NOTE

Studies on the constituents from the fruits of *Phaleria macrocarpa*

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Abstract From the fruits of *Phaleria macrocarpa*, icariside C_3 (**1**), phalerin (**2**), and mangiferin (**3**) were isolated and their structures were identified on the basis of spectroscopic data. Icariside C_3 (**1**) showed a slow vasorelaxant activity against noradrenaline-induced contraction of isolated rat aorta. The structure of phalerin (**2**) was revised as 2,4′,6-trihydroxy-4-methoxybenzophenone-2-*O*-β-D-glucoside.

Keywords *Phaleria macrocarpa* · Vasorelaxant activity · Icariside C_3 · Phalerin · Mangiferin

Introduction

Phaleria macrocarpa (Thymelaeaceae), known as Mahkota Dewa, has been used traditionally for treatment of cancers in Indonesia [1]. So far, 3,4,5-trihydroxy-4′-methoxy-benzophenone-3-*O*-β-D-glucoside, named as phalerin, has been isolated from the leaves of *P. macrocarpa*, which showed cytotoxicity against myeloma cell line (NS-1) [2].

On the other hand, phenylpropanoids in many plants exhibit various bioactivities such as anti-bacterial, cytotoxicity, anti-oxidant, enzyme inhibition, and immuno-modulator properties. We reported that some phenylpropanoids, alkaloids, and peptides in plants showed the vasodilator activity on rat aorta [3, 4]. On continuing the

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search for chemical constituents with vasorelaxant activity in medicinal plants, we examined isolation of chemical constituents with vasorelaxant activity from the fruits of *Phaleria macrocarpa*. The extract prepared from *P. macrocarpa* showed a moderate vasorelaxant activity. The present paper deals with the isolation and identification of chemical constituents from the fruits of *P. macrocarpa*.

The dried fruits of *P. macrocarpa* (400 g) were extracted with $CHCl_3$ and then MeOH to yield $CHCl_3$ (15.4 g) and MeOH (96 g) extracts, respectively. The $CHCl_3$ extract (8 g), which showed a moderate vasorelaxant activity against isolated rat aortic strips, was subjected to a silica gel column ($CHCl_3$/MeOH, 1:0 → 0:1) and then purified by HPLC (MeOH/H_2O) to give compounds **1** (10.3 mg) and **2** (1.6 mg).

Compound **1** showed the pseudomolecular ion peak at *m/z* 441 (M+Na)$^+$ in ESIMS. Compared the NMR data of **1** with those of a known sesquiterpene glycoside, icariside C_3, isolated from *Epimedium grandiflorum* var. *thunbergianum* [5], the similar chemical shift pattern was observed. Detailed analysis of HSQC and HMBC correlations, compound **1** was identified with icariside C_3 [5] (Fig. 1).

Compound **2** showed the pseudomolecular ion peak at *m/z* 423 (M+H)$^+$ in ESIMS, and the molecular formula, $C_{20}H_{22}O_{10}$, was established by HRESIMS [*m/z* 423.1290 (M+H)$^+$ Δ −0.1 mDa]. ^{1}H and ^{13}C NMR data suggested the presence of one carbonyl, six sp^2 quaternary carbons, six sp^2 methines, one sp^3 methyl, one sp^3 methylene, and five sp^3 methines. Among them, signals due to six oxygen-bearing carbons at δ_c 62.6, 71.3, 74.8, 77.8, 78.4, and 102.7, which were assignable to glucose carbons appeared. Detailed analysis of 2D NMR correlations, **2** was identified with 2,4′,6-trihydroxy-4-methoxybenzophenone-2-*O*-β-D-glucoside, which has been isolated from *Gnidia involucrate* belonging

to Thymelaeaceae [6]. So far, phalerin was isolated as a benzophenone glycoside from the leaves of *Phaleria macrocarpa* [2]. Compared the NMR data of **2** with those of phalerin, the same chemical shifts were observed and therefore **2** and phalerin were the same compounds, although the proposed two structures were not identical as shown in Fig. 2. The difference between **2** and phalerin was the substituent pattern of the aromatic rings of the benzophenone. HMBC correlations as shown in Fig. 3 and NOESY correlations between the methoxy methyl and two aromatic protons of H-3 and H-5 clearly indicated the structure of **2** and phalerin to be 2,4′,6-trihydroxy-4-methoxybenzophenone-2-*O*-β-D-glucoside.

The MeOH extract was subjected to a Diaion HP-20 column (H_2O/MeOH, 1:0 → 0:1) and then purified by HPLC (MeOH/H_2O) to give a *C*-glucosylxantone (**3**) together with a benzophenone glycoside (**2**). Compound **3**
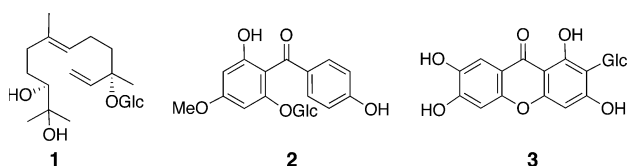

Fig. 1 Structures of icariside C_3 (**1**), 2,4′,6-trihydroxy-4-methoxy-benzophenone-2-*O*-β-D-glucoside (**2**), and mangiferin (**3**)

2, 4', 6-trihydroxy-4-methoxy-benzophenone-2-*O*-β-D-glucoside (**2**)
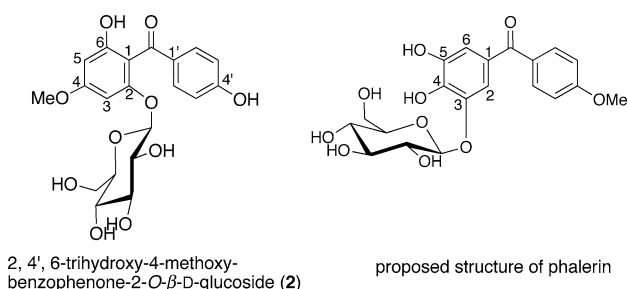
proposed structure of phalerin

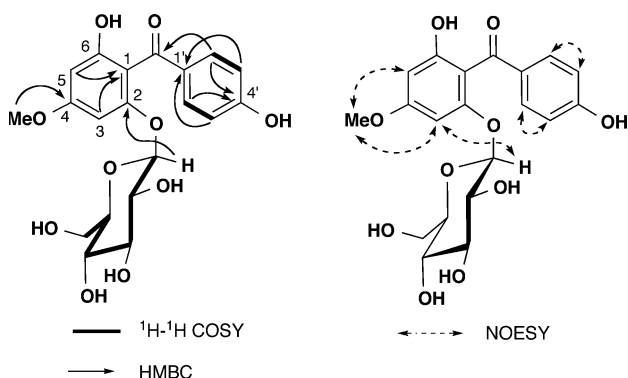**Fig. 2** Structures of **2** and the proposed structure of phalerin

Fig. 3 2D NMR correlations for **2**

was identified with mangiferin by comparison of spectral data with those described in the literature [7].

The $CHCl_3$ extract from *P. macrocarpa* showed a moderate vasorelaxant activity. When noradrenaline (NA) 3×10^{-7} M was applied to thoracic aortic rings with endothelium after achieving a maximal response, we added **1–3** at 10^{-4} M [8]. Icariside C_3 showed a slow vasorelaxant effect, whereas **2** and **3** were found to have no vasorelaxant effect.

Calcium ion can contract aortic rings concentration dependently in Ca^{2+}-free Krebs–Henseleit solution (KHS) after depolarization with isotonic high K^+ (60 mM) by the influx via voltage-dependent Ca^{2+} channels (VDCs); this contraction was not inhibited by icariside C_3.

Before NA 3×10^{-7} M was applied to thoracic aortic rings, the aortic rings were exposed to icariside C_3 (**1**:10^{-4} M) for one hour. The NA-induced contractions were divided into two phases. The first contraction is called the phasic phase, characterized by an initial rapid and transient contraction, and the second is the tonic phase, exhibiting a long-lasting contraction. Compound **1** did not inhibit the phasic contractions, while it clearly inhibited the tonic contractions, indicating **1** did not affect receptor of NA (NAα1) (Figs. 4, 5).

On the other hand, concentration-response relationships for relaxant responses of isoproterenol on the aortic rings precontracted with 3×10^{-7} M NA was shown in Fig. 6. As icariside C_3 (**1**) enhanced vasorelaxant responses of isoproterenol, **1** inhibited NA-induced vasocontractions, which might be in part attributable to the involvement of an increase of the second messenger such as cAMP and cGMP, inhibition of phosphodiesterase, and activation of adenylate cyclase.
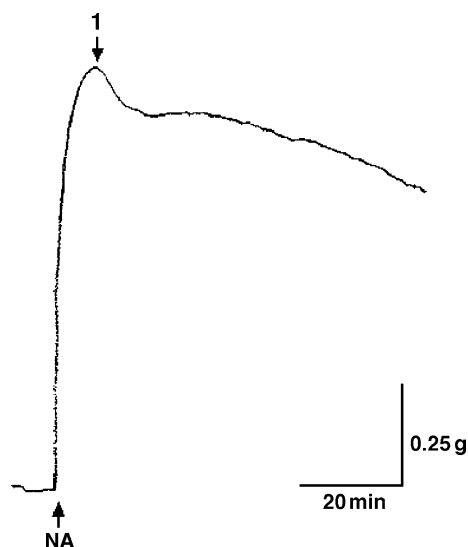

Fig. 4 Typical effect of 10^{-4} M of icariside C_3 (**1**) on aortic rings precontracted with 3×10^{-7} M noradrenaline (*NA*)
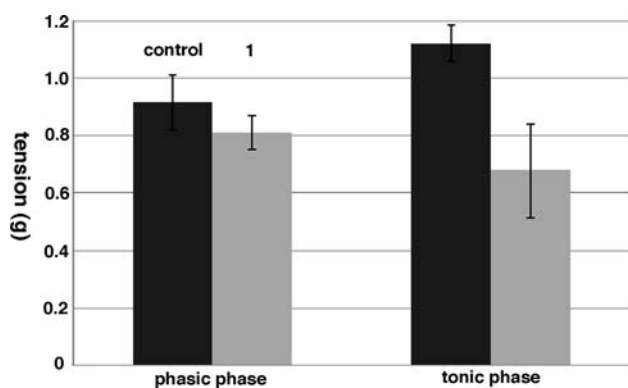
Fig. 5 Effects of **1** (10^{-4} M) on the NA-induced phasic and tonic contractions of rat aortic rings
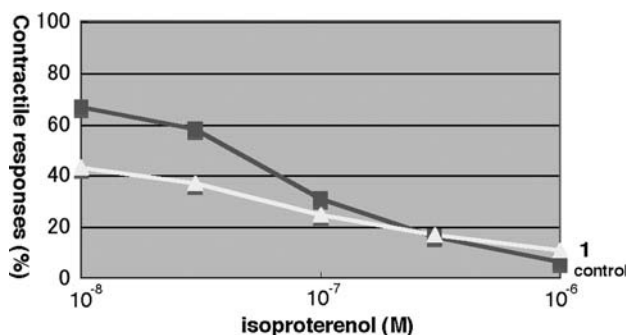

Fig. 6 Concentration-response relationships for relaxant responses of isoproterenol on the aortic rings precontracted with 3×10^{-7} M NA

Experimental section

General experimental procedures

Optical rotations were measured on a JASCO DIP-4 polarimeter. UV spectra were recorded on a Shimadzu UV1600PC spectrophotometer and IR spectra on a Perkin–Elmer 1710 spectrophotometer. ^{1}H and 2D NMR spectra were recorded on a 600 MHz spectrometer at 300 K, while ^{13}C NMR spectra were measured on a 150-MHz spectrometer. Chemical shifts were reported using residual CD$_3$OD (δ_H 3.31 and δ_C 49.0) as internal standard. Standard pulse sequences were employed for the 2D NMR experiments. Mass spectra were obtained using a Micromass LCT spectrometer.

Plant material

The mature fruits of *Phaleria macrocarpa* (Thymelaeaceae) were collected at Pasuruan (ca. 300 m a.s.l), East Java, Indonesia in 2005. The botanical identification was made by Mr. Abdul Rahman, MSi, Department of Natural Products, Faculty of Pharmacy, Airlangga University,

Indonesia. Voucher specimens have been deposited at the Department of Natural Products, Faculty of Pharmacy, Airlangga University.

Extraction and isolation

The sun-dried fruits of *Phaleria macrocarpa* (400 g) was crushed and macerated with CHCl$_3$ (24 h) at room temperature (800 ml×6), then the residue was macerated (24 h) with MeOH (800 ml×6). The extracts were then evaporated in rotary evaporator (40°C) yield CHCl$_3$ extract (15.4 g) and MeOH extract (96 g). The CHCl$_3$ extract was chromatographed on silica gel using CHCl$_3$/MeOH (5:2) and then the fourth fraction was purified by HPLC (60% MeOH/H$_2$O) to give icariside C$_3$ (**1**, 0.005%) [5]. The first fraction was purified by HPLC (35% MeOH/H$_2$O) to give 2,4′,6-trihydroxy-4-methoxybenzophenone-2-*O*-β-D-glucoside (**2**, 0.0008%). The MeOH extract was chromatgraphed on Diaion HP-20 using MeOH/H$_2$O and then the fraction eluted from 60% MeOH was purified by HPLC (35% MeOH/H$_2$O) to give 2,4′,6-trihydroxy-4-methoxybenzophenone-2-*O*-β-D-glucoside (**2**, 0.007%) and mangiferin (**3**, 0.008%) [7].

2,4′,6-Trihydroxy-4-methoxybenzophenone-2-*O*-β-D-glucoside (2)

Colorless solid; $[\alpha]_D^{20}$ +4° (*c* 0.9, MeOH); UV (MeOH) λ_{max} 205 nm (ε 3,400), 225 nm (ε 1,300), and 290 nm (ε 1,200); IR (KBr) ν_{max} 3,380, 2,940, 2,880, 1,620, 1,510, 1,420, 1,390, 1,320, 1,280, 1,200, 1,160, 1,080, and 920 cm^{-1}; ^{1}H NMR (CD$_3$OD); δ3.25 (1H, dd, *J* =9.2, 9.2 Hz, H-4″), 3.38 (2H, m, H-3″ and H-5″), 3.64 (1H, dd, *J* =6.0, 11.9 Hz, H-6″), 3.80 (3H, s, OMe), 3.85 (2H, dd, *J*=2.3, 11.9 Hz, H-2″ and H-6″), 4.86 (1H, d, *J* =7.8 Hz, H-1″), 6.16 (1H, d, *J* =2.0 Hz, H-5), 6.38 (1H, d, *J* = 2.0 Hz, H-3), 6.68 (2H, d, *J* =8.7 Hz, H-3′ and H-5′), and 7.65 (2H, d, *J* =8.7 Hz, H-2′ and H-6′). ^{13}C NMR (CD$_3$OD); δ55.9 (OMe), 62.6 (C-6″), 71.3 (C-4″), 74.8 (C-2″), 77.8 (C-3″), 78.4 (C-5″), 94.8 (C-5), 97.0 (C-3), 102.7 (C-1″), 112.4 (C-1), 117.5 (C-3′ and C-5′), 129.3 (C-1′), 134.0 (C-2′ and C-6′), 158.3 (C-6), 158.9 (C-2), 163.9 (C-4), 169.0 (C-4′), and 196.7 (C=O); ESIMS *m/z* 423 (M+H)$^+$; HRESIMS *m/z* 423.1290 (M+H; calcd for C$_{20}$H$_{23}$O$_{10}$, 423.1291).

Vasodilator assay

A male Wistar rat weighting 340 g was killed by bleeding from carotid arteries under an anesthetization [8]. A section of the thoracic aorta between the aortic arch and the

diaphragm was removed and placed in oxygenated, modified Krebs-Henseleit solution (KHS: 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, and 11.0 mM glucose). The aorta was cleaned of loosely adhering fat and connective tissue and cut into ring preparations 3 mm in length. The tissue was placed in a well-oxygenated (95% O_2, 5% CO_2) bath of 10 ml KHS solution at 37°C with one end connected to a tissue holder and the other to a force-displacement transducer (Nihon Kohden, TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g. During this time, the KHS in the tissue bath was replaced every 20 min.

After equilibration, each aortic ring was contracted by treatment with 3×10^{-7} M NA. The presence of functional endothelial cells was confirmed by demonstrating relaxation to 10^{-5} M acetylcholine (Ach), and aortic ring in which 80% relaxation occurred, were regard as tissues with endothelium. When the NA-induced contraction reached plateau, each sample was added.

These animal experimental studies were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, and under the supervision of the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Science, Sports Culture, and Technology of Japan.

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