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Research article

In vitro inhibitory effects of flavonoids from the extracts of *Artocarpus* species on prostaglandin E_2(PGE_2) production in human plasma

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Key words: Anti-inflammatory, Moraceae, Radioimmunoassay, Cyclooxygenase-2.

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Abstract

In this study, seven flavonoids from several *Artocarpus* species, artocarpin (**1**), isobavachalcone (**2**), cycloheterophyllin (**3**), 2',4'-dihydroxy-4-methoxy-3'-prenyldihydrochalcone (**4**), 4',5-dihydroxy-6,7-(2,2-dimethylpyrano)-2'-methoxy-8-γ,γ –dimethylallylflavone (**5**), artonin E (**6**) and oxyresveratrol (**7**) were investigated for their inhibitory effects on the production of prostaglandin E_2 (PGE_2) in human plasma.The inhibitory effect of seven compounds isolated from several *Artocarpus* species was determined by measuring the PGE_2 level in plasma using radioimmunoassay (RIA) technique. Radiolabelled antigen, [^{3}H]-PGE_2 that bound with antibody was displaced by unlabelled antigen present in plasma. The unbound radiolabelled antigen was calculated using liquid scintillation counter. Among the seven flavonoids, artocarpin (**1**) showed the highest inhibition of 68.1%, followed by artonin E (**6**) and isobavachalcone (**2**) with 66.8% and 63.9% inhibition respectively. Meanwhile, cycloheterophyllin (**3**) exhibited the lowest inhibition with 21.5%. Compound **1**, **4**, **5** and **6** were the most potent inhibitor in inhibit the biosynthesis of PGE_2 with IC_{50} value of 11.66 µM, 8.99 µM, 7.04 µM and 8.98 µM, respectively. These results showed that compound **1**,**4**, **5** and **6** inhibited the activity of PGE_2 production in human blood induces by lipopolysaccharide (LPS), indicating that it might directly inhibit cyclooxygenase-2 (COX-2) enzymatic activity. Different substitution pattern of the flavonoids may contribute in different range of inhibitory activity.

Introduction

Inflammation is a complex biological response that can be aroused by a variety of stimuli including pathogens, noxious mechanical and chemical agents, and autoimmune responses. The symptoms of inflammation are characterized by the heat, redness, swelling, pain and loss of function. The inflammatory response occurs in the vascularized connective tissue, including plasma, circulating cells, blood vessels, and cellular and extracellular components. These responses will increase micro vascular caliber, enhance vascular permeability, leukocyte recruitment, and release of inflammatory mediators [1].

Cyclooxygenase (COX) is an enzyme that plays an important role as inflammatory mediator and involved in the release of arachidonic acid, a precursor for biosynthesis of eicosanoids like prostaglandins and prostacyclin [2]. PGE_2 is a metabolite of arachidonic acid through the COX-2 pathway. It has an established role in causing inflammation and also contributes to other functions such as febrile responses, vasodilation and alteration of micro vascular permeability, blood clotting, and initiation of labor, bone metabolism, blood healing and immune responses [3].

Medicinal plants have been source of wide variety of biologically active compounds for many centuries and have been used extensively as crude material or as pure compounds for treating various disease conditions [4]. Presently, the uses of herbal medicines have been increased due to toxicity and side effects gave by synthetic drugs. Medicinal plants such as *Artocarpus* species may play an important role in the development of potent therapeutic agents. *Artocarpus* is the most important genus in Moraceae family apart from *Ficus*.

There are 47 species of *Artocarpus* and only 20 species could be found in Malaysia including the cultivated plants [5]. This genus becomes the important source of edible fruit, good timber and widely used in folk medicine.

Artocarpus species have been investigated for various biological activities. The methanolic extracts of *A. heterophyllus* showed anti-bacterial activity as the isophenyl flavones in the extract inhibited the growth of primary cariogenic bacteria [6]. The extract also exhibited excellent toxicity on cancer cells and was non-toxic to normal cells [7]. Besides that, the ethyl acetate fraction of

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A. heterophyllus leaf extract exerted strong hypoglycemic activity in both normoglycemic and diabetic rats [8]. *Artocarpus* species were also reported to possess anti-inflammatory [9], anti-fungal [10], immunomodulatory [11], anti-cholinergic [12], protease inhibitory [13], melanin biosynthesis inhibitory [14] and wound healing properties [15].

Artocarpus species is well known as source of the isoprenoid-substituted phenolic compounds, especially flavonoids [16]. Flavonoids come from a group of natural substances with variable phenolic structures and are found in fruit, vegetables, grains, bark, roots, stems, flowers, tea and wine [17]. The flavonoids are usually subdivided into nine sub-classes including flavonols, flavones, flavanones, flavanols, isoflavones, anthocyanidins, proanthocyanidins, aurones and chalcones [18]. The flavonoids have been reported to possess anti-inflammatory [19], hepatoprotective[18], anti-thrombotic [20], anti-viral [21] and anti-carcinogenic activities [22].

In the present study, we report the inhibitory effects of seven flavonoids from *Artocarpus* species, artocarpin (**1**), isobavachalcone (**2**), cycloheterophyllin (**3**), 2',4'-dihydroxy-4-methoxy-3'-prenyldihydrochalcone (**4**), 4',5-dihydroxy-6,7-(2,2-dimethylpyrano)-2'-methoxy-8-γ,γ-dimethylallylflavone (**5**), artonin E (**6**) and oxyresveratrol (**7**) on the production of PGE_2 in human plasma.

Experimental

Materials and Methods

General

Centrifuge: Hettich (Tuttlingen, Germany). Liquid Scintillation Counter: Perkin Elmer (Waltham, Massachusetts, U.S.). Radiolabelled PGE_2 ([^{3}H]-PGE_2, 50 μCi mmol^{-1}) was purchased from Perkin Elmer (Waltham, Massachusetts, U.S.). Unlabelled PGE_2 and anti-PGE_2 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BSA (Bovine Serum Albumin) was purchased from Boehringer Manheim Co. (Germany). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lipopolysaccharide from *E. coli* (LPS) 1 mg/mL was used as prostaglandin endoperoxide synthesis induction in whole blood. Phosphate buffer solutions (PBS) 0.01 M, pH 7.4 was used as assay buffer. Dextran-coated charcoal (0.4% dextran, 2% charcoal) was used to separate the free and bound ligands. Commercial LSC cocktail (Ultima Gold MV) purchased from Perkin Elmer (Waltham, Massachusetts, U.S.) was used as scintillation cocktail.

Flavonoids from *Artocarpus* species

Four flavonoids, namely, artocarpin (**1**), isobavachalcone (**2**), cycloheterophyllin (**3**) and 2',4'-dihydroxy-4-methoxy-3'-prenyldihydrochalcone (**4**) were isolated from *A. lowii*, while another three flavonoids, 4',5-dihydroxy-6,7-(2,2-dimethylpyrano)-2'-methoxy-8-γ,γ dimethylallyl flavone (**5**), artonin E (**6**) and oxyresveratrol (**7**) were isolated from *A. scortechinii.* The extraction and isolation procedures, as well as the spectroscopic data of these flavonoids were described and reported previously [23, 24, 25]. The purity of the flavonoids was > 95 % as assessed by MS and NMR.

Radioimmunoassay for Prostaglandin E_2

Radioimmunoassay was carried out to determine the levels of PGE_2 productions in human plasma according to the modified method of Patrignani*et al.* [26] as described previously by Saadawi*et al.* [27]. The use of human blood was approved by the Ethics Committee of Universiti Kebangsaan Malaysia (UKM) (approval no. NF-052-15). Radioimmunoassay procedures were carried out in triplicate for each compound. A series of concentrations of PGE_2 standard were prepared, ranging from 2.45-400 pg/0.1 mL. One hundred μL of PGE_2 standard solution was added to 100 μL of anti-PGE_2 and 100 μL of [^{3}H] -PGE_2. The mixtures were incubated at 4°C for 18-24 h. After 24 h incubation, 200 μL of dextran-charcoal were added to the mixtures and incubated again for 10 min. After centrifugation at 3000 × g for 15 min at 4°C, 300 μL of supernatant were added to 3 mL of liquid scintillation cocktail. The radioactivity was measured by a liquid scintillation analyzer.

Each sample (1 mg) was dissolved in 1 mL dimethyl sulfoxide (DMSO) and ethanol (1:1) to form a stock solution of 1 mg/mL, DMSO and ethanol (1:1) was used as negative control and indomethacin as positive control. Blood was drawn by aseptic vein puncture of healthy volunteers who fulfilled the following inclusion criteria: good health, non-smoker, had not taken any medications of drugs and alcohol within the last 2 weeks and must be fasting within the last 8 hours. One mL of blood was incubated for 24 h at 37°C with 10 μL of LPS and 10 μL of serial dilutions of each compound in DMSO and ethanol (1:1) (0.625-10 μg/mL). The mixture was further centrifuged at 2600 × g for 15 min at 4°C. The reaction mixtures consisted of 100 μL of plasma, 100 μL of anti-PGE_2 and 100 μL of [^{3}H] -PGE_2 were incubated for 18 to 24 h at 4°C. After incubation, 200 μL of dextran-charcoal were added to the mixtures and incubated again for 10 min. After centrifugation at 3000 × g for 15 min at 4°C, 300 μL of supernatant were added to 3 mL of liquid scintillation cocktail. The radioactivity was measured by a liquid scintillation analyzer.

Calculating unknown

The data obtained for each set of triplicate were averaged. The net counts for all standards and samples were calculated by subtracting the value of 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) was calculated as follows:

$$\%\frac{B}{Bo} = \frac{Bx - Nc}{B0 - Nc} \times 100$$

% B/Bo = percent bound
Bx= antibody binding to the antigen in the test samples and controls
Bo= total binding between the antibody and tracer
Nc= non-specific binding

The % B/Bo for each standard versus the corresponding concentration of PGE_2 was plotted. The concentration in each sample was determined by interpolation from the standard curve. Percentage inhibition of the sample was obtained by the following equation:

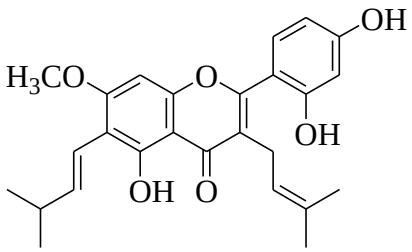
$$\%\ inhibition = 100 - \{\frac{(PGE2\ in\ sample)}{(PGE2\ in\ control)} \times 100\}$$

Statistics

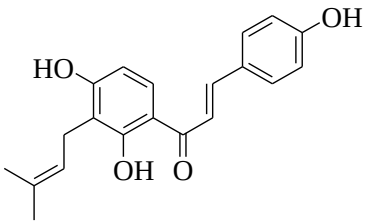
The percentage inhibition values for each compound are presented as means ± standard error mean (SEM). Data were analyzed using one way of analysis variance test (ANOVA) to determine differences between groups. Graph pad prism is used to determine the IC_{50} values for the active compounds.

Results and Discussion

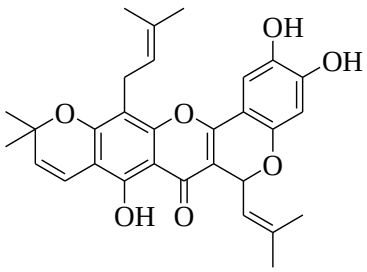
Seven flavonoids from *Artocarpus* species (Figure 1) have been examined for their inhibitory effects on LPS-induced PGE_2 production in human plasma by using radioimmunoassay (RIA) technique. Flavonoids can regulate arachidonic acid metabolism via inhibition of cyclooxygenase activity from different sources, as well as on their anti-inflammatory effect. Therefore, the compounds which inhibit the production of PGE_2 may reduce the inflammation responses. Arbitrarily classified, percentage inhibition that is greater or equal than 70% is strong for inhibition, 50 - 70% is moderate for inhibition, and less than 50% is weak inhibitor [28].



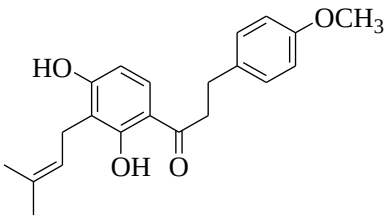
(**1**)



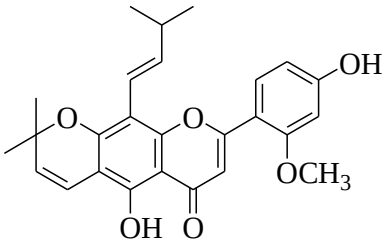
(**2**)



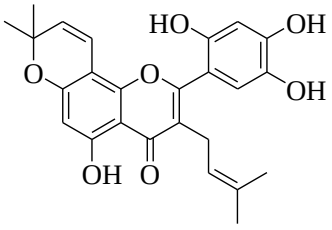
(**3**)



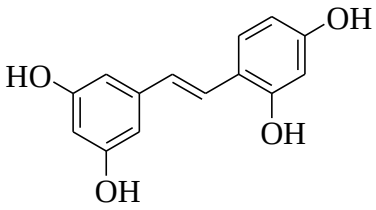
(**4**)



(**5**)



(**6**)



(**7**)

Figure 1. Chemical structures of flavonoids isolated from *Artocarpus* species.

The inhibition of the seven flavonoids at the final concentration of 10 µg/mL is showed in Table 1. Among the seven flavonoids, compound **1** showed the highest inhibition of 68.1% as compared with positive control (Indomethacin), followed by compound **6**, compound **2**, compound **4**, compound **5** and compound **7** with 66.8%, 63.9%, 62.5%, 61.8% and 55.7% inhibition, respectively. All these compounds showed moderate inhibitor against PGE_2 production in human plasma. Meanwhile, the lowest inhibition of 21.5% was exhibited from compound **3**. Compounds which showed inhibitory activity of more than 50% were subsequently tested at serial concentrations to obtain their IC_{50} values.

Table 1. Percentage inhibition (%) of the compounds isolated from *Artocarpus* species at a concentration of 10 µg/mL on PGE_2 production in human plasma induced by LPS

Compound	% inhibition
1	*68.1±3.8
2	*63.9±3.9
3	*21.5±4.3
4	*62.5±3.8
5	*61.8±5.2
6	*66.8±5.9
7	*55.7±6.0
Indomethacin (positive control)	**79.2±2.1**

Values are presented as mean ± SEM (n=3); *P <0.05 as compared with control

Six compounds were further investigated at various concentrations ranging from 10 to 0.625 µg/mL. All compounds showed dose-dependent response, as the concentration of the compound increased, the percentage of inhibition increased (Figure 2). Compound **1**, **4**, **5** and **6** (Table 2) were identified as promising inhibitor with IC_{50} value of 11.66 µM, 8.99 µM, 7.04 µM and 8.98 µM, respectively. Compound **1** is comparable to indomethacin (12.27 µM), a potent cyclooxygenase inhibitor [29] while compound **4**, **5** and **6** are more potent inhibitor.

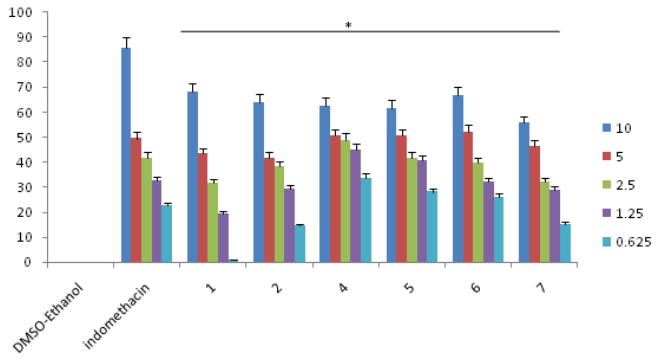


Figure 2. Inhibitory effects of compounds 1, 2,4,5,6 and 7 at different final concentration on PGE_2 production in human plasma induced by LPS (value given in mean ± SEM; n=3;*P <0.05 as compared with control).

Table 2. IC_{50} values of compounds 1, 2, 4, 5, 6 and 7 on PGE_2 production by LPS-stimulated human plasma.

Compound	IC_{50} µg/mL (µM)
1	5.09 ± 1.78 (11.66)*
2	5.37 ± 1.49 (16.55)*
4	3.16 ± 0.75 (8.99)*
5	3.06 ± 1.00 (7.04)*
6	3.92 ± 0.90 (8.98)*
7	6.15 ± 1.68 (25.18)*
Indomethacin (positive control)	4.39 ± 0.77 (12.27)

Values are presented as mean ± SEM (n=3); IC_{50} values in µM are presented in parentheses; *P <0.05 as compared with control

Compound **5** was found to be the most effective in inhibiting the biosynthesis of PGE_2 with IC_{50} value of 7.04 µM. This compound had been reported to show moderate inhibition of anti-tyrosinase activity with IC_{50} value of 312.5 µg/mL [30]. Jamil *et al.* [31] reported anti-microbial activity of compound **5** in which it showed broad spectrum activity in inhibiting the growth of all bacteria and fungi with inhibition zone diameter in the range of 9.0 – 13.7 mm. Compound **6** is the second effective in the inhibition of PGE_2 production. Previous study on compound **6** isolated from *A. communis* Forst stated that it exhibited the most potent inhibition on arachidonate 5-lipoxygenase with IC_{50} value of 0.36 uM [32]. Lipoxygenase is one of the regulatory enzymes that are essential to inflammation and the immune response [2]. Flavonoids affect arachidonic acid metabolism in different ways. Some flavonoids specifically block cyclooxygenase or lipoxygenase, whereas others block both enzymes [33]. Compound **6** isolated from *A. communis* also showed stronger effect against cancer cells, mouse L-1210 and colon 38 as compared to the aflavin, vital drug [16]. This compound found to be the most efficacious inhibitor that inhibited the TNF-α release induced by okadoic acid at low concentration [34, 35]. Compound **4** is the third effective with IC_{50} value of 8.99 µM and was reported as a new prenylateddihydrochalcone that showed strong scavenging action against the DPPH radical [23]. Compound **4** exhibited low inhibition on platelet activating factor receptor binding to rabbit platelets with 32% as compared to cedrol (standard antagonists). This compound also exhibited moderate anti-tyrosinase activity and low anti-microbial activity [30, 31].

The strong to moderate inhibition showed by the seven flavonoids may depend on their chemical structures. The relationship between their activity and the presence of specific functional groups in the molecules is undeniable. Different groups attached to flavonoids may contribute in different range of inhibitory activity. All flavonoids share a basic C6-C3-C6 phenyl-benzopyran backbone. The position of the phenyl ring relative to the benzopyran

moiety allows a broad separation of these compounds into flavonoids (2-phenyl-benzopyrans), isoflavonoids (3-phenyl-benzopyrans) and neoflavonoids (4-phenyl-benzopyrans). The structure analysis relationship study has been done on chalcones (compound **2** and **4**). Several naturally occurring chalcones have been reported to show anti-inflammatory properties. The presence of methoxy group at the A ring of compound **4** and the reduction of double bond between α,β-position slightly decrease the inhibitory effects by 1.4 %. In chalconoid type of structure, hydroxyl group (as in compound **2**) at position C-4 in ring B is also an important requirement. When the hydroxyl group is methylated and the reduction of the double bond at α,β-position as in compound **4**, the antimicrobial activity decreased [16]. The presence of lipophilic group in compound **2** may be the reason that it has potential in anti-microbial activity [36]. Whereas for the flavone (compound **3,5** and **6**), the presence of ether group between ring B and C in the compound **3**, significantly decreases the inhibition of PGE_2 biosynthesis by 41.1 % as compared to compound **5**. The presence of three hydroxyl group in compound **6** exhibited strong activity with percentage inhibition of 66.8%. Compound **1** is a flavanone and gave the highest percentage of inhibition and the presence of hydroxyl (C-2 and C-4) and lipophilics (C3 and C6) group might contribute to the inhibitory activity.

Conclusion

Among the compounds isolated from *Artocarpus* species, compound **1**, **4**, **5** and **6** showed a potent dose-dependent inhibitory activity on PGE_2 production in human blood induced by LPS, indicating that it might directly inhibit COX-2 enzymatic activity. Further studies are necessary to elucidate the mechanisms behind this effect and to identify the lead compounds for the development of anti-inflammatory therapeutic agents.

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