Phalerin, a new benzophenoic glucoside isolated from the methanolic extract of Mahkota Dewa \textit{[Phaleria macrocarpa (scheff). Boerl.]} leaves

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Abstract

Mahkota Dewa \textit{[Phaleria macrocarpa (Scheff). Boerl.]} is used traditionally to treat cancers in Indonesia. Extract methanol of the leaves displayed a small LC50 value (63.16 µg/ml) on BST (Brine Shrimp Lethality test) assay; therefore a phytochemical study of this extract was undertaken. A new benzophenoic glucoside was isolated from the methanol extract and identified as 4,5-dihydroxy,4'-methoxybenzophenone-3-O-β-D-glucoside (Phalerin) based on its spectroscopic data. Phalerin was cytotoxic to myeloma cell line (NS-1) \textit{(in vitro)} having IC50 of 83 µg/ml or 1.9 x 10^{-1} mM.

Key words: \textit{Phaleria macrocarpa}, Phalerin, myeloma

Introduction

The plant Mahkota Dewa \textit{[Phaleria macrocarpa (Scheff.) Boerl.]} is utilized as alternative medicine to treat cancers in Indonesia (Harmanto, 1998). Preliminary study indicated that methanol extract of the leaves of \textit{P. macrocarpa} displayed a small LC50 value (63.16 µg/ml) on the BST screening model that was associated with cytotoxic activity (Mae et al, 2002; McLaughlin and Rogers, 1998). Therefore this study was aimed to isolate and identify compounds present in the methanolic extract, and then to determine their cytotoxicity \textit{(in vitro)} on myeloma cell line (NS-1). Now we report isolation, identification of Phalerin from the methanol extract of the leaves of \textit{P. macrocarpa} and its cytotoxicity \textit{(in vitro)} on myeloma cell line (NS-1).

Methodology

General experimental procedure. The optical rotation ([α]D) was taken on Polaxc-D, Atago, Austria and the UV spectrum was measured on a Cary 1Bio UV-Visible spectrophotometer. The IR spectrum was obtained using a PYE-UNICAM Sp-
Extraction, partition and Isolation of Phalerin:
The leaves of Phaleria macrocarpa (Scheff), Boerl. were collected from Kalurahan Wedomartani, district of Sleman, Province of Yogyakarta, Indonesia in November 2001. The plant species was identified by Department of Pharmacognosy, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia. The voucher specimen (BF 102, code PM) was deposited in the department cited above. The leaves were air-dried and ground. The powdered material (0.5 kg) was macerated with methanol (3 L) at room temperature for 24 hours, and then filtered to separate filtrate from residue. The residue was macerated 3 times, and all the filtrates were combined and dried in vacuo to dryness to give methanol extract (61.2 g). The methanol extract was triturated with CHCl₃ to give CHCl₃ soluble (10.1 g) and CHCl₃ insoluble (50.5 g) fractions. The CHCl₃ insoluble fraction was fractionated by vacuum liquid chromatography [SiO₂ Prep. grade (5 g); EtOAc with increasing amount of methanol] to give 14 fractions that were combined to 5 fractions (I-V) on the basis of their tlc profiles similarities. Phalerin was isolated by preparative thin layer chromatography [SiO₂ prep. grade, CHCl₃:EtOAc (1:4 v/v) developed twice]

Cytotoxicity assay: The test was performed in 96 wells tissue culture plates by MTT assay (Moosman, 1983). Initially, stock solution was made by dissolving Phalerin (5.0 mg) in DMSO (1.0 ml), the stock solution concentration was 5 mg/ml.

The myeloma cells (NS-1) were maintained in a continuous logarithmic culture in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand island, New York, USA), streptomycin (100 µg/ml), penicillin (100 units/ml) and glutamin (2 mM). The cell was cultured at 37°C in a humidified incubator with 5% CO₂.

The experiment was started with 50 µl tumor cell suspensions (2x10⁴ cells/well) that was plated in 96 then well flatbottom microtiter plates. Phalerin was added in a two-fold dilution sequence of six steps, made in a full medium, started with the 500 µg/ml of stock solution. Every dilution was used in quadruplicate by adding 50 µl to a column of four wells. Column 1 was used as untreated cells, and column 2 as control medium. The plates were pre-incubated for 24, 48 and 72 hours at 37°C, 5% CO₂, and then MTT solution (5 mg/ml in PBS) was added (10 µl/well) to the cells. The plates were pre-incubated for 4 hours at 37°C, 5% CO₂, and then 10 µl/well was added to the cells and mixed thoroughly. The density was measured at 540 nm using an automated microplate reader (ELISA plate reader) after 24, 48 and 72 hours incubation. The doses tested for Phalerin were 6.94, 20.83, 62.50, 125, 250 and 500 µg/ml.

Result and Discussion
Phalerin appeared as reddish brown flakes having optical rotation [α]D25 +0.53 (ε, 0.15, EtOH), showed UV absorption at 292 nm
indicating the presence of C=O substituted aromatic ring (Silverstein et al., 1981). IR spectra of Phalerin showed absorption band at 3387 cm⁻¹ (broad) indicating the present of –OH group, absorption band at 2922 cm⁻¹ indicating the present of saturated –C-H group, while the unsaturated H-C was showed at 2698 cm⁻¹. Characteristic absorption band at 1616 cm⁻¹ strongly indicated the present of α-β unsaturated –C = O and the bands at 1512,
1436 cm\(^{-1}\) indicated the present of aromatic ring due to -C=C- (Silverstein \textit{et al}, 1981) (Figure 1). This data gave an idea that Phalerin was an aromatic compound.

EI-MS spectra of Phalerin displayed the molecular ion peak at m/z 445 [M + + 23 (Na)], therefore the molecular weight of Phalerin was 422; and the EI-HRMS of Phalerin determined the molecular formula of Phalerin was C\(_{20}\)H\(_{22}\)O\(_{10}\). It was clear that compound Phalerin was not a diterpenoid type compound although Phalerin consisted of 20 carbons, but instead Phalerin was an aromatic compound attached to a sugar as a glycoside. This assumption was confirmed further by the degrees of unsaturation value of Phalerin that was equal to 9.

The \(^{13}\)C-NMR (Figure 2) (Table I) spectra of Phalerin confirmed further the EI-HRMS data as it showed that Phalerin consisted of 20 carbons. Based on their chemical shift signals and APT technique data, the 20 carbons identities were determined as 12 Aromatic carbons [6 unsubstituted (HC=), and 6 substituted (–C=)], 1 –C=O, 5 HC-O- and 1 –CH\(_2\)-O- of sugar, and 1 CH\(_3\)O- groups (Silverstein and Webster, 1998).

\(^1\)H-NMR spectra data of Phalerin (Figure 4) (Table I) indicated the presence of aromatic protons (δ, 7.52, d, \(J=\) 8 Hz; and 6.60, d, \(J=\)8 Hz ), and each signal was integrated for 2 protons. Two other aromatic proton singlet signals (δ, 6.00 and 6.19) were also observed which indicated that these two protons were fairly separated from the neighbouring protons. Signal at δ, 3.55 ppm (s, integrated 3 Hs) was a methoxy group and signals between δ, 3.40 - 4.70 ppm was identified as HC-O- signals correspond to sugar moiety that was very much resemble to signals reported for β-D-glucose (Chulia and Kaouadji, 1985; Bloor and Qi, 1994; Wang \textit{et al}, 2003). Furthermore, the \(J (7 \text{ Hz})\) value between H-1” and H-2” indicated that the two protons coupling was axial-axial (β) as sugar molecules tends to form a stable conformation in which the –OH groups are nearly equatorial (Bruneton, 1999). Hydroxyl signal was observed as a broad signal at δ, 4.90 ppm.
corresponded to –OH of sugar as it was shown by the absence of carbon directly attached to –H signal (δ 4.90 ppm) on the 13C–1H 2D-NMR (Hetcor) spectra (Figure 5). Phalerin was deduced as 4,5-dihydroxy,4'-methoxybenzophenon-3-O-β-D-glucoside and it was named as Phalerin. In addition, the chemical shifts of carbons and protons of Phalerin (Table I) were assigned by 2D-NMR techniques (Cosy and Noesy) and the molecular model (Figure 7) was established, calculated using Hyperchem software.

As Mahkota Dewa was reported to be active as anticancer, then cytotoxic property of phalerin at various doses was tested on myeloma cell line (NS-1). The result was observed after 24, 48 and 72 hours incubation. Phalerin was cytotoxic to myeloma cell line...
Table II. Cytotoxic effect of Phalerin on mieloma cell line (in vitro) after 24, 48, and 72 hours incubation (Dose vs viability %)

<table>
<thead>
<tr>
<th>Dose (µg/ml)</th>
<th>Viability (%) (24 hours)</th>
<th>Viability (%) (48 hours)</th>
<th>Viability (%) (72 hours)</th>
</tr>
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<tbody>
<tr>
<td>6.94</td>
<td>97.84</td>
<td>74.57</td>
<td>63.51</td>
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<td>20.83</td>
<td>96.46</td>
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</tr>
<tr>
<td>500</td>
<td>7.60</td>
<td>7.11</td>
<td>2.33</td>
</tr>
</tbody>
</table>

Figure 6. Myeloma cells viability (%) due to Phalerin at various concentrations (6.94 – 500 µg/ml) in vitro.

Figure 7. Molecular model of Phalerin

(in vitro), and its toxicity was dose dependent (Table II). Smaller doses (µg/ml) of Phalerin, higher the myeloma cell viability (%) and it occurred on all period of times of incubation (24, 48, and 72 hours). The IC$_{50}$ of phalerin was calculated by probit analysis as 1.9 x 10$^{-1}$ M, furthermore cells viability (%) values of 72 hours period of incubation at the dose of 500 µg/ml were almost the same. This was possibly due to the lack of nutrition in the medium therefore the IC$_{50}$ was calculated at the 48 hours of incubation (Figure 6).

The IC$_{50}$ value of phalerin is considered high and said to be low cytotoxicity on myeloma cell line; however, cytotoxicity properties benzophenone derivatives have been reported lately. All the isolated benzophenone derivatives from Garcinia species display a strong apoptosis-inducing effect against human leukaemia cell line, and these compounds act
on growth suppression due to apoptosis mediated by the activation of Caspase-3 (Matsumoto et al., 2003). William et al. (2003) also reported that benzophenone derivatives (Guttiferone A, G) were cytotoxic to A2780 human ovarian cell line (IC\textsubscript{50} = 6.8 and 8.0 µg/ml, respectively). Phalerin is a glucoside compound but those reported compounds are not glycoside compounds; therefore it is possible that phalerin will be activated in the body by cleaving the glucoside bond (by glucosidase) to give the active product. This model of mechanism is known as enzyme activated substrate approach.

**Conclusion**

The major compound present in the methanolic extract of the leaves of Mahkota Dewa \([Phaleria macrocarpa\) (Scheff.) Boerl.] was identified as 4,5-dihydroxy,4'-methoxybenzophenone-3-O-β-D-glucoside or Phalerin. Phalerin was cytotoxic to myeloma cell line (NS-1) \((in vitro)\) having IC\textsubscript{50} = 83 µg/ml or 1.9 x 10\textsuperscript{-1} mM.

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**References**


