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Antioxidative polyphenols from Nigerian mistletoe *Loranthus micranthus* (Linn.) parasitizing on *Hevea brasiliensis*

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ABSTRACT

Two new phenolic glycosides, linamarin gallate (**1**) and walsuraside B (**2**), together with nine known compounds, catechin (**3**), epicatechin (**4**), epicatechin 3-*O*-gallate (**5**), epicatechin 3-*O*-(3-*O*-methyl) gallate (**6**), epicatechin 3-*O*-(3,5-*O*-dimethyl)gallate (**7**), epicatechin 3-*O*-(3,4,5-*O*-trimethyl)gallate (**8**), quercetin 3-*O*-β-D-glucopyranoside (**9**), rutin (**10**), and peltatoside (**11**), were isolated from the leafy twigs of Nigerian mistletoe *Loranthus micranthus* (Linn.) parasitic on *Hevea brasiliensis*. Compound **1** was characterized as an unusual cyanogenic glycoside, while compound **8** was isolated for the first time from a natural source. This is the first report of a cyanogenic glycoside from mistletoes. The structures of the new compounds were unambiguously elucidated by 1D (^{1}H, ^{13}C), 2D NMR (COSY, HSQC, and HMBC) and by mass spectroscopy. The antioxidant activities of the isolated compounds (**1**–**11**) were evaluated using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay.

1. Introduction

Mistletoes are hemi parasitic plants growing on different host trees and shrubs. They depend on their host plant for water and mineral nutrition, even though they produce their own carbohydrates through photosynthesis [1]. Mistletoes are widely distributed in Nigeria with their local names depending on the region where they occur. Eastern Nigeria mistletoes grow in the South-eastern part of the country and are represented by the species *Loranthus micranthus* (Linn.). *L. micranthus* grows on many host trees like *Kola acuminata*, *Baphia nitida*, *Persia americana*, *Irvingia gabonensis*, *Citrus simensis*, *Pentacletra macrophylla*, *Treculiar africana*, and *Ficus exaperata* [1,3]. The leaves of mistletoes are traditionally used in folkloric medicine of Nigeria for the treatment of diarrhea, epilepsy, hypertension and rheumatism [2]. Methanolic extracts of mistletoes parasitic on different host trees have been reported to have antidiarrhea [3], antidiabetic [4], antimicrobial [5] and immunomodulatory activities [6]. Steroids and triterpenoids with immunomodulatory potentials have previously been isolated from Eastern Nigeria mistletoe *L. micranthus* parasitic on *Kola accuminata* [7]. In this study we describe the isolation and structure elucidation of two new phenolic glycosides (**1**–**2**) and nine known polyphenols (**3**–**11**) from the leafy twigs of *L. micranthus* parasitic on the rubber tree *Hevea brasiliensis*. The antioxidant activities of the isolated compounds were tested using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay and employing propylgallate as a positive control.

2. Results and discussion

Semi-preparative HPLC separation of the EtOAc and n-BuOH extracts of the leafy twigs of *L. micranthus* parasitic on *H. brasiliensis* led to the isolation of eleven polyphenols (Fig. 1).

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Compound **1** was obtained as a pale pink amorphous powder. The HR-ESIMS spectrum of **1** exhibited a pseudomolecular peak at *m/z* 398.10925 $[M–H]^-$, indicating a molecular formula of $C_{17}H_{21}NO_{10}$, containing eight degrees of unsaturation. The ^1H NMR spectrum showed one singlet at 7.08 ppm integrated as 2H, suggesting the presence of a tetrasubstituted benzene ring. The occurrence of an anomeric proton at δ_H 4.65 d ($J = 7.7$ Hz) was indicative of a sugar unit, which was identified as a glucosyl residue, by analysis of the coupling constants (Table 1) together with the ^1H–^1H COSY correlations (Fig. 2). In the COSY spectrum, the anomeric proton (δ_H 4.65 d, H-1′) was found to correlate with H-2′ (δ_H 3.23, dd), which further coupled to H-3′ (δ_H 3.45, t). The latter proton showed correlation to H-4′ (δ_H 3.39, dd), which in turn correlated to H-5′ (δ_H 3.64, ddd), and H-5′ was further coupled to H_2-6′ (δ_H 4.52, 4.39, each dd), thus revealing a typical coupling pattern attributable to a glucosyl residue. In addition, two methyl singlets were found at δ_H 1.61 (s, CH_3-3), and 1.63 (s, CH_3-4) in the ^1H NMR spectrum.

The ^{13}C NMR spectrum showed 17 carbon signals, which were divided into 1 carbonyl carbon, 6 sp^2-hybridized carbons, 7 oxygenated sp^3-hybridized carbons (one methylene, five methines, and one quaternary carbon), 1 sp-hybridized carbon as well as two methyls, as aided by the DEPT-135 experiment. The six sp^2-hybridized carbons include two chemically equivalent methines (δ_C 110.2, CH-2″/6″), and two equivalent oxygenated quaternary carbons (δ_C 146.5, C-3″/5″), as well as two further quaternary carbons at δ_C 121.4 (C-1″) and 139.9 (C-4″), and their chemical shifts were indicative of a galloyl group when the carbonyl group (δ_C 168.3, C-7″) was taken into consideration. This hypothesis was supported by the HMBC spectrum, in which the correlations from the aromatic proton(s) H-2″/6″ (δ_H 7.08) to C-1″, C-3″/5″, C-4″, CH-6″/CH-2″, and C-7″ were observed (Fig. 2). As expected, the five oxygenated methines [δ_C 101.3 (CH-1′), 74.8 (CH-2′), 77.8 (CH-3′), 71.6 (CH-4′), 75.6 (CH-5′)], and one oxygenated methylene (δ_C 64.8, CH_2-6′) were assigned to the glucosyl, in which C-1′ was connected to the remaining oxygenated quaternary carbon (δ_C 73.1, C-1) through an ether bond, while C-6′ was linked to the galloyl group via an ester bond, as evidenced by the HMBC correlations from H-1′ to C-1, and from H_2-6′ to C-7″ (Fig. 2). Moreover, the interactions from both methyls (CH_3-3, and CH_3-4) to C-1, and the quaternary carbon C-2 (δ_C 121.8) were observed in the HMBC spectrum. Since the

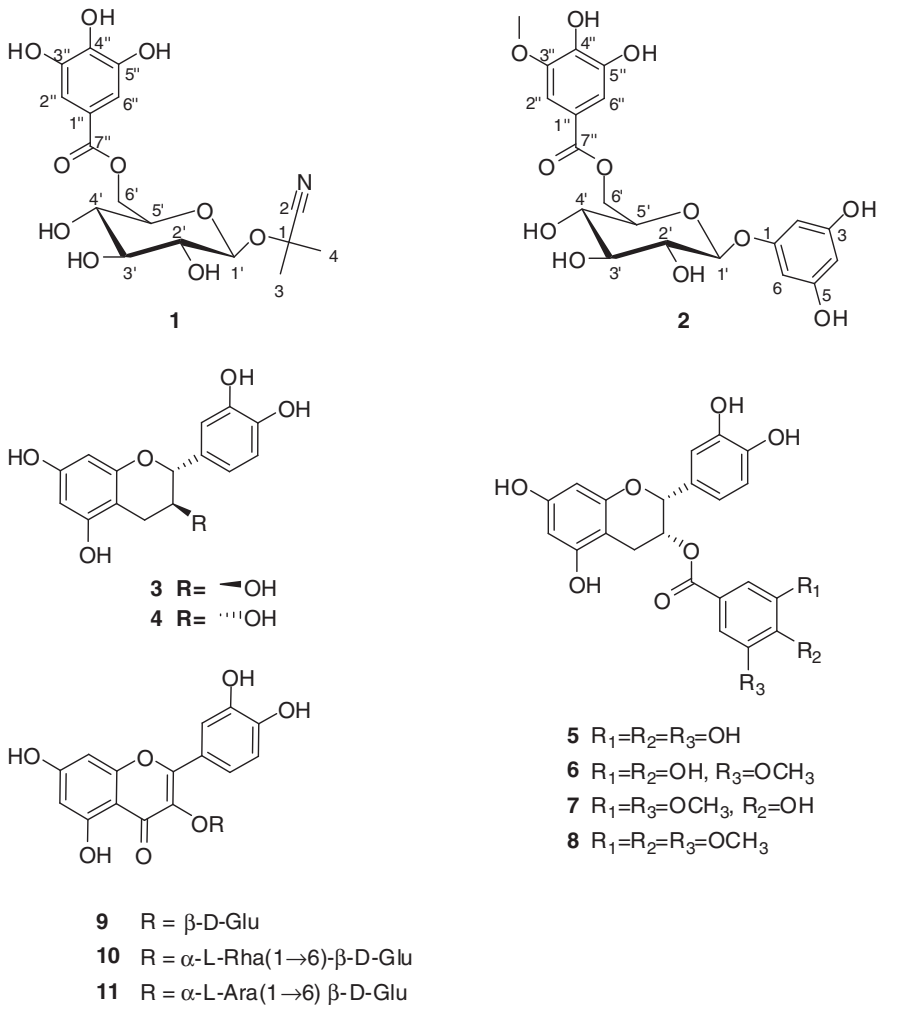

Fig. 1. Structures of the isolated compounds (**1**–**11**).

Table 1
^{1}H (600 MHz) and ^{13}C(150 MHz) NMR Data of compound **1** (CD_3OD).[a]

Position	δ_C	δ_H (*J* in Hz)	HMBC (H→C) [b]
1	73.1 s		
2	121.8 s		
3	28.4 q	1.61 s	1, 2, 4
4	27.9 q	1.63 s	1, 2, 3
1′	101.3 d	4.65 d (7.7)	1
2′	74.8 d	3.23 dd (9.1, 7.8)	1′, 3′
3′	77.8 d	3.45 t (9.0)	2′, 4′
4′	71.6 d	3.39 dd (9.6, 9.1)	
5′	75.6 d	3.64 ddd (9.7, 6.3, 2.1)	
6′	64.8 t	4.52 (dd, 11.9, 2.1) 4.39 (dd, 11.9, 6.4)	5′, 7″
1″	121.4 s		
2″	110.2 d	7.08 s	1″, 3″, 4″, 6″, 7″
3″	146.5 s		
4″	139.9 s		
5″	146.5 s		
6″	110.2 d	7.08 s	1″, 2″, 4″, 5″, 7″
7″	168.3 s		

[a] Assignments were based on DEPT-135, COSY, HSQC, and HMBC experiments.
[b] HMBC correlations are from proton(s) stated to the indicated carbon.

above moieties only accounted for 6° of unsaturation, the remaining two degrees of unsaturation and one nitrogen atom required by the molecular formula could only be rationalized by a nitrile group involving C-2 and N. This was confirmed by comparing the NMR data of **1** with those of the known cyanogenic glycoside linamarin [8]. The β-configuration of the anomeric carbon was inferred by the large coupling constant ($J = 7.7$ Hz) [8], and the common D-configuration for glucose was assumed. Therefore, compound **1** was elucidated as 2-methylpropionitrile-2-*O*-(6-*O*-galloyl)-β-D-pyranoglucoside, and named linamarin gallate.

This is the first report of cyanogenic glycosides from mistletoes whereas these compounds are typical metabolites of the host plant *H. brasiliensis* [9–12]. It can then be assumed that the linamarin core structure of **1** is taken up by the mistletoe from its host plant via the xylem whereas esterification with gallic acid probably occurs in the mistletoe based on the frequent occurrence of gallic acid moieties in several of the other isolated metabolites (e.g. **2** and **5**–**8**). It is worth to mention that the transfer of secondary metabolites produced by a host plant to the parasitic species was reported previously, such as the transfer of qinolizidine alkaloids from various host plants of the Fabaceae to parasitizing *Cuscuta* species [13].

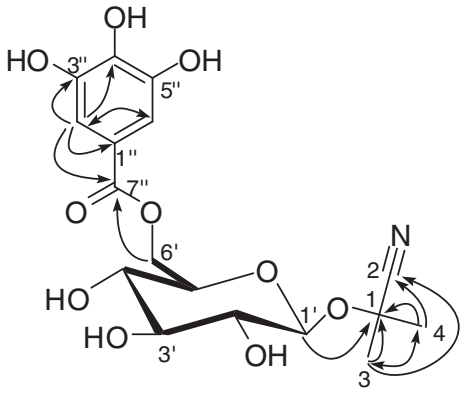

Fig. 2. Key ^{1}H–^{1}H COSY (bold line) and HMBC (H→C) correlations of compound **1**.

Compound **2** was obtained as a pinkish amorphous powder. Its molecular formula was determined to be $C_{20}H_{22}O_{12}$ by HR-ESIMS. The ^{1}H and ^{13}C NMR data of **2** (Table 2) were similar to those of **1**, both indicating the presence of a glucosyl, and a galloyl group. However, the methyl signals in the ^{1}H NMR spectrum of **1** were replaced by three additional aromatic protons in that of **2**. These protons include two chemically equivalent ones at δ_H 6.08 (2H, d, $J = 2.1$Hz), and one proton split to a triplet at δ_H 5.95 (1H, t, $J = 2.1$Hz), showing meta coupling with the former protons, thus revealing a 1,3,5-trioxygenated benzene moiety. This moiety was connected to the glucosyl unit via a C1-O-C1′ ether bond, as supported by the HMBC correlation from the anomeric proton (δ_H 4.88, d, H-1′) to C-1 (δ_C 160.9). Apart from those signals, one methoxy group (δ_H 3.85, s; δ_C 56.7, q) was observed in the NMR spectrum of **2**, which was located at C-3″ of the galloyl unit, as this methoxyl showed a cross-peak to C-3″ (δ_C 149.2) in the HMBC spectrum. This was consistent with the occurrence of two chemically inequivalent protons at δ_H 7.18 (d, $J = 1.9$ Hz), and 7.23 (d, $J = 1.9$ Hz) resulting from the introduction of a 3″-OMe in the galloyl group. Therefore, compound **2** was characterized as 3,5-dihydroxyphenyl-1-O-(6-O-(3-O-methyl)galloyl)-β-D-pyranoglucoside, which was structurally similar to the reported walsuraside, and differed from the latter by an additional methyl substitution in the galloyl residue [14]. Hence, the new compound was designated as walsuraside B.

The known compounds were readily identified by comparison of their spectroscopic data with those reported in the literature. The known compounds were catechin (**3**) [15], epicatechin (**4**) [16], epicatechin 3-*O*-gallate (**5**) [17], epicatechin 3-*O*-(3-*O*-methyl)gallate (**6**) [18], epicatechin 3-*O*-(3,5-*O*-dimethyl)gallate (**7**) [16], epicatechin 3-*O*-(3,4,5-*O*-trimethyl)gallate (**8**) [19], quercetin 3-*O*-β-D-glucopyranoside (**9**) [20], rutin (**10**) [21], and peltatoside (**11**) [22]. Compound **8** was isolated for the first time from a natural source.

Table 2
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data of compound **2** (CD_3OD).[a]

Position	δ_C	δ_H (*J* in Hz)
1	160.9 s	
2/6	96.8 d	6.08 d (2.1)
3/5	160.1 s	
4	98.2 d	5.95 t (2.1)
1′	102.1 d	4.88 d [b]
2′	74.8 d	3.44 m
3′	77.8 d	3.49 m
4′	71.6 d	3.48 m
5′	75.6 d	3.72 m
6′	64.9 t	4.65 (dd, 11.9, 2.1) 4.38 (dd, 12.0, 5.8)
1″	121.0 s	
2″	106.3 d	7.18 d (1.9)
3″	149.2 s	
4″	139.2 s	
5″	146.3 s	
6″	112.0 d	7.23 d (1.9)
7″	168.3 s	
3″-OMe	56.7 q	3.85 s

[a] Assignments were based on DEPT-135, COSY, HSQC, and HMBC experiments.
[b] Overlapped with water peak.

All the isolated compounds were examined for their effects on the growth of the L5178Y mouse lymphoma cell line using the MTT assay. However, none of them exhibited inhibitory activity ($IC_{50} > 10$ μM).

The antioxidant effects of the isolated compounds were assessed using the DPPH assay (Table 3). For comparison, the known naturally occurring antioxidant chlorogenic acid was included. All isolated compounds ($IC_{50} = 23.8–50.1$ μM) were found to be more active than the reference compound ($IC_{50} = 67.9$ μM) in the DPPH assay. Among them, compounds **10**, and **11**, both flavonol-3-*O*-disaccharides, showed the strongest antioxidant activities with IC_{50} (μM) values of 23.8, and 24.6, respectively. On the other hand, catechin (**3**) and epicatechin (**4**) showed the weakest active, both having an IC_{50} value of 50.1 μM. However, the introduction of 3-*O*-galloyl moieties significantly improved the antioxidant effect, as in the case of compounds **5**–**8** ($IC_{50} = 31.0–35.3$ μM). It was reported that the antioxidant activity of the flavonoids is correlated to their electron delocalization potentials [23]. For example, the *ortho*-dihydroxy structure in B ring of flavonoids is capable of delocalizing the electron, and the C2/C-3-double bond in conjugation with a 4-oxo function in the C ring provides further delocalization from the ring B [23]. This was consistent with our observation that the flavonol derivatives (**9**–**11**) were generally more active than the catechin derivatives (**3**–**8**) in the DPPH assay.

3. Experimental section

3.1. General experimental procedures

The optical rotations were recorded on a Perkin-Elmer 241 MC polarimeter.1D (^{1}H,^{13}C, DEPT-135) and 2D (COSY, HSQC, HMBC) NMR spectra were recorded using Bruker ARX 500 and DMX 600 NMR spectrometers, respectively. The chemical shifts are given in ppm (δ) referring to the solvent peak of CD_3OD at δ_H 3.31, δ_C 49.0 and coupling constants (*J*) are in Hz. HR-ESIMS measurements were carried out using a Micromass Q-Tof 2 mass spectrometer while HPLC-ESIMS measurements were performed on a Thermofinnigan LCQ DECA mass spectrometer coupled to an Agilent 1100 HPLC system that included an on-line photodiode array detector. Analytical HPLC analysis was performed with a HPLC system (Dionex, Munich, Germany) equipped with a photodiode-array detector, employing a linear gradient of MeOH in H_2O (adjusted to pH 2 with 0.1% formic acid) from 5% to 100% in 35 min. Routine detection was set at 235, 254, 280, and 340 nm. The separation column (i.d. 4×125 mm) was pre-filled with 5 μm Eurosphere 100 C18 (Knauer, Berlin, Germany). Semi-preparative HPLC was performed on a C18 column (300×8 mm, Eurosphere 100–10) on a MERCK HITACHI system equipped with a UV Detector L-7400 and a Pump L-7100 connected to a Kipp&Zonen Flatbed Recorder. The flow rate was 5 mL/min. Vacuum liquid chromatography (VLC) was performed on silica gel (230–400 mesh, Merck) using a glass column (i.d. 3×30 cm). Gel permeation column chromatography (CC) was performed on Sephadex LH-20 (Merck, Germany) using a glass column (i.d. 3×110 cm). TLC was performed on TLC plates pre-coated with silica gel 60 F_{254} (0.2 mm thickness, Merck, Darmstadt, Germany) using various solvent combinations as the mobile phase.

Table 3
Radical scavenging (DPPH) activities of compounds **1**–**11**.

Compound	IC_{50} (μM)
1	36.9
2	34.6
3	50.1
4	50.1
5	34.5
6	31.0
7	35.3
8	32.2
9	31.3
10	23.8
11	24.6
Chlorogenic acid	67.9

3.2. Plant material

Fresh leafy twigs of mistletoe *L. micranthus* (Linn.) parasitic on *H. brasiliensis* were collected from Enugu-Ezike in Enugu State, South-eastern Nigeria in January 2012. The mistletoe was identified by Mr. A. O. Ozioko of the Bioresources Conservation and Development Program (BDCP), Nsukka. A voucher specimen (LM1610) was deposited at the herbarium of the Institute.

3.3. Extraction and isolation

The air dried leafy twigs (500 g) were macerated with 3.0 L of methanol and extracted at room temperature for 48 h with agitation. The filtrate was evaporated *in vacuum* (40 °C) to yield a rusty brown residue (50 g), which was suspended in 400 mL of 10% methanol in water and the resulting mixture successively partitioned against n-hexane, EtOAc and n-BuOH to obtain n-hexane (HF, 3.90 g), EtOAc (EF, 13.8 g), n-BuOH (BF, 12.6 g) and water (WF, 1.02 g) fractions, respectively.

Part of the EtOAc fraction (5 g) was purified by vacuum liquid chromatography using silica gel (230–400 mesh, 3.0×30 cm, 500 g) as the stationary phase and eluted with a gradient of n-hexane in EtOAc (10:0, 8:2, 6:4, 4:6, 2:8, 0:10, each 500 mL) and of dichloromethane (DCM) in methanol (9:1, 7:3, 5:5, 3:7,1:9, each 1000 mL) to afford 11 sub-fractions (EF1-EF11). Fraction EF6 (348.5 mg) was further fractionated on Sephadex LH-20 (100% MeOH) to afford seven sub-fractions ($EF6_A$–$EF6_G$). Fraction $EF6_D$ (43.2 mg) was separated by semi-preparative HPLC using MeOH–H_2O as mobile phase to give compounds **3** (16.5 mg) and **4** (11.3 mg). Similarly, fraction $EF6_E$ (36.3 mg) was purified using semi-preparative HPLC with MeOH–H_2O as the mobile phase to yield compounds **7** (3.0 mg) and **8** (2.9 mg). Fractions $EF6_F$ and $EF6_G$ were purified in a similar way to afford compounds **6** (15.8 mg), and **5** (5.9 mg), respectively. Fraction EF9 (600 mg) was subjected to Sephadex LH-20 column chromatography (3×110 cm) eluted with 100% MeOH to give nine sub-fractions ($EF9_A$–$EF9_I$). Sub-fraction $EF9_A$ (83.0 mg) was purified by semi-preparative HPLC using MeOH–H_2O as the mobile phase to give compound **1** (6.2 mg). Likewise, compounds **10** (5.6 mg), and **9** (5.8 mg) were obtained respectively from sub-fractions $EF9_B$ (77.3 mg), and $EF9_D$ (43.2 mg) by semi-preparative HPLC. The n-BuOH fraction

(10 g) was subjected to vacuum liquid chromatography over silica gel (230–400 mesh, 3.0×30 cm, 800 g) and eluted with a gradient of DCM in MeOH (9:1, 8:2, 7:3, 6:4, 5:5,4:6, 3:7, 2:8, 1:9,0:10, each 1000 mL) to give 10 fractions (BF1-BF10). Sub-fraction BF3 was subjected to Sephadex LH-20 column chromatography eluting with 100% MeOH (3×110 cm) to afford seven sub-fractions ($BF3_A$–$BF3_G$). Fraction $BF3_A$ (65.6 mg) was purified by semi-preparative HPLC (MeOH–H_2O) to give compound **11** (2.6 mg), and sub-fraction $BF3_G$ (58.8 mg) was separated in a similar manner to afford compound **2** (1.5 mg).

*3.3.1. Linamarin gallate (**1**)*

Pale pink amorphous powder; $[\alpha]_D^{20}$-5.5 (c 0.10, MeOH); UV (MeOH) λ_{max} 215.3, 274.3 nm; ^1H (600 MHz, CD_3OD), and ^{13}C (150 MHz, CD_3OD) NMR data: see Table 1; HR-ESIMS: *m/z* 398.10925 $[M–H]^-$ (calcd. for $C_{17}H_{20}NO_{10}$ 398.10927).

*3.3.2. Walsuraside B (**2**)*

Pink amorphous powder; $[\alpha]_D^{20}$-23.2 (c 0.10, MeOH); UV (MeOH) λ_{max} 201.4, 275.2 nm; ^1H (600 MHz, CD_3OD), and ^{13}C (150 MHz, CD_3OD) NMR data: see Table 2; HR-ESIMS: *m/z* 453.10377 $[M–H]^-$ (calcd. for $C_{20}H_{21}O_{12}$ 453.10385).

3.4. Cytotoxic assay

Cytotoxicity was tested against L5178Y mouse lymphoma cells using the microculture tetrazolium (MTT) assay and compared to that of untreated controls, as described previously [24].

3.5. Antioxidant activities

The DPPH assay was performed according to the procedure described by Tsevegsuren et al. [25]. The compounds were dissolved in methanol to give a concentration of 1 mg/1000 μL stock solution. Ten microliter (10 μL) of the test samples was added to 490 μL DPPH solution (4.5 mg/100 mL) in an ependorf vial. The mixture was incubated for 3 min and the color change (from deep violet to light yellow) of the DPPH free radical was measured by recording the absorbance using a UV/Visible spectrophotometer (Perkin Elmer, Lambda 25) at 517 nm. Prior to the measurement, the difference in absorption between a DPPH blank solution and the positive control (propylgallate, 76 μM) was determined. This difference was then taken as 100% antioxidative activity. The percent antioxidative activity was determined from the difference in absorption between the samples at 76 μM and the DPPH blank as follows:

$$a_A(\%) = \left[\left(A_B - A_p\right)/\left(A_B - A_{pos}\right)\right] \times 100$$

where a_A = % antioxidative activity compared to the positive control, A_B = absorption of the DPPH blank solution, A_p = absorption of the sample, and A_{pos} = absorption of the positive control (propylgallate). Measurements were done in triplicates, and the IC_{50} values were determined by linear regression.

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