An Investigation of Anti-Inflammatory Properties of Methanol Extract of *Syzygium malaccense* on Lipopolysaccharide-Stimulated Raw 264.7 Macrophages

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Abstract—*Syzygium malaccense* is reported to contain flavonoids which contribute to anti-oxidant, anti-microbial and anti-cancer properties. Nevertheless, anti-inflammatory activities of *S. malaccense* fruits have not been validated scientifically. Therefore, this study was aimed to investigate the anti-inflammatory activities of methanol extracts of *S. malaccense* fruits on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Maximum non-toxic dosage (MNTD) of *S. malaccense* methanol extracts was determined via MTT assay, followed by assessing nitric oxide (NO) with Griess reagent and measuring pro-inflammatory and anti-inflammatory mediators with ELISA in LPS-stimulated cells. MNTD and ½MNTD were determined to be 4.50±0.5 µg/mL and 2.25 µg/mL, respectively. Studies revealed that pro-inflammatory mediators (NO, prostaglandin E₂ (PGE₂), tumour necrosis factor-α (TNF-α) and interleukin (IL)-6) and anti-inflammatory cytokine (IL-10) were increased in pro-inflammatory cytokines such as interleukins (IL-6, IL-1β) and anti-inflammatory cytokine (IL-10) were increased in LPS-stimulated cells pre-treated with MNTD and ½ MNTD extracts. Methanol extract of *S. malaccense* fruits did not possess anti-inflammatory activities when tested on LPS-stimulated RAW 264.7 macrophages experimental model.

Keywords—Inflammation, Interleukin-6, Interleukin-10, Nitric oxide, Prostaglandin E₂, *Syzygium malaccense*, Tumour necrosis factor-α

I. INTRODUCTION

Inflammation is a defensive response of the immune system, cells within the damaged tissue and local vascular system to microbial infection or physical and chemical irritants [1, 2]. Inflammation is categorised as either acute or chronic inflammation. Acute inflammation occurs in relatively short duration (minutes or days). However, continuing inflammation leads to chronic inflammation which occurs in longer duration (weeks, months or years). Inflammation is characterised by heat, pain, redness, swelling and loss of function [3]. Macrophages involves in the inflammation process by over-production of pro-inflammatory cytokines such as interleukins (IL-6, IL-1β) and tumour necrosis factor (TNF-α) [1, 3]. Pro-inflammatory cytokines worsen inflammation by up-regulating the inflammatory reactions [3]. Besides, inflammation leads to up-regulation of inflammatory mediators such as nitric oxide (NO), prostaglandin E₂ (PGE₂) and reactive oxygen species (ROS) by activated macrophages in response to microbial such as lipopolysaccharide (LPS) [1, 3]. However, the action of pro-inflammatory cytokines is controlled by immune-regulatory molecules which are anti-inflammatory cytokines such as IL-4, IL-10, IL-13 and interferon-γ (IFN-γ) [3, 4]. IL-10 has the potent anti-inflammatory properties in suppressing the pro-inflammatory cytokines expression among the anti-inflammatory cytokines [3]. NO provides anti-inflammatory effect under physiological condition, but it is classified as pro-inflammatory mediator that worsen inflammation due to up-regulation in abnormal condition [5, 6]. In addition, PGE₂ is a crucial mediator in regulating the immune responses and other biological activities under physiological conditions [7]. PGE₂ participates in the inflammatory responses and causes the signs of inflammation such as redness, pain and swelling [7].

Increase in pro-inflammatory mediators may lead to an increased risk of diseases such as atherosclerosis, arthritis and neuroinflammation [1]. Non-steroidal anti-inflammatory drug (NSAID) is used widely in treating inflammation by inhibiting the generation of cyclooxygenase enzymes, COX-1 and COX-2 which are responsible to produce inflammatory mediators and prostaglandins [8, 9]. However, long-term administration of NSAID brings adverse effects such as stomach ulcer, gastrointestinal bleeding and renal failure due to the inhibition of COX-1 enzyme which is responsible to maintain the integrity of stomach lining and normal renal function [8, 10, 11]. Thus, medicinal plants have been one of the alternatives to treat inflammation as they are believed to bring comparable therapeutic effects yet with fewer side effects and less expensive.

*Syzygium malaccense* belongs to Myrtaceae family and also known as ‘Malay Apple’ or ‘Mountain Apple’ [12]. *S. malaccense* is probably originated from lowland rainforest of Malaysia, Sumatra and Java [13]. *S. malaccense* is claimed to contain flavonoids [13]. Flavonoids exhibit anti-oxidant, anti-microbial, anticancer, anti-viral, immunomodulatory, anti-thrombictic and anti-inflammatory properties as well [13, 14, 15, 16]. Gonzalez-Gallego *et al.* (2007) stated that dietary flavonoids exhibited anti-inflammatory properties by inhibiting...
enzymes and mediators of the inflammatory process [17]. Apart from that, Savitha et al. (2011) stated that the methanolic leaf extract of *S. malaccense* possessed strong antioxidant activity [13]. Anti-oxidant is correlated with anti-inflammatory as excess production of ROS causes cellular and tissue damage, thus, worsen inflammation [18]. Moreover, Dunstan et al. (1997) reported that the leaves of *S. malaccense* have been used to treat inflammation in Western Samoa [15]. However, the anti-inflammatory activity of the fruits of *S. malaccense* is yet to be scientifically validated. Therefore, this study was conducted to determine the anti-inflammatory properties of methanol extract of *S. malaccense* using the in vitro model of lipopolysaccharide-stimulated RAW 264.7 macrophages.

**II. METHODS**

**A. Preparation of Stock Solution**

The stock solution of methanol extract of *S. malaccense* was prepared by dissolving 1 mg of extracts in 20 µL of DMSO (Sigma Aldrich, USA).

**B. Cell Culture**

The RAW 264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, UK) supplemented with 10% of Fetal Bovine Serum (FBS) (GIBCO, South America), penicillin (100 units/mL) and streptomycin (100 µg/mL) (GIBCO, South America) and fungizone (0.1%) (GIBCO, South America).

**C. Determination of Maximum Non-Toxic Dose (MNTD)**

RAW 264.7 cells were seeded in 96 well flat-bottom plates (Corning, USA) at a density of 8 x 10^3 cells/mL. When the cells reached 70% to 80% confluency, the cells were treated with various concentrations of extracts (250, 125, 31.25, 7.81, 1.95, 0.49, and 0 mg/mL) and further incubated at 37°C with 5% CO_2_. After 24 hours of incubation, the cell viability was assessed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The absorbance was measured at 570 nm with 630 nm as reference wavelength using microplate reader (Dynex, USA). The percentage of viability and cytotoxicity was then calculated.

**D. Cell Stimulation and Treatment**

RAW 264.7 cells were seeded in 24-well plate (Corning, USA) at a density of 1 x 10^4 cells/mL. When the cells reached 70% to 80% confluency, the cells were stimulated and/or treated with LPS and MNTD or ½MNTD of the extracts. Treatment was initiated by adding in extract at MNTD and 1/2MNTD for three hours. After three hours, 1µg/mL of LPS was added to induce inflammation and further incubated for 24 hours. In this study, indomethacin was used as positive drug control. After 24 hours of incubation, the supernatant was collected for the measurement of nitric oxide, prostaglandin and cytokines level.

**E. Measurement of Nitric Oxide Formation**

The cell culture supernatant was evaluated by Griess reaction by adding 50 µL of Griess reagent (Sigma Aldrich, USA) to 50 µL supernatant in 96 well flat-bottom plates in dark condition. In Griess deazotization reaction, red pink colour was formed in the presence of nitrite. In this study, fresh culture medium was used as reference wavelength using microplate reader (Tecan®, Austria) after 30 minutes of incubation at room temperature. The amount of nitrite was calculated from a sodium nitrite (John Kollin, UK) standard curve constructed from 0 to 250 µM.

**F. Determination of Prostaglandin E2 and Cytokines Level**

The concentrations of PGE_2_ and cytokines (TNF-α, IL-6 and IL-10) in 150 µL and 50 µL of supernatant, correspondingly were also assessed using Quantikine Enzyme-linked Immunosorbent Assay (ELISA) kits (R&D Systems, USA) with sensitivity of 41.4, 5.1, 1.8 and 4 pg/mL, respectively. The measurement was conducted based on manufacturer’s instruction.

**G. Statistical Analysis**

The experiment for the determination of MNTD was conducted in five replicates. However, the experiment for cell stimulation and treatment was conducted in triplicates. The results presented were from independent experiments and represented as mean ±SD (standard deviation). The significant differences between the treatments was assessed using Student’s t-test with SPSS 11.0 software at p<0.05.

**III. RESULTS AND DISCUSSION**

**A. Determination of Maximum Non-Toxic Dose (MNTD)**

In this study, the MNTD of *S. malaccense* fruits was determined by treating RAW 264.7 cells with various concentrations of methanol extract. In order to reach a precise cytotoxic concentration, the MNTD of *S. malaccense* was obtained through optimisation. Basically, the MNTD for the methanol extract of *S. malaccense* discovered was 4.5±0.5 µg/mL, which showed 0% toxicity to the cells. Studies revealed that extract at the concentration below 4.5±0.5 µg/mL did not bring any cytotoxic effect towards the cells. Conversely, extract concentration higher than 4.5 µg/mL would kill the cells due to cytotoxicity, and eventually led to cell death.

The cytotoxicity effects of methanol extract of *S. malaccense* could be attributed to the presence of flavonoids in the extract. Methanol extract of *S. malaccense* fruits might possess anti-oxidant activity as the methanol leaf extract of *S. malaccense* has been reported to possess strong antioxidant activity due to the presence of high amount of flavonoids and phenolic [13]. Flavonoids at specific concentration have the potential to prevent lipid peroxidation, chelate redox-active metals and reduction of inflammatory processes involving ROS through the radical scavenging and chelating effect [19]. However, the flavonoids exhibit significant cytotoxicity at high concentrations corresponding to dose-dependent manner [20].

**B. Measurement of Nitric Oxide Formation**

In order to determine the anti-inflammatory properties of methanol extract of *S. malaccense*, MNTD and ½MNTD extracts were used to pre-treat the cells prior to LPS stimulation. The NO formation was then measured with Griess reagent. Indomethacin at 25µM, which is commonly used as anti-inflammatory agent, was used as a positive drug control.

This study discovered that untreated cells showed a low
amount of NO production (Fig. 1). This might be due to NO plays a dual role in cell-mediated immune response. NO brings both anti-and pro-inflammatory effects and involves in immunity and inflammation [5, 6]. Under normal condition, NO exhibits anti-inflammatory effects. However, the level of NO elevated in response to LPS in activated macrophages upon stimulation with LPS [6]. This is in accordance with the present study, whereby it was observed that stimulation of the cells with LPS alone (6.03±0.63 μM) (negative control) resulted in the concentration of NO to be increased significantly as compared to the control (1.37±0.50 μM). LPS is a major component of Gram negative bacteria outer membrane, which is able to stimulate inflammation by inducing inducible nitric oxide synthase (iNOS) transcription factor and subsequently resulting in NO increase [21].

An anti-inflammatory agent should have the potential to inhibit iNOS enzyme and subsequently suppressing NO production via down-regulation of nuclear factor-κB (NF-κB) [22]. However, this is not the case in the present studies, in which the methanol extract of S. malaccense fruits caused an increase in NO level. In LPS-stimulated cells pre-treated with MNTD, the NO level gave a significant increase to 38.81% compared to the cells stimulated with LPS alone (Fig. 1). Similarly, cells pre-treated with ½MNTD extracts produced the highest concentration of NO (8.85±0.41 μM) among all the treatment groups. Thus, this indicated that both MNTD and ½MNTD of extracts did not show inhibitory effect on NO production, yet the extracts even cause inductive effect of NO.

C. Determination of Prostaglandin E\(_2\) Level

The anti-inflammatory ability of S. malaccense extracts was further investigated on other inflammatory mediators such as PGE\(_2\), using ELISA. PGE\(_2\) is an important inflammatory mediator and is generated upon inflammation by activated COX-2 [7]. As presented in Fig. 2, cells stimulated with LPS (2049.63 pg/mL±78.08 pg/mL) showed PGE2 concentration that was 49.05% higher than the untreated cells (1375.12±63.48 pg/mL). These results further prove the theory that prostaglandin generation is low in uninflamed tissue, therefore, untreated cells showed lower PGE2 concentration compared to cells stimulated with LPS only [7]. However, prostaglandin level raises immediately in acute inflammation, hence, cells stimulated with LPS only showed elevation of PGE2 level compared to untreated cells with LPS only [7]. Ideal anti-inflammatory agents have the ability to reduce PGE2 level by inhibiting COX-2 expression. However, this condition was not observed in the present studies. Methanol extract of S. malaccense fruits showed an increase level of PGE2 alike NO production effects. LPS-stimulated cells treated with MNTD (2450.06±195.46 pg/mL) and ½MNTD extracts (2364.12±86.00 pg/mL) showed increasing level of PGE2 by 19.54% and 15.34%, correspondingly. Thus, S. malaccense did not display inhibitory effects against PGE2. This could be due to high concentration of NO present in the cells. Cyclooxygenases activity might be increased due to the interaction of NO and superoxide ions which subsequently resulted in the production of peroxynitrite [22]. Peroxynitrite is a crucial modulator in cyclooxygenases activity in inflamed cells and lead to biosynthesis of prostaglandin [22]. Based on the studies conducted by Yoon et al. (2009), D-limonene not only inhibited PGE2 production on LPS-stimulated RAW 264.7 cells, it also inhibits NO production [23]. This could be due to the presence of peroxynitrite that acted as a modulator of prostaglandin. Hence, it is suggested that D-limonene showed the correlation of the NO and PGE2 in reducing both NO and PGE2.

D. Determination of Cytokines Level

The anti-inflammatory ability of S. malaccense extract was further investigated on other inflammatory mediators such as TNF-α, IL-6 and IL-10 using ELISA. TNF-α is one of the pro-inflammatory cytokines which contributes to the inflammatory process. NF-κB is a transcription factor which participates in gene expression towards immune and inflammatory responses [24]. Activated NF-κB leads to the expression of the pro-inflammatory mediators such as TNF-α and IL-6 [24]. Thus, upon stimulation with LPS, the cells triggered the signalling cascades and eventually cause the elevation level of TNF-α as compared to the untreated cells. As shown in Fig. 3(A), stimulation of the cells with LPS alone (820.68±5.54 pg/mL) resulted in an increase in the concentration of TNF-α as compared to untreated cells (809.73±4.97 pg/mL).

Anti-inflammatory agents should have the potential to suppress NF-κB, which eventually inhibit the expression of pro-inflammatory cytokines such as TNF-α. However, the findings in this study were contradicted to the condition mentioned above. This study exhibited that the methanol extract of S. malaccense fruits demonstrated an increase level of TNF-α. LPS-stimulated cells pre-treated with MNTD (828.92±22.17 pg/mL) and ½MNTD (822.84±10.13 pg/mL) extracts showed slight increase in TNF-α level by 1% and 0.26 % respectively. Therefore, S. malaccense failed to reduce the TNF-α production, which indicated that the extracts did not possess anti-inflammatory properties. Similar findings were reported in the studies conducted by Xagorari et al. (2001), whereby myricetin and catechin showed no effect on LPS-induced TNF-α release [25]. On the contrary, the flavonoids such as quercetin and luteolin were proven to be effective in suppressing the action of LPS which eventually blocking 80% of the TNF-α release [26]. Therefore, it could be hypothesised that the methanol extract of S. malaccense fruits might contain higher amount of myricetin and catechin than quercetin and luteolin.

Besides TNF-α, IL-6 is also one of the pro-inflammatory cytokines which contributes to the inflammation process. Similar to TNF-α, activation of NF-κB leads to the expression of IL-6 [24]. Therefore, macrophages response actively in the presence of LPS, which leads to secretion of IL-6. As shown in Fig. 3(B), cells stimulated with LPS only (142.12±15.77 pg/mL) showed elevation of IL-6 concentration compared to untreated cells (16.83±1.22 pg/mL). This condition was similar to TNF-α production. Anti-inflammatory agents should have the ability to suppress the activation of NF-κB to inhibit the expression of pro-inflammatory cytokines such as IL-6 and subsequently resulting in reduction of IL-6 level. However, current findings were opposed to the condition mentioned above as the methanol extract of S. malaccense fruits caused an increase level of IL-6. LPS-stimulated cells pre-treated with
whereby the water extract of *S. malaccense* did not exhibit anti-inflammatory properties. However, studies using other *S. malaccense* extracts such as ethanol, hexane, ethyl acetate or water extracts should be further carried out prior to the confirmation of no anti-inflammatory properties in *S. malaccense* fruits. This is crucial as different bioactive compounds could be extracted from different solvent systems. Apart from that, *S. malaccense* should be further isolated as the concentrated bioactive compounds could possess stronger anti-inflammatory effect than the crude extracts.

In anti-inflammatory model, IL-10 contributes in modulating the function adaptive immune-related cells by acting as immunosuppressive and anti-inflammatory cytokines. Upon inflammation, IL-10 down-regulate pro-inflammatory responses through the inhibition of NF-κB activity [28]. As illustrated in Fig. 3(C), cells treated with LPS only (181.56±3.98 pg/mL) showed the level of IL-10 to be elevated dramatically as compared with the untreated cells (80.21±0.44 pg/mL). An increase in IL-10 concentration in cell stimulated with LPS only indicating that the anti-inflammatory cytokines presented regulatory action after inflammatory reaction.

A potential anti-inflammatory agent should be capable to increase the concentration of IL-10 to eliminate the pro-inflammatory cytokines. The IL-10 concentration in this study was found to increase as well. IL-10 concentration in both LPS-stimulated cells pre-treated with MNTD (215.31±1.33 pg/mL) and ½ MNTD extracts (228.44±4.86 pg/mL) were increased significantly by 18.59% and 25.82%, correspondingly. However, the increasing level of NO, PGE₂, TNF-α and IL-6 might indicate that methanol extract of *S. malaccense* fruits did not possess anti-inflammatory properties. Therefore, elevation of IL-10 concentration in LPS-stimulated cells pre-treated with both MNTD and ½ MNTD extracts could be due to the presence of high level of TNF-α, which eventually resulted in the production of IL-10 to mitigate the harmful effects of TNF-α [29].

Based on the hypothesis mentioned above, the *S. malaccense* fruits might be containing higher amount of myricetin or catechin than quercetin or luteolin. Thus, the extracts might not be able to provide sufficient amount of IL-10 that could reduce the effects of TNF-α. Similar phenomenon was noted in the studies conducted by Yoon et al. (2009), whereby the water extract of *Scutellaria baicalensis* exhibited anti-inflammatory activities in LPS-stimulated RAW 264.7 cells by inhibiting the production of pro-inflammatory mediators such as IL-3, IL-6 and NO but inhibitory effect was shown in IL-10 as well [30]. Besides, Lee et al. (2011) reported that myristicin significantly reduced the pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, COX-2 but also inhibit the anti-inflammatory IL-10 on polyinosinic-polycytidylic acid-stimulated RAW 264.7 cells [31].

IV. CONCLUSIONS

In conclusion, the present studies revealed that the methanol extract of *S. malaccense* did not possess inhibitory effects on the production of NO, PGE₂, TNF-α and IL-6, and the level of IL-10 was increased as well in LPS-stimulated RAW 264.7 macrophages. Hence, these findings suggested that *S. malaccense* fruits did not exhibit anti-inflammatory properties.
Fig 3. The effects of methanol extract of S. malaccense fruits on (A) TNF-α (B) IL-6 (C) IL-10 production in LPS-stimulated RAW 264.7 cells. Bars indicate the mean±SD. * denotes significantly different as compared to untreated control at p<0.05 using Student’s t-test; # denotes significantly different as compared to the cells stimulated with LPS only at p<0.05 using Student’s t-test.

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