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Anti-inflammatory Activity of the Major Compound from Methanol Extract of *Phaleria macrocarpa* Leaves

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Abstract: *Phaleria macrocarpa* or mahkota dewa is a plant originally found in Papua. The variety usages of this plant such as treatment for cancer, impotency, diabetes mellitus and skin diseases lead to the studies of verification and validation of the claims made by the Indonesian traditional medicine practitioners. This study was done by targeting the chemical marker compound, phalerin, to ensure whether this particular compound contribute to the anti-inflammatory activity of the plant. The phalerin was extracted from *Phaleria macrocarpa* leaves using methanol and the compound was verified by the HPLC, NMR and LCMS. The anti-inflammatory potential of phalerin was evaluated using three *in vitro* assays which are Lipoxygenase (LOX), Hyaluronidase (HYA) and Xanthine Oxidase (XO) assays. The phalerin was identified as mild anti-inflammatory compound with percentage inhibition of 34.83 ± 4.64 and $23.47 \pm 9.43\%$ in XO and LOX assay, respectively. However, phalerin does not showing significant activity on HYA assay with $1.34 \pm 0.57\%$ of inhibition. This study verified that phalerin does have effects on inflammation and functions moderately on XO and LOX assays.

Key words: *Phaleria macrocarpa*, phalerin, anti-inflammatory

INTRODUCTION

Phaleria macrocarpa (Scheff) Boerl. or God's crown plant (mahkota dewa) is known by Indonesians for its usages in treating various diseases. Almost all parts of this plant including fruits, seeds, stems and leaves can be used in treatment. However, mahkota dewa cannot be consumed directly as it may cause swollen, numb and unconsciousness (Harmanto, 2003). Traditionally, the leaves of mahkota dewa have been used to treat dysentery, allergy, tumor and impotency. The fruit is used to treat flu, rheumatism, heart diseases and cancer. On the other hand, the seed is toxic to be consumed and thus can be used as insecticide and for treating skin diseases (Harmanto, 2003).

Studies have been done to prove the medicinal effects of the mahkota dewa plant. Recent studies have shown that the anti-hyperglycemic (Sugiwati *et al.*, 2009) and anti-nephropathy (Triastuti *et al.*, 2009) of mahkota dewa may be correlated to the increased renal antioxidant enzyme activity in the kidney. Moreover, fruits, leaves and stems of the plant which contain phenolic compounds show antioxidant activity (Irianti *et al.*, 2008; Soeksmanto *et al.*, 2007). It is also confirmed that the Mahkota Dewa extract has cytotoxic activities against Leukemia L1210 cell line (Winarno and Katrin, 2009), myeloma cells (Saifudin *et al.*, 2006) and human cancer cell line Ec-9706 (Zhang *et al.*, 2007). Moreover, ethanol

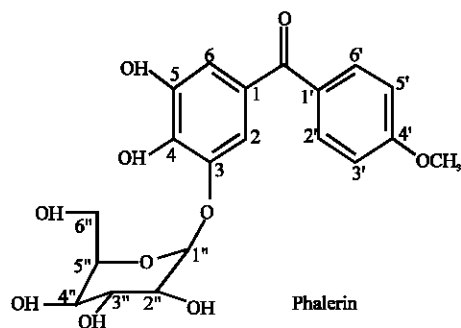


Fig. 1: Chemical structure of phalerin

extract of mahkota dewa has been studied and been reported having an effect to augment splenic NK1.1 cell activities by increasing their killing activity, expression of surface molecules and IFN- γ production (Ghufron *et al.*, 2008).

Thus, *Phaleria macrocarpa* has been proven to have medicinal effect such as anticancer, antitumor and antioxidant. However, there is no study done yet to determine the specific compound that contribute these medicinal effects. Phalerin is one of the major compound found in Mahkota Dewa leaves and is believed to have medicinal effect. Therefore, this study aims to investigate the activity of the major component known as phalerin (Fig. 1) extracted from Mahkota Dewa leaves.

MATERIALS AND METHODS

Materials: The *Phaleria macrocarpa* leaves were obtained from Yaacob Berkat Enterprise, Bukit Pulau, Melaka. Dried and fine powdered leaves were used in this experiment.

Extraction process: The extraction process is done by referring to Hartati *et al.* (2005) with some modifications. Further details of the extraction processes to extract the phalerin can be referred to Ismail *et al.* (2011).

Chemicals and reagents: Soybean lipoxygenase type-IB, linoleic acid, sodium salt, xanthine, nordihydroguaiaretic acid (NDGA), allopurinol, hyaluronidase and hyaluronic acid were purchased from Sigma Chemical Co., St. Louis, U.S.A. Xanthine oxidase was purchased from Roche, U.S.A. UV 96-well plate obtained from Greiner.

Preparation of test sample: Stock solution of test sample for hyaluronidase assay was solubilized in dimethylsulphoxide (DMSO) to concentration of 5 mg mL⁻¹. Stock solution of test sample for lipoxygenase and xanthine oxidase assays were prepared in methanol (MeOH)/DMSO at a concentration of 20 mg mL⁻¹. Stock solution have to be diluted in phosphate buffer pH 8 to give concentration of 2 mg mL⁻¹.

Apigenin, allopurinol and NDGA were used as reference compound for hyaluronidase, xanthine oxidase and lipoxygenase assays, respectively.

In vitro anti-inflammatory assay

Assay for inhibition of hyaluronidase activity (HYA): The assay was performed according to Sigma protocol with slight modifications (Ling *et al.*, 2003). The assay medium consisting of 80 U hyaluronidase in 100 µL 20 mM sodium phosphate buffer was preincubated with 25 µL of test sample (in DMSO) for 10 min at 37°C. Then the assay was commenced by adding 100 µL hyaluronic acid and incubated for a further 45 min at 37°C. The undigested hyaluronic acid was precipitated with 1 mL acid albumin solution. After standing at room temperature for 10 min, the absorbance of the reaction mixture was measured at 600 nm. The absorbance in the absence of enzyme was used as reference value for maximum inhibition. The inhibitory activity of test sample was calculated as the percentage ratio of the absorbance in the presence of test sample vs. absorbance in the absence of enzyme. All

reactions were performed in triplicate in 96-well microplate in Tecan Infinite M200 Microplate Reader (Tecan, Austria).

Calculation of inhibition:

$$\text{Inhibition (\%)} = \frac{A_s}{A_{\text{ref}}} \times 100 \quad (1)$$

where, A_s and A_{ref} are the absorbance of the sample and absorbance of the reference, respectively. The results were expressed as the mean of inhibitory value of three separate experiments measured in triplicates.

Assay for inhibition of xanthine oxidase activity (XO):

Xanthine oxidase inhibiting activity was conveniently measured by slightly modifying the spectrometric method developed by Noro *et al.* (1983). Xanthine oxidase was purchased from Roche, U.S.A. and xanthine (substrate) was purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade. In assay protocol, 130 µL of 0.05 M potassium phosphate buffer (pH 7.5), 10 µL of test-compound solution and 10 µL of xanthine oxidase enzyme solution were mixed and incubated for 10 min at 25°C. The reaction was then initiated by the addition of 100 µL xanthine (substrate) solution. The enzymatic conversion of xanthine from uric acid and hydrogen peroxides measured at absorbance of 295 nm. Test compounds and reference standards were dissolved in DMSO. All reactions were performed in triplicates in 96-well UV microplate in Tecan Infinite M200 Microplate Reader (Tecan, Austria).

Calculation of inhibition:

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100 \quad (2)$$

where, A_c and A_s are the absorbance of the control and absorbance of the sample respectively. The results were expressed as the mean of inhibitory value of three separate experiments measured in triplicates.

Assay for inhibition of lipoxygenase activity (LOX):

Lipoxygenase activity was conveniently measured by slightly modifying the spectrometric method developed by Azhar-Ul-Haq *et al.* (2004). Soybean lipoxygenase (1.13.11.12) type I-B and linoleic acid were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade. In assay protocol, 160 µL of 100 mM sodium phosphate buffer (pH 8.0), 10 µL of test-compound solution and 20 µL of lipoxygenase enzyme

solution were mixed and incubated for 10 min at 25°C. The reaction was then initiated by the addition of 10 µL linoleic acid (substrate) solution, with the formation of (9Z,11E)-(13S)-13-hydroperoxyoctadeca-9,11-dienoate, the change in the absorbance at 234 nm. Test compound and the positive control were dissolved in DMSO. All reactions were performed in triplicate in 96-well UV microplate in Tecan Infinite M200 Microplate Reader (Tecan, Austria).

Calculation of inhibition:

$$\text{Inhibition (\%)} = \frac{A_c - A_s}{A_c} \times 100 \quad (3)$$

where, A_c and A_s are the absorbance of the control and absorbance of the sample, respectively. The results were expressed as the mean of inhibitory value of three separate experiments measured in triplicates.

RESULTS AND DISCUSSION

Table 1 shows the inhibition effects of phalerin from mahkota dewa leaves extract on hyaluronidase, xanthine oxidase and lipoxigenase assays. The major component, phalerin, obtained from methanol extract of mahkota dewa leaves showed low effect on the anti-inflammatory activity in all three assays studied with the percentage of inhibition below 50%.

Hyaluronidase is an enzyme that degrades hyaluronic acid and known to be involved in allergic effects, migration of cancer and inflammation. In this study, HYA assay showed very low inhibition effect with 1.34±0.57% of inhibition. On the other hand, inhibition xanthine oxidase reduces the production of uric acid which relates to medical condition such as gout (Pacher *et al.*, 2006). However XO assay and LOX assay did show mild effect as anti-inflammatory agent with percent of inhibition of 34.83±4.64 and 23.48±9.43, respectively. This implies that phalerin may be used as anti-inflammatory to treat mild inflammation. Moreover, this study implies that other compound in mahkota dewa leaves might contribute to the anti-inflammatory activity. Thus, further study need to be done to identify and verify the specific compound or may be the synergy effect of the compounds in mahkota dewa leaves that contribute to the anti-inflammatory activity.

Table 1: Inhibitory effects of phalerin from Mahkota Dewa leaves extract on anti-inflammatory assays

Assays	Inhibition (%)	Positive controls	Inhibition (%)
HYA	1.34±0.57	Apigenin	75.48±4.08
XO	34.83±4.64	Allopurinol	98.47±0.99
LOX	23.47±9.43	NDGA	97.85±1.87

CONCLUSION

The phalerin was identified as a mild anti-inflammatory compound with percentage of inhibition of 34.83±4.64 and 23.47±9.43% in XO and LOX assay respectively. However phalerin does not significantly affect against HYA assay with 1.34±0.57% of inhibition. This study verifies that phalerin can be used as anti-inflammatory agent which functions moderately on XO and LOX assays.

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