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Investigating Prostaglandin E_2 and Intracellular Oxidative Stress Levels in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages upon Treatment with *Strobilanthes crispus*

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***Abstract*—Background:** Uncontrolled inflammation may cause serious inflammatory diseases if left untreated. Non-steroidal anti-inflammatory drug (NSAIDs) is commonly used to inhibit pro-inflammatory enzymes, thus, reduce inflammation. However, long term administration of NSAIDs leads to various complications. Medicinal plants are getting more attention as it is believed to be more compatible with human body. One of them is a flavonoid-containing medicinal plants, *Strobilanthes crispus* which has been traditionally claimed to possess anti-inflammatory and antioxidant activities. Nevertheless, its anti-inflammatory activities are yet to be scientifically documented. **Objectives:** This study aimed to examine the anti-inflammatory activity of *S. crispus* by investigating its effects on intracellular oxidative stress and prostaglandin E_2 (PGE_2) levels. **Materials and Methods:** In this study, the Maximum Non-toxic Dose (MNTD) of methanol extract of both leaves and stems of *S. crispus* was first determined using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenytetrazolium Bromide (MTT) assay. The effects of *S. crispus* extracts at MNTD and half MNTD (½MNTD) on intracellular ROS as well as PGE_2 levels in 1.0 µg/mL LPS-stimulated RAW 264.7 macrophages were then be measured using DCFH-DA and a competitive enzyme immunoassay kit, respectively. **Results:** The MNTD of leaf extract was determined as 700µg/mL while for stem was as low as 1.4µg/mL. When LPS-stimulated RAW 264.7 macrophages were subjected to the MNTD of *S. crispus* leaf extract, both intracellular ROS and PGE_2 levels were significantly reduced. In contrast, stem extract at both MNTD and ½MNTD did not significantly reduce the PGE_2 level, but significantly increased the intracellular ROS level. **Conclusion:** The methanol leaf extract of *S. crispus* may possess anti-inflammatory properties as it is able to significantly reduce the intracellular ROS and PGE_2 levels of LPS-stimulated cells. Nevertheless, further studies such as investigating the interleukin, nitric oxide and cytokine tumor necrosis factor-α (TNFα) levels has to be conducted to further confirm the anti-inflammatory properties of *S. crispus.*

***Keywords*—**Anti-inflammatory, natural products, prostaglandin E_2, reactive oxygen species.

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I. Introduction

INFLAMMATION is part of our body immune's response which is easily triggered by foreign body or pathogens. To eliminate these stimulating agents, immune system is activated by starting up the inflammatory and healing process. This will up-regulate a series of pro-inflammatory enzymes, cytokines, reactive oxygen/nitrogen species (RO/NS) and signaling protein at the site of infection [1]. Mediators and transcription factors that regulate expression of gene encoding pro-inflammatory cytokine, chemokines, growth factors and inducible enzymes are also produced [2]. Inducible enzyme such as cyclooxygenase-2 (COX-2) is expressed during inflammation by cytokines and bacterial product (lipopolysaccharide), then, prostaglandin will be produced from arachidonic acid metabolism [2]. Prostaglandin is important in contributing to the pain and swelling in inflammation. However, inflammation is not as conservatory as we know, it can become chronic and uncontrolled if prolonged inflammation. Chronic inflammation can be pathophysiological process in leading to serious disease development and progression such as atherosclerosis, rheumatoid arthritis, asthma globally, diabetes and other gastrointestinal diseases [1]. Thus, blocking the COX-2 enzymes can be one of the therapeutic solutions to inhibit further inflammation. Corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) are drugs used to inhibit COX enzymes, but long term drug administration may cause adverse effects that are detrimental to health. For example, low-dose aspirin was associated with an increased risk of upper gastrointestinal bleeding [3].

The search of plant-derived bioactive compounds is getting more attention because it is believed to interfere with inflammatory mediators and its expression, have better compatibility with human body, yet with fewer side effects. Among all the possible bioactive compounds, the flavonoids found in medicinal plants showed excellent anti-inflammatory characteristic in both *in vitro* and *in vivo* studies [4]-[6]. *Strobilanthes crispus* (Acanthaceae family) also known as 'Pecah beling' is a useful Chinese herb native to countries from Madagascar, Malaysia to Indonesia. It is used as diuretic, anti-diabetic and laxatives among traditional practitioners. It has been proven scientifically as antioxidant, anti-diabetic, anti-obesity, anti-fungal, anti-viral and anti-bacterial agent [7].

In addition, *S. crispus* contains flavonoids such as catechin which may act as the general inhibitors of inflammation–induced cell activation [4]. Although with various traditional claims and presence of known bioactive compounds, the research on *S. crispus* has been mainly focused on its anti-cancer properties. The effects of methanolic extract and its regulation on inflammatory mediator's level on normal cell lines are still remained unclear. Thus, this study focused on the effects of methanolic extract of *S. crispus* on the production of main inflammatory mediators in cellular models of inflammation (LPS-stimulated RAW 264.7 cells).

II. Materials and Methods

A. Preparation of Methanol Extracts

Strobilanthes crispus plants were cleaned, separated into leaf and stem parts prior to air dried. A total of 100g of leaves and stems were then weighed separately, ground to fine powder and extracted with 500mL of 95% methanol (Fisher, UK). The powder was soaked and kept in dark condition at room temperature for 3 days. The suspension was then filtered and the filtrate was concentrated using rotary evaporator (Buchi, USA). To prepare the stock solution, 0.0005g of the leaf and stem extracts were dissolved in 60µL and 80µL of dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA), respectively. The extracts were then further diluted to the desired concentrations with the culture medium prior to addition into the cells.

B. Cell Culture

The RAW 264.7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, UK) with 10% Fetal Bovine Serum (FBS) (GIBCO, South America), penicillin (100 units/mL) and streptomycin (100 µg/mL) (GIBCO, South America). The cultures were incubated at 37ºC with 5% CO_2 and allowed to grow until reaching 70%-100%.

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

To determine the MNTD of *S. crispus* extracts, a total of 8 × 10^3 cells/well were seeded into flat bottom 96-well plate (Corning, USA) and incubated in 5% CO_2 at 37°C until it was more than 70% confluent. Then, the filter sterilised extracts (at the concentration of 2500µg/mL for leaf extract and 400µg/mL for stem extract) were two-fold serially diluted several times to obtain the concentrations ranging from 0-2500µg/mL and 0-400 µg/mL for leaf and stem extracts, respectively. The serially diluted extracts were then added into the cells and incubated for 24 hours.

After 24 hours, the cell viability was measured using MTT assay. Firstly, 10µL of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenytetrazolium bromide (MTT) solution was added into each wells. After 4 hours of incubation in dark, the resulting intracellular purple formazan was solubilised by DMSO (Sigma Aldrich, USA). After proper mixing, the absorbance was measured using microplate reader at 570nm with the reference wavelength of 630nm. The effects of various concentrations of extract on cell cytotoxicity (%) were plotted and the maximum non-toxic doses (MNTD) of both extracts were then determined.

D. Cell Treatments

A total of 1×10^5 cells/well were seeded into flat bottom 24-well plate (Corning, USA) and incubated in 5% CO_2 incubator at 37°C until it was at least 70% confluent, after which, the cells were pre-treated with 12 treatment groups as shown in Table I. In this study, 25µM of indomethacin acted as the positive drug control. After 3 hours of pre-treatment, cells were exposed to 1µg/mL LPS for 24 hours. After 24 hours, the intracellular ROS level was measured using DCFH-DA while the prostaglandin E_2 concentration in cell culture supernatant were quantified using Prostaglandins E_2 Express EIA kit (Cayman, USA) according to the manufacturer's instruction.

TABLE I
Treatment Group in Investigating the Anti-Inflammatory of *Stobilanthes crispus* Extracts on LPS-Stimulated RAW 264.7 Cells

Group	Treatments
1	control (untreated cells)
2	1µg/mL LPS only
3	Indomethacin (25µM)
4	Leaf extract at MNTD (700µg/mL) + 1µg/mL LPS
5	Leaf extract at ½MNTD (350µg/mL) + 1µg/mL LPS
6	Stem extract at MNTD (1.4µg/mL) + 1µg/mL LPS
7	Stem extract at ½MNTD (0.7µg/mL) + 1µg/mL LPS
8	Indomethacin (25µM) + 1µg/mL LPS

E. Statistical Analysis

All the experiments were conducted with at least three replicates and three independent experiments. All data are expressed as means ± S.D. Significant differences were examined using Student's *t*-test, in which value of $p<0.05$ was considered significant.

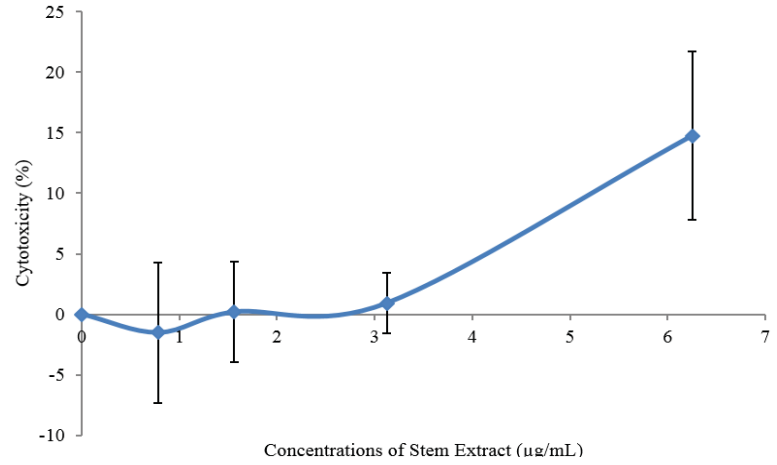
III. Results and Discussion

A. Determination of MNTD

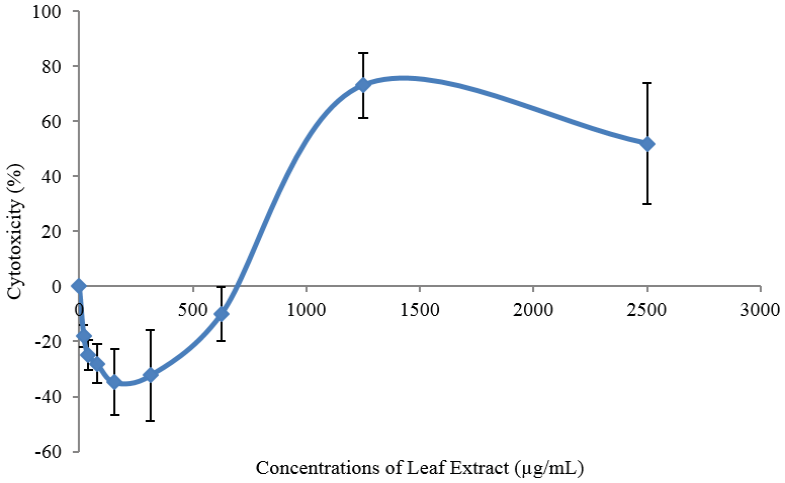
MNTDs were determined to ensure that the methanolic extract did not exhibit cytotoxic response towards RAW 264.7 cells. In fact, previous studies have indicated that methanolic leaf extract of *S. crispus* showed modest cytotoxic effect on certain cancer cell lines such as HepG2 (liver), Caco-2 (colon) and MDA-MB-231 (breast) [8], [9]. As shown in Fig. 1 (a), the MNTD of leaf extract determined in this study was 700 µg/mL while the MNTD of stem extract was significantly lower, which was only 1.4µg/mL (Fig. 1 (b)). Thus, it could be concluded that the methanolic stem extract of *S. crispus* could be more toxic to the corresponding cell line as compared to its leaf counterpart. As a comparison with other studies on anti-inflammatory properties of plants on RAW 264.7 cells, the methanolic plants extracts such as avocado, taro, red turnip, sereves, komatsuna, basil, mitsuba and Chinese mustard exhibited 30% or less with significant cell cytotoxcity (<50%) at a concentration of 200µg/mL [10]. Therefore, current studies indicated that the leaves of *Strobilanthes crispus* could be less toxic and safer to be consumed.

B. Determination of ROS Levels upon Treatments

Fig. 2 shows the relative ROS level per 1×10^4 cells after subjected to different treatments. All the treatments showed a value that was significantly different from the untreated cells that recorded a value of 253.70 ± 30.86 RFU. This value was 50% lower than the cells stimulated with 1µg/mL alone (550.88 ± 73.05 RFU). The presence of ROS in untreated cells is common under cell culture conditions. In fact, oxidative stress can be found in every cell culture and is imposed on every cell which can be probably caused by an increase in oxidant generation, decrease in antioxidant protection, or a repair oxidative damage [11]. The higher level of ROS in LPS-stimulated cells could be contributed by aerobic respiration, peroxisomal β-oxidation of fatty acids, microsomal cytochrome P450, metabolism of xenobiotic compounds, arginine metabolism and tissue specific enzymes [11].



(a)



(b)

Fig. 1 Effects of various concentrations of (a) leaf extract (b) stem extract of *Strobilanthes crispus* on RAW 264.7 cells after incubated *in vitro* for 24 hours. Bar indicates the mean ± standard deviation

Meanwhile, the presence of 25µM indomethacin, which acted as the positive drug control caused a 21.25% of decrease in RFU as opposed to the cells stimulated with LPS alone. Nevertheless, the value recorded was not significantly different from the LPS-stimulated cells. Indomethacin is a non-selective non-steroidal anti-inflammatory drug (NSAIDs) used to reduce inflammation by inhibiting cyclooxygenase enzyme (COX) activity, thereby reduce pain and swelling [12]. However, indomethacin treatment may also cause increasing in depletion of mitochondrial glutathione (antioxidant) content, mitochondrial lipid peroxidation and mitochondrial total protein carbonyl formation, which results in inhibition of mitochondrial respiration, thus, leads to the enhancement of ROS generation [13]. This may explain why indomethacin failed to reduce the ROS levels effectively.

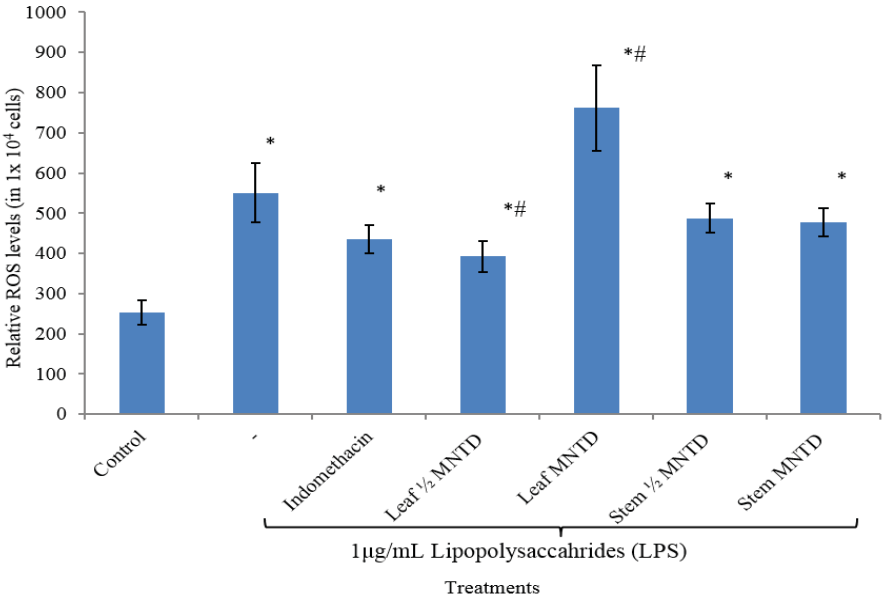


Fig. 2 Intracellular ROS levels in LPS-stimulated cells upon treatment with leaf and stem extracts of *Strobilanthes crispus*. Bars indicate the means ± S.D. '*' indicates that the treatment was significantly different from the untreated cells using student *t*-test at $p<0.05$. '#' denotes the treatment was significantly different from the LPS-stimulated cells using student *t*-test at $p<0.05$

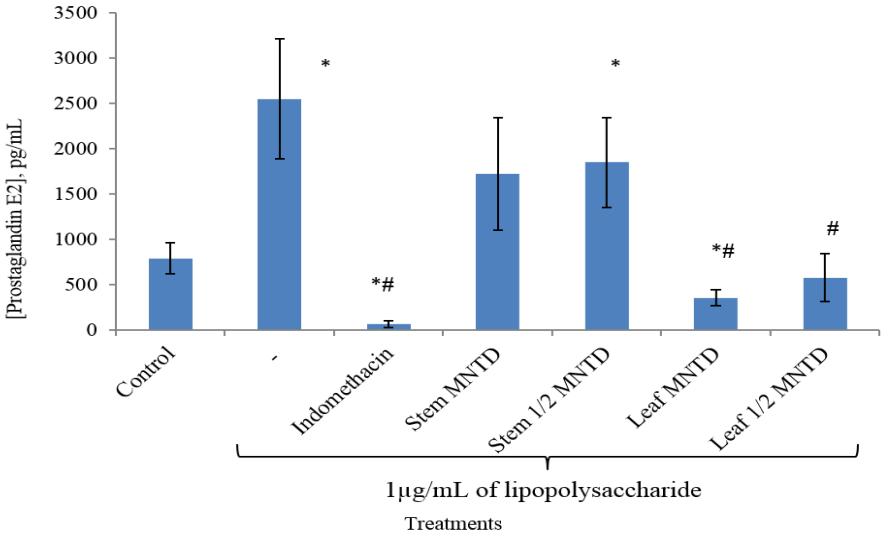


Fig. 3 The concentration of prostaglandin E_2 in LPS-stimulated cells upon treatment with leaf and stem extracts of *Strobilanthes crispus*. Bars indicate the means ± S.D. '*' indicates that the treatment was significantly different from the untreated cells using student *t*-test at $p<0.05$. '#' denotes the treatment was significantly different from the LPS-stimulated cells using student *t*-test at $p<0.05$

Studies showed that the leaf extracts of *Strobilanthes crispus* particularly at ½MNTD was capable in reducing the oxidative stress in LPS stimulated cells. As illustrated in Fig. 2, treatments with ½ MNTD of leaf extract gave a significant RFU of 392.63 ± 38.69 with low cell cytotoxicity (14.26±1.92%) as compared to both untreated cells and LPS-stimulated cells. This could be due to the fact that *S. crispus* contains flavonoids such as catechins, alkaloids, caffeine and tannin; antioxidant phytosterols such as α-sitosterol, campesterol, phytol and stigmasterol, which contributed to the total antioxidant activity [8], [14]. Studies have shown that flavonoid functions effectively on mediating ROS-induced signaling cascades by regulating the activities of different protein kinases [15]. For example, quercetin (one of the flavonoids) can reduce oxidative damage by augmenting the concentration of gluthathione, inhibiting Ca^{2+}-influx which allows cells to survive at low concentration of cytoplasmic Ca^{2+}, thus, blocking Ca^{2+}-channels responsible for cell death [15]. On the other hand, other treatments tested (MNTD of leaf extract, MNTD and ½MNTD of stem extract) recorded a

total RFU of 762.21 ± 106.37, 487.78 ± 35.84 and 477.84 ± 34.63, respectively. The ROS level in the treatment with MNTD of leaf extract increased drastically and significantly as compared to the LPS-stimulated cells by causing the highest cytotoxicity (34.09±9.64%) among all the treatments (Fig. 3). This might be due to the maximum dose of leaf extract which provides high level of flavonoid contents. Studies have shown that excessive flavonoid intake may cause toxic side effects, which flavonoids may act as mutagens and pro-oxidants that generate free radicals [16]. Although the MNTD of leaf extract determined should not cause cell death, long exposure of high dose leaf extract may also cause cell death, thus resulted in high ROS level. As for stem extract, although it managed to reduce the ROS level lower than the LPS-stimulated cells, both treatments were considered less effective as the reduction was not significantly different from the LPS-treated cells. The reason of less effectiveness of stem extract should be further studied as phytochemical profile of stem extract could be different from that of leaf extract.

C. Determination of Prostaglandin E_2 Level upon Treatments

Prostaglandins (PGEs) are small-molecule derivatives of arachidonic acid that is degraded from phospholipids at the site of tissue damage. Arachidonic acid is metabolized into two unstable prostaglandins (prostaglandin G_2 and H_2) and the process is catalysed by cyclooxygenases enzyme (COX) [17]. These prostaglandins are later transformed by isomerases into prostacyclin, thromboxane A_2, prostaglandin D_2, E_2, and $F_{2\alpha}$. [17]. This study focused on the prostaglandin E_2 because of its pyrogenic action and its relationship to pain sensitivity acted on inflammation [18].

Fig. 3 represents the prostaglandin E_2 concentration in treated cells measured by competitive enzyme immunoassay kit. Undoubtedly, the cells stimulated with LPS alone have the highest concentration of prostaglandin E_2 (2552.36 ± 664.02pg/mL) while a low concentration of prostaglandin E_2 (789.51 ± 169.91pg/mL) was detected in the untreated cells. The detection of prostaglandin E_2 even in unstimulated cells could be due to the cellular regeneration or autoimmunity system in the cells [18]. Meanwhile, treatment with indomethacin recorded the least concentration of prostaglandin E_2 (60.87±36.77pg/mL), a value which was even lower than in untreated cells. This could indicate the high efficiency of NSAIDs in treating inflammation and preventing formation of prostaglandin E_2.

As for extracts of *S. crispus*, only treatments with ½MNTD and MNTD of leaf extract showed a lower production of prostaglandin E_2, 353.57 ±91.04pg/mL and 575.12 ±263.15pg/mL, respectively while the prostaglandin E_2 level in cells treated with ½MNTD and MNTD of stem extract was not significantly different from those produced in LPS-stimulated cells (1721.8 ±618.32pg/mL and 1848.22 ±496.41pg/mL, respectively). This indicated that leaf extracts of *S. cirspus* could contain anti-inflammatory compounds as opposed to its stem extract. *Strobilanthes crispus* exhibits anti-inflammatory activity possibly by reducing the expression of inducible COX-2 in cells such as macrophages, which is only present at site of inflammation [17]. When there is less gene expression on COX-2 gene, less COX-2 enzyme is produced and thus fewer prostaglandins E_2 is generated [17].

IV. Conclusion

In this study, the intracellular oxidative stress level and prostaglandin E_2 concentration of LPS-stimulated RAW 264.7 macrophages has been significantly reduced after treated with methanolic leaf extract of *S. crispus*. Contrarily, these similar capabilities were not observed in cells treated with stem extracts. With the presence of stem extracts, the oxidative stress level was relatively high while the concentration of prostaglandin E_2 was not significantly reduced. Apart from that, the chemical compounds that contributed to the reduction in prostaglandin E_2 levels in leaf extract should also be determined. In addition, other pro-inflammatory mediators such as interleukin, nitric oxide and cytokine tumor necrosis factor-α (TNFα) should also be evaluated to further confirm the anti-inflammatory properties of *Strobilanthes crispus.*

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