International Scientific Journal
Journal of Medical and Biological Sciences
http://bioscience.scientific-journal.com

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

Ying Pei Wong*, Jeya Seela A/P Anandha Rao, Anna Pick Kiong Ling, Rhun Yian Koh
Department of Applied Biomedical Science and Biotechnology, School of Health Sciences, International Medical University, Bukit Jalil, 57000 Kuala Lumpur, Malaysia.
*YingPei_Wong@imu.edu.my

***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 significantly reduced while MNTD of stem extract resulted in an increase in IL-10 level. On the other hand, no significant reduction of IL-6 production was seen upon treatment except for indomethacin, which acted as the positive control drug. The present results showed that *S. crispus* could possess anti-inflammatory properties on lipopolysaccharide-stimulated RAW 264.7 macrophages through suppression of NO production and increase in IL-10 level.

***Keywords*—** *inflammation;interleukins;lipopolysacharride; nitric oxide; Strobilanthes crispus*

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 (TNF α) , 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 gastro-duodenal 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].

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 has 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 sufoxide (DMSO) (Sigma Aldrich), Dulbecco's Modified Eagle's Medium (DMEM) (Gibco), foetal bovine serum (FBS) (Gibco), penicilin (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% CO_2, 37°C.

D. Determination of maxiumum 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 10^5 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 maxiumum 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. cirspus* [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 involves directly in LPS-stimulated NO synthesis [29]. This could be due to the suppression of iNOS gene induction by flavanoids such as catechins, epicatechin and quercetin which can scavenge NO [30-31]. These flavonoids are found in *S. crispus*. Meanwhile, an insignificant amount of NO reduction by MNTD and ½ MNTD of *S. crispus* stem extract could be due to the reversible binding of the plant active compound to their molecular targets as plant extract is used as pre-treatment before inflammation is induced [29].

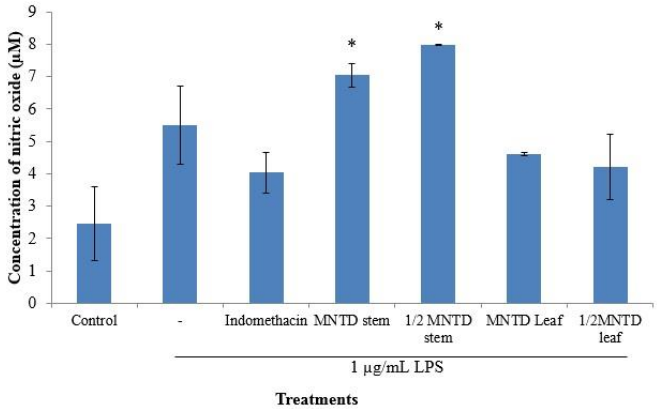

Figure 1: Effect of different treatments on nitric oxide level produced by RAW 264.7 macrophages. '*' denotes significantly different from control at $p<0.05$ using Student's *t*-test.

The anti-inflammatory activity of *S. crispus* was also examined based on its ability in suppressing or promoting 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.

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 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 flavnoids to the cytokine receptors.

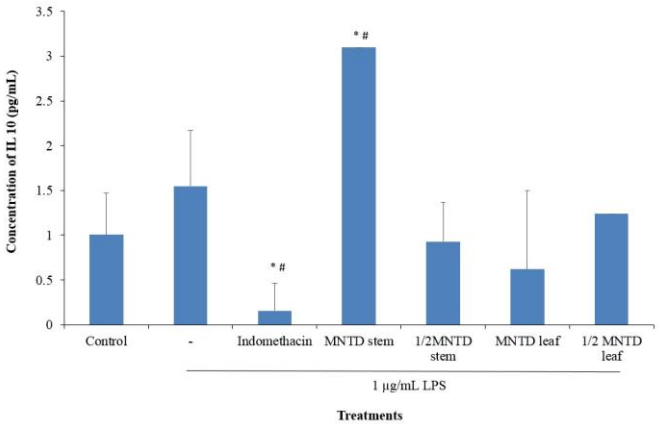

Figure 2: Effect of various treatments on concentration of Interleukin-10 produced by RAW 264.7 macrophages. '*' denotes significantly different from control at $p<0.05$ using student *t*-test. '#' denotes significantly different from LPS treatment at $p<0.05$ using Student's *t*-test

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*.

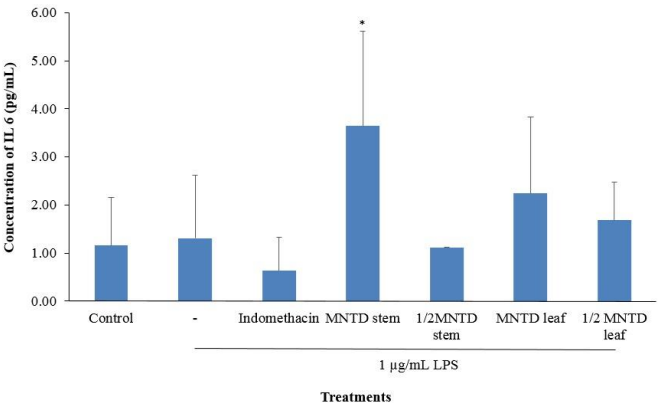

Figure 3: Effect of various treatments on concentration of Interleukin-6 produced by RAW 264.7 macrophages.'#' denotes significantly different from LPS treatment at $p<0.05$ using Student's *t*-test

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

REFERENCES

[1] Neville A.P, Cliff J.W and Ian A: The Journal of Inflammation. *BioMed Central* 2004, 1(1):1-14.
[2] Feghali, C.A, Wright, T.M: Cytokines in acute and chronic inflammation. *Front. Biosci* 1997, 2:12-26.
[3] Inflammation: Acute. 2001. Retrieved 5 April 2013 from http://immuneweb.xxmu.edu.cn/reading/innate/15.pdf
[4] Steven M.O, Vera A.D: Anti-Inflammatory Cytokines. *Chest Journal* 2000, 117(4):1162-1172.
[5] Giuseppe C, Eleonora D and John L. W: Nitric Oxide and Inflammation. *Bentham Science* 2006, 5(2):115-119.
[6] Gil Ã. Polyunsaturated fatty acids and inflammatory diseases: *Biomedicine and Pharmacotherapy* 2002, 56(8):388-396.
[7] Pan MH, Chiou YS, Tsai ML, Ho Ct: Anti-inflammatory activity of traditional Chinese medicinal herbs. *Journal of Traditional and Complementary Medicine* 2011, 1(1): 8-24.
[8] Mueller M, Hobiger S, Jungbauer A.: Anti-inflammatory activity of extrats from fruits, herbs and spices. *Food Chemistry* 2010, 122:987-996.
[9] Kazuhide H, Eiji U, Toshio W, Yukiko Y, Eijiro M, Mitsuyuki M, Satoshi T, Tetsuo A.: Present status and strategy of NSAIDs-induced small bowel injury. *J Gastroenterol* 2009, 44(9):879-888.
[10] Sven T, Stephan R, Simon W, Pius H, Beatrice T, Peter M. V, Matthias E, Peter J: Cardiovascular safety of non-steroidal anti-inflammatory drugs: Network meta-analysis. *BMJ* 2011, 342: 1-11.

[11] Hamid Reza N.P, Perrine B, Odile S, Anick B: Use of nonaspirin nonsteroidal anti-inlammatory drugs during pregnancy and the risk of spontaneous abortion. *Canadian Medical Association Journal* 2011, 183(15):1713-1720.

[12] Ejaz P, Bhojani K, Joshi VR: NSAIDs and Kidney. *Journal of the Association of Physicians of India* 2004, 52:632-640.

[13] Analytical, Bioactivity and Stability Studies On *Strobilanthes*.sp. 2008. Retrieved 1 April 2013 from http://eprints.usm.my/9532/1/ANALYTICAL,_BIOACTIVITY_AND_STABILITY_STUDIES.pdf

[14] Mohd Fadzelly A.B., Asmah R, Fauziah O: Effect of *Strobilanthes crispus* Tea Aqueous Extract on Glucose and Lipid Profile in Normal and Stretozotocin-Induces Hyperglycemic Rats. *Plant Foods for Human Nutrition* 2006, 61:7-12.

[15] Muslim N.S, Ng K.W, Itam A, Nassar Z.D, Ismail Z. and Abdul Majid A.M.S: Evaluation of Cytotoxic, Anti-angiogenic and Antioxidant Properties of Standardized Extracts of *Strobilanthes crispus* Leaves. *International Journal of Pharmacology* 2010, 6(5):591-599.

[16] Mohd Fadzelly Abu Bakar , Arnida Hani Teh , Asmah Rahmat , Fauziah Othman ,Normah Hashim and Sharida Fakurazi: Antiproliferative Properties and Antioxidant Activity of Various Types of *Strobilanthes crispus* Tea. *International Journal of Cancer Research* 2006, 2: 152-158.

[17] Liza M.S, Abdul Rahman R., Mandana B, Jinap S, Rahmat A, Zaidul I.S.M, Hamid A: Supercritical carbon dioxide extraction of bioactive flavonoid from *Strobilanthes crispus* (Pecah Kaca). *ScienceDirect* 2012, 88: 319-326.

[18] Al-Henhena N, Mahmood A. A, Al-magrami A, A Nor Syuhada. B, Zahra A. A., Summaya M. D, Suzi M. S. and Salmah I: Histological study of wound healing potential by ethanol leaf extract of *Strobilanthes crispus* in rats. *Journal of Medicinal Plants Research* 2011, 5(16):3660-3666.

[19] Miean KH, Mohamed S: Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants. *J Agric Food Chem* 2001,49(6):3106-12.

[20] Pusztai R, Beladi I and Bakai M: Study of the effect of flavonoids and related substances I. The effect of Quercetin on different virus. *Acta Microbiol Acad Scientiarum Hungaricae* 1968, 13:113-118.

[21] Profiling Flavonoid Cytotoxicity in Human Breast Cancer Cell Lines. 2012. Retrieved on 10 April 2013 from http://www.academia.edu/1958482/Profiling_Flavonoid_Cytotoxicity_in_Human_Breast_Cancer_Cell_Lines

[22] Tapas AR, Sakarkar DM and Kakde RB: Flavonoids as Nutraceuticals: A Review. *Tropical journal of Pharmaceutical Research* 2008, 7(3): 1089-1099.

[23] McDonald C, Inohara N and Nunez G: Peptidoglycan Signaling in Innate Immunity and Inflammatory Disease. *The Journal of Biological Chemistry* 2005, 280: 20177-20180.

[24] Kang HO, Kim SB, Joung DK, Mun SH, Seo YS, Kim MR, Shin DW, Choi HG, Kweon KT and Kwon DY: Anti-inflammatory effect of *Papufussiella kuromo* in lipopolysaccharide or peptidoglycan-induced macrophages cells. *African journal of Pharmacy and Pharmacology* 2012, 6(40): 2791-2797.

[25] Koh YJ, Cha DS, Ko JS, Park HJ and Choi HD: Anti-inflammatory Effect of *Taraxacum officinale* Leaves on Lipopolysaccharide-Induced Inflammatory Responses in RAW 264.7 Cells. *Journal of Medicinal Food* 2010,13(4):870-878.

[26] Amin AR, Vyas P, Attur M, Piziak JL, Patel IN, Weissmann G and Abramson SB: The mode of action of aspirin-like drugs: Effect on inducible nitric oxide synthase. *Proc. Natl. Acad. Sci* 1995, 92: 7926-2930.

[27] Reeves KJ, Reed MW, Brown NJ. *Is nitric oxide important in photodynamic therapy*? *Journal of Photochemistry and Photobiology* 2009, 95(3):141-147.

[28] Bai JY, Liu BH, Zhao DY, Cheng GF: Effect of indomethacin on expression of interleukin-6 caused by lipopolysaccharide in rheumatoid arthritic patients' synoviocyte. *Yao Xue Xue Bao* 2003, 38(11):809-12.

[29] David R. Burk1, Zbigniew A. Cichacz and Sasha M. Daskalova: Aqueous extract of *Achillea millefolium* L. (Asteraceae) inflorescences suppresses lipopolysaccharide-induced inflammatory responses in RAW 264.7 murine macrophages. *Journal of Medicinal Plants Research* 2010, 4(3): 225-234.

[30] Selvakkumar C, Gayathri B, Sanathkumar KV, Subhadra Lakshmi B and Balakrishnan: Potential Anti-inflammatory properties of Crude Alcoholic Extract of *Ocimim basilicum L*. in Human Peripheral Blood Mononuclear Cells. *Journal of Health Science* 2007, 53(4):500-507.

[31] Sutherland BA, Rosanna M.A, Appleton I: Mechanisms of action of green tea catechins, with a focus on ischemia-induced neurodegeneration. *Journal of Nutritional Biochemistry* 2006, 17 :291–306.

[32] Gomez CR, Karavitis J, Palmer JL, Faunce DE, Ramirez L, Nomellini V and Kovacs EJ: Interleukin-6 Contributes to Age-Related Alteration of Cytokine Production by Macrophages. *Mediators of Inflammation* 2010, 2010:1-7