Antidesma thwaitesianum Müll. Arg. fruit extract rich in 5-hydroxymethylfurfural exhibits anti-inflammatory effects in lipopolysaccharide-stimulated RAW264.7 macrophages

Benchaluk Thongchuai1, Supaporn Khamchun1, Wimonrut Insuan2, Daoyot Daorueang1, Plubplung Sansai1, Orapin Insuan1*

1Department of Medical Technology, School of Allied Health Sciences, University of Phayao, Phayao 56000, Thailand
2Department of Veterinary Technology, Faculty of Veterinary Technology, Kasetsart University, Bangkok 10900, Thailand

Introduction: Antidesma thwaitesianum Müll. Arg is a tropical fruit, which has been commonly used for healthy food and traditional herbal medicine. This study aimed to investigate the anti-inflammatory effects of A. thwaitesianum fruit extract (AFE) rich in 5-hydroxymethylfurfural (5-HMF) in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages.

Methods: The chemical composition of AFE was analysed by gas chromatography/mass spectrometry (GC/MS). RAW264.7 cells were used as an in vitro inflammatory response model. RAW264.7 cells were pre-treated with various concentrations of AFE or 5-HMF and subsequently treated with LPS. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The production levels of pro-inflammatory cytokines and mediators, including nitric oxide (NO), interleukin (IL)-1β, and tumour necrosis factor (TNF)-α were determined by the Griess assay and enzyme-linked immunosorbent assay. The protein expression of inducible nitric oxide synthase (iNOS) was examined by western blot analysis.

Results: AFE had a high content of 5-HMF (61.03% ± 0.49%). Pre-treatment with AFE and 5-HMF markedly reduced LPS-induced pro-inflammatory mediators and cytokines, namely NO, IL-1β, and TNF-α, in RAW264.7 cells; this reduction correlated with downregulation of iNOS expression.

Conclusion: This study suggests that A. thwaitesianum fruit extract containing 5-HMF could modulate the LPS-induced inflammatory response by inhibiting NO, IL-1β, and TNF-α production and INOS expression. A. thwaitesianum fruit extract rich in 5-HMF could be considered a potential therapeutic agent for the prevention of inflammation.

Implication for health policy/practice/research/medical education: Antidesma thwaitesianum fruit extract rich in 5-HMF attenuated LPS-stimulated inflammatory response by inhibiting NO, IL-1β and TNF-α production and iNOS expression. Therefore, A. thwaitesianum rich in 5-HMF might be considered a potential therapeutic agent for inflammatory disease treatment.

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Introduction
Inflammation is a normal self-protective response to foreign pathogens or tissue injury. However, chronic inflammation leads to the pathogenesis of several diseases, such as arthritis, cardiovascular diseases, diabetes mellitus, metabolic syndromes, and cancers (1). Macrophages are major mononuclear phagocytic cells that play vital roles in the pathogenesis of inflammatory diseases. During the inflammatory process, pro-inflammatory cytokines and inflammatory mediators, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, nitric oxide (NO), and prostaglandin E2 (PGE2), are overproduced by macrophages.
A commonly used in vitro inflammatory response model is lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages; it allows researchers to assess the anti-inflammatory activities of naturally derived compounds (1). LPS, a component of the gram-negative bacterial cell wall, is one of the most potent activators of macrophages. Activated macrophages transcriptionally express inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, which are the key regulator enzymes associated with the production of NO and PGE$_2$, respectively (5). iNOS catalyses the oxidative deamination of L-arginine to produce NO. Excessive NO generation leads to the accumulation of reactive oxygen species (ROS) and can cause harmful effects, including inflammatory diseases (6). COX-2 synthesises prostaglandins (PGs), which function as mediators of the inflammatory response to induce pain, fever, and other symptoms (7). The LPS-mediated expression of inflammatory cytokines involved in the expression of specific genes is regulated by nuclear transcription factor kappa B (NF-kB) and the mitogen-activated protein kinase (MAPK) signalling pathways (8,9). NF-kB, a transcription factor composed of the p65 and p50 subunits, regulates the transcription of multiple genes, various inducible enzymes (COX-2 and iNOS), and pro-inflammatory cytokines (10,11). MAPKs, including extracellular signal-regulated kinase (ERK), c-Jun N terminal kinase (JNK), and p38, control the expression of pro-inflammatory mediators and cytokines by sequential phosphorylation (12,13). They are involved in the LPS-induced expression of iNOS and COX-2 in activated macrophages (1,14). Thus, regulating the expression of pro-inflammatory mediators and cytokines is one of the most effective strategies to treat inflammation.

**Antidesma thwaitesianum** Müller Arg (family: Euphorbiaceae), commonly known as mamao luang, is a tropical fruit that is typically used for soft drinks and healthy food. Moreover, it has been used as a traditional herbal medicine because it contains high amounts of phenolic acids, flavonoids, and anthocyanins. Vanillic acid, gallic acid, caffeic acid, p-coumaric acid, ferulic acid, sinapinic acid, and cinnamic acid are the phenolic acids in mamao (15). The most abundant flavonoids are (-)-epicatechin and (+)-catechin (flavan-3-ols), Cyanidin-3-O-glucoside, cyanidin 3-rutinoside, and malvidin 3,5-diglucoside are the main anthocyanin glycosides presented in mamao fruits (15). Fresh and dried fruits of *A. thwaitesianum* have many biological properties, including antioxidant, anticancer, antimicrobial, and anti-inflammatory activities (16-20). Several studies have reported that *A. thwaitesianum* have many biological properties, including antioxidant, anticancer, antimicrobial, and anti-inflammatory activities (16-20). Several studies have reported that *A. thwaitesianum* have many biological properties, including antioxidant, anticancer, antimicrobial, and anti-inflammatory activities (16-20). Several studies have reported that *A. thwaitesianum* have many biological properties, including antioxidant, anticancer, antimicrobial, and anti-inflammatory activities (16-20). Several studies have reported that *A. thwaitesianum* have many biological properties, including antioxidant, anticancer, antimicrobial, and anti-inflammatory activities (16-20). Several studies have reported that *A. thwaitesianum* have many biological properties, including antioxidant, anticancer, antimicrobial, and anti-inflammatory activities (16-20).
by using split injection (split ratio 5:1) for a 1 µL sample, with the following temperature settings: injector set at 250°C, column set at 60°C, heating ramp of 10°C/min, final temperature of 280°C, and detector set at 200°C. Helium was used as the carrier gas at a flow rate of 1.5 mL/min. Fragmentation was performed by electron impact (70 eV), and the mass range was between 40 and 500 amu. Identification of compounds was based on comparisons of the mass spectra with those of the NIST (NIST14) mass spectral library data standard of the GC/MS system.

Cell culture
The RAW264.7 mouse macrophage cell line was obtained from American Type Culture Collection (ATCC number TIB-71) and cultured in DMEM supplemented with 10% fetal bovine serum and 100 units/mL penicillin–streptomycin under a humidified (95%) atmosphere of 5% CO₂ at 37°C.

Cell viability test
RAW264.7 cells were plated into a 96-well plate at a density of 2 × 10⁴ cells/well (cell viability >95%). After 24 hours, the cells were pre-treated with various concentrations of AFE or 5-HMF for 2 hours and subsequently treated with 1 µg/mL LPS for 22 hours. Next, the cells were incubated with MTT solution at the final concentration of 0.5 mg/mL for 4 hours at 37°C. Then, the supernatant was removed, 100 µL of dimethyl sulfoxide was added to dissolve the formazan crystals, and absorbance at 570 nm was determined by using a microplate reader. Untreated cells with a cell viability of 100% were used as the control. The cell viability of the treated wells was calculated as a percentage relative to that of the control.

Measurement of NO and cytokine production
RAW264.7 cells (1 × 10⁴ cells/well) were plated into a 6-well plate and treated with various concentrations of AFE or 5-HMF. After 2 hours of treatment, the cells were incubated with LPS (1 µg/mL) for 22 hours. The NO production in the cell culture supernatant was measured by using a Griess reagent kit (Invitrogen, Thermo Fisher Scientific, Inc., Eugene, OR, USA), whereas the levels of IL-1β and TNF-α were detected by using ELISA kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions.

Western blot analysis
RAW264.7 cells were plated into 6-well plate at a concentration of 1 × 10⁴ cells/mL. After 24 hours, the cells were treated with different concentrations of AFE or 5-HMF for 2 hours and subsequently treated with LPS (1 µg/mL) for 22 hours. The cells were harvested and washed with cold phosphate buffer saline. Total proteins were extracted from the cells using Laemmli lysis buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the protein concentration was quantified by using a Bradford assay kit (Thermo Fisher Scientific, Inc., IL, USA). Western blot analysis was performed according to a standard protocol. Briefly, 30 µg of proteins was separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes. The membranes were incubated in 5% non-fat dry milk in Tris-buffered saline (TBS) to block nonspecific protein binding. The membranes were subsequently incubated with primary rabbit monoclonal antibodies against iNOS and β-actin, the latter served as a loading control, at 4°C overnight. The membranes were then washed three times with 0.05% Tween 20-TBS and incubated with horseradish peroxidase–conjugated secondary antibodies at room temperature for 1 hour. Protein bands were detected by using an enhanced chemiluminescence reagent (Bio-Rad Laboratories) and then exposed to X-ray film. Band intensity data were obtained by using ImageJ software (https://imagej.nih.gov/ij).

Statistical analysis
All results are expressed as mean ± standard deviation (SD). Differences between the treatments were determined by one-way analysis of variance (ANOVA). Statistical significance was accepted for P values < 0.05.

Results
Chemical characteristics of A. thwaitesianum fruit extract
The chemical composition of A. thwaitesianum fruit extract (AFE) is shown in Table 1. The AFE contained 22 compounds; the main compound was 5-HMF (61.03% ± 0.49%). Other abundant compounds were D-allose (aldohexose) (9.16% ± 0.33%), 3-methyl-2,5-furandione (6.25% ± 0.09%), 1,6-anhydro-beta-D-glucofuranose (5.22% ± 0.25%), and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (4.72% ± 0.15%).

Cytotoxic effects of A. thwaitesianum fruit extract and 5-HMF on RAW264.7 cells
The MTT assay was used to determine the cytotoxicity of AFE and 5-HMF in RAW264.7 cells. Treatment with both AFE (12.5–400 µg/mL) and 5-HMF (12.5–400 µg/mL) with or without LPS (1 µg/mL) had no effect on the viability of RAW264.7 cells. The percentage of cell survival under all conditions relative to the control exceeded 80% (Figure 1). Therefore, the 100–400 µg/mL AFE and 50–200 µg/mL 5-HMF were used in the subsequent experiments.

Effects of A. thwaitesianum fruit extract and 5-HMF on LPS-induced NO production and iNOS expression in RAW264.7 cells
To investigate the anti-inflammatory properties of AFE and 5-HMF, we initially measured the production of NO in LPS-stimulated RAW264.7 cells. As shown in Figures 2A and 2B, the NO level in the LPS-treated group showed a significant increase relative to that in the control group...
Anti-inflammatory effects of Antidesma thwaitesianum

NO release was inhibited dramatically by both AFE and 5-HMF in a dose-dependent manner \(^{(P < 0.005)}\). NO production is related directly to upregulation of the expression of iNOS. Therefore, the effect of AFE and 5-HMF on LPS-induced iNOS protein expression was examined by western blot analysis. The iNOS protein level was markedly increased by LPS stimulation and suppressed by AFE (400 µg/mL) and 5-HMF (100 and 200 µg/mL) treatment (Figure 2C-F).

Effects of A. thwaitesianum fruit extract and 5-HMF on LPS-induced IL-1β and TNF-α in RAW264.7 cells

As the treatment of AFE and 5-HMF inhibited the expression of the pro-inflammatory mediators NO

Table 1. Chemical constituents and their percentages in Antidesma thwaitesianum fruit extract identified by gas chromatography/mass spectrometry (GC/MS)

| Retention time (min) | Peak area (%) | Chemical formula | Chemical composition |
|----------------------|---------------|------------------|----------------------|
| 3.52                 | 6.25 ± 0.09   | C\(_5\)H\(_4\)O\(_3\) | 3-Methyl-2,5-furandione |
| 3.78                 | 0.89 ± 0.03   | C\(_6\)H\(_6\)O\(_2\) | 5-Methyl-2-furancarboxaldehyde |
| 4.00                 | 0.89 ± 0.11   | C\(_3\)H\(_4\)NO\(_3\) | 2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one |
| 4.36                 | 0.21 ± 0.02   | C\(_3\)H\(_3\)N \(\cdot\)O \(\cdot\)H | 2-Butyl-\(\cdot\)methyl ester, L-1,3,2-oxazaborolane-4-carboxylic acid |
| 4.61                 | 1.34 ± 0.10   | C\(_5\)H\(_4\)O \(\cdot\)N | Dihydro-3-methylene-2,5-furandione |
| 4.82                 | 0.91 ± 0.01   | C\(_6\)H\(_6\)O \(\cdot\) | 4-Oxo-pentanoic acid |
| 5.21                 | 0.27 ± 0.02   | C\(_6\)H\(_8\)N | 1,3,5-Triazine-2,4,6-triamine |
| 5.24                 | 0.44 ± 0.01   | C\(_6\)H\(_6\)O | 2,5-Furandicarboxaldehyde |
| 5.33                 | 1.50 ± 0.08   | C\(_6\)H\(_6\)O | Furfuryl hydroxymethyl ketone |
| 5.70                 | 0.21 ± 0.01   | C\(_6\)H\(_6\)O | Butyl acetate |
| 5.72                 | 0.29 ± 0.02   | C\(_6\)H\(_6\)O | Levoglucosenone |
| 6.19                 | 4.72 ± 0.15   | C\(_6\)H\(_8\)O | 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one |
| 6.61                 | 0.43 ± 0.05   | C\(_6\)H\(_8\)O | Ethyl propionylacetate |
| 6.95                 | 0.43 ± 0.03   | C\(_6\)H\(_8\)O | 2-Pentanol, propanoate |
| 7.05                 | 0.26 ± 0.01   | C\(_6\)H\(_4\)O \(\cdot\) | 1,2,5,6-dianhydrogalactitol |
| 7.35                 | 61.03 ± 0.49  | C\(_6\)H\(_8\)O | 5-Hydroxymethylfurfural |
| 7.84                 | 1.38 ± 0.08   | C\(_6\)H\(_8\)O | Methylene-butanedioic acid |
| 9.40                 | 2.84 ± 0.11   | C\(_6\)H\(_12\)O \(\cdot\) | Succinic acid, 3-methylbutyl pentyl ester |
| 10.26                | 0.79 ± 0.01   | C\(_6\)H\(_10\)NO \(\cdot\) | 2-(hydroxymethyl)-2-nitro-1,3-propanediol |
| 10.91                | 9.16 ± 0.33   | C\(_6\)H\(_10\)O \(\cdot\) | D-allose |
| 11.68                | 0.57 ± 0.09   | C\(_6\)H\(_10\)NO \(\cdot\) | 1-[Beta-d-ribofuranosyl]-5-fluoro-4-O-difluoromethyl uracil |
| 12.12                | 5.22 ± 0.25   | C\(_6\)H\(_10\)O | 1,6-Anhydro-beta-D-glucofuranose |

Each value is expressed as mean ± SD of three measurements.

Figure 1. The effect of Antidesma thwaitesianum fruit extract (AFE) and 5-hydroxymethylfurfural (5-HMF) on the viability of RAW264.7 macrophages. The cells were treated with indicated concentrations of A: AFE and B: 5-HMF in the absence or presence of lipopolysaccharide (LPS). The results are expressed as the mean ± SD (n = 3).
and iNOS, the effects of AFE and 5-HMF on the pro-inflammatory cytokines, including IL-1β and TNF-α, were examined by ELISA. The levels of IL-1β and TNF-α were significantly higher in the LPS-treated group than in the control group (Figure 3). Pre-treatment with various concentrations of 5-HMF (50-200 μg/mL) significantly decreased LPS-stimulated IL-1β and TNF-α production in a dose-dependent manner (Figure 3B and 3D). The produced level of TNF-α was dramatically and dose-dependently reduced by AFE relative to that in the LPS-treated group (Figure 3C). However, the production level of IL-1β was not changed (Figure 3A).

Discussion

Dietary components have notable biological effects, including the ability to ameliorate inflammation. While steroids and anti-inflammatory non-steroidal compounds are commonly used to treat chronic inflammatory diseases, these drugs may have undesirable side effects (27,28). Research on the anti-inflammatory effects of natural compounds aims to provide alternative therapies. There have been a number of investigations on Antidesma thwaitesianum fruit and it anti-inflammatory actions. In this study, we focussed on the anti-inflammatory effects of AFE rich in 5-HMF in LPS-stimulated macrophages. GC/MS was used to analyse the chemical constituents of AFE. The major constituent detected in AFE was 5-HMF (61.03% ± 0.49%). 5-HMF can be found naturally in honey and processed foods, including fruit juices, UHT milk, and baked foods (29). Besides, it can be produced by acid-catalysed thermal dehydration of fructose (30). Thus, our result demonstrated the potential of Antidesma thwaitesianum fruit as a natural source of 5-HMF. Several beneficial effects of 5-HMF have been reported, including anti-allergic, antioxidative, anticancer, and anti-inflammatory properties (25,31). Maillard reaction products containing 5-HMF exert antioxidant and anti-inflammatory effects in interferon-γ and phorbol ester–induced Caco-2 cells (32). Researchers have also explored the anti-inflammatory activities of 5-HMF in LPS-induced RAW264.7 cells via suppression of MAPK, NF-κB, and Akt/mTOR signalling pathways.

Macrophages are innate immune cells that play a role as sensors and responders to inflammation (33,34). The model of LPS-induced inflammatory responses in RAW264.7 cells has been widely used for anti-inflammatory drug...
screening. In the present study, we confirmed that AFE rich in 5-HMF had an anti-inflammatory effect on the LPS-stimulated inflammatory response of RAW 264.7 cells. During inflammation, macrophage activation results in increased production of NO, a reactive free radical, by iNOS. High NO levels are cytotoxic in inflammation (35). Therefore, the inhibition of NO and iNOS overexpression is important for anti-inflammation. We found that AFE rich in 5-HMF and 5-HMF alone effectively inhibited NO production by downregulating iNOS protein expression in LPS-stimulated RAW 264.7 cells. Hence, AFE rich in 5-HMF and 5-HMF can inhibit the production of inflammatory mediators. LPS-induced macrophage activation can lead to the production of pro-inflammatory cytokines and mediators, including IL-1β, IL-6, and TNF-α (1). TNF-α is central in the inflammatory process and is normally found in chronic inflammatory diseases (36), while IL-1β is important for the initiation and enhancement of the inflammatory response (37). Therefore, blocking pro-inflammatory cytokine production is one of the most effective strategies for the treatment of inflammation. Our results support the anti-inflammatory effects of AFE rich in 5-HMF and 5-HMF in LPS-stimulated RAW264.7 cells. AFE and 5-HMF significantly inhibited the release of TNF-α, whereas only 5-HMF inhibited IL-1β production. The potential of AFE rich in 5-HMF to reduce LPS-induced inflammation was confirmed by its ability to reduce NO, TNF-α, and IL-1β, which are central in inflammation.

**Conclusion**

The fruit extract of *A. thwaitesianum* containing 5-HMF and 5-HMF alone exerted anti-inflammatory properties by suppressing the production of NO, TNF-α, and IL-1β, reductions that correlated with downregulation of iNOS expression. *A. thwaitesianum* rich in 5-HMF might be considered a potential therapeutic agent for inflammatory disease treatment. Despite the fact that we have established the anti-inflammatory effect of *A. thwaitesianum* rich in 5-HMF, the molecular mechanisms underlying the inhibitory effect of the extract need to be elucidated.

**Authors’ contributions**

BT and WI performed the experiments, analysed the data, and wrote the manuscript. SK, DD, and PS performed the experiments and analysed the data. OI designed and performed the experiments, analysed the data, and wrote and edited the manuscript. All co-authors revised and approved the final version of the manuscript.

**Conflict of interests**

The authors declare no conflict of interest.

**Ethical considerations**

Ethical issues, including text plagiarism, misconduct, data fabrication, falsification, double submission or publication, redundancy have been carefully observed by authors.

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