Natural Product Sciences

- **Manuscript ID** : NPS-15-0065
- **Manuscript Type** : Regular Article
- **Manuscript Title** : Anti-inflammatory Effect of Mangosteen (Garcinia mangostana L.) Peel Extract and its Compounds in LPS-induced RAW264.7 Cells

Dear Dr. Wahyu Widowati:

Your revised manuscript titled `Anti-inflammatory Effect of Mangosteen (Garcinia mangostana L.) Peel Extract and its Compounds in LPS-induced RAW264.7 Cells` has been submitted successfully.

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Evaluations (1st Review) :

Editorial Comment

Recommendation Major Revision

Ms. Ref. No.: NPS-15-0065

Title: Anti-inflammatory Effect of Mangosteen (Garcinia mangostana L.) Peel Extract and its Compounds in LPS-induced RAW264.7 Cells

Comments to the Author

Dear Professor Wahyu Widowati,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below.

READ THESE INSTRUCTIONS AND FOLLOW THEM CAREFULLY.

In addition to submitting a clean copy of your revised manuscript through On-line submission of http://www.ksp.or.kr/, please also upload a copy with the changes highlighted in yellow or typed in red. Do not show deleted material or track changes. (This means you have to submit two separate files: one clean copy of the revised manuscript and one with changes highlighted.).

The revision of your manuscript is due no later than one month after you got the Revision Request letter.

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Do not hesitate to contact me if I can be of any assistance.

With kind regards,

● Reviewer A :

This manuscript describes anti-inflammatory effect of mangosteen peel extract and mangostin isolated from mangosteen. authors evaluated cytotoxicity and anti-inflammatory effect using in vitro model. In RAW cell, concentration of inflammatory mediators such as COX-2, IL-6, IL-1b and NO were measured to evaluate anti-inflammatory effect. Activities of mangosteen and mangostin were very potent. experiment design is very good. logical explanation is also very clear.

However, anti-inflammatory effect of mangosteen and mangostin was previously reported several times other papers. So originality of this paper is a little bit low. Please refer the papers listed below.

- β Mangostin suppress LPS-induced **inflammatory** response in RAW 264.7 macrophages in vitro and carrageenan-induced peritonitis in vivo.
- α-Mangostin: **anti-inflammatory** activity and metabolism by human cells.
- Anthelmintic, **anti-inflammatory** and antioxidant effects of **Garcinia mangostana** extract in hamster opisthorchiasis.....

In this reason, I think that this manuscript should not be published in Natural Product Sciences.

● Reviewer B :

The authors report the anti-inflammatory effects of Garcinia mangostana and its major compounds in LPS-activated RAW 264.7 cells.

1. As authors described in Discussion, the exposure of macrophage to the exogenous stimulators like LPS induces the expression of inducible NO synthase (iNOS) followed by the overproduction of nitric oxide. In the present study, Garcinia mangostana and its active compounds a-mangostin and r-mangostin were found to inhibite nitric oxide production in LPS-activated RAW264.7 cells. The evaluation of inhibitory activities of the samples on the expression of iNOS is strongly proposed.

2. The previous reports on the protective effects of Garcinia mangostana or its compounds on inflammation or related diseases need to be discussed in the manuscript.

3. Indicate the specimen number of the extract of Garcinia mangostana.

4. The name and affiliation of the institute where the plant material was identified was indicated but the information of the person was not shown.

5. The method for the quantification of COX-2, IL-1b and IL-6 were very similar. To combine each paragraph into one is proposed. Also, Table 1 and 2 are recommended to combine into one.

6. For the quantification of COX-2 expression in cells, the test sample is generally prepared from cell lysate while for cytokines, cell culturing media where the produced cykines from cells were relased. Eventhough the authors used ELISA kit and followed the manufactural's protocol, give the method for the preparation of samples from cells and assay method briefly.

7. In results section (page 9), use comma instead of semicolon in the sentence of "GMPE treatment showed low viability at 100;75;50;25 ug/mL" and the other sentences.

8. In results section (page 9), the indication of concentration of each sample needs to be modified to avoid confusion. "…three concentration of GMPE (20, 10, 5 ug/ml), a-mangostin (75,50, 25 ug/ml)…"

9. Give the formula how the inhibitory activity of the samples on COX-2, IL-1b, IL-6 and NO was calculated in Table 3-6.

10. The authors used 20% FBS supplemented DMEM for culture of RAW264.7 cell but for the assays, 10% FBS-DMEM was used. Why?

11. Unite the units; hours, hr, h

12. Too many grammatically incorrect English throughout the manuscript. To proofread the manuscript by native speaker is recommended.

Dear Editor of NPS

Thank you very much for your review of my manuscript

Herewith I attach the revised manuscript

● **Reviewer A :**

This manuscript describes anti-inflammatory effect of mangosteen peel extract and mangostin isolated from mangosteen. authors evaluated cytotoxicity and anti-inflammatory effect using in vitro model. In RAW cell, concentration of inflammatory mediators such as COX-2, IL-6, IL-1b and NO were measured to evaluate anti-inflammatory effect. Activities of mangosteen and mangostin were very potent. experiment design is very good. logical explanation is also very clear.

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- Anthelmintic, **anti-inflammatory** and antioxidant effects of **Garcinia mangostana** extract in hamster opisthorchiasis.....

In this reason, I think that this manuscript should not be published in Natural Product Sciences.

Comment :

Thank you very much for your highly review to correct my manuscript, thank you very much your evaluation that my experiment design very good, logical explanation is very clear but you mentioned that my research was reported previously.

1. I would like to explain that the first article as you mentioned (β Mangostin suppress LPS-induced **inflammatory** response in RAW 264.7 macrophages *in vitro* and carrageenan-induced peritonitis in vivo), this article mentioned that β mangostin suppress LPS-induced inflammatory in RAW 264.7. My research was done to evaluate the anti-inflammatory effect of α-mangostin, γ-mangostin the most compounds of mangosteen peel extract (my previous paper entitled "High Performance Liquid Chromatography (HPLC) Analysis, Antioxidant, Antiaggregation of Mangosteen Peel Extract (Garcinia mangostana L.) and I continue to know the anti-inflammatory potency of the most compounds of mangosteen extract, so I continue my research to measure the anti-inflammatory effect of α-mangostin, γ-mangostin and mangosteen peel extract.
2. The second article you mentioned the article entitle α-Mangostin : **anti-inflammatory** activity and metabolism by human cells. This research using cell line THP-1 (monocyte-like leukemia) for IL-8 level as inflammatory marker, HepG2 (hepatocellular carcinoma) for TNF-α level as inflammatory marker, Caco-2 HTB-37 (colorectal adenocarcinoma cells that spontaneously differentiate to enterocyte-like phenotype) for IL-8 level as inflammatory marker, and HT-29 (colorectal adenocarcinoma) for IL-8 level as inflammatory marker, RAW 264.7 for NO level as inflammatory level. My research using LPS–induced-RAW 264.7 were treated with α-mangostin, γ-mangostin and mangosteen peel extract with inflammatory marker COX-2, IL-6, IL-1β, NO and cytotoxic assay. This research report you mentioned was different with my research both measured-parameter and anti-inflammatory agent

- The third article you mentioned the article entitle Anthelmintic, **anti-inflammatory** and antioxidant effects of **Garcinia mangostana** extract in hamster opisthorchiasis. This research was specific inflammatory model that inflammatory in hamster opisthorchiasis infected with Opisthorchis viverrini alone (OV). The result showed that G. mangostana had no renal toxic effect. ABTS radical-scavenging assay indicated that the extract had antioxidant property. Reduction in aggregation of inflammatory cells surrounding the hepatic bile duct, especially at the hilar region, was found in the OVGM. This research report used *in vivo* study with hamsters as the research subject while our paper used *in vitro* study with RAW 264.7 cells as the subject.

For the case of reviewer B, we would like to thank for giving constructive comments to make our paper better and suitable for publish. Responding to the reviewer B's comments, we have made several changes in our article, including :

- The correlation of NO production and iNOS has added.

- Previous reports of G mangostana against inflammation has mentioned several times in discussion, but we added more reports now.

- The information of the person identified the plant has been added.

- The method for quantification of COX-2, IL-1β, and IL-6 has changed. The ELISA protocol for COX-2 was slighty different with IL-1β and IL-6, therefore the assay protocol for COX-2 was separated alone. Whilst for IL-1β and IL-6, we used same assay protocol, hence the method of IL-1β and IL-6 quantification was combined into one paragraph. The assay protocol now explained clearly.

- Table 1 and 2 now combined into one table, Table 1.

- The semicolon in several places in the article has changed to comma to separate the concentration of samples used.

- Some sentences has changed to avoid the confusion, regarding of explaining the concentration used for the treatments.

- The mistakes of using 20% FBS in the method was corrected, to 10% FBS-DMEM for culturing RAW 264.7 cells.

- The units are now united, all in "hours".

- The grammar has been checked by a grammar app, and several sentences has changed.

For some comments, we decided to not making any changes, which are:

- The reviewer ask for the specimen number, but there was no data of specimen number, therefore the specimen number couldn't be mentioned.

- - The reviewer ask for method in preparation of samples for COX-2 expression quantification. For COX-2 concentration quantification, the samples used were the cell free supernatant. The method for preparing the cell free supernatant was mentioned in the method of Pro-inflammatory activation of cells and treatment, “The cell free supernatant was taken for the next assay by centrifugation at 2000 g for 10 minutes. The supernatant was stored at -79°C for the COX-2, IL-6, IL-1β, and NO concentration and inhibitory activity assay.”

All in all, we have made several changes to improve our article and we hope we can get positive feedbacks and get published.

Anti-inflammatory Effect of Mangosteen (*Garcinia mangostana* L.) Peel Extract and its Compounds in LPS-induced RAW264.7 Cells

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Abstract - Inflammation plays an important role in host defense against external stimuli such as infection by pathogen, endotoxin or chemical exposure by the production of the inflammatory mediators that produced by macrophage. Anti-inflammatory factor is important to treat the dangers of chronic inflammation associated with chronic disease. This research aims to analyze the anti-inflammatory effects of *Garcinia mangostana* L. peel extract (GMPE), α-mangostin, and γ-mangostin on LPS-induced murine macrophage cell line (RAW 264.7) by inhibiting the production of inflammatory mediators. The cytotoxic assay of *G. mangostana* L. extract, α-mangostin, and γ-mangostin was performed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) to determine the safe and non-toxic concentration in RAW 264.7 for the further assay. The concentration of inflammatory mediators (COX-2, IL-6, IL-1β, and NO) were measured by the ELISA-based assay in treated LPS-induced RAW 264.7. The inhibitory activity was determined by the reducing concentration of inflammatory mediators in treated LPS-induced RAW 264.7 over the untreated cells. This research revealed that GMPE, α-mangostin, and γ-mangostin possess the anti-inflammatory effect by reducing COX-2, IL-6, IL-1β, and NO production in LPS-induces RAW 264.7 cells.

Keywords: anti-inflammatory, GMPE, α-mangostin, γ-mangostin, macrophages, inflammatory mediator.

Introduction

Natural products have long been over the years contributed to the development of new therapeutic drugs for a variety of human diseases.[1] Many tropical plants have interesting biological activities with their therapeutic potential, including *Garcinia mangostana* L. (mangosteen). Mangosteen has been used for hundreds of years around the world, mostly in Southeast Asia, as a medicine for a great variety diseases.[2] Many studies have shown that the various parts extract contain varieties of secondary metabolites such as prenylated and oxygenated xanthones. Xanthones as the major bioactive secondary metabolites were reported to have many pharmacological activities including antioxidant, antifungal, anti-bacteria, cytotoxic, anti-inflammatory, antihistamine, anti-HIV, antimalarial and other activities.[3-5] The previous study confirmed that the *G. mangostana* peel extract (GMPE) contained α-mangostin (105 ppm), γ-mangostin (7.20 ppm), garcinone C (3.50 ppm), and garcinone D (9.92 ppm) based on high performance liquid chromatography (HPLC) analysis.[6] In this study, the anti-inflammatory potential of *G.mangostana* L. peel extract (GMPE) and its compound were observed.

Inflammation plays an important role in host defense encompasses multiple processes against external stimuli such as infection by pathogen, exposure to bacterial endotoxin or chemical exposure.[7] The inflammation process involves changes in blood flow, increased vascular permeability, destruction of tissue via the activation and migration of leukocytes with the synthesis of reactive oxygen derivatives (oxidative burst) and synthesis of local inflammatory mediators.[8] The secretion of the inflammatory mediators both of proinflammatory mediators such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, and Nitric Oxide (NO) and anti-inflammatory such as IL-10 are the primary response to inflammation in addition to leukocyte recruitment.[9] Inflammation relates to various diseases such as rheumatoid arthritis, inflammatory bowel disease, artherosclerosis, Alzheimer's, and

has a role in various kinds of cancer.[10] Anti-inflammatory is important to treat the danger of chronic inflammation associated with chronic disease.[11]

Several mechanisms of action have been proposed to describe the phytochemical potential for anti-inflammatory, such as: 1) antioxidant and radical scavenging activity; 2) modulation of cellular activities of inflammation-related cells (mast cells, macrophages, lymphocytes, and neutrophils); 3) modulation of proinflammatory enzyme activities; 4) modulation of the production of other proinflammatory molecules; and 5) modulation of proinflammatory gene expression.[12] This study focused on the potential of GMPE, α-mangostin, and γ-mangostin in modulation of the proinflammatory molecules production by inhibiting the proinflammatory cytokines production including COX-2, IL-6, IL-1β, and NO in LPS-induced murine macrophage cell line model (RAW 264.7). The RAW 264.7 murine macrophage cell line is widely used as an inflammatory model *in vitro*.[13]

Experimental

General Experimental Procedure – *Garcina mangostana* L. was collected from Cisalak-Subang, West Java, Indonesia plantation and identified by a staff of herbarium of the Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. The murine macrophage cell line RAW 264.7 (ATCC® TIB-71™) was obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia.

α-mangostin (95-99% purity), and γ-mangostin (95-99% purity) were bought from Biopurify Phytochemical Ltd. (Chengdu, China). The medium and its component for cell culture such as Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), penicillin, streptomycin, and Trypsin-EDTA were purchased from Biowest. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)

used for cell viability assay was purchased from Promega (Madison, WI, USA). COX-2 ELISA kit and protocol (E-EL-M0959) was purchased from Elabscience. IL-6 and IL-1β ELISA kit and protocol (431301, and 432601) were purchased from BioLegend. Nitrate/Nitrite colorimetric assay kit (KA1342) was purchased from Abnova. MultiSkan Go (Thermo Scientific), a microplate reader, was used for viability assay, measuring COX-2, IL-6, IL-1β, and NO concentration.

Plant Extract preparation - The peels of *Garcina mangostana* L. were collected, chopped, and kept in drier tunnel service. Extraction was performed based on the maceration method using ethanol 70% as the solvent for collecting *Garcinia mangostana* L. peel extract (GMPE).[14,15]

RAW 264.7 Cells Culture and Viability Assay - The murine macrophage cells were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin. The cells then maintained at the 37°C humidified atmosphere incubator with 5% CO_2 until the cells were confluence. The cells were washed and harvested using Trypsin-EDTA and centrifuged at 2500 rpm for 4 minutes.[16-19]

Viability assay - The viability assay was performed to determine the safe and nontoxic concentration for the next assay, evaluated by MTS assay. Briefly, 100 µL cells (5 x 10^3 cells per well) in medium (DMEM supplemented with 10% FBS and 100 U/mL penicillin and streptomycin) were plated in 96-well plate and incubated for 24 hours at 37°C in a humidified atmosphere incubator with 5% CO_2. The medium then washed and added with 99 µL new medium and 1 µL of GMPE, α-mangostin, and γ-mangostin in different concentration and DMSO in triplicate then the plate were incubated for 24 hours. Untreated cells were served as the control. Briefly, 20 µL MTS was added to each well. The plate was incubated in 5% CO_2 at 37°C incubator for 4 hours. The absorbance was measured at 490 nm on a microplate reader.[18-21]

Proinflammatory activation of cells and treatment - The pro-inflammatory activation of cells was performed based on Yoon, *et al* (2009) and modified method.[17-19,22] The cells were seeded in 6-well plate in density of 5 x 10^3 cells per well and incubated for 24 hours at 37°C in a humidified atmosphere and 5% CO_2.[23] The medium (DMEM supplemented with 10% FBS and 100 U/mL penicillin and streptomycin) then washed and supplemented with 1600 µL growth medium and 200 µL GMPE (20, 10, 5 µg/mL), α-mangostin, and γ-mangostin (75, 50, 25 µM). Around 1-2 hours later, the medium was added with 200 µL LPS (1 µg/mL) and incubated for 24 hours at 37°C in a humidified atmosphere and 5% CO_2.[18,19] After incubation of RAW 264.7 cells with LPS for 24 hours, the quantity of COX-2, IL-6, IL-1β, and NO was accumulated in the cell-free supernatant. The cell-free supernatant then was taken for the next assay by centrifugation at 2000 g for 10 minutes. The supernatant was stored at -79°C for the COX-2, IL-6, IL-1β, and NO concentration and inhibitory activity assay.

Quantification of COX-2 concentration and inhibitory activity assay after treatment - The quantitative determination of COX-2 concentration in the cell-free supernatant was performed using COX-2 ELISA Kit Elabscience (E-EL-M0959). Briefly, 100 µL of standard, blank, and sample solution was added into each well then sealed and incubated for 90 minutes at 37°C. The cell-free supernatant after treated with GMPE, α-mangostin, and γ-mangostin were served as the sample. The LPS-induced cells free supernatant without extract and compounds were used as positive control. The normal cell or untreated cell was used as negative control. Subsequently, the liquid of each well removed and 100 µL of Biotinylated Detection AB working solution was added into each well, incubated for 1 hour at 37°C. The plate then washed prior to the addition of 100 µL HRP conjugate, incubated for 30 minutes at 37°C. The plate was washed again and 90 µL of substrate solution was added into each well, incubated for about 15 minutes at 37°C.

Following that, 50 µL of stop solution was added and the absorbance measured using microplate reader at 450 nm.[24] The concentration of COX-2 was determined from COX-2 standard curve, the regression equation obtained was y = 0.09543x – 0.01039 with $r^2 = 0.99$. The percentage of inhibitory activity was calculated using following equation:

$$\text{COX2 inhibitory activity } (\%) = \frac{(COX2p - COX2s)}{COX2p} \times 100$$

where *COX2p* : COX-2 concentration of positive control (ng/mL); *COX2s* : COX-2 concentration of samples (ng/mL).

Quantification of IL-6 and IL-1β concentration and inhibitory activity assay after treatment - The quantitative determination of IL-6 and IL-1β concentration in the cell-free supernatant was performed using Mouse IL-6 ELISA MAX Standard Sets (BioLegend. 431301) and Mouse IL-1β ELISA MAX Standard Sets (BioLegend 432601), respectively. Approximately 100 µl of diluted capture antibody solution was added to each well, incubated overnight at 4°C. Following that, the plate was washed 4 times and 200 µL of assay diluent added into each well, incubated at room temperature for 1 hour with shaking. The plate then washed again 4 times, prior to addition of the diluted standards and samples. The cell-free supernatant after treated with GMPE, α-mangostin, and γ-mangostin were served as the sample. The LPS-induced cells free supernatant without extract and compounds were used as positive control. The normal cell was used as negative control. The plate was incubated for 2 hours at room temperature with shaking, then washed for 4 times. Subsequently, 100 µL of diluted detection antibody solution was added into each well and the plate was incubated for 1 hour with shaking at room temperature. After the plate was washed again for 4 times, 100 µl of diluted Avidin-HRP solution was added, incubated for 30 minutes with shaking at room temperature. The plate was washed 5 times, then 100 µL of TMB substrate solution was added to each well and incubated for 15 minutes in the dark. Finally, 100 µL of stop solution was added and the absorbance was read at 450 nm using microplate reader.[17-19,25] The

quantity of IL-6 and IL-1β were determined from the standard curve. The regression equation obtained from IL-6 standard curve was y = 0.0007x – 0.0412 with r^2 = 0.98, and from IL-1β standard curve was y = 0.0013x – 0.0157 with r^2 = 0.99. The percentage of inhibitory activity was calculated using following equation:

$$\text{Inhibitory activity } (\%) = \frac{(Cp - Cs)}{Cp} \times 100$$

where *Cp* : IL-6 or IL-1β concentration of positive control (pg/mL); *Cs* : IL-6 or IL-1β concentration of samples (µM).

Quantification of nitrite associated with NO concentration and inhibitory activity assay after treatment - The determination of nitrite associated with NO production was performed using Nitrate/Nitrite colorimetric assay.[24] The cell-free supernatant after treated with GMPE, α-mangostin, and γ-mangostin were served as the sample. The LPS-induced cells without GMPE or compound free supernatant was used as positive control. The normal cell-free supernatant was used as negative control. The samples were read in 540 nm of wavelength in a microplate reader. The quantity of nitrite was determined from the sodium nitrite standard curve, the regression equation obtained was y = 0.0253x – 0.0550 with r^2 = 0.98. The percentage of inhibitory activity was calculated using following equation:

$$\text{NO inhibitory activity } (\%) = \frac{(NOp - NOs)}{NOp} \times 100$$

where *NOp* : NO concentration of positive control (µM); *Nos* : NO concentration of samples (µM).

Statistical analysis - All data were derived from three independent experiments. Statistical analysis was performed using SPSS software (version 20.0). The data were presented as mean ± standard deviation. Significant differences between the groups were determined using the Analysis of Variance (ANOVA) followed by Duncan Post Hoc Test $P<0.05$ were considered as statistical significance.[18,19]

Results and Discussion

Natural plant compounds are now gaining more pharmacological attention as many plant products still unexplored and show a wide range of activities.[26] Major compounds of several commonly used botanicals, including mangostin have been reported to have anti-inflammatory actions. The xanthones, such as α-mangostin and γ-mangostin are major bioactive compounds found in the fruit peel of mangosteen.[6,27] In this study, we evaluated the biological effects of GMPE, α-mangostin, and γ-mangostin on inflammatory mediators production in LPS-induced RAW 264.7 cell line as the model. The cytotoxic assay was performed to determine the safe and nontoxic concentration of GMPE and compounds for the next assay. Nontoxicity of the substrate was indicated by over 90% of cells viability by MTS assay. Viability test is an important aspect of pharmacology which deals with the adverse effect of bioactive substance on living organism prior to the use of substances as drug or chemical in clinical use.[28-30] The α-mangostin and γ mangostin in the concentration of 100 µM were toxic toward RAW 264.7 cells, therefore the respective concentration was not used for the treatments and the concentration of 75, 50, and 25 was chosen instead (Table 1). In other hand, GMPE treatment showed low viability at 100, 75, 50, and 25 µg/mL. Hence, the lower concentration of GMPE were tested. Finally, the cells in the concentration of 20, 10, and 5 µg/mL of GMPE treatment showed high viability and nontoxic to the cells (Table 1).

A stimuli of LPS can activate the macrophages that involved in the pathological processes in several acute and chronic disorders by secreting several inflammatory mediators.[7,18,19,31] The overproduction of the inflammatory mediators contributes to the pathogenesis of several diseases such as sepsis, rheumatoid arthritis, atherosclerosis, pulmonary fibrosis, and chronic hepatitis.[32] Several nonsteroidal anti-inflammatory drugs

were the currently available drugs to reduce the inflammation, but it pose a side effect and major problem.[33] Therefore, the development of new anti-inflammatory agents from natural sources that more active and have fewer side effects become important.

Based on cytotoxic result, three concentration of GMPE (20, 10, 5 μg/mL), α-mangostin (75, 50, 25 μg/mL), and β-mangostin (75, 50, 25 μg/mL) were applied for IL-6, IL-1β, NO, and COX-2 assay. All of the anti-inflammatory assays showed that GMPE possessed potent COX-2, IL-1β, IL-6, and NO inhibitory activity. The isolated compound from GMPE including α-mangostin and γ-mangostin also possessed the same activity. Inhibiting the synthesis of mediators that plays a role in inflammation will be useful for autoimmune diseases and inflammation treatment. COX-2 is a key regulatory enzyme of the prostaglandin/eicosanoid pathway that highly induced by pro-inflammatory cytokines in an NF-κβ dependent manner.[34] COX-2 induced several stimuli and is responsible for the pro-inflammatory cytokine at the inflammatory sites.[35] COX-2 is highly induced by pro-inflammatory cytokine (IL-1β and IL-6) that serve as endogenous pyrogens that causes fever during inflammation by up-regulating the inflammatory responses and stimulating the production of acute phase reactans.[36] In addition to COX-2 inhibitory activity, NO inhibitory activity may be as attractive as one of anti-inflammatory agent screening indicator. NO plays a significant role in host immune defense, vascular regulation, neurotransmission, and other system in normal condition. In human body, a family of nitric oxide synthase (NOS) enzyme is responsible for catalyzing the synthesis of NO.[37] The expression of inducible NOS (iNOS) in various inflammatory and tissue cells can be induced by LPS or proinflammatory cytokines such as interleukin (IL-1).[37] Overproduction of NO and iNOS are especially related to various human diseases including inflammation.[16,18,19,38]

GMPE and α-mangostin in the highest concentration showed the highest inhibitory activity against COX-2 production (Table 2). COX-2 with COX-1 are the key players in the

inflammatory response which catalyze the conversion of arachidonic acid into pro-inflammatory prostaglandins and triggers the production of other pro-inflammatory mediators.[34] IL-6 is a pleiotropic cytokine which modulates inflammatory responses.[39] GMPE in the concentration of 20 µg/mL showed the highest inhibitory activity (Table 3). Inhibiting the production of IL-1β was important in finding the anti-inflammatory agent. GMPE, α-mangostin, and γ-mangostin inhibited the production of IL-1β in a concentration-dependent manner. The highest concentration of GMPE, α-mangostin and γ-mangostin possessed the highest inhibitory activity with no significant differences observed among them (Table 4). IL-1β is a potent proinflammatory cytokine released by macrophages in systemic inflammatory responses that regulate inflammatory reaction and immune response.[40] In this study, the nitrite concentration was associated with NO production. Excessive levels of NO can mediate proinflammatory and have destructive effects, thus lowering the NO concentration can be used as an anti-inflammatory action. The GMPE showed the highest NO inhibitory activity, meanwhile the positive control showed the highest concentration of NO (Table 5). This research result indicated that the LPS successfully induced the inflammation of the RAW 264.7 cells[41] and GMPE had anti-inflammatory properties by reducing the NO production. The inhibition of NO production might related to suppression of iNOS expression, as NO synthesis is catalyzed by iNOS.

GMPE possessed the highest inhibitory activity against COX-2, IL-6, and NO production, but all of the tested concentration both for GMPE and its compounds have no significant differences in inhibitory activity of IL-1β. This research was consistent with previous research that *G. mangostana* fruit hull inhibited the inflammatory related diseases through NO and PGE2 releases.[42] Likewise, two compounds α-mangostin and γ-mangostin inhibited NO and PGE2 production and COX-2 activity in LPS-induced RAW 264.7 cells according to Chen, *et al* (2007) study.[27] The inhibitory activity of α-mangostin and γ-

mangostin from GMPE against IL-6 also revealed by Bumrungpert, *et al* (2010) study.[43] Furthermore, the α-mangostin and γ-mangostin revealed to attenuated LPS-induced inflammatory gene expression of TNF-α, IL-1β, IL-6, IL-8, MCP-1, and Toll-like receptor-2.[44] The effect of *G. mangostana* and its compounds against inflammation in this study also in line with Chomnawang, *et al* (2007) study which reported anti-inflammatory activity of *G. mangostana* on inflammation caused by *Propionibacterium acnes* through suppression of pro-inflammatory cytokines, [45] as well as Lee, *et al* (2013) study which revealed that *G. mangosteen* and its compounds has great potential in the treatment and prevention of rheumatoid arthritis, a chronic inflammatory disease, showed by inhibition of TNF-α and IL-6 production in LPS-stimulated mice, reduction of paw edea in the carrageenan-induced rats, and reduction of arthritis score in the CIA rats.[46]

Conclusion

This research revealed that GMPE, α-mangostin, and γ-mangostin possess the anti-inflammatory potential by inhibiting COX-2, IL-6, IL-1β, and NO. These extracts may have therapeutic potential for the modulation and regulation of macrophage activation, and may provide safe and effective treatment option for various inflammation-mediated diseases. However, the therapeutic potential of these plant extracts will be further clear after preclinical and clinical test were conducted.

Acknowledgements

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Table and Figure Legends

Table 1. Effect various concentrations of GMPE and mangostins toward RAW 264.7 cell viability

The data are presented as mean ± standard deviation. Different superscript letters (^{a,b,c,cd,cde,de,def,efg,efgh,fgh,gh,h}) in the same column (the viability among concentrations of the samples) are significant at $P < 0.05$ based on Duncan's *post-hoc* comparisons ($P < 0.05$). The experiment was conducted in triplicate replication

Table 2. Effect various concentrations of GMPE and mangostins toward COX-2 concetration and COX-2 inhibitory activity in RAW 264.7 cell

The data are presented as mean ± standard deviation. Different letters (^{a,b,bc,c,cd,d,de,e,f}) in the same coloumn (among various concentrations of GMPE, mangostins in COX-2 concentrations and inhibitory activity) are significant at $P < 0.05$ based on Duncan's *post-hoc* comparisons ($P < 0.05$). The experiment was conducted in triplicate replication

Table 3. Effect various concentrations of GMPE and mangostins toward IL-6 in RAW 264.7 cell

The data are presented as mean ± standard deviation. Different letters (^{a,ab,bc,abc,cd,ef,fg,efg,f,g}) in the same coloumn (among various concentrations of GMPE, mangostins in IL-6 concentrations and inhibitory activity) are significant at $P < 0.05$ based on Duncan's *post-hoc* comparisons ($P < 0.05$). The experiment was conducted in triplicate replication

Table 4. Effect various concentrations of GMPE and mangostins toward IL-1β in RAW 264.7 cell

The data are presented as mean ± standard deviation. Different letters (^{a,ab,b,c,bc}) in the same coloumn (among various concentrations of GMPE, mangostins in IL-1β concentrations and inhibitory activity) are significant at $P < 0.05$ based on Duncan's *post-hoc* comparisons ($P < 0.05$). The experiment was conducted in triplicate replication

Table 5. Effect various concentrations of GMPE and mangostins toward NO in RAW 264.7 cell

The data are presented as mean ± standard deviation. Different letters (^{a,ab,b,c,bc}) in the same coloumn (among various concentrations of GMPE, mangostins in NO concentrations and inhibitory activity) are significant at $P < 0.05$ based on Duncan's *post-hoc* comparisons ($P < 0.05$). The experiment was conducted in triplicate replication

Figure 1. Chemical Structure of (a) α-mangostin and (b) ϒ- mangostin

Table 1.

Samples	Viability (%)
Control	100.00±0.00^{def}
GMPE 100 µg/mL	7.36±2.67^{a}
GMPE 75 µg/mL	8.13±1.69^{a}
GMPE 50 µg/mL	48.86±12.81^{b}
GMPE 25 µg/mL	102.49±8.14^{efg}
GMPE 20 µg/mL	100.80±4.45^{def}
GMPE 10 µg/mL	115.23±16.03^{fgh}
GMPE 5 µg/mL	123.10±12.93^{h}
α-mangostin 100 µM	85.81±4.75^{cd}
α-mangostin 75 µM	98.50±6.36^{de}
α-mangostin 50 µM	112.76±8.86^{efgh}
α-mangostin 25 µM	116.95±9.43^{gh}
γ-mangostin 100 µM	83.30±11.55^{c}
γ-mangostin 75 µM	97.23±3.70^{cde}
γ-mangostin 50 µM	103.38±6.54^{efg}
γ-mangostin 25µM	105.34±5.43^{efg}

Table 2.

Samples	COX-2	
	COX-2 concentration (pg/mL)	COX-2 inhibitory activity (%)
Negative control	0.81 ± 0.03^{a}	72.93 ± 0.97^{f}
Positive control	2.98 ± 0.170^{g}	0.11 ± 5.72^{a}
GMPE 20 μg/mL	1.35 ± 0.18^{b}	54.59 ± 6.09^{e}
GMPE 10 μg/mL	2.02 ± 0.12^{de}	32.21 ± 3.87^{cd}
GMPE 5 μg/mL	2.16 ± 0.07^{ef}	27.40 ± 2.28^{bc}
α-mangostin 75 μM	1.43 ± 0.08^{b}	52.13 ± 2.61^{e}
α-mangostin 50 μM	1.75 ± 0.12^{c}	41.16 ± 4.00^{d}
α-mangostin 25 μM	2.06 ± 0.05^{de}	30.87 ± 1.78^{bc}
γ-mangostin 75 μM	1.89 ± 0.03^{cd}	36.58 ± 0.89^{cd}
γ-mangostin 50 μM	1.98 ± 0.03^{d}	33.56 ± 1.16^{cd}
γ-mangostin 25μM	2.31 ± 0.03^{f}	22.48 ± 0.89^{b}

Table 3.

Samples	IL-6	
	IL-6 concentration (pg/mL)	IL-6 inhibitory activity (%)
Negative control	176.57±5.14^{a}	73.87±0.76^{g}
Positive control	675.43±4.58^{g}	0.00±0.68^{a}
GMPE 20μg/mL	304.33±55.85^{b}	54.95±8.27^{f}
GMPE 10 μg/mL	351.52±33.57^{bc}	47.96±4.97^{ef}
GMPE 5 μg/mL	602.48±7.83^{efg}	10.80±1.16^{abc}
α-mangostin 75 μM	337.62±57.83^{bc}	50.02±8.56^{ef}
α-mangostin 50 μM	422.34±55.88^{cd}	37.48±8.28^{de}
α-mangostin 25 μM	567.52±66.90^{ef}	15.98±9.91^{bc}
γ-mangostin 75 μM	500.24±53.87^{de}	25.94±7.98^{cd}
γ-mangostin 50 μM	582.14±90.57^{efg}	13.81±13.41^{abc}
γ-mangostin 25μM	645.14±92.87^{fg}	4.49±13.75^{ab}

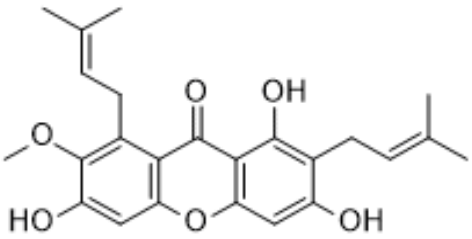
Table 4.

Samples	IL-1β	
	IL-1β concentration (pg/mL)	IL-1β inhibitory activity (%)
Negative control	841.44±18.01 a	28.87±1.52^{c}
Positive control	1183.03±35.09^{c}	0.00±2.97^{a}
GMPE 20μg/mL	894.31±77.23^{a}	24.41±6.53^{c}
GMPE 10 μg/mL	950.70±115.33^{ab}	19.64±9.75^{bc}
GMPE 5 μg/mL	951.72±45.98^{ab}	19.55±3.89^{bc}
α-mangostin 75 μM	877.28±35.87^{b}	25.84±3.03^{c}
α-mangostin 50 μM	910.43±79.98^{ab}	23.04±6.76^{bc}
α-mangostin 25 μM	942.64±121.95^{ab}	20.32±10.31^{bc}
γ-mangostin 75 μM	817.69±3.80^{a}	30.88±0.32^{c}
γ-mangostin 50 μM	936.41±43.20^{ab}	20.85±3.65^{bc}
γ-mangostin 25μM	1041.41±110.80^{b}	11.97±9.37^{b}

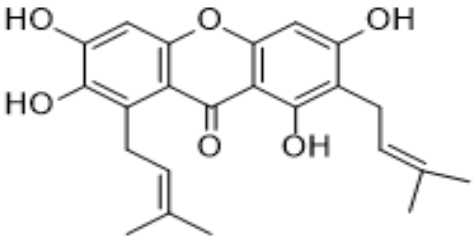
Table 5.

Samples	NO	
	NO concentration (pg/mL)	NO inhibitory activity (%)
Negative control	6.06±0.17[a]	82.74±0.50[j]
Positive control	35.10±0.08[j]	0.01±0.23[a]
GMPE 20μg/mL	23.29±0.07[b]	33.66±0.19[i]
GMPE 10 μg/mL	23.92±0.04[c]	31.86±0.12[h]
GMPE 5 μg/mL	27.07±0.07[f]	22.89±0.19[e]
α-mangostin 75 μM	24.55±0.03[d]	30.07±0.09[g]
α-mangostin 50 μM	25.86±0.02[e]	26.33±0.07[f]
α-mangostin 25 μM	28.88±0.12[g]	17.73±0.34[d]
γ-mangostin 75 μM	26.94±0.05[f]	23.24±0.14[e]
γ-mangostin 50 μM	29.37±0.08[h]	16.32±0.23[c]
γ-mangostin 25μM	30.26±0.07[i]	13.78±0.21[b]

Figure 1.



(a)



(b)

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- **Manuscript Type** : Regular Article
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Anti-inflammatory Effect of Mangosteen (*Garcinia mangostana* L.) Peel Extract and its Compounds in LPS-induced RAW264.7 Cells

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Abstract – Inflammation plays an important role in host defense against external stimuli such as infection by pathogen, endotoxin or chemical exposure by the production of the inflammatory mediators that produced by macrophage. Anti-inflammatory factor is important to treat the dangers of chronic inflammation associated with chronic disease. This research aims to analyze the anti-inflammatory effects of *Garcinia mangostana* L. peel extract (GMPE), α-mangostin, and γ-mangostin on LPS-induced murine macrophage cell line (RAW 264.7) by inhibiting the production of inflammatory mediators. The cytotoxic assay of *G. mangostana* L. extract, α-mangostin, and γ-mangostin was performed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) to determine the safe and non-toxic concentration in RAW 264.7 for the further assay. The concentration of inflammatory mediators (COX-2, IL-6, and IL-1β) were measured by the ELISA-based assay and NO by the nitrate/nitrite colorimetric assay in treated LPS-induced RAW 264.7 cells. The inhibitory activity was determined by the reducing concentration of inflammatory mediators in treated LPS-induced RAW 264.7 over the untreated cells. This research revealed that GMPE, α-mangostin, and γ-mangostin possess the anti-inflammatory effect by reducing COX-2, IL-6, IL-1β, and NO production in LPS-induces RAW 264.7 cells.
Keywords – Anti-inflammatory, GMPE, α-Mangostin, γ-Mangostin, Macrophages, Inflammatory mediator

Introduction

Natural products have long been over the years contributed to the development of new therapeutic drugs for a variety of human diseases.[1] Many tropical plants have interesting biological activities with their therapeutic potential, including *Garcinia mangostana* L. (mangosteen). Mangosteen has been used for hundreds of years around the world, mostly in Southeast Asia, as a medicine for a great variety diseases.[2] Many studies have shown that the various parts extract contain varieties of secondary metabolites such as prenylated and oxygenated xanthones. Xanthones as the major bioactive secondary metabolites were reported to have many pharmacological activities including antioxidant, antifungal, anti-bacteria, cytotoxic, anti-inflammatory, antihistamine, anti-HIV, antimalarial and other activities.[3-5] The previous study confirmed that the *G. mangostana* peel extract (GMPE) contained α-mangostin (105 ppm), γ-mangostin (7.20 ppm), garcinone C (3.50 ppm), and garcinone D (9.92 ppm) based on high performance liquid chromatography (HPLC) analysis.[6] In this study, the anti-inflammatory potential of *Gmangostana* L. peel extract (GMPE) and its compound were observed.

Inflammation plays an important role in host defense encompasses multiple processes against external stimuli such as infection by pathogen, exposure to bacterial endotoxin or chemical exposure.[7] The inflammation process involves changes in blood flow, increased vascular permeability, destruction of tissue via the activation and migration of leukocytes with the synthesis of reactive oxygen derivatives (oxidative burst) and synthesis of local inflammatory mediators.[8] The secretion of the inflammatory mediators both of proinflammatory mediators such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, and Nitric Oxide (NO) and anti-inflammatory such as IL-10 are the primary response to inflammation in addition to leukocyte recruitment.[9] Inflammation relates to various diseases such as rheumatoid arthritis, inflammatory bowel disease, artherosclerosis, Alzheimer's, and has a role in various kinds of cancer.[10] Anti-inflammatory is important to treat the danger of chronic inflammation associated

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with chronic disease.[11]

Several mechanisms of action have been proposed to describe the phytochemical potential for anti-inflammatory, such as: 1) antioxidant and radical scavenging activity; 2) modulation of cellular activities of inflammat;ion-related cells (mast cells, macrophages, lymphocytes, and neutrophils; 3) modulation of proinflammatory enzyme activities; 4) modulation of the production of other proinflammatory molecules; and 5) modulation of proinflammatory gene expression.[12] This study focused on the potential of GMPE, α-mangostin, and γ-mangostin in modulation of the proinflammatory molecules production by inhibiting the proinflammatory cytokines production including COX-2, IL-6, IL-1β, and NO in LPS-induced murine macrophage cell line model (RAW 264.7). The RAW 264.7 murine macrophage cell line is widely used as an inflammatory model *in vitro*.[13]

Experimental

General Experimental Procedure – *Garcina mangostana* L. was collected from Cisalak-Subang, West Java, Indonesia plantation and identified by a staff of herbarium of the Department of Biology, School of Life Sciences and Technology, Bandung Institute of Technology, Bandung, West Java, Indonesia. The murine macrophage cell line RAW 264.7 (ATCC® TIB-71™) was obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia.

α-mangostin (95-99% purity), and γ-mangostin (95-99% purity) were bought from Biopurify Phytochemical Ltd. (Chengdu, China). The medium and its component for cell culture such as Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), penicillin, streptomycin, and Trypsin-EDTA were purchased from Biowest. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) used for cell viability assay was purchased from Promega (Madison, WI, USA). COX-2 ELISA kit and protocol (E-EL-M0959) was purchased from Elabscience. IL-6 and IL-1β ELISA kit and protocol (431301, and 432601) were purchased from BioLegend. Nitrate/Nitrite colorimetric assay kit (KA1342) was purchased from Abnova. MultiSkan Go (Thermo Scientific), a microplate reader, was used for viability assay, measuring COX-2, IL-6, IL-1β, and NO concentration.

Plant Extract preparation – The peels of *Garcina mangostana* L. were collected, chopped, and kept in drier tunnel service. Extraction was performed based on the maceration method using ethanol 70% as the solvent for collecting *Garcinia mangostana* L. peel extract (GMPE).[14,15]

RAW 264.7 Cells Culture and Viability Assay – The murine macrophage cells were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin. The cells then maintained at the 37 °C humidified atmosphere incubator with 5% CO_2 until the cells were confluence. The cells were washed and harvested using Trypsin-EDTA and centrifuged at 2500 rpm for 4 minutes.[16-19]

Viability assay – The viability assay was performed to determine the safe and nontoxic concentration for the next assay, evaluated by MTS assay. Briefly, 100 μL cells (5×10^3 cells per well) in medium (DMEM supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin) were plated in 96-well plate and incubated for 24 hours at 37 °C in a humidified atmosphere incubator with 5% CO_2. The medium then washed and added with 99 μL new medium and 1 μL of GMPE, α-mangostin, and γ-mangostin in different concentration and DMSO in triplicate then the plate were incubated for 24 hours. Untreated cells were served as the control. Briefly, 20 μL MTS was added to each well. The plate was incubated in 5% CO_2 at 37 °C incubator for 4 hours. The absorbance was measured at 490 nm on a microplate reader.[18-21]

Proinflammatory activation of cells and treatment – The pro-inflammatory activation of cells was performed based on Yoon, *et al.* (2009) and modified method.[17-19, 22] The cells were seeded in 6-well plate in density of 5×10^5 cells per well and incubated for 24 hours at 37 °C in a humidified atmosphere and 5% CO_2.[18,19-23] The medium (DMEM supplemented with 10% FBS and 100 U/mL penicillin and 100 μg/mL streptomycin) then washed and supplemented with 1,600 μL growth medium and 200 μL GMPE (20, 10, 5 μg/mL), α-mangostin, and γ-mangostin (75, 50, 25 μM). Around 1-2 hours later, the medium was added with 200 μL LPS (1 μg/mL) and incubated for 24 hours at 37 °C in a humidified atmosphere and 5% CO_2.[18,19] After incubation of RAW 264.7 cells with LPS for 24 hours, the quantity of COX-2, IL-6, IL-1β, and NO was accumulated in the cell-free supernatant. The cell-free supernatant then was taken for the next assay by centrifugation at 2,000 g for 10 minutes. The supernatant was stored at −79 °C for the COX-2, IL-6, IL-1β, and NO concentration and inhibitory activity assay.

Quantification of COX-2 concentration and inhibitory activity assay after treatment – The quantitative determination of COX-2 concentration in the cell-free supernatant was performed using COX-2 ELISA Kit Elabscience (E-EL-M0959). Briefly, 100 μL of standard, blank, and sample solution was added into each well then sealed and

incubated for 90 minutes at 37 ºC. The cell-free supernatant after treated with GMPE, α-mangostin, and γ-mangostin were served as the sample. The LPS-induced cells free supernatant without extract and compounds were used as positive control. The normal cell or untreated cell was used as negative control. Subsequently, the liquid of each well removed and 100 µL of Biotinylated Detection AB working solution was added into each well, incubated for 1 hour at 37 ºC. The plate then washed prior to the addition of 100 µL HRP conjugate, incubated for 30 minutes at 37 ºC. The plate was washed again and 90 µL of substrate solution was added into each well, incubated for about 15 minutes at 37 ºC. Following that, 50 µL of stop solution was added and the absorbance measured using microplate reader at 450 nm.[24] The concentration of COX-2 was determined from COX-2 standard curve, the regression equation obtained was $y = 0.09543x - 0.01039$ with $r^2 = 0.99$. The percentage of inhibitory activity was calculated using following equation:

$$\text{COX2 inhibitory activity (\%)} = \frac{(COX2p - COX2s)}{COX2p} \times 100$$

where *COX2p* : COX-2 concentration of positive control (ng/mL); *COX2s* : COX-2 concentration of samples (ng/mL).

Quantification of IL-6 and IL-1β concentration and inhibitory activity assay after treatment – The quantitative determination of IL-6 and IL-1β concentration in the cell-free supernatant was performed using Mouse IL-6 ELISA MAX Standard Sets (BioLegend. 431301) and Mouse IL-1β ELISA MAX Standard Sets (BioLegend 432601), respectively. Approximately 100 µl of diluted capture antibody solution was added to each well, incubated overnight at 4 ºC. Following that, the plate was washed 4 times and 200 µL of assay diluent added into each well, incubated at room temperature for 1 hour with shaking. The plate then washed again 4 times, prior to addition of the diluted standards and samples. The cell-free supernatant after treated with GMPE, α-mangostin, and γ-mangostin were served as the sample. The LPS-induced cells free supernatant without extract and compounds were used as positive control. The normal cell was used as negative control. The plate was incubated for 2 hours at room temperature with shaking, then washed for 4 times. Subsequently, 100 µL of diluted detection antibody solution was added into each well and the plate was incubated for 1 hour with shaking at room temperature. After the plate was washed again for 4 times, 100 µl of diluted Avidin-HRP solution was added, incubated for 30 minutes with shaking at room temperature. The plate was washed 5 times, then 100 µL of TMB substrate solution was added to each well and incubated for 15 minutes in the dark. Finally, 100 µL of stop solution was added and the absorbance was read at 450 nm using microplate reader.[17-19,25] The quantity of IL-6 and IL-1β were determined from the standard curve. The regression equation obtained from IL-6 standard curve was $y = 0.0007x - 0.0412$ with $r^2 = 0.98$, and from IL-1β standard curve was $y = 0.0013x - 0.0157$ with $r^2 = 0.99$. The percentage of inhibitory activity was calculated using following equation:

$$\text{Inhibitory activity (\%)} = \frac{(C_p - C_s)}{C_p} \times 100$$

where *Cp* : IL-6 or IL-1β concentration of positive control (pg/mL); *Cs* : IL-6 or IL-1β concentration of samples (pg/mL).

Quantification of nitrite associated with NO concentration and inhibitory activity assay after treatment – The determination of nitrite associated with NO production was performed using Nitrate/Nitrite colorimetric assay kit (Abnova KA1342).[24] The cell-free supernatant after treated with GMPE, α-mangostin, and γ-mangostin were served as the sample. The LPS-induced cells without GMPE or compound free supernatant was used as positive control. The normal cell-free supernatant was used as negative control. The samples were read in 540 nm of wavelength in a microplate reader. The quantity of nitrite was determined from the sodium nitrite standard curve, the regression equation obtained was $y = 0.0253x - 0.0550$ with $r^2 = 0.98$. The percentage of inhibitory activity was calculated using following equation:

$$\text{NO inhibitory activity (\%)} = \frac{(NO_p - NO_s)}{NO_p} \times 100$$

where *NOp* : NO concentration of positive control (µM); *NOs* : NO concentration of samples (µM).

Statistical analysis – All data were derived from three independent experiments. Statistical analysis was performed using SPSS software (version 20.0). The data were presented as mean ± standard deviation. Significant differences between the groups were determined using the Analysis of Variance (ANOVA) followed by Duncan Post Hoc Test $P < 0.05$ were considered as statistical significance.[18,19]

Result and Discussion

Natural plant compounds are now gaining more pharmacological attention as many plant products still

Table 1. Effect various concentrations of GMPE and mangostins toward RAW 264.7 cell viability

Samples	Viability (%)
Control	100.00±0.00^{def}
GMPE 100 μg/mL	7.36±2.67^{a}
GMPE 75 μg/mL	8.13±1.69^{a}
GMPE 50 μg/mL	48.86±12.81^{b}
GMPE 25 μg/mL	102.49±8.14^{efg}
GMPE 20 μg/mL	100.80±4.45^{def}
GMPE 10 μg/mL	115.23±16.03^{fgh}
GMPE 5 μg/mL	123.10±12.93^{h}
α-mangostin 100 μM	85.81±4.75^{cd}
α-mangostin 75 μM	98.50±6.36^{de}
α-mangostin 50 μM	112.76±8.86^{efgh}
α-mangostin 25 μM	116.95±9.43^{gh}
γ-mangostin 100 μM	83.30±11.55^{c}
γ-mangostin 75 μM	97.23±3.70^{cde}
γ-mangostin 50 μM	103.38±6.54^{efg}
γ-mangostin 25 μM	105.34±5.43^{efg}

The data are presented as mean ± standard deviation. Different superscript letters (^{a,b,c,cd,cde,de,def,efg,efgh,fgh,gh,h}) in the same column (the viability among concentrations of the samples) are significant at $p < 0.05$ based on Duncan's *post-hoc* comparisons ($p < 0.05$). The experiment was conducted in triplicate replication

unexplored and show a wide range of activities.[26] Major compounds of several commonly used botanicals, including mangostin have been reported to have anti-inflammatory actions. The xanthones, such as α-mangostin and γ-mangostin are major bioactive compounds found in the fruit peel of mangosteen.[6,27] In this study, we evaluated the biological effects of GMPE, α-mangostin, and γ-mangostin on inflammatory mediators production in LPS-induced RAW 264.7 cell line as the model. The cytotoxic assay was performed to determine the safe and nontoxic concentration of GMPE and compounds for the next assay. Nontoxicity of the substrate was indicated by over 90% of cells viability by MTS assay. Viability test is an important aspect of pharmacology which deals with the adverse effect of bioactive substance on living organism prior to the use of substances as drug or chemical in clinical use.[28-30] The α-mangostin and γ mangostin in the concentration of 100 μM were toxic toward RAW 264.7 cells, therefore the respective concentration was not used for the treatments and the concentration of 75, 50, and 25 was chosen instead (Table 1). In other hand, GMPE treatment showed low viability at 100, 75, 50, and 25 μg/mL. Hence, the lower concentration of GMPE were tested. Finally, the cells in the concentration of 20, 10, and 5 μg/mL of GMPE treatment showed high viability and nontoxic to the cells (Table 1).

A stimuli of LPS can activate the macrophages that involved in the pathological processes in several acute and chronic disorders by secreting several inflammatory mediators.[7,18,19,31] The overproduction of the inflammatory mediators contributes to the pathogenesis of several diseases such as sepsis, rheumatoid arthritis, atherosclerosis, pulmonary fibrosis, and chronic hepatitis.[32] Several nonsteroidal anti-inflammatory drugs were the currently available drugs to reduce the inflammation, but it pose a side effect and major problem.[33] Therefore, the development of new anti-inflammatory agents from natural sources that more active and have fewer side effects become important.

Based on cytotoxic result, three concentration of GMPE (20, 10, 5 μg/mL), α-mangostin (75, 50, 25 μg/mL), and β-mangostin (75, 50, 25 μg/mL) were applied for IL-6, IL-1β, NO, and COX-2 assay. All of the anti-inflammatory assays showed that GMPE possessed potent COX-2, IL-1β, IL-6, and NO inhibitory activity. The isolated compound from GMPE including α-mangostin and γ-mangostin also possessed the same activity. Inhibiting the synthesis of mediators that plays a role in inflammation will be useful for autoimmune diseases and inflammation treatment. COX-2 is a key regulatory enzyme of the prostaglandin/eicosanoid pathway that highly induced by pro-inflammatory cytokines in an NF-κβ dependent manner.[34] COX-2 induced several stimuli and is responsible for the pro-inflammatory cytokine at the inflammatory sites.[35] COX-2 is highly induced by pro-inflammatory cytokine (IL-1β and IL-6) that serve as endogenous pyrogens that causes fever during inflammation by up-regulating the inflammatory responses and stimulating the production of acute phase reactans.[36] In addition to COX-2 inhibitory activity, NO inhibitory activity may be as attractive as one of anti-inflammatory agent screening indicator. NO plays a significant role in host immune defense, vascular regulation, neurotransmission, and other system in normal condition. In human body, a family of nitric oxide synthase (NOS) enzyme is responsible for catalyzing the synthesis of NO.[37] The expression of inducible NOS (iNOS) in various inflammatory and tissue cells can be induced by LPS or proinflammatory cytokines such as interleukin (IL-1).[37] Overproduction of NO and iNOS are especially related to various human diseases including inflammation.[16,18,19,38]

GMPE and α-mangostin in the highest concentration showed the highest inhibitory activity against COX-2 production (Table 2). COX-2 with COX-1 are the key players in the inflammatory response which catalyze the conversion of arachidonic acid into pro-inflammatory

Table 2. Effect various concentrations of GMPE and mangostins toward COX-2 concentration and COX-2 inhibitory activity in RAW 264.7 cell

Samples	COX-2	
	COX-2 concentration (ng/mL)	COX-2 inhibitory activity (%)
Negative control	0.81±0.03[a]	72.93±0.97[f]
Positive control	2.98±0.170[g]	0.11±5.72[a]
GMPE 20 μg/mL	1.35±0.18[b]	54.59±6.09[e]
GMPE 10 μg/mL	2.02±0.12[de]	32.21±3.87[cd]
GMPE 5 μg/mL	2.16±0.07[ef]	27.40±2.28[bc]
α-mangostin 75 μM	1.43±0.08[b]	52.13±2.61[e]
α-mangostin 50 μM	1.75±0.12[c]	41.16±4.00[d]
α-mangostin 25 μM	2.06±0.05[de]	30.87±1.78[bc]
γ-mangostin 75 μM	1.89±0.03[cd]	36.58±0.89[cd]
γ-mangostin 50 μM	1.98±0.03[d]	33.56±1.16[cd]
γ-mangostin 25 μM	2.31±0.03[f]	22.48±0.89[b]

The data are presented as mean ± standard deviation. Different letters ([a,b,bc,c,cd,d,de,e,f]) in the same coloumn (among various concentrations of GMPE, mangostins in COX-2 concentrations and inhibitory activity) are significant at $p < 0.05$ based on Duncan's *post-hoc* comparisons ($p < 0.05$). The experiment was conducted in triplicate replication

Table 3. Effect various concentrations of GMPE and mangostins toward IL-6 in RAW 264.7 cell

Samples	IL-6	
	IL-6 concentration (pg/mL)	IL-6 inhibitory activity (%)
Negative control	176.57±5.14[a]	73.87±0.76[g]
Positive control	675.43±4.58[g]	0.00±0.68[a]
GMPE 20 μg/mL	304.33±55.85[b]	54.95±8.27[f]
GMPE 10 μg/mL	351.52±33.57[bc]	47.96±4.97[ef]
GMPE 5 μg/mL	602.48±7.83[efg]	10.80±1.16[abc]
α-mangostin 75 μM	337.62±57.83[bc]	50.02±8.56[ef]
α-mangostin 50 μM	422.34±55.88[cd]	37.48±8.28[de]
α-mangostin 25 μM	567.52±66.90[ef]	15.98±9.91[bc]
γ-mangostin 75 μM	500.24±53.87[de]	25.94±7.98[cd]
γ-mangostin 50 μM	582.14±90.57[efg]	13.81±13.41[abc]
γ-mangostin 25 μM	645.14±92.87[fg]	4.49±13.75[ab]

The data are presented as mean ± standard deviation. Different letters ([a,ab,bc,abc,cd,ef,fg,efg,f,g]) in the same coloumn (among various concentrations of GMPE, mangostins in IL-6 concentrations and inhibitory activity) are significant at $p < 0.05$ based on Duncan's *post-hoc* comparisons ($p < 0.05$). The experiment was conducted in triplicate replication

prostaglandins and triggers the production of other pro-inflammatory mediators.[34] IL-6 is a pleiotropic cytokine which modulates inflammatory responses.[39] GMPE in the concentration of 20 μg/mL showed the highest inhibitory

Table 4. Effect various concentrations of GMPE and mangostins toward IL-1β in RAW 264.7 cell

Samples	IL-1â	
	IL-1β concentration (pg/mL)	IL-1β inhibitory activity (%)
Negative control	841.44±18.01[a]	28.87±1.52[c]
Positive control	1,183.03±35.09[c]	0.00±2.97[a]
GMPE 20 μg/mL	894.31±77.23[a]	24.41±6.53[c]
GMPE 10 μg/mL	950.70±115.33[ab]	19.64±9.75[bc]
GMPE 5 μg/mL	951.72±45.98[ab]	19.55±3.89[bc]
α-mangostin 75 μM	877.28±35.87[b]	25.84±3.03[c]
α-mangostin 50 μM	910.43±79.98[ab]	23.04±6.76[bc]
α-mangostin 25 μM	942.64±121.95[ab]	20.32±10.31[bc]
γ-mangostin 75 μM	817.69±3.80[a]	30.88±0.32[c]
γ-mangostin 50 μM	936.41±43.20[ab]	20.85±3.65[bc]
γ-mangostin 25 μM	1,041.41±110.80[b]	11.97±9.37[b]

The data are presented as mean ± standard deviation. Different letters ([a,ab,b,c,bc]) in the same coloumn (among various concentrations of GMPE, mangostins in IL-1β concentrations and inhibitory activity) are significant at $p < 0.05$ based on Duncan's *post-hoc* comparisons ($p < 0.05$). The experiment was conducted in triplicate replication

activity (Table 3). Inhibiting the production of IL-1β was important in finding the anti-inflammatory agent. GMPE, α-mangostin, and γ-mangostin inhibited the production of IL-1β in a concentration-dependent manner. The highest concentration of GMPE, α-mangostin and γ-mangostin possessed the highest inhibitory activity with no significant differences observed among them (Table 4). IL-1β is a potent proinflammatory cytokine released by macrophages in systemic inflammatory responses that regulate inflammatory reaction and immune response.[40] In this study, the nitrite concentration was associated with NO production. Excessive levels of NO can mediate proinflammatory and have destructive effects, thus lowering the NO concentration can be used as an anti-inflammatory action. The GMPE showed the highest NO inhibitory activity, meanwhile the positive control showed the highest concentration of NO (Table 5). This research result indicated that the LPS successfully induced the inflammation of the RAW 264.7 cells[41] and GMPE had anti-inflammatory properties by reducing the NO production. The inhibition of NO production might related to suppression of iNOS expression, as NO synthesis is catalyzed by iNOS.

GMPE possessed the highest inhibitory activity against COX-2, IL-6, and NO production, but all of the tested concentration both for GMPE and its compounds have no significant differences in inhibitory activity of IL-1β. This research was consistent with previous research that *G. mangostana* fruit hull inhibited the inflammatory related

Table 5. Effect various concentrations of GMPE and mangostins toward NO in RAW 264.7 cell

Samples	NO	
	NO concentration (μM)	NO inhibitory activity (%)
Negative control	6.06±0.17[a]	82.74±0.50[j]
Positive control	35.10±0.08[j]	0.01±0.23[a]
GMPE 20 μg/mL	23.29±0.07[b]	33.66±0.19[i]
GMPE 10 μg/mL	23.92±0.04[c]	31.86±0.12[h]
GMPE 5 μg/mL	27.07±0.07[f]	22.89±0.19[e]
α-mangostin 75 μM	24.55±0.03[d]	30.07±0.09[g]
α-mangostin 50 μM	25.86±0.02[e]	26.33±0.07[f]
α-mangostin 25 μM	28.88±0.12[g]	17.73±0.34[d]
γ-mangostin 75 μM	26.94±0.05[f]	23.24±0.14[e]
γ-mangostin 50 μM	29.37±0.08[h]	16.32±0.23[c]
γ-mangostin 25 μM	30.26±0.07[i]	13.78±0.21[b]

The data are presented as mean ± standard deviation. Different letters ([a,ab,b,c,bc]) in the same coloumn (among various concentrations of GMPE, mangostins in NO concentrations and inhibitory activity) are significant at $p < 0.05$ based on Duncan's *post-hoc* comparisons ($p < 0.05$). The experiment was conducted in triplicate replication

diseases through NO and PGE2 releases.[42] Likewise, two compounds α-mangostin and γ-mangostin inhibited NO and PGE2 production and COX-2 activity in LPS-induced RAW 264.7 cells according to Chen, *et al.* (2007) study.[27] The inhibitory activity of α-mangostin and γ-mangostin from GMPE against IL-6 also revealed by Bumrungpert, *et al.* (2010) study.[43] Furthermore, the α-mangostin and γ-mangostin revealed to attenuated LPS-induced inflammatory gene expression of TNF-α, IL-1β, IL-6, IL-8, MCP-1, and Toll-like receptor-2.[44] The effect of *G. mangostana* and its compounds against inflammation in this study also in line with Chomnawang, *et al.* (2007) study which reported anti-inflammatory activity of *G. mangostana* on inflammation caused by *Propionibacterium acnes* through suppression of pro-inflammatory cytokines, [45] as well as Lee, *et al.* (2013) study which revealed that *G. mangosteen* and its compounds has great potential in the treatment and prevention of rheumatoid arthritis, a chronic inflammatory disease, showed by inhibition of TNF-α and IL-6 production in LPS-stimulated mice, reduction of paw edema in the carrageenan-induced rats, and reduction of arthritis score in the CIA rats.[46]

This research revealed that GMPE, α-mangostin, and γ-mangostin possess the anti-inflammatory potential by inhibiting COX-2, IL-6, IL-1β, and NO. These extracts may have therapeutic potential for the modulation and regulation of macrophage activation, and may provide safe and effective treatment option for various inflammation-mediated diseases. However, the therapeutic potential of these plant extracts will be further clear after preclinical and clinical test were conducted.

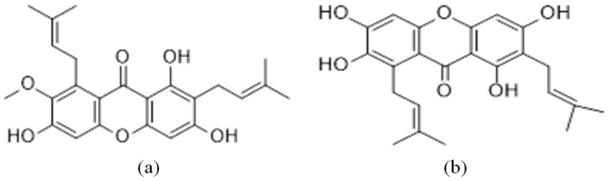

Fig. 1. Chemical structure of (a) α-mangostin and (b) γ-mangostin.

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