Anti-inflammatory Effect of *Strobilanthes crispus* methanolic extract on Lipopolysaccharide-stimulated RAW 264.7 Macrophages

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Abstract— Inflammation is rapid response by body to deal with injuries, foreign particles and damaged cells. An unattended inflammation could lead to complication in cerebrovascular, cardiovascular system, joint and intestines. However, currently available conventional drugs exhibited adverse effects on many organ systems besides treating inflammation. *Strobilanthes crispus*, a native plant is believed to have anti-inflammatory property as it has been used in folk medicine to treat various diseases. Nevertheless, no scientific studies have been conducted to prove this traditional claim. Hence, this study focused on investigating the anti-inflammatory property of *S. crispus* on lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages experimental model. The maximum non-toxic dose (MNTD) of *S. crispus* methanol extracts and optimum LPS concentration were determined prior to determination of anti-inflammatory effect of *S. crispus*. MNTD of *S. crispus* was determined using MTT assay and the optimum LPS was determined based on the production of nitric oxide (NO) using Griess reaction. Finally, the anti-inflammatory effect of *S. crispus* was determined by examining the NO and cytokines levels, namely interleukin-6 (IL-6) and interleukin-10 (IL-10) using Procarta immunoassay kit. The MNTD for *S. crispus* leaves and stem extracts was 160 µg/mL and 1.5 µg/mL, respectively. The optimum LPS needed to induce maximum inflammation was 1 µg/mL. Upon pre-treatment with half MNTD (1/2MNTD) of leaf extract, the production of NO was increased accumulation of fluid at the inflammation site, and untreated, it will alter the vascular permeability that inflammation is rapid response by body to deal with 

expression regulations associated with the immunogens. Pro-inflammatory cytokines including Interleukin-1 (IL1), Tumour necrosis factor-α (TNFα), Interleukin-6 (IL6), Interleukin-11 (IL11) and Interleukin-8 (IL8) will be up-regulated inflammation while anti-inflammatory cytokines such as Interleukin-4 (IL4), Interleukin-13 (IL13), Interleukin-11 (IL11) and Interleukin-10 (IL10) act as antagonist against inflammation [4]. Besides cytokines, nitric oxide (NO) is often related to inflammation as it plays a vital role as cytokines. NO acts as either pro-inflammatory or anti-inflammatory. Low level of NO mediates host defence by acting as anti-inflammatory agent whereas high level of NO causes inflammation [5].

Although inflammation is primarily a defensive response by body, when it develops into chronic phase and left untreated, it becomes detrimental to tissues and cells [6]. Studies stating that chronic inflammation might lead to complications in human organ systems including cerebrovascular, cardiovascular, joint and intestines [7]. Besides that, chronic inflammation is often related to rheumatoid arthritis, atherosclerosis and asthma [8].

Current available conventional treatments used for inflammation are corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs). These drugs act against inflammation by blocking the production of prostaglandin and cyclooxygenase (COX). However, blockage of the production of these compounds has vast impact on other vital mechanisms such as blood pressure control which eventually might lead to cardiovascular disease. Besides, NSAIDs might cause gastrointestinal mucosal lesions due to the complication in gastric acid secretion. Moreover, there is also risk of major congenital malformations, spontaneous abortion and renal failure [9-12]. Hence, medicinal plants have been given high priority to be chosen as alternative treatment for variety of diseases as the treatment provided has no or fewer side effects compared to conventional medicine. Furthermore, treatment using medicinal plants is less expensive. Nevertheless, to enable the usage of medicinal plants as alternative medicine, researches and developments are very important to trace the advancement of traditional medicines [13].

I. INTRODUCTION

Inflammation describes as the any defensive response by body towards foreign particles, injuries and cellular damage. There are two major phases of inflammatory effects i.e. acute phase and chronic phase. Once the immune responses has been triggered, it will alter the vascular permeability that increased accumulation of fluid at the inflammation site, and stimulate the production of enzymes, leukocytes, reactive nitrogen species, and inflammatory mediators such as cytokines [1-3]. Upon inflammation, different types of cytokines will be released as the results of their gene expression regulations associated with the immunogens. Pro-inflammatory cytokines including Interleukin-1 (IL1), Tumour necrosis factor-α (TNFα), Interleukin-6 (IL6), Interleukin-11 (IL11) and Interleukin-8 (IL8) will be up-regulated inflammation while anti-inflammatory cytokines such as Interleukin-4 (IL4), Interleukin-13 (IL13), Interleukin-11 (IL11) and Interleukin-10 (IL10) act as antagonist against inflammation [4]. Besides cytokines, nitric oxide (NO) is often related to inflammation as it plays a vital role as cytokines. NO acts as either pro-inflammatory or anti-inflammatory. Low level of NO mediates host defence by acting as anti-inflammatory agent whereas high level of NO causes inflammation [5].

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Strobilanthes crispus is one of the most commonly used traditional medicines from Madagascar to Malaysia. S. crispus, classified under the Family of Acanthaceae and locally known as ‘daun pecah kaca’ or ‘jin batu’ is a type of native plant that grows easily in the forest, riverbanks and abandoned fields [14]. A study on S. crispus revealed that its leaves have anti-diabetic property, water extract of fermented and unfermented leaves reduce blood glucose level in hyperglycemic rats and unfermented leaves reduce blood glucose level in normal rat [14]. Meanwhile, in South Asia region, S. crispus is widely used to treat cancer. Researchers have found that methanol and aqueous extracts of S. crispus are cytotoxic to human cancer cell lines such as breast carcinoma, colon carcinoma, hepatocellular carcinoma and many more [15]. In addition to that, studies on anti-proliferative properties and antioxidant activity of various types of S. crispus tea stated that tea has high anti-oxidant and anti-proliferative properties on cancer cell lines [16].

The above mentioned previous studies showed that till this most recent time, most of the researches on S. crispus are mainly focusing on its anti-diabetic and anti-cancer property. There are no studies reporting on the anti-inflammatory effect of S. crispus leaves and stem to date which have been a reason for this study to be conducted. Furthermore, S. crispus have been reported to contain some flavonoids such as apigenin, quercetin, (+)-catechin, kaempferol, naringenin (-)-epicatechin, rutin, luteolin, and myricetin [17], which have been found in other medicinal plants that exhibiting anti-inflammatory property. Thus, this present study was conducted to identify the anti-inflammatory property of S. crispus based on inflammatory mediators such as IL-6, and IL-10 and NO levels.

II. MATERIALS AND METHODS

A. Materials

Methanol (Fisher), Dimethyl sulfoxide (DMSO) (Sigma Aldrich), Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco), foetal bovine serum (FBS) (Gibco), penicillin (Gibco), Streptomycin (Gibco), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma), indomethacin (Sigma), Griess reagent (Sigma Aldrich), Procarta Immunoassay Kit (Panomics Inc.).

B. Preparation of methanolic extract

Leaves and stems of S. crispus were washed and cleaned before air-dried at room temperature for a week. Upon drying, S. crispus leaves and stems were grounded into powder prior to the extraction with methanol at room temperature for 3 days in dark condition. The resulting suspension was filtered and the filtrate was left to be concentrated through evaporation.

C. Cell cultures

RAW 267.4 macrophages from murine mouse were maintained and incubated in DMEM medium supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 µg/mL) at 5% CO₂, 37°C.

D. Determination of maximum non-toxic dose (MNTD)

RAW 267.4 cells were seeded in 96-well plates until achieved at least 70% confluent. Various concentrations of S. crispus stem and leaf methanol extracts were prepared using serial dilution. Incubated RAW 264.7 cells were then treated with various concentrations of S. crispus extract and further incubated at 37°C for another 24 hours. The treated cells were then subjected to MTT assay and MNTD was determined. The MTT solution (5mg/mL) was added and the absorbances were measured at 570nm using microplate reader after incubation.

E. Determination of NO, IL-6 and IL-10

Cells with the density of 1 x 10⁴ cells/well were seeded in 12-well plate and incubated for 24 hours. S. crispus methanol extracts at MNTD or half MNTD (1/2MNTD) and 25µM of indomethacin were added. After incubation, inflammation was stimulated using LPS. The supernatant was collected for the measurement of both nitric oxide and cytokines levels by Griess reagent and Procarta Immunoassay kit, respectively.

F. Statistical Analysis

All the results obtained from three independent experiments were summarised and presented using Student’s t-test with SPSS 11.0 software with p < 0.05 as significant value.

III. RESULTS AND DISCUSSION

A. Determination of maximum non-toxic dose

Determination of MNTD is crucial to identify the maximum concentration of plant extract that is non-toxic to RAW 264.7 cells upon treated with plant extracts. The MNTD obtained for S. crispus leaves methanol extract was 160 µg/mL while 1.5 µg/mL was obtained as MNTD for S. crispus stem methanol extract. At these concentration, the extracts exhibited the promising cell viability. It has been suggested that the growth promoting property of the flavonoids found in S. crispus [18]. Flavonoids such as quercetin, myricetin and rutin are reported to have strong anti-oxidant property, which can inhibit the formation of free radicals in the cells that might cause oxidative stress and lead to cell death [19]. These flavonoids are found in S. crispus [17].

In contrast, the increased cytotoxicity due to increased concentrations of S. crispus leaves and stem methanol extract could possibly be explained by auto-oxidation caused by the flavonoids which leads to cell apoptosis [20]. Studies have reported that high dose of catechin down regulates expression of apoptosis suppressor proteins Bcl-2 and Bcl-xL, which results in cell apoptosis [21]. Besides that, the high cytotoxicity might be contributed by high dose of quercetin in S. crispus. Quercetin at high dose has been reported to cause the formation of tumours in mice model [22]. Studies have reported that many flavonoids show biphasic effects on cell death. Apigenin at low concentration promotes cell growth by activation of
ERα-mediated gene transcription. However, at high concentrations, apigenin inhibits growth by down regulating expressions of ERα-mediated gene transcription and protein kinases which are essential for cell growth [21].

B. Determination of NO, IL-6 and IL-10

In present study, the anti-inflammatory activity was examined by pre-treatment the RAW 264.7 macrophages with methanol extract of *S. crispus* leaf and stem prior to induction of inflammation by LPS. The levels of NO and cytokines were measured on LPS-stimulated RAW 264.7 macrophages.

It has been a well-known fact that NO is an important mediator in inflammation and high levels of NO causes variety of pathological processes including various forms of inflammation, circulatory shock, and carcinogenesis [23-24]. NO is produced from an amino acid precursor known as L-arginine by nitric oxide synthase (NOS). NOS is an enzyme that plays a vital role in converting L-arginine into NO [25]. This enzyme occurs in two forms in the cells: constitutive NOS (cNOS) and inducible NOS (iNOS). cNOS is responsible to produce NO under normal conditions to maintain homeostasis whereas iNOS produces NO when there is stimulation by foreign particles to elicit immune response such as host defense, inflammation and tissue repair [26-27].

As shown in Figure 1, the control group which was not treated with either LPS or extracts could also produce NO. This might be due to the RAW 264.7 macrophages produce NO through cNOS to maintain homeostasis. Present studies also revealed that cells treated with ½MNTD of *S. crispus* leaf extract reduce production of NO about 23%. Nevertheless, this reduction is not as low as those recorded in cells treated with positive drug control, indomethacin. It is well studied that indomethacin acts to reduce NO and IL-6 production through inhibition of synthesis of COX [26, 28]. Reduction of NO upon pre-treatment with MNTD and ½ MNTD of *S. crispus* leaf extract seemed to indicate that there are flavonoids in *S. crispus* that inhibit protein levels of iNOS, an crucial enzyme that plays a vital role in converting L-arginine into NO [25]. As illustrated in Figure 2, a high production of IL-10 by cells treated with MNTD of *S. crispus* stem extract was not significant, which was 0.6 pg/mL and 1.2 pg/mL for MNTD of leaf and ½ MNTD of leaf, respectively. This might show that three hours of pre-treatment was insufficient for the binding of extracts or flavonoids to the cytokine receptors.

As illustrated in Figure 2, a high production of IL-10 by cells treated with MNTD of *S. crispus* stem extract was observed, in which signified that flavonoids present in the extract could have contributed to this anti-inflammatory property. A study on anti-inflammatory action of green tea has reported that catechins enhanced the production of anti-inflammatory cytokines such as IL-10 [31], which are in accordance with the current findings. Catechin is also found in *S.crispus* [17]. It was also found that ½ MNTD of *S. crispus* stem reduced the production of IL-10 by 40%. In contrast, the production of IL-10 by *S. crispus* leaf extract was not significant, which was 0.6 pg/mL and 1.2 pg/mL for MNTD of leaf and ½ MNTD of leaf, respectively. This might show that three hours of pre-treatment was insufficient for the binding of extracts or flavonoids to the cytokine receptors.

As shown in Figure 2 and Figure 3, the untreated control cells showed production of both IL-10 and IL-6. Figure 3 shows that the untreated control group was also capable in producing IL-6. This might be due to the aged serum usage that stimulated the baseline inflammatory state. Hence, it is suggested that variation in environment of the cell culture might contribute to the variation in results [32]. On the other hand, cells treated with indomethacin showed a total reduction of 90% in IL-10 and 52% reduction in IL-6 as compared to LPS group, respectively. This might be due to the improper binding of drug on cytokine receptors [29]. Besides that, indomethacin is a non-selective NSAID, which is capable of suppressing the production of any inflammatory mediators.

As shown in Figure 3, the production of IL-6, which is a pro-inflammatory mediator, was also found that ½ MNTD of *S. crispus* leaf reduced the production of IL-6 and IL-10. During inflammation, IL-10 acts as anti-inflammatory mediator whereas IL-6 is a pro-inflammatory mediator.

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Figure 2: Effect of various treatments on concentration of Interleukin-10 produced by RAW 264.7 macrophages. ‘*’ denotes significantly different from control at p<0.05 using student t-test. ‘#’ denotes significantly different from LPS treatment at p<0.05 using Student’s t-test.

From the results obtained, no significant reduction in IL-6 was observed (Figure 3). This shows that anti-inflammatory property exhibited by S. crispus is not by the reduction of IL-6 but could be acting on other inflammatory mediators. The reason for this might be due to the antagonist action by IL-10 against IL-6. In this part, only treatment using ½MNTD of S. crispus stem slightly reduced the production of IL-6 by 14.5% as compared to the LPS treatment group. Nuclear Factor-κB (NF-κB) is a type of pro-inflammatory transcription factor that regulates expressions of various genes involved in apoptosis, cell proliferation and inflammation. Up-regulations of pro-inflammatory cytokines take place upon stimulation by LPS or any other foreign materials. Flavonoids such as luteolin, apigenin, kaempherol and quercetin have been identified to inhibit the expression of NF-κB and further leads to the inhibition of IL-6 production [25]. Thus, this could possibly explain the ineffectiveness of S. crispus in suppressing the production of IL-6 as the above mentioned flavonoids were found in S. crispus.

Figure 3: Effect of various treatments on concentration of Interleukin-6 produced by RAW 264.7 macrophages. ‘*’ denotes significantly different from LPS treatment at p<0.05 using Student’s t-test.

IV. CONCLUSION

Based on the present experimental model, it can be concluded that Strobilanthes crispus could possess anti-inflammatory properties on lipopolysaccharide-stimulated RAW 264.7 macrophages through suppression of nitric oxide production while promoting Interleukin-10 production. However, future investigations should be conducted to further confirm the anti-inflammatory effect of S. crispus. Initiation of treatment using plant extract can be done simultaneously with inflammation induction or after inflammation induction. In addition, the anti-inflammatory study of S. crispus can also be done using other solvent extracts such as hexane, chloroform, ethanol and water as different extracts might contain different compounds. Besides that, investigation of the involvement of other anti-inflammatory mediators such as reactive oxygen species, tumour necrosis factor α, prostaglandin and cyclooxygenase should be done extensively to further verify the anti-inflammatory effect of S. crispus.

ACKNOWLEDGMENT

The author would like to thank International Medical University for the financial support under MBI01/2010(03)/2012.

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