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# *Immunostimulant Activity of Flavonoid Isolated from The Acetone Extract of Silver Fern (Pityrogramma calomelanos)*

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**Abstract**—A flavonoid compound namely kaemferol had been separated from the acetone extract of the silver fern (*Pityrogramma calomelanos*). It was obtained as yellow needles crystal with m.p. of 271-273 °C. Determination of its structure was conducted by the spectroscopic method and by comparison with reported literature data. Based on the carbon clearance method, isolated flavonoid showed immunostimulant activity with phagocytosis index of 1.846.

**Keywords**—*Pityrogramma calomelanos, kaemferol, immunostimulat activity*

## I. INTRODUCTION

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

Many flavonoids in dihydrochalcone type had been separated from the fern species in *Pityrogramma* genus. For examples, from *Pityrogramma triangularis* had been separated 2',6',4'-trihydroxy-3',5'-dimethyl-4'-methoxy dihydrochalcone [4]. Previous work, we had reported secondary metabolites isolated from the dichloromethane extract of *P. calomelanos*' aerial part [5]. Many secondary metabolites from plants have properties as immunostimulants. It can increase the work of the components of the immune system [6]. The secondary metabolites that have bioactivity as immunostimulant agents are terpenoids, steroids, alkaloids and polyphenols [7]. In this paper, we reported the isolation of kaemferol from acetone extract of *P. calomelanos* and assay of its immunostimulat activity using carbon clearance method .

## II. MATERIALS AND METHODS

### A. Material

The materials used in this research were the aerial part of silver fern, *n*-hexane, dichloromethane, chloroform, ethyl acetate, acetone, methanol, FeCl<sub>3</sub>, HCl, Mg ribbon, kieselgel 60 GF-254 (Merck), precoated silica gel 60 F-254 (Merck), the male mice, tween 80, physiological NaCl 0.9 %, Na-EDTA, Na-CMC 0.3%, acetic acid 1%, Na-Citri, and chinese ink

### B. Instrument

The instruments used in this research were a set of extraction tools with maceration method, Buchner funnel, *rotary vacuum evaporator* (Heidolp laborata 4001), a set of thin layer chromatography (TLC) and vacuum liquid chromatography (VLC), Fisher John melting point apparatus, UV spectrophotometer (Shimadzu Pharma Spec. UV-1700), IR spectrophotometer (Perkin Elmer USA 89485), mass spectrophotometer (Shimadzu QP-2010S), and other glass tools.

### C. Sample Preparation

Silver fern was obtained from Kletak forest, Nongkojajar, Pasuruan, East Java. Before the research it was identified by the Indonesian Institute of Sciences of the Purwodadi Botanical Garden, East Java, Indonesia. Furthermore the sample of the aerial part of the silver fern was cleaned, then dried at room temperature. After that it was milled into a fine powder ready to be extracted.

### D. Extraction and Isolation

The dried powder of The aerial part dried powdered of *P. calomelanos* (5 kg) was exhaustively extracted successively with *n*-hexane (6 L x 3) , ethyl acetate (6 L x 3), and acetone (6 L x 3) at room temperature. The acetone extract were evaporated in vacuo, revealed 76 g (blackish brown) residue).

A portion of acetone extract (5.0 g) was chromatographed by VLC and eluted with solvents of increasing polarity (chloroform, chloroform-methanol, methanol) produced 127

fractions (15 mL each). Removal of the solvent under reduced pressure of the combined fractions of 60-100 gave the brown solid (1,2 g). It was rechromatographed by FC with chloroform-acetone (9:1) as eluent, obtained 60 fractions (10 mL each). The fractions 12-30 were collected, recrystallized in chloroform-methanol yielded a flavonoid 3, 5, 7, 4'-tetrahydroxy flavone (kaempferol) (**1**) (30 mg).

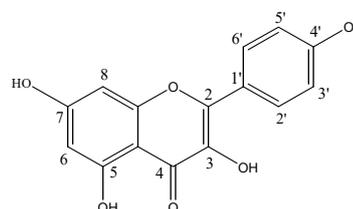
#### E. Immunostimulant activity using carbon clearance method

Ten male mice were divided into 2 groups, namely negative control group (Na-CMC 0.3%) and treatment group (given kaempferol isolate from silver ferns with a dose of 4 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  $\mu$ L, 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  $\mu$ L 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) [8]

### III. RESULTS AND DISCUSSIONS

Kaempferol (**1**) was obtained as yellow needles crystal ( $\text{CHCl}_3$ -acetone), mp. 271-273°C, which gave positive test (green) with  $\text{FeCl}_3$  and Shinoda test (Mg-HCl) (orange). It showed one spot on TLC using three eluents system with  $R_f$  of 0.36 (chloroform-acetone = 3 : 1), 0.44 (chloroform-ethyl acetate = 1 : 1), and 0.73 (chloroform-methanol = 5 : 1). UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) : 267, 367 nm; (MeOH + NaOH): 275, 322 (sh), 405 nm; (MeOH+ $\text{AlCl}_3$ ): 270, 353 (sh), 421 nm; (MeOH+ $\text{AlCl}_3$ +HCl): 269, 351 (sh), 421 nm; (MeOH+NaOAc): 276, 343 (sh), 426 nm; (MeOH+NaOAc+ $\text{H}_3\text{BO}_3$ ): 270, 343 (sh), 423 nm. IR (KBr)  $\nu_{\text{max}}$  : 3414 (OH), 3036 (aromatic C-H), 1659 (chelated C=O), 1613, 1567, 1510 (aromatic C=C), 1381, 1308, 1249, 1178  $\text{cm}^{-1}$ .  $^1\text{H-NMR}$  (500 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  (ppm) : 6.17 (1H, *d*,  $J = 1.9$  Hz, H-6), 6.38 (1H, *d*,  $J = 1.9$  Hz, H-8), 6.89 (2H, *d*,  $J = 9.2$  Hz, H-3',5'), 8.07 (2H, *d*,  $J = 8.6$  Hz, H-2',6').  $^{13}\text{C-NMR}$  (125,8 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  (ppm) : 94.5 (C-8), 99.3 (C-6), 104.6 (C-10), 116.4 (C-3', C-5'), 123.8 (C-1'), 130.8 (C-2', C-6'), 137.2 (C-3), 148.1 (C-2), 158.3 (C-5), 160.6 (C-4'), 162.6 (C-9), 165.7 (C-7), 177.4 (C-4).

**Compound 1** The positive results on the test using  $\text{FeCl}_3$  reagent (yellowish green) and Shinoda test (Mg + HCl) (yellow) of compound **1** indicated that it was a flavonoid compound [9]. The absorption maxima at 267 (band II) and 367 nm (band I) in the UV spectrum supported that **1** was a flavonol with a free 3-hydroxyl group. The bathochromic shift of band I on adding NaOH reagent (38 nm) and  $\text{AlCl}_3$  + HCl reagent (54 nm) indicated the presence of a hydroxyl group at C-4' and C-5, respectively. The presence of a hydroxyl group at C-7 was exhibited by bathochromic shift of band II (9 nm) on adding NaOAc reagent. No bathochromic shift on adding NaOAc +  $\text{H}_3\text{BO}_3$  reagent supported that **1** didn't have ortho-di hydroxyl group at A and B rings. The IR spectrum of **1** clearly disclosed absorption bands for OH group ( $3414 \text{ cm}^{-1}$ ), chelated carbonyl group ( $1659 \text{ cm}^{-1}$ ), and aromatic C=C ( $1613, 1567, 1510 \text{ cm}^{-1}$ ). The  $^1\text{H-NMR}$  spectrum of **1** exhibited four doublet proton signals at  $\delta_{\text{H}}$  6.17, 6.38, 6.89 and 8.07. Two doublet proton signals at  $\delta_{\text{H}}$  6.17 ( $J = 1.9$  Hz) and 6.38 ( $J = 1.9$  Hz) due to a pair of meta coupled protons H-6 and H-8 in the A-ring, respectively, supported the presence of a hydroxyl group at C-5 and C-7. While two doublet proton signals at  $\delta_{\text{H}}$  6.89 ( $J = 9.2$  Hz, H-3',5') and 8.07 ( $J = 9.2$ , H-2',6') due to two pairs of ortho-coupled protons in the B-ring, confirmed the presence of a hydroxyl group at C-4'. The  $^{13}\text{C-NMR}$  spectrum exhibited 15 carbon signals which corresponded to **1**, containing five oxygen aryl carbons [ $\delta_{\text{C}}$  148.1 (C-2), 158.3 (C-5), 160.6 (C-4'), 162.6 (C-9), and 165.7 (C-7)], one oxygenolefin carbon [ $\delta_{\text{C}}$  137.2 (C-3)], and one carbonyl carbon [ $\delta_{\text{C}}$  177.4 (C-4)]. The correlation spectroscopy (HMQC and HMBC) spectral data supported complete assignment of all proton-bearing carbon signals of **1**. Further supporting evidence of structure **1** for kaempferol came from comparison of the  $^1\text{H-NMR}$  and  $^{13}\text{C-NMR}$  spectral data with those of reported data in literature [10, 11] From the above results, compound **1** was proposed for the structure of kaempferol ([3,5,7,4'-tetrahydroxy flavone)



(1)

Based on the immunostimulant activity assay known that kaempferol had phagocytosis index (FI) of 1.846. The isolated flavonoid could be categorized as a strong immunostimulant because its FI was bigger than 1.5 [12]. The kaempferol is one of the flavonoid compounds predicted to have immunostimulant activity because it could improve the

body immune system induced by cyclophosphamide so percentage of T CD8<sup>+</sup> cell increase [13]. Flavonoid was predicted to increase the proliferation of B cells and lymphocyte T cells, release of specific cytokines such as TNF- $\alpha$ , IFN- $\gamma$  and IL-4. In addition, it was also able to stimulate phagocytic activity of macrophages, lysosomal enzyme activity, and release of nitric oxide by macrophages [14]. Thus the kaemferol had potency to be developed as immunostimulant agent.

#### IV. CONCLUSIONS

It had been separated the phenolic compound, namely kaemferol from the acetone extract of *P. calomelanos*'s aerial part. It was obtained as yellow needles crystal with m.p. of 271-273 °C. It could be categorized as a strong immunostimulant because its FI was bigger than 1.5 namely 1.846. Thus the kaemferol had potency to be developed as immunostimulant agent.

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