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New neolignans from the seeds of *Myristica fragrans* that inhibit nitric oxide production

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ABSTRACT

Five new 8-*O*-4′ type neolignans, named myrifralignan A–E (**1**–**5**), together with five known analogues (**6**–**10**), were isolated from the seeds of *Myristica fragrans* Houtt. Their chemical structures were determined using several spectroscopic methods. Compounds **3**–**10** exhibited potent inhibitory activity against the production of nitric oxide (NO) in the RAW264.7 cell line stimulated by lipopolysaccaride. Myrislignan (**7**) and machilin D (**10**) were the most potent inhibitors of NO production amongst these compounds. The IC_{50} values of myrislignan and machilin D were 21.2 and 18.5 μM. And, their inhibitory activity was more than *L*-N^6-(1-iminoethyl)-lysine, a selective inhibitor of inducible nitric oxide synthase (IC_{50} = 27.1 μM). Furthermore, real-time PCR analysis revealed that these neolignans could significantly suppress the expression of inducible nitric oxide synthase mRNA. These results demonstrated that the 8-*O*-4′ type neolignans are promising candidates as anti-inflammatory agents.

1. Introduction

Myristica fragrans Houtt. (Myristicaceae) is an aromatic evergreen tree indigenous to the Maluku Province of Indonesia, formerly known as the Spice Islands (van Gils & Cox, 1994). The seeds of *M. fragrans* are known as nutmeg and the scarlet aril surrounding the seed is named mace. Nutmeg was introduced into Europe during the 12th century by Arab merchants. Nutmeg has been used as the spice in sweet and savoury cooking as well as a medicine. Studies reported that nutmeg exhibits a broad range of pharmacological properties, including anti-inflammatory (Olajide et al., 1999), antibacterial (Narasimhan & Dhake, 2006), antioxidant, antiangiogenic (Piaru, Mahmud, Abdul Majid, & Mahmoud Nassar, 2012), anticarcinogenic (Lee et al., 2006), antidiarrhoeal (Lima et al., 2000) and antiplatelet aggregation (Janssen et al., 1990) activities. Nutmeg is added to the prescriptions or individually used for the treatment of stomach cramps, diarrhoea, rheumatism, psychosis, nausea and flatulence (van Gils & Cox, 1994). Also, nutmeg has been used as an aphrodisiac and an abortifacient. Lignans are the major active components in *M. fragrans* and possess various bioactivities, such as anti-inflammation (Cao, Yang, Xu, & Li, 2013), antioxidant, anti-cytotoxicity (Duan, Tao, Hao, Gu, & Zhu, 2009), inhibition of protein tyrosine phosphatase 1B (Yang et al., 2006), anti-platelet (Kang, Min, & Lee, 2013) and antifungal activities (Cho et al., 2007).

The discovery that mammalian cells can produce the free radical nitric oxide (NO) has drawn the attentions of investigators in all the fields of biology and medicine (Rubbo, Darley-Usmar, & Freeman, 1996). NO regulates many critical aspects of cellular function (Soloviev, Lehen'kyi, Zelensky, & Hellstrand, 2004). However, excessive production of NO by nitric oxide synthase (NOS) is involved in many diseases, as well as inflammation that can

Abbreviations: CC, column chromatography; RP-SP-HPLC, reversed phase semi-preparative high-performance liquid chromatography; t_R, retention time; MeOH, methanol; DMSO, dimethyl sulfoxide; $CDCl_3$, deuterated chloroform; TMS, tetramethylsilane; IR, infrared; UV, ultraviolet; CD, circular dichroism; EI–MS, electron ionisation mass spectrometry; HR-ESI–MS, high-resolution electron spray ionisation mass spectrometry; NMR, nuclear magnetic resonance; ^1H–^1H COSY, ^1H–^1H correlation spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; LPS, lipopolysaccharide; *L*-NIL, *L*-N^6-(1-iminoethyl)-lysine; IND, indomethacin; NO, nitric oxide; iNOS, inducible nitric oxide synthase; RT-PCR, reverse transcription-polymerase chain reaction; IC_{50}, half maximal inhibitory concentration.

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ultimately cause tissue injury. Several studies reported that excessive NO generation is associated with shock (Nava, Palmer, & Moncada, 1991), inflammatory diseases (Molero et al., 1995), liver cirrhosis (Soderman, Leone, Furst, & Persson, 1997), asthma (Stirling et al., 1998), juvenile parkinsonism (Hyun et al., 2002). Thus, the discovery of inhibitors of NO production from natural products is an active area of interest around the world.

A previous study reported that some dihydrobenzofuran type neolignans isolated from nutmeg showed inhibitory activity on NO production induced by lipopolysaccharide (LPS) (Cao et al., 2013). In the current study, eight 8-*O*-4′ type neolignans (three of them of which are novel) isolated from nutmeg exhibited potent inhibitory effects against NO production, and suppressed the expression level of inducible nitric oxide synthase.

2. Materials and methods

2.1. General

Optical rotation was measured on an Autopol III polarimeter (Rudolph Research Analytical, Flanders, NJ, USA) with chloroform ($CHCl_3$) as a solvent. IR spectra were recorded on a Nicolet™ 470 FT-IR spectrometer (Thermo Nicolet, Inc., Madison, WI, USA) with KBr discs. Ultraviolet (UV) data were recorded on a Varian Cary 300 ultraviolet–visible spectrophotometer (Varian Inc., Palo Alto, CA, USA) in methanol (MeOH). Circular dichroism (CD) spectra were recorded with MeOH as the solvent on a JASCO J-810 spectropolarimeter (Jasco, Hachioji, Tokyo, Japan). Electron ionisation mass spectrometry (EI–MS) data were obtained using a TRACE 2000 mass spectrometer (Finnigan, Silicon Valley, CA, USA). High-resolution electron spray ionisation mass spectrometry (HR-ESI–MS) data were obtained using a Daltonics APEX IV Fourier Transform ICR high-resolution mass spectrometer (Bruker, Karlsruhe, Baden-Wuerttemberg, Germany). NMR data were acquired on a Bruker AV400 spectrometer (Bruker, Karlsruhe, Baden-Wuerttemberg, Germany); 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR) using deuterated chloroform ($CDCl_3$) as the solvent, with TMS as an internal standard. Open column chromatography (CC) separation was carried out on silica gel (200–300 mesh; Qingdao Marine Chemical Co., Qingdao, China). Thin layer chromatography (TLC) was conducted on silica gel GF_{254} plates (Merck, Darmstadt, Germany). Spots were visualised under UV light or by spraying with 10% H_2SO_4 in 95% ethanol followed by heating. Reversed phase semi-preparative HPLC (RP-SP-HPLC) was carried out on a instrument including an LC P600 pump, a UV600 UV–Vis detector and Labtech Chromsoftware (LabTech Co., Beijing, China), equipped with a Phenomenex Luna 10 C_{18} column (21.2 mm × 250 mm, 10 μm, Phenomenex Inc., Torrance, CA, USA) at a flow rate of 5 ml/min and all UV detection was set up at 210 nm. cDNA synthesis was carried out using a MyCycler PCR instrument (Bio-Rad Laboratories, Inc., Hercules, CA, USA). A MX3005P Real Time PCR instrument was used to amplify and detect DNA (Agilent Technologies Inc., Wilmington, DE, USA).

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), Griess reagent, phosphate buffered saline (PBS), lipopolysaccharide (LPS), *L*-N^6-(1-iminoethyl)-lysine (*L*-NIL), indomethacin (IND), and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco™ (Grand Island, NY, USA). 96-Well plates were obtained from Corning Costar (Corning Costar, Cambridge, MA, USA). Chromatographic grade MeOH was purchased from Tianjin Xihua Special Type Reagent Factory (Tianjin, China). Deionised water (H_2O) was purchased from Wahaha Co., Ltd. (Hangzhou, China). Milli-Q grade H_2O was used for the bio-assay. Other analytical grade reagents were purchased from Beijing Chemical Works (Beijing, China). Trizol reagent kit was purchased from Life Technologies Co (San Diego, CA, USA). Reverse Transcription System kit and GoTaq® qPCR Master Mix kit were purchased from Promega Co (Madison, Wisconsin, USA). All of the primers were synthesised by Beijing Liuhe Genomics Technology Co. Ltd. (Beijing, China).

The murine macrophage cell line RAW264.7 was obtained from the Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China). Cell culture was carried out in a Sanyo MCO-15 AC carbon dioxide (CO_2) incubator (Sanyo Electric Co., Ltd., Osaka, Japan), and MTT assay was taken on Thermo Multiskan MK 3 Automated Microplate Reader (Thermo-Labsystems, Franklin, MA, USA).

2.2. Plant material

The dried ripe seeds of *M. fragrans* (nutmeg) were purchased from Indonesia in 2011 and identified by Professor Xiu-Wei Yang of School of Pharmaceutical Sciences, Peking University Health Science Center, Peking University. A voucher specimen (No. 6396121RDK) was deposited in State Key Laboratory of Natural and Biomimetic Drugs (Peking University).

2.3. Extraction and isolation

The extraction of nutmeg (24.00 kg) was performed using CO_2 supercritical extraction at 20 Mpa and 50 °C for 2 h under CO_2 with a flow rate of 280 kg h^{-1}. The separation pressure was 8 Mpa and separation temperature was 50 °C. 8.02 kg of CO_2 extract was obtained and 4.00 kg of the extract (4.00 kg) was dissolved in MeOH (13 l). After six times extraction using microwaves, it resulted in a red-brown viscous oil of 1450 g and an insoluble residue. The oil (797 g) was subjected to a silica gel CC, eluted with a gradient solvent system of cyclohexane (CHA)–ethyl acetate (EtOAc) (60:1 → 1:1, *v*/*v*), EtOAc, and MeOH to give fractions A to M. The fraction D (126 g) was purified by medium pressure CC over silica gel H using a gradient solvent system of CHA–acetone (ACE) (1:0 → 50:1, *v*/*v*) to yield nine fractions (Fr.D_1 to Fr.D_9). The Fr.D_4 (30.8 g) was separated by RP-SP-HPLC (80% aqueous MeOH) to give twenty-five (Fr.D_4-1 to Fr.D_4-25). By further purification of RP-SP-HPLC, compounds **6** (5.0 mg, 70% aqueous MeOH, t_R = 61 min) from the Fr.D_4-5 (18.6 mg) and **1** (1.1 mg, 58% aqueous CH_3CN, t_R = 120 min) from the Fr.D_4-11 (73.1 mg) were afforded. Fr.G (39.3 g) was separated by CC on a silica gel and eluted with petroleum ether (PE)–ACE (5:1, *v*/*v*) to yield nine subfractions. Fr.G_1 to Fr.G_9, and Fr.G_4 (15.2 g) was further separated by RP-SP-HPLC to give compound **2** (0.6 mg, 55% CH_3CN, t_R = 38 min). The Fr.I (43 g) was separated by CC on a silica gel eluted with PE–ACE (5:1, *v*/*v*) to give eleven fractions (Fr.I_1 to Fr.I_{11}). By further purification of RP-SP-HPLC, compound **7** (14.2 mg, 65% aqueous MeOH, t_R = 60 min) was purified from a part of Fr.I_8 (4.7 g) and compound **8** (7.8 mg, 56% MeOH, t_R = 56 min) was separated from Fr.I_9 (12.1 g). Fr.K (6.0 g) was subjected to CC over silica gel and eluted with PE–ACE (9:2, *v*/*v*). The eluate was collected in portions of 100 ml and eluates containing similar components by TLC detection were combined to yield 11 fractions (Fr.K_1 to Fr.K_{11}). Then Fr.K_7 (1.0 g) was separated by RP-SP-HPLC (60% MeOH) to yield a residue (72.2 mg), which was further purified by RP-SP-HPLC (45% CH_3CN) to give compounds **3** (3.8 mg, t_R = 48 min) and **4** (3.5 mg, t_R = 58 min). Fr.L (35.1 g) was chromatographed over silica gel and eluted with PE–ACE (4:1, *v*/*v*) to generate Fr.L_1 to Fr.L_{11}, and Fr.L_{10} (9.0 g) was further separated through RP-SP-HPLC (70% aqueous MeOH) to generate eight subfractions, Fr.L_{10}-1 to Fr.L_{10}-8. Subfractions Fr.L_{10}-4 (123.1 mg), Fr.L_{10}-5 (39.3 mg) and Fr.L_{10}-7 (13.0 mg) were further purified by RP-SP-HPLC resulted in the isolation of compounds **9** (18.1 mg, 63% aqueous MeOH for Fr.L_{10}-4, t_R = 58 min),