

## Effects of Diterpene Acids on Malondialdehyde Generation during Thrombin Induced Aggregation of Rat Platelets

Soleh Kosela\*, Asri Rasad\*, Sjamsul Arifin Achmad\*\*, Wahyudi Wicaksonon, Soung Kyung Baik\*\*\*, Yong Nam Han\*\*\* and Byung Hoon Han\*\*\*

\*Department of Chemistry, Universitas Indonesia, Jakarta, Indonesia

\*\* Chemistry Department, Insitutute Technology Bandung, Indonesia

\*\*\* Natural Products Research Institute, Seoul National University, Seoul 110 Korea

**Abstract** □ The effects of diterpene acids (i.e, pimaradienoic acid, kaurenoic acid, hydroxycembratrienoic acid and dihydroxycembratetraenoic acid) on malondialdehyde generation by rat platelets in response to thrombin were studied. All the compounds inhibited the generation of MDA.

**Keywords** □ Diterpene acids, platelet, inhibition of malondialdehyde generation, *Aralia continentalis*, *Cleome viscosa*.

The measurement of MDA in platelets can be used as the indicator of platelet prostaglandin synthesis, and it is simpler than the measurement of prostaglandin (1, 2). MDA is concomitantly released with 12-L-hydroxy-5, 8, 10-heptadecatrienoic acid (12-HHT) and thromboxane  $A_2$  when human platelets resuspended in artificial media are treated with thrombin or other reagents (2). In platelets,  $PGH_2$  is mainly converted into MDA, 12-HHT and  $TXA_2$  (2). MDA generation by human platelets is known to be prevented by the inhibitors of cyclooxygenase or thromboxane synthase such as non-steroidal anti-inflammatory agents or imidazole derivatives (2-6).

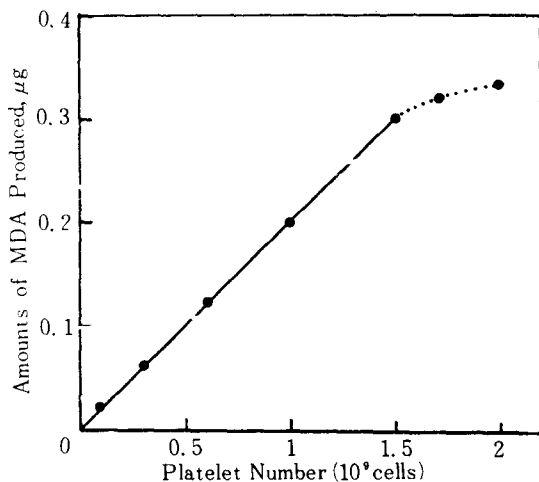
Two diterpene acids (I, II) were isolated as the active principles of *Aralia continentalis* K., which has been used as a folk medicine for anti-inflammation and anti-rheumatic (7). We found that I and II showed more stronger anti-inflammatory activities than phenylbutazone, utilizing the carrageenin-induced edema test in rat (7, 10). Both structures were identified as (-)-pimara-8 (14), 15-dien-19-oic acid (I) and (-)-11,16-en-19-oic acid (II).

Recently, we have also isolated two new unsaturated cembrane acids from *Cleome viscosa* L. (8). Their structures have been established to be (1R\*, 3E, 7Z, 12R\*)-20-hydroxycembra-3, 7, 15-trien-19-oic acid (III) and (3E, 7Z, 11Z)-17,

20-dihydroxycembra-3, 7, 11, 15-tetraen-19-oic acid (IV). *Cleome viscosa* (syn. *C. icosandra*, *Polanisa viscosa*; Cappariadaceae), a common weed found in the tropics of both hemispheres, has been used in herbal folk-medicine for headache relieving and wounds healing (9). III or IV belonging to a diterpene acid, was supposed to exhibit anti-inflammatory activity, since the plant has been used for wounds healing. Their anti-inflammatory activities were assessed by investigating their effects on malondialdehyde generation by rat platelets, and were compared with I and II.

Washed rat platelets also released MDA in response to thrombin as like human platelets, as shown in Fig.1. Amounts of MDA produced were proportionally increased according to platelet concentration within  $0, 2$  to  $1, 5 \times 10^9$  cells/ml.

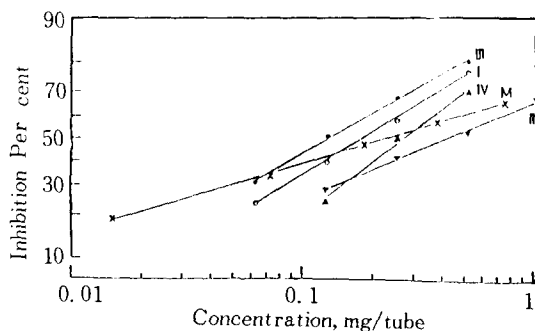
The inhibition of diterpene acids I to IV and imidazole on MDA generation during thrombin-induced aggregation of rat platelets were observed as shown in Fig.2. All the samples strongly inhibited MDA production. In order to determine 50% inhibition concentration ( $IC_{50}$ ), the platelet concentration was given to  $1 \times 10^9$  cells/ml, and the inhibitor concentrations were varied. Linearization of inhibition per cent was achieved on a logit-log paper. Values for  $IC_{50}$  were drawn from Fig.2 as following: I, 0, 18; II, 0, 40; III, 0, 13; IV, 0, 26; imidazole, 0, 21 mg/ $10^9$  cells platelets/ml.



**Fig. 1.** Assay of malondialdehyde produced in various concentration of rat platelets in response to bovine thrombin.

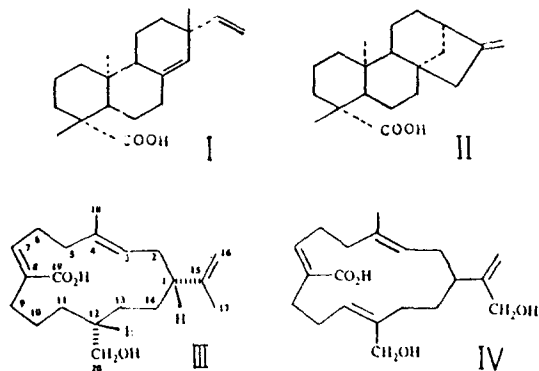
Platelets prepared from nine rats were pooled. One ml of the platelets was treated with  $150 \mu$ l (10 units) of bovine thrombin at  $37^\circ\text{C}$  for 30 min. Amounts of MDA produced were determined by TBA method. Each point was duplicate data.

Although III and IV showed the inhibition of MDA generation as well as I and II, it could not conclude that III and IV may possess anti-inflammatory action. After isolating large amounts of III and IV, further studies need to clarify their pharmacological actions.



**Fig. 2.** Inhibition of MDA formation by diterpene acids and imidazole.

One ml of washed rat platelets ( $1 \times 10^9$  cells/ml) was preincubated with 0.1 ml of inhibitor solution at  $37^\circ\text{C}$  for 30 min and then  $50 \mu$ l (10 Units) of bovine thrombin was added to aggregate platelets. The reaction mixture was further incubated at  $37^\circ\text{C}$  for 30 min. I to IV, compounds I to IV; M, imidazole.



## EXPERIMENTAL METHODS

### Preparation of platelet suspensions

Male Sprague-Dawley rats (250-300g) were anaesthetized with pentobarbital (60mg/kg, i.p.) and blood was collected by heart puncture into plastic injectors containing 3.13% sod. citrate to provide a 1 in 10 dilution by blood. Platelet-rich plasma (PRP) was obtained by centrifugation at 140g and  $10^\circ\text{C}$  for 20 min. PRP was centrifuged at 600g and  $10^\circ\text{C}$  for 20 min. Platelets were suspended in phosphate buffered saline (PBS, pH 7.4). Platelets prepared from nine rats were pooled and the platelet number was adjusted to  $1 \times 10^9$  cells/ml (TOA Automatic Platelet Counter, PL-100).

### MDA formation during rat platelet aggregation

Suspended platelet (1 ml) was warmed to  $37^\circ\text{C}$  in small polypropylene tubes and  $100 \mu$ l of inhibitor suspension was added. The mixture was preincubated at  $37^\circ\text{C}$  for 30 min and then 50ml (10U) of bovine thrombin was added. The tubes were further incubated at  $37^\circ\text{C}$  for 30 min. One ml of 3.0% thiobarbituric acid plus 0.4% sod. dodecyl sulfate in 7.5% acetic acid buffer (pH 4.0) was added to the reaction mixture, and heated in a boiling water bath for 1hr. After cooling to room temperature, extraction with butanol (2 ml) was carried out. Optical density of butanol layer was determined at 534 nm in a UV/visible spectrophotometer (Gilford, 2600).

### Chemicals

Bovine thrombin (229 units per mg protein), 2-thiobarbituric acid, imidazole and sod. dodecyl sulfate were purchased from Sigma. Inhibitors were suspended in PBS by a homogenizer.

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