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THE INHIBITORY ACTIVITY IN 5-LIPOXYGENASE PATHWAY OF HISPIDULIN FROM *MILLINGTONIA HORTENSIS* LINN. F.

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ABSTRACT

Hispidulin, a bioactive flavonoid isolated from the flower of Millingtonia hortensis *Linn. f., was tested for anti-phlorgistic effect by observing the inhibitory activity in 5-lipoxygenase pathway. The test was performed by incubating the hispidulin with 1-^{14}C-arachidonic acid and porcine leukocyte suspension containing 5-lipoxygenase. After the incubation, the 1-^{14}C-arachidonic acid and its metabolite were separated and quantified by RP-HPLC. Hispidulin showed inhibition of 65% at 64 μM.*

INTRODUCTION

Millingtonia hortensis Linn. f. is an ornamental plant with Thai local name "peep" The dried flowers can be used as a remedy for asthma in Thai traditional medicine[1-3]. The methanol extract of the flowers after fractionating by partition with chloroform and then separation by silica gel chromatography, yielded a bioactive flavonoid, hispidulin, as described in detail by Anulakanapakorn et al.[4]

The previous study indicated that the antiasthmatic efficacy of "peep" was due to the bronchodilating effect of hispidulin[4]. However, the exact mechanism of action and/or other activities of this flavonoid were not elucidated. In this study, hispidulin was tested for its activity in 5-lipoxygenase (5-LO) pathway.

The 5-LO pathway produces leukotrienes, i.e., LTC_4, LTD_4 and LTE_4 as well as 5-hydroxyeicosatetraenoic acid (5-HETE)[5, 6]. Most of these metabolites play important roles in processes associated with inflammation including asthma and allergic response.[7, 8]. The formation of these metabolites can be inhibited by specific inhibitors of 5-LO. In this study, hispidulin was observed for its inhibitory activity in 5-LO by incubating with 1-^{14}C-arachidonic acid (AA) and purified porcine leukocyte suspension containing 5-LO activity. After incubation, AA and its metabolites were separated and quantified by RP-HPLC. The inhibitory activity was determined by calculating the amounts of AA and its metabolites comparing with the control.

MATERIALS AND METHODS

Isolation of hispidulin[4]

Briefly, the dried coarsely-powdered flowers of *M. hortensis* Linn.f. were exhaustively extracted with methanol by marceration. After concentrating, the extract was evaporated to dryness. The remaining methanol extract was successively partitioned with petroleum ether, chloroform and n-butanol. The dried chloroform fraction was then submitted to a short column chromatography using silica gel G as adsorbent and chloroform as eluent. Fraction Q36-38 was concentrated and yellow precipitates were obtained. Recrystallization in methanol yielded hispidulin as a yellow precipitate.

Isolation of porcine leukocytes[9, 10]

Fresh porcine blood (450 ml) was mixed with 50 ml of 0.15 M NaCl –0.14 M sodium citrate solution containing 100 IU of heparin, and to this suspension were added 100 ml of 0.16 M NaCl containing 6% (w/w) of Dextran T-500. The solution was allowed to stand for 1 hour and then centrifuged at 300g for 7 min at 4°C. The pellet, containing mostly leukocytes, was suspended in a small volume of 0.14 M Tris buffer, pH 7.6, containing 6 mM D-glucose, 50 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM KCl and centrifuged once more. The pellet was suspended in 40 ml of 17 mM Tris buffer, pH 7.2, containing 32 mM NH_4Cl and left to stand at room temperature for 5 min to allow the remaining red cells to lyse. The cells were sedimented as described above and washed with 40 ml of 25 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl. The cells were again sedimented and resuspended in 40 ml of the phosphate buffer. Cell concentration was adjusted to 15×10^6 cells/ml.

In vitro 5-lipoxygenase inhibition assay

The incubation mixture contained 2.5 ml of leukocyte cell suspension, 25 μl of 0.2 M $CaCl_2$, 10 μl of ETYA (5,8,11,14-eicosatetrayonic acid) solution (0.2 mg/ml ethanol), 12 μl of ionophore A 23187 solution (2 mg/ml ethanol), 0.1 μCi of 1-^{14}C-arachidonic acid (65 m ci/m mol, NEN) and 50 μl of hispidulin dissolved in ethanol. Control solution contained ethanol in place of hispidulin and the concentration of ethanol was never permitted to be greater than 5%. The solution was incubated at 37°C for 5 min and the reaction terminated by adding 15 μl of 25% (w/v) NaOH and 525 μl of 1% formic acid.

Analysis of arachidonic acid and metabolites

The reaction mixture was extracted with 4 ml of ethyl acetate which was evaporated to dryness and the residue taken up in 0.1 ml of ethanol. Arachidonic acid and its metabolites were separated by RP-HPLC, equipped with a radioactive monitoring system, using a Hibar Lichrosher 100 Ch 18/2 column (125×44 mm ID, 0.5 micron, Merck), and a linear gradient

5-LIPOXYGENASE INHIBITION TEST

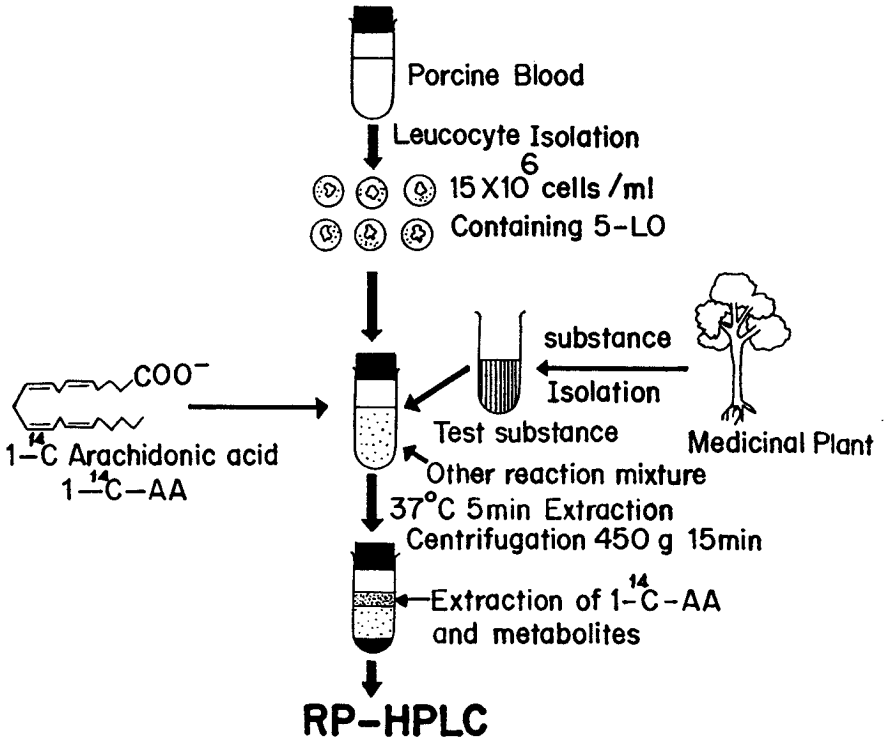

Fig. 1. Summary of the *in vitro* test for 5-lipoxygenase inhibition

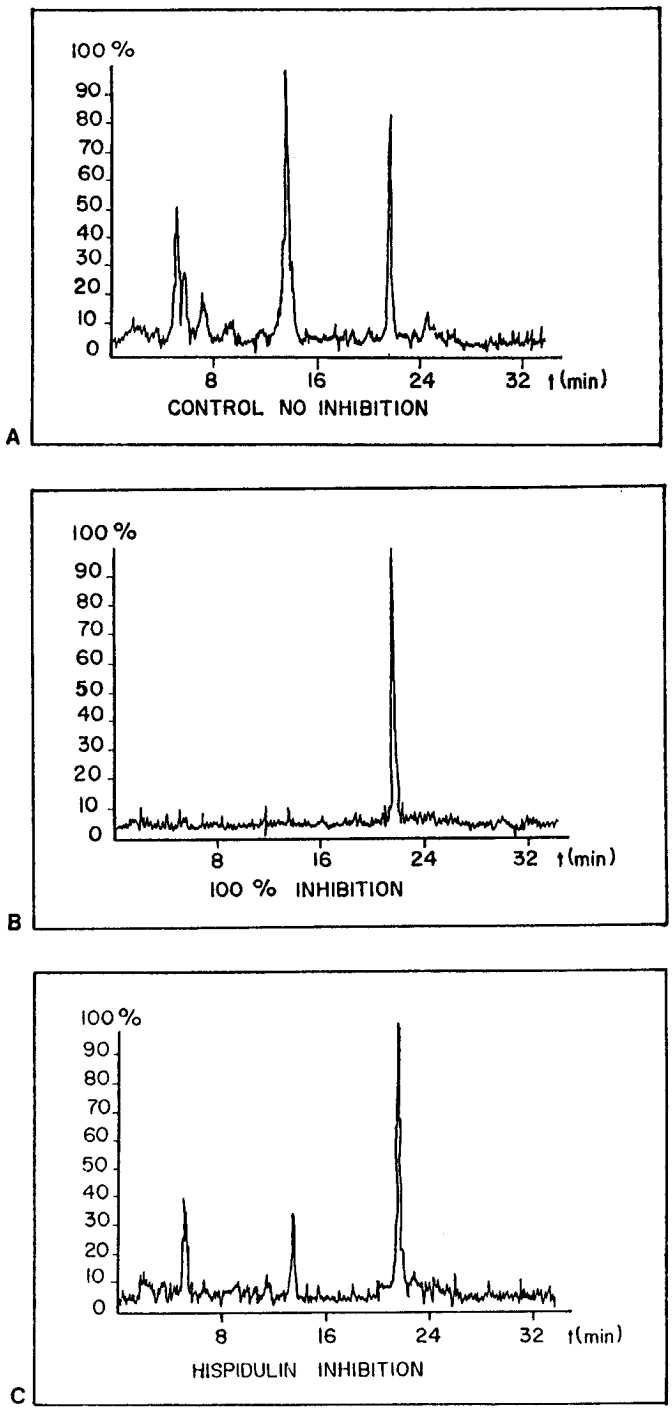

Fig. 2. HPLC-elution of 1−^{14}C-arachidonic acid and its metabolites in 5-lipoxygenase pathway. (A) After incubating 1−^{14}C-arachidonic acid with 5-lipoxygenase. (B) Showing 100% inhibition; arachidonic acid was not degraded as shown by the main peak. (C) After incubating 1−^{14}C-arachidonic acid with 5-lipoxygenase and hispidulin, showing 65% inhibition at 64 μM.

of 09-100% eluent B (eluent A: 1:1 (v/v) of acetonitrile:water; eluent B: 9:1 (v/v) of acetonitrile:water containing 0.1% of 1N phosphoric acid) at a flow rate of 1 ml/min for 20 min followed by an elution with eluent B for a further 10 min. The injection volume was 30 μl.

RESULTS AND DISCUSSION

Flavonoids are chemical constituents widely present in plants and possess many pharmacological activities[11, 12]. Recently hispidulin, a natural flavone, isolated from *Arnica montana,* L. (Asteraceae) has been reported to inhibit platelet aggregration[13]. In this study hispidulin isolated from *M. hortensis Linn.* f. (Bignoniaceae) was tested for inhibitory activity in the 5-LO pathway. The inhibition assay is shown diagramatically in Fig. 1.

Fig. 2 shows the results of the quantitative assay for 1-^{14}C-AA and its metabolites in the 5-LO pathway. In Fig. 2A is shown the pattern of a control reaction indicating the products of many kinds of leukotrienes generated from 1-^{14}C-AA whose elution location is shown in Fig. 2B (100% inhibition of 5-LO activity). Fig. 2C shows the profile obtained in the presence of 64 μM hispidulin which inhibited 65% of 5-LO activity.

These results clearly demonstrated that hispidulin possessed the ability to inhibit the 5-LO pathway to metabolise 1-^{14}C-AA. It can be postulated that hispidulin may serve as an anti-inflammatory or antiphlorgistic agent and thus may be an effective drug in the treatment of asthma or allergic reactions. However further studies are needed, such as its effect on cyclooxygenase pathway, on histamin or serotonin release. The toxicity of hispidulin has to be evaluated before it can be considered being applied in clinical use.

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บทคัดย่อ

Hispidulin เป็นสาร flavonoid สำคัญที่แยกได้จากดอกปีบ ซึ่งเป็นสมุนไพรที่รู้จักกันดีว่านำมาใช้แก้อาการหอบหืด ได้มีผู้รายงานฤทธิ์การขยายหลอดลม ซึ่งเกี่ยวข้องกับการรักษาหอบหืด ในการศึกษานี้ได้นำ hispidulin ที่แยกได้บริสุทธิ์มาตรวจสอบกลไกที่ยับยั้งสารสำคัญที่มีผลก่อให้เกิดอาการหอบหืดคือยับยั้งการเกิดสาร leukotrienes ใน 5-lipoxygenase pathway พบว่าที่ความเข้มข้น 64 uM hispidulin สามารถยับยั้งการแตกสลายของ arachidonic acid ไปเป็น leukotrienes ได้ถึง 65% จึงเชื่อว่าการนำ hispidulin หรือดอกปีบมาประยุกต์ใช้ให้เหมาะสมจะเป็นแนวทางหนึ่งในการรักษาและบรรเทาอาการหอบหืด