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Tyrosinase Inhibitory Activities of Safrole from *Myristica fragrans* Houtt.

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Abstract Five phenylpropanoids (**1-5**), a benzofuran neolignan (**6**), two 8-*O*-4'-neolignans (**7-8**), and five tetrahydrofuran lignans (**9-13**) were isolated from a methanol extract of *Myristica fragrans* seeds. The structures of **1-13** were determined by ^{1}H- and ^{13}C-NMR spectroscopic data analyses and a comparison with the literature data. Compound **3** was isolated for the first time from this plant. All the isolated compounds were evaluated for their inhibitory activity against tyrosinase. Among them, safrole (**1**) showed significant inhibitions against both the monophenolase (IC_{50}=32.11 μM) and diphenolase (IC_{50}=27.32 μM) activities of tyrosinase. The kinetic analysis shows that safrole (**1**) is competitive inhibitors for both monophenolase and diphenolase. The apparent inhibition constant (K_i) for safrole (**1**) binding with free enzyme was determined to be 16.05 and 13.66 μM for monophenolase and diphenolase, respectively.

Keywords 8-*O*-4'-neolignans · benzofuran neolignan · *Myristica fragrans* · phenylpropanoids · tetrahydrofuran lignans

Introduction

Myristica fragrans Houtt. (Myristicaceae) is an aromatic evergreen tree cultivated in South Africa, India, and other tropical countries. The nutmeg refers to the dried kernels of this plant. It was imported into Europe at the 12th century and, has been used indigenously as a spice in many Western foods. *M. fragrans* is also prescribed for medicinal purposes in Asia to treat many diseases such as rheumatism, muscle spasm, decreased appetite, and diarrhea (Nguyen et al., 2010). It contains 25–30% fixed oils, 5–15% volatile oils, phenylpropanoids, lignans, and neolignans (Davis and Graham, 1982; Hattori et al., 1987; Hada et al., 1988) and has antitumor, antimicrobial, antiviral, antifungal, anti-atherosclerotic and anti-inflammatory properties (Orabi et al., 1991; Yang et al., 2006; Cho et al., 2007). Previously, novel dibenzylbutane lignan, 7-methyl ether dibenzylbutane lignan, and six known lignans, were characterized as LDL antioxidant agents from the seed of *M. fragrans* (Kwon et al., 2008).

Tyrosinase is a copper-containing enzyme, widely distributed in microorganisms, animals and plants and is a key enzyme in melanin biosynthesis. Melanin plays a crucial protective role against skin photo carcinogenesis. However, the production of abnormal melanin pigmentation is a serious esthetic problem in human beings (Sabudak et al., 2013). Furthermore, tyrosinase inhibitors may be clinically used for the treatment of some skin disorders associated with melanin hyperpigmentation and are also important in cosmetics for skin whitening effects (Maeda and Fukuda, 1991; Seiberg et al., 2000). In recent years, tyrosinase inhibitors have attracted concern owing to the hyperpigmentation (Friedman, 1996), resulting from the increased use of tyrosinase enzyme in medicinal and cosmetic products (Maeda and Fukuda, 1991), and their identification and isolation from natural sources have been also increased (Son et al., 2000). Natural tyrosinase inhibitors are generally considered to be free of harmful side effects and can be produced at reasonable low costs. Therefore, the development and utilization of more effective tyrosinase inhibitors of natural origin are desired.

In our continuous search for new tyrosinase inhibitors from *M. fragrans*, the MeOH extracts were subsequently partitioned and isolated. As a result, five phenylpropanoids (**1-5**), a benzofuran neolignan (**6**), two 8-*O*-4'-neolignans (**7** and **8**) and five tetrahydrofuran lignans (**9-13**) were isolated from *M. fragrans*. In this study, the isolation and structural determinations of these thirteen compounds are described. All the isolated compounds were

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evaluated for their inhibitory activities towards both the monophenolase and diphenolase activities of tyrosinase.

Materials and Methods

Plant material. The seeds of *M. fragrans* Houtt. were purchased from the Kyungdong Oriental Herbal Market at Korea, in November 2012.

Instruments. NMR experiments were conducted on a Bruker AM 300 or 500 MHz FT-NMR instrument with tetramethylsilane as internal standard. EIMS was collected on Jeol JMS-700 spectrometer (Japan). Optical rotations were measured on Perkin-Elmer 343 polarimeter (USA). Silica gel (230-400 mesh, Merck, Germany), RP-18 (ODS-A, 12 nm, S-150 m, YMC, Japan) and Sephadex LH-20 (Amersham Biosciences, USA) were used for column chromatography. Thin-layer chromatographic analysis was performed on Kieselgel 60 F_{254} (silica gel, 0.25 mm layer thickness, Merck, Germany) and RP-18 F_{254S} (Merck) plates.

Extraction and isolation. The dried seeds (500 g) of *M. fragrans* were chopped and extracted three times with 100% methanol (1 L×3) for 7 days at room temperature. The combined methanol extract was concentrated *in vacuo* to yield a brown gum (11.3 g). The methanol extract was suspended in H_2O (500 mL), then partitioned in turn with *n*-Hexane, EtOAc and BuOH (each 3×500 mL), to afford *n*-Hexane extract (1.8 g), EtOAc extract (3.5 g), and BuOH extract (1.5 g). The *n*-Hexane-soluble extract was silica gel column chromatography (230–400 mesh) using *n*-Hexane/EtOAc (100:1→1:1) mixture to yield eight subfractions (A1-A8). Fraction A3-A6 (1 g) was subjected to silica gel column chromatography (230–400 mesh) with a gradient of *n*-Hexane/EtOAc (99:1→1:1) to yield six subfractions (B1-B6). Subfraction B2 was separated by silica gel column chromatography using *n*-Hexane/EtOAc (99:1→49:1) then purified by Sephadex LH-20 column chromatography elution with 100% methanol to afford compounds **1** (30 mg). Fraction B4 was silica gel column chromatography with *n*-Hexane/EtOAc (99:1→49:1) to produce nine subfractions (C1-C9). Futher fraction C8 was RP-C_{18} column chromatography, with 70% methanol to give compounds **2** (35 mg) and **4** (20 mg). The EtOAc-soluble extract was silica gel column chromatography (230-400 mesh) using $CHCl_3$/acetone (100:1→1:1) mixtures to yield seven subfraction (D1-D7). Fraction D1 (900 mg) was applied to silica gel column (230–400 mesh) chromatography with *n*-Hexane/EtOAc (49:1→1:1) to yield 14 subfractions (E1-E14). Subfractions E6-E8 was subjected to silica gel column (230–400 mesh) chromatography with *n*-Hexane/EtOAc (49:1→9:1) to afford compounds **6** (8 mg) and **9** (12.3 mg). Fractions D2-D3 (290 mg) was silica gel column chromatography, with 100% $CHCl_3$ to yield 18 fractions (F1-F18). Fraction F11 was RP-C_{18} column chromatography, with 70% MeOH to yield ten subfractions (G1-G10). Fractions G3-G6 was further purified on Sephadex LH-20, with 100% MeOH to give compounds **3** (10.6 mg), **5** (14.3 mg) and **10** (7.5 mg). Fraction F15 was silica gel column chromatography, with 100% $CHCl_3$ to yield twenty subfractions (H1-H20). Fractions H13-H15 was purified through a RP-C_{18} column, with 70% MeOH to yield five subfractions (I1-I5). Fractions I3-I4 was repeatedly Sephadex LH-20 column chromatography, with 100% MeOH to afford compounds **11** (4.5 mg) and **12** (7 mg). Fraction D5-D6 (730 mg) was silica gel column chromatography, with $CHCl_3$-Me_2CO gradient (99:1→1:1) to yield sixteen subfractions (J1-J16). Of these, fractions J10-J15 was chromatographed using Sephadex LH-20 to give compounds **7** (19.7 mg)**, 8** (10 mg) and **13** (215.8 mg).

Compound 1: slightly yellow oil; EIMS *m/z* 162; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.30 (2H, d, *J*=6.7 Hz, H-7), 5.06 (1H, m, H-9a), 5.08 (1H, m, H-9b), 5.90 (2H, s, OCH_2O), 5.93 (1H, m, H-8), 6.64 (1H, m, H-6), 6.68 (1H, m, H-2), 6.74 (1H, d, *J*=7.9 Hz, H-5); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 39.9 (C-7), 100.8 (OCH_2O), 108.2 (C-2), 109.1 (C-5), 115.7 (C-9), 121.3 (C-6), 133.9 (C-1), 137.6 (C-8), 145.9 (C-4), 147.7 (C-3).

Compound 2: colorless oil; EIMS *m/z* 192; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 3.29 (2H, d, *J*=6.7 Hz, H-7), 3.88 (3H, s, OCH_3-3), 5.07 (1H, m, H-9a), 5.10 (1H, m, H-9b), 5.92 (2H, s, OCH_2O), 5.95 (1H, m, H-8), 6.35 (1H, s, H-6), 6.38 (1H, s, H-2); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 40.6 (C-7), 57.0 (OCH_3-3), 101.6 (OCH_2O), 103.1 (C-2), 108.2 (C-6), 116.2 (C-9), 133.9 (C-5), 135.0 (C-1), 137.8 (C-8), 143.9 (C-3), 149.3 (C-4).

Compound 3: colorless needles; EIMS *m/z* 178; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 4.22 (2H, dd, *J*=1.4, 5.9 Hz, H-9), 5.88 (2H, s, OCH_2O), 6.15 (1H, m, H-8), 6.46 (1H, d, *J*=15.8 Hz, H-7), 6.69 (1H, d, *J*=8.0 Hz, H-5), 6.75 (1H, dd, *J*=1.6, 8.0 Hz, H-6), 6.86 (1H, d, *J*=1.7 Hz, H-2); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 62.7 (C-9), 100.1 (OCH_2O), 104.8 (C-2), 107.3 (C-5), 120.1 (C-6), 125.7 (C-8), 130.0 (C-7), 130.1 (C-1), 146.3 (C-4), 147.0 (C-3).

Compound 4: white powder; EIMS *m/z* 180; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 6.26 (1H, d, *J*=26.4 Hz, H-8), 6.80 (1H, d, *J*=13.6 Hz, H-5), 6.95 (1H, dd, *J*=3.3, 13.7 Hz), 7.06 (1H, d, *J*=3.3 Hz, H-2), 7.57 (1H, d, *J*=26.4 Hz, H-7); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 113.7 (C-2), 114.1 (C-8), 115.1 (C-5), 121.5 (C-6), 126.4 (C-1), 145.3(C-4), 145.7 (C-3), 146.0 (C-7), 169.7 (C-9).

Compound 5: white powder; EIMS *m/z* 194; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 3.35 (2H, d, *J*=6.6 Hz, H-7), 3.89 (6H, s, OCH_3-3, 5), 5.14 (2H, m, H-9a, 9b), 6.02 (1H, m, H-8), 6.43 (2H, s, H-2, 6); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 40.3 (C-7), 56.2 (OCH_3-3,5), 105.1 (C-2,6), 115.7 (C-9), 131.1 (OH-4), 132.9 (C-1), 137.6 (C-8), 147.0 (C-3,5).

Compound 6: Colorless transparent prism; $[\alpha]_D^{25}$ –45.4 ° (*c* 0.1 in $CHCl_3$); EIMS *m/z* 324; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.38 (3H, d, *J*=6.8 Hz, 9-CH_3), 1.87 (3H, d, *J*=5.6 Hz, 9'-CH_3), 3.41 (1H, m, H-8), 3.89 (3H, s, OCH_3-5'), 5.10 (1H, d, *J*=8.9 Hz, H-7), 5.94 (2H, s, OCH_2O), 6.12 (1H, m, H-8'), 6.37 (1H, d, *J*=15.4 Hz, H-7'), 6.75 (1H, s, H-2'), 6.77 (1H, s, H-5), 6.78 (1H, s, H-6'), 6.88 (1H, d, *J*=7.8 Hz, H-6), 6.92 (1H, s, H-2); ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 18.3 (CH_3-9), 18.8 (CH_3-9'), 46.2 (C-8), 56.4 (OCH_3-5'), 93.8 (C-7), 101.5 (OCH_2O), 107.2 (C-2), 108.5 (C-5), 109.8 (C-6'), 113.8 (C-2'), 120.6 (C-6), 123.9 (C-8'), 131.4 (C-7'), 132.7 (C-1'), 133.5 (C-3'), 134.8 (C-1), 144.5 (C-5'), 146.9 (C-4'), 148.0 (C-4), 148.3 (C-3).

Compound 7: Colorless oil; $[\alpha]_D^{20}$ +10.7° (c 0.4 in $CHCl_3$); EIMS *m/z* 374; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.12 (3H, d, *J*=6.4 Hz, 9-CH_3), 3.37 (2H, d, *J*=6.7 Hz, H-7'), 3.87 (6H, s, OCH_3-3' and OCH_3-5'), 3.89 (3H, s, OCH_3-3), 4.07 (1H, d, *J*=2.4 Hz, OH-7), 4.36 (1H, m, H-8), 4.79 (1H, t, *J*=2.4 Hz, H-7), 5.15 (2H, m, H-9'), 5.51 (1H, s, OH-4), 6.0 (1H, m, H-8'), 6.45 (2H, s, H-2' and H-6'), 6.69 (1H, d, *J*=8.1 Hz, H-6), 6.84 (1H, d, *J*=8.1 Hz, H-5), 6.97 (1H, s, H-2); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 12.8 (C-9), 40.6 (C-7'), 56.0 (OCH_3-3), 56.2 (OCH_3-3', 5'), 72.8 (C-7), 82.3 (C-8), 105.6 (C-2', 6'), 108.6 (C-2), 113.9 (C-5), 116.2 (C-9'), 118.8 (C-6), 132.1 (C-1), 133.1 (C-4'), 136.1 (C-1'), 137.1 (C-8'), 144.5 (C-4), 146.5 (C-3), 153.5 (C-3', 5').

Compound 8: Colorless oil; $[\alpha]_D^{20}$ –25.0° (c 0.25 in $CHCl_3$); EIMS *m/z* 344; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.91 (3H, d, *J*=6.5 Hz, 9'-CH_3), 1.20 (3H, d, *J*=6.4 Hz, 9-CH_3), 3.51 (1H, s, OH-7), 3.92 (6H, s, OCH_3-3 and OCH_3-3'), 4.39 (1H, m, H-8), 4.84 (1H, s, H-7), 5.57 (1H, s, OH-4), 6.24 (1H, m, H-8'), 6.41 (1H, d, *J*=15.8 Hz, H-7'), 6.78 (1H, d, *J*=8.1 Hz, H-6), 6.86 (1H, s, H-5), 6.93 (1H, d, *J*=8.5 Hz, H-6'), 6.94 (1H, s, H-2'), 6.97 (1H, s, H-5'), 6.99 (1H, s, H-2); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 13.5 (CH_3-9), 18.4 (CH_3-9'), 55.9 (OCH_3-3), 56.0 (OCH_3-3'), 73.6 (C-7), 82.6 (C-8), 108.9 (C-2), 109.4 (C-2'), 114.0 (C-5), 119.0 (C-6'), 119.2 (C-6), 120.1 (C-5'), 125.0 (C-8'), 130.5 (C-7'), 131.9 (C-1), 133.8 (C-1'), 144.9 (C-4), 145.7 (C-3'), 146.5 (C-3), 151.6 (C-4').

Compound 9: white crystalline solid; $[\alpha]_D^{20}$ –90° (*c* 0.13 in $CHCl_3$); EIMS *m/z* 340; IR (KBr) ν_{max} 3440, 1625, 1558, 1507, 1456, 976 cm^{-1}, UV λ_{max} nm 236, 289 (MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.62 (3H, d, *J*=6.9 Hz, 9'-CH_3), 1.00 (3H, d, *J*=6.4 Hz, 9-CH_3), 2.43 (2H, m, H-8 and H-8'), 4.61 (1H, d, *J*=9.4 Hz, H-7), 5.40 (1H, d, *J*=4.4 Hz, H-7'), 5.94 (4H, s, OCH_2O×2), 6.76 (1H, s, H-6'), 6.78 (2H, s, H-5 and H-5'), 6.82 (1H, d, *J*=8.1 Hz, H-6), 6.85 (1H, s, H-2'), 6.91 (1H, s, H-2); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 9.8 (C-9'), 12.2 (C-9), 43.8 (C-8'), 47.9 (C-8), 85.1 (C-7'), 86.1 (C-7), 101.2 (OCH_2O), 101.3 (OCH_2O), 106.9 (C-2), 107.2 (C-2'), 108.3 (C-5'), 108.4 (C-5), 119.4 (C-6), 119.9 (C-6'), 134.9 (C-1'), 137.4 (C-1), 146.7 (C-4'), 147.3 (C-4), 147.8 (C-3), 148.2 (C-3').

Compound 10: colorless amorphous; $[\alpha]_D^{20}$ –53 ° (*c* 0.4 in $CHCl_3$); EIMS *m/z* 342; IR (KBr) ν_{max} 3495, 1610, 1520, 980 cm^{-1}, UV λ_{max} nm 233, 284 (MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.64 (3H, d, *J*=6.9 Hz, 9'-CH_3), 1.03 (3H, d, *J*=6.3 Hz, 9-CH_3), 2.46 (2H, m, H-8 and H-8'), 4.65 (1H, d, *J*=9.1 Hz, H-7), 5.45 (1H, d, *J*=4.1 Hz, H-7'), 5.55 (1H, s, OH-4'), 6.0 (2H, s, OCH_2O), 6.78 (1H, s, H-6'), 6.81 (1H, s, H-5), 6.86 (1H, d , *J*=8.0 Hz, H-6), 6.89 (1H, s, H-5'), 6.92 (1H, s, H-2'), 6.94 (1H, s, H-2); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 9.4 (C-9'), 11.8 (C-9), 43.5 (C-8'), 47.7 (C-8), 56.0 (OCH_3-3'), 84.8 (C-7'), 85.7 (C-7), 100.9 (OCH_2O), 106.5 (C-2), 108.0 (C-5), 108.7 (C-2'), 113.9 (C-5'), 118.8 (C-6'), 119.5 (C-6), 132.5 (C-1'), 137.2 (C-1), 144.3 (C-4'), 146.3 (C-3'), 146.9 (C-4), 147.9 (C-3).

Compound 11: colorless oil; $[\alpha]_D^{20}$ 0 ° (*c* 0.11 in $CHCl_3$); EIMS *m/z* 342; IR (KBr) ν_{max} 3495, 1610, 1520, 980 cm^{-1}, UV λ_{max} nm 233, 284 (MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.02 (6H, d, *J*=6.5 Hz, 9-CH_3 and 9'-CH_3), 2.30 (2H, m, H-8 and H-8'), 3.90 (3H, s, OCH_3-3'), 4.46 (2H, dd, *J*=3.2, 6.6 Hz, H-7 and H-7'), 5.56 (1H, s, OH-4'), 5.95 (2H, s, OCH_2O), 6.79 (1H, d, *J*=7.9 Hz, H-5), 6.87 (1H, s, H-6'), 6.89 (1H, s, H-6), 6.90 (1H, s, H-5'), 6.97 (2H, s, H-2 and H-2'); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 12.9 (C-9, 9'), 44.5 (C-8'), 44.6 (C-8), 55.9 (OCH_3-3'), 87.4 (C-7), 87.5 (C-7), 101.0 (OCH_2O), 106.8 (C-2'), 108.0 (C-5), 109.0 (C-2), 114.1 (C-5'), 119.4 (C-6), 119.9 (C-6'), 134.0 (C-1'), 136.2 (C-1), 145.1 (C-4'), 146.5 (C-3'), 147.0 (C-4), 147.8 (C-3).

Compound 12: colorless oil; $[\alpha]_D^{20}$ +0.7 ° (*c* 0.5 in $CHCl_3$); EIMS *m/z* 358; IR (KBr) ν_{max} 3400, 1610, 1520 cm^{-1}, UV λ_{max} nm 231, 278 (MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.05 (6H, dd, *J*=2.7, 6.6 Hz, 9-CH_3 and 9'-CH_3), 2.35 (2H, m, H-8 and H-8'), 3.88 (9H, d, *J*=4.3 Hz, OCH_3-3, 3', 4'), 4.52 (2H, t, *J*=6.2 Hz, H-7 and H-7'), 5.55 (1H, s, OH-4), 6.86 (1H, d, *J*=8.1 Hz, H-5'), 6.90 (1H, d, *J*=8.1 Hz, H-5), 6.93 (1H, d, *J*=8.1 Hz, H-6), 6.96 (1H, s, H-6'), 6.97 (1H, s, H-2), 7.0 (1H, s, H-2'); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 12.9 (CH_3-9'), 13.0 (CH_3-9), 44.3 (C-8'), 44.4 (C-8), 55.9 (OCH_3-3', 4'), 56.0 (OCH_3-3), 87.3 (C-7'), 87.4 (C-7), 109.2 (C-2), 109.9 (C-2'), 111.1 (C-5'), 114.1 (C-5), 118.6 (C-6'), 119.3 (C-6), 134.2 (C-1), 134.9 (C-1'), 145.1 (C-4), 146.5 (C-3), 148.5 (C-4'), 149.0 (C-3').

Compound 13: colorless oil; $[\alpha]_D^{20}$ 0° (*c* 0.37 in $CHCl_3$); EIMS *m/z* 344; IR (KBr) ν_{max} 3400, 1620, 1520 cm^{-1}, UV λ_{max} nm 230, 278 (MeOH); ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.03 (6H, d, *J*=6.5 Hz, 9-CH_3 and 9'-CH_3), 2.34 (2H, m, H-8 and H-8'), 3.87 (6H, s, OCH_3-3 and OCH_3-3'), 4.50 (2H, d, *J*=6.3 Hz, H-7 and H-7'), 5.63 (2H, s, OH-4 and OH-4'), 6.90 (2H, d, *J*=8.1 Hz, H-5 and H-5'), 6.93 (2H, d, *J*=8.1 Hz, H-6 and H-6'), 6.96 (2H, s, H-2 and H-2'); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 12.9 (C-9, 9'), 44.3 (C-8, 8'), 55.9 (OCH_3-3, 3'), 87.3 (C-7, 7'), 109.2 (C-2, 2'), 114.2 (C-5, 5'), 119.3 (C-6, 6'), 134.2 (C-1, 1'), 145.1 (C-4, 4'), 146.5 (C-3, 3').

Tyrosinase inhibitory activity. Mushroom tyrosinase (EC 1.14.18.1) was assayed as described previously with slight modifications (Ha et al., 2005), using L-tyrosinase or L-DOPA as substrate. In a spectrophotometric experiment, the enzyme activity was monitored by dopachrome formation at 475 nm with a Spectra MAX plus spectrophotometer (Molecular device, USA) at 30°C. All test samples were dissolved in DMSO and used for the experiment with dilution. The final concentration of DMSO in the test solution was 1.5%. First, 200 μL of a 4.5 mM L-tyrosine or 12 mM L-DOPA aqueous solution was mixed with 2785 μL of 0.25 M phosphate buffer (pH 6.8) and 10 μL of the test sample, incubated at 30°C for 10 min. Then, 5 μL of tyrosinase solution (130 units) was added to the phosphate buffer and incubated for additional 20 min. DMSO without test samples was used as the control, and kojic acid was used as a positive control. The assay was conducted in triplicate of separate experiments. The data analysis was performed by using Sigma Plot 2000 (SPSS Inc., USA). The inhibitory concentration leading to 50% activity loss (IC_{50}) was obtained by fitting experimental data to the logistic curve by the equation as follows:

$$\text{Activity (\%)}=100[1/(1+([I]/IC_{50}))]$$

Inhibition mode was analyzed by Enzyme Kinetics Module 1.0

(SPSS Inc.) equipped with Sigma Plot 2000

Results and Discussions

Five phenylpropanoids (**1-5**), a benzofuran neolignans (**6**), two 8-*O*-4'-neolignans (**7** and **8**), and five tetrahydrofuran lignans (**9-13**) were isolated from the MeOH extract of *M. fragrans* seeds by repeated column chromatography (Fig. 1).

Compound **1** was obtained as slightly yellow oil. The EIMS of **1** had a molecular ion peak at *m/z* 162 [M^+], consistent with the molecular formula of $C_{10}H_{10}O_2$. The ^{1}H-NMR spectrum of **1** showed three aromatic protons δ_H 6.64 (1H, m, H-6), 6.68 (1H, m, H-2) and 6.74 (1H, d, *J*=7.9 Hz, H-5), a methylenedioxyl group δ_H 5.90 (2H, s, OCH_2O), two methylene protons δ_H 3.30 (2H, d, *J*=6.7 Hz, H-7), 5.06 (1H, m, H-9a) and 5.08(1H, m, H-9b), and a methine proton δ_H 5.93 (1H, m, H-8). These spectral data suggested **1** to be a phenylpropanoid. Based on of the spectral data, compound **1** was identified as 3,4-methylenedioxy-allylbenzene (safrole) by comparing its spectroscopic data with literature data (Cavalcante et al., 1985).

Compound **3** was obtained as colorless needles. The EIMS of **3** had a molecular ion peak at *m/z* 178 [M^+], consistent with the molecular formula of $C_{10}H_{10}O_3$. The ^{1}H- and ^{13}C-NMR data of **3** were very similar to those of **1**, except for the presence of a methylene proton δ_H 4.22 (2H, dd, *J*=1.4, 5.9 Hz, H-9), and two methine protons δ_H 6.15 (1H, m, H-8) and 6.46 (1H, d, *J*=15.8 Hz, H-7). Based on the spectral data, compound **3** was identified as 3,4-methylenedioxycinnamyl alcohol by comparing its spectroscopic data with the literature data (Benevides et al., 1999). This compound was isolated from this plant for the first time.

Compound **9** was obtained as a white crystalline solid, with a molecular formula of $C_{20}H_{20}O_5$ based on the EIMS peak at *m/z* 340 [M]$^+$. The ^{1}H-NMR spectrum was observed a symmetric tetrahydrofuran lignan signals, as two methyl groups at δ_H 0.62 (3H, d, *J*=6.9 Hz, H-9) and 1.00 (3H, d, *J*=6.4 Hz, H-9) in addition to two-oxybenzyl methine protons at δ_H 4.61 (1H, d, *J*=9.4 Hz, H-7) and 5.40 (1H, d, *J*=4.4 Hz, H-7). In particular, the sharp singlet signal at δ_H 5.94 (4H, s) indicated the presence of two methylenedioxyl groups. The ^{13}C-NMR and HMBC spectra confirmed the presence of the piperonyl groups at C-7 and C-7,

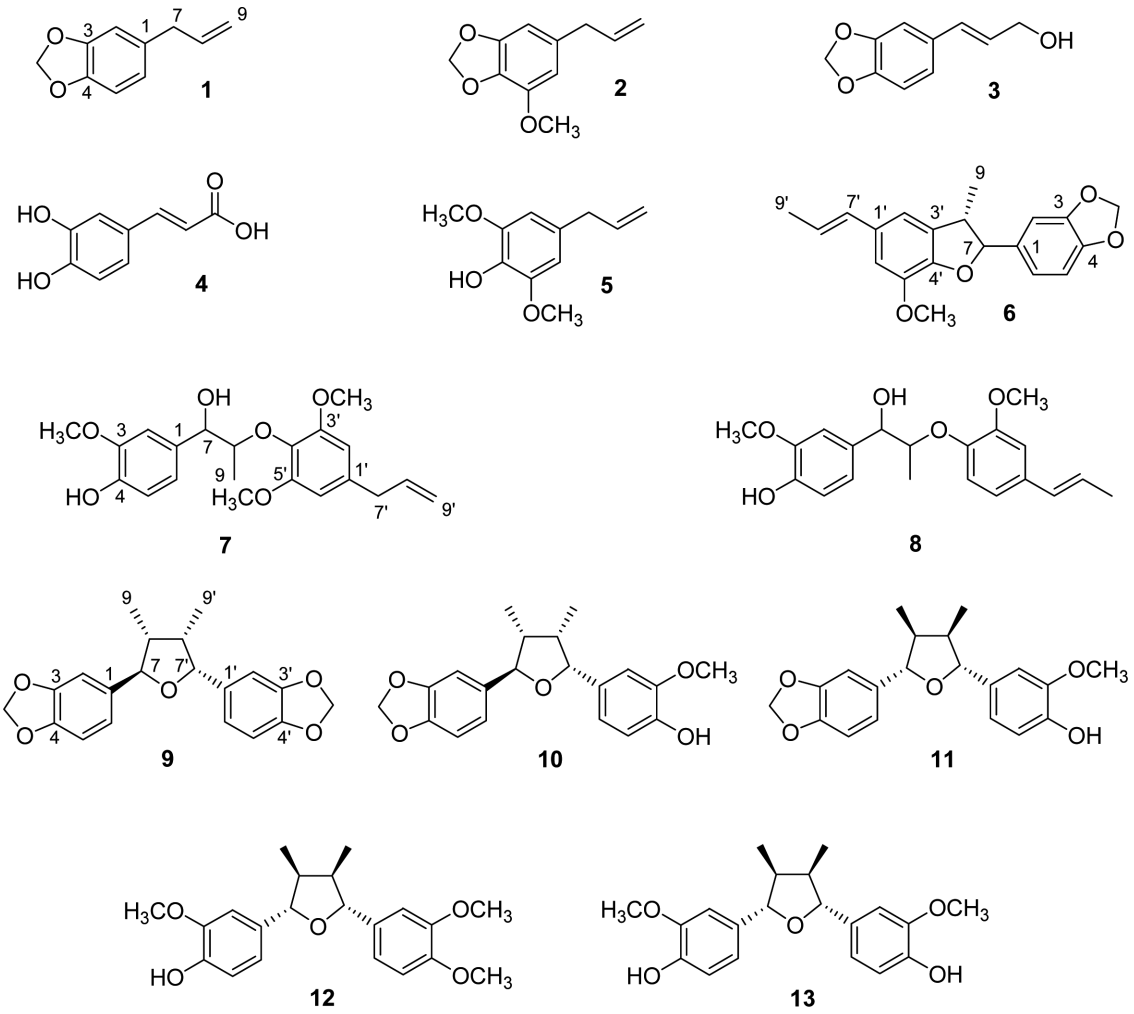

Fig. 1 Structures of compounds **1–13** from *M. fragrans*.

Table 1 Kinetics and Inhibition Constants of mushroom tyrosinase by safrole

Parameter	Substrate	
	L-Tyrosine	L-DOPA
IC_{50}	32.11 μM	27.32 μM
K_m	388.36 μM	984.67 μM
V_{max}	3.2 μmol/min	61.02 μmol/min
Inhibition Type	Competitive	Competitive
K_i	16.05 μM	13.66 μM

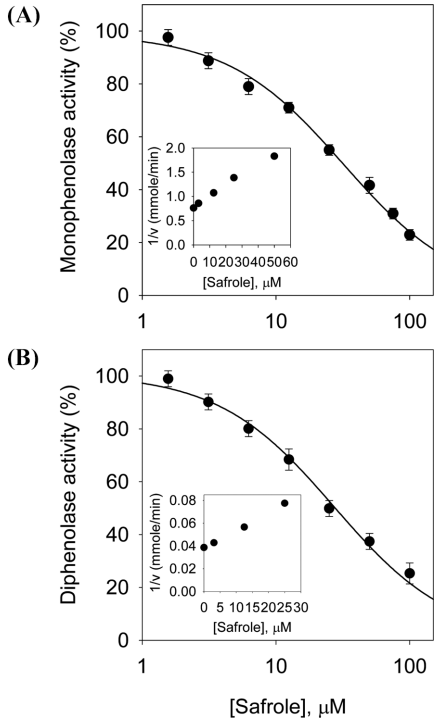

Fig. 2 (A) Effect of safrole (1) on the tyrosinase catalyzed oxidation of L-tyrosine. Inset: Plot of 1/υ vs. concentration of 1, (B) Effect of safrole (1) on the tyrosinase catalyzed oxidation of L-DOPA. Inset: Plot of 1/υ vs. concentration of 1.

respectively. The optical rotation of **9** ($[\alpha]_D^{20}$ –90.0°) showed the same negative sign as that of the literature ($[\alpha]_D^{25}$ –93.0°). Therefore, based on all the above mentioned evidence and reference data (Urzua et al., 1987; Shimomura et al., 1987; Bandara Herath and Anoma Priyadarshani, 1996), the structure of compound **9** was established as (7S,8S,7S,8R)-3,4,3',4'-dimethylenedioxy-7,7'-epoxylignan [(−) zuonin A].

Compound **10** was obtained as a colorless amorphous, with a molecular formula of $C_{20}H_{22}O_5$ based on the molecular ion peak in the EIMS spectrum at *m/z* 342 $[M]^+$. The ^{1}H-NMR spectrum showed a nonsymmetric tetrahydrofuran lignan signals, as two methyl groups at δ_H 0.64 (3H, d, *J*=6.9 Hz, H-9') and 1.03 (3H, d, *J*=6.3 Hz, H-9) in addition to two-oxybenzyl methine protons at δ_H 4.65 (1H, d, *J*=9.1 Hz, H-7) and 5.45 (1H, d, *J*=4.1 Hz, H-7'). ^{13}C-NMR and HMBC spectra confirmed the presence of the piperonyl and guaiacyl groups at C-7 and C-7', respectively. These

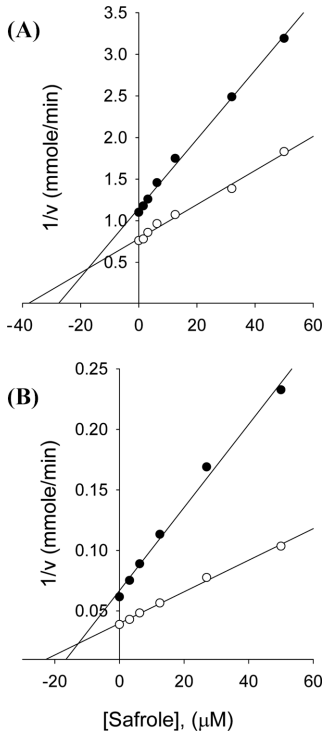

Fig. 3 Dixon plots for the inhibition of the monophenolase and diphenolase activities of tyrosinase by safrole (1). (A) Concentrations of substrates for curves were 150 (●) and 300 (○) μM, respectively. (B) Concentrations of substrates for curves were 400 (●) and 800 (○) μM, respectively.

data of **10** were very similar to those of **9**, except for the presence of a hydroxyl group at δ_H 5.55 (1H, s, OH-4) and methoxy group at δ_H 3.91 (3H, s, OCH_3-3') instead of 3',4'-methylenedioxyl group. These observations indicate that the relative structure of **10** is the same as that of chicanine. The optical rotation of **10** showed a negative value ($[\alpha]_D^{20}$ –53.0°), confirming that the structure of **10** is an enantiomer of chicanine ($[\alpha]_D^{31}$ +118.8°). Therefore, based on all the spectroscopic and reference data (Sadhu et al., 2003; Konishi et al., 2005), the structure of **10** was established as (7R,8S,7'R,8'R)-3,4-methylenedioxy-4'-hydroxy-3'-methoxy-7,7'-epoxylignan [(−)chicanine].

The isolated compounds were evaluated for their inhibitory activities on tyrosinase. As shown in Table 1 and Fig. 2A, safrole (**1**) exhibited a dose-dependent inhibitory effect on the monophenolase activity of mushroom tyrosinase (IC_{50}=32.11 μM). Compound **1** also significantly inhibited mushroom tyrosinase diphenolase activity with an IC_{50} of 27.32 μM (Fig. 2B). The result obtained indicates that the phenylpropanoid (**1**) exhibited significantly higher inhibitory activity than a benzofuran neolignan (**6**, IC_{50} >100 μM), two 8-*O*-4'-neolignans (**7** and **8**, IC_{50} >100 μM), and five tetrahydrofuran lignans (**9**-**13**, IC_{50}>100 μM). In the previous reports, phenylpropanoids, aldehydes, and other derivatives, including 3,4-dihydroxycinnamic acid (Lee, 2002), anisaldehyde (Ha et al., 2005), 2*E*-alkenals (Kubo and Kinst-Hori, 1999b) were identified as tyrosinase inhibitors. Among them, phenylpropanoids

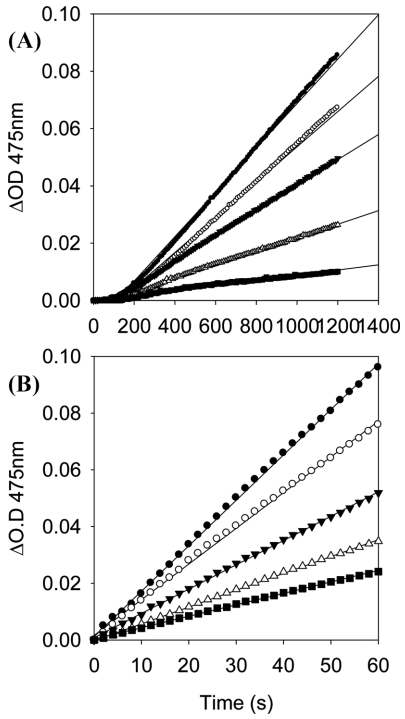

Fig. 4 Time dependent inhibition of tyrosinase in the presence of safrole (1). (A) Concentrations of safrole (1) from top to bottom were 0 (●), 6.25 (○), 25 (▼), 75 (△), 200 (■) μM, (B) Concentrations of safrole (1) from top to bottom were 0 (●), 12.5 (○), 25 (▼), 50 (△), 100 (■) μM

containing a hydroxyl group possess potent tyrosinase inhibitory activity (Seo et al., 2003). In the case of (2*E*)-alkenals the hydrophobic alkyl chain length from the hydrophilic enal group seems to be related to their inhibitory potency. In this study as well, we suggest that the inhibitory effect of **1** was determined by the propenyl group. In contrast, **4** was oxidized by enzyme as the substrate.

The kinetic behavior of mushroom tyrosinase during the oxidation of L-tyrosine and L-DOPA was studied. Under the condition used in the present investigation, the oxidation of L-tyrosine and L-DOPA by mushroom tyrosinase followed the Michaelis-Menten kinetics. The kinetic parameters for mushroom tyrosinase were obtained from the Dixon plot. The results illustrated in Fig. 3 show that safrole (**1**) is a competitive inhibitor because increasing the safrole (**1**) concentration resulted in a family of lines with a common intercept on the 1/υ axis but with different slopes. The equilibrium constant for inhibitor binding, K_i, of L-tyrosine and L-DOPA obtained from the Dixon plot were 16.05 and 13.06 μM, respectively, as listed in Table 1. The time dependence of **1** on the tyrosinase catalyzed oxidation of L-tyrosine and L-DOPA was studied. Compound **1** showed time dependent inhibition (Fig. 4). As shown in Fig. 4A, the lag time is known for the oxidation of monophenol substrates such as L-tyrosine and this lag can be shortened. This lag time can be extended by monophenolase inhibitors such as tropolone (Kahn and Andrawis, 1985) and galangin (Kubo and Kinst-Hori, 1999a). Compound **1** did not extend this lag phase, indicating that **1** dose not inhibit the hydroxylation of L-tyrosine.

In summary, thirteen kinds of phenolic compounds were isolated from the *n*-hexane and EtOAc-soluble fractions of *M. fragrans*. The structures were identified as safrole (**1**), myristicin (**2**), 3,4-methylenedioxycinnamyl alcohol (**3**), caffeic acid (**4**), methoxyeugenol (**5**), licarin B (**6**), *erythro*-(7S,8R)-$\Delta^{8'}$-4,7-dihydroxy-3,3',5'-trimethoxy-8-*O*-4'-neolignan (**7**), machilin C (**8**), (-) zuonin A (**9**), (-) chicanine (**10**), (−) machilin F (**11**), nectandrin A (**12**) and nectandrin B (**13**) by the physicochemical and spectroscopic data. In particular, compound **3** was isolated for the first time from this plant. The isolated compounds were evaluated for their tyrosinase inhibitory activities. Among them, safrole (**1**) inhibited the oxidation of L-tyrosine and L-DOPA catalyzed by mushroom tyrosinase. The inhibition mechanism obtained from the Dixon plot show that **1** is a competitive inhibitor. As **1** only binds the free enzyme to form an EI complex rather than bind the ES complex.

In this regard, safrole showed significant tyrosinase inhibitory activity among the tested phenylpropanoids; however, the reason for this difference is still largely unknown. There still seems a lack of important knowledge for designing effective tyrosinase inhibitors. Further studies are ongoing to investigate the biological significance of safrole (**1**) as a tyrosinase inhibitor in the living systems.

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