D^{20}$ +28 (*c* 0.32, MeOH), ESI−MS *m*/*z* 291 $[M + H]^+$] (*7*) were obtained from fraction **3d** by RP column chromatography using an eluent of MeOH/H_2O (6:5). Quercitrin [(**1**, 28 mg, yellow powder, mp 234 °C, $[\alpha]_D^{20}$ −158 (*c* 0.62, MeOH), ESI−MS *m*/*z* 471 $[M + Na]^+$] (*13*) was isolated from fraction **3e** by means of a RP column eluted with MeOH/H_2O (1:1). Fraction **2e** was subjected to a silica gel column using an eluent of $CHCl_3$/MeOH (9:2) to give three subfractions (**4a**−**c**). Consequently, fraction **4b** was further purified using a RP column with an eluent of MeOH/H_2O (2:3) to yield hyperin [**3**, 22 mg, yellow powder, mp 232−233 °C, $[\alpha]_D^{20}$ −55 (*c* 0.5, MeOH), ESI−MS *m*/*z* 465 $[M + H]^+$] (*14*); gallocatechin [**9**, 11.6 mg, colorless needles, mp 189−191 °C, $[\alpha]_D^{20}$ +32 (*c* 0.6, MeOH), ESI−MS *m*/*z* 307 $[M + H]^+$] (*15*); 4-hydroxy-phenylethyl-*O*-β-D-glucopyranose [**12**, 50 mg, colorless needles, mp 220 °C, $[\alpha]_D^{20}$ +17 (*c* 0.4, MeOH), ESI−MS *m*/*z* 301 $[M + H]^+$] (*7*); and 6′-*O*-galloylsalidroside [**13**, 23 mg, colorless needles, mp 116−117 °C, $[\alpha]_D^{20}$ +15 (*c* 0.48, MeOH), ESI−MS *m*/*z* 453 $[M + H]^+$] (*16*). Fraction **1e** was chromatographed on a silica gel column with $CHCl_3$/MeOH/H_2O (10:3:0.4) to afford three subfractions (**5a**−**c**). Phenylethyl-*O*-[β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside] [**15**, 4.3 mg, colorless syrup, $[\alpha]_D^{20}$ +13 (*c* 0.4, MeOH), ESI−MS *m*/*z* 433 $[M + H]^+$] (*17*) was obtained from fraction **5c** using a RP column with MeOH/H_2O (1:2). Next, fraction **5b** was rechromatographed on a RP column with MeOH/H_2O (3:5) to give three subfractions (**6a**−**c**). Myricitrin [**5**, 5.5 mg, yellow powder, $[\alpha]_D^{20}$ −17 (*c* 0.3, MeOH), ESI−MS *m*/*z* 465 $[M + H]^+$] (*18*) and 6-hydroxy-quercetin-3-*O*-galactose [**6**, 6.2 mg, yellow powder, $[\alpha]_D^{20}$ +37 (*c* 0.6, MeOH), ESI−MS *m*/*z* 481 $[M + H]^+$] (*19*) were obtained from subfraction **6a** by RP column chromatography using MeOH/H_2O as the eluent. Finally, quercetin-3-*O*-[β-D-xylopyranosyl-(1→2)-β-D-glucopyranoside] [**4**, 5.1 mg, yellow syrup, $[\alpha]_D^{20}$ +17 (*c* 0.3, MeOH), ESI−MS *m*/*z* 619 $[M + Na]^+$] (*20*) and 3′-*O*-galloylsalidroside [**14**, 22 mg, white powder, $[\alpha]_D^{20}$ +4.5 (*c* 0.5, MeOH), ESI−MS *m*/*z* 453 $[M + H]^+$] (*16*) were isolated from subfraction **6c** using a RP column with MeOH/H_2O (1:3).

TOSC Assay. The TOSC assay was used to evaluate the peroxyl radical-scavenging capacity of the isolated compounds (*4*). Basically, peroxyl radicals were generated by thermal homolysis of ABAP at 35 °C. The assay conditions used were 0.2 mM KMBA and 20 mM ABAP

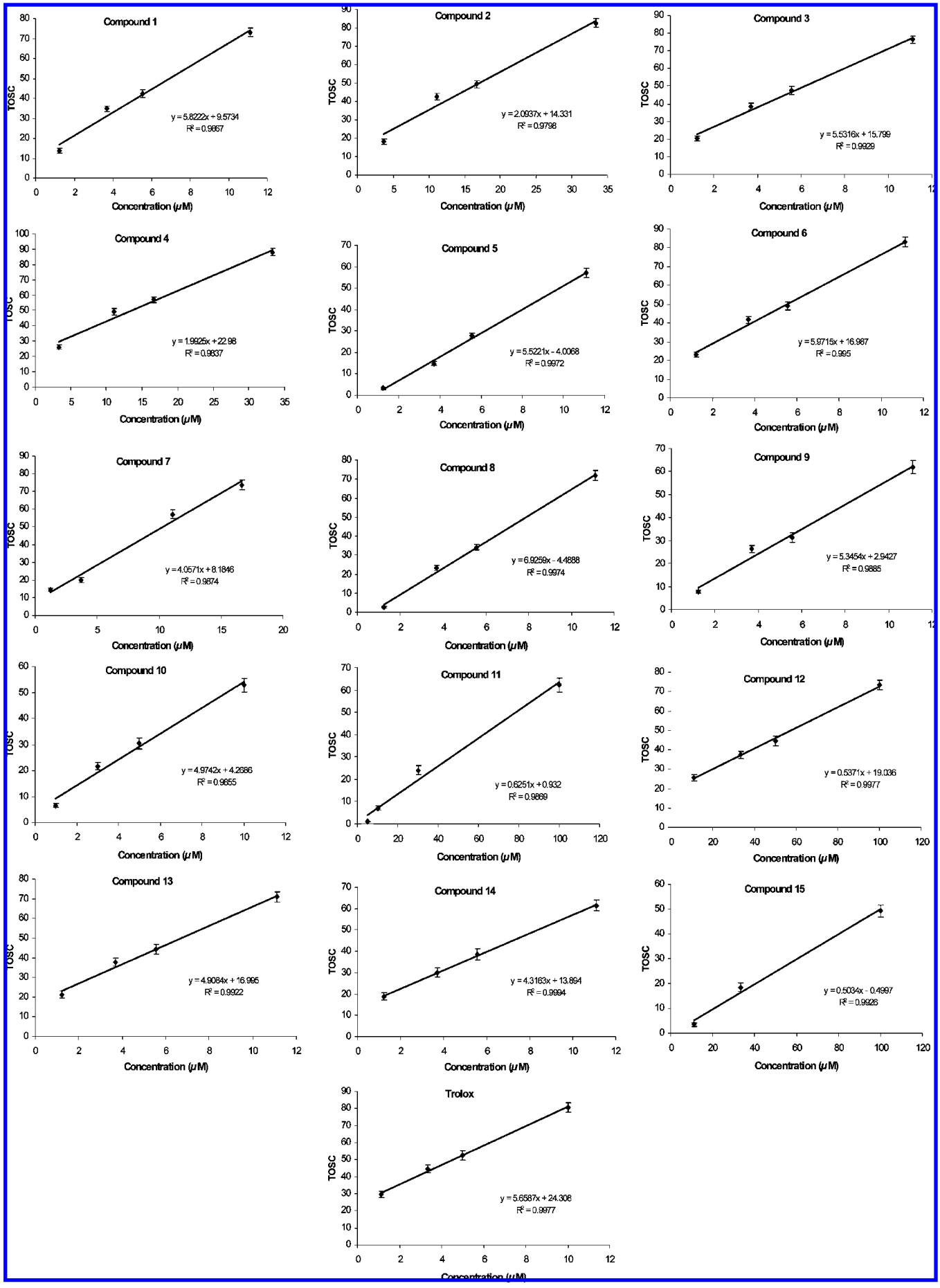

Figure 2. TOSC versus concentration for the samples.

in 100 mM potassium phosphate buffer (pH 7.4). Under these conditions, the rate of radical input in the reaction is 1.66×10^{-8} M s^{-1}. Reactions were carried out in 10 mL rubber septa-sealed vials in a final reaction volume of 1 mL. The reactions were initiated by the injection of 100 μL of 200 mM ABAP in water directly through the rubber septum. Ethylene production was measured by the gas chromatographic analysis of 150 μL aliquots taken directly from the head space of the reaction vial. Samples were monitored in sequence at 15 min intervals within a time course of 60 min. Analyses were performed with a Shimadzu-2010 (Shimadzu Corp., Japan) gas chromatograph equipped with a DB-05 capillary column (30 m length $\times$ 0.32 mm inner diameter). The oven, injection, and flame ionization detector (FID) temperatures were 60, 180, and 180 °C, respectively. Helium, at a flow rate of 30 mL/min, was used as the carrier gas.

Quantification of TOSC. The TOSC value for each concentration of sample was calculated as follows:

$$\mathrm{TOSC} = 100 - \left(\frac{\int \mathrm{SA}}{\int \mathrm{CA}} \times 100 \right)$$

Here, $\int$SA and $\int$CA are the integrated areas from the sample reaction and control reaction. Thus, a sample that displays no peroxyl radical-scavenging capacity would give an area equal to the control ($\int$SA/$\int$CA = 1) and a resulting TOSC value of 0. On the other hand, as the $\int$SA approaches 0, the hypothetical TOSC value approaches 100. Relative TOSC (rTOSC) and comparative TOSC (cTOSC) values were calculated using the method of Winston et al. (*4*). Briefly, rTOSC values were obtained from the slope of the linear regression lines for the TOSC curves. cTOSC values were quantified by dividing the rTOSC value of the sample by that obtained of Trolox, as shown below

$$\mathrm{cTOSC} = \frac{\mathrm{rTOSC(compound)}}{\mathrm{rTOSC(Trolox)}}$$

Statistical Analysis. Statistical analysis was performed using the spreadsheet program Excel (Microsoft Office 2003). The data are the mean of three repeated experiments in triplicate. Values varied by no more than 5% between experiments.

RESULTS AND DISCUSSION

By various column chromatography, 15 compounds (**1**−**15**) (**Figure 1**) were obtained from the EtOAc extract. Their structures were characterized by the comparison of their physical properties, NMR spectra (^{1}H and ^{13}C NMR), and electrospray ionization mass spectrometry (ESI−MS) to those published in the literature. It is the first time that compounds **4**−**7**, **9**−**11**, and **13**−**15** have been isolated from this plant.

The TOSC assay exploits the oxidation of KMBA to ethylene gas by the peroxyl radicals generated by the thermal homolysis of ABAP at 35 °C (see the Materials and Methods). All of the samples were analyzed for at least five concentrations. In the presence of each sample, individually, ethylene production was found to decrease proportionally because of increasing of concentration (data not shown). TOSC values were calculated as described in the Materials and Methods. It has been found that, the larger the TOSC value, the higher the degree of inhibition. The TOSC values of the different concentrations of the compounds are shown in **Figure 2**. Experimental TOSC values were then used to determine rTOSC values. rTOSC values are defined by the assay conditions used; that is, the temperature and ABAP concentration (*4*). From the TOSC graphs, the rTOSC values can be calculated by obtaining the slope of the regression line within the linear range. In addition, cTOSC values were then inferred and can be used as a mean of comparison between the isolated compounds relative to Trolox, a standard antioxidant. The rTOSC and cTOSC values of the compounds are shown in **Table 1**.

Table 1. Relative and Comparative TOSC Values for the Samples

compound	rTOSC	cTOSC
Trolox (positive control)	5.66 ± 0.11	1.00
1	5.82 ± 0.09	1.03
2	2.10 ± 0.10	0.37
3	5.53 ± 0.14	0.98
4	1.99 ± 0.07	0.35
5	5.52 ± 0.16	0.98
6	5.98 ± 0.12	1.05
7	4.06 ± 0.10	0.72
8	6.93 ± 0.13	1.22
9	5.35 ± 0.16	0.95
10	4.97 ± 0.14	0.88
11	0.63 ± 0.03	0.11
12	0.54 ± 0.07	0.10
13	4.91 ± 0.14	0.87
14	4.32 ± 0.10	0.76
15	0.50 ± 0.02	0.09

It can be seen that, among the isolated compounds, (+)-catechin (**8**) is the most active, followed by 6-hydroxy-quercetin-3-*O*-galactose (**6**) and quercitrin (**1**), respectively. Compounds **11**, **12**, and **15** were considered to have no activity. Of the 15 isolates, compounds **1**, **6**, and **8** were more active than the known antioxidant Trolox, while compounds **3** and **9** were roughly as active as Trolox.

Structurally, all 15 compounds (apart from compound **11**) belong to flavonoids [flavonols (**1**−**7**) and flavanols (**8**−**10**)] and phenyl ethyl glycosides (**12**−**15**). The TOSC result suggested that the peroxyl radical-scavenging capacity of the active compounds decreased in the order: flavanols (**8**−**10**) > flavonols (**1**−**7**) > phenylethyl glycosides (**12**−**15**). This finding was consistent with the recognition that flavonoids have been shown to scavenge various ROS having peroxyl radicals (*21*−*23*). In addition, (+)-catechin (**8**) and gallocatechin (**9**) have been isolated from many plants, especially from green tea (*Camellia sinensis*), and they are considered as potent antioxidant components (*24*).

Further structural analysis relative to the TOSC results found that, of the isolated flavonoids, the compounds with a quercetin-type B ring showed good activity. Consequently, the number of hydroxyl groups plays an important role in the antioxidant capacity of the compound. It is generally accepted that increasing the number of phenolic hydroxyl groups will increase the antioxidant activity of the flavonoid (*23*). Accordingly, in our present study, compounds **5** and **6** are relatively more active than compounds **1** and **3**, which are more active than compound **2**. Of the four phenylethyl glycosides, only compounds **13** and **14**, which both have a galloyl group, displayed antioxidant activity. Hence, the presence of the galloyl group definitely influenced their peroxyl radical-scavenging capacities.

The scavenging of peroxyl radicals, which breaks the chain of propagation in free-radical reactions, is a key step in the prevention of lipid peroxidation. It is suggested that a dietary intake of antioxidant-containing foods may be beneficial because it may lower the risk of certain pathophysiologies that have been associated with ROS, including heart disease, liver injury, etc. (*25*). Therefore, this study has also helped to explain, in part, why this plant has been used for the treatment of hepatic disorders in folk medicine.

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