*Malaysian Journal of Science* **26** (1): 79 – 83 (2007)

# Evaluation of *Piper betle* on Platelet Activating Factor (PAF) Receptor Binding Activities

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Received 9th August 2006, accepted in revised form 16th April 2007.

**ABSTRACT** Betel vine (*Piper betle*) is a tropical Asian plant belonging to the family Piperaceae. It is a vine that has long held a place in Asian history, religion and culture. Its medicinal uses are many and all parts of the plants are utilized. As part of our research programme into the exploitation of natural sources for their biological activities, various extracts of *P. betle* from 2 different locations (i.e. Pahang and Melaka) were screened for antagonist activity towards [$^3$H]-PAF (platelet activating factor) in washed rabbit platelet receptor binding studies. The methanolic extract of leaves from each location were suspended in water and partitioned with hexane, dichloromethane and ethyl acetate. Our results showed that the activity towards inhibition of [$^3$H]-PAF receptor binding was pinpointed to be in dichloromethane fraction from Melaka. Further purification of this fraction revealed hydroxychavicol as the main component involved in its anti-inflammatory activity.

**ABSTRAK** Sireh (*Piper betle*) adalah sejenis tumbuhan tropika yang tergolong dalam famili Piperaceae. Dari segi sejarah, keagamaan dan kebudayaan, ia merupakan tumbuhan yang telah lama mendapat tempat di dalam masyarakat Asia. Tumbuhan ini mempunyai nilai-nilai perubatan dan biasanya keseluruhan bahagian tumbuhan digunakan. Sebagai salah satu program kajian untuk mengenalpasti aktiviti biologi daripada sumber semulajadi, pelbagai ekstrak *P. betle* dari 2 lokasi terpilih (Pahang dan Melaka) digunakan dalam kajian ikatan faktor pengaktif platelet dengan reseptornya di platelet arnab. Ekstrak metanol daun dari setiap lokasi direndam di dalam air dan disaring dengan menggunakan heksana, diklorometana dan etil asetat. Fraksi diklorometana dari Melaka mempunyai aktiviti penghalangan [$^3$H]-PAF pada reseptor yand lebih ketara. Komponen utama yang dihasilkan dari proses penulenan fraksi ini dikenali sebagai hidroksikavikol yang juga merupakan komponen aktif antiinflamasi.

(*Piper betle,* platelet activating factor, receptor binding, dichloromethane fraction)

## INTRODUCTION

*Piper betle* Linn. or locally known as sireh belongs to the family Piperaceae. It is a perennial dioecious creeper native to the central and eastern regions of the Malay Archipelago [1]. The plant is cultivated from East Africa through all of tropical Asia to Oceania. The leaves have a clove like flavour and been chewed (as a masticate) by many people in the Asian region. It is still a common practice in rural Sri Lanka and in some other Asian countries. The chewing of sireh acts as a gentle stimulant, but beyond all other uses, has that of sweetening the breath.

Medicinally *P. betle* is an aromatic, carminative, stimulant and astringent used as a preventive for worms and in snake bites. The leaves are used in various ways to relieve constipation in children and also used for poulticing ulcerated noses and applied to the body after childbirth. The juice is dropped into the ears for wounds; into eyes in painful infection and night blindness and in a decoction used as a lotion after childbirth. Essential oil from leaves of this plant has been used for the treatment of respiratory catarrhs and as antiseptic and the fruit is employed with honey as a remedy for cough [2, 3].

Platelet activating factor (PAF) is a potent phospholipid mediator that possess many

biological activities. It functions in normal physiological processes such as inflammation, haemostasis and several aspects of reproduction. However, its role in the mediation of pathological responses including asthma, ischemia, gastric and pulmonary distress, allergy and shock has made it the focus of intense research. PAF is active at nanomolar concentrations and induces responses in target cells by binding to specific receptors that are coupled to several signalling mechanisms [4, 5]. Pertinent to an understanding of the mechanism of the receptor–mediated action of PAF on cells, it is important to have antagonists to the binding site of this potent lipid chemical mediator. Many antagonists of the PAF receptor have been described including structural analogs and several classes of compounds without any structural relationship. The latter often were discovered by screening natural products and traditional therapies for asthma and inflammatory disorders [6].

As part of a program oriented towards the discovery of bioactive natural products, extracts from *Piper betle* collected from Melaka and Pahang were tested for anti-PAF activity using rabbit platelets in vitro, focussing on platelet activating factor as a mediator involved in the inflammatory activity.

## MATERIALS AND METHODS

### Extraction and fractionation

Fresh samples of *Piper betle* were collected from Maran, Pahang (1 kg) and Bukit Senggeh, Melaka (4 kg). The plant materials were air dried and ground to mesh size 40 - 60. The dried plant materials were then extracted with methanol using cold extraction method at room temperature for one month. The methanol extracts were then evaporated under vacuum pressure by rotary evaporator at 45°C to yield dark green crude methanolic extracts of 47.5g and 190g, respectively. Distilled water was added to the crude methanolic extracts and the mixtures were fractionated by liquid-liquid partitioning using n-hexane, dichloromethane and ethyl acetate successively. Each solvent was removed under pressure to give hexane soluble extract, dichlromethane soluble extract, EtoAc soluble extract and residue as aqueous soluble extract. All fractions were concentrated in vacuo and stored prior to testing. All fractions were evaluated for anti-inflammatory activity on [$^3$H]-PAF receptor binding assay. The dicholoromethane extract from Melaka was found to be the most active towards inhibition of [$^3$H]-PAF receptor binding. Therefore this extract was selected for further fractionation. Separation of dichloromethane soluble extract (20g) by column chromatography was done on silica gel 60 (230 - 400 mesh) as adsorbent by eluting with solvent gradient grade of n-hexane, ethyl acetate and finally methanol. Fifty fractions were collected and were monitored by TLC and recombined based on the Rf values. Fractions 19-34 contained major component and showed as single spot under UV 254nm and give purplish red colour after treatment with 10% of $H_2SO_4$. This fraction was further purified by column chromatography using chloroform: methanol (9:1) to afford white crystal. The isolated compounds were then analyzed by spectroscopic methods using Nuclear Magnetic Resonance (NMR) and compared with literature data.

### Animals

New Zealand white rabbits of both sexes weighing from 2.5 – 5.0 kg.

### Preparation of platelets

Whole blood samples were drawn by cardiac-puncture from healthy New Zealand white strain rabbit (3 - 4 kg). Six volumes of blood were mixed with one volume of ACD solution (0.15M trisodium citrate, 0.075M citric acid, pH 5.2). The blood was centrifuged at 270 x g for 10 min at room temperature, and the top platelet rich plasma was removed carefully. The latter was further centrifuged at 500 x g for 15 min. The platelet pellets were then washed twice by means of centrifugation at 500 x g (15 min) in Buffer A (20% ACD solution, 60% $K_2HPO_4$ buffer, 20% sodium citrate, PH 6.8) followed once at 150 x g (10 min) in Buffer B (50 ml $K_2HPO_4$, 0.1gm bovine serum albumin, PH 7.0). The top whitish layer was removed and centrifuged at 500 x g (15 min) to obtain the platelets. Platelets from different rabbits were usually pooled, a procedure that has not led to any behavioral differences from platelets prepared from a single animal. The final concentration for the platelets was adjusted to 3 X $10^8$ platelets/ml.

### PAF receptor binding inhibitory assay

The assay was carried out in triplicate according to the modified method [7]. The reaction mixture consisted of 200 µl of washed rabbit suspension, 25 µl of $^3$H-PAF (2.0nM) with or without unlabeled PAF (2.0 µM) and 25 µl of sample.

The final concentration of sample in the reaction mixture was 18.2 µg/ml. Cedrol, a known PAF receptor antagonist was used as a standard in this bioassay. These reaction mixtures were incubated at room temperature for 1 h. The free and platelet bound ligands were then separated by filtration technique using a glass microfibre filter in a cell harvester. Radioactivity was measured by scintillation counting. Specific binding of radiolabeled is defined as the difference between total radioactivities of bound $^3$H-PAF in the reaction mixture with the absence and presence of excess unlabeled PAF. Percentage inhibition of the sample was determined according to the equation used by [8]. The results of the assay are expressed as the mean of 3 distinct experiments.

$$\% \text{ Inhibition} = \frac{(Tc - Nc) - (Ts - Ns)}{Tc - Nc} \times 100$$

Where,
Tc = total binding of control
Ts = total binding of sample
Nc = non-specific binding of control
Ns = non-specific binding of sample

## RESULTS AND DISCUSSION

The phospholipid-derived inflammatory mediators - prostaglandins, leukotriens and platelet activating factor (PAF) have been at the focus of inflammatory research over the last decade. PAF in particular has been linked to conditions including asthma, septic shock, ischemia, reperfusion injury and pancreatitis, among many others [9]. Since the discovery and structural identification of PAF, its specific receptor has been given much attention. The use of a receptor binding method in search of PAF antagonists is motivated when the exact nature of the response is to be elucidated. The most common cell type used for binding studies with PAF is platelets and in most cases membrane fractions of the cells are prepared [10].

In this study, the total and specific activities of the PAF receptor binding antagonist activity of *P. betle* were measured using rabbit platelets as the receptor source and [$^3$H]-PAF as a ligand. The data are listed in Table 1 and for comparison the percentage inhibition of Cedrol, a known PAF antagonist from natural product [8] is also shown. Four different levels of activity have been determined. An inhibition below 20% is considered to be insignificant at the dose tested. Sample which showed inhibition between 20 and 40% is regarded to be low while inhibition between 40 and 60% as moderate and above 60% is expressed as high. The results obtained in this study indicate a considerable difference in antiinflammatory activity between extracts collected from Melaka and Pahang with crude methanolic extract from Melaka being more active than the other extract. Subsequent fractionation from both crude methanolic extracts exhibited various degree of inhibition, dicloromethane extract from Melaka being the most active with inhibition of 59.8%. Therefore, the dicloromethane extract from Melaka was further subjected to column and thin layer chromatography. The use of mobile phase hexane/ethyl acetate = 7:3 for all extracts (Hexane, dichloromethane, ethyl acetate and methanol) gave a separation that is equally spread over the plate. Purification of the dichloromethane fraction resulted in obtaining major compound, identified as hydroxychavicol (Figure 1) based on their $^{13}$C and $^1$H NMR and also by comparison with the literature. This compound was further evaluated using the same biological assay system. Thus, as shown in Table 2, the test compound at various concentrations revealed it to be active with $IC_{50}$ value of 71.32 µM ± 2.3. The compound showed dose-dependent responses, i.e. as the concentration of the compound increased the percentage inhibition increased. The active compound showed comparable $IC_{50}$ value but higher than that of Cedrol (22.5 ± 1.2 µM). Hydroxychavicol (HC), a major phenolic compound in *P. betle* leaf, has been shown to inhibit the nitrosation reaction [11], and enhance mouse liver glutathione S-transferase activity in vivo [12]. The chemical species 1'-hydroxychavicol, structurally similar to hydroxychavicol inhibits the TPA-induced $H_2O_2$ production and inflammatory response in mouse skin [13].

The results indicate that the anti-inflammatory action of the active compound seems to be directly or indirectly associated with its ability to inhibit PAF at their receptor, and to a lesser extent, PAF-mediated inflammatory responses. The precise mechanism through which the compound exerts its anti-inflammatory effect is currently not completely clear. Therefore further studies need to be carried out to investigate the structure activity relationship of the active compound and to elucidate the precise mechanism underlying this activity.

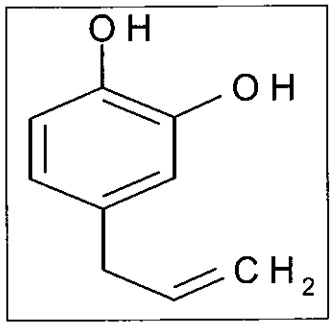


**Figure 1.** Chemical structure of hydroxychavicol

**Table 1.** Inhibitory profile of various extracts of *Piper betle* L. on PAF receptor binding to platelets at concentration of 200µg/ml.

| EXTRACT | INHIBITORY POTENTIAL OF SAMPLE (%) | |
|---|---|---|
| | PAHANG (MARAN) | MELAKA (BUKIT SENGGEH) |
| Methanol | 50.4 | 71.9 |
| Hexane | 37.7 | 59.4 |
| Dichloromethane | 38.8 | 59.8 |
| Ethyl acetate | 47.2 | 47.1 |

Cedrol (Positive control) = 77.8%

**Table 2.** Percentage inhibition by hydroxychavitol on PAF receptor binding to platelets at various concentrations and its $IC_{50}$ value.

| Compound | 18.2 | 9.1 | 4.5 | 1.8 | $IC_{50}$ (µM) |
|---|---|---|---|---|---|
| | | (µg/ml) | | | (mean ± SD) |
| Hydroxychavicol | 69.3 | 43.6 | 30.3 | 15.4 | 71.2 ± 2.3 |
| Cedrol | 79.4 | 59.0 | 47.8 | 29.3 | 22.5 ± 1.2 |

Data represent mean ± SD of two or three independent experiments performed in triplicate.

## ACKNOWLEDGEMENTS

This project was financially supported by the Malaysian Government (IRPA, Project No. 30300403006).

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