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Antioxidation and Tyrosinase Inhibition of Polyphenolic Curcumin Analogs

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A series of polyphenolic curcumin analogs were synthesized and their inhibitory effects on mushroom tyrosinase and the inhibition of 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical formation were evaluated. The results indictated that the analogs possessing *m*-diphenols and *o*-diphenols exhibited more potent inhibitory activity on tyrosinase than reference compound rojic acid, and that the analogs with *o*-diphenols exhibited more potent inhibitory activity of DPPH free-radical formation than reference compound vitamin C. The inhibition kinetics, analyzed by Lineweaver–Burk plots, revealed that compounds B_2 and C_2 bearing *o*-diphenols were non-competitive inhibitors, while compounds B_{11} and C_{11} bearing *m*-diphenols were competitive inhibitors. In particular, representative compounds C_2 and B_{11} showed no side effects at a dose of 2,000 mg/kg in a preliminary evaluation of acute toxicity in mice. These results suggest that such polyphenolic curcumin analogs might serve as lead compounds for further design of new potential tyrosinase inhibitors.

Key words: tyrosinase inhibition; antioxidation; polyphenolic curcumin analogs; inhibition kinetics

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase, is a multifunctional copper-containing enzyme widely distributed in nature.[1)] It catalyzes two distinct reactions of melanin biosynthesis: the *o*-hydroxylation of monophenols to *o*-phenols (monophenolase activity), and the oxidation of *o*-phenols to *o*-quinones (diphenolase activity).[2)] These quinones are highly reactive compounds that can polymerize spontaneously to form melanins, which determine the color of mammalian skin and hair.[3)] Various dermatological disorders, such as age spots, freckle, melasma, and ephelide, are caused by the accumulation of excessive levels of epidermal pigmentation.[4)] Hence, tyrosinase inhibitors have become increasingly important in medication[5–7)] and the cosmetics of industry[8,9)] to prevent hyperpigmentation.

In recent years, a large number of naturally occurring and synthetic tyrosinase inhibitors have been reported,[4,10,11)] but some of these inhibitors are either not potentially active enough to be of practical use, or not compatible with safety regulations for medicines and cosmetics. The potentially active inhibitors, such as kojic and arbutin, have not yet been demonstrated to be clinically efficient inhibitors.[12,13)] Hydroquinone, a widely used skin-lightening agent, is still controversial as to its biosafety due to its cytotoxic and mitogenic properties.[14)] Hence, it is still necessary to search for and develop novel tyrosinase inhibitors with potent activities and smaller side effects.

In addition, antioxidant therapies are increasingly recognized as a potential strategy to prevent many diseases, including inflammation, acute CNS injury, cardiovascular disease, and asthma. Applications of antioxidants as preservatives in skin-protective ingredients in cosmetics are also receiving increasing attention and interest.[15,16)] Developing tyrosinase inhibitors with potent antioxidation may be a feasible way to balance skin brightening and side effects.

Curcumin (diferuloylmethane, Scheme 1), a well-known antioxidant polyphenol from the rhizome of *Curcuma longa* Linn, is a major ingredient of turmeric. It is used in the therapy of inflammatory and infectious diseases in ayurvedic medicine.[17)] Many studies indicate that curcumin has cancer preventive,[18)] anti-inflammatory,[19)] antioxidative,[20)] and antiviral activities.[21)] In addition, the safety of curcumin is evident from its consumption for centuries at levels of up to 100 mg/d by people in many countries.[22)] These beneficial properties have attracted numerous efforts for the development of curcumin as a safe therapeutic agent. Curcumins have been approved as additives in cosmetics and as a natural yellow colorants and antioxidants for many years.[23)] Recently, it was found to be beneficial in treating certain skin diseases,[24)] and one of its derivatives, tetrahydrocurcumin, has been recommended for use in cosmetics as a lighting agent.[25)] Furthermore, Lee *et al.* reported recently that some curcumin analogs exhibited inhibitory activity against tyrosinase.[26)] This attracted our interest to further study of the inhibitory effect on tyrosinase and the antioxidation of curcumin analogs.

In this study, considering the important roles of polyphenolic hydroxyls in certain reported tyrosinase inhibitors,[27–30)] a series of curcumin analogs bearing polyphenols were synthesized (Scheme 2), and their inhibitory activities against tyrosinase and free-radical

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scavenging properties on DPPH were evaluated. In addition, the inhibition mechanism and preliminary acute toxicity of the potent active compounds were also investigated, with the aim of developing novel tyrosinase inhibitors with potent activities, together with smaller side effects.

Results and Discussion

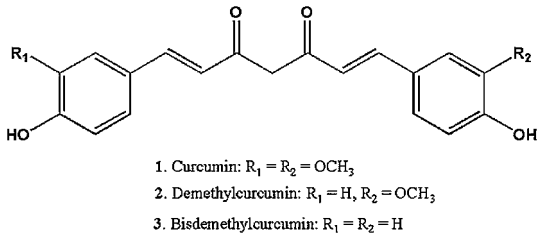
Chemistry

Natural curcuminoids (**1–3**) were isolated from turmeric, and 19 new compounds and 41 known compounds were synthesized through condensation of the appropriate aromatic aldehydes and cyclohexanone ($\mathbf{A_{1–11}}$), cyclopentanone ($\mathbf{B_{1–11}}$), acetone ($\mathbf{C_{1–11}}$), tetrahydrothiopyran-4-one ($\mathbf{D_{1–10}}$), tetrahydropyran-4-one ($\mathbf{E_{1–10}}$), and tetrahydropyran-4-one ($\mathbf{F_{1–10}}$) under acidic conditions at room temperature following published procedures.[31] These polyphenolic curcumin analogs were synthesized by a one-step procedure without phenolic hydroxy protection, and the final products were purified by recrystallization. The structures were determined using various spectroscopic methods, including ^{1}H NMR and EI-MS, and purity was confirmed by elemental analysis.

Inhibitory effects on mushroom tyrosinase

Natural curcuminoids (**1**, **2**, and **3**) isolated from *Curcuma longa* and synthetic curcumin analogs ($\mathbf{A_{1–11}}$, $\mathbf{B_{1–11}}$, $\mathbf{C_{1–11}}$, $\mathbf{D_{1–7}}$, $\mathbf{E_{1–10}}$, and $\mathbf{F_{1–10}}$) were subjected to tyrosinase inhibitory assay by measuring the oxidation of L-DOPA according to a published protocol.[29] As Fig. 1 indicates, the remaining enzyme activity rapidly decreased with increasing concentrations of compound $\mathbf{C_{11}}$. Enzyme inhibition data were expressed as IC_{50} values as summarized in Table 1. Kojic acid, a commercial whitening ingredient, was used as reference compound.

Based on the data shown in Table 1, it was found that natural curcuminoids **1–3** showed potent inhibitory activities against tyrosinase, and that compound **3** exhibited the highest inhibitory activity, with an IC_{50} value of 33.50 μM, among curcuminoids. In the synthetic curcumin analogs, compounds with *o*-diphenols ($\mathbf{A_2}$-$\mathbf{F_2}$) and *m*-diphenols ($\mathbf{A_{11}}$-$\mathbf{C_{11}}$) exhibited much higher inhibitory activities than others. Compound $\mathbf{C_{11}}$ ($IC_{50} = 0.65$ μM) was the strongest inhibitor of all the observed compounds, and it was much stronger than the reference kojic acid ($IC_{50} = 28.59$ μM).



1. Curcumin: $R_1 = R_2 = OCH_3$
2. Demethylcurcumin: $R_1 = H$, $R_2 = OCH_3$
3. Bisdemethylcurcumin: $R_1 = R_2 = H$

Scheme 1. Chemical Structures of Curcuminoids.

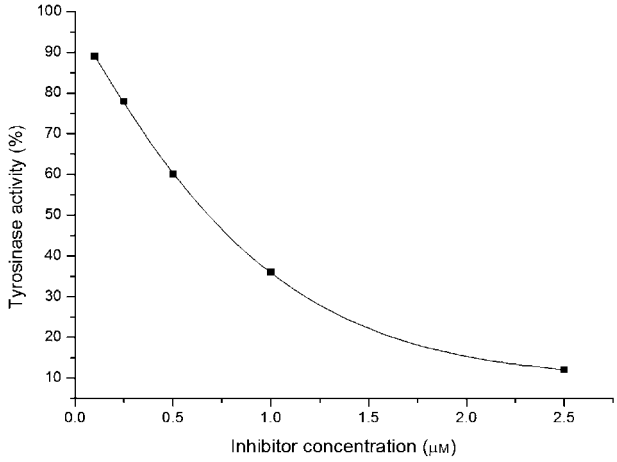
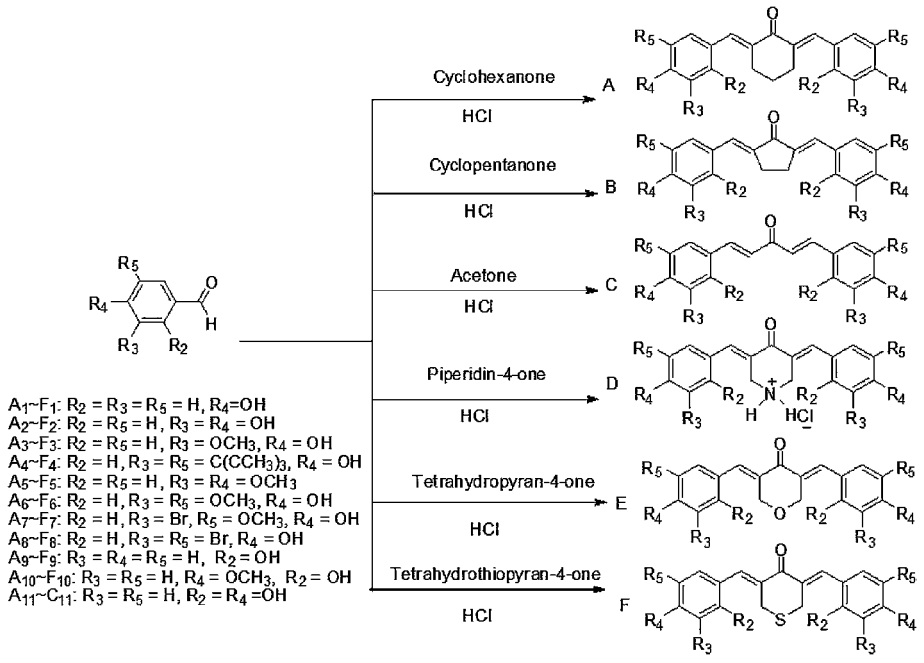


Fig. 1. Effects of Compound $\mathbf{C_{11}}$ on Mushroom Tyrosinase for the Catalysis of L-DOPA.



Scheme 2. Synthesis of Curcumin Analogs.

Table 1. Antioxidative and Tyrosinase Inhibitory Effects of Curcumin Analogs

Compound	Inhibition (IC_{50}/μM)[a]		Compound	Inhibition (IC_{50}/μM)[a]	
	DPPH	Tyrosinase		DPPH	Tyrosinase
A_1	>300	64.48 ± 3.42	D_1	>300	171.60 ± 8.02
A_2	16.82 ± 0.87	2.16 ± 0.58	D_2	29.26 ± 1.42	11.32 ± 1.60
A_3	48.40 ± 2.06	68.26 ± 3.96	D_3	65.18 ± 3.72	>200
A_4	70.10 ± 3.65	>200	D_4	71.56 ± 3.83	>200
A_5	NI[b]	NI	D_5	NI	NI
A_6	53.68 ± 1.68	>200	D_6	85.10 ± 4.32	>200
A_7	73.80 ± 3.98	>200	D_7	103.20 ± 5.12	>200
A_8	>300	193.22 ± 7.43	E_1	>300	47.28 ± 2.17
A_9	>300	>200	E_2	18.80 ± 0.51	1.36 ± 0.68
A_{10}	216.81 ± 11.36	>200	E_3	56.90 ± 3.18	62.37 ± 3.84
A_{11}	50.60 ± 3.23	0.93 ± 0.22	E_4	53.70 ± 2.32	>200
B_1	>300	41.87 ± 1.38	E_5	NI	NI
B_2	5.79 ± 0.12	1.24 ± 0.22	E_6	49.06 ± 1.87	>200
B_3	48.90 ± 1.69	56.40 ± 2.89	E_7	82.78 ± 4.67	>200
B_4	59.20 ± 3.01	>200	E_8	>300	177.11 ± 5.78
B_5	NI	NI	E_9	>300	>200
B_6	17.90 ± 0.54	>200	E_{10}	>300	>200
B_7	78.06 ± 3.28	>200	F_1	>300	72.60 ± 3.68
B_8	>300	172.32 ± 5.11	F_2	21.10 ± 0.86	2.64 ± 0.83
B_9	>300	>200	F_3	53.90 ± 2.37	79.80 ± 4.77
B_{10}	124.62 ± 6.78	>200	F_4	52.20 ± 2.13	>200
B_{11}	47.10 ± 4.33	0.78 ± 0.13	F_5	NI	NI
C_1	>300	31.26 ± 1.32	F_6	58.70 ± 2.86	>200
C_2	6.73 ± 0.18	1.19 ± 0.21	F_7	83.64 ± 3.54	>200
C_3	40.20 ± 2.03	41.25 ± 1.73	F_8	>300	>200
C_4	65.72 ± 2.87	116.44 ± 3.76	F_9	>300	>200
C_5	NI	NI	F_{10}	>300	>200
C_6	38.40 ± 1.65	176.32 ± 5.61	1	21.62 ± 1.21	94.73 ± 3.76
C_7	96.4 ± 5.40	162.83 ± 3.81	2	37.80 ± 1.67	53.03 ± 2.17
C_8	>300	102.43 ± 2.53	3	34.64 ± 1.53	33.50 ± 1.26
C_9	>300	>200	Vc	21.14 ± 0.82	ND[c]
C_{10}	186.42 ± 8.21	>200	Kojic acid	ND	28.59 ± 1.30
C_{11}	48.24 ± 3.78	0.65 ± 0.12			

[a]Values are means with standard deviation for three different experiments.
[b]NI, No Inhibition.
[c]ND, no data.

Compared with the inhibitory activities of curcumin analogs bearing 4-phenolic hydroxyls (**A_1**-**E_1**), the activities of these compounds decreased when R_3 and R_5 were replaced by bromine, methoxyl, or *t*-butyl. When only one side of *p*-phenolic hydroxyl, R_3 or R_5, was replaced by methoxyl (**A_3**-**F_3**), the inhibitory activities decreased slightly, whereas, when both sides of *p*-phenolic hydroxyl, R_3 and R_5, were replaced by non-hydroxylic groups, the inhibitory activities decreased sharply. In addition, the activities of curcumin analogs bearing 2-phenolic hydroxyls (**A_9**-**F_9**) were much weaker than those of curcumin analogs bearing 4-phenolic hydroxyls (**A_1**-**F_1**). In a comparison between compounds **A_{10}**-**C_{10}** and compounds **A_{11}**-**C_{11}**, only 4-phenolic hydroxyls was replaced by methoxyls, and the activities of **A_{10}**-**C_{10}** almost dispeared, whereas the activities of **A_{11}**-**C_{11}** were the strongest of all the compounds. It suggested that the 4-phenolic hydroxyls in curcumin analogs play a crucial role in inhibitory activity against tyrosinase. Nerya and his coworkers also found that the OH group substation at the *para* position of the chalcone benzene ring was the major factor affecting inhibitory potency.[31)]

In addition, the different ketone linkers between two aromatic rings of curcumin analogs showed different active trend, although there was no big difference between different linkers. The activities of compounds with acetone linkers (series **C**, compounds **C_{1-11}**) exhibited the strongest activities, and those of compounds with cyclopentanone (series **B**, compounds **B_{1-11}**), tetrahydropyran-4-one (series **E**, compounds **E_{1-10}**), cyclohexanone (series **A**, compounds **A_{1-11}**), tetrahydrothiopyran-4-one (series **F**, compounds **F_{1-10}**) and piperidin-4-one (D_{1-7}) were weaker in that order, which hints that the smaller bulk of these linkers exhibited stronger activity than the others.

Inhibition mechanism of the selected compounds as to mushroom tyrosinase

Lineweaver-Burk plots of the inhibition kinetics of tyrosinase for the selected compounds, **B_2**, **C_2**, **B_{11}**, and **C_{11}**, are shown in Fig. 2. Figure 2A and B are for selected compounds **B_2** and **C_2** with *o*-diphenols, and the straight lines crossing the x axis at the same point show unchanged K_m and decreased V_{max} with increasing inhibitor concerntrations, which is typical for non-competitive inhibition. In Fig. 2C and D, for selected compounds **B_{11}** and **C_{11}** with *m*-diphenols, the straight lines crossing the y axis at the same point show unchanged V_{max} and decreased K_m with increasing inhibitor concentrations, which is typical for competitive inhibition. The inhibition constants K_i (Table 2) were calculated from the plots of the slope *versus* the concentration of selected compounds.

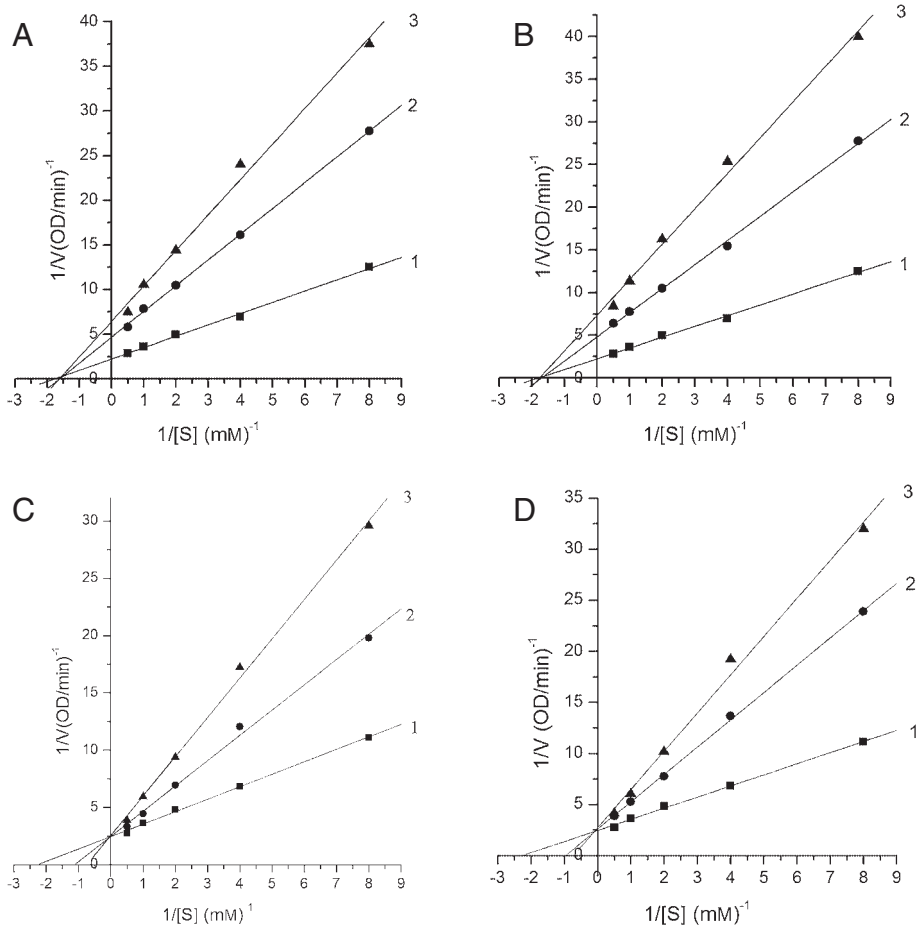


Fig. 2. Lineweaver–Burk Plots of the Inhibition of Selected Compounds $\mathbf{B_2}$, $\mathbf{C_2}$, $\mathbf{B_{11}}$, and $\mathbf{C_{11}}$ on Mushroom Tyrosinase for the Catalysis of L-DOPA.

The concentrations of $\mathbf{B_2}$ $\mathbf{C_2}$, $\mathbf{B_{11}}$, and $\mathbf{C_{11}}$ for curves 1–3 were 0, 0.4 μM, 0.8 μM; 0, 0.3 μM, 0.6 μM; 0, 0.6 μM, 1.2 μM; 0, 0.6 μM, and 1.2 μM respectively.

Most competitive inhibitors of tyrosinase have molecular structures that closely resemble that of the products of the various steps. Base on the structures of synthetic curcumin analogs, the compounds with *o*-diphenols were similar to the substrate L-DOPA, but the mechanisms of curcumin analogs $\mathbf{B_2}$ and $\mathbf{C_2}$ with *o*-diphenols showed non-competitive inhibition, and compounds $\mathbf{B_{11}}$ and $\mathbf{C_{11}}$ with *m*-diphenols showed competitive inhibition, which indicates that curcumin analogs with *o*-diphenols combined with both the free enzyme and the enzyme-substrate complex, and that curcumin analogs with *m*-diphenols combined only with free enzyme molecule. Baek *et al.* have reported that tyrosinase inhibitors 1,3-diphenylpropanes with *m*-diphenols showed competitive inhibition,[32] and Song *et al.* have reported that 3,5-dihydroxystilbenes with *m*-diphenols also exihibited competitive inhibition.[33] The details of the inhibition mechanisms of curcumin analogs as to tyrosinase call for further study.

Effects on free radical scavenging

Table 1, columns 2 and 5, shows the DPPH radical scavenging activities of curcumin analogs. Compounds with *o*-diphenols ($\mathbf{A_2}$-$\mathbf{F_2}$) showed more active DPPH radical scavenging than the other curcumin analogs. Generally, in a comparision within the same series of curcumin analogs, the introduction of methoxyl and *t*-butyl to the adjacent of 4-phenols strengthens the activities of DPPH radical scavenging, whereas the introduction of bromines weakens their activities. In addition, in a comparison with diffenent linkers between the two aromatic rings, cylopentanone (series B) and actone (series C) showed stronger DPPH radical scavenging properties than the others.

Table 2. Kinetics and Inhibition Constants of Compounds $\mathbf{B_2}$, $\mathbf{C_2}$, $\mathbf{B_{11}}$, and $\mathbf{C_{11}}$ in the Activity of Mushroom Tyrosinase

Compound	Inhibition type	Inhibition onstant (K_i) (μM)
B_2	Non-competetive	0.50
C_2	Non-competetive	0.45
B_{11}	Competetive	0.37
C_{11}	Competetive	0.24

Evaluation of acute toxicity in mice

Safety is a primary consideration for tyrosinase inhibitors, especially for those materials used in medicine and cosmetic products. In this study, $\mathbf{C_2}$ and $\mathbf{B_{11}}$ were selected as representative compounds to evaluate toxicity in mice. Clinical symptoms were measured for 14 d after a single oral gavage administration in accord with the OECD 423 Guideline. The results indictated that none of the mice after administration at a dose of 2,000 mg/kg of body weight per d showed any mortality, and autopsy of the animals at the end of the experimental period (14 d) revealed no apparent changes

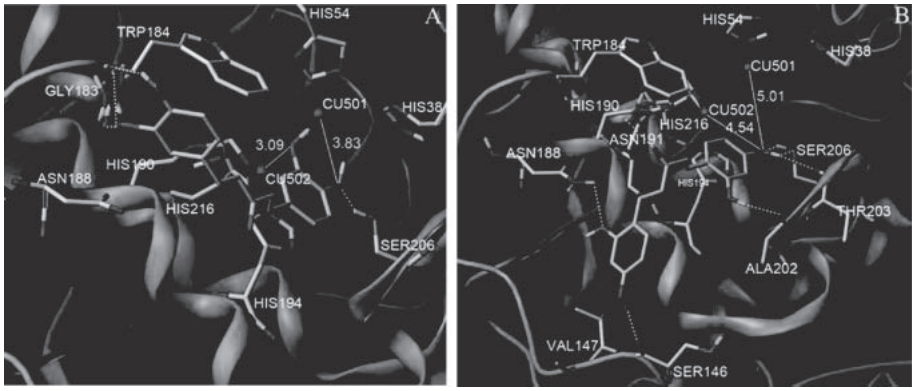


Fig. 3. Proposed Binding Modes of $\mathbf{C_2}$ (A) and $\mathbf{C_{11}}$ (B) in the Active Site of the Tyrosinase (PDB accession code 2ZWE).
The inhibitor molecules are colored yellow for carbon atoms. The broken lines show hydrogen-bonding, and the solid lines show the distance of metal-coordination interactions. The docking models were generated using Surflex-Dock.

in any organs. This indicates that compounds $\mathbf{C_2}$ and $\mathbf{B_{11}}$ had no acute toxicity at 2,000 mg/kg in mice.

Molecular docking study

Recently, the crystallographic structure of tyrosinase was determined. The three-dimensional structure of tyrosinase make it possible to gain a better understanding of the tyrosinase inhibition mechanism. Considering that compounds $\mathbf{C_2}$ and $\mathbf{C_{11}}$ exhibited potent inhibition against tyrosinase and that their inhibition mechanisms were different, we selected them to study the interaction mode by docking. Although the structure of mushroom tyrosinase has not yet been determined, we borrowed the crystallographic data because there is high homology for the active center of most tyrosinases of different origins. It was found that $\mathbf{C_2}$ and $\mathbf{C_{11}}$ both formed a π-π stack between one of aromatic rings of the ligands and His 194 of tyrosinase, and multiple hydrogen bonds between the phenolic hydroxyls of the compounds and the residues of the active site of tyrosinase (Fig. 3). In Fig. 3A, $\mathbf{C_2}$ with *o*-diphenols formed four hydrogen bonds with Gly 183, Trp 184 and Ser 206, and the distances between the 3,4-phenolic hydroxyls of the compound and dinuclear copper ions were 3.09 Å and 3.83 Å respectively (Fig. 3A). $\mathbf{C_{11}}$ with *m*-diphenols formed seven hydrogen bonds, with Ser 206, Ala 202, Asn 191, Asn 188, Thr 203, and Ser 146, and the distances between the 2,4-phenolic hydroxyls and the dinuclear copper ions were 4.54 Å and 5.01 Å respectively (Fig. 3B). The different interactions of $\mathbf{C_2}$ and $\mathbf{C_{11}}$ with tyrosinase might explain why the inhibitory avtivity of the curcumin analogs with *m*-diphenols was stronger than that of the curcumin analogs with *o*-diphenols. Their modes of inhibition of tyrosinase were entirely different.

Conclusion

In this study, a series of polyphenolic curcumin analogs were synthesized, and their effects on mushroom tyrosinase inhibition and free-radical scaveraging were evaluated. The results indicated that the analogs possessing *m*- or *o*-diphenols exhibited more potent activity inhibition of tyrosinase than the others. Analysis of the inhibition kinetics revealed that the analogs with *o*-diphenols were non-competitive inhibitors, while the analogs with *m*-diphenols were competitive. The docking study that the analogs with *m*-diphenols and those with *o*-diphenols were different. The primary evaluation of acute toxicity showed that $\mathbf{C_2}$ and $\mathbf{B_{11}}$ were not toxic to the mice. These results suggest that with the advantage of easily synthesizable small molecules, polyphenolic curcumin analogs can be developed as pharmacological tyrosinase inhibitors.

Experimental

Reagents and general procedures. Melting points were determined on a Yanagimoto micro-melting apparatus (MP 500D), and are uncorrected. The ^{1}H NMR spectra were measured on a 300 mhz NMR Spectrometer (Varian Gemini-2000) using DMSO as solvent, unless otherwise specified. Chemical shifts for ^{1}H NMR (300 MHz) were expressed as ppm with TMS as internal standard. Multiplicities were recorded as singlet, broad singlet, doublet, triplet, quartet, and multiplet. Mass spectra were obtained on a mass spectrometer (LCMS-2010A). Elemental analyses were performed on a elemental analyzer (Perkin Elmer 240C). Curcuminoids (**1–3**) were isolated from an extract of *Curcuma longa*, and compounds A_{1-7}, B_{1-7}, C_{1-6}, and D_{1-7}, were synthesized, purified and characterized as previously described.[34,35] Tyrosinase and L-3,4-dihydroxy phenylalanine (L-DOPA) were purchased from Sigma–Aldrich (St. Louis, Mo). Other chemicals were purchased from commercial suppliers and were dried and purified when necessary. The water used was re-distillated and ion-free.

General procedure for the synthesis of curcumin analogs. Sixty curcumin analogs (series A, B, C, D, E, and F) were synthesized as previously described, with modifications.[34,35] A mixture of the appropriate aldehyde (0.01 mol) and the ketone (0.005 mol) was dissolved in glacial acetic acid or ethanol saturated with anhydrous hydrogen chloride and this was heated in a water bath at 25–30 °C for 2 h. After standing for 2–5 d, the mixture was treated with cold water and filtered. The solid obtained was then washed and dried. The crude product was recrystallized from appropriate solvents (methanol or ethanol).

2,6-Bis(3,5-dibromo-4-hydroxylbenzylidene)cyclohexanone (A_8). Yield 83%. mp 238–239 °C. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 10.32 (brs, 2H, –OH), 7.77 (s, 4H, ArH), 7.46 (s, 2H, –CH=C–), 2.85 (m, 4H, $-CH_2-C-CH_2-$), 1.75 (m, 2H, C–CH_2–C). LC–MS (*m*/*z*): 621 $[M-1]^-$. Anal. Calc. for $C_{20}H_{14}Br_4O_3$: C 38.62, H 2.27. Found: C 38.59, H 2.30.

2,6-Bis(2-hydroxybenzylidene)cyclohexanone (A_9). Yield 75%. mp 147–149 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 9.87 (brs, 2H, –OH), 7.79 (s, 2H, ArH), 7.30 (s, 2H, –CH=), 7.18 (m, 2H, ArH), 6.89 (d, J = 8.1 Hz, 2H, ArH), 6.83 (d, J = 8.1 Hz, 2H, ArH), 2.81 (m, 4H,

–CH_2–C–CH_2–), 1.67 (m, 2H, C–CH_2–C). LC–MS (m/z): 305 $[M-1]^-$. Anal. Calc. for $C_{20}H_{18}O_3$: C 78.41, H 5.92. Found: C 78.21, H 6.01.

2,6-Bis(2-hydroxy-4-methoxybenzylidene)cyclohexanone (A_{10}). Yield 72%, mp 206–208 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 9.80 (brs, 2H, –OH), 7.21 (d, $J = 8.1$ Hz, 2H, ArH), 6.73 (s, 2H, –CH=), 6.61 (d, $J = 8.1$ Hz, 2H, ArH), 6.47 (s, 2H, ArH), 3.68 (s, 6H, $-OCH_3$), 2.81 (m, 4H, –CH_2–C–CH_2–), 1.74 (m, 2H, C–CH_2–C). LC–MS (m/z): 366. Anal. Calc. for $C_{22}H_{22}O_5$: C 72.12, H 6.05. Found: C 72.21, H 6.08.

2,6-Bis(2,4-dihydroxybenzylidene)cyclohexanone (A_{11}). Yield 51%, mp >300 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.00 (s, 2H, –OH), 9.80 (s, 2H, –OH), 7.16 (d, $J = 8.1$ Hz, 2H, ArH), 6.33 (s, 2H, –CH=), 6.26 (d, $J = 8.1$ Hz, 2H, ArH), 6.19 (s, 2H, ArH), 2.80 (m, 4H, –CH_2–C–CH_2–), 1.72 (m, 2H, C–CH_2–C). LC–MS (m/z): 338. Anal. Calc. for $C_{22}H_{22}O_5$: C 70.99, H 5.36. Found: C 70.83, H 5.42.

2,6-Bis(3,5-dibromo-4-hydroxylbenzylidene)cyclopentanone (B_8). Yield 85%. mp >300 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.55 (brs, 2H, –OH), 8.07 (s, 4H, ArH), 7.32 (s, 2H, –CH=C–), 3.05 (s, 4H, –CH_2–CH_2–). LC–MS (m/z): 607 $[M-1]^-$. Anal. Calc. for $C_{19}H_{12}Br_4O_3$: C 37.54, H 1.99. Found: C 37.49, H 2.02.

2,5-Bis(2-hydroxybenzylidene)cyclopentanone (B_9). Yield 81%. mp 224–226 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.09 (brs, 2H, –OH), 7.77 (s, 2H, –CH=C–), 7.53 (d, $J = 8.1$ Hz, 2H, ArH), 7.22 (m, 4H, ArH), 6.88 (m, 4H, ArH), 3.01 (s, 4H, –CH_2–CH_2–). LC–MS (m/z): 291 $[M-1]^-$. Anal. Calc. for $C_{19}H_{16}O_3$: C 78.06, H 5.52. Found: C 77.93, H 5.46.

2,5-Bis(2-hydroxy-4-methoxybenzylidene)cyclopentanone (B_{10}). Yield 74%. mp 212–213 °C. ^{1}H NMR (MSDO-d_6, 400 MHz) δ (ppm): 10.23 (s, 2H, –OH), 7.74 (s, 2H, ArH), 7.50 (d, $J = 8.1$ Hz, 2H, –C=CH–), 6.51 (d, $J = 8.1$ Hz, 4H, ArH), 3.76 (s, 6H, $-OCH_3$), 2.96 (s, 4H, –CH_2–CH_2–). LC–MS (m/z): 351 $[M-1]^-$. Anal. Calc. for $C_{21}H_{20}O_5$: C 71.58, H 5.72. Found: C 71.45, H 5.55.

2,5-Bis(2,4-dihydroxybenzylidene)cyclopentanone (B_{11}). Yield 52%. mp >300 °C. ^{1}H NMR (MSDO-d6, 300 MHz) δ (ppm): 10.00 (s, 2H, –OH), 9.80 (s, 2H, –OH), 7.69 (s, 2H, –CH=C–), 7.37 (d, $J = 8.7$, 2H, arom), 6.38 (s, 2H, arom), 6.32 (d, $J = 8.7$ Hz, 2H, arom), 2.92 (s, 4H, –CH_2–CH_2–). ESI-MS (m/z): 325 ($[M+1]^+$). Anal. Calc. for $C_{19}H_{16}O_5$: C 70.36, H 4.97. Found: C 70.25, H 5.04.

1,5-Bis(3-bromo-4-hydroxy-5-methoxyphenyl)penta-1,4-dien-3-one (C_7). Yield 85%. mp 171–172 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.05 (brs, 2H, –OH), 7.63 (d, $J = 15.9$, 2H, –CH=C–), 7.54 (d, $J = 8.1$ Hz, 2H, ArH), 7.39 (d, $J = 8.1$ Hz, 2H, ArH), 7.20 (d, $J = 15.9$, 2H, –CH=C–), 3.90 (s, 6H, OCH_3). LC–MS (m/z): 481 $[M-1]^-$. Anal. Calc. for $C_{19}H_{16}Br_2O_5$: C 47.14, H 3.33. Found: C 47.10, H 3.28.

1,5-Bis(3,5-dibromo-4-hydroxylphenyl)penta-1,4-diene-3-one (C_8). Yield 92%. mp 280–281 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.53 (brs, 2H, –OH), 8.02 (s, 4H, ArH), 7.65 (d, $J = 15.9$ Hz, 2H, –CH=C–), 7.25 (d, $J = 15.9$ Hz, 2H, –C=CH–). LC–MS (m/z): 581 $[M-1]^-$. Anal. Calc. for $C_{17}H_{10}Br_4O_3$: C 35.09, H 1.73. Found: C 35.06, H 1.74.

1,5-Bis(2-hydroxyphenyl)penta-1,4-dien-3-one (C_9). Yield 81%. mp 224–226 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.21 (brs, 2H, –OH), 7.90 (d, $J = 15.9$ Hz, 2H, –CH=C–), 7.66 (d, $J = 8.1$ Hz, 2H, ArH), 7.24 (m, 4H, ArH), 6.87 (m, 4H, ArH). LC–MS (m/z): 265 $[M-1]^-$. Anal. Calc. for $C_{17}H_{14}O_3$: C 76.68, H 5.30. Found: C 76.56, H 5.23.

1,5-Bis(2-hydroxy-4-methoxyphenyl)penta-1,4-dien-3-one (C_{10}). Yield 68%. mp 149–151 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.28 (brs, 2H, –OH), 7.82 (d, $J = 15.9$, 2H, –CH=C–), 7.60 (d, $J = 8.1$, 2H, ArH), 7.10 (d, $J = 15.9$ Hz, 2H, –CH=C–), 6.46 (d, $J = 8.1$ Hz, 2H, ArH), 3.74 (s, 6H, OCH_3). LC–MS (m/z): 325 $[M-1]^-$. Anal. Calc. for $C_{19}H_{18}O_5$: C 69.93, H 5.56. Found: C 69.74, H 5.60.

1,5-Bis(2,4-dihydroxybenzylidene)penta-1,4-3-one (C_{11}). Yield 42%. mp >300 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.08 (brs, 2H, –OH), 9.80 (brs, 2H, –OH), 7.62 (d, $J = 15.9$, 2H, –CH=C–), 7.40 (d, $J = 8.1$, 2H, ArH), 6.48 (d, $J = 15.9$ Hz, 2H, –CH=C–), 6.40 (d, $J = 8.1$ Hz, 2H, ArH), LC–MS (m/z): 299 $[M-1]^-$. Anal. Calc. for $C_{17}H_{14}O_5$: C 68.45, H 4.73. Found: C 68.24, H 4.80.

3,5-Bis(4-hydroxybenzylidene)-tetrahydropyran-4-one (D_1). Yield 82%. mp 226–228 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.03 (brs, 2H, –OH), 7.55 (s, 2H, –CH=), 7.28 (d, $J = 8.1$, 4H, ArH), 6.85 (d, $J = 8.1$, ArH), 4.85 (s, 4H, –CH_2–O–CH_2–). LC–MS(m/z): 307 $[M-1]^-$. Anal. Calc. for $C_{19}H_{16}O_4$: C 74.01, H 5.23. Found: C 74.00, H 5.24.

3,5-Bis(3,4-dihydroxybenzylidene)-tetrahydropyran-4-one (D_2). Yield 90%. mp >300 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 9.50 (brs, 2H, –OH), 9.18 (brs, 2H, –OH), 7.45 (s, 2H, –CH=), 6.81 (s, 2H, ArH), 6.76 (m, 4H, ArH), 4.83 (s, 4H, –CH_2–O–CH_2–). LC–MS (m/z): 339 $[M-1]^-$. Anal. Calc. for $C_{19}H_{16}O_6$: C 67.05, H 4.74. Found: C 67.01, H 4.79.

3,5-Bis(4-hydroxy-3-methoxybenzylidene)-tetrahydropyran-4-one (D_3). Yield 82%. mp 226–228 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 9.54 (brs, 2H, –OH), 7.52 (s, 2H, –CH=), 7.07 (s, 2H, ArH), 7.00 (m, 2H, ArH), 6.83 (m, 2H, ArH), 4.98 (s, 4H, –CH_2–O–CH_2–), 3.81 (s, 6H, OCH_3). LC–MS (m/z): 367 $[M-1]^-$. Anal. Calc. for $C_{21}H_{20}S_6$: C 68.47, H 5.47. Found: C 68.45, H 5.49.

3,5-Bis(3,5-ditertbutyl-4-hydroxylbenzylidene)-tetrahydropyran-4-one (D_4). Yield 65%. mp 236–238 °C. ^{1}H NMR (DMSO, 300 MHz) δ (ppm): 7.59 (brs, 2H, –OH), 7.51 (s, 2H, –C=), 7.13 (s, 4H, –ArH), 4.90 (s, 4H, –CH_2–O–CH_2–), 1.49 (s, 36H, C–CH_3). LC–MS (m/z): 531 $[M-1]^-$. Anal. Calc. for $C_{35}H_{48}O_4$: C 78.91, H 9.08. Found: C 78.92, H 9.13.

3,5-Bis(3,4-dimethoxybenzylidene)-tetrahydropyran-4-one (D_5). Yield 62%. mp 134–135 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 7.78 (s, 2H, –CH=CH–), 6.91 (dd, $J = 8.1$, 4H, arom), 6.87 (s, 2H, arom), 4.96 (s, 4H, –CH_2–O–CH_2–), 3.93 (s, 6H, $-OCH_3$), 3.91 (s, 6H, $-OCH_3$). LC–MS (m/z): 625 $[M+1]^+$. Anal. Calc. for $C_{23}H_{24}O_6$: C 69.68, H 6.10. Found: C 69.61, H 6.13.

3,5-Bis(4-hydroxy-3,5-dimethoxybenzylidene)-tetrahydropyran-4-one (D_6). Yield 82%. mp 226–228 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 9.03 (brs, 2H, –OH), 7.58 (s, 2H, –CH=), 6.70 (s, 4H, ArH), 4.95 (s, 4H, –CH_2–O–CH_2–), 3.81 (s, 12H, OCH_3). LC–MS (m/z): 411 $[M-1]^-$. Anal. Calc. for $C_{23}H_{24}O_8$: C 64.48, H 5.65. Found: C 64.43, H 5.68.

3,5-Bis(3-bromo-4-hydroxy-5-methoxybenzylidene)-tetrahydropyran-4-one (D_7). Yield 45%. mp 171–172 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.09 (brs, 2H, –OH), 7.55 (s, 2H, –CH=), 7.16 (s, 2H, ArH), 7.03 (s, 2H, ArH), 4.91 (s, 4H, –CH_2–O–CH_2–), 3.88 (s, 6H, $-OCH_3$). LC–MS (m/z): 525 $[M-1]^-$. Anal. Calc. for $C_{21}H_{18}Br_2O_6$: C 47.94, H 3.45. Found: C 47.87, H 3.49.

3,5-Bis(3,5-dibromo-4-hydroxylbenzylidene)-tetrahydropyran-4-one (D_8). Yield 62%. mp 134–135 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.50 (br, 2H, OH); 7.61 (s, 4H, ArH), 7.50 (s, 2H, CH=CH); 4.86 (s, 4H, –CH_2–O–CH_2–). LC–MS (m/z): 623 $[M-1]^-$. Anal. Calc. for $C_{19}H_{12}Br_4O_4$: C 36.58, H 1.94. Found: C 36.52, H 1.98.

3,5-Bis(2-hydroxybenzylidene)-tetrahydropyran-4-one (D_9). Yield 74%. mp 167–168 °C. ^{1}H NMR (DMSO, 300 MHz) δ (ppm): 10.09 (brs, 2H, –OH), 7.89 (s, 2H, ArH), 7.24 (m, 2H, –ArH), 7.07 (m, 2H, –ArH), 6.91 (m, 2H, –ArH), 6.85 (m, 2H, –ArH), 4.79 (s, 4H, –CH_2–O–CH_2–). LC–MS (m/z): 307 $[M-1]^-$. Anal. Calc. for $C_{19}H_{16}O_4$: C 74.01, H 5.23. Found: C 74.00, H 5.25.

3,5-Bis(2-hydroxy-4-methoxybenzylidene)-tetrahydropyran-4-one (D_{10}). Yield 65%. mp >300 °C. ^{1}H NMR (DMSO, 300 MHz) δ (ppm): 9.89 (brs, 2H, –OH), 7.32 (d, $J = 8.1$ Hz, 2H, ArH), 6.73 (s, 2H, –CH=), 6.61 (d, $J = 8.1$ Hz, 2H, ArH), 6.47 (s, 2H, ArH), 4.38 (s, 4H, $–CH_2–O–CH_2–$), 3.71 (s, 6H, $–OCH_3$). LC–MS (m/z): 367 $[M-1]^-$. Anal. Calc. for $C_{22}H_{20}O_6$: C 68.47, H 5.47. Found: C 68.41, H 5.52.

3,5-Bis(4-hydroxybenzylidene)-tetrahydrothiopyran-4-one (E_1). Yield 95%. mp >300 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 9.95 (brs, 2H, –OH), 7.51 (s, 2H, –CH=), 7.38 (d, $J = 8.1$ Hz, 4H, ArH), 6.83 (d, $J = 8.1$ Hz, 4H, ArH), 3.92 (s, 4H, $–CH_2–S–CH_2–$). LC–MS (m/z): 323 $[M-1]^-$. Anal. Calc. for $C_{19}H_{16}O_3S$: C 70.35, H 4.97. Found: C 70.31, H 5.03.

3,5-Bis(3,4-dihydroxybenzylidene)-tetrahydrothiopyran-4-one (E_2). Yield 90%. mp >300 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 9.43 (brs, 2H, –OH), 9.14 (brs, 2H, –OH), 7.41 (s, 2H, –CH=), 6.92 (s, 2H, ArH), 6.83 (d, $J = 8.1$ Hz, 2H, ArH), 6.80 (d, $J = 8.1$ Hz, 2H, ArH), 3.93 (s, 4H, $–CH_2–S–CH_2–$). LC–MS (m/z): 355 $[M-1]^-$. Anal. Calc. for $C_{19}H_{16}O_5S$: C 64.03, H 4.53. Found: C 64.01, H 4.55.

3,5-Bis(4-hydroxy-3-methoxybenzylidene)-tetrahydrothiopyran-4-one (E_3). Yield 82%. mp 98–100 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 9.64 (brs, 2H, –OH), 7.57 (s, 2H, –CH=), 7.00 (s, 4H, ArH), 6.85 (m, 4H, ArH), 3.89 (s, 4H, $–CH_2–S–CH_2–$), 3.81 (s, 6H, OCH_3). LC–MS(m/z): 383 $[M-1]^-$. Anal. Calc. for $C_{21}H_{20}O_5S$: C 65.61, H 5.24. Found: C 65.60, H 5.26.

3,5-Bis(3,5-ditertbutyl-4-hydroxylbenzylidene)-tetrahydrothiopyran-4-one (E_4). Yield 65%. mp 248–249 °C. ^{1}H NMR (DMSO, 300 MHz) δ (ppm): 7.54 (brs, 2H, –OH), 7.42 (s, 2H, –C=), 7.23 (s, 4H, –ArH), 3.98 (s, 4H, $–CH_2–S–CH_2–$), 1.40 (s, 36H, $C–CH_3$). LC–MS (m/z): 547 $[M-1]^-$. Anal. Calc. for $C_{35}H_{48}O_3S$: C 76.60, H 8.82. Found: C 76.58, H 8.79.

3,5-Bis(3,4-dimethoxybenzylidene)-tetrahydrothiopyran-4-one (E_5). Yield 62%. mp 134–135 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 7.73 (s, 2H, CH=CH), 7.03 (dd, $J = 8.1$, 2H, arom), 6.93 (s, 2H, arom), 6.91 (dd, $J = 8.1$, 2H, arom), 3.96 (s, 4H, $–CH_2–S–CH_2–$), 3.93 (s, 6H, $–OCH_3$), 3.91 (s, 6H, $–OCH_3$). LC–MS (m/z): 413 $[M+1]^+$. Anal. Calc. for $C_{23}H_{24}O_5S$: C 66.97, H 5.86. Found: C 66.86, H 5.90.

3,5-Bis(4-hydroxy-3,5-dimethoxybenzylidene)-tetrahydrothiopyran-4-one (E_6). Yield 82%. 149–151 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 8.93 (brs, 2H, –OH), 7.53 (s, 2H, –CH=), 6.80 (s, 4H, ArH), 4.03 (s, 4H, $–CH_2–S–CH_2–$), 3.80 (s, 12H, $–OCH_3$). LC–MS (m/z): 411 $[M-1]^-$. Anal. Calc. for $C_{23}H_{24}O_7S$: C 62.15, H 5.44. Found: C 62.13, H 5.47.

3,5-Bis(3-bromo-4-hydroxy-5-methoxybenzylidene)-tetrahydrothiopyran-4-one (E_7). Yield 48%. mp 224–225 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.09 (brs, 2H, –OH), 7.48 (s, 2H, –CH=), 7.26 (s, 2H, ArH), 7.11 (s, 2H, ArH), 3.99 (s, 4H, $–CH_2–S–CH_2–$), 3.87 (s, 6H, $–OCH_3$). LC–MS (m/z): 541 $[M-1]^-$. Anal. Calc. for $C_{21}H_{18}Br_2O_5S$: C 46.52, H 3.35. Found: C 46.43, H 3.40.

3,5-Bis(3,5-dibromo-4-hydroxylbenzylidene)-tetrahydrothiopyran-4-one (E_8). Yield 95%. mp 240–242 °C. ^{1}H NMR (DMSO-d6, 300 MHz) δ (ppm): 10.47 (br, 2H, OH); 7.82 (s, 4H, ArH), 7.45 (s, 2H, –CH=C–), 3.92 (s, 4H, $–CH_2–S–CH_2–$). LC–MS (m/z): 639 $[M-1]^-$. Anal. Calc. for $C_{19}H_{12}Br_4O_3S$: C 35.66, H 1.89. Found: C 35.60, H 1.91.

3,5-Bis(2-hydroxybenzylidene)-tetrahydrothiopyran-4-one (E_9). Yield 74%. mp 171–172 °C. ^{1}H NMR (DMSO, 300 MHz) δ (ppm): 9.98 (brs, 2H, –OH), 7.73 (s, 2H, –CH=), 7.27 (d, $J = 8.1$, 2H, –ArH), 7.12 (m, 2H, –ArH), 6.90 (d, $J = 8.1$, 2H, ArH), 6.84 (m, 2H, –ArH), 3.87 (s, 4H, $–CH_2–S–CH_2–$). LC–MS (m/z): 323 $[M-1]^-$. Anal. Calc. for $C_{19}H_{16}O_3S$: C 70.35, H 4.97. Found: C 70.30, H 5.06.

3,5-Bis(2-hydroxy-4-methoxybenzylidene)-tetrahydrothiopyran-4-one (E_{10}). Yield 57%. mp 220–222 °C. ^{1}H NMR (DMSO, 300 MHz) δ (ppm): 9.85 (brs, 2H, –OH), 7.26 (d, $J = 8.1$ Hz, 2H, ArH), 6.98 (s, 2H, –CH=), 6.64 (d, $J = 8.1$ Hz, 2H, ArH), 6.47 (s, 2H, ArH), 3.71 (s, 6H, $–OCH_3$), 3.58 (s, 4H, $–CH_2–S–CH_2–$). LC–MS (m/z): 383 $[M-1]^-$. Anal. Calc. for $C_{21}H_{20}O_5S$: C 65.61, H 5.24. Found: C 65.55, H 5.30.

Tyrosinase inhibition assay. A spectrophotometric assay for tyrosinase was performed according to a method reported[36] by Yi W *et al.* with little modification. Measurement was performed in triplicate for each concentration. IC_{50} values were determined by interpolation of the dose-response curves.The inhibition types of the inhibitors were determined by Lineweaver–Burk plots and a replot of enzyme activity *versus* the reciprocal of the substrate L-DOPA concentration.

DPPH radical scavenging assay. The Free radical scavenging activities of curcum analogs were assayed using A stable DPPH, following standard method[37] with little modification. Briefly, 1 mL of 0.1 mM DPPH radical solution was mixed with 3 mL of various concentrations of curcumin analogs or vitamin C dissolved in methanol. The mixture was vortexed vigorously and left for 30 min at 40 °C in the dark. For baseline control, 3 mL of methanol was used. The absorbance was measured at 517 nm.

Acute toxicity study in mice. The acute toxicity of **C_2** and **B_{11}** was examined according to the OECD 423 Guideline for Testing of Chemicals of Acute Oral Toxicity–Acute Toxic Class Method (OECD, 2001).[38] Since the available information suggested that curcumin analogs have low toxicity potential and that mortality was unlikely, the test procedure was applied with a starting dose of 2,000 mg/kg of body mass. Male and female Chinese Kun Ming (KM) mice (n = 12, six males and six females, 7 weeks old, 18–22 g), obtained from Center for Animal Tests of Sun Yat-sen University, China, were housed in the university-approved animal facility in rooms maintained at 22 ± 2 °C, at 55–60% humidity under a 12-h photoperiod. After 5 d of adaptation to laboratory conditions, the mice were divided into four equal groups (controls, male and female, and treated, male and female, three animals in each group, equal body masses) and given by gavage either a single dose of 2,000 mg of sample (dispersed in 3% Tween 80 aqueous solution)/kg of body mass and an equal volume of distilled water containing 3% Tween 80 for the control groups, using a suitably graduated syringe and a stainless steel intubation cannula. The mice had free access to distilled water and a commercial standard diet. They were observed individually at least once during the first 30 min after dosing, and periodically during the first 24 h, with special attention during the first 4 h, and daily thereafter, for a total of 14 d. All observations were systematically recorded and records were maintained for each animal. The individual body masses of animals were determined shortly before the test substance was administered and weekly thereafter. All the animals were sacrificed at the end of the observation period and subjected to necropsy.

Molecular modeling. To date, all attempts to determine experimentally the X-ray structure of human tyrosinase have failed. To overcome this problem, a homology model was made with the crystal structure of a bacterium tyrosinase taken from *Streptomyces castaneoglobisporus* as template (PDB accession code 2ZWE). The crystal structure of tyrosinase 2ZWE was a complex with caddie protein ORF378 which bound in the active site of tyrosinase, and hence the ORF378 was removed according to the method reported by Matoba Y *et al.* [39] For a docking study, all water molecules were removed, AMBER charges were assigned, the orientations of the side chain amides were corrected, and hydrogen atmos were added and their positions were optimized by energy minimization using the AMBER7 FF99 force field. Compounds C2 and C11 were selected and built using Sybyl 8.0 (Tripos, St. Louis, MO). After sketching of the molecules, Gasteiger-Hückel partial charges were automatically assigned. Energy evaluations were made using the Triopos force-filed. Geometry optimizations were performed using 20 iterations of simplex followed by 500 steps of steepest descent protocol and then 2,000 steps of Powell algorithm minimization. The molecular energies of all compounds always converged within a gradient displacement criterion of 0.001 kcal/Å^2. Docking calculations were performed with Surflex-Dock on the Red Hat workstation. "Protomol" of Surflex-Dock was used to guide molecular docking. The protomol was defined by setting

the threshold value and the bloat value at 0.66 and 2 Å respectively. The binding pocket of tyrosinase has been defined with the residues surrounding the dicopper ions, since they are implied in the recognition of the L-DOPA substrate.[39] The values of additional starting conformations per molecule and maximum number of poses per ligand were both expanded to 40 to increase the accuracy of the binding mode. Other parameters were based on the software default setting. Then the ligands were docked into the active side of tyrosinase under the same conditions.

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