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ORIGINAL ARTICLE

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Antioxidant activity and constituents of extracts from the root of *Garcinia multiflora*

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Abstract The methanolic extract of the root of *Garcinia multiflora* and its derived soluble fractions, namely those soluble in ethyl acetate (EtOAc), *n*-butanol, and water, were screened for their antioxidant activities. Among them, the EtOAc-soluble fraction exhibited the highest scavenging activity against the 1,1-dipheny1–2-picrylhydrazyl radical, the highest superoxide radical scavenging activity, and the strongest reducing power. In addition, according to the bioactivity-guided isolation, 26 specific phytocompounds, including 3 aromatics, 3 benzophenones, 3 flavonoids, 3 isocoumarins, 1 phloroglucinol, 6 steroids, and 7 xanthones, were isolated from the EtOAc-soluble fraction and identified. Of these, 2,4,3′,4′-tetrahydroxy-6-methoxybenzophenone and 1,3,6,7-tetrahydroxyxanthone were found to be the major bioactive constituents, present in the crude extract in concentrations of 3.9 and 15.6 mg/g, respectively. These two compounds had similar antioxidant activities to (+)-catechin, a well-known antioxidant.

Key words Antioxidant · Bioactivity-guided isolation · *Garcinia multiflora* · Phytocompound · Reactive oxygen species

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Introduction

Reactive oxygen species (ROS) including superoxide radical, hydroxyl radical, singlet oxygen, and hydrogen peroxide are often generated as by-products of biological reactions or from exogenous factors and these molecules are responsible for cellular injury and aging processes.[1,2] Electron acceptors, such as molecular oxygen, react rapidly with free radicals to become radicals themselves, and these are also referred to as reactive oxygen species.[3] The role of free radicals (or ROS) in the pathogenesis of various serious diseases, including neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, and inflammation, has been recognized.[4,5] Thus, a potent scavenger of these species may serve as a possible preventive intervention for free radical-mediated diseases.[6]

Garcinia multiflora Champ., a dioecious tree, is distributed in southern mainland China, Hong Kong, and the southern part of Taiwan. This plant is used as a traditional Chinese medicine (TCM),[7] and its stems are reported to contain garcinianones A, garcinianones B, 4,6,4′-trihydroxy-2,3′-dimethoxy-3-prenylbenzophenone, 4,6,3′,4′-tetrahydroxy-2-methoxybenzophenone, (1*E*,22*Z*)-1,22-diferuloyloxydocosane, (1*E*,24*Z*)-1,24-diferuloyloxyteracosane, 3,8-dihydroxy-2,4,6-trimethoxyxanthone, 6,3′-dihydroxy-2,4-dimethoxybenzophenone, maclurin, and 2,4,6,3′-tetrahydroxybenzophenone.[8] It is also reported that bioflavonoids isolated from *G. multiflora* possess anti-HIV activity against HIV-1 reverse transcriptase.[9] However, to date the potential health benefits of root extracts of *G. multiflora* have not been studied. It is well known that prenylated xanthones and their structurally related benzophenones often exhibit a wide range of biological and pharmacological activities, such as antioxidant, cytotoxic, anti-inflammatory, antimicrobial, and antifungal effects.[10] Therefore, *G. multiflora* may be a good candidate for further development as an antioxidant remedy. In this study, according to the bioactivity-guided isolation, column chromatography (CC) and high performance liquid chromatography (HPLC) were employed to separate and purify

phytocompounds from the root of *G. multiflora*, and their antioxidant activities were evaluated by various in vitro assays. In addition, the chemical structures of antioxidant phytocompounds were identified by mass spectrometry (MS) and nuclear magnetic resonance (NMR) analyses.

Materials and methods

Chemicals

1,1-Dipheny1–2-picrylhydrazyl (DPPH), hypoxanthine, xanthine oxidase, nitroblue tetrazolium chloride (NBT), 2-thiobarbituric acid (TBA), trichloracetic acid (TCA), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4′-disulfonic acid sodium salt (Ferrozine), Folin-Ciocalteu reagent, and (+)-catechin were purchased from Sigma (St. Louis, MO). All other unlabeled chemicals and reagents were of analytical grade.

Plant materials

Garcinia multiflora root was collected in Pin-Ton County, in the south of Taiwan. The plant species was identified by Mr. Muh-Tsuen Gun, formerly a technician of the Department of Botany, National Taiwan University, and a voucher specimen (voucher no. 183837) was deposited at the Herbarium of the Department of Botany, National Taiwan University, Taipei, Taiwan.

Extraction and isolation

The dried samples (10.5 kg) were cut into small pieces and soaked in methanol at ambient temperature (25°C) for 7 days. The extract was decanted, filtered under vacuum, concentrated in a rotary evaporator, and then lyophilized. The resulting methanolic crude extract (601.3 g) was fractionated successively with ethyl acetate (EtOAc), *n*-butanol (BuOH), and water to yield soluble fractions of EtOAc (398.5 g), BuOH (81.6 g), and H_2O (100.2 g). The EtOAc-soluble fraction (293.8 g) was loaded onto a chromatography column (Geduran Si-60, Merck, Darmstadt, Germany) and eluted with EtOAc/*n*-hexane (gradient elution was performed by changing from 2/98 to 100/0) and 15 subfractions (EA1-EA15) were collected. Twenty-six pure constituents (Fig. 1) were isolated from EA7-EA11 by semipreparative HPLC using a PU-980 pump (Jasco, Japan) equipped with a RI-1530 refractive index detector (Jasco, Japan) and a 250 × 10.0 mm i.d., 5 μm Luna silica column (Phenomenex, Torrance, CA). Separation was achieved using an isocratic system of EtOAc/*n*-hexane at a flow rate of 4 ml/min. All structures of obtained constituents were determined by MS (Finnigan MAT-95S, Germany) and NMR (Varian Unity Plus 400, USA) analyses, and their spectral data were consistent with the literature.[8,11–33]

Fig. 1. Chemical structures of phytocompounds isolated from the subfractions EA7-EA11: syringaldehyde (**1**), 3,4-dihydroxybenzoic acid methyl ester (**2**), 4-methoxybenzoic acid (**3**), 2,4,6,3′-tetrahydroxybenzophenone (**4**), 2,4,3′,4′-tetrahydroxy-6-methoxybenzophenone (**5**), 2,4,6,3′,4′-pentahydroxybenzophenone (**6**), 4′,5,7-trihydroxyflavanone (**7**), luteolin (**8**), apigenin (**9**), 5-carboxymellein (**10**), 5-formylmellein (**11**), 5-hydroxymethylmellein (**12**), isoxanthochymol (**13**), 3*β*-hydroxystigmast-5-en-7-one (**14**), 3*β*-hydroxystigmasta-5,22-dien-7-one (**15**), stigmastanol (**16**), 5,6-dihydrostigmasterol (**17**), *β*-sitosterol (**18**), stigmasterol (**19**), 1,3,6,7-tetrahydroxyxanthone (**20**), 1,3,7-trihydroxyxanthone (**21**), 1,3,5,6-tetrahydroxyxanthone (**22**), 1,7-dihydroxyxanthone (**23**), 1,5-dihydroxyxanthone (**24**), 1,5,6-trihydroxyxanthone (**25**), and 1,6-dihydroxy-3,5,7-trimethoxyxanthone (**26**)

1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay

The DPPH radical scavenging activity of the test samples was examined according to the method reported by Gyamfi et al.[34] First, 50 μl of the test samples in methanol (final concentrations were 1, 5, 10, 50, and 100 µg/ml, respectively) was mixed with 450 µl of 50 mM Tris-HCl buffer (pH 7.4) and 1000 µl of 0.1 mM DPPH ethanol solution. After 30 min of incubation at ambient temperature, the reduction of the DPPH free radical was measured by reading the absorbance at 517 nm. (+)-Catechin was used as the positive control. The inhibitory activity was calculated according to the following equation: % inhibition = [(absorbance of control – absorbance of sample)/absorbance of control] × 100.

Superoxide radical scavenging assay (NBT assay)

Measurement of superoxide radical scavenging activity was carried out according to the method of Kirby and Schmidt.[35] First, 20 µl of 15 mM ethylenediaminetetraacetic acid diso-

dium salt (Na_2EDTA) in buffer (50 mM KH_2PO_4/KOH, pH 7.4), 50 µl of 0.6 mM NBT in buffer, 30 µl of 3 mM hypoxanthine in 50 mM KOH, 5 µl of the test samples in methanol (final concentrations were 1, 5, 10, 50, and 100 µg/ml, respectively), and 145 µl of buffer were mixed in 96-well microplates. The reaction was started by adding 50 µl of xanthine oxidase (1 unit in 10 ml buffer) to the mixture. Then the reaction mixture was incubated at ambient temperature, and the absorbance at 570 nm was determined every 1 min up to 8 min using the ELISA reader (Labsystems Multiskan, USA). (+)-Catechin was used as the positive control. The inhibitory activity was calculated according to the following equation: % inhibition = [(rate of control reaction – rate of sample reaction)/rate of control reaction] × 100.

Reducing power assay

Reducing power was determined according to the method described by Oyaizu.[36] Briefly, 1 ml of reaction mixture, containing the test samples (final concentrations were 1, 5, 10, 50, and 100 µg/ml, respectively) in phosphate buffer (0.2 M, pH 6.6), was incubated with 500 µl of potassium ferricyanide (1%, w/v) at 50°C for 20 min. The reaction was terminated by adding 500 µl of trichloroacetic acid (10%, w/v), and then the mixture was centrifuged at 3000 rpm for 10 min. The upper layer of the solution (500 µl) was mixed with 500 µl of distilled water and 100 µl of ferric chloride (0.1%, w/v) solution, and the absorbance was measured at 700 nm. (+)-Catechin was used as the positive control. Increased absorbance of the reaction mixture indicated increased reducing power.

Ferrous ion-chelating ability assay

The ferrous ion-chelating potential of the test samples was evaluated according to the method of Dinis et al.[37] Briefly, 200 µl of test sample in methanol (final concentrations were 25, 125, 250, 1250, and 2500 µg/ml, respectively) and 740 µl of methanol were added to 20 µl of 2 mM $FeCl_2$. The reaction was initiated by the addition of 40 µl of 5 mM ferrozine into the mixture, which was then shaken vigorously and left to stand at ambient temperature for 10 min. The absorbance of the reaction mixture was measured at 562 nm. (+)-Catechin was used as the positive control. The percent of inhibition of ferrozine-Fe^{2+} complex formation was calculated according to the following equation: % inhibition = [(absorbance of control – absorbance of sample)/absorbance of control] × 100.

Determination of total phenolics

Total phenolic contents were determined according to the Folin-Ciocalteu method,[38] using gallic acid as the standard. The test samples were dissolved in 5 ml of methanol/water (50%, v/v). The sample solution (500 µl) was mixed with 500 µl of 1 N Folin-Ciocalteu reagent. The mixture was allowed to stand for 5 min, which was followed by the addition of 1 ml of 20% Na_2CO_3. After 10 min of incubation at ambient temperature, the mixture was centrifuged for 8 min (12 000 *g*), and the absorbance of the supernatant was measured at 730 nm. The total phenolic contents were expressed as gallic acid equivalent (GAE) in milligrams per gram of sample.

Statistical analyses

All results were expressed as mean ± standard deviation ($n = 3$). The significance of difference was calculated by Scheffe's test, and values of $P < 0.05$ were considered to be significant.

Results and discussion

DPPH radical scavenging activity of *Garcinia multiflora* extracts

Various methods have been designed to measure antioxidant activities of plant extracts or pure compounds. Some involve production of transient radical species under steady state or pulse conditions. Indeed, stable organic radicals that produce color changes upon reaction with antioxidants have received much attention. The commercially available DPPH radical is now widely used; it is reduced by antioxidants to the hydrazine form (DPPH–H). In fact, DPPH radical is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[39] In the present study, the free radical scavenging activity of *Garcinia multiflora* root extract was assessed by DPPH assay. Accordingly, as shown in Fig. 2A, the DPPH radical scavenging activity of the methanolic extract and its derived soluble fractions from *G. multiflora* root, including EtOAc-, BuOH-, and water-soluble fractions, was shown in a dose-dependent manner. Of these, the EtOAc fraction showed the strongest scavenging activity. Except for the water-soluble fraction, all extracts showed a good inhibitory activity against DPPH radical. The IC_{50} values of the crude extract, EtOAc fraction, BuOH fraction, and water fraction were 16.0, 14.1, 29.9, and >100 µg/ml, respectively. For (+)-catechin, a well-known antioxidant compound used as a reference control in this study, the IC_{50} value was 2.9 µg/ml. These results indicate that the free radical scavenging activity of root extracts of *G. multiflora* can be effectively enriched in the EtOAc fraction.

Superoxide radical scavenging activity of *G. multiflora* extracts

Superoxide radical is known to be very harmful to cellular components as a precursor of many ROS.[40] Additionally, superoxide radical is biologically important because it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals.[41] Figure 2B shows the

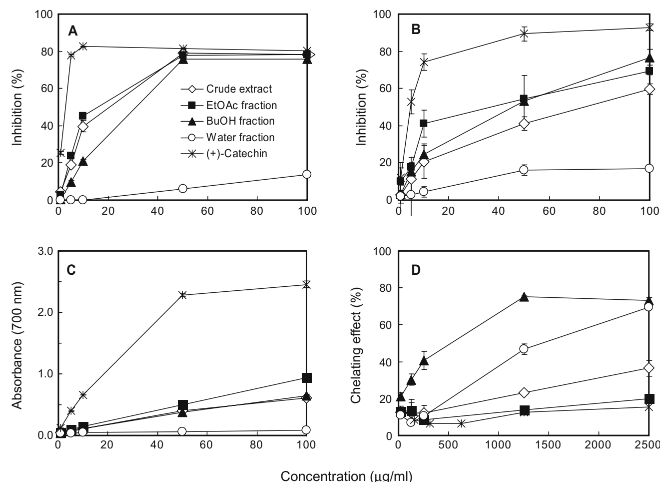

Fig. 2A–D. Antioxidant activities of methanolic extract and its derived soluble fractions from the root of *Garcinia multiflora*. **A** 1,1-Dipheny1–2-picrylhydrazyl (DPPH) radical scavenging activity, **B** superoxide radical scavenging activity, **C** reducing power, **D** ferrous ion-chelating ability. Data are given as mean ± standard deviation (n = 3)

superoxide radical scavenging activity of the methanolic extract and its derived soluble fractions compared with (+)-catechin. Accordingly, the inhibitory activity of all tested samples was observed in a dose-dependant manner, and the EtOAc fraction exhibited the highest activity. The IC_{50} values of (+)-catechin, methanolic crude extract, EtOAc fraction, BuOH fraction, and water fraction were 4.2, 71.1, 37.5, 42.4, and >100 μg/ml, respectively. This result was similar to that of DPPH assay; of all the fractions tested, the EtOAc-soluble fraction exhibited the strongest free radical scavenging activity, whereas the water-soluble fraction was the weakest.

Reducing power of *G. multiflora* extracts

Fe(III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action.[42] In general, the reducing properties are associated with the presence of reductones,[43] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom.[44] In this study, as shown in Fig. 2C, the reducing power of methanolic crude extract and its derived soluble fractions decreased in the following order: EtOAc fraction > methanolic crude extract = BuOH fraction > water fraction. Again, of these, the EtOAc-soluble fraction revealed the best reducing power. On the other hand, at a concentration of 100 μg/ml, the absorbances (at 700 nm) of methanolic crude extract, EtOAc, BuOH, and water fractions were 0.60, 0.94, 0.64, and 0.08, respectively. However, under the same experimental conditions, Shon et al.[45] reported that EtOAc extracts of red, yellow, and white onions showed absorbances around 0.6–0.7 at a concentration of 5000 μg/ml. In comparison with these onion extracts, which are declared as antioxidants, about 50-fold higher activities were found for both EtOAc and BuOH fractions of *G. multiflora* root. These results indicated that the reducing power of root extracts of *G. multiflora*, especially the EtOAc-soluble fraction, was much better than that of onion EtOAc extracts. Furthermore, many studies reported a direct correlation between antioxidant activities and reducing power in various plant extracts.[43,46,47] In the current study, a similar correlation was also observed.

Ferrous ion-chelating effect of *G. multiflora* extracts

It is well known that ferrozine can quantitatively form complexes with Fe(II). However, in the presence of chelating agents, the complex formation is limited and further results in a decrease in the red color of the complex. The chelating effect of the test samples on ferrous ions is shown in Fig. 2D. As can be seen, the chelating ability of test samples was increased with increasing concentration from 250 to 2500 μg/ml. Meanwhile, the BuOH and water fractions exhibited excellent chelating ability. The IC_{50} values of methanolic crude extract, EtOAc, BuOH, and water fractions were >2.5, >2.5, 0.6, and 1.6 mg/ml, respectively. With a dosage as low as 1.25 mg/ml, the chelating ability of the BuOH-soluble fraction reached approximately 80%, whereas (+)-catechin only reached approximately 10%. These results demonstrated that most fractions divided from the root of *G. multiflora* showed a better chelating ability than (+)-catechin. Furthermore, it has been reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion.[44] According to the data shown in Fig. 2, the EtOAc-soluble fraction showed similar character to that of (+)-catechin; it was not a good secondary antioxidant due to its poor capacity for metal ion binding, but it was an excellent primary antioxidant (or free radical scavenger).

Total phenolic contents of *G. multiflora* extracts

The Folin-Ciocalteu method is not an antioxidant test but rather is an assay for the quantity of oxidizable substance, such as phenolic phytocompounds.[48] Correlation between the contents of phenolic compounds and antioxidant activities has been described in many studies.[48–50] Phenolic compounds are very important plant constituents because of their scavenging ability due to their hydroxyl groups. Figure 3 shows the contents of total phenolics in the crude extract and its derived fractions calculated as gallic acid equivalent (GAE). As can be seen, the total phenolic content of the EtOAc fraction (225.4 mg/g) was higher than that of crude extract (189.0 mg/g), BuOH fraction (182.4 mg/g), and water fraction (56.2 mg/g). According to the above results, except for the ferrous ion-chelating effect, there was a high correlation for all samples between the total phenolic contents and antioxidant activities, including DPPH radical scavenging activity, superoxide radical scavenging activity, and reducing power.

Identification and DPPH radical scavenging activity of major antioxidant phytocompounds from *G. multiflora* extracts

Based on the bioactivity-guided isolation principle, the root extract of *G. multiflora*, especially the EtOAc-soluble fraction, might be a good candidate for development as a novel natural antioxidant. Therefore, in this study, the EtOAc-soluble fraction was further divided into 15 subfractions by column chromatography. Table 1 shows the elution solvent, collected weight, and DPPH radical scavenging activity for these 15 subfractions. Of these, EA11 was eluted with 50% EtOAc/*n*-hexane (v/v) and exhibited the strongest inhibitory activity against DPPH radical (IC_{50} = 3.1 µg/ml). In addition, three specific antioxidant phytocompounds, including 2,4,6,3′-tetrahydroxybenzophenone (**4**), 2,4,3′,4′-tetrahydroxy-6-methoxybenzophenone (**5**), and 1,3,6,7-tetrahydroxyxanthone (**20**), were further isolated by HPLC separation from the EA11 subfraction, and their contents were determined as 3.1, 3.9, and 15.6 mg/g of methanolic crude extract, respectively. To determine the antioxidant activities of these major active compounds, DPPH, NBT, and reducing power assays were performed. (+)-Catechin was used as the positive control. As shown in Table 2, the IC_{50} values for DPPH radical scavenging activity of these major phytochemicals (compounds **4**, **5**, and **20**) were 29.1, 10.0, and 14.6 µM, respectively. These results not only suggested that catechol skeletons had better antioxidant activity, but also revealed that the DPPH radical scavenging activities of 2,4,3′,4′-tetrahydroxy-6-methoxybenzophenone and 1,3,6,7-tetrahydroxyxanthone were the same as (+)-catechin, a well-known antioxidant. Similarly, these compounds showed the same order of compound **5** > compound **20** > compound **4** for the reducing power with CE values (catechin equivalent in mM/M of sample) of 866.8, 833.6, and 62.4 mM/M, respectively. However, for superoxide radical, of these, compound **20** showed the best scavenging activity. The IC_{50} values of (+)-catechin, compound **20**, compound **5**, and compound **4** were 7.4, 13.4, 27.9, and 112.3 µM, respectively.

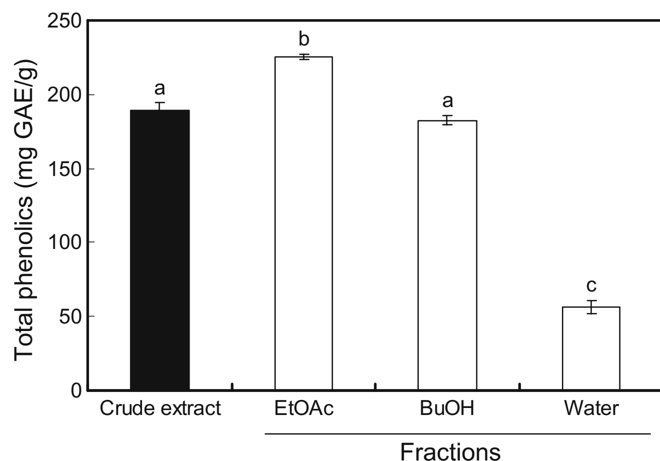

Fig. 3. Total phenolic contents of methanolic extract and its derived soluble fractions from the root of *G. multiflora*. Data are given as mean ± standard deviation (n = 3). Bars marked by *different letters* are significantly different at the level of $P < 0.05$ according to the Scheffe test

Table 1. Elution solvent, collected mass and 1,1-dipheny1–2-picrylhydrazyl (DPPH) radical scavenging activity of 15 subfractions from ethyl acetate (EtOAc)-soluble fraction

Subfraction	Mobile phase[a]	Mass (g)	IC_{50} (µg/ml)
EA1	2%	10.0	>100
EA2	5%	11.9	>100
EA3	5%	5.3	>100
EA4	10%	15.4	9.3
EA5	10%	13.0	>100
EA6	20%	6.2	>100
EA7	20%	9.4	5.9
EA8	20%	6.5	9.4
EA9	30%	4.8	20.9
EA10	50%	8.3	10.6
EA11	50%	10.0	3.1
EA12	50%	19.1	25.2
EA13	70%	55.2	41.3
EA14	70%	57.7	6.5
EA15	100%	41.6	8.4
Total		274.3[b]	

[a] Ratio of EtOAc/*n*-hexane (v/v)
[b] Recovery: 93.4%

On the other hand, according to the results of Table 1, subfractions eluted out with 20%–50% EtOAc/*n*-hexane (v/v) (EA7-EA11) generally exhibited the stronger inhibitory activity against DPPH radical. The following 26 specific phytocompounds were isolated by HPLC from those subfractions, and their chemical structures were elucidated by MS and NMR analyses: syringaldehyde (**1**),[11] 3,4-dihydroxybenzoic acid methyl ester (**2**),[12] 4-methoxybenzoic acid (**3**),[13] 2,4,6,3′-tetrahydroxybenzophenone (**4**),[14] 2,4,3′,4′-tetrahydroxy-6-methoxybenzophenone (**5**),[8] 2,4,6,3′,4′-pentahydroxybenzophenone (**6**),[15] 4′,5,7-trihydroxyflavanone (**7**),[16] luteolin (**8**),[17] apigenin (**9**),[18] 5-carboxymellein (**10**),[19] 5-formylmellein (**11**),[19] 5-hydroxymethylmellein (**12**),[20] isoxanthochymol (**13**),[21] 3*β*-hydroxystigmast-5-en-7-one (**14**),[22]

Table 2. Antioxidant activities and contents of major phytocompounds from the EA11 subfraction

Phytocompound	IC_{50} (µM)		Reducing power (CE)[a]	Content (mg/g methanolic extract)
	DPPH radical	Superoxide radical		
2,4,6,3′-Tetrahydroxybenzophenone (**4**)	29.1	112.3	62.4	3.1
2,4,3′,4′-Tetrahydroxy-6-methoxybenzophenone (**5**)	10.0	27.9	866.8	3.9
1,3,6,7-Tetrahydroxyxanthone (**20**)	14.6	13.4	833.6	15.6
(+)-Catechin (positive control)	10.0	7.4	–	–

[a] (+)-Catechin equivalent (mM/M sample)

3β-hydroxystigmasta-5,22-dien-7-one (**15**),[23] stigmastanol (**16**),[24] 5,6-dihydrostigmasterol (**17**),[25] β-sitosterol (**18**),[26] stigmasterol (**19**),[26] 1,3,6,7-tetrahydroxyxanthone (**20**),[27] 1,3,7-trihydroxyxanthone (**21**),[28] 1,3,5,6-tetrahydroxyxanthone (**22**),[29] 1,7-dihydroxyxanthone (**23**),[30] 1,5-dihydroxyxanthone (**24**),[31] 1,5,6-trihydroxyxanthone (**25**),[32] and 1,6-dihydroxy-3,5,7-trimethoxyxanthone (**26**).[33] This is the first study to investigate the effects of phytocompounds of *G. multiflora* root on antioxidant activities, and the data obtained clearly establish the antioxidant potency of extracts of *G. multiflora*.

Conclusions

It is well known that free radicals are one of the causes of several diseases. This study demonstrated for the first time that, among the crude extract and its derived soluble fractions from *Garcinia multiflora* root, the EtOAc-soluble fraction possessed the highest antioxidant activities and free radical scavenging activities. Of 15 subfractions from the EtOAc-soluble fraction, the EA11 subfraction exhibited the strongest DPPH radical scavenging capacity. Meanwhile, three major constituents were isolated and identified from the EA11 subfraction. All these constituents showed a significant inhibitory activity against the DPPH radical. Accordingly, the root of *G. multiflora* contained abundant antioxidants and showed significant antioxidant activities. This result implies that the extracts or the derived phytocompounds from *G. multiflora* root have great potential to prevent diseases caused by the overproduction of radicals, and it may be suitable for the treatment of degenerative diseases. Future studies should focus on the employment of modern medical chemical techniques to modify the structures of specific plant ingredients into better agents with high efficacy and activity. In addition, in vivo pharmacological research should also be conducted.

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