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Inhibitory Effect of Leaf, Bark and Twig of *Dipterocarpus alatus* on the Inflammation Mediators, Nitric Oxide, PGE_2, IL-1β and TNF-α in Macrophage RAW 264.7

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ABSTRACT

Dipterocarpus alatus has been used in traditional medicine to treat leprosy and gonorrhoea and for relief of pain but there are limited studies investigating the mechanisms of this activity. In this work, we screened *D. alatus* leaf, bark and twig extracts for anti-inflammatory activity using lipopolysaccharide (LPS) stimulated RAW 264.7 cells and determined their effects on inflammatory mediators and cytokines. *D. alatus* extracts were tested for cytotoxicity and effects on NO, PGE_2, IL-1*β* and TNF-α production in LPS-stimulated RAW 264.7 macrophages using Griess reaction and ELISA test kits. All extracts were non-toxic to RAW 264.7 cells at a concentration of 100 µg/mL. Extracts from all tested parts of *D. alatus* reduced levels of NO, PGE_2, and IL-1β but did not affect TNF-α in LPS-stimulated RAW 264.7 cells. Thus, *D. alatus* extracts show specific anti-inflammatory activity with good potential as a treatment for inflammatory diseases.

Keywords: Dipterocarpaceae, ELISA, Cytokines, Nitric oxide, Prostaglandin E2.

Introduction

Plants of the Dipterocarpaceae family are known to be sources of sesquiterpenes, triterpenes, flavonoids, and resveratrol oligomers.[1-5] Many of them display bioactivities such as anti-inflammatory, antioxidant, anticancer, immunosuppressive, anti-bacterial and anti-HIV effects.[4-8] *Dipterocarpus alatus* is a tree in the Dipterocarpaceae family that is usually found in tropical areas.[1] This plant has been widely used in traditional medicines. In Ayurvedic medicine, *D. alatus* oleo-resin has been used to treat leprosy and gonorrhoea. The bark can be used as ingredient for the relief of pain while the leaf and twig can be used as a haemagogue. A previous study identified potential anti-hyperuricaemic and anti-inflammatory activities of vaticaffinol, a resveratrol tetramer isolated from *D. alatus*.[3] Furthermore, *D. alatus* twig extract and dipterocarpol showed antibacterial effects against MSSA and MRSA *in vitro*, and improved wound healing in an MRSA-induced superficial skin infection model in mice.[6] Leaf, bark and twig extracts of *D. alatus* also exhibited strong antioxidant activity.[5]

Many diseases, such as Acquired Immunodeficiency Syndrome (AIDS), asthma, cancer, congestive heart failure and neurological diseases, have an association with the actions of inflammatory cytokines.[9] Inflammation is a complex process, initiated by several factors such as bacterial infection and chemical injury, that can result in cell injury or death.[10] Macrophages play a major role in the innate immune response by secreting pro-inflammatory cytokines including several interleukins (IL) and tumor necrosis factor-α (TNF-α). Pro-inflammatory cytokines in-turn induce expression of iNOS and cyclooxygenase-2 (COX-2), increasing the formation of nitric oxide and prostaglandins (PGs), and increasing signs of inflammation. Phenolic components from natural products have been reported to have many biological activities, including anti-inflammation.[11] In particular, polyphenol components isolated from Korean *Lonicera japonica* have been shown to inhibit COX-2, iNOS, and cytokines such as TNF-α, IL-1 β, and IL-6 by suppressing the p38 MAPK and NF-κB pathways.[12] Gallic acids and ellagic acids from *Radix Sanguisorbae* exhibited inhibitory effects on prostaglandin E2 (PGE_2) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages, with no cytotoxicity.[13] Moreover, ellagic acid, gallic acid and punicalagin A&B isolated from *Punica granatum* fruit inhibit LPS-induced NO, PGE_2 and IL-6 production.[14] Finally, methanol extracts of *Reaumuria vermiculata* shoot showed antioxidant activity and dichloromethane extracts displayed anti-inflammatory activity, inhibiting NO release by over 100% at 80 µg/mL in LPS-stimulated RAW 264.7 macrophages.

Our previous study showed that the bark extract of *D. alatus* had the highest antioxidant activity and the highest total phenolic content and all extracts showed cytotoxic activity against the U937 cell line.[5] However, there is a lack of biological activity studies of *D. alatus* that support traditional use. The current study examined the effect of *D. alatus* extracts on inflammatory mediators and cytokines using lipopolysaccharide (LPS) stimulated RAW 264.7 cells.

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Materials and Methods

Plant material

The leaf, twig, and bark of *D. alatus* were collected from Khon Kaen province, Thailand in June 2014. The *D. alatus* herbarium specimen (No. PSKKF03682) was identified by Assoc. Prof. Suppachai Tiyaworanant, Faculty of Pharmaceutical Sciences, Khon Kaen University. The plant materials were cut and mashed into powder. The dry powder of leaves (1,300 g), twigs (700 g) and bark (1,900 g) was macerated with methanol (2L × 3 times) for 24 h at room temperature. The solvent was removed after filtration and evaporated under reduced pressure followed by freeze-drying. Dipterocarpol was isolated from oleo-resin of *D. alatus* as described previously.[6]

Reagents and materials

Lipopolysaccharide (LPS, from *E. coli* O111:B4), Roswell Park Memorial Institute medium (RPMI 1640), fetal bovine serum (FBS) and 0.5% trypsin-EDTA were purchased from Gibco Inc., NY, USA. Sulfanilamide, naphthylethylenediamine dihydrochloride and phosphoric acid were obtained from Sigma Ltd. (USA). 3-(4, 5-dimethyl-2-thiazolyl) 2, 5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from R&D Systems (Minnesota, USA). ELISA test kits for PGE2 (ab133021), murine IL-1β (ab100705) and TNF-α (ab100747) were purchased from Abcam (USA).

Cell culture

The mouse macrophage cell line (RAW 264.7) was purchased from Cell Lines Service (Germany) and cultured in RPMI 1640 medium with 10% FBS and 1% (v/v) penicillin–streptomycin under 5% CO_2 incubator. For the experiments, the number of cells was calculated by haemocytometer with Trypan blue staining before seeding into 24-well plates.

MTT assay

RAW 264.7 cells (10^5 cells/well) were treated with leaf, twig, and bark extracts of *D. alatus* in 24 well plates at the various concentrations and incubated for 24 h. MTT (0.5 mg/mL) was added into the plates and incubated for another 30 minutes. The culture medium was removed and the cells were dissolved in DMSO. The optical density (OD) was measured at 550 nm with a microplate reader. Fresh culture medium was used as a blank for background subtraction.[15]

Gallic acid and protocatechuic acid content

The gallic acid content of *D. alatus* extracts was determined by HPLC (Agilent 1260 Infinity II LC Systems) with a C_{18} column (HP hypersil ODS 5 µm particle size C_{18}, 250 × 4.0 mm). The isocratic system for mobile phase was 20% MeOH in 1% acetic acid at a flow rate of 1.0 mL/min, column temperature 25°C, injection volume 20 µL and UV detection at 280 nm. The HPLC chromatograms of the extracts and standard compounds were compared. The presence of gallic acid and protocatechuic acid in the extracts was determined based on their retention times at 3.8 and 6.8 min, respectively.[16]

Dipterocarpol content

Dipterocarpol content was determined by HPLC auto injection (Primaide 1000 series, Hitachi, Japan) coupled with a photodiode array detector and separation was carried out using a reversed phase column (Kinetex® 2.6 µm particle size C_{18}, 100 × 4.6 mm) (Phenomenex, Torrance, USA). The isocratic system used a flow rate of 0.8 mL/min, a mobile phase of acetonitrile (solvent A) and purified water with 0.05% trifluoroacetic acid (v/v) (solvent B) in the ratio 7:3 (solvent A: solvent B), an injection volume of 20 µL, and column temperature 25 °C. The wavelength for detection of dipterocarpol was 210 nm. The presence of dipterocarpol in the extract was determined based on retention time at 30.4 min.[17]

Total flavonoid content

The flavonoid contents of leaf, bark and twig were determined using a slightly modified method.[18] The sample and 2%$AlCl_3$ (1:1) were added to 96-well plates. The solution was left to stand at room temperature for 20 min. After incubation, the absorbance was read at 415 nm using a microplate reader. The total flavonoid contents were calculated as quercetin equivalent units (mg QE/g DW).

Determination of inflammatory mediators and cytokines in RAW 264.7 cell line.

RAW 264.7 cells (2×10^5 cells/well) were incubated with either leaf, twig, or bark extracts of *D. alatus*, or aspirin (ASA, positive control), along with LPS (1 µg/mL) at 37 °C for 24 h. Culture medium was collected to determine levels of inflammatory markers and cytokines. The quantity of accumulated nitrite was determined as an indicator of NO production. Briefly, the mixture of 100 µL of cell culture medium and 100 µL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) was incubated at room temperature for 10 min, and the absorbance was then measured at 550 nm by microplate reader. For PGE_2 and the pro-inflammatory cytokines (IL-1 β, and TNF-α), levels were measured using ELISA kits according to the manufacturer's protocol.

Statistical analysis

All experimental data are expressed as the mean ± SD. Analysis of variance (ANOVA) was used to analyse group differences between treated samples and the control *via* SigmaStat for Windows version 3.11 software. A *p*-value lower than 0.05 ($p < 0.05$) indicated statistical significance.

Results and Discussion

Macrophages play an important role in inflammatory processes in both the early and late stages of inflammation and in various organs and connective tissue.[19] Screening for the toxicity of crude extracts is important before determination of anti-inflammatory activity. The RAW 264.7 murine macrophage cell line was treated with various concentrations of *D. alatus* extract for 24 hours and %cell viability was determined via MTT assay. The crude extract of leaf was not toxic to RAW 264.7 cells up to 200 µg/mL, whereas bark and twig extracts caused reductions in %cell viability to 80.21 ± 17.27 and 80.42 ± 7.94 percent, respectively, at the 200 µg/mL concentration. The isolated bioactive compound, dipterocarpol was also not toxic to RAW 264.7 at 25 µg/mL and %cell viability was reduced to 58.38 ± 2.60 at 50 µg/mL (Table 1). From the cytotoxicity study, non-toxic concentrations of crude extracts and dipterocarpol between 50-100 µg/mL and 25-50 µg/mL were selected for the anti-inflammatory experiments.

Nitric oxide (NO) and prostaglandin E2 (PGE_2) are important mediators of the inflammatory process and produce symptoms of inflammation. So, lower levels of these two mediators can prevent inflammatory reactions.[20] In contrast, increases in NO and PGE_2 production have been implicated in the pathology of cancer and neuronal diseases.[19,21] The current study evaluated NO levels indirectly, via analysis of nitrite concentrations using the Griess reaction, as an indicator of NOS activity.[22] PGE_2 production was used as an indirect determination of COX-2 activity.[23] The results show that leaf, twig and bark crude extracts at concentrations of 50 and 100 µg/mL reduced production of both NO and PGE_2. At 100 µg/mL, leaf and bark extracts inhibited nitrite levels by 47.37% and 32.46%, respectively, while twig extract reduced nitrite levels by 15.69% (Table 2). For PGE_2, the bark extract of *D. alatus* at 100 µg/mL markedly reduced PGE_2 production (by 89.09%), while leaf and twig extracts reduced PGE_2 production by 37.16% and 12.34%, respectively (Table 2). Aspirin (ASA), a nonsteroidal anti-inflammatory drug (NSAID), was used as a positive control in these experiments. It showed nearly 100% inhibition of PGE_2 production but did not affect nitric oxide level.[13] Interestingly, dipterocarpol exhibited a similar effect to ASA, not affecting NO but reducing PGE_2 levels.

Cytokines are mediators of cell growth, differentiation, repair, and inflammation, so they define the magnitude and nature of the immune response. TNF-α and IL-1β are important inflammatory cytokines that are produced by various cell types, including macrophages. They act

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as B cell growth factors, activate T cells, and induce protein synthesis in the inflammatory phase. Moreover, TNF-α directly stimulates the vascular endothelium to synthesise PGE_2.[22, 24] IL-1β is a pro-inflammatory cytokine that has been implicated in pain, inflammation and autoimmune conditions. Over production of IL-1β is involved in the pathophysiological changes that occur in diseases such as rheumatoid arthritis, neuropathic pain, inflammatory bowel disease, osteoarthritis, vascular disease, multiple sclerosis, and Alzheimer's disease.[25] Therefore, inhibition of IL-1β could be a successful method for managing pain and inflammation. *D. alatus* crude extracts significantly reduced IL-1β levels. At the tested concentration of 100 µg/mL, leaf, twig and bark extracts reduced IL-1β levels in LPS-stimulated RAW 264.7 cells by 78.08%, 68.49% and 64.38%, respectively (Table 2). In contrast, all three *D. alatus* crude extracts did not show a significant reduction in TNF-α levels (Table 2). Aspirin inhibited IL-1β by 43.83% and TNF- α by 27.24%. Overall, these results suggest that the *D. alatus* extracts contain bioactive compounds that might exert their anti-inflammatory effects through the same mechanism as NSAID drugs.[26-28] Also, dipterocarpol might be the main compound responsible for this anti-inflammatory activity as 50 µg/mL dipterocarpol reduced IL-1β and TNF- α levels by 100.00% and 99.35%, respectively.

According to the study of Yongram et. al. (2019),[5] the total phenolic content in the different parts of *D. alatus* is an important determinant for their antioxidant activity. This is in accordance with the study of Chen et al. (2017),[3] who isolated a resveratrol tetramer from *D. alatus* that reduced the levels of inflammatory cytokines in hyperuricemic mice. Therefore, the chemical contents of the *D. alatus* leaf, bark and twig extracts were determined and are shown in Table 3. The results show that two phenolic compounds, gallic acid and protocatacechuic acid, were found in the bark extract at yields of 5.09 and 7.18 mg/g extract, respectively. Dipterocarpol, a triterpenoid, was found in twig extract at 16.46 mg/g extract. This variation in the types of anti-inflammatory compounds found in the different parts of *D. alatus* is interesting for further development.

The tested crude extracts of *D. alatus*, which contain multiple bioactive components with multiple biological activities, are interesting for development as multitarget therapy drug. This plant showed low toxicity in cytotoxicity screening on RAW 264.7 cells and appeared to act on specific types of pro-inflammatory cytokines. This is in accordance with the study of Chen et al. (2017)[3] who isolated a resveratrol tetramer from *D. alatus* that reduced inflammatory cytokines in hyperuricaemic mice. From the results, *D. alatus* shows good potential as an anti-inflammatory herbal medicine. In the future, the active compounds in *D. alatus* should be isolated as chemical markers of anti-inflammatory activity and investigated for other biological activities.

Table 1: The %viability of *D. alatus* crude extracts in RAW 264.7 cells

Crude extract	Concentration (µg/mL)			
	25	50	100	200
Leaf	92.40 ± 11.04	101.65 ± 1.25	93.37 ± 7.06	96.99 ± 1.28
Twig	94.66 ± 7.23	94.92 ± 2.51	100.32 ± 3.14	80.42 ± 7.94*
Bark	100.64 ± 7.70	90.38 ± 13.45	103.05 ± 9.08	80.21 ± 17.27
Dipterocarpol	104.60 ± 5.86	58.38 ± 2.60	-	-

* $p < 0.05$ Compare to control group (vehicle treated)

Table 2: %Inhibition of IL-1 β and TNF- α by *D. alatus* extract in LPS-stimulated RAW 264.7 cells.

Crude extract	Concentration	%Inhibition			
	(µg/mL)	NO	PGE_2	IL-1β	TNF-α
Leaf	50	46.46 ± 0.83*	13.12 ± 6.03*	73.97 ± 2.19*	11.25 ± 2.49
	100	47.37 ±1.28*	37.16 ± 4.87*	78.08 ± 2.16*	8.87 ± 3.16
Twig	50	14.38 ± 2.13*	20.01 ± 2.97*	52.05 ± 0.63*	< 1
	100	15.69 ± 0.99*	12.34 ± 2.94*	68.49 ± 2.42*	< 1
Bark	50	31.87 ± 0.64*	40.83 ± 0.91*	38.35 ± 14.68*	< 1
	100	32.46 ± 0.40*	89.09 ± 6.60*	64.38 ± 0.63*	< 1
Dipterocarpol	25	0.88 ± 0.91	31.68 ± 9.48*	97. 91± 2.*42	74. 06±6 .*07
	50	0 ± 1.32	33.48 ± 10.16*	100.00 ±3 .*16	99. 35±4 .*52
ASA	1 mM	0 ± 2.24	96.98 ± 15.11*	43.83 ± 1.79	27.24 ± 1.38

* $p < 0.05$ Compare to control group (vehicle treated)

Table 3: The Chemical composition of *D. alatus* extracts

Crude extract	Chemical contents				
	TPC[5] (mg GAE/g DW)	TFC (mg QE/g DW)	Gallic acid (mg/g extract)	Protocatechuic acid (mg/g extract)	Dipterocarpol (mg/g extract)
Leaf	308.60 ± 4.32	14.40 ± 0.21	3.84 ± 0.02	-	< 2
Twig	128.29 ± 3.89	4.39 ± 0.71	3.13 ± 0.03	-	16.46 ± 0.14
Bark	366.43 ± 11.52	< 1	5.09 ± 0.15	7.18 ± 0.09	< 2

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Conclusion

Crude extracts of *D. alatus* leaf, twig, and bark reduced production of the inflammatory mediators NO and PGE_2 in LPS-stimulated RAW 264.7cells. Therefore, *D. alatus* extract has excellent potential for development as an herbal product for inflammatory diseases. Future studies of the effects of *D. alatus* in animal models would be of importance to confirm the mechanism and efficacy of *D. alatus* anti-inflammatory activity.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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