Monoamine Oxidase B and Free Radical Scavenging Activities of Natural Flavonoids in *Melastoma candidum* D. Don

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Monoamine oxidase type B (MAO-B) activity and free radicals are elevated in certain neurological diseases. Four natural flavonoids, quercitrin, isoquercitrin, rutin, and quercetin, were isolated for the first time from the leaves of *Melastoma candidum* D. Don. They exhibited an inhibitory effect on MAO-B. These potent flavonoids were purified using bioassay-guided fractionation and were separated by Diaion, Sephadex LH-20, and MCI CHP20P columns. The IC_{50} values of the four potent flavonoids, quercitrin, isoquercitrin, rutin, and quercetin on monoamine oxidase were 19.06, 11.64, 3.89, and 10.89 μM and enzyme kinetics analysis revealed apparent inhibition constants (K_i) of 21.01, 2.72, 1.83, and 7.95 μM, respectively, on the substrate, benzylamine. The four potent compounds also exhibited hydroxyl radical scavenging activity as determined using a spin trapping electron spin resonance method. This suggests that the four flavonoids from *M. candidum* possess both MAO-B inhibitory and free radical scavenging activities. These important properties may be used for preventing some neurodegenerative diseases in the future.

Keywords: *Melastoma candidum; monoamine oxidase B; quercitrin; isoquercitrin; quercetin; rutin; hydroxyl radical scavenging*

INTRODUCTION

The problems of aging-related diseases, such as neurodegenerative diseases (e.g., Alzheimer's, Parkinson's, and Huntington's diseases), have been emphasized recently. The intricate causes of the aging process are still a matter of extensive speculation giving rise to many theories; in particular, the role of reactive oxygen species (ROS) is a prerequisite nowadays for understanding this process (*1*−*4*). Another prominent feature accompanying aging, an increase in catecholamine metabolism, has also attracted attention, and monoamine oxidase (MAO), a key enzyme in this process, has been extensively studied. MAO is a flavoenzyme located on the outer membranes of mitochondria; it catalyzes the oxidative deamination of biogenic amines, such as dopamine (DA), serotonin (5-HT), and norepinephrine, accompanied by a release of NH_3 and H_2O_2 (*5*). The enzyme exists in two subtypes, MAO A and MAO B, which can be distinguished by their substrate specificities and selectively to their inhibitors (*6*). The regulation of MAO activity was important for the treatment of depression (*7*) and Parkinson's disease (*8*). Oxidation of dopamine by MAO can lead to production of neurotoxic compounds such as hydrogen peroxide (9, *10*), semiquinones (*11*), hydroxyl radicals (*12*, *13*), and superoxide (*14*−*16*). The formation of these radicals, in conjunction with an environment rich in iron as is often described for Parkinson's disease, can preempt potent OH formation through the Fenton reaction and induce subsequent lipid peroxidation and neurodegeneration. Several pieces of evidence also suggest that free radical formation is another of the major pathogenic factors in neurodegenerative disorders (*17*, *18*). Therefore, inhibition of MAO activity and free radical scavenging might be important for the treatment or prevention of neurodegenerative disorders.

Melastoma candidum is a medicinal plant used in China for activating the blood and eliminating stasis, for clearing heat and toxins, for treating traumatic injuries, and for activating vital energy. It has been reported to lower blood pressure through decreasing the sympathetic tone and causing direct vasodilatation in adult hypertensive rats (SHR) (*19*). To date, there is no report on its inhibition of MAO or free radical scavenging activities. In the present study, the activity of MAO was measured by a fluorometric method based on the detection of H_2O_2 in a horseradish peroxidase-coupled reaction using *N*-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) in 96-well microplates (*20*). By using this bioguide, we investigated the active principles present in *M. candidum* and evaluated their inhibition of MAO B with a fluorometric spectrophotometer and their free radical scavenging activity by electron spin resonance (ESR).

MATERIALS AND METHODS

Plant Material. The fresh green leaves of *M. candidum* D. Don were collected from Taipei Botanical Garden in September 1998. The plant was identified by H. L. Chiang, Taipei Botanical Garden, Taiwan, ROC.

Chemicals. Monoamine oxidase, horseradish peroxidase, and benzylamine were purchased from Sigma (St. Louis, MO). Amplex Red (catalog no. A-6550) was from Molecular Probes

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(Eugene, OR). The spin trapping reagent, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), was supplied by Sigma Industries (St. Louis, MO). The following are the sources of hydroxyl radical and their corresponding supplier: the source of the hydroxyl radical, ferrous sulfate ($FeSO_4 \cdot 7H_2O$), was purchased from Sigma Industries; hydrogen peroxide (H_2O_2) was from Wako (Osaka, Japan). The other chemicals and reagents used in the experiment were of the highest grade commercially available.

Fluorometric Spectrophotometric Assay. The fluorometric assay was conducted in a 96-well microplate. The fluorescence was measured by a fluorescence microplate reader with the filter set for excitation at 473 ± 10 and emission at 580 ± 10 nm.

Sample Preparation. Each stock solution of test sample was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in each well was maintained below 0.01%.

MAO-B Inhibitory Activity of Test Samples. MAO-B inhibitory activity was determined by fluorometric spectrophotometry. The reaction mixture contained 2×10^{-4} units/mL MAO-B, 1 unit/mL HRP, 1 mM benzylamine, and 50 μM Amplex Red. After incubation for 60 min at room temperature, MAO-B activity was measured with a fluorometric method (*20*). MAO-B activity was expressed as the percentage of activity relative to the control experiment conducted simultaneously without addition of these inhibitory compounds.

HPLC Analysis. An LC-6A isocratic pump (Shimadzu Corp. Chromatographic Instruments Division, Kyoto, Japan) was connected to an SPD-6A ultraviolet spectrophotometric detector set at UV 280 nm. Columns consisted of (i) a LiChrospher Si-60 5 μm, 4.0 × 250 mm, and (ii) a Licrospher RP-18e, 4.0 × 250 mm (Merck, Darmstadt, Germany). The solvent systems were *n*-hexane/methanol/tetrahydrofuran/formic acid (47:42:10:1) containing 450 mg/L oxalic acid for column i and 0.05 M H_3PO_4/0.05 M KH_2PO_4/acetonitrile (40:40:20) for column ii. The flow rate was 1.0 mL/min. The solvent was degassed prior to use.

Isolation and Identification of Active Components of *M. candidum*. The dried leaves of *M. candidum* were homogenized with 70% acetone. After filtration and acetone evaporation, the aqueous concentrate was partitioned by ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) saturated with H_2O, respectively. All three fractions were subjected to bioactive evaluation on the inhibitory models of MAO to identify the active fraction. The EtOAc and *n*-BuOH extracts showed the stronger activities. Repeated chromatography of the fractions with monitoring of the inhibitory activity and HPLC analysis led to the isolation of active constituents. The EtOAc extract was chromatographed on Diaion HP-20 and Sephadex LH-20 columns and eluted with a gradient solvent system of methanol in H_2O (H_2O → 20% MeOH → 40% MeOH → 60% MeOH → 100% MeOH), respectively. After HPLC monitoring and bioactivity evaluation, fractions were combined and rechromatographed on an MCI CHP20P column developed with aqueous methanol in a stepwise gradient mode (H_2O → 10% MeOH → 20% MeOH → 30% MeOH → 40% MeOH → 100% MeOH), giving compounds **1** and **2**. The *n*-BuOH extract was also submitted to column chromatography on Sephadex LH-20 and MCI CHP 20P columns, which are as described above. This allowed isolation of compounds **3** and **4**.

Identification of Isolated Compounds. Structures of purified compounds were identified by direct comparisons of their retention times, melting points, and spectral data (^{1}H NMR and ^{13}C NMR) with those of literature data (*21*−*24*).

Assessment of Inhibition Kinetics. The assays were carried out with the IC_{50} values of tested samples and various concentrations of benzylamine. The apparent inhibition constants (K_i) for these four compounds were calculated by Lineweaver−Burk plots for the tested samples by respectively plotting the slope of each double-reciprocal plot versus the corresponding inhibitor concentration at which it was obtained (*25*).

Evaluation of Scavenging Activity on Hydroxyl Radical (HO•). The hydroxyl radical (HO•) was generated by the Fenton reaction consisting of 0.1 mM H_2O_2, 0.1 mM $FeSO_4$, and DMPO. The spectrum of DMPO-OH was measured at 20 s after the addition of $FeSO_4$, and it lasted for 15 s. The scavenging activity was calculated as described above. The scavenging activity is reported as the percent inhibition of the peak intensity of the control in which phosphate buffer solution was used instead of sample solution (*26*).

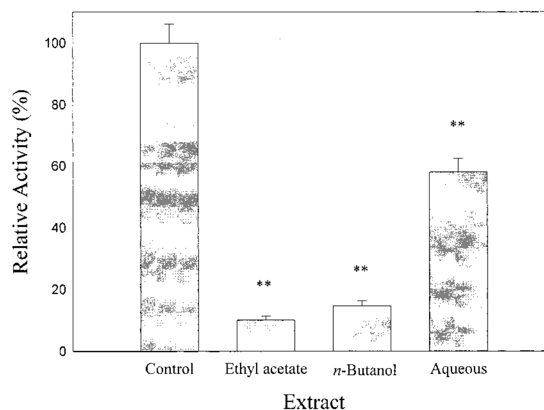
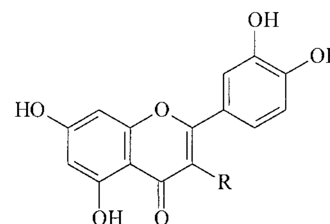


Figure 1. Inhibitory effect of each fraction (100 μg/mL) of *M. candidum*: control; ethyl acetate extract; *n*-butanol extract; aqueous extract. **, differs significantly from the control, no inhibition, at $p < 0.05$.



R	Name
-O-Rhamnose	Quercitrin
-O-Glucose	Isoquercitrin
-O-Glucose-Rhamnose	Rutin
-OH	Quercetin

Figure 2. Structures of compounds isolated from *M. candidum*.

ESR Measurements. All measurements were carried out immediately after the reaction mixture was prepared. All ESR spectra were recorded at ambient temperature (25 °C) on a Bruker EMX-6/1 electron paramagnetic resonance (EPR) spectrometer WIN-EPR. SimFonia software version 1.2 was used to record the spectra. ESR instrument settings were as follows: center field, 345.4 ± 5.0 mT; microwave power, 8 mW (9.416 GHz); modulation frequency, 100 kHz; modulation amplitude, 5 G; time constant, 0.6 s; and conversion time, 83 ms.

Data Analysis. The data are presented as the mean ± standard deviation (SD) of each triplet test. The Mann−Whitney *U* test was used to analyze the significance of differences between blank and test groups. The enzyme kinetics were analyzed by Lineweaver−Burk plots.

RESULTS AND DISCUSSION

The dried leaves of *M. candidum* were homogenized in 70% acetone. After the evaporation of acetone, the 70% acetone extract was partitioned by EtOAc and *n*-BuOH. The MAO-B inhibitory activity of these fractions, EtOAc, *n*-BuOH, and aqueous, was evaluated.

Table 1. Inhibitory Effect of Isolated Compounds on MAO-B

compound	inhibition[a] (%)				IC_{50}[b] (μM)
	12.5 μg/mL	6.25 μg/mL	3.125 μg/mL	1.5625 μg/mL	
l-deprenyl	6.59 ± 0.51	2.12 ± 0.33	–	–	–
quercitrin	55.1 ± 3.87	48.6 ± 1.65	41.5 ± 0.13	38.9 ± 1.04	19.06 ± 0.001
isoquercitrin	65.3 ± 2.01	55.8 ± 0.78	43.7 ± 1.07	39.4 ± 1.44	11.64 ± 0.001
rutin	63.2 ± 2.48	57.3 ± 3.38	50.4 ± 3.58	48.4 ± 3.87	3.89 ± 0.001
quercetin	57.4 ± 3.70	53.8 ± 4.56	48.9 ± 3.56	44.6 ± 3.35	10.89 ± 0.003

[a] Values represent the mean ± SD, $n = 3$. The inhibition percentage of each test sample was calculated as follows: inhibitory percentage (%) = [1 − (fluorescence of test sample − fluorescence of blank)/(fluorescence of control − fluorescence of blank)] × 100%. [b] Molecular weights of quercitrin, isoquercitrin, rutin, and quercetin are 449 $(M + H)^+$, 465 $(M + H)^+$, 611 $(M + H)^+$, and 303 $(M + H)^+$, respectively. *l*-Deprenyl is used as the positive control.

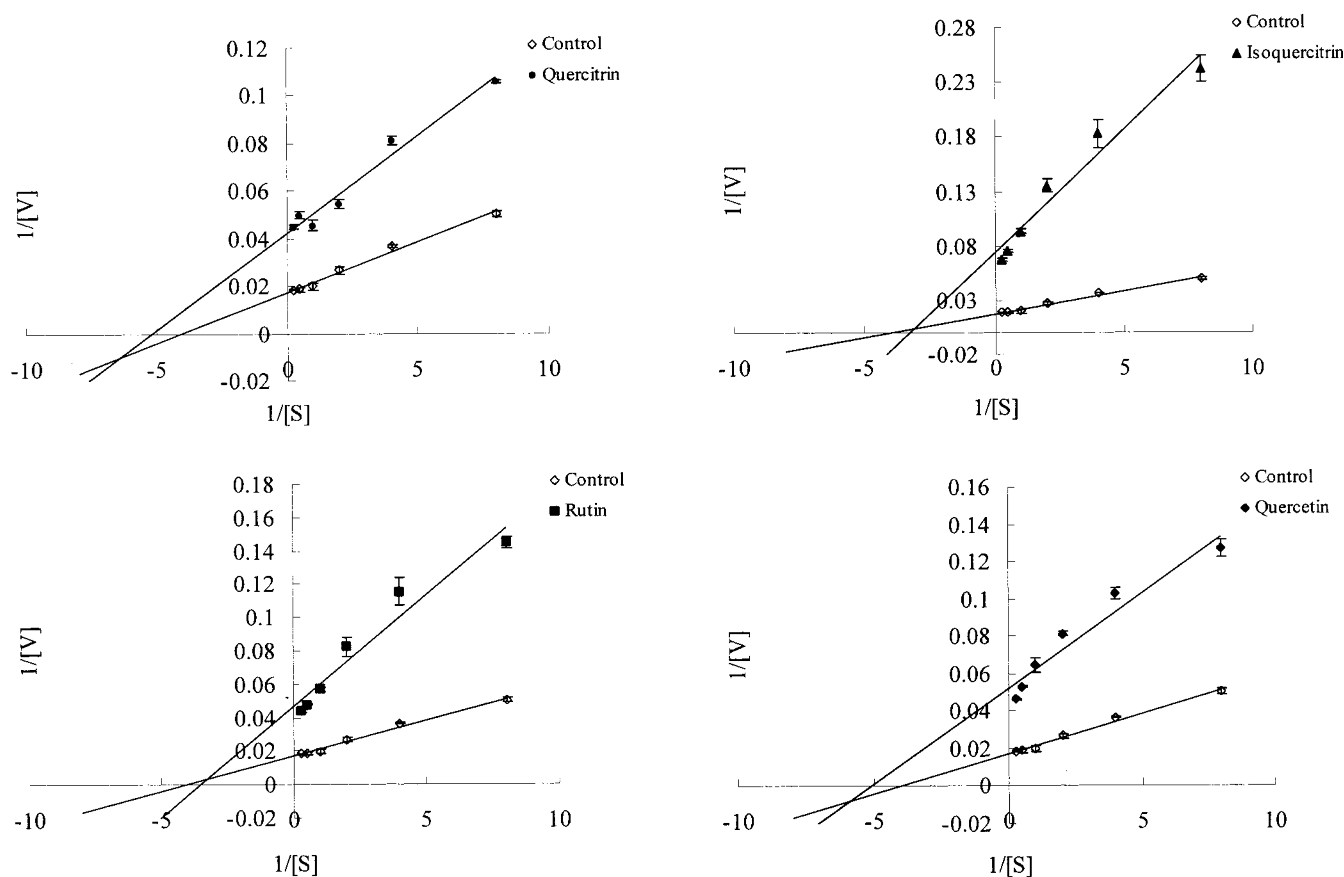


Figure 3. Inhibitory effects of quercitrin, isoquercitrin, rutin, and quercetin (IC_{50} values) on MAO-B as shown by Lineweaver−Burk plots in the absence (control) and in the presence of test samples with benzylamine as the substrate.

When the concentration of the extracts was 100 μg/mL, the activities relative to control (100%) were 89.9, 85.3, and 42.0%, respectively. The prominent activity was found in the EtOAc and *n*-BuOH extracts (Figure 1). Both monoamine oxidase activity and HPLC-directed purification of the EtOAc and *n*-BuOH extracts by various chromatographic methods, that is, Diaion HP-20, Sephadex LH-20, and MCI CHP-20P columns, led to isolation of the principal active components. By correlating with the retention times of HPLC, melting points, and NMR spectra of literature data, compounds **1**−**4** were identified as quercitrin (quercetin 3-*O*-β-L-rhamnoside) (*21*), isoquercitrin (quercetin 3-*O*-β-D-glucoside) (*22*), rutin (quercetin 3-*O*-β-D-glucose-[1,6]-*O*-α-L-rhamnoside) (*23*), and quercetin (*24*), respectively. The structures of these compounds are shown in Figure 2, and this is the first time that they have been isolated from this plant.

Compounds **1**−**4** exhibited dose-dependent inhibitory effects on MAO-B activity. IC_{50} values for MAO-B inhibition by quercitrin, isoquercitrin, rutin, and quercetin were 19.06, 11.64, 3.89, and 10.89 μM, respectively. Compared with the positive control, *l*-deprenyl, an MAO-B inhibitor, the four flavonoids are more effective in the experiment. The inhibitory potency on MAO-B is shown in Table 1. Furthermore, we performed an enzyme kinetics study of the four active components in each IC_{50} concentration with various concentrations of benzylamine substrate (0.125, 0.25, 0.5, 1, 2, and 4 mM). The Lineweaver−Burk plot of the data is shown in Figure 3. The results indicate that the four isolated active components, quercitrin, isoquercitrin, rutin, and quercetin, acted as mixed-type inhibitors with respect to the substrate benzylamine, and K_i values against this substrate were calculated to be 21.01, 2.72, 1.83, and 7.95 μM, respectively (Table 2).

The structures of the four isolated compounds are those of flavonoids. Differences among the four compounds are related to the 3-substituted flavonoid skeleton. Quercetin has a free hydroxyl group in the C3

Table 2. Apparent Inhibition Constants (K_i) and Inhibitory Modes of Each Tested Compound

compound	inhibition mode	K_i[a] (μM)
quercitrin	mixed (noncompetitive−uncompetitive)	21.01
isoquercetrin	mixed (competitive−noncompetitive)	2.72
rutin	mixed (competitive−noncompetitive)	1.83
quercetin	mixed (noncompetitive−uncompetitive)	7.95

[a] The apparent inhibition constant (K_i) of tested compounds was calculated using Lineweaver−Burk plots.

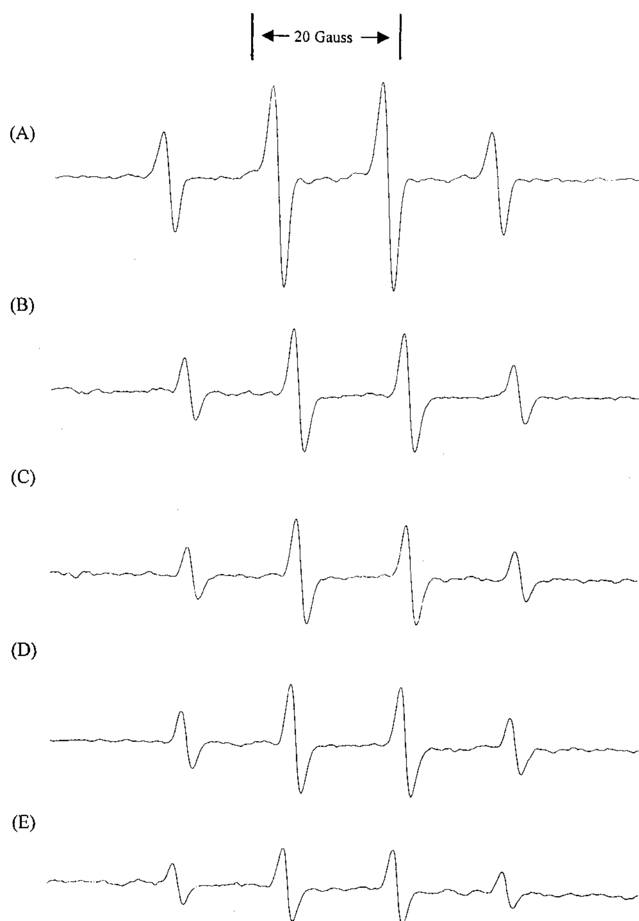


Figure 4. ESR spectra of scavenging activity of isolated flavonoids at 10 μg/mL against hydroxyl radicals: (A) control; (B) quercitrin; (C) isoquercitrin; (D) rutin; (E) quercetin.

position of the flavonol skeleton. Quercitrin, isoquercitrin, and rutin are flavonol glycosides, and they have the same aglycon quercetin in their structures. Also, quercitrin and isoquercitrin possess one sugar in the molecule, whereas there are two sugars in rutin. The potent MAO-B inhibitory activity may be attributed to the structures of flavonoids, and the sugar portions of the structures are also important features in the increase of the MAO-B inhibitory activity.

Under controlled conditions, the hydroxyl radical ($OH^{\bullet}$) was generated by the Fenton reaction to form DMPO-OH adducts. The adducts gave a characteristic ESR spectrum, which consisted of a quartet signal with an intensity ratio of 1:2:2:1 (*27*). Inhibitory effects on the hydroxyl radical were shown by various intensities of the DMPO-OH signal. When each isolated active component was treated with 10 μg/mL, respectively, the four natural flavonoids also exhibited hydroxyl radical scavenging activity against the hydroxyl radical as shown by ESR spectrometry (Figure 4).

A previous study corroborated a distinct relationship between MAO activity, free radicals, and the development of neurodegenerative diseases with aging (*28*). Increased oxidative deamination of dopamine enhances the production of hydrogen peroxide, which in the presence of transition metals can be converted to harmful cytotoxic hydroxyl free radicals. It has been postulated that excessive oxidative deamination of dopamine and the resultant enhanced oxidative stress may be involved in the pathogenesis of Parkinson's disease (*29*). Inhibition of deamination-induced oxidative stress by blocking monoamine oxidase activity and inhibiting the formation of free radicals might have a beneficial effect on aging and age-related neurodegenerative disorders.

Naturally occurring flavonoids have been reported to possess several activities, for instance, antioxidant activity (*30*), platelet aggregation, anti-HIV activity (*31*), antibacterial activity (*32*), and tumor cell growth inhibition (*33*, *34*). Therefore, flavonoids have attracted attention because of their beneficial effects. The present study indicates that four active flavonoids, quercitrin, isoquercitrin, rutin, and quercetin, are contained in *M. candidum*. This is the first report of anti-MAO-B activity and direct hydroxyl radical scavenging activity by ESR spectroscopy for the four flavonoid compounds from *M. candidum*. They may potentially prove to have a preventative value of neurodegeneration in the future.

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