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Tyrosinase inhibitory activities of cinnamic acid analogues

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The aim of this study was to show how tyrosinase inhibitory activity is correlated with the structure of cinnamic acid derivatives. We synthesized cinnamic acid derivatives, and investigated their tyrosinase inhibitory and DPPH radical scavenging activities. The results show that reduction of C=C double bonds and the substituent group of cinnamic acid derivatives have an effect on antioxidant activity and tyrosinase inhibitory activity. Among these compounds, compounds **2**, **6** and **6a** showed a potent tyrosinase inhibitory activity with IC_{50} (50% inhibitory concentration) values of 115.6 μM, 114.9 μM and 195.7 μM, respectively. The results obtained provide a useful clue for the design and development of new tyrosinase inhibitors.

1. Introduction

The tyrosinases are responsible not only for melanization in animals but also for browning in plants. Browning is caused by the oxidation of phenolic compounds containing two *o*-dihydroxy groups to the corresponding *o*-quinone. This reaction produces undesirable changes in color, flavour and nutritive value of the products. The prevention and control of enzymatic browning has been reported (Labuza et al. 1992). In addition, tyrosinase inhibitors have become increasingly important in cosmetic products in relation to hyperpigmentation (Maeda and Fukuda 1991). Many tyrosinase inhibitors find applications in cosmetic products as whitening agents and for depigmentation after sunburn (Lee 2002). Therefore, there is a concerted effort to search for naturally occurring tyrosinase inhibitors from plants, because plants constitute a rich source of bioactive chemicals and many of them are largely free from harmful adverse effects (Kubo et al. 1995). It is well known that tyrosinase can be inhibited by aromatic aldehydes (Jimenez et al. 2001) and aromatic acids (Valero et al. 1991). For example, benzoic acid, 4-substituted benzaldehydes and cuminaldehyde have been identified as potent tyrosinase inhibitors (Liu et al. 2003; Kubu and Kinst Hori 1998). In particular, naturally occurring hydroxycinnamic acid derivatives, such as caffeic acid, ferulic acid and sinapic acid, appear to be tyrosinase inhibitors (Kim et al. 2005; Matsuda et al. 1995) which is attributed to their structural resemblance to and L-DOPA and L-tyrosine, natural tyrosinase compounds (Briganti et al. 2003). Furthermore, it has been observed that *para*-hydroxycinnamic acid inhibited both monophenolase and diphenolase activities and the polar hydroxy group at the *para* position increased monophenolase inhibitory activity, whereas, it decreased diphenolase inhibitory activity (Lee 2002).

These cinnamic acid derivatives have been considered as attractive potential antioxidants due to their natural origin (Silva et al. 2000; Teixeria et al. 2005; Siquet et al. 2006). Also, these compounds are well known to have a variety of biological activities, such as anticancer (Zhang and Ji 1992), antihepatoxic (Peter Alvarez et al. 1996) and antibacterial activities (Ramos Nino et al. 1996). In particular, several hydroxycinnamic acid derivatives have been found to possess strong antioxidant activities as radical scavengers, their antioxidant activity being strongly related to their structural features and the presence of a hydroxyl function in the aromatic structure (Bran Williams et al. 1995). However, strucure-activity relationship (SAR) studies of the tyrosinase inhibitory and radical scavenging activities of cinnamic acid derivatives (Table 1) have not been reported in detail. The aim of this study is to show the tyrosinase inhibitory activities and structure-activity relationships (SAR) of cinnamic acid derivatives.

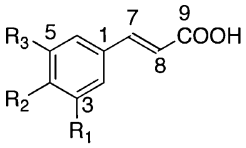
2. Investigations, results and discussion

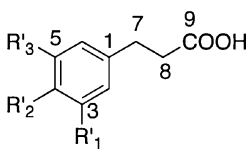
2.1. Antioxidant activities of cinnamic acid derivatives 1-8 and dihydrocinnamic acid derivatives 1a-8a

The antioxidant activities of compounds **1–8** were evaluated by EC_{50} (50% effective concentration) in DPPH radical scavenging as shown in Fig. 1. The results indicated that an OH group at the 4-position of cinnamic acid derivatives (caffeic acid, ferulic acid and sinapic acid) effects DPPH radical scavenging activity, but 4-hydroxycinnamic acid (*p*-coumaric acid) exhibited weak radical scavenging capacity in the DPPH assay. The radical-scavenging activities were higher when a catechol group was present, but when the *meta*-hydroxy function was substituted by a methoxy group (ferulic acid), activity was weakened; furthermore, the introduction of another methoxy group in an *ortho*-position to a hydroxy group (sinapic acid) led to an increase in antioxidant activity relative to ferulic acid.

The antioxidative activity of polyphenols is generally ascribed to their hydroxyl groups. The antioxidative efficiency of monophenols is increased substantially by one or two electron donor substitutions at the *ortho* position to the hydroxyl (Cuvelier et al. 1992; Terao et al. 1993). In other words, the presence of electron-donating groups attached to the aromatic ring such as $-CH_3$, -OMe and -OH ought to increase the ease of hydrogen atom abstraction and, consequently, increase the antiradical performance. Furthermore, the presence of a second hydroxyl group in the *ortho* or *para* position is known to increase the

Table 1: The structure of cinnamic acid derivatives (1-8) and dihydrocinnamic acid derivatives (1a-8a)





compound	R_1	R_2	R_3	compound	R'_1	R'_2	R'_3
Cinnamic acid (**1**)	H	H	H	Dihydro cinnamic acid (**1a**)	H	H	H
p-Coumaric acid (**2**)	H	OH	H	Dihydro-*p*-cinnamic acid (**2a**)	H	OH	H
p-Methoxy cinnamic acid (**3**)	H	OMe	H	Dihydro-*p*-methoxy cinnamic acid (**3a**)	H	OMe	H
Caffeic acid (4)	OH	OH	H	Dihydro caffeic acid (**4a**)	OH	OH	H
Ferulic acid (**5**)	OMe	OMe	H	Dihydro ferulic acid (**5a**)	OMe	OH	H
Isoferulic acid (**6**)	OH	OMe	H	Dihydro isoferulic acid (**6a**)	OH	OMe	H
3,4 Dimethoxy-cinnamic acid (**7**)	OMe	OMe	H	3,4 Dimethoxy-dihydro cinnamic acid (**7a**)	OMe	OMe	H
Sinapic acid (**8**)	OMe	OH	OMe	Dihydro sinapic acid (**8a**)	OMe	OH	OMe

formation of antioxidative *o*-quinone or *p*-quinone (Bran Williams et al. 1995; Graf 1992).

Next, the results for dihydro cinnamic acid derivatives (**1a–8a**) are shown in Fig. 2. Dihydro sinapic acid (**8a**) was the most potent compound having a greater effect on radicals than the other compounds. The results show that the reduction of the double bond of cinnamic acid derivatives improves antioxidative activity (caffeic acid (**4**), ferulic acid (**5**), isoferulic acid (**6**) and sinapic acid (**8**)). The results are consistent with those of Silva et al. (2001). Dihydrocinnamic derivatives have side bonds, which allow the phenyl group to have a certain flexibility to rotate. Furthermore, Guyton et al. (1991) have reported that the antioxidative mechanism for BHT (2,6-di-*tert*-butyl-4-methyl-phenol) is that BHT can be transformed to the quinone methide by two-electron oxidation because it is possible for an aryl proton at the 4-methyl group to be abstracted by a free radical. Therefore, dihydrocinnamic derivatives would show more potent radical scavenging activity than cinnamic acid derivatives. EC_{50} values for these compounds are summarized in Table 2.

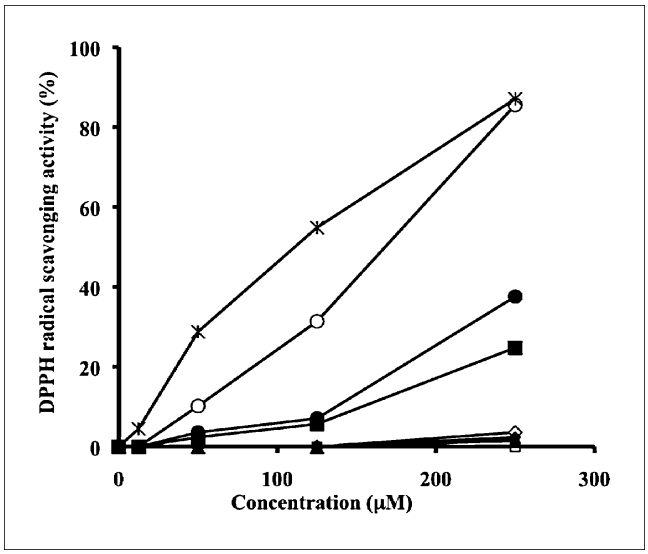


Fig. 1: DPPH radical scavenging activities of cinnamic acid derivatives (**1**-**8**), Cinnamic acid (1), *p*-Coumaric acid (2), *p*-Methoxy cinnamic acid (3), Caffeic acid (4), Ferulic acid (5), Isoferulic acid (6), 3,4-Dimethoxy cinnamic acid (7), Sinapic acid (8), BHT

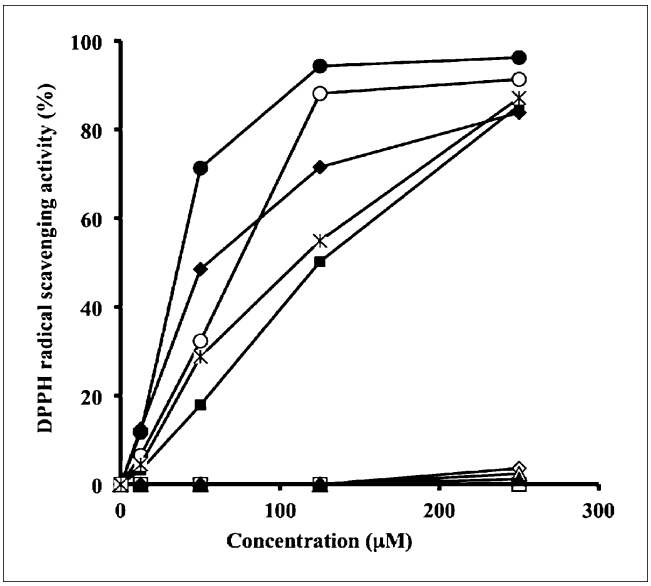


Fig. 2: DPPH radical scavenging activities of dihydro cinnamic acid derivatives (**1a**-**8a**), Dihydro cinnamic acid (1a), Dihydro-*p*-coumaric acid (2a), Dihydro-*p*-methoxy cinnamic acid (3a), Dihydro caffeic acid (4a), Dihydro ferulic acid (5a), Dihydro isoferulic acid (6a), 3,4-Dimethoxy-dihydro cinnamic acid (7a), Dihydro sinapic acid (8a), BHT

2.2. Inhibitory activity on tyrosinase of cinnamic acid derivatives 1-8 and dihydrocinnamic acid derivatives 1a-8a

Considering cinnamic acid derivatives (**1–8**) and dihydrocinnamic acid derivatives (**1a–8a**), as inhibitors, we studied their effects on the activity of mushroom tyrosinase in the oxidation of L-tyrosine. The results are shown in Fig. 3 and Fig. 4.

Compounds **2** and **6** had inhibitory effects on the activity of the enzyme, compounds **1** and **3** inhibited weakly, and compounds **4, 5, 7** and **8** had no inhibitory activity. Furthermore, among the dihydrocinnamic acid derivatives, compounds **2a** and **3a** showed weak inhibitory activity, whereas compound **6a** exhibited strong inhibition of tyrosinase (Fig. 4). The inhibitory activity of compound **6a** has not been reported to date, but this study showed for the first time that compound **6a** had an inhibitory effect on tyrosinase. The IC_{50} values of compounds **2**, **3**, **6** and **6a** were 115.6 μM, 861.7 μM, 114.9 μM and 195.7 μM, respectively. The conjugated double bond of cinnamic acid derivatives

Table 2: EC_{50} of compounds 1-8a on DPPH radical scavenging activity

compound	EC_{50} (μM)	compound	EC_{50} (μM)
1	>250	**1a**	>250
2	>250	**2a**	>250
3	>250	**3a**	>250
4	155.3	**4a**	64.6
5	>250	**5a**	124.4
6	>250	**6a**	52.9
7	>250	**7a**	>250
8	>250	**8a**	32.1
BHT*	104.8		

* reference sample

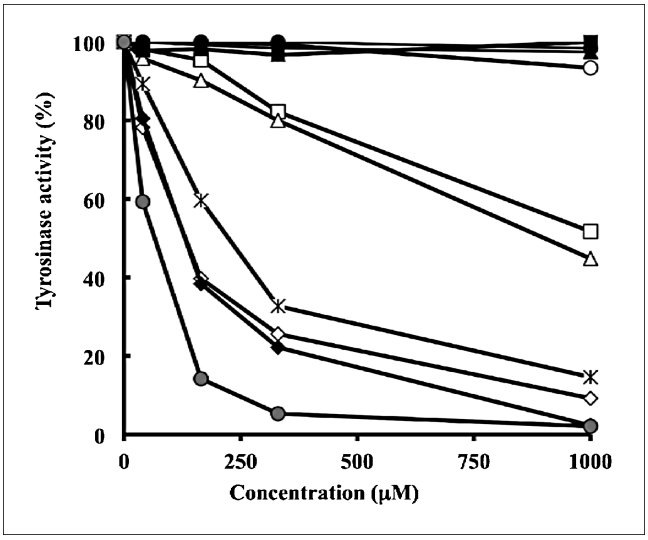


Fig. 3: Inhibitory effects of cinnamic acid derivatives **1**-**8** against tyrosinase activity, Cinnamic acid (1), *p*-Coumaric acid (2a), *p*-Methoxy cinnamic acid (3) Caffeic acid (4) Ferulic acid (5), Isoferulic acid (6), 3,4-Dimethoxy cinnamic acid (7), Sinapic acid (8), Arbutin, Kojic acid

was found to be essential, dihydro *p*-coumaric acid (**2a**), the single bond derivative of **2**, especially, showing almost no activity. This double-bond moiety would cause a steric interaction with the tertiary structure of the active site of tyrosinase when the carboxylic acid part is coordinated to the active site, therefore, the structural restriction observed around the double bond would be required to increase the inhibitory activity of cinnamic acid derivatives. Furthermore, cinnamic acid derivatives which have a hydroxyl group or a methoxy group at the 4-position, inhibit tyrosinase activity. This is consistent with the general observation that methoxylated and hydroxylated cinnamic acid derivatives serve as inhibitors of tyrosinase (Billaud et al. 1996). Thus, methoxylation and hydroxylation of cinnamic acid derivatives may play an important role in determining the tyrosinase inhibitory activity. In the case of *p*-coumaric acid, a methoxy or hydroxyl group at the 3-position decreases tyrosinase inhibitory activity, while in the case of *p*-methoxy cinnamic acid, the hydroxyl group at the 3-position increases inhibitory activity, and to the contrary, methoxylation at the 3-position decreases inhibitory activity. The IC_{50} values of compounds **1-8** and **1a-8a** are summarized in Table 3. In addition**,** the inhibitory mechanism of compounds **2, 3, 6** and **6a** on mushroom tyrosinase, during the oxidation of L-tyrosine, was analyzed by a Lineweaver-Burk plot as shown in Fig. 5. The three or four lines representing the uninhibited enzyme and different concentrations of *p*-coumaric acid, *p*-methoxy cinnamic acid, isoferulic acid and dihydro isoferulic acid intersected on the horizontal axis. These results indicate that these compounds exhibit non-competitive inhibition of L-tyrosine oxidation by mushroom tyrosinase. This observed behavior indicates that the inhibitor can bind not only with free enzyme, but also with the enzyme-substrate complex. The reaction catalyzed by tyrosinase is the oxidation of L-tyrosine to dopaquinone which occurs through two steps; hydroxylation of L-tyrosine to L-DOPA, then oxidation of the latter to dopaquinone, and it is observed that

Table 3: Inhibitory effects of compound 1-8a on mushroom tyrosinase

compound	concentration (μM)	inhibition (%)	IC_{50} (μM)	compound	concentration (μM)	inhibition (%)	IC_{50} (μM)
1	165	4.6		**1a**	165	6.6	
	330	17.8			330	14.4	
	1000	49.3	>1000		1000	40.5	>1000
2	40	21.8		**2a**	165	4.8	
	165	60.3			330	6.1	
	330	74.4	115.6		1000	19.6	>1000
3	165	9.8		**3a**	165	19.2	
	330	20.0			330	28.3	
	1000	55.2	861.7		1000	46.4	>1000
4	330	0.6	>1000	**4a**	330	1.6	
	1000	6.6			1000	2.7	>1000
5	330	3.3		**5a**	330	15.8	
	1000	0			1000	17.9	>1000
6	40	19.5	>1000	**6a**	40	27.5	
	165	61.6			165	46.5	
	330	77.8	114.9		330	60.6	195.7
7	165	0.8		**7a**	165	14.6	
	330	1.6			330	19.2	
	1000	2.5	>1000		1000	20.2	>1000
8	330	0		**8a**	330	11.6	
	1000	1.6	>1000		1000	22.6	>1000
arbutin*			210.5	kojic acid*			51.6

* reference sample

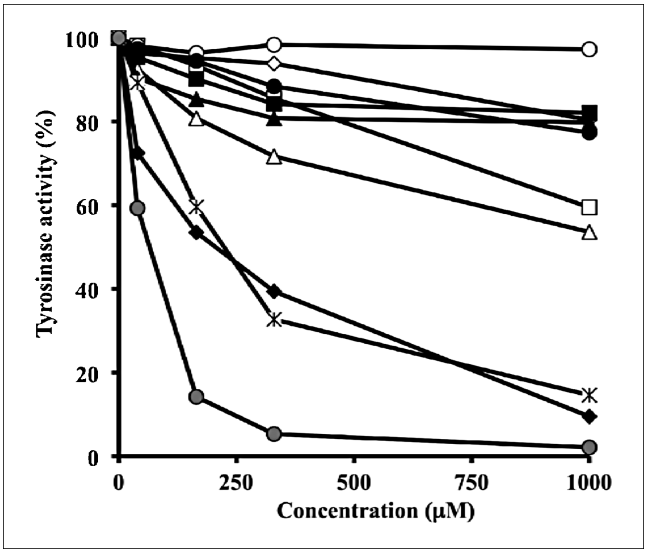


Fig. 4: Inhibitory effects of dihydro cinnamic acid derivatives **1a**-**8a** against tyrosinase activity, Dihydro cinnamic acid (1a), Dihydro-*p*-coumaric acid (2a), Dihydro-*p*-methoxy cinnamic acid (3a) Dihydro caffeic acid (4a), Dihydro ferulic acid (5a), Dihydro isoferulic acid (6a), 3,4-Dimethoxy-dihydro cinnamic acid (7a), Dihydro sinapic acid (8a), Arbutin, Kojic acid

antioxidants may inhibit the oxidation step, without interacting with tyrosinase. Compounds **2** and **6** showed the highest tyrosinase inhibitory activity with IC_{50} values of 115.6 μM and 114.9 μM. but these compounds showed almost no free radical scavenging activity. The inhibition data combined with the free radical scavenging activity indicated that two activities are not directly correlated. However, the substituent groups of cinnamic acid, and C=C double bond of cinnamic acid derivatives effect both tyrosinase inhibitory activity and radical scavenging activity. The results obtained have provided a useful clue to the design and development of new tyrosinase inhibitors.

3. Experimental

3.1. Materials

Thin layer chromatography (TLC) was performed on precoated plates (silica gel 60 F_{254}, 0.25 mm, Merck, Darmstadt, Germany). Column chromatography was carried out using 70 – 230 mesh silica gel (Kieselgel 60, Merck, Germany). Melting points (m.p.) were measured on a MP-5000D melting-point apparatus. UV spectra were measured on a Hitachi U-1500 spectrophotometer. ^{1}H and ^{13}C NMR data were all obtained on a JEOL ECA-400 (400 MHz) in $CDCl_3$ and DMSO-d_6 with TMS as internal standard. Tyrosinase (E.C. 1.14.18.1) was purchased from Sigma-Aldrich (St. Louis, Mo), L-tyrosine, cinnamic acid, *p*-coumaric acid, ferulic acid, caffeic acid, sinapic acid, *p*-methoxy-cinnamic acid, 3,4-dimethoxy cinnamic acid, CuCl, $NaBH_4$, DPPH (1,1-diphenyl-2-picrylhydrazyl), BHT(2,6-di-*tert*-butyl-4-methyl- phenol), and *p*-toluenesulfonic acid monohydrate were purchased from Wako Pure Chemistry (Osaka, Japan), arbutin, kojic acid and palladium 10% on carbon (wetted with ca. 55% water) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and isoferulic acid and all solvents were purchased from Kanto Chemical (Tokyo, Japan).

3.2. Synthesis of dihydro cinnamic acid derivatives 1a - 8a

3.2.1. Synthesis of dihydro cinnamic acid derivatives 1a, 3a – 8a

Compounds **1**, **3–8** (2 mmol) were dissolved in 8 ml of methanol, palladium on carbon (10%, wet, 200 mg) was added and the mixture was stirred under H_2 at atmospheric pressure at room temperature for 2 h. The catalyst was filtered through a bed of Celite® and solvent was evaporated under reduced pressure to yield 78.7%-98.0%.

*Dihydro cinnamic acid (***1a***)***:** White crystals; Yield 95.0%; m.p. 46–49 °C. IR ν_{max} (KBr): 3029, 1698, 1454, 754 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.19 – 7.31 (5H, m, aromatic), 2.96 (2H, t, 8.0 Hz, H-7), 2.68 (2H, t, 8.0 Hz, H-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ 30.6 (C-7), 35.6 (C-8), 126.3 (C-4), 128.2 (C-3 and C-5), 128.5 (C-2 and C-6), 140.1 (C-1), 179.2 (C-9).

*Dihydo p-methoxy cinnamic acid (***3a***)*: Light yellow powder; Yield 95.4%; m.p. 96–98 °C. IR ν_{max} (KBr): 2931, 1701, 1613, 1513, 821 cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.12 (2H, d, 8.4 Hz, H-2 and H-6), 6.83 (2H, d, 8.4 Hz, H-3 and H-5), 3.78 (3H, s, 4-OMe), 2.89 (2H, t, 8.0 Hz, H-7), 2.64 (2H, t, 8.0 Hz, H-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.7 (C-7), 35.9 (C-8), 55.2(4-OMe), 113.9 (C-3 and C-5), 129.2 (C-2 and C-6), 132.2(C-1), 158.1 (C-4), 178.9 (C-9).

*Dihydro caffeic acid (***4a***)*: Light yellow crystals; Yield 98.0%; m.p. 130–131 °C. IR ν_{max} (KBr) 3367, 1681, 1604, 1526, 820 cm^{-1}. ^{1}H NMR (400 MHz, DMSO) δ 6.62 (1H, d, 8.0 Hz, H-5), 6.59 (1H, d, 2.0 Hz, H-2), 6.44 (1H, dd, 2.0 Hz, 8.0 Hz, H-6), 2.64 (2H, t, 7.2 Hz, H-7), 2.42 (2H, t, 7.2 Hz, H-8); ^{13}C NMR (100 MHz, DMSO) δ 29.8 (C-7), 35.8 (C-8), 115.5 (C-5), 115.7 (C-2), 118.8 (C-6), 131.7 (C-1), 143.4 (C-3), 145.0 (C-4), 174.0 (C-9).

*Dihydro ferulic acid (***5a***)***:** Light beige crystals; Yield 98.0%; m.p. 86–87 °C. IR ν_{max} (KBr): 3423, 2938, 1702, 1605, 819 cm^{-1}. ^{1}H NMR (400 MHz, DMSO) δ 6.84 (1H, d, 7.2 Hz, H-5), 6.71 (1H, d, 2.0 Hz, H-2), 6.70 (1H, dd, 2.0 Hz, 7.2 Hz H-6), 3.87 (3H, s, 3-OMe), 2.89 (2H, t, 8.0 Hz, H-7), 2.65 (2H, t, 8.0 Hz, H-8); ^{13}C NMR (100 MHz, DMSO) δ 30.1 (C-7), 35.8 (C-8), 55.5 (3-OMe), 112.5 (C-2), 115.3 (C-5), 120.3 (C-6), 131.7 (C-1), 144.7 (C-3), 147.4 (C-4), 174.0 (C-9).

*Dihydro isoferulic acid (***6a***)***:** White crystals; Yield 94.6%; m.p. 142–143 °C. IR ν_{max} (KBr): 3396, 2939, 1702, 1590, 1518, 808 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.79 (1H, d, 1.6 Hz, H-2), 6.77 (1H, d, 8.4 Hz, H-5), 6.68 (1H, dd, 8.4 Hz, 1.6 Hz, H-6), 3.86 (3H, s, 4-OMe), 2.87 (2H, t, 7.6 Hz, H-7), 2.64 (2H, t, 7.6 Hz, H-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ 30.0 (C-7), 35.6 (C-8), 56.0 (4-OMe), 110.7 (C-5), 114.5 (C-2), 119.6 (C-6), 133.5 (C-1), 145.1 (C-3), 145.6 (C-4), 178.2 (C-9).

*3,4-Dimethoxy dihydro cinnamic acid (***7a***)***:** Light beige crystals; Yield 78.7%; m.p. 89–90 °C. IR ν_{max} (KBr): 2934, 1699, 1591, 1517, 841 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.80 (1H, d, 8.8 Hz, H-5), 6.75 (1H, dd, 8.8 Hz, 2.0 Hz, H-6), 6.74 (1H, d, 2.0 Hz H-2), 3.86 (3H, s, 4-OMe), 3.87 (3H, s, 3-OMe), 2.91 (2H, t, 8.0 Hz, H-7), 2.67 (2H, t, 8.0 Hz, H-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ 30.2 (C-7), 35.8 (C-8), 55.8 (4-OMe), 55.9 (3-OMe) 111.3 (C-2), 111.7 (C-5), 120.0 (C-6), 132.8 (C-1), 147.5 (C-4), 148.9 (C-3), 178.2 (C-9).

*Dihydro sinapic acid (***8a***)***:** Beige crystals; Yield 93.2%; m.p. 97–98 °C. IR ν_{max} (KBr): 3457, 2948, 1703, 1621, 804 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.43 (2H, s, H-2, 6), 3.85 (6H, s, 3-OMe and 5-OMe), 2.88 (2H, t, 8.0 Hz, H-7), 2.65 (2H, t, 8.0 Hz, H-8)^{13}C-NMR (100 MHz, $CDCl_3$) δ 30.8 (C-7), 36.0 (C-8), 56.2 (3-OMe and 5-OMe), 104.9 (C-2,6), 131.3 (C-1), 133.1 (C-4), 147.0 (C-3,5), 178.9 (C-9).

*3.2.2. Synthesis of dihydro p-coumaric acid (***2a***)*

Compound **2** (200 mg, 1.2 mmol) was dissolved in 4 ml of methanol and then *p*-toluene sulfonic acid monohydrate (8.0 mg, 0.05 mmol) was added and the mixture was refluxed for 2 h. After addition of H_2O, the mixture was made neutral by adding prydine and extracted with EtOAc. The extract was washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure to leave white crystals, which were column-chromatographed on SiO_2 with hexane – EtOAc (6:1, v/v) to give *p*-methyl coumarate (172 mg, 79.2%). To a stirred solution of *p*-methyl coumarate (142 mg, 0.8 mmol) and CuCl (238 mg, 2.4 mmol) in methanol (15 ml) was added $NaBH_4$ (304 mg, 8 mmol) in small portions over a period of 30 min at 0 °C (Narisada et al. 1989). Then the resulting black residue was removed by filtration, and the filtrate was acidified with 5% aqueous HCl and extracted with EtOAc. The extract was washed successively with saturated aqueous $NaHCO_3$ and brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure, to give white crystals. The white crystals were identified as dihydro *p*-methyl coumarate (134 mg, 93.3%) by NMR data. The base hydrolysis of dihydro *p*-methyl coumarate gave compound **2a** (87 mg, 70.2%).

*Dihydro p-coumaric acid (***2a***)***:** White crystals; Yield 51.9%; m.p. 118–120 °C. IR ν_{max} (KBr): 3393, 3026, 1703, 1599, 828 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.07 (2H, d, 8.0 Hz, H-2 and H-6), 6.76 (2H, d, 8.0 Hz, H-3 and H-5), 2.89 (2H, t, 8.0 Hz, H-7), 2.64 (2H, t, 8.0 Hz, H-8); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.8 (C-7), 35.5 (C-8), 115.4 (C-3 and C-5), 129.4 (C-2 and C-6), 131.1 (C-1), 155.6 (C-4), 179.9 (C-9).

3.3. Methods

3.3.1. DPPH radical scavenging activity

The scavenging activity of cinnamic acid derivatives for the DPPH radical was monitored according to the method of Gaspar et al. (2009). A quantity of 500 μL of a 0.5 mM methanoic DPPH solution was mixed in a cuvette with 500 μL of cinnamic acid derivatives at different concentration levels. These cuvettes were shaken vigorously. The cuvettes were allowed to stand at 27 °C for 30 min, and the absorbance was measured at 517 nm using a U-1500 spectrophotometer. The percentage of radical scavenging activity was

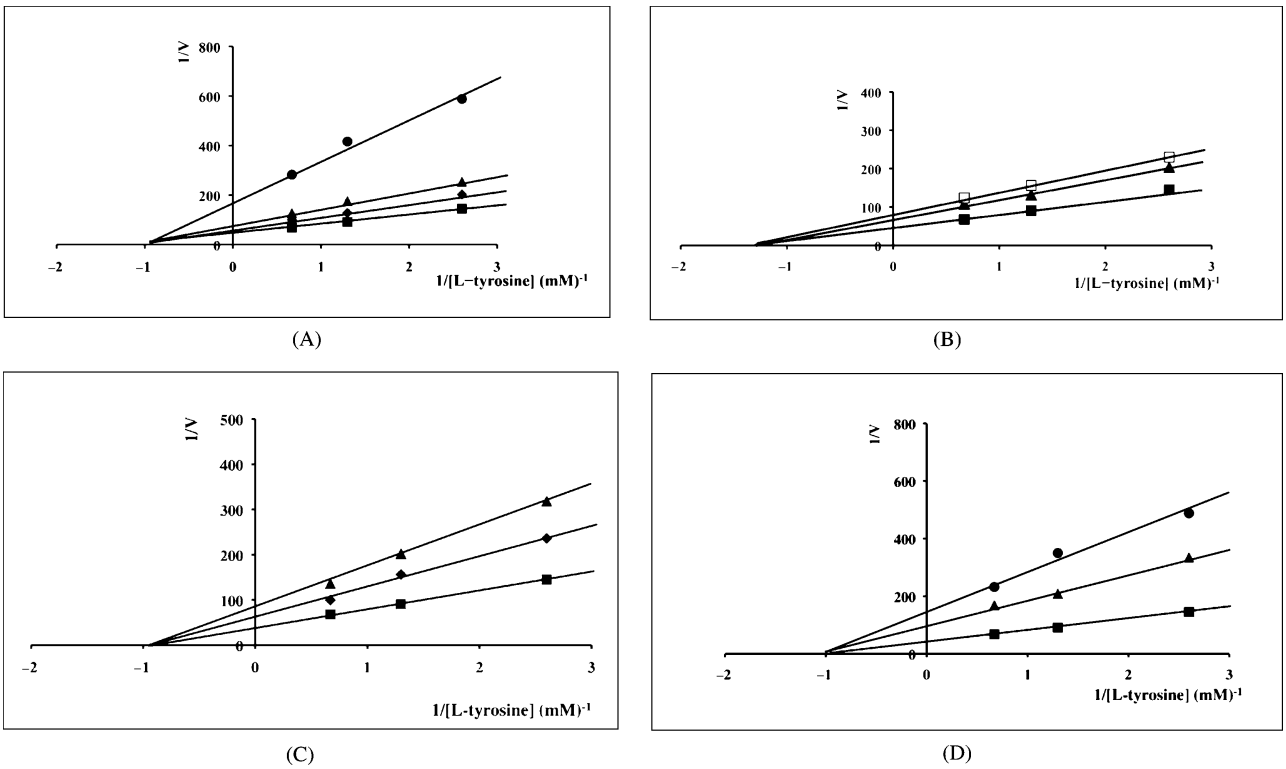


Fig. 5: Lineweaver-Burk Plots of mushroom Tyrosinase and L-Tyrosine; (A) *p*-Coumaric acid: (■) 0 μM, (♦) 40 μM, (▲) 160 μM, (●) 330 μM, (B) *p*-Methoxy cinnamic acid: (■) 0 μM, (▲) 160 μM, (□) 1000 μM (C) Isoferulic acid: (■) 0 μM, (♦) 40 μM, (▲) 160 μM (D) Dihydoisoferulic acid (■) 0 μM, (▲) 160 μM, (●) 330 μM. 1/V: 1/Δ492 nm/min

calculated using the equation:Radical scavenging activity (%) = (control OD – sample OD/control OD) × 100
All tests were performed in triplicate. BHT was used as a reference standard for the investigation of radical scavenging activity.

3.3.2. Tyrosinase assay

The tyrosinase assay was performed by the method of Baek et al. (2008) with slight modifications, using L-tyrosine as the substrate. 140 μL of 0.1 M phosphate buffer (pH 7.0), 36 μL of 1.5 mM L-tyrosine and 13 μL of sample solution were added to each well of a 96-well plate and then incubated at 37 °C for 10 min. Then 16 μL of mushroom tyrosinase (500 unit/ml, 0.1 M phosphate buffer at pH 7.0) was added, and the assay mixture was incubated at 37 °C for 20 min. Before and after incubation, the amount of dopachrome produced in the reaction mixture was measured at 492 nm in a microplate reader (Corona Electric Co., Ltd). Arbutin and kojic acid were used as a positive control. The extent of tyrosinase inhibition by the different compounds added was calculated and expressed as the percentage necessary for 50% inhibition concentration (IC_{50}).
The percentage of tyrosinase activity was calculated as follows:
Tyrosinase activity (%) = [(*C*-*D*)/(*A*-*B*)] × 100, where *A* is the absorbance at 492 nm without test sample, *B* is the absorbance at 492 nm without test sample and substrate, *C* is the absorbance at 492 nm with test sample, *D* is the absorbance at 492 nm with test sample, but without substrate.

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