

Secondary Metabolites Isolated from The Dichloromethane Extract of Silver Fern (Pityrogramma Calomelanos)

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**Secondary Metabolites Isolated from The Dichloromethane Extract of
Silver Fern (*Pityrogramma Calomelanos*).**

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ABSTRACT

Three secondary metabolites namely n-tetratetracontane, β -sitosterol, and 2',6'-dihydroxy-4'-methoxydihydrochalcone had been isolated from the dichloromethane extract of the silver fern's aerial part (*Pityrogramma calomelanos*). The separation were conducted by vacuum liquid chromatography and flash chromatography techniques, while purification by recrystallization. The n-tetratetracontane, β -sitosterol, and 2',6'-dihydroxy-4'-methoxydihydrochalcone were obtained as colorless amorphous solid (m.p.54-55 oC), colorless crystal (136-135 oC), and pale yellow crystal (m.p. 169 – 171 oC), respectively. Their structures were identified base on the spectroscopic data (UV, IR, MS, and NMR) and by comparison with reported literature data.

Keywords: Pityrogramma calomelanos, n-tetratetracontane, β -sitosterol, 2',6'-dihydroxy-4'-methoxydihydrochalcone

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INTRODUCTION

Pityrogramma calomelanos was one of the ferns belonging the Polypodiaceae family widely distributed in tropical Asia, especially Indonesia. It usually grew in open region, near streams, slope of mountain, and old wall [1]. This fern was used as the ornamental plant and phytoremediation land polluted arsenic (As), zink (Zn), lead (Pb), and mercury (Hg) [2]. This fern had been used for traditional medicine. The leaves were used externally to heal wounds and stop bleeding. An infusion of the whole plant was used to increase male sexual stamina and to treat female haemorrhaging. An aqueous extract was drunk or applied locally to treat venereal disease in Guyana. It was also used for asthma, cough, cold, pneumonia, tuberculosis, and whooping cough. An infusion was used to treat pulmonary conditions [3].

Several flavonoid compounds in dihydrochalcone type had been separated from the fern species in *Pityrogramma* genus. For examples the flavonoid 2',6'-dihydroxy-4,3'-dimethoxy-4',5'-metilendioxy dihydrochalcone was isolated from *Pityrogramma ebenea* [4]. While from *Pityrogramma triangularis* had been separated dihydrochalcone namely 2',6',4-trihydroxy-3'-methyl-4'-methoxy dihydrochalcone and 2',6',4-trihydroxy-3',5'-dimethyl-4'-methoxy dihydrochalcone [5]. Therefore, the chemical constituents of *P. calomelanos* had not been reported. In the course of our studies, three secondary metabolites namely n-tetratetracontane (1), β -sitosterol (2), and 2',6'-dihydroxy-4'-methoxydihydrochalcone (3) had been isolated from the aerial part of *P. calomelanos*. In this paper, we reported the isolation and structure determination of those isolates.

MATERIALS AND METHODS

The aerial part of *P. calomelanos* was collected from the Kletak forest, Nangkajajar village, Pasuruan, East Java, Indonesia in March 2017. The n-hexane, benzene, dichlorometane, chloroform, ethyl acetate, methanol, ethanol, and sulphuric acid were obtained from local sources and were analytical grade (Merck). Kieselgel 60 GF-254 (Merck) and silica gel G 60 63-200 μ m (Merck) were used for vacuum liquid chromatography (VLC) and flash chromatography (FC), respectively. Precoated silica gel 60 F-254 (Merck) 0.25 mm, 20 x 20 cm was used for thin layer chromatography (TLC) and spots were detected by spraying with the sulphuric acid solution 5% (v/v) in ethanol followed by heating.

The equipments utilized in this experiment were Fisher John melting point apparatus, UV-Vis spectrophotometer (Shimadzu Pharmaspec UV-1800), infra red spectrophotometer (Shimadzu FTIR-8400S), NMR spectrophotometer (JEOL JNM ECA-500, operating at 500 MHz (1 H) and 125.7 MHz (13 C)), mass spectrometer (Shimadzu QP-2010S using electron impact, EI, ion mode).

29 Extraction and isolation

The aerial part dried powdered of *P. calomelanos* (5 kg) was exhaustively extracted successively with n-hexane and dichloromethane at room temperature. The dichloromethane extract was evaporated in vacuo, revealed 98 g (blackish green).

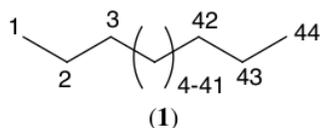
A portion of dichloromethane extract (5 g) was chromatographed by VLC and eluted with solvents of increasing polarity (n-hexane, n-hexane-ethyl acetate, ethyl acetate) yielded 155 fractions (15 mL each). Removal of the solvent under reduced pressure of the combined fractions of 4-9 gave the white solid (1,2 g). It was recrystallized in acetone yielded n-tetratetracontane (1) (131 mg). The combined fractions of 50-55 (165 mg) was fractionated sequentially by FC eluting with n-hexane-ethyl acetate (85:15) yielded 20 fractions. The combined fractions of 10-13 from FC was recrystallized in methanol gaved β -sitosterol (2) (80 mg). While the combined fractions of 68-78 (525 mg) from VLC was recrystallized in chloroform-n-hexane yielded 2',6'-dihydroxy-4'-methoxydihydrochalcone (3).

RESULTS AND DISCUSSION

n-Tetratetracontane (1) was obtained as colorless amorphous solid, mp. 54-55 $^{\circ}$ C, which gave negative test with Liebermann-Burchard reagent. UV (n-hexane) λ_{max} (log ϵ) : 207 (3.99). IR (KBr) ν_{max} : 2955, 2920 (alkyl C-H stretching), 1464, 1377 (alkyl C-H bending) cm^{-1} . EIMS, m/z (rel. int., %): 618 (M^+ , not appear), 253 (2), 239

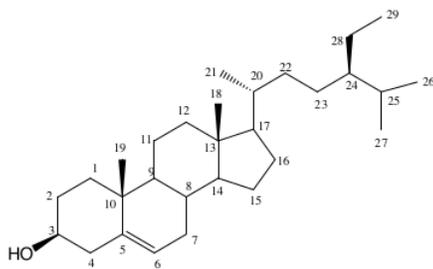
(2), 225 (2), 211 (3), 197 (3), 183 (3), 169 (3), 155 (7), 141 (10), 127 (10), 113 (13), 99 (23), 85 (52), 71 (74), 57 (100, base peak), 43 (61).

Compound **1** showed the negative results on the test using Liebermann-Burchard reagent. It indicated that isolate was not steroid or triterpene [6]. A absorption peak at 207 nm in the UV spectrum showed that **1** had not conjugation double bond. The absorption peak at 2955, 2920 (alkyl C-H stretching), 1464 and 1377 (alkyl C-H bend) cm^{-1} at the IR spectrum supported that **1** was long chain hydrocarbon in lipid group. The EIMS spectrum of **1** showed a molecular ion peak at m/z 618, corresponding a molecular formula $\text{C}_{44}\text{H}_{90}$ (DBE = 0). The release of the methylene fragment ions ($m/z = 14$) start from m/z 253 until m/z 43 also supported the presence of straight long chain hydrocarbon in **1**. Based on the GC-MS database library, compound **1** was predicted n-tetratetracontane. From the above results, compound **1** was identified as n-tetratetracontane. The presence of **1** is the first time reported from *P. calomelanos* and the fern in *Pityrogramma* genus. However its presence was ever reported from the *Cassia fistula* oil [7].



β -Sitosterol (2) was obtained as colorless amorphous powder, mp. 136-137 °C, which gave positive test with Liebermann-Burchard reagent (blue). It showed one spot on TLC using three eluents system with R_f of 0.29 (n-hexane-ethyl acetate = 9 : 1), 0.71 (n-hexane-ethyl acetate = 4 : 1), and 0.74 (chloroform-ethyl acetate = 9 : 1). UV (n-hexane) λ_{max} (log ϵ) : 207 (3.71). IR (KBr) ν_{max} : 3426 (O-H stretching), 2939, 2851 (alkyl C-H stretching), 1651 (C=C stretching), 1464, 1379 (alkyl C-H bending), 1053 (C-O stretching) cm^{-1} . $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ (ppm) : 0.68 (s, H-18), 0.81 (d, $J = 6.8$ Hz, H-27), 0.84 (d, $J = 6.8$ Hz, H-9), 0.85 (t, $J = 8.0$ Hz, H-29), 0.92 (d, $J = 6.4$ Hz, H-21), 1.01 (s, H-19), 3.52 (m, H-3), 5.35 (brd, $J = 5.2$ Hz, H-6). $^{13}\text{C-NMR}$ (125.8 MHz, CDCl_3) δ (ppm) : 11.8 (C-18), 12.0 (C-29), 18.8 (C-21), 19.0 (C-27), 19.4 (C-19), 19.8 (C-26), 21.1 (C-11), 33.0 (C-28), 24.3 (C-15), 26.0 (C-23), 28.2 (C-16), 29.1 (C-25), 31.6 (C-2), 31.8 (C-7), 31.9 (C-8), 33.9 (C-22), 36.1 (C-20), 36.5 (C-10), 37.2 (C-1), 39.7 (C-12); 42.3 (C-13), 42.3 (C-4), 45.8 (C-24), 50.1 (C-9), 56.0 (C-17), 56.7 (C-14), 73.5 (C-3), 123.5 (C-6), 142.4 (C-5). EIMS, m/z (rel. int. %): 414 (M^+ , 38), 396 (21), 381 (17), 329 (31), 273 (14), 255 (21), 231 (17), 213 (28), 119 (34), 95 (52), 43 (100, base peak).

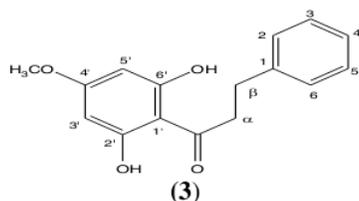
Compound **2** showed the positive results on the test using Liebermann-Burchard reagent. It showed that isolate was a steroidal [6]. A absorption peak at 207 nm in the UV spectrum showed that **2** had not the conjugation double bond. The EIMS spectrum of **1** showed a molecular ion peak at m/z 414, corresponding a molecular formula $\text{C}_{29}\text{H}_{50}\text{O}$ (DBE = 5). The fragment ion at m/z 255 was characterized for β -sitosterol (DBE = 5), resulted from releasing the side chain and a water molecule [8]. The absorption peak at 3426 (O-H), 2934, 2851 (alkyl C-H stretching), 1651 (C=C), 1464, 1379 (alkyl C-H bending), 1053 (C-O) supported that **2** was β -sitosterol. The existence of hydroxyl group at **2** also supported by the presence of a fragment ion at m/z 396 ($\text{M}-\text{H}_2\text{O}$)⁺ and multiplet proton signal at δ_{H} 3.53 ppm due to oxyalkyl proton (H-3). Based on the above results, compound **2** was identified as β -sitosterol. The proton signal at δ_{H} 3.53 ppm due to olefinic proton (H-6) and a carbon signal at δ_{C} 121.7 ppm (C-5) and 140.8 ppm (C-6), supported the presence of double bond (C=C) at **2**. The $^{13}\text{C-NMR}$ spectrum of **2** showed 29 proton signals containing of one oxyalkyl carbon (δ_{C} 71.8), two olefinic carbons (δ_{C} 121.7 and 140.8), and the others were alkyl carbon signals. The $^1\text{H-NMR}$, $^{13}\text{C-NMR}$, and EIMS spectral data of **2** corresponded with reported data of β -sitosterol in literature [8,9,10]. From the above results, **2** was proposed for the structure of β -sitosterol. This steroid is the first time reported from *P. calomelanos* and the fern in *Pityrogramma* genus. However its presence was ever reported from the other fern, i.e. *Chingia sakayensis* [10].



(2)

2',6'-dihydroxy-4'-methoxydihydrochalcone (3) was obtained as pale yellow crystal (chloroform-n-hexane), mp. 169-171°C, which gave positive test with FeCl_3 (greenish yellow) and Shinoda test (Mg-HCl) (yellow). It showed one spot on TLC using three eluents system with R_f of 0.86 (chloroform-ethyl acetate = 9 : 1), 0.44 (n-hexane-ethyl acetate = 4 : 1), and 0.31 (n-hexane-ethyl acetate = 9 : 1). UV (MeOH) λ_{max} (log ϵ) : 285 (3.72), 330 (sh) (2.89) nm; (MeOH + NaOH): 295 (3.68), 364 (sh) (3.21) nm; (MeOH+ AlCl_3): 305 (3.76), 374 (sh) (2.83) nm; (MeOH+ AlCl_3 +HCl): 288 (3.68), 375 (sh) (2.50) nm; (MeOH+NaOAc): 285 (3.75) nm; (MeOH+NaOAc+ H_3BO_3): 285 (3.76) nm. IR (KBr) ν_{max} : 3269 (OH stretching), 3023 (aromatic C-H stretching), 2918, 2849 (alkyl C-H stretching), 1643 (chelated C=O stretching), 1595, 1528 (aromatic C=C stretching), 1427, 1366 (alkyl C-H bending), 1213, 1080 (C-O stretching) cm^{-1} . $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ (ppm) : 3.02 (2H, t, J = 7.9 Hz, H- β), 3.40 (2H, t, J = 7.3 Hz, H- α), 3.79 (3H, s, 4'-OCH₃), 5.93 (2H, s, H-3' and H-4'), 7.25 (5H, m, H-2,3,4,5,6). $^{13}\text{C-NMR}$ (125.8 MHz, CDCl_3) δ (ppm) : 30.7 (C- β), 45.8 (C- α), 55.7 (4'-OCH₃), 94.6 (C-3',5'), 104.9 (C-1'), 126.1 (C-4), 128.6 (C-2,6), 128.7 (C-3,5), 141.8 (C-1), 165.7 (C-2',4',6'), 204.7 (C=O). EIMS, m/z (rel. int., %): 272 (25), 255 (6), 177 (3), 167 (100, base peak), 140 (38), 136 (3), 124 (3), 111 (6), 104 (6), 91 (22), 77 (6), 69 (6), 51 (6), 39 (6).

Compound **3** showed the positive results on the test using FeCl_3 reagent (yellowish green) and Shinoda test (Mg + HCl) (yellow). It indicated that isolate was a flavonoid compound [6]. The absorption bands of IR spectrum at 3269 (OH), 3023 (aromatic C-H), 2918, 2849 (alkyl C-H), 1643 (chelated C=O), 1595, 1528 (aromatic C=C) supported that isolate was a flavonoid. The UV spectrum of **3** indicated absorption characteristic of dihydrochalcone-type compounds at 285 nm (band II) and 330 nm (sh) (band I) [6,11]. No bathochromic shift of band I on adding of NaOH and NaOAc reagents indicated that the isolates did not have a free OH group at C-4'. The bathochromic shift of band II on adding of AlCl_3 + HCl reagent supports the existence of an OH group free at C-6'. While the addition of NaOAc + H_3BO_3 did not cause the bathochromic shift of band II. This showed the absence of ortho-dihydroxy group at A ring in flavonoid isolate. Two triplet proton signal at δ_{H} 3.02 and 3.40 ppm due to H- α and H- β , respectively, supported that **1** had a basic skeleton of dihydrochalcone. While the presence of singlet proton signal at δ_{H} 3.79 ppm indicated that C-4' binded a methoxy group. Multiplet proton signal at δ_{H} 7.25 showed that **3** had a structure similar to the B ring of pinocembrine [12,13] that was not substituted. The $^{13}\text{C-NMR}$ spectrum of **3** exhibited 11 carbon signals represented 16 carbon signals consisted of alkyl carbon [δ_{C} 30.7 (C- β), 45.8 (C- α)], methoxy carbon [δ_{C} 55.7 (4'-OCH₃)], aryl carbon [δ_{C} 94.6 (C-3',5'), 104.9 (C-1'), 126.1 (C-4), 128.6 (C-2,6), 128.7 (C-3,5), 141.8 (C-1)], oxyaryl carbon [δ_{C} 165.7 (C-2',4',6')] and carbonyl carbon [δ_{C} 204.7 (C=O)]. The EIMS spectrum of **3** showed a molecular ion peak at m/z 272, corresponding a molecular formula $\text{C}_{16}\text{H}_{16}\text{O}_4$. From the above results, compound **3** was identified as 2',6'-dihydroxy-4'-methoxy-dihydrochalcone. This flavonoid is the first time reported from P. calomelanos and the fern in Pityrogramma genus. However its existence was ever reported from the Notholaena sulphurea and Populus spp [14].



CONCLUSION

It had been separated the three secondary metabolites namely n-tetradetracontane, β -sitosterol, and 2',6'-dihydroxy-4'-methoxydihydrochalcone from the dichloromethane extract of *P. calomelanos*'s aerial part. Three isolates were obtained as colorless amorphous solid (m.p.54-55 °C), colorless crystal (136-137 °C), and pale yellow crystal (m.p. 169-171 °C), respectively. This is the first time reported for existence of three isolates from *P. calomelanos* and the fern in *Pityrogramma* genus bigger percent yield.

ACKNOWLEDGEMENTS

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