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Anti-inflammatory Activity of Sacha Inchi (*Plukenetia volubilis* L.) Oil in LPS-Stimulated RAW 264.7 Cells

Narumes Suppasawat[1], Narawat Nuamnaichati[2], Supachoke Mangmool[2], Vimol Srisukh[1], Pattamapan Lomarat[1,*]

[1] *Department of Food Chemistry, Faculty of Pharmacy, Mahidol University, Sri-Ayuthaya Road, Rajathevi, Bangkok Bangkok, Thailand*

[2] *Department of Pharmacology, Faculty of Pharmacy, Mahidol University, Sri-Ayuthaya Road, Rajathevi, Bangkok Bangkok, Thailand*

* Corresponding author: Tel. +66(0)26448704 ext. 5729; E-mail address: pattamapan.lom@mahidol.ac.th

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Introduction

Nowadays, people consume a lot of unhealthy diet which causes nutritional imbalance leading to chronic diseases. Large amounts of omega-6 fatty acids (linoleic acid, LA, 18: 2n-6) [1] that produce pro-inflammatory metabolic products contributed to the formation of thrombus, atheroma and inflammatory disorders. On the other hand, omega-3 fatty acids (alpha-linolenic acid, ALA, 18:3n-3) play an important role in the prevention and treatment of coronary artery disease, diabetes, other inflammatory diseases, autoimmune disorders and cancer. Omega-3 fatty acids also reduce the production of omega-6 fatty acids.[2] The imbalance ratio of omega-6 to omega-3 fatty acids may lead to inflammatory diseases including cardiovascular and neuronal diseases and obesity.[3] Several sources of information suggest that a diet with a ratio of omega-6 to omega-3 fatty acids of approximately 1 is recomomended for an average adult[4], whereas in Western diets the ratio is 15/1–16.7/1.[5] Moreover, the mediterranean-inspired diets (MIDs) which contain fatty fish and flax seed are reported that fatty acids and mediators derived from them are important for the inflammatory process.[6] However, increasing the intake of oils rich in omega-3 fatty acids such as canola, flaxseed, chia seed and olive oils which are low in omega-6 fatty acids are recommended.[7] Sacha Inchi (*Plukenetia volubilis* L.) seed, a native plant of the Amazon rainforest, has been used traditionally by the Peruvian Andes to provide nutrients and as a remedy since the ancient time.[8] The Sacha Inchi seed contain higher fat composition (~42%) than other parts, along with high carbohydrate (~30%) and protein (~24%) which has been reported to possess anti-inflammatory activity.[9,10] Moreover, the oil from ripe seeds showed good fatty acid profile, especially poly-unsaturated fatty acid (83%) which comprised of LA (35%) and ALA (48%).[11]

Macrophage cells play an important role in inflammatory diseases through the release of factors that are involved in the inflammation response such as reactive oxygen species (ROS) and inflammatory mediators including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β).[12]

The aim of this work is to investigate the anti-inflammatory activity of Sacha Inchi oil (SIO) that cultivated in Thailand by using the RT-qPCR method. The level of mRNA expressions of inflammatory mediators (COX-2, iNOS, TNF-α and TGF-β) in lipopolysaccharide (LPS)-stimulated mouse macrophage RAW 264.7 cells were analyzed.[13]

Materials and Methods

Chemicals and reagents AR grade methanol (Burdick & Jackson, MI, USA), Tween 20 (Merck, Germany) and DMSO (Fisher Scientific) was used for all experiments. The cell culture media including Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from Gibco (USA). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (St. Louis, MO).

Plant Material Sacha Inchi (*Plukenetia volubilis* L.) seeds were collected from Nakhon Ratchasima, Thailand. Voucher specimens were deposited at Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University. The Sacha Inchi oil was extracted with hexane by oil extractor (SER 148/6 Series, VELP Scientifica, Italy) and kept in the -20°C freezer for further analysis.

Cell culture Mouse macrophage RAW 264.7 cells were provided by Assoc. Prof. Dr. Supachoke Mangmool, Department of Pharmacology, Faculty of Pharmacy, Mahidol University.

Cell viability testing The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium reduction assay was the modified method that was used to investigate cell viability. Mouse macrophage RAW 264.7 cells (1×10^4 cells/well) were cultured in 96-well plate with 200 µL of 1% FBS, 1% penicillin-streptomycin in DMEM and incubated in incubator (37°C, 5% CO_2) for 24 hours. The mixture containing, 72% of 50% methanol, 20% tween and 8% DMSO was used to dissolve the oil. The cells were treated with either vehicle or SIO solution in various concentrations (0.5, 1, 5, 10, 50, 100, 200 and 500 µg/mL; each condition was done in triplicate) and incubated for 24 hours. After treatment, the medium was removed and replaced with MTT solution. MTT was dissolved in DMEM at 1 mg/mL. Two hundred microliters of MTT solution was then added into each well and incubated for 4 hours. After that MTT was removed. One hundred microliters of DMSO was added to each well to dissolve formazan crystals and mixed to ensure complete solubilization. The absorbance was measured at 570 nm by UV spectrophotometer microplate reader (Tecan Infinite M200, Tecan, Austria). The effect of cell viability in macrophage RAW 264.7 cells was calculated using the following equation.[13, 14]

$$\% \text{ Cell viability} = (\text{Absorbance of treated cell} / \text{Absorbance of control cell}) \times 100$$

Treatment and cell induction by LPS First, the mouse macrophage RAW 264.7 cells (1×10^5 cells/well) were cultured in 6-well plate with 1500 µL of 1% FBS and 1% penicillin-streptomycin in DMEM and then incubated for 24 hours. The cell culture was treated with 0.005 mg/mL of oil solution and incubated for 3 hours. After that, 5 µL of 1 mg/mL of LPS was added into the untreated and treated cell and incubated for 6 hours. The culture medium was removed and washed with PBS. The mRNA of cell was extracted by the protocol of GeneJET RNA Purification Kit (Thermo Scientific, Lituania). The mRNA samples were kept in the -80°C freezer (Thermo Fisher Scientific, USA). The gene expression of cell was detected by using KAPA SYBR FAST One-Step qRT-PCR Kit (KAPA biosystems, USA) and analyzed by Mx 3005P Real Time PCR system (Agilent Technologies, USA).[13]

Results

Cell viability testing

After treatment of vehicles and samples in various concentrations, the absorbance was measured by UV spectrophotometer microplate reader (Tecan Infinite M200, Tecan, Austria) and calculated. The results showed that the concentrations of SIO at 0.1 and less than 0.1 mg/mL had % cell viability more than 80% and the concentrations of SIO more than 0.1 mg/mL showed the cytotoxicity both in vehicles (controls) and samples (Figure 1). Therefore, the highest concentration of SIO used in the next experiment was 0.1 mg/mL or lower than 0.1 mg/mL.

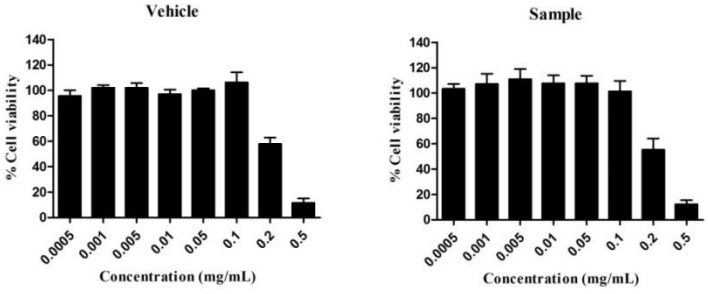

Figure 1 Cell viability of SIO on mouse macrophage RAW 264.7 cells. Cells were treated with SIO at various concentrations (0.0005-0.5 mg/mL) for 24 hours. Cell viability was measured and calculated as %cell viability. Both vehicles and samples were shown as the mean ± SEM (n=3).

The mRNA expressions of inflammatory mediators measurement

After the cell viability testing, 0.005 mg/mL concentration of oil solution was used for the mRNA expression of inflammatory mediators testing on mouse macrophage RAW 264.7 cells. The cells were treated with SIO solution for 3 hours and then stimulated with 1 µg/mL of LPS for 6 hours. After that the cells were extracted to obtain the mRNA. The mRNA expressions of inflammatory mediators of SIO were analyzed by gene specific primers and determined by using RT-qPCR. The results of the mRNA expression of inflammatory mediators (COX-2, iNOS, TNF-α and TGF-β1) were shown in Figure 2 (A-D, respectively). The mRNA expression of inflammatory mediators of cells induced with LPS was increased compared with control. Treatment of SIO (5 µg/mL) on LPS-induced RAW 264.7 cells demonstrated that the mRNA expression of inflammatory mediators including to COX-2, iNOS, TNF-α and TGF-β1 tended to be decreased compared with untreated sample. Especially, COX-2 and TNF-α mRNA expression were significantly decreased in LPS-induced RAW 264.7 cells (Figure 2C).

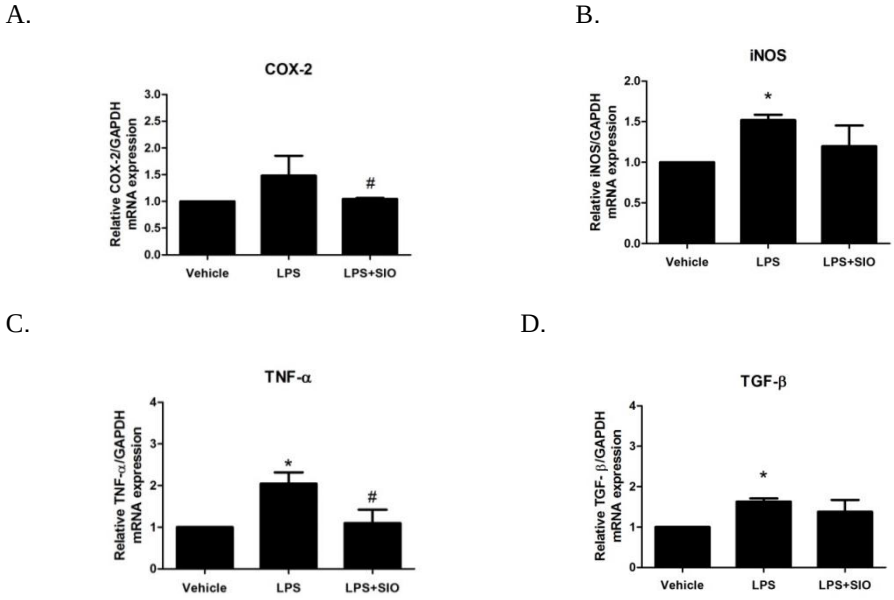

Figure 2 mRNA expressions of inflammatory mediators of SIO on LPS-induced mouse macrophage RAW 264.7 cells. Cells were treated with SIO (5 µg/mL) for 3 hours and then stimulated with LPS (1 µg/mL) for 6 hours. After that the mRNA were extracted from cell and was analyzed by gene specific primers and determined by using RT-qPCR. The level of mRNA expressions of inflammatory mediators including to COX-2, iNOS, TNF-α and TGF-β1 were shown as the mean ± SEM (n=3). **P*<0.05 vs. control (vehicle); #*P*<0.05 vs. LPS

Discussion

In this study, the mixture containing 72% of 50% methanol, 20% tween and 8% DMSO was used to dissolve SIO. Thus, this mixture was used in cell viability testing as the vehicle (control) and sample in various concentrations to investigate the concentration that was toxic to RAW 264.7cells using MTT assay. The results showed that the concentrations of both vehicle and sample at 0.0005-0.1 mg/mL did not produce toxicity to the cells. Therefore, the concentration used in the next experiment was 5 µg/mL. The mRNA expressions of inflammatory mediators measurement was the next experiment performed to investigate the anti-inflammatory activities of SIO. After the treatment on LPS-induced RAW 264.7 cells, the results showed that the level of mRNA expressions of inflammatory mediators (COX-2, iNOS, TNF-α and TGF-β1) tended to be decreased compared with untreated. A significant decrease in TNF-α mRNA expression of SIO in this study was in accordance with the result from previous study in which SIO decreased LPS-induced expression of TNF-α (0.72-fold) in human endothelial vein cells (HUVEC).[15] In addition, a study on toxicity of SIO in mice revealed that SIO was nontoxic and exhibited significant anti-inflammatory effect.[16] Moreover, the active compounds that showed the anti-inflammatory activities could be good fatty acids in SIO, especially omega-3 fatty acids.[11] There were strong evidences indicated that omega-3 fatty acids was beneficial as a dietary supplement in chronic inflammatory diseases such as rheumatoid arthritis, asthma and cardiovascular disease.[17] Moreover, flax seed oil, the vegetable-based oils which was rich in ALA and has the ratio of omega-6 to omega-3 fatty acids (0.3)[18] showed a decrease in TNF-α protein expression in animals fed with fish oil and flax seed oil enriched diets compared to control.[19] Interestingly, SIO also has the suitable ratio of omega-6

to omega-3 fatty acids of approximately 1 which led to the decrease in the amounts of omega-6 fatty acids products such as arachidonic acid (AA, 20:4n-6), which are pro-inflammatory mediator. [4, 20]

Conclusion

This study demonstrated that SIO that cultivated in Thailand exhibited the anti-inflammatory activities on LPS-induced mouse macrophage RAW 264.7 cells by suppressing the level of mRNA expressions of inflammatory mediators including to COX-2, iNOS, TNF-α and TGF-β. Thus, SIO has potential for inhibition of the inflammatory process. However, further studies such as preclinical and clinical studies on SIO are needed to prove it's anti-inflammatory activities. In addition, it is interesting to develop novel functional foods from SIO which could be an alternative to edible oil products commercially available in the market.

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