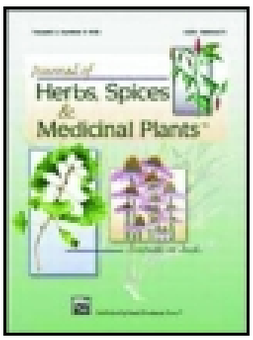

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Inhibitory Effects of *Mitrella kentii* Extracts on Inflammatory Mediators' Biosynthesis and Binding

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ABSTRACT

The inhibitory effects of *Mitrella kentii* leaf and stem extracts on the production prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) and antagonist effect on platelet-activating factor (PAF) receptor binding were evaluated. The inhibition of PGE_2 and TXB_2 productions were determined using the radioimmunoassay technique, and the inhibitory effect of PAF receptor binding to rabbit platelet was determined using the ^{3}H-PAF as a ligand. Among the extracts tested, ethyl acetate, methanol and hexane leaf extracts, and methanol stems extract showed inhibitory effects on PGE_2 and TXB_2 productions and antagonistic effect on PAF receptor binding. *M. kentii* extracts may have the potential of being developed as supplements for inflammatory conditions.

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KEYWORDS
Prostaglandin E; thromboxane B_2; platelet activating factor; radioimmunoassay; blood platelets

Introduction

Inflammation is an immediate body response to cells and tissues injury caused by various exogenous or endogenous inducers. The response is achieved through complex regulatory pathways which involve numerous inflammatory mediators, and among those are the prostanoids, prostaglandin E_2 (PGE_2) and thromboxane A_2 (TXA_2) and the platelet-activating factor (PAF). PGE_2 and TXA2 are metabolites of arachidonic acid which utilize the cyclooxygenase (COX) pathway[1] whilst PAF is synthesized by either the remodeling pathway[2] or the *de novo* pathway.[3] PGE_2 enhances formation of edema [4] while TXA_2 is a vasoconstrictor and a promoter of platelet aggregation.[5] PAF once bound to the receptors will stimulate aggregation, degranulation of platelets and granulocytes, chemotaxis of neutrophils, and macrophages respiratory burst.[6] Although inflammation is viewed as the body defense and repair mechanism, the overproduction of these mediators will lead to pathological conditions such as Crohn's disease[7], atherosclerosis[8], and septic shock.[6]

Since the discovery of aspirin which was derived from a willow bark in 1897[9], the quest to find anti-inflammatory drugs which give optimum

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therapeutic effect with minimum side effects is ongoing. Early generation of non-steroidal anti-inflammatory drugs (NSAIDs) which include aspirin, indomethacin, and ibuprofen inhibit both COX-1 and COX-2 iso-enzymes resulting in the erosion of gastric mucosa.[10] Partially selective COX-2 inhibitor, meloxicam, was introduced which causes less gastrointestinal problems than nonselective NSAIDs.[11] Selective COX-2 inhibitors; celecoxib and rofecoxib, were approved by the United States Food and Drug administration in 1988[12] and 1999[12], respectively. However, rofecoxib was withdrawn from the market in 2004 due to cardiovascular risk.[13] Celecoxib is under scrutiny ever since and newer agents were sought.

Many plant species from Annonaceae such as *Dennettia tripetal*a[14], *Annona muricata*[15], *Xylopia parviflora*[16], *Annona vepretorum*[17], and *Xylopia aethiopica*[18] have been reported to possess anti-inflammatory activity. *Mitrella kentii* (Annonaceae) is commonly found in the tropics especially in the Asia-Pacific. The plant is also called *Melodorum pisocarpum* and *M. elegans* or commonly as 'kiawi' (Indonesian) and have been used traditionally as a remedy for fever.[19] Phytochemical compounds isolated from the bark of this plant; liriodenine, anonaine, and asimilobinem and aequaline[20] and from the stem; (-)-neolinderatin, (-)-linderatin and 2′,6′-dihydroxy-4′ methoxydihydrochalcone, and (+)-catechin[21] may contribute to its anti-pyretic effect.

In this study, the inhibition of PGE_2 and TXB_2 productions in human whole blood by *M. kentii* extract was determined using the radioimmunoassay technique. The inhibitory effect of platelet activating factor receptor (PAFr) binding to rabbit platelet was determined using the 3H-PAF as a ligand. Ethyl acetate and methanol extracts showed strong inhibitory activities on PGE_2 and TXB_2 productions, respectively, while hexane extract displayed strong PAF antagonistic effect.

Materials and Methods

General

Hexane, ethyl acetate, and methanol used were of analytical grades. Radiolabeled PGE_2 (^{3}H-PGE_2, 50 μCi/mmol) and TXB_2 (^{3}H-TXB_2, 25 μCi/mmol) were purchased from Amersham (Buckinghamshire, UK). Unlabeled PGE_2, unlabeled TXB_2, anti-PGE_2, and anti-TXB_2 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Radiolabeled PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, 125 Ci/mmol) was purchased from Amersham. Unlabeled PAF and cedrol were obtained from Sigma Chemical Co. Bovine serum albumin was purchased from Boehringer Mannheim Co. (Mannheim, West Germany). Other chemicals were purchased from Merck Co. (Darmstadt, Germany) and BDH Laboratory Supplies (Poole, UK).

Ethylenediamine tetraacetic acid (EDTA) 2% was used as an anticoagulant. Lipopolysaccharide (LPS) 1 mg mL^{-1} was used to induce PGE endoperoxide synthesis in whole blood. Phosphate buffer solution 0.01 M, pH 7.4, was used as a buffer for assays. Dextran charcoal (0.4% dextran, 2% charcoal) was used to separate the free and bound ligand. Scintillation cocktail was made up of 2,5-diphenyloxazole (0.26%), 2,2′-*p*-phenylene-bis-5-phenyloxazole (0.006%), toluene (500 mL), and Triton X (250 mL). Radioactivity was measured by a liquid scintillation counter (Packard Tri-Carb, models 2100TR/2300TR, Hamburg, Germany).

Plant Material

Fresh leaves of *M. kentii* were collected from the Angsi mountain forest in Negeri Sembilan, Malaysia, in July 2008 and identified by Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). A voucher specimen (AZ 69) was deposited at the Herbarium of Faculty of Science and Technology, UKM.

Preparation of Plant Extracts

Dried ground leaves and stems of *M. kentii* (1,000 g) were extracted successively with hexane (3 × 2.5 L, 24 h each), ethyl acetate (3 × 2.5 L, 24 h each), and methanol (3 × 2.5 L, 24 h each) by maceration. The solvents were then evaporated using a rotatory evaporator to yield hexane (14.9 g, 1.5%), ethyl acetate (29.5 g, 3.0%), and methanol (67.8 g, 6.8%) extracts, respectively.

Preparation of Blood Sample

The use of human blood in this study was approved by the Ethics Committee of UKM (approval no. FF-168-2007). Briefly, venous blood (30 mL from each volunteer) was obtained by an aseptic vein puncture from healthy human volunteers who fulfilled the following inclusion criteria: nonsmoker, fasted overnight, and did not take any medicine or supplements within the last two weeks and transferred to a polypropylene tube containing 10% (v/v) of 2% EDTA for the PGE_2 radioimmunoassay and allowed to clot for the TBX_2 radioimmunoassay

Cell Viability

Cell viability was determined by the standard trypan blue exclusion method. Blood cells (1 × 106/mL) were incubated with 1.25 and 10.0 μg mL^{-1} of extracts each in triplicate at room temperature (27°C) for 24 h. The blue dye

uptake was an indication of cell death. The percentage viability was calculated from the total cell counts.

Radioimmunoassay for PGE_2 and TXB_2

Radioimmunoassay was carried out to determine the levels of PGE_2 and TXB_2 productions by blood cells following incubation with compounds and coagulation as described.[22] Radioimmunoassay procedures were carried out in triplicate for each compound.

Preparation of Standards

A series of PGE_2 and TXB_2 standards concentrations were prepared, ranging from 2.45 to 240 and from 2.05 to 500 pg/0.1 mL, respectively. PGE_2 standard solution (100 µL) was added to 100 µL of anti-PGE_2 and 100 µL of ^{3}H-PGE_2. Meanwhile, 100 µL of TXB_2 standard solution was added to 100 µL of anti-TXB_2 and 100 µL of ^{3}H-TXB_2. The mixtures were incubated at 4°C for 18–24 h. After incubation, the mixtures were added to 200 µL of dextran charcoal and were incubated again for 10 min. After centrifugation at 2000 × g for 15 min at 4°C, 3 mL of liquid scintillation cocktail was added to 300 µL of supernatant. The radioactivity was measured by a liquid scintillation counter.

PGE_2 Radioimmunoassay

Blood (1 mL) was incubated at 37°C for 24 h with 10 µL of LPS and 10 µL of serial dilutions of each compound in dimethyl sulfoxide (DMSO) and ethanol (1:1 ratio) (1.25–10 µg mL^{-1}) or control. A solution containing DMSO and ethanol (1:1 ratio) was used as a negative control and indomethacin, a known COX inhibitor was used as a positive control. After incubation, the blood was centrifuged at 2,000 × g for 10 min at 4°C to separate the plasma. The reaction mixtures consisted of 100 µL of plasma, 100 µL of anti-PGE2 and 100 µL of 3H-PGE2 were incubated at 4°C for 18–24 h. After incubation, the mixtures were added to 200 µL of dextran charcoal and incubated again for 10 min. Final concentrations of samples in the mixture were 10.0, 5.0, 2.5 and 1.25 µg mL^{-1}. After centrifugation at 3,000 × g for 15 min at 4°C, 3 mL of liquid scintillation cocktail was added to 300 µL of supernatant. The radioactivity was measured by a liquid scintillation counter.

TBX_2 Radioimmunoassay

Thromboxane B_2 assay was carried out similar to the PGE_2 assay. In this assay, 1 mL of blood was mixed with 10 µL of serial dilutions of each

compound in DMSO and ethanol (1:1 ratio) (1.25–10 μg mL^{-1}) or control and was allowed to clot for 60 min at 37°C. A solution containing DMSO and ethanol (1:1 ratio) was used as a negative control and indomethacin was used as a positive control. The blood was centrifuged at 2,000 × g for 10 min at 4° C to separate the serum as supernatant. The reaction mixtures consisted of 100 μL of serum, 100 μL of anti-TXB2, and 100 μL of 3H-TXB2 and were incubated at 4°C for 18–24 h. After incubation, 200 μL of dextran charcoal was added to the mixture and was incubated further for 10 min. The final concentrations of the samples in the mixture were 10.0, 5.0, 2.5, and 1.25 μg mL^{-1}. After centrifugation at 3,000 × g for 15 min at 4°C, 3 mL of liquid scintillation cocktail was added to 300 μL of supernatant. The radioactivity was measured by a liquid scintillation counter.

Calculation of PGE_2 and TXB_2 Concentrations

The readings obtained for each set of triplicates were averaged. The net counts for all standards and samples were calculated by subtracting the value of the antibody binding to the antigen in the sample (Bx) with non-specific binding (Nc). The normalized percentage bound (% B/Bo) for each standard and sample (Bx) were calculated as follows:

$$\left\{ \%\mathrm{B/Bo} = \frac{\mathrm{Bx} - \mathrm{Nc}}{\mathrm{Bo} - \mathrm{Nc}} \times 100\% \right\} \tag{1}$$

The % *B*/*Bo* for each standard versus the corresponding picogram (pg) concentration of PGE_2 and TXB_2 were plotted using semi-logarithmic graph. The concentrations of PGE_2 and TXB_2 in each sample were determined by interpolation from the standard curve. Percentage inhibition of samples was obtained as follows:

$$\%\mathrm{inhibition} = 100 - \frac{[\mathrm{PGE_2 \text{ or } TXB_2 \text{ in sample}}]}{[\mathrm{PGE_2 \text{ or } TXB_2 \text{ in control}}]} \times 100 \tag{2}$$

PAF Receptor Binding Assay

The assay was carried out as described.[23] The procedure was approved by the Animal Ethics Committee of UKM (approval no. FSKB/2007/Juriyati/10-July/192). The reaction mixtures consisted of 200 μL of washed rabbit platelet suspension, 25 μL of 3H-PAF (2.0nM) with or without unlabeled 25 μL of PAF (2.0 μM), and 25 μL of compound (200 μg mL^{-1}) or control solution. The final concentration of compounds in the reaction mixtures were 18.2, 9.1, 4.5, 2.3 μg mL^{-1}. Cedrol, a known PAF antagonist, was used as a positive control and 0.1% DMSO in saline was used as a negative control. The final concentration of DMSO in the reaction mixture was fixed at 0.1% to avoid

interference with the receptor-binding studies. The reaction mixture was incubated at room temperature (27°C) for 1 h. The free and bound ligands were separated by a filtration using Whatman GF/C glass fiber filters. The radioactivity was measured by a scintillation counter. The difference between total amounts of bound 3H-PAF in the absence and the presence of excess unlabeled PAF is defined as specific binding of the radiolabeled ligand. The IC50 values of the extracts were obtained from at least three independent determinations. Percentage inhibition of the sample was obtained as follows:

$$\%\text{Inhibition} = \frac{(\text{Tc} - \text{Nc}) - (\text{Ts} - \text{Ns})}{\text{Tc} - \text{Nc}} \times 100 \quad (3)$$

Tc = total binding of control; *Ts* = total binding of sample
Nc = nonspecific binding of control; *Ns* = nonspecific binding of sample

Statistical Analysis

Data were analyzed using the Statistical Package for Social Sciences software. Each sample was measured in triplicate and the data were presented as means ± standard deviation (SD) and analyzed by one-way analysis of variance ($P < .05$). Probit program was used to determine the IC_{50} values for the active extracts.

Results

Cell viability test carried out to evaluate the cytotoxicity of the extracts on blood cells at 1.25 and 10.0 µg mL^{-1} showed that the blood cells were viable (>95%) after 24 h of incubation at both concentrations. Among the extracts tested for their inhibitory effects on PGE_2 and TBX_2 productions, ethyl acetate leaves extract exhibited the strongest inhibitory effect on the production of PGE_2 (64.4% inhibition) induced by LPS (Table 1) whereas in the TXB_2 assay, methanol leaves extract displayed the highest percentage inhibition (41.0%) (Table 2). The inhibition of TXB_2 production in human whole

Table 1. Percentage inhibition (%) of *Mitrella kentii* extracts at 10 µg mL^{-1} on PGE_2 production in human whole blood induced by Lipopolysaccharide (LPS).

Extract	Plant parts	% Inhibition
Hexane	Leaves	11.6 ± 4.4*
Ethyl acetate	Leaves	64.4 ± 8.1
Methanol	Leaves	3.5 ± 3.5*
Hexane	Stems	40.9 ± 2.6*
Ethyl acetate	Stems	37.3 ± 17.8*
Methanol	Stems	8.4 ± 3.9*
Indomethacin	–	83.8 ± 4.9

Data are mean ± SD ($n = 3$); *$P < 0.05$ as compared with control.

Table 2. Percentage inhibition (%) of *Mitrella kentii* extracts at 10 μg mL^{-1} on TXB_2 production in human whole blood.

Extract	Plant parts	% Inhibition
Hexane	Leaves	28.8 ± 6.2*
Ethyl acetate	Leaves	21.5 ± 9.6*
Methanol	Leaves	41.0 ± 3.4
Hexane	Stems	33.9 ± 4.1*
Ethyl acetate	Stems	25.5 ± 16.6*
Methanol	Stems	36.1 ± 10.4*
Indomethacin	–	73.9 ± 6.5

Data are mean ± SD (n = 3); *P < 0.05 as compared with control.

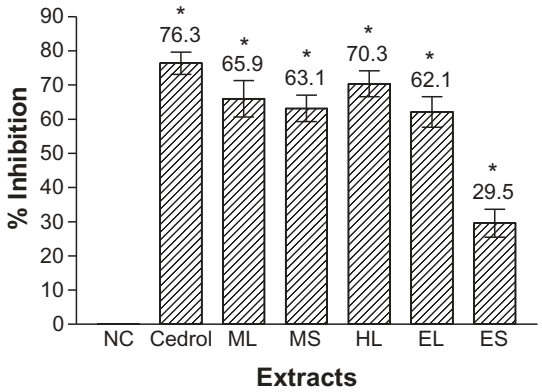

Figure 1. Percentage inhibition (%) of the *Mitrella kentii* extracts on PAF receptor binding to rabbit platelets at 18.2 μg mL^{-1}.

blood was in a dose-dependent manner. However, none of the extracts showed more than 50% inhibition on the TBX_2 production.

Hexane extract of *M. kentii* leaves showed inhibitory effect on the PAFr binding with percentage inhibition of 70.3% followed by methanol leaves extract (65.9%), methanol stems extract (63.1%), and ethyl acetate leaves extract (62.1%) (Fig. 1). The inhibitory effect of hexane leaves extract (70.3%) was comparable to that of the positive control, cedrol (76.3%), a known PAF antagonist from nature.[24] The inhibitory effects of these extracts were then evaluated at various concentrations, and the IC_{50} values of hexane leaves extract and methanol stems extract were determined by probit analysis as 7.0 and 7.2 *M. kentii*, respectively (Table 3).

Discussion

In this study, six solvent extracts from *M. kentii* leaves and stems were examined for their inhibitory effects on prostanoids biosynthesis and PAF binding to the PAFr. The strong inhibition of PGE_2 production by ethyl

Table 3. IC_{50} values of the *Mitrella kentii* extracts on PAF receptor binding to rabbit platelets.

Extract	Plant parts	IC_{50} µg mL^{-1}
Hexane	Leaves	7.0 ± 1.0
Ethyl acetate	Leaves	9.6 ± 1.7
Methanol	Leaves	8.6 ± 0.6
Methanol	Stems	7.2 ± 0.7
Cedrol	–	6.0 ± 0.7

Data are mean ± SD (n = 3).

NC, negative control; ML, methanol leave extract; MS, methanol stem extract; HL, hexane leave extract; EL, ethyl acetate leave extract; ES, ethyl acetate stem extract.

acetate leaves extract may be due to direct inhibition of COX-2 enzymatic activity, possibly by the antioxidant effects of the extract or related to its capacity to bind COX-2 active site. Moreover, phospholipase A_2 (PLA_2) may also be as a potential target of the extract. Moderate activity observed in the inhibition of TXB_2 production might be due to direct inhibition of COX-1 enzymatic activity. This is because the measurement of serum TXB_2 production by the platelets following blood coagulation is a specific test for assessment of COX-1 activity.[25] Information on the biological activities of bioactive compounds isolated from *M. kentii* was scarce nonetheless, reports on other plants of Annonaceae were abundant[26], and a study demonstrated PAFr inhibition by *Enicosanthellum pulchrum*.[27]

Extracts which showed promising activities in the inhibition of PGE_2 and TXB_2 productions and antagonistic activity on PAFr may be further investigated for their active constituents and potential in the development of potent anti-inflammatory agents.

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Conflicts of Interest

The authors declare no conflicts of interest in this study.

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