Fitoterapia 81 (2010) 185–189

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Chemical constituents of *Baeckea frutescens* leaves inhibit copper-induced low-density lipoprotein oxidation

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ARTICLE INFO

Article history:
Received 25 May 2009
Accepted in revised form 12 August 2009
Available online 21 August 2009

Keywords:
Baeckea frutescens
Myrtaceae
Arteriosclerosis
Low-density lipoprotein
Biflavonoid
Chromone

ABSTRACT

Oxidative modification of LDL plays an important role in the genesis of arteriosclerosis. This study focused on the effects of the leaves of *Baeckea frutescens* in the prevention of arteriosclerosis. The leaves of *B. frutescens* have afforded, besides known flavonoid and chromone glycosides, a novel biflavonoid glycoside, characterized as 3-*O*-α-L-rhamnopyranosylmyricetinyl-(I-2″,II-2″)-3-*O*-α-L-rhamnopyranosylmyricetin on the basis of chemical and spectral evidence. This compound exhibited marked inhibition of copper-induced LDL oxidation.

1. Introduction

Baeckea frutescens L. (Myrtaceae) is a shrub or small tree that grows widely from Australia to South China. The leaves are needle-like and aromatic. In China, the leaves are used as a medicinal tea, which is drunk to aid recovery from fever associated with disease. In Indonesia, the leaves of *B. frutescens* are referred to as "Jungrahab" and are one of the raw materials of traditional folk medicine, Jamu [1]. Chemical studies on the leaves of *B. frutescens* have indicated the presence of an essential oil [2,3], in addition to sesquiterpene [4] phloroglucinol [5], flavanone [6,7], flavonol [8], chromone [9,10] and chromanone [9] derivatives. So far, however, pharmacological studies on this plant have not been carried out.

During the course of our search for natural products capable of preventing lifestyle-related diseases, we have investigated the ability of chemical constituents of *B. frutescens* leaves to prevent arteriosclerosis. Arteriosclerosis is seemingly initiated by the oxidation of low-density lipoprotein (LDL) [11]. LDL is taken up from plasma into endothelial cells (ECs), where it is oxidized by reactive oxygen species or the transition metals copper or iron, producing oxidized LDL (ox-LDL). Ox-LDL stimulates the adhesion and entry of monocytes into ECs, where the monocytes are subsequently differentiated to macrophages by the action of monocyte colony-stimulating factor. Ox-LDL is endocytosed by macrophages via scavenger receptors to form foam cells [12]. These foam cells become aggregated into fatty streaks and are deposited on the ECs, where they form the key component of arteriosclerosis.

In this study, we have investigated the inhibitory effect of chemical constituents from *B. frutescens* leaves on copper-induced LDL oxidation, a model reaction for the early stage of the development of arteriosclerosis.

2. Experimental

2.1. General procedures and plant material

Optical rotations were measured by a Jasco DIP-1000 digital polarimeter. HR-FAB-MS was performed with a JEOL GCmate. IR and UV spectra were measured on a Shimadzu FT-IR 8300 infrared spectrometer and a Hitachi U-3000 spectrometer, respectively. The NMR spectra were recorded on a Bruker DPX-400 instrument. The plant materials were purchased in Jakarta, Indonesia and the plant was identified by Dr. Suhardjono, the Botanical Garden, Bogor, Indonesia. The voucher specimen has

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doi:10.1016/j.fitote.2009.08.021

been deposited at the Botanical Museum of Kobe Gakuin University [10].

2.2. Extraction and isolation

The leaves of *B. frutescens* (3.8 kg) were extracted with hot MeOH. The solvent was removed under reduced pressure to yield a MeOH extract (1448 g). The MeOH extract was suspended in a MeOH/H_2O (400/1200 ml) mixture and then extracted successively with $CHCl_3$, EtOAc and *n*-BuOH (each 1600 ml × 3). Each solvent was removed under reduced pressure to yield $CHCl_3$ (507 g), EtOAc (408 g), *n*-BuOH (270 g) and H_2O (263 g) soluble phases. The EtOAc soluble phase was subjected to chromatography on Sephadex LH-20 using MeOH to give a flavonoid-containing fraction. This fraction was subjected repeatedly to silica gel column chromatography using an EtOAc–MeOH–H_2O (90:7:3) solvent system and to Rp-18 column chromatography using a MeOH–H_2O (1:4) solvent system, and was finally purified by column chromatography on Sephadex LH-20 using MeOH to give compound 1. The *n*-BuOH-soluble phase was subjected to chromatography on silica gel using a gradient of $CHCl_3$ and MeOH (100:0–0:100) to give flavonoid and chromone-containing fractions. These fractions were then subjected repeatedly to silica gel column chromatography using an EtOAc–MeOH–H_2O (95:3.5:1.5, 90:7:3, 85:10:5) solvent system and to Rp-18 or Rp-8 column chromatography using a MeOH–H_2O (1:4 for Rp-18, 1:3 for Rp-8) solvent system, and were finally purified by column chromatography on Sephadex LH-20 using MeOH to give compounds 2–10.

Compound 2 (270 mg): Yellow needles from MeOH; $[\alpha]_D^{23}$ −250.9 (c = 0.55, MeOH); HR-negative-FAB-MS m/z: 925.1668 $[M-H]^-$ (Calcd for $C_{42}H_{37}O_{24}$, 925.16733); IR ν_{max}^{KBr} cm^{-1}: 3400, 1653, 1613, 1577, 1500, 1450, 1301, 1191, and 1045; UV λ_{max} nm (log ε): 360 (4.25), 306 (4.18), 248 (4.64), 234 (4.63), and 210 (4.77). ^1H and ^{13}C NMR (400 and 100 MHz, respectively, DMSO-d_6): Table 1.

Acid hydrolysis of 2: Compound 2 (50 mg) was treated with 5% HCl (10 ml) under reflux for 3 h. The residue was purified by column chromatography on Sephadex LH-20 using MeOH to yield 2a (30 mg). The solution was neutralized with Amberlite IRA and the resin was filtered off. The filtrate was purified by column chromatography on Sephadex LH-20 using MeOH–H_2O (2:1) to afford L-rhamnose (5 mg).

Compound 2a: ^1H NMR (400 MHz, DMSO-d_6): 12.49 (s, 5, 5‴-OH), 6.04 (d, J = 2.1 Hz, H-6, 6‴), 5.27 (d, J = 2.1 Hz, H-8, 8‴), and 6.66 (s, H-6‴, 6⁗); ^{13}C NMR (100Mz, DMSO-d_6): 149.75 (C-2, 2‴), 135.92 (C-3, 3‴), 175.70 (C-4, 4‴), 160.41 (C5, 5‴), 97.65 (C-6, 6‴), 163.11 (C-7, 7‴), 92.90 (C-8, 8‴), 156.12 (C-9, 9‴), 103.14 (C-10, 10‴), 120.65 (C-1‴, 1⁗), 116.58 (C-2′, 2⁗), 144.79 (C-3′, 3⁗), 134.64 (C-4′, 4⁗), 143.56 (C-5′, 5⁗), and 108.99 (C-6′, 6⁗).

Acetylation of 2a: Compound 2a (4 mg) was acetylated with Ac_2O-pyridine, and the product was purified by column chromatography on Sephadex LH-20 using $CHCl_3$–MeOH (1:2) to yield the dodecaacetate 2b (6 mg).

Compound 2b: ^1H NMR (400 MHz, DMSO-d_6): 7.29 (s), 6.96 (d, J = 2.1 Hz), 6.77 (d, J = 2.1 Hz), 2.38, 2.30, 2.29, 2.23, 2.14, and 1.95 (each s, $COCH_3$).

2.3. HPLC analysis

HPLC was performed with an ODS column (CAPCELL PAK C18 Type MG 5 μm, 4.6 × 250 mm, SHISEIDO). The detection wavelength was 254 nm. Elution was carried out with MeOH–H_2O containing 0.1% phosphoric acid (32.5:67.5) at a flow rate of 1 ml/min. The injection volume was 10 μl.

2.4. Evaluation of the inhibitory effects on LDL oxidation

The extent of LDL oxidation was assessed by using a modified thiobarbituric acid reactive substances (TBARS) method, as previously described [13]. The results were analyzed by one-way ANOVA followed by Dunnett's test.

3. Results and discussion

The inhibitory effects of the MeOH extract and the $CHCl_3$, EtOAc, *n*-BuOH and H_2O soluble phases obtained from *B. frutescens* leaves on copper-induced LDL oxidation was measured by using the TBARS method [13]. LDL was incubated in PBS containing cupric ion in the presence of the MeOH extract and the above four soluble phases. At the end of the incubation period, TBARS reagent was added and the formation of TBARS was measured at 515 nm. As shown in Fig. 1, the MeOH extract exhibited marked activity, and this activity was concentrated into the EtOAc- and *n*-BuOH-soluble phases. To determine the chemical constituents of the biologically active EtOAc and *n*-BuOH soluble phases, HPLC analysis was

Table 1
^1H and ^{13}C NMR spectral data for 2 in DMSO-d_6.

No.	^{13}C	^1H	No.	^{13}C	^1H	No.	^{13}C	^1H
2, 2‴	158.90		1′, 1⁗	120.74		1″, 1⁗′	101.88	5.06 (brs)
3, 3‴	135.26		2′, 2⁗	117.17		2″, 2⁗′	69.90	3.72 (brs)
4, 4‴	177.83		3′, 3⁗	144.26*		3″, 3⁗′	70.00	3.57 (brd, 9.5)
5, 5‴	161.02		4′, 4⁗	135.17		4″,4⁗′	71.60	3.05 (t, 9.5)
6, 6‴	98.33	6.01 (d, 2.0)	5′, 5⁗	144.05*		5″,5⁗′	70.21	3.44 (m)
7, 7‴	163.78		6′, 6⁗	108.73	6.59 (s)	6″,6⁗′	17.71	0.85 (d, 5.8)
8, 8‴	92.80	5.41 (d, 2.0)						
9, 9‴	156.42							
10, 10‴	104.18							
5, 5‴-OH		12.66 (s)						

Coupling patterns and coupling constants (J) in Hz are given in parentheses.
* May be interchanged.