Immunostimulant activity of steroid compound from the indonesian silver fern (P. calomelanos)

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Immunostimulant activity of steroid compound from the indonesian silver fern (*P. calomelanos*)

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Abstract. A secondary metabolite in steroid compound, namely β-sitosterol had been isolated from the n-hexane extract of the Indonesian silver fern. Extraction was conducted by maceration method, separation by chromatography techniques (vacuum liquid chromatography and flash chromatography), purification by recrystallization, and identification of its molecular structure by the spectroscopic methods and by comparison with reported literature data. The steroid isolate was yielded as colorless amorphous powder (melting point of 136– 137 °C). Based on the carbon clearance method, isolated steroid showed immunostimulant activity with phagocytosis index of 1.625.

1. Introduction

Immunostimulants are ingredients that can increase the work of the components of the immune system It is able to modulate the human immune system by acting to improve immune system imbalances [1]. The use of immunostimulants in therapy is often hampered by the high cost of immunostimulants available in the patented drug market, the majority of which are imported from abroad. Therefore, the use of immunostimulants from natural ingredients is indispensable, in addition to relatively low prices, the side effects are relatively low [2,3]. Many secondary metabolites from plants have properties as immunostimulants. The secondary metabolites that have bioactivity as immunostimulant agents are terpenoids, steroids, alkaloids and polyphenols [4].

The Indonesia silver fern (*P. calomelanos*) was one of the ferns widespead in Indonesia. It was usually found in open area, mountain slope, old wall, and near streams [5]. People used this fern as the decorative plants for a long time ago. In addition, it had been used for phytoremediation of soil contaminated by arsenic (As) and heavy metals (Hg, Zn, and Pb) [6]. This fern had been used for traditional medicine. An infusion of the whole plant was utilized to treat female haemorrhaging and to enhance the male sexual stamina. The leaves were used externally to heal wounds and stop bleeding. In Guyana, an aqueous extract was drunk or applied locally to treat venereal disease. An infusion was used to treat pulmonary conditions, asthma, cough, cold, tuberculosis, and pneumonia [7].

Wollenweber, *et al* reported that several flavonoids in dihydrochalcone type had been isolated from the fern species in *Pityrogramma* genus. For examples, the dihydrochalcone compound namely 2',6',4-trihydroxy-3'-methyl-4'-methoxy dihydrochalcone had been isolated from *P. triangularis* [8]. Previous work, we had reported secondary metabolites separated from the dichloromethane extract of the silver fern 's aerial part [9]. In this paper, we reported the isolation of steroid from *n*-hexane extract of P. *calomelanos* and assay of its immunostimulant activity using carbon clearance method.

2. Materials and methods

2.1. Materials and Equipments

The Indonesian silver fern's aerial part was obtained from the Kletak forest, Nangkajajar village, Pasuruan, East Java, Indonesia in March 2017. The n-hexane, ethyl acetate, methanol, ethanol and suphuric acid were obtained in the form of an analytical grade (Merck). Kieselgel 60 GF-254 (Merck) was used for vacuum liquid chromatography, while the silica gel G 60 63-200 µm (Merck) was used for flash chromatography. Thin layer chromatography was conducted using the precoated silica gel 60 F-254 (Merck) 0.25 mm, 20 x 20 cm. The detection of spot at TLC was carried out using the sulphuric acid solution 5% (v/v) in ethanol. The male mice, tween 80, physiological NaCl 0.9 %, Na-EDTA, Na-CMC 0.3%, acetic acid 1%, and chinese ink for immunostimulant activity.

The equipments utilized in this experiment were the electrothermal melting point apparatus, UV-Vis spectrophotometer (Shimadzu Pharmaspec UV-1700), infra-red spectrophotometer (Shimadzu FTIR-8400S), NMR spectrometer [JEOL JNM ECA-500 spectrometer, operating at 500 MHz (^{1}H) and 125.7 MHz (^{13}C)], and mass spectrometer (Shimadzu QP-2010S) using electron impact (EI).

2.2. Extraction and isolation

The silver fern's aerial part (10 kg) was dried at room temperature for ten days. Then sample was milled so that it produced the dried powder which was ready to be extracted. As much as 5 kg of the dried powder of silver fern's aerial part was extracted with n-hexane at room temperature using maceration method. Removal of the solvent in vacuo, resulted 113 g of blackish green residue. As much as 5 g of n-hexane extract was separated by vacuum liquid chromatography (VLC) and eluted with solvents of increasing polarity (n-hexane, n-hexane-ethyl acetate mixtures, ethyl acetate) produced 122 fractions (the volume of each fraction of 15 mL). The combined fractions of 48-57 (345.4 mg) was fractionated sequentially by flash chromatography (FC) eluting with n-hexane-ethyl acetate (85 :15) resulted 22 fractions (the volume of each fraction of 10 mL). The combined fractions of 8-15 (88.4 mg) from FC was recrystalized in methanol yielded β-sitosterol (**1**) (29.4 mg).

2.3. Immunostimulant activity assay using the carbon clearance method

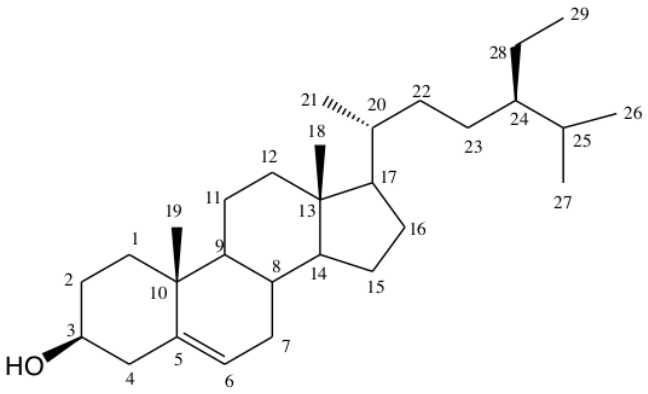
Ten male mice were divided into 2 groups, namely negative control group (Na-CMC 0.3%) and treatment group (given steroid isolate from silver ferns with a dose of 1 mg / kg BW). Each group consisted of 5 test animals were given a solution preparation test orally once a day, for 6 consecutive days. On the 7th day the mice from all groups were taken blood through the tail vein, put it in the drop plate which already contained sodium citrate (anticoagulant). Piped blood as much as 25 µL, lysed with the addition of 4 mL of 1% acetic acid, then the absorbance was measured by UV-Vis spectrophotometer at a wavelength of 650 nm. This first blood sample is used as blank (0th minute). Mice from all groups were injected with a carbon suspension of 0.1 mL / 10 g BW of mice intravenously at the tail. A total of 25 µL of mouse blood were taken at 3, 6, 9, 12, and 15 minutes, after carbon injection, and treated in the same way as blood taken before carbon injection (0th minute). The data obtained were analyzed using linear regression analysis to determine phagocytic constants and phagocytosis index (IF) [10]

3. Results and analysis

Isolate **1** was yielded as colorless amorphous powder, melting point of 136-137 °C, which gave positive test with Liebermann-Burchard reagent (blue). It gave one spot on TLC using three elution system with Rf of 0.74 (chloroform-ethyl acetate = 9: 1), 0.71 (*n*-hexane-ethyl acetate = 4: 1), and 0.29 (*n*-hexane-ethyl acetate = 9: 1). The UV spectrum of **1** (n-hexane) gave the maximum wavelength at 207 nm (log ε = 3.71). The IR spectrum of **1** (KBr) showed the absorption peaks at 3426 (O-H stretching), 2934, 2851 (alkyl C-H stretching), 1651 (C=C stretching), 1464, 1379 (alkyl C-H bending), 1053 (C-O stretching) cm^{-1}. The ^{1}H-NMR spectrum of **1** ($CDCl_3$) gave the proton signals

a 0.68 (*s*, H18), 0.81 (*d, J* = 6.8 Hz, H27), 0.84 (*d, J* = 6.8 Hz, H26), 0.85 (*t, J* = 8.0 Hz, H29), 0.92 (*d, J* = 6.4 Hz, H21), 1.01 (*s*, H19), 3.52 (*m*, H3), 5.35 (*brd, J* = 5.2 Hz, H6) ppm. While the ^{13}C-NMR spectrum of **1** ($CDCl_3$) showed the carbon signals at 11.8 (C18), 12.0 (C29), 18.8 (C21), 19.0 (C27), 19.4 (C19), 19.8 (C26), 21.1 (C11), 23.0 (C28), 24.3 (C15), 26.0 (C23), 28.2 (C16), 29.1 (C25), 31.6 (C2), 31.8 (C7), 31.9 (C8), 33.9 (C22), 36.1 (C20), 36.5 (C10), 37.2 (C1), 39.7 (C12); 42.3 (C13), 42.3 (C4), 45.8 (C24), 50.1 (C9), 56.0 (C17), 56.7 (C14), 73.5 (C3), 123.5 (C6), 142.4 (C5) ppm. The EIMS spectrum of **1** gave peaks at m/z (rel. int., %) of 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).

Isolate **1** gave the positive results on the test using Liebermann-Burchard reagent. It showed that isolate was a steroid [11]. A absorption peak at 207 nm in the UV spectrum showed that **1** had not the conjugation double bond. A molecular ion peak of m/z 414 at EIMS spectrum of **1** corresponded a molecular formula $C_{29}H_{50}O$ (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 [12]. The absorption peak at 3426 (O-H), 2934, 2851 (alkyl C-H starching), 1651 (C=C), 1464, 1379 (alkyl C-H bending), 1053 (C-O) supported that **1** was β-sitosterol. The presence fragment ion at m/z 396 $(M-H_2O)^+$ and multiplet proton signal at δ_H 3.53 ppm due to oxyalkyl proton (H-3) supported the existence of hydroxyl group at **1.** Based on the above results, isolate **1** was identified as β-sitosterol. The olefinic proton signal (H-6) at δ_H 3.53 ppm and carbon signal at δ_C 121.7 ppm (C-5) dan 140.8 ppm (C-6), supported the presence of double bond (C=C) at **1.** The ^{13}C-NMR spectrum of **1** 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}H-NMR, ^{13}C-NMR, and EIMS spectral data of **1** corresponded with reported data of *β*- sitosterol in literature [12,13]. From the above results, **1** 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* [14] and *Cassia fistula* [15].



(**1**)

Figure 1. The ^{13}C-NMR spectrum of sample

Based on the immunostimulant activity assay known that β-sitosterol had phagocytosis index (FI) of 1.625. The isolated steroid could be categorized as a strong immunostimulant because its FI was bigger than 1.5 [4]. The *β*- sitosterol is one of the steroid compound predicted had immunostimulant activity because it could improve the body immune system induced by cyclophosphamide so percentage of T CD8+ cell increase [16]. Thus the *β*- sitosterol had potency to be developed as immunostimulant agent.

4. Conclusion

It had been separated a secondary metabolite namely β-sitosterol from the n-hexane extract of silver fern 's aerial part. It was yielded as colorless amorphous powder with melting point of 136-137 ºC. It could be categorized as a strong immunostimulant because its FI was bigger than 1.5 namely 1.625. Thus the *β*- sitosterol had potency to be developed as immunostimulant agent.

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