ฤทธิ์ต้านการอักเสบของสารสกัดจากเถาวัลย์เปรียง

ANTI-INFLAMMATORY ACTIVITY OF *Derris scandens* Benth. EXTRACT

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ฤทธิ์ต้านการอักเสบของสารสกัดจากเถาวัลย์เปรียง

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ANTI-INFLAMMATORY ACTIVITY OF *Derris scandens* Benth. EXTRACT

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BY

KANLAYANEE SRIKLUNG

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The dried stem of *Derris scandens* Benth. (Leguminosae) is well known as an Asian herbal medicinal plant used to treat a variety of ailments. The *Derris scandens* ethanol extract has been found to have both antioxidant and anti-cancer properties. However, the anti-inflammatory activity and the molecular mechanism of *Derris scandens* extract is still unknown. The study was initiated by MTT assay to define the non-cytotoxic in a Raw 264.7 macrophages cell. Lipopolysaccharide (LPS) was used to induce cell inflammation. The *Derris scandens* extract in sunflower oil, light mineral oil and rice bran oil at 50 μg/mL and 100 μg/mL were used for this study. The results showed that at 100 μg/mL of *Derris scandens* in sunflower oil (DSF), *Derris scandens* mineral oil (DSL) and *Derris scandens* rice bran oil (DSR) effectively inhibited nitric oxide production. In addition, 1.25 μM and 2.5 μM of lupalbigenin, a pure compound from *Derris scandens* ethanol extract showed anti-inflammatory properties by down-regulated iNOS, IL-6 and TNF-α gene expression by Real-Time RT-PCR and also decreased COX-2, iNOS and TNF-α protein expression by western blot analysis. Moreover, lupalbigenin also showed significantly decreased phosphorylation of p65 in NF-kB translocation and exerted p38 and JNK phosphorylation MAPKs pathway. This finding indicated that *Derris scandens* in sunflower oil (DSF), *Derris scandens* in mineral oil (DSL), *Derris scandens* rice bran oil (DSR) and lupalbigenin could be used as anti-inflammatory agents.

Keyword : Derris scandens extract, anti-inflammatory, lupalbigenin, NF-kB translocation

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Kanlayanee Sriklung

KANLAYANEE SRIKLUNG

F

TABLE OF CONTENTS

Page

LIST OF TABLE

Page

LIST OF FIGURE

Page

CHAPTER 1

INTRODUCTION

Derris Sandens Benth. or *Tao-Wan-Priang,* it belongs to the family *Leguminosae* and well known as a Thai medicinal plant. It is used for various treatments such as expectorant, antitussive, diuretic, and anti-dysentery agents and the treatment of muscle aches and pains Its dried stem in the form of the hydroalcoholic extract was reported to have both antimicrobial[1] and immunostimulating activities[2]. In a pharmacological study, the polar fractional applied, resulted in a marked decrease in blood pressure and heart rate[3]. Coumarins, isoflavones, and isoflavone glycosides have also been previously reported as chemical constituents of the stems of *D. Scandens* [4-11]. The isolated flavonoids from leaf and root extracts of *D. Scandens* exhibited significant anti-inflammatory activity in Rat when compared to the standard drug[12]. *Derris Scandens Benth.* the extract is the one of Thai famous herbal which general using as traditional medicine, however, the biological molecular cascade and cellular signaling pathway of anti-inflammatory have not been reported.

Objective

This study was created by a Thai pharmaceutical company who is the partial funded of my Ph.D. scholarship. The company aims to investigate whether *Derris Scandens* extract in three different formative solvents which are sunflower oil, light mineral oil, and rice bran oil can inhibit inflammatory cellular marker expression and cell signaling pathways. The outcome results may confirm their products for anti-inflammation cream or balm and allow the consumer to be confident to use traditional herbal to treat in the proper product. On the other hand, the company also provides 50% ethanol *D. Scandens* Benth. extract for purifying to get the hits compound and use for anti-inflammatory activity study. Raw 264.7 macrophages cell has been chosen for this experiments and lipopolysaccharide (LPS) will be performing to induce inflammatory cell change. Firstly, the cytotoxicity of *D. Scandens* Benth. extract with Raw 264.7 cell

will be determined by MTT assay to find the non-toxic concentration. And then determine the nitric oxide concentration by Greiss reagent assay. The molecular study will determine the expression of mRNA induction by molecules such as iNOS COX-2 Interleukins by real-time polymerase chain reaction (PCR) and the expression of proteins including COX-2, iNOS, TNF-**α,** and NF-kB by western blotting. Finally, the anti-inflammatory molecular signaling pathway will be determined such as MAPK and NF-**κ**B mechanism. The data may show the anti-inflammatory signaling pathway of *D. Scandens* extract in 3 differences solvent which is DS in sunflower oil, DS in light mineral oil and DS in rice bran oil including the purification compound from *D. Scandens* extract and these finding will be beneficial for developing anti-inflammatory drug without or less side effect in the future.

In addition, we performed the vascular activity study of a prenylated metabolite of genistein, lupalbigenin, is a key component of *Derris scandens*, and we compared the vascular activity of this isoflavone with that of genistein, in porcine splenic artery either short term and over long term period. This experiment can account for the beneficial effects attributed to *Derris scandens* extracts in musculoskeletal conditions.

CHAPTER 2

LITERATURE REVIEW

2.1 Inflammation

The inflammation is the immune body response when the tissue damaged or infection which is related to molecular and cellular signal cascade that can cause physiology responses. There are two types of inflammation divided by timing responses[13, 14].

2.1.1 Acute inflammation

Acute inflammation is a typical response by the short term that occurs before the immune response becomes established. In the area of the injury, the molecular signals have released from cells and causing several changes in the affected area such as vasodilation, increased vascular permeability, increased blood flow. An exudation of fluids containing proteins like antibodies, and invasion by several different types of leukocytes, granulocytes, monocytes, and lymphocytes [15, 16].

2.1.2 Chronic inflammation

Chronic inflammation is a more prolonged duration and the presence of lymphocytes and macrophages, resulting in increased expression of pro-inflammatory mediators which activates inflammatory cells by increasing the expression of pro-inflammatory cytokines, up-regulating genes that produce NF kappa B, NADPH oxidase, phospholipase A_2, *COX-1* and *-2*, 5-LOX, myeloperoxidase, *iNOS*, increasing oxygen consumption and producing many oxygen-free radicals that can finally lead to certain degenerative diseases[17]

2.3 Mediators of inflammation

There are numerous mediators involved in inflammatory responses and have different activities upon a variety of chemical groups include mast cells, neutrophils, eosinophils, macrophages, lymphocytes, endothelial cells, and platelets. These cells react to a variety of signals, stimuli, and irritants to orchestrate the inflammatory response.

2.3.1 Cyclooxygenase and Arachidonics metabolites

Cyclooxygenase (COX) is prostaglandins involved in the inflammatory response and produced by arachidonic acid which is released from the plasma membrane by phospholipases (PLAs) and metabolized by the sequential actions of prostaglandin G/H synthase, or cyclooxygenase (COX), and respective synthases[18]. Prostaglandin production depends on the activity of prostaglandin G/H synthases, COX-1, expressed constitutively in most cells, is the dominant source of prostanoids that subserve housekeeping functions, such as gastric epithelial cytoprotection and homeostasis. COX-2, induced by inflammatory stimuli, hormones and growth factors, is the more important source of prostanoid formation in inflammation and in proliferative diseases, such as cancer [19-21].

2.3.2 Cytokines

Cytokines are the soluble glycoproteins that have the role of the regulated immune response. The anti-inflammatory cytokines are a series of immune regulatory molecules that control the pro-inflammatory cytokine response. Cytokines act in concert with specific cytokine inhibitors and soluble cytokine receptors to regulate the human immune response. Specific cytokine receptors for IL-1, IL-6, and TNF-α [22-24].

2.3.3 Nitric oxide

NO is an important cell-signaling messenger in a wide range of physiological and pathophysiologic processes. Small amounts of NO play a role in maintaining resting vascular tone, vasodilation, and anti-aggregation of platelets. In response to cytokines

(TNF-α, IL-1) and other inflammatory mediators, the production of relatively large quantities of NO is stimulated. In larger quantities, NO is a potent vasodilator, facilitates macrophage-induced cytotoxicity, and may contribute to joint destruction in some types of arthritis[25].

2.4 Nuclear factor-κB (NF-κB) in the inflammatory response signaling pathway

Activation of the NF-κB proteins plays a central role in inflammation through the regulation of genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes such as cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS)[26]. This pathway is activated upon appropriate extracellular stimulation, most often by stress or pro-inflammatory cytokines, including TNF and IL-1, and by pathogens such as bacterial components including lipopolysaccharide (LPS) through the Toll-like receptors [27-29].

2.5 MAP kinase pathway and inflammation

MAP kinase is a type of transcription factor in the cytoplasm and nuclease that can activate cell signaling by phosphorylation to induced gene and protein expression. There are three major groups The activation of MAP kinases cascades which are known in humans that lead to altered gene expression [30-32]. The ERK signaling module was the first MAP kinase cascade to be characterized, is a vital mediator of a number of cellular fates including growth, proliferation, and survival [33, 34]. The c-Jun NH2-terminal kinases (JNKs) signaling pathway has been implicated in a large variety of pathological conditions, including cancer, stroke, ischemic heart disease, and inflammatory disorders, and has therefore been appreciated as an attractive candidate for drug development [35, 36]. The p38 Mitogen-Activated Protein , the main biological response of p38 activation involves the production and activation of inflammatory mediators to initiate leucocyte recruitment and activation. Furthermore, it was demonstrated that LPS stimulation of neutrophils resulted in p38 activation, leading to cell adhesion and regulation of TNF-α synthesis. Also, p38 can regulate TNF-α induced expression of

vascular cell adhesion molecule in endothelial cells and chemoattractants such as N-formyl-methionylleucyl-phenylalanine, platelet-activating factor, and TGF-b can induce neutrophil chemotaxis through p38 activation [37-40].

2.6 Vascular smooth muscle cells

Vascular smooth muscle cells (VSMCs) is a main structural component of the vessel wall and play a key role in maintaining the vascular structure and facilitate contractile [41]. Smooth muscle cells can be typically found in cardiac and skeletal muscle and controlled by the autonomic nervous system. Smooth muscle cells also develop tonic and phasic contractions in response to changes in load or length. For the stimuli, smooth muscle cells use cross-bridge cycling between actin and myosin to develop force, and calcium ions (Ca^{2+}) serve to initiate contraction[42].

Receptor regulation is associated with vascular smooth muscle contraction, the estrogen receptor is the important one and has several studies about[43]. Some research has been found estrogen receptor α and β are played an important role involving vascular smooth muscle contraction[44, 45].

Phytoestrogens are found in natural plant compounds that are structurally and functionally similar to mammalian estrogens and their active metabolites[46]. Genistein and daidzein are the two most well-characterized isoflavones and human exposure to these compounds occurs primarily through the consumption of soy-based food and beverage products[47]. Some of the research has been reported isoflavone can inhibit vascular smooth muscle contraction via estrogen receptor either α and β, therefore, it can also call phytoestrogens [48]. 17β-oestradiol is one of an estrogen steroid hormone and involved in the regulation of female hormone reproductive cycles which can inhibit VSMC proliferation via cytosolic/nuclear ERs and transcriptional genomic effects[49].

Vascular smooth muscle contraction is associated with increases in calcium ion [Ca^{2+}] upon the amount of Ca^{2+} release from the sarcoplasmic reticulum (SR) and Ca^{2+} flux between extracellular space. Oestradiol mainly inhibits Ca^{2+} influx rather than Ca^{2+} release from the intracellular stores and may also have a direct effect on Ca^{2+} channels

[50, 51]. Phytoestrogens may inhibit VSM contraction by inhibiting Ca^{2+} influx or Ca^{2+} release from the SR by decreasing the Ca^{2+} sensitivity of the contractile apparatus [52]. However, the mechanisms by which phytoestrogens decrease Ca^{2+} may vary in different blood vessels. Some animal phytoestrogen such as genistein can cause vascular relaxation by blocking Ca^{2+} entry[53]. Also, in the porcine coronary artery, genistein and oestradiol cause relaxation of KCl-, 5HT- and $CaCl_2$-induced contractions mainly by inhibiting Ca^{2+} influx, and these effects may not be related to ER or classical genomic activities. Other studies suggest that the vasorelaxant effects of phytoestrogens involve inhibition of intracellular Ca^{2+} release [54]. Phytoestrogens and Vascular Inflammation can be reduced inflamed implicated in atherogenesis, and oestradiol may affect the course of atherosclerosis by inhibiting vascular inflammation[55]. Oestradiol also exerts vascular anti-inflammatory effects and attenuates TNF-α-induced mRNA expression of inflammatory mediators[56, 57]. Genistein protects against inflammatory factor-induced EC dysfunction and inhibits leukocyte-endothelium interaction and decrease TNF-α -induced secretion of monocyte chemotactic protein-1, a cytokine recruiting white blood cells to sites of inflammation[58, 59]. Genistein reduced mRNA expression levels of E-selectin, CAM-1 and P-selectin which are elicited by the proinflammatory bacterial LPS and also inhibits the activity of the key inflammatory enzyme secretory phospholipase A_2 in mice [57, 60].

2.6 *Derris Scandens Benth.* and its bioactivity

Derris Scandens Benth (family : Leguminosae) or the local name is Tao-Wan-Priang , belongs to the family of Leguminosae. A variety of biologically active compounds have been identified from the extract of this plant. The major active constituents of *D. Scandens* are benzyls and isoflavones, including genistein, coumarins, scandinone, scandenin, prenylated isoflavones, and isoflavone glycosidesa woody vine growing throughout Southeast Asia, including Thailand. The main compounds showing intestinal α-glucosidase inhibitory and free radical scavenging activity are scandinone, scadenone, scandenin A, scandenin B, and 4', 5', 7-trihydroxybiprenyl isoflavone. The extract from the stem of this plant has been a long

term used in traditional remedies throughout Southeast Asia. It has been used for the treatment of numerous diseases such as arthritis, muscular pain, diabetes, inflammation, and hypertension. It was reported that *D.scandens* extracts were effective and safe for the treatment of patients with knee osteoarthritis [61-63].

FIGURE 1 Derris Scandens Benth. (Leguminosae)

FIGURE 2 Dried stem of Derris Scandens Benth.

Insecticidal constituents, viz., rotenone and lonchocarpic acid, were reported also from the roots of *D. Scandens*[62]. The use of *D. scandens* as folk medicine and the antimicrobial activity of the crude extract prompt us to investigate phytochemical investigation[63]. Previous studies indicated the presence of coumarins, isoflavones, flavones, isoflavone glycosides and phenyl coumarins as chemical constituents from *D. Scandens*[64]. Extracts using organic solvents have revealed the presence of compounds that appear to modify inflammatory processes. For example, ethanolic has been reported to enhance NK cell activity in peripheral blood mononuclear cells and possess hypotensive activity [65, 66]. Chemical analysis of *D. Scandens* has revealed the presence of numerous isoflavone derivatives, including scandione and scandenal and up to 15 related derivatives. Interestingly, many of these compounds are to know to possess one or two prenyl groups [61].

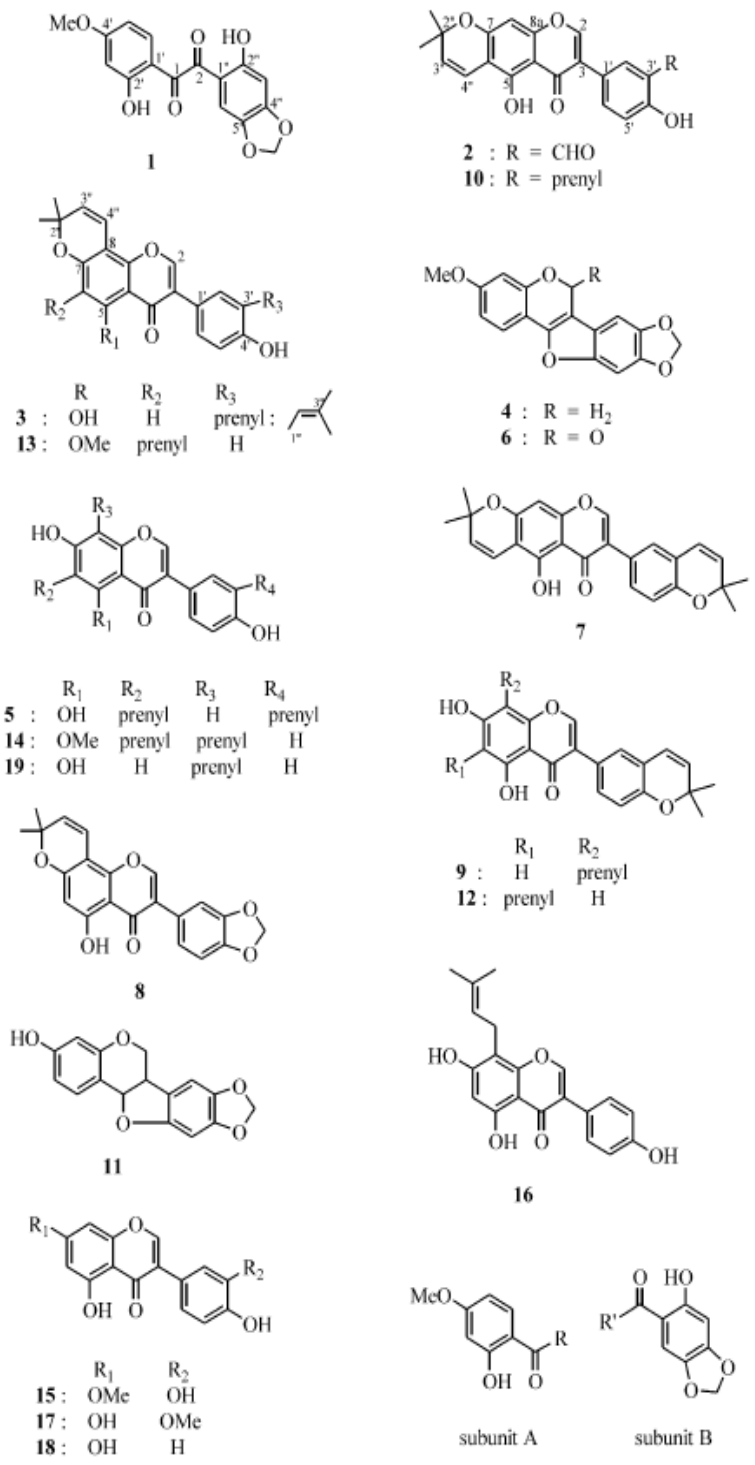


FIGURE 3 Compound, isolated from Derris scandens with different prenyl groups.

In 2010, Mahabusarakam and colleagues had researched a bioactivity-directed investigation of root extract of *Derris Scandens* the result has shown led to the isolation and characterization of a new benzil derivative[61, 62].

Over the past decade, it has become increasingly apparent that the prenylation of flavonoids is impacted upon both the biological activity and duration of action of these molecules[67]. For example, prenylation of the isoflavone genistein has been reported to convert phyto-estrogenic activity of the parent compound into anti-estrogen activity and influence the ability of naringenin to promote bone resorption[68, 69]. Interestingly, some of the compounds reported by Mahabusarakam and colleagues (2003) exhibit marked free radical scavenging activity, antibacterial activity and, uniquely, hypertensive activity when injected into anesthetized rats[62]. The study of anti-cancer activity of *Derris Scandens* extract with Hepatocellular carcinoma (HCC), a primary liver cancer, is one of the most prevalent causes of cancer-associated mortality globally and originates from hepatocyte cells in the liver have been found can affect the cell proliferation of HCC-S102 via an apoptotic pathway[70]. Moreover, Hematulin and colleagues (2012) have been investigated the activity of *Derris Scandens* extract as radiosensitizers to increase the efficacy of radiotherapy. The result shown extract was found to enhance radiation-induced G2/M phase arrest, induce Akt activation, and increase motility of Hep-2 Laryngeal cancer cells[71]. However, the *Derris Scandens* extract on the anti-inflammatory signaling pathway to suppress inflammatory processes induced by Lipopolysaccharide (LPS) in RAW 264.7 macrophages cell has not been reported.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

All chemicals used in this study were analytical grade and were listed alphabetically as follows.

Name of chemicals	Company
Acrylamide	BioRad
Agarose gel	BMA product
Ammonium persulfate	USB
Bovine serum albumin	USB
Bromophenol blue	Sigma
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma
Dimethylsulfoxide (DMSO)	Amresco
Dulbecco's modified Eagle's medium (DMEM)	Gibco BRL
EDTA (Ethylenediaminetetraacetic acid)	Sigma
Fetal bovine serum (FBS)	PAA
Glycerol	Sigma
Glycine	Sigma
Griess reagent	Sigma
HEPES (N-[2-hydroxyethyl] piperazine-N' [2-ethanesulfonic acid]	USB
2-Mercaptoethanol	Sigma
Magnesium chloride ($MgCl_2.6H_2O$)	Sigma
Methanol	Merck
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide)	Sigma
Penicillin/streptomycin	PAA
Potassium chloride (KCl)	Merck

Potassium phosphate (KH_2PO_4)	Merck
Sodium chloride (NaCl)	Merck
Sodium citrate	Biorad
Sodium dodecyl sulfate (SDS)	Merck
Sucrose	Merck
TEMED (N,N,N',N'-Tetramethylethylene diamine)	USB
Tris ([Tris Hydroxymethyl] aminomethane)	Sigma
Triton-x 100	Phamacia
Trypan blue	Sigma
Trypsin	PAA
Tween-20	Phamacia

3.1.2 Solutions and drugs

The composition of K-H solution was (in mM): NaCl, 118; KCl, 4.8; MgSO4.7H2O, 1.2; CaCl2.2H2O, 1.3; NaHCO3, 25.0; KH2PO4, 1.2. Benzyl penicillin, streptomycin sulphate, ficoll, genistein, 17β-estradiol and ICI 182-780 were all obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK). U46619 was obtained from Alexis Coporation (Nottingham, UK). genistein, and ICI 182-780 were dissolved in 100% DMSO at a concentration of 10mM (< 0.1% DMSO in final incubation medium), whereas 17β-estradiol was dissolved in absolute ethanol at a concentration of 10mM, all other drugs were dissolved in distilled water.

3.1.3 Instruments

Name of instrument	**Company**
Centrifuge-HSC 10K	Vant
Centrifuge-MIKRO 20	Hettich
Centrifuge ICE centra-4R	Hettich
CO_2 incubator (water-jacketed)	NuAire
Fluorescence microscope	Olympus
Inverted microscope	Olympus

Lamina flow-Nu-440	NuAire
Western blot apparatus	BioRad
Shaking-Mini Rocker MR-I	Biosan
Microplate reader	BioTek

3.1.4 Cell culture

The Macrophages cell, Raw 264.7, has been obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cell line will have maintained as a monolayer in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The medium will be refreshed every 2-3 days. After about 90% of confluence. Subcultures were prepared by scraping, the cultured cells were detached with a cell scraper and sub-cultured. Cells that were used in the study area in the exponential phase of growth and at passage 2^{nd} to 25^{th} after thawing.

3.1.5 *Derris Scanden* Benth. Crude extractions

Derris Scanden Benth. extracts were obtained from Bangkok Lab and Cosmetic Co., Ltd. Ratchaburi province. The stem was chopped into small pieces. The sample was dried at 60 °C then powdered in a mixer grinder. The extraction process was performed by macerated the dried powder in sunflower oil, light mineral oil, rice bran oil and 50% ethanol for 3 days. The extracts were subsequently filtered, evaporated till dryness under reduced pressure. The plants' extract was kept for further experiments at -20°C.

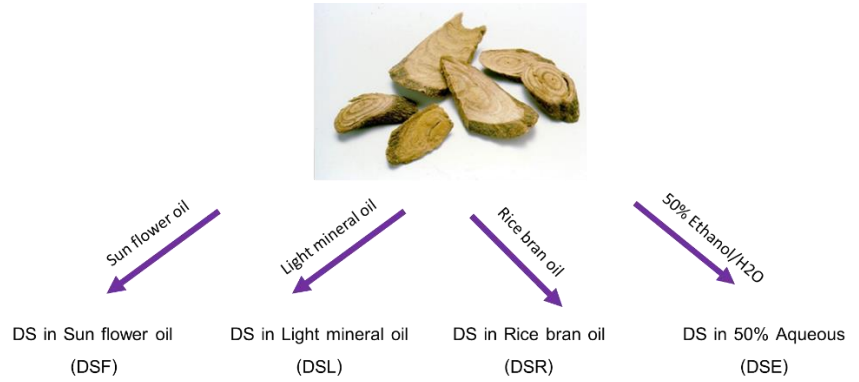


FIGURE 4 Derris scandens in 4 differents extractions, sunflower oil, light mineral oil, rice bran oil and 50% aqueous extracts.

3.2 Methods

3.2.1 *Derris Scanden* Benth. 50% crude extract purification

Two types of chromatography had been choose for this study. Firstly we start from partition chromatography, which is basically understood as a method of separation of solutes utilizing the partition of the solutes between two liquid phases. The individual differences in partition coefficients are the principal to distinguish in which the components present in the mixture get distributed more likely into two liquid phases. In Partition chromatography, the molecules are separated in between two phases i.e. both stationary phase and mobile phase are in the same phase. So molecules get dispersed into either phase preferentially. The partition chromatography is the basic principle involved in many separation techniques like high-performance liquid chromatography and gas chromatography. In this study, we used the paper chromatography works on the principle to separate fractions from *Derris Scanden* Benth. 50% crude extract. Lupalbigenin, purification from 50%Ethanol aqueous stems of *Derris Scandens* Benth.

The column chromatography with a fine adsorbent solid; a solid had been used on the next step of purification which can be able to hold onto liquid particles on its outer surface. The column chromatography looks similar to a Pasteur pipette. The narrow exit of the column is first plugged with glass wool or a porous plate in order to support the column packing material and keep it from escaping the tube. Then the adsorbent solid (usually silica) is tightly packed into the glass tube to make the separating column. The solvent had been used as the mobile phase is passed through the dry column to finish preparing the column. Once the column is correctly prepared, the sample to be separated is placed at the top of the wet column.

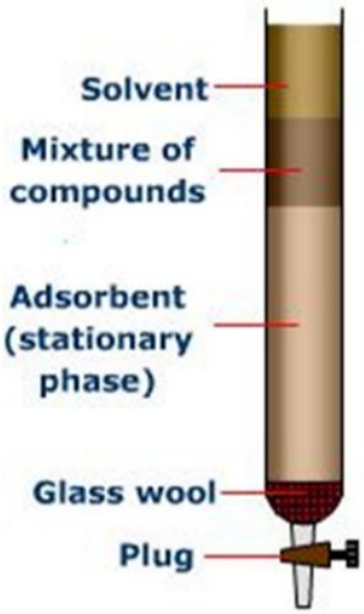


FIGURE 5 Adsorption Column Chromatography

3.2.2 Cell viability assay

Cells were seeded at a density of 1×10^4 cells/well in a 96-well plate and allowed to grow for 24 h. Cells will be then treated with compound at various concentrations whereas the control group was treated with DMSO. After incubation for 24 h, 100 µl of 0.5 mg/ml MTT solution were added to each well and the plate was further incubated for 2 h at 37°C. The supernatant was aspirated and 100 µL of DMSO will be added to each well to solubilize water-insoluble purple formazan crystals. The absorbance at 570 nm will be measured using a microplate reader. (BioTek,USA) and the inhibitory concentration 50% (IC_{50}) value will be calculated using GraphPad Prism 3.03 (GraphPad Software Inc., San Diego, CA).

3.2.3 Detection of morphology change

About 100,000 Raw cells will be plated in 6-well plate (Nunc,USA), Cell were pre-treated with *DS* extracts, solvents, and compounds for 1 h and then incubated with 1 µg/mL of LPS for 18 h. The morphological changes in the RAW cells will be observed by using phase-contrast microscopy (Olympus, USA).

3.2.4 DPPH assay

DPPH is a common abbreviation for an organic chemical compound 2,2-diphenyl-1-picrylhydrazyl. It is a dark-colored crystalline powder composed of stable free-radical molecules. DPPH has two major applications, one is a monitor of chemical reactions involving radicals, most notably it is a common antioxidant assay and another is a standard of the position and intensity of electron paramagnetic resonance signals

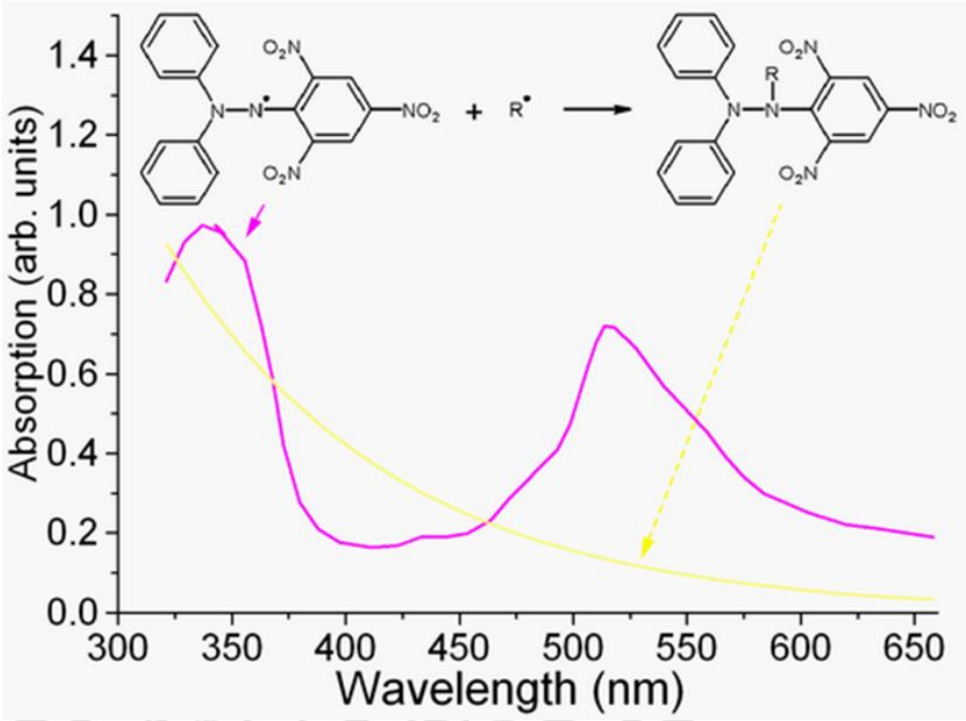


FIGURE 6 DPPH reaction represented by absorption vs wavelength

3.2.5 Nitrite determination

RAW 264.7 cells (10^5 cells/well) were seeded in 24-well plates and allowed to adhere overnight. Cell were pre-treated with *DS* extracts, solvents, and compounds for 1 h and then incubated with 1 μg/mL of LPS for 24 h. The cell-free culture medium was collected and100 μL were used for NO determination. The nitrite accumulated in the culture medium was measured as an indicator of NO production based on the Griess reaction. Briefly, 100 μL cell culture medium will be mixed with an equal volume of the Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamide- HCl), incubated at room temperature for 15 min, and the absorbance was measured at 540 nm using an ELISA microplate reader (Nunc, USA). The amount of nitrite present in the samples was calculated by means of a standard curve generated using serial dilutions of $NaNO_2$ in the fresh culture medium.

3.2.6 RNA extraction and cDNA synthesis

The cells were seeded in 6-well plates (5.0×10^5 cells/well) and incubated with or without DS extract (100 μg/ml and 50 μg/mL) prior to LPS stimulation (1 μg/mL). After 24 h, total RNA was isolated from the cells using the TRIzol® Reagent (Thermo Fisher Scientific, USA). Accurate nucleic acid quantification is essential for gene expression analysis, especially when total RNA amounts are used to normalize target gene expression. The RNA concentration and purity are commonly determined by measuring the ratio of UV absorbance at 260 nm and 280 nm. With NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). One microgram of RNA will be reversed transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) to obtain cDNA according to the manufacturer's protocol of T100 ™ Thermal Cycler (Bio-Rad, USA)

3.2.7 Real-time PCR

Real-time PCR was performed with CFX96 Touch Real-Time PCR Detection System (Bio-rad, USA) using Power SYBR® Green PCR Master Mix (Bio-rad, USA) according to the manufacturer's protocol. Briefly, PCR was performed in a final volume of 20 μL including 10 ng sample cDNA, 5 μM specific forward and reverse primers, and

10 μL Power SYBR® Green PCR Master Mix. PCR reactions consisted of an initial denaturing cycle at 95°C for 10 min, followed by 40 amplification cycles of 15 s at 95°C and 1 min at 60°C. The primers will be purchased from Macrogen (Seoul, Korea). The expression levels of TNF-α, iNOS, and IL-6 will be normalized using β-actin as an internal control. The analysis will be carried out in triplicate. The primers for iNOS, COX-2, TNF-α, IL-6 and β-active were used as shown in TABLE 1

TABLE 1. The primers used for RT-PCR analysis

	Sense Primer Sequence 5'-3	Antisense Primer Sequence5'-3
TNF-α	GCGACGTGGAACTGGCAGAA	CAGTAGACAGAAGAGCGTGGTG
IL-6	GTTGCCTTCTTGGGACTGAT	CATTTCCACGATTTCCCAGA
iNOS	TGGAGCGAGTTGTGGATTGT	CTCTGCCTATCCGTCTCGTC
β-actin	TGCTGTCCCTGTATGCCTCTG	GCTGTAGCCACGCTCGGTCA

3.2.8 Western blot analysis

Western blot is a powerful technique for identifying specific proteins in the given sample of tissue homogenate or extract. It uses sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate proteins according to their molecular weight. SDS binds to hydrophobic portions of a protein, disrupting its folded structure and allowing it to exist stably in solution in an extended conformation. As a result, the length of the SDS-protein complex is proportional to its molecular weight. The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are probed using an antibody (primary antibody) specific to the target protein. To detect the antibody-antigen complex, a secondary antibody tagged with a biochemically detectable marker is used. In this work, the enhanced chemiluminescence system (ECL) was used of which secondary antibody is conjugated with horseradish-peroxidase. The band on the membrane was visualized by loaded with ECL reagent results in the chemiluminescence signal that can be captured on the film.

1) Sample preparation

To prepare samples for gel electrophoresis, cells need to be lysed to release the proteins of interest. After treatments, cells were harvested and washed once with ice-cold PBS. Then, 2×10^6 cells were lysed for 30 min on ice in 50 µl of RIPA lysis buffer containing a complete mini protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The cell lysate was centrifuged at 14,000 rpm at 4°C for 30 min. The supernatant was transferred to a new tube and the protein content was determined before loading into the gel.

2) Protein determination

The concentration of protein in the cell lysate was determined by using Bio-Rad protein assay (Bio-Rad Laboratories, USA) which based on the method of Bradford's. Bovine serum albumin (BSA) was used as a standard protein. To determine the protein concentration, BSA were diluted with distilled water to 1, 0.5, 0.25, 0.125, 0.0625 and 0 mg/ml. The cell lysate was also diluted to 1/10. After that, 100 µl of each

standard protein and samples were mixed with 900 µl of dye solution and incubated at room temperature for 5 min. The protein concentration was determined at 595 nm by using a spectrophotometer.

3) Preparation of SDS-PAGE

The gels consist of acrylamide, bisacrylamide, SDS, and a Tris-HCl buffer with adjusted pH. In general, two-part discontinuous gels are used. The samples are loaded onto the upper portion (stacking gel), which has a low acrylamide concentration, low pH, and low resolving ability. When samples run through the stacking gel, all proteins are concentrated into a narrow band. That narrow band then enters the lower portion (separating gel) that separates proteins by size.

4) Separating gel

The acrylamide concentration chosen for the separating gel depends on the sizes of proteins to be separated. Smaller proteins are resolved at higher acrylamide concentrations and vice versa. The separating gel was prepared according to table 1. The separating gel solution was loaded to about 2 cm below the top of the glass plates. A thin layer of butanol was overlaid onto the surface of separating gel to prevent oxygen exposure to the gel and made the gel surface to smooth. The separating gel was allowed to polymerize for 40 min.

5) Running condition

The total protein will be mixed with a 5X sample buffer and boiled for 5-7 min. Protein samples were loaded into the gel. The gel was run at 100 V for 1 h in running buffer. After that, the gel was transferred onto polyvinylidene fluoride (PVDF) membranes (Pall Corporation, USA) for 2 h at 100 V with the use of a Mini Trans-Blot Cell® (Bio-Rad).

6) Protein detection

After protein transfer, the membrane was blocked with TBST containing 5% non-fat milk for 1 h at room temperature. The blots were incubated overnight at 4°C with primary antibody; rabbit polyclonal COX-2, iNOS, NF-κB, and antibody (all diluted 1:1000, from Cell signaling Technology and Merck Millipore). To remove excess primary antibody, membranes were washed in TBST for 10 min 3 times followed by incubation

with the appropriate secondary antibody conjugated with horseradish peroxidase (diluted 1:5000) for 1 h at room temperature. To remove excess secondary antibody, the washing step is required as for the primary antibody. Immunoreactive protein bands were detected by chemiluminescence using enhanced chemiluminescence reagent (ECL) and exposed to CCD camera (BioTek Instrument, Winooski, VT).

3.2.9 Immunofluorescence

Raw 264.7 cells were seeded at $2x10^5$ cells/well on cover glass-bottom dishes for 24 h. After 14 h incubation, cells were treated on compounds for 2 h and then stimulated or not with 1 ug/mL of LPS for 3 h. Cells were fixed in 4 % formaldehyde for 15 min at room temperature and permeated with methanol at -20 °C for 10 min. Cells were then blocked for 1 h with 1 % BSA in PBS and permeabilized by incubation with 0.4 % Triton X-100 for 30 min. Subsequently, cells were washed with PBS and incubated with anti-p65 primary antibody (1:400) at 4 °C overnight. After washing with PBS, cells were incubated with a fluorescence secondary antibody (1:200) at 4 °C for 1.5 h. at RT in the dark and cells were then washed with PBS. Stained with Hoechst for 10-15 mins and cells were then washed with PBS. Slides were mounted and counter-stained using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and visualized under a laser scanning microscope (LSM 700; ZEISS, Jena, Germany).

3.2.9 Tissue preparation

Porcine splenic were obtained from a local abattoir and placed in modified Krebs-Henseleit (K-H) solution maintained at 4°C before being transported to the laboratory. The anterior descending branch of the splenic artery was dissected from the splenic, cleaned of connective tissue, and then divided into 4 mm long segments.

FIGURE 7 Tissue dissected after overnight incubated with 4°C in modified Krebs-Henseleit (K-H) solution

3.2.10 Contraction Studies

For contraction experiments, After overnight storage, segments were removed from the incubation solution and placed in K-H solution (maintained at 37°C and gassed with 95% O_2 and 5% CO_2) in a 15 ml isolated organ bath, prepared for isometric tension recordings as previously described and allowed to equilibrate for 60 min. Contractions of the segment were measured using a Grass FT03 isometric force transducer connected to a MacLab unit coupled to a Macintosh LC4 computer running Chart 3.5. Segments were repeatedly exposed to 60mM KCl for 15 min until reproducible contractions were observed. The various concentrations of genistein, lupalbigenin, and 17β-estradiol were added 60 min before exposed to cumulatively increasing concentrations of either KCl (6-60mM) or U46619 (a stable thromboxane-mimetic analog, 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin F2α, 1-200 nM) or phenylephrine, a direct-acting sympathomimetic amine chemically related to adrenaline and ephedrine with potent vasoconstrictor property. Phenylephrine is a post-synaptic alpha-adrenergic receptor agonist that causes vasoconstriction, increases systolic/diastolic pressures, reflex bradycardia, and stroke output (0.05-1mM). Recorded each preparation was exposed to maximally-effective concentration-response curves of KCl, U46619, and phenylephrine.

In our study, we performed a short-term experiment with pre-incubated splenic artery segments with a specific concentration of genistein, lupalbigenin and 17β-estradiol prior 45 min and then contracted the tissues with KCl, U46619, and phenylephrine in a short period time around 4- 5 h. In term of a longer period, time incubation after each segment were repeatedly exposed to 60mM KCl for 15 min until reproducible contractions the specific concentrations of genistein, lupalbigenin and 17β-estradiol were added and incubated for 14 h.

FIGURE 8 Porcine splenic artery segments (5 mm) were prepared

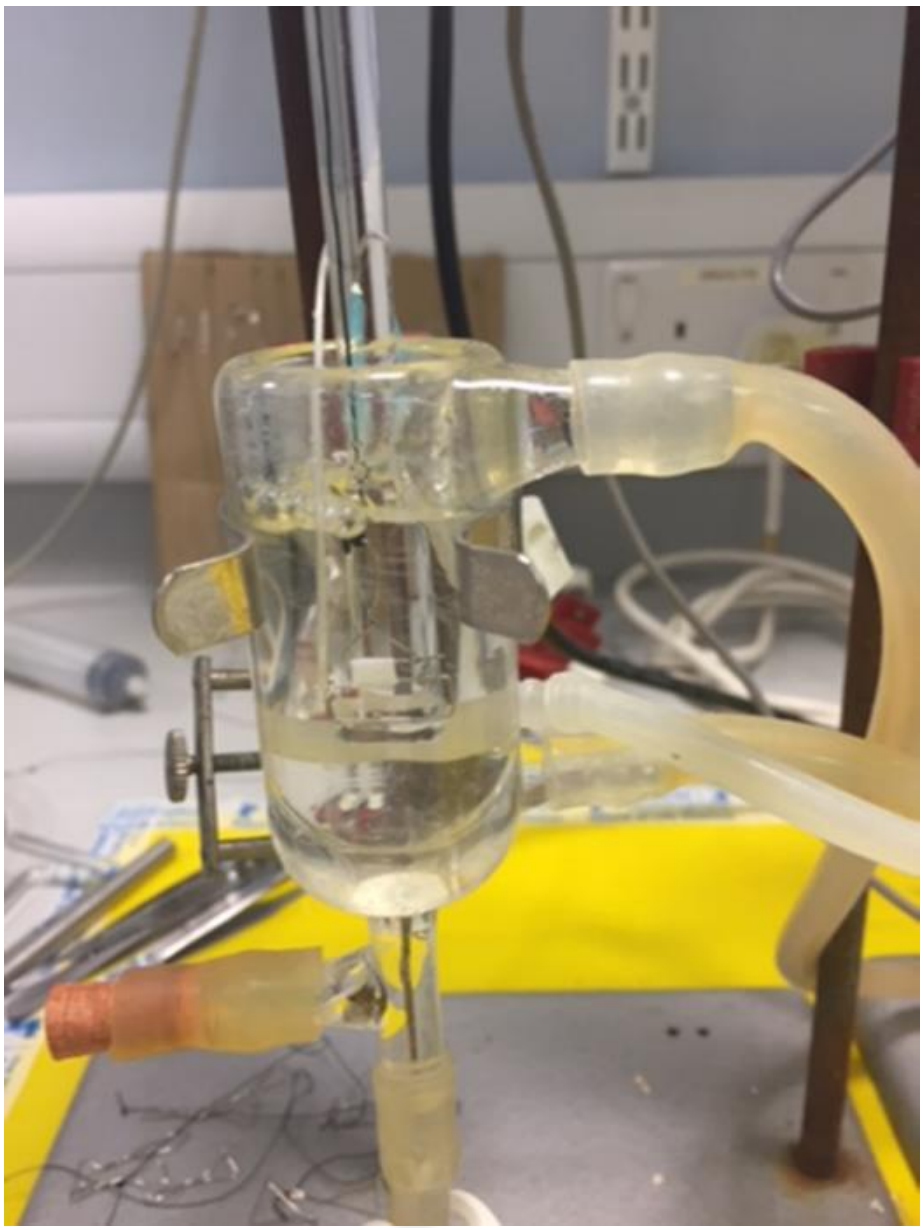


FIGURE 9 Ring segments (5mm) were prepared for isometric tension recording in Krebs-Henseleit solution and gassed with 95%O_2/5%CO_2 held at 37°C.

3.2.11 Data analysis and Statistic analysis

All data from results of Raw 264.7 cells values are presented as mean ± SD. Differences between the mean values of the normally distributed data were assessed by one-way Analysis of Variance (ANOVA) (Dundette's and Sidak's multiple comparisons test). The criterion for differences was considered significant at $p < 0.05$. A test where appropriate using GraphPad Prism version 8.3 (GraphPad Software, San Diego, CA, USA).

The results from contractions produced by Phenylephrine, KCl, and U46619 were measured as gram weight (g wt.). All the tissue responses after a cumulative addition of genistein, lupalbigenin, 17β-estradiol, and fulvestrant were recorded either as a percentage of the third 60mM KCl contraction or as a percentage of the maximum response produced of U-46619 using Microsoft Excel software. All the results were expressed as mean ± SEM [72]. The number of observations in different animals for each experiment was expressed as (n) and this usually ranged from 4-12 for each experiment. In all experiments, the differences between the mean values of the normally distributed data were assessed by one-way Analysis of Variance (ANOVA) , Wilcoxon Rank Sign Test for paired data and Bonferroni's All Pairs Comparison two groups and considered significant if the P-value was <0.05%.

Chapter 4

Result

4.1 Effect of DS extracts on the viability of RAW 264.7 macrophages

Cells were treated with various concentrations of DS in sunflower oil, light mineral oil, rice bran oil (10, 50 and 100 µg/mL) and then measured the cytotoxicity using MTT assay to determine the non-cytotoxic concentration (Figure 10). As a result showed *DS* extracts in sunflower oil, light mineral oil, and rice bran oil have not seen any cytotoxicity from 10, 50 and 100 µg/mL contrastingly there are slightly induce cell growth including the cytotoxicity of three formative solvents. Whereas, the result of DS in 50% aqueous had high toxicity with Raw 264.7 cells after a 24-h treatment.

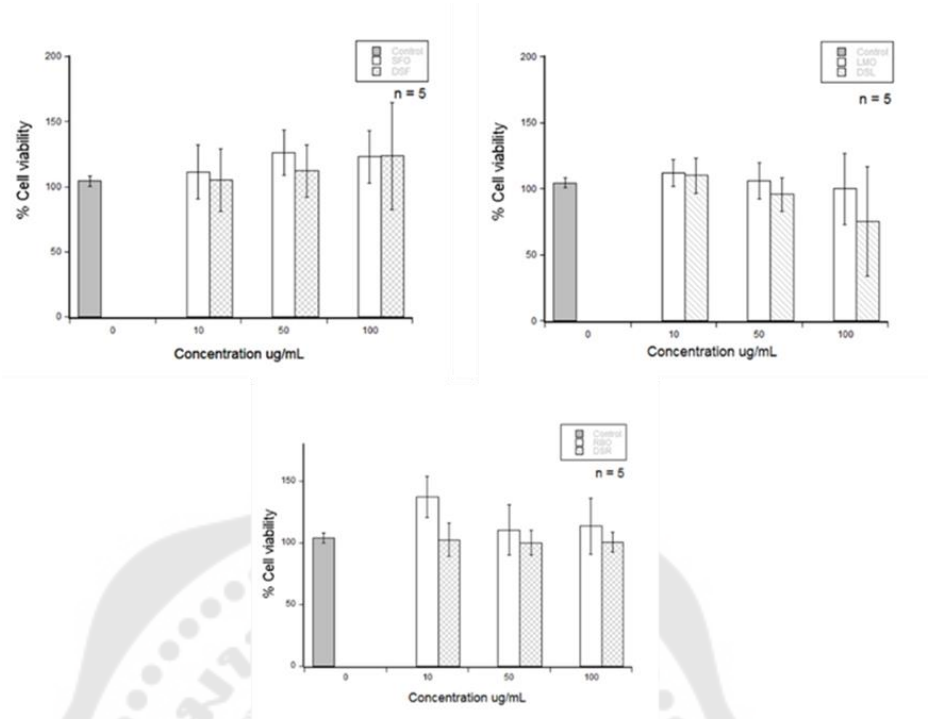


FIGURE 10 Effect of DS extracts on cell viability. Cells were treated with three different solvents of DS extract in RAW 264.7 cells at the concentrations of 10, 50 and 100 µg/ml and detected by MTT assay. Data are the mean ± SD (n= 5)

4.2 Morphological change of *DS* extracts

The cell morphology of the macrophage RAW 264.7 cells under *DS* extract treatment in the presence or absence of LPS (1 μg/mL). The cells were monitored under optical microscopy (100×) for 18 hours; see Figure 11-15. In control or untreated cells , the cell morphology generally showed a round form whereas LPS-activated RAW 264.7 cells had changed to an irregular form with accelerated spreading and forming pseudopodia and vesicles. The co-treatment of LPS with *DS* in sunflower oil, light mineral oil, and rice bran oil reduced the level of vesicles while *DS* in 50% ethanol caused cell death whether 50 μg/mL and 100 μg/mL. However, pre-treated cells with *DS* extracts can not stop cells transforming after LPS-induced. the results don't clear *DS* extracts can be protected cell change from LPS-indued in RAW 264.7 cells.

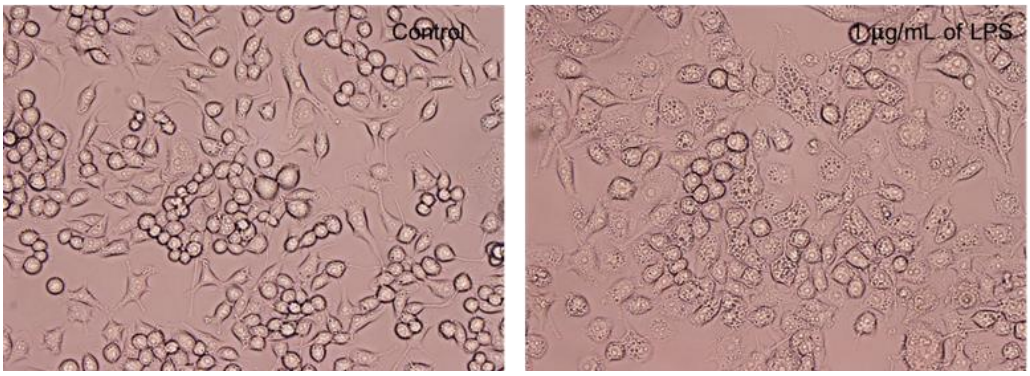


FIGURE 11 Morphology of macrophage RAW 264.7 cell with and without 1 µg/mL of LPS visualized by optical microscopy (×100). This data is represented by n = 10.

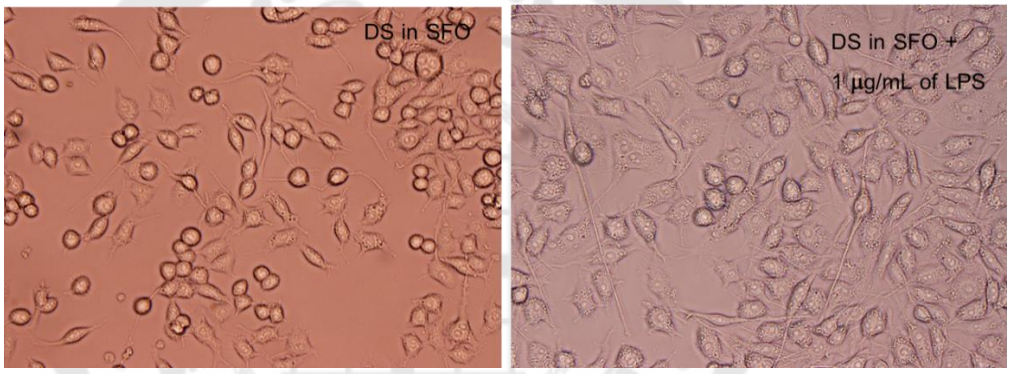


FIGURE 12 Morphology of macrophage RAW 264.7 cell 100 µg/mL of DS in sunflower oil with and without 1 µg/mL of LPS visualized by optical microscopy (×100). This data is represented by n = 5.

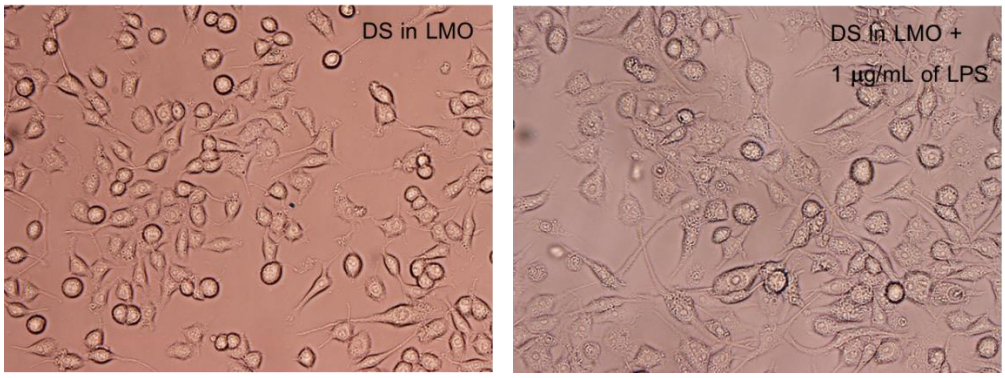


FIGURE 13 FIGURE 14 Morphology of macrophage RAW 264.7 cell 100 µg/mL of DS in light mineral oil with and without 1 µg/mL of LPS visualized by optical microscopy (×100). This data is represented by n = 5.

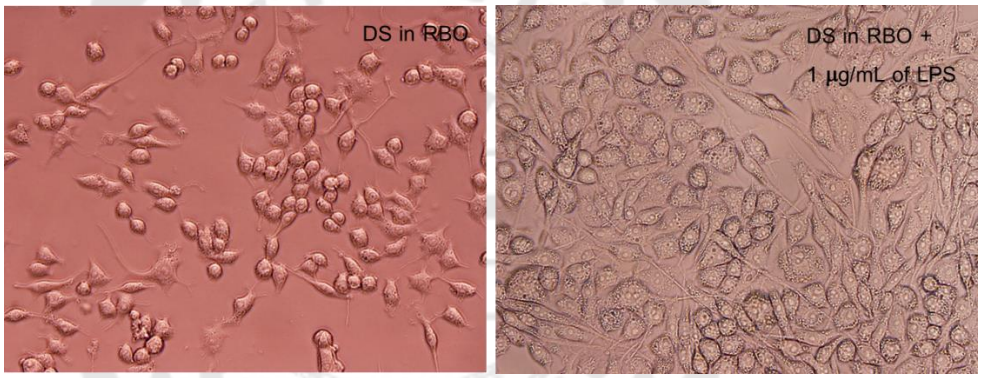


FIGURE 15 Morphology of macrophage RAW 264.7 cell 100 µg/mL of DS in rice bran oil with and without 1 µg/mL of LPS visualized by optical microscopy (×100). This data is represented by n = 5.

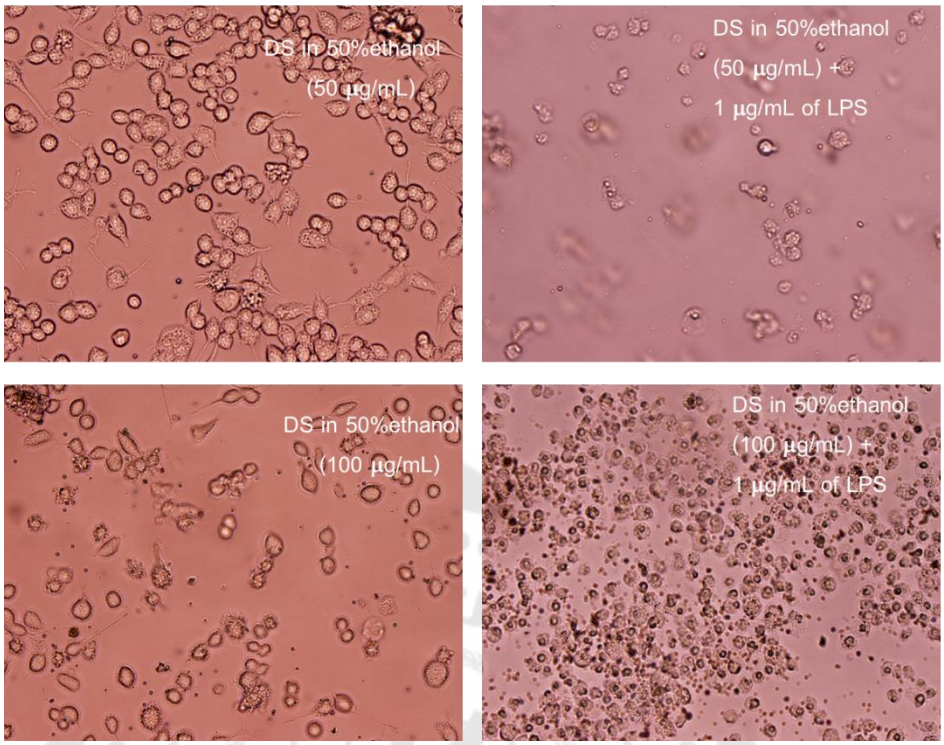


FIGURE 16 Morphology of macrophage RAW 264.7 cell of 50 µg/mL of DS in 50% ethanol (upper) and 100 µg/mL in 50% ethanol [73] with and without 1 µg/mL of LPS visualized by optical microscopy (×100). This data is represented by n = 3.

4.3 DPPH antioxidant activity of *DS* extracts

The DPPH assay had performed to study the ability of *DS* in three different solvents can be the anti-oxidative agent. The result showed the percentage of scavenging of the compound from 1-50 μg/mL and L-ascorbic acid was used as a standard agent. DS in sunflower oil has potent anti-oxidative ability similarity with L-ascorbic acid to scavenge free radical in the cell stress. (Figure 16-17)

Figure 17 is showed only the positive of scavenging percentage of L-ascorbic acid, DS in sunflower oil and DS in sunflower oil. As a result, L-ascorbic acid is the standard using anti-oxidant can be 80% scavenging while DS in sunflower oil has increasing potent anti-oxidant as dose manner. However, DS in light mineral oil (DSL) also is a good potential for oxidative stress without dose-dependent.

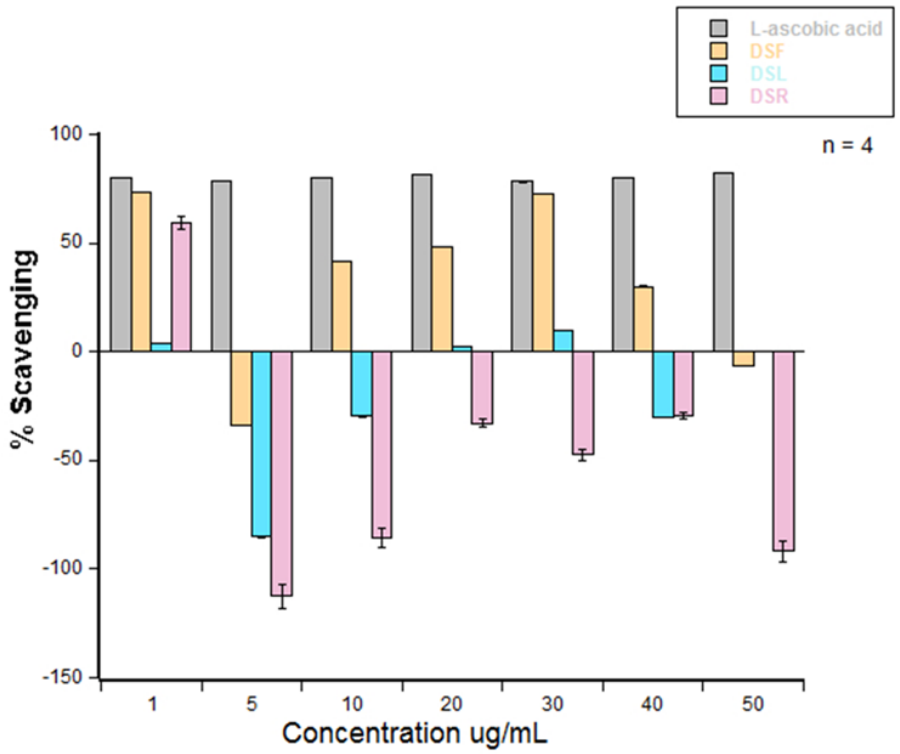


FIGURE 17 The percentage of scavenging of DS extract in sunflower oil, light mineral oil and rice bran oil from 1-50 µg/mL detect by DPPH assay. Data are the mean± SD (n= 4).

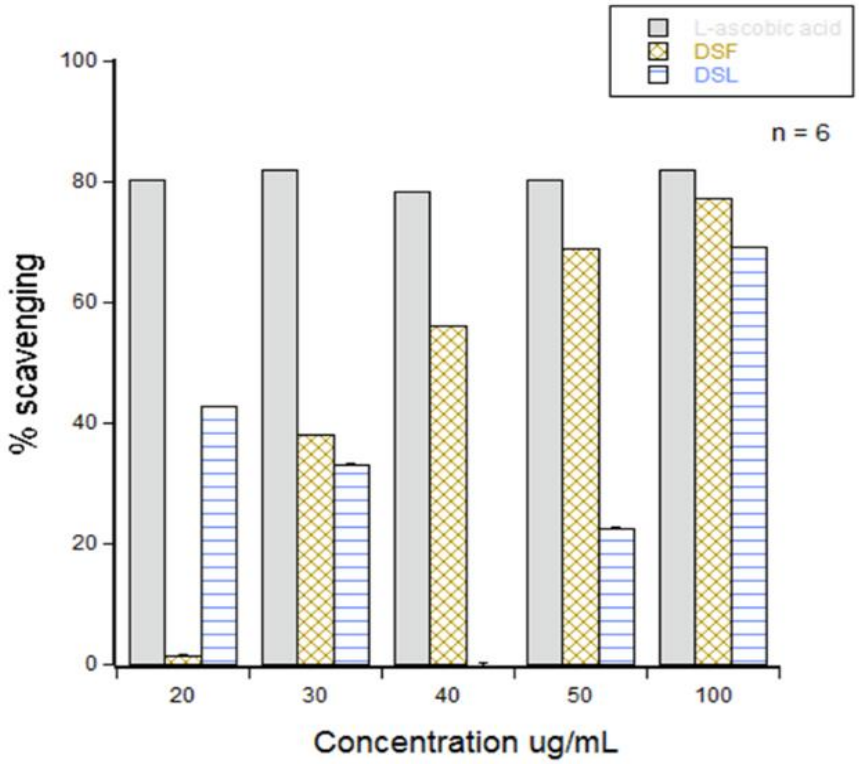


FIGURE 18 The percentage of scavenging of DS extract in sunflower oil and light mineral oil from 20-100 µg/mL detect by DPPH assay. Data are the mean ± SD (n= 6).

4.4 Effect of DS extract on Nitrite Production

Nitrite (NO) is a major inflammatory mediator during the immune-inflammatory response and can be able to measure in culture medium after incubation with stimuli by Griess reaction. For *DS* extracts and solvents are using 50 and 100 µg/mL (Fig 18-19). Figure.18 showed NO concentration of *DS* in 3 different solvents and solvents only treated, the result founded that *DS* extracts and solvents not induce NO secretion in Raw 264.7 cell when comparing at control.

Figure. 19 showed the results from DS extract and solvents with 1 µg/mL of LPS treated. Surprisingly, at 50 and 100 µg/mL of DS in sunflower oil (DSF) and at 100 a µg/mL of rice bran oil (RBO) and DS in rice bran oil (DSR) founded significant decreased NO concentration. However, DS in light mineral oil and its solvent doesn't have NO inhibitory activity.

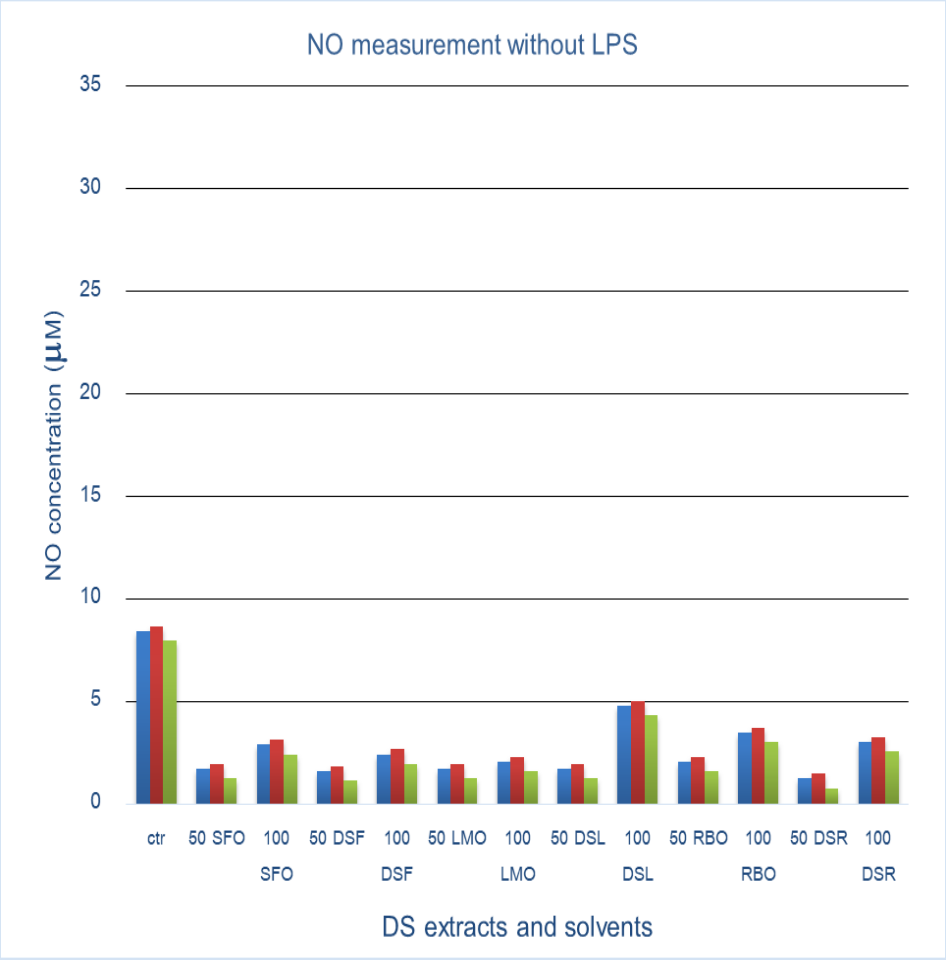


FIGURE 19 NO production and values are mean ± standard deviation of three (n=3) independent experiment

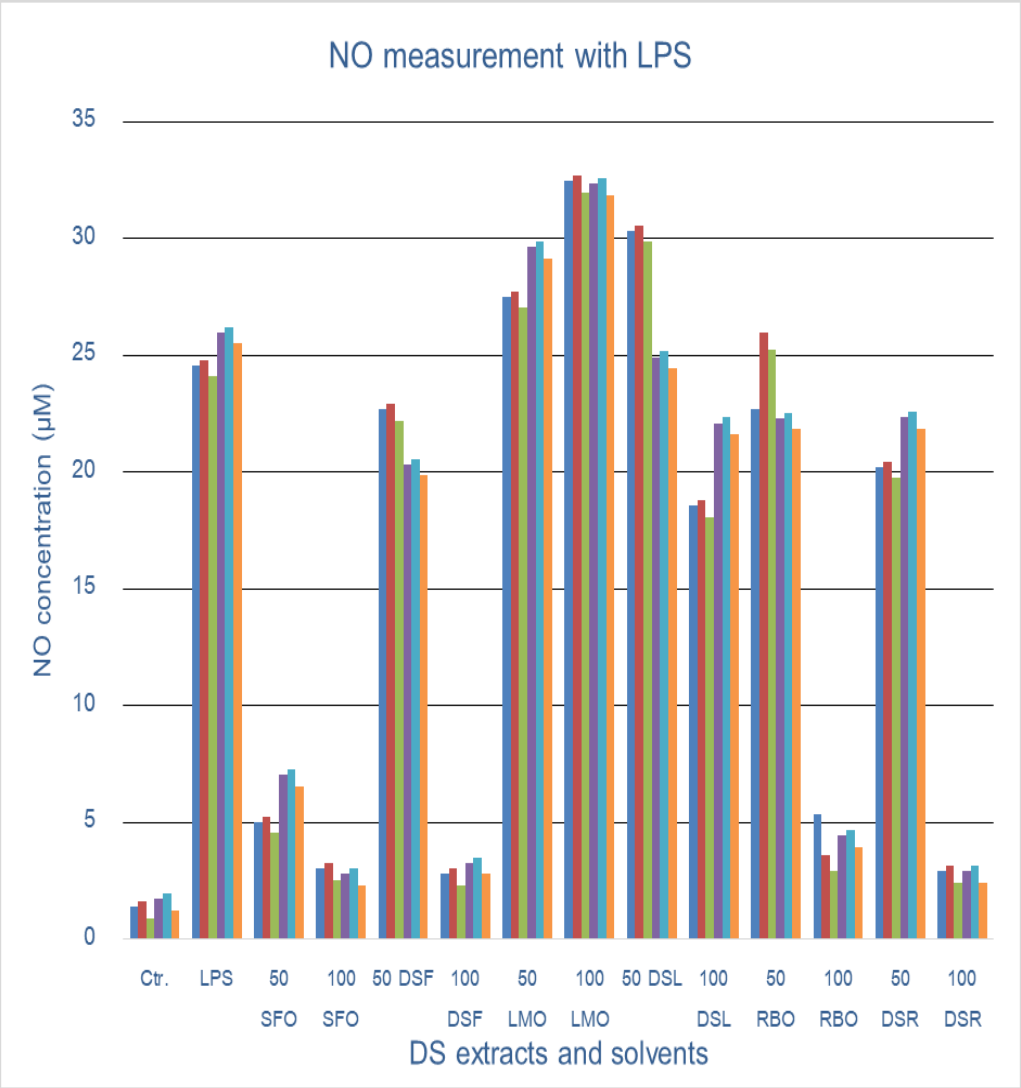


FIGURE 20 NO production and values are mean ± standard deviation of sixth (n=6) independent experiment

TABLE 2. NO concentration of DS extracts and solvents without 1 μg/mL of LPS and values are mean ± standard deviation of three (n=3) independent experiment

		NO concentration (mM)			AVE	SD
Ctr.		8.43345	8.66902	7.96231	8.35493	0.35984
SFO	50	1.71967	1.95524	1.24853	1.64115	0.35984
	100	2.89753	3.1331	2.42638	2.819	0.35984
DSF	50	1.60188	1.83746	1.13074	1.52336	0.35984
	100	2.42638	2.66196	1.95524	2.34786	0.35984
LMO	50	1.71967	1.95524	1.24853	1.64115	0.35984
	100	2.07303	2.3086	1.60188	1.9945	0.35984
DSL	50	1.71967	1.95524	1.24853	1.64115	0.35984
	100	4.7821	5.01767	4.31095	4.70357	0.35984
RBO	50	2.07303	2.3086	1.60188	1.9945	0.35984
	100	3.48645	3.72203	3.01531	3.40793	0.35984
DSR	50	1.24853	1.4841	1.77739	1.50334	0.26495
	100	3.01531	3.25088	2.54417	2.93679	0.35984

TABLE 3. NO concentration of DS extracts and solvents with 1 μg/mL of LPS and values are mean ± standard deviation of sixth (n=6) independent experiment

		NO concentration (μM)						AVE	SD
Ctr.		1.36631	1.60188	0.89517	1.71967	1.95524	1.24853	1.46447	0.34284
	1 mg/mL of LPS	24.5701	24.8057	24.0989	25.9835	26.2191	25.5124	25.1983	0.76535
SFO	50	5.01767	5.25324	4.54653	7.02002	7.25559	6.54888	5.94032	1.0434
	100	3.01531	3.25088	2.54417	2.77974	3.01531	2.3086	2.819	0.31654
DSF	50	22.6855	22.9211	22.2144	20.3298	20.5654	19.8587	21.4291	1.21395
	100	2.77974	3.01531	2.3086	3.25088	3.48645	2.77974	2.93679	0.37659
LMO	50	27.5147	27.7503	27.0436	29.6349	29.8704	29.1637	28.4963	1.10003
	100	32.4617	32.6973	31.9906	32.3439	32.5795	31.8728	32.3243	0.29965
DSL	50	30.3416	30.5771	29.8704	24.9234	25.159	24.4523	27.554	2.72496
	100	18.563	18.7986	18.0919	22.0966	22.3322	21.6254	20.2513	1.79105
RBO	50	22.7244	25.96	25.2532	22.3086	22.5442	21.8375	23.438	1.57061
	100	5.36867	3.60424	2.89753	4.42874	4.66431	3.9576	4.15351	0.78855
DSR	50	20.212	20.4476	19.7409	22.3322	22.5677	21.861	21.1936	1.10003
	100	2.89753	3.1331	2.42638	2.89753	3.1331	2.42638	2.819	0.29381

4.5 Inhibitory effect of *DS* extracts on iNOS, IL-6 and TNF-α gene expressions

From the results of NO inhibition, 100 µg/mL of *DS* in sunflower oil (DSF), sunflower oil [74], *DS* in rice bran oil (DSR) and rice bran oil (RBO) had chosen for iNOS and TNF-α gene expression study by using real-time PCR, β-active has been used to normalize gene expression. The result showed at the 100 µg/mL of *DS* in sunflower oil (DSF) and sunflower oil [74] were founded markedly inhibited iNOS and TNF-α gene expression compared with cells were infected by only 1 µg/mL of LPS. (figure.20)

Figure 21 demonstrated the result of the cell were pretreated with 100 µg/mL of DS in rice bran oil (DSR) slightly decreased iNOS and TNF-α gene expression but only solvent had found more induced comparing with cell were infected by only 1 µg/mL of LPS.

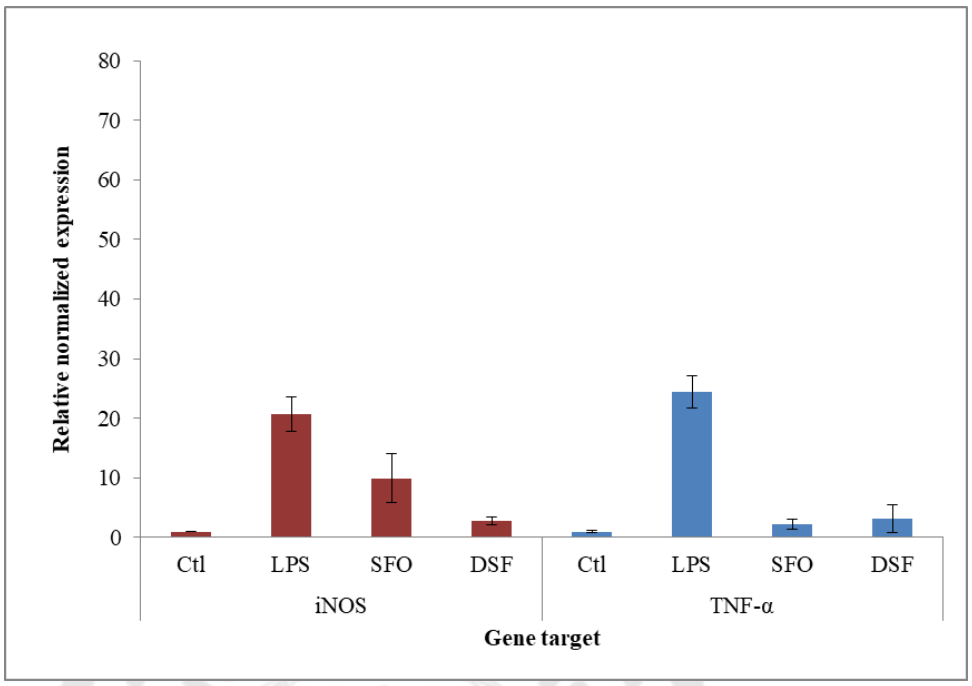


FIGURE 21 The analysis of gene expression by Real-time PCR of iNOS and TNF-α. Values are mean ± standard deviation of three independent experiment

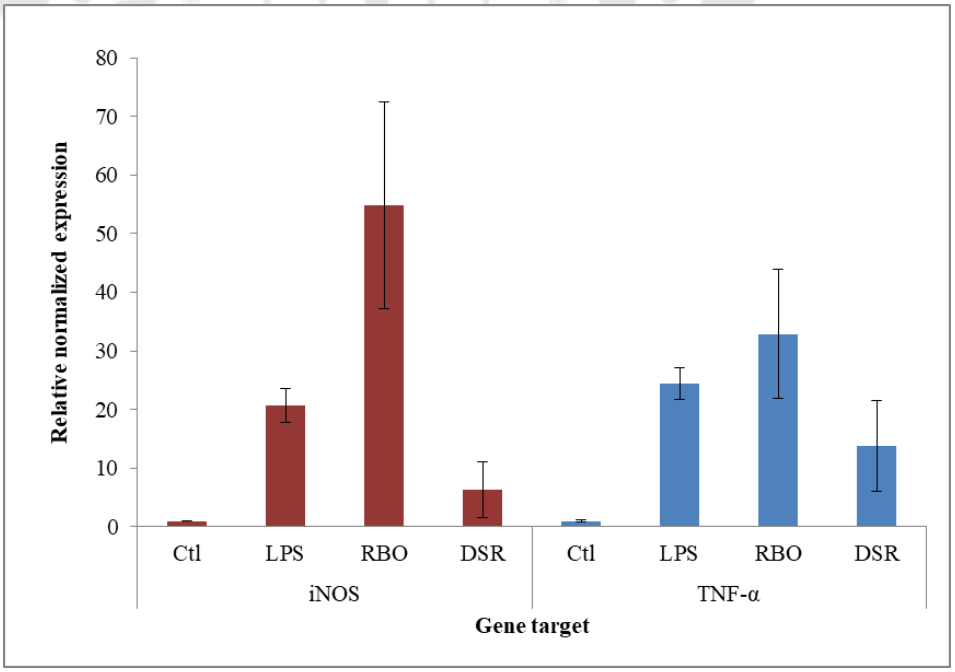


FIGURE 22 the analysis of gene expression by Real-time PCR of iNOS and TNF-α. Values are mean ± standard deviation of three independent experiment

4.6 Inhibition of iNOS, COX-2 and TNF-α protein expressions by western blotting

Raw 264.7cells were treated with 100 µg/mL of *DS* in sunflower oil (DSF), sunflower oil [(74)], *DS* in rice bran oil (DSR) and rice bran oil (RBO). The results showed *DS* extracts had slightly decreased COX-2 protein expression while the solvents doesn't.

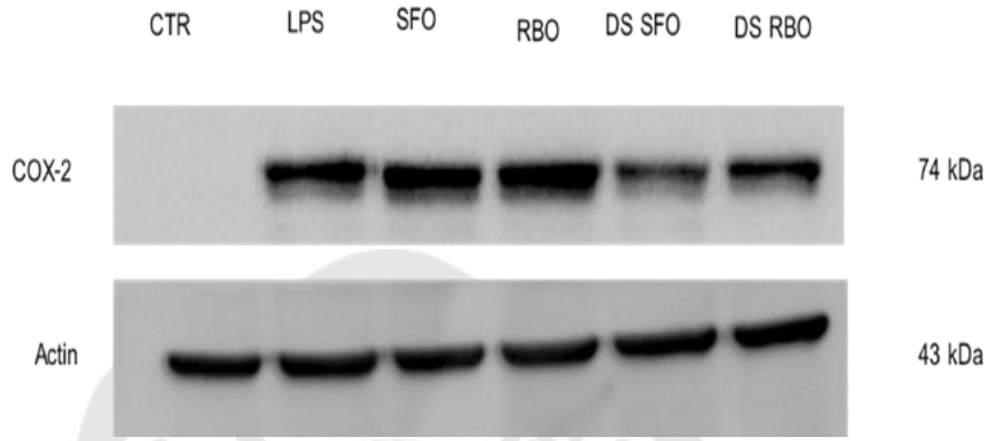


FIGURE 23 Effect of Lupalbigenin on COX-2 by western blotting

4.7 Lupalbigenin purification from 50%Ethanol aqueous stems of *Derris Scandens* Benth.

Lupalbigenin was isolated from 50% Ethanol aqueous stems of *Derris Scandens* Benth. The extract was obtained from Bangkok Lab and Cosmetic Co., Ltd. Ratchaburi province. Staring by 20 g of the crude extract was partitioned with 5% Methanol/95% Dichloromethane to get 2° crude extract in 5%MeOH/DCM. (Figure. 23-26)

FIGURE 24 Partition Chromatography

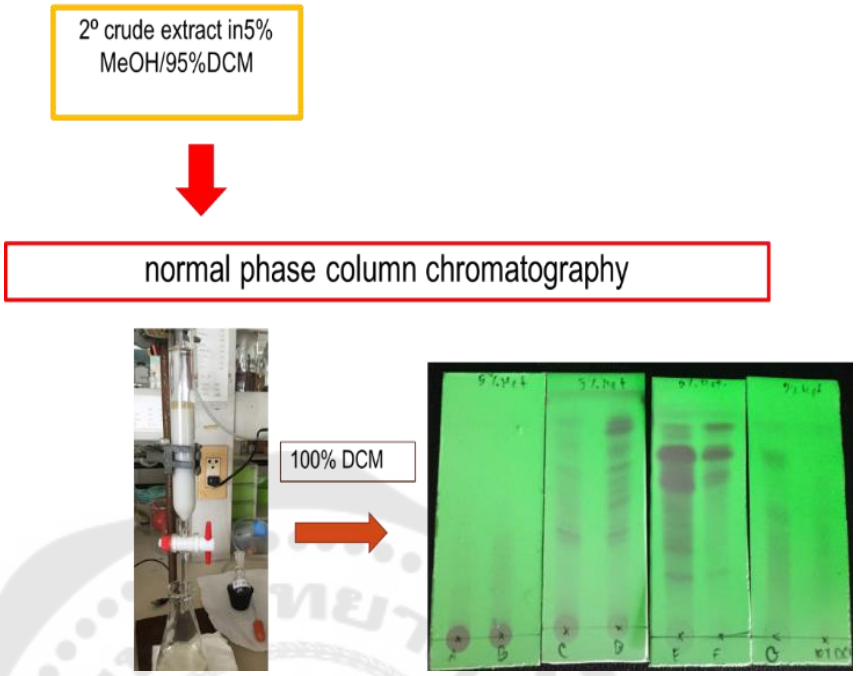


FIGURE 25 First running to vacuum-liquid chromatography on silica gel (100%DCM)

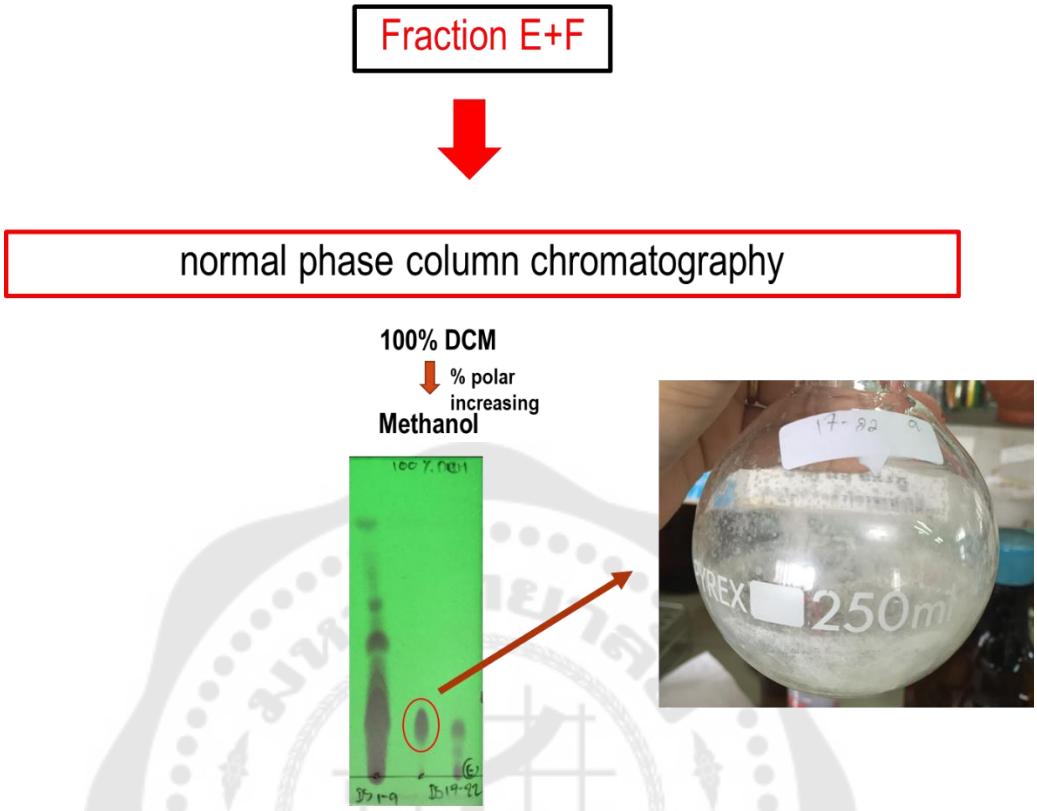
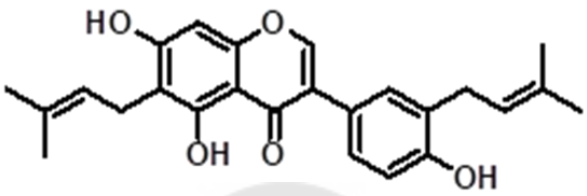


FIGURE 26 Repeated normal phase column chromatography

Fraction E and F (121 mg) were separated by normal phase chromatography on silica gel (MeOH-DCM, gradient) to give Lupalbigenin (29.5 mg) and the structure was determined through analysis of its spectroscopic data.

Lupalbigenin



MW. 406.5

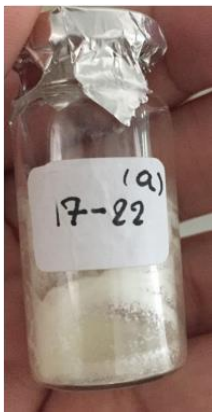


FIGURE 27 Lupalbigenin structure and 98% purification form

4.8 Effect of lupalbigenin on the viability of RAW 264.7 macrophages

Cells were treated with various concentrations of lupalbigenin (1.25-75 μM) and then measured the cytotoxicity using the MTT assay to determine the non-cytotoxic concentration (Figure 27). As results lupalbigenin at the concentration of 12.5 μM and lower were not significantly toxic with macrophages cells after a 24-h treatment.

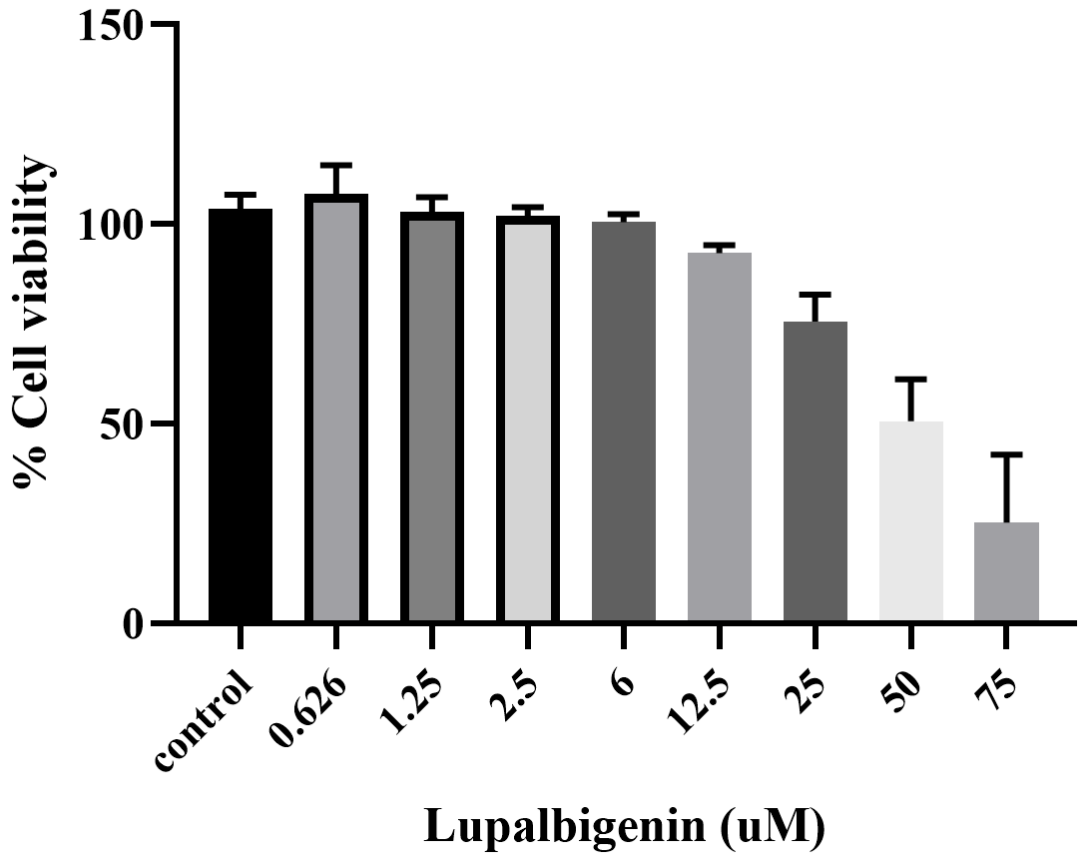


FIGURE 28 Effect of lupalbigenin on RAW 264.7 macrophage cell viability. Cells were treated with lupalbigenin at the concentrations of 0.625, 1.25, 2.5, 6, 12.5, 25, 50 and 75 μM and detected by MTT assay. Data are the mean ± SD (n= 6).

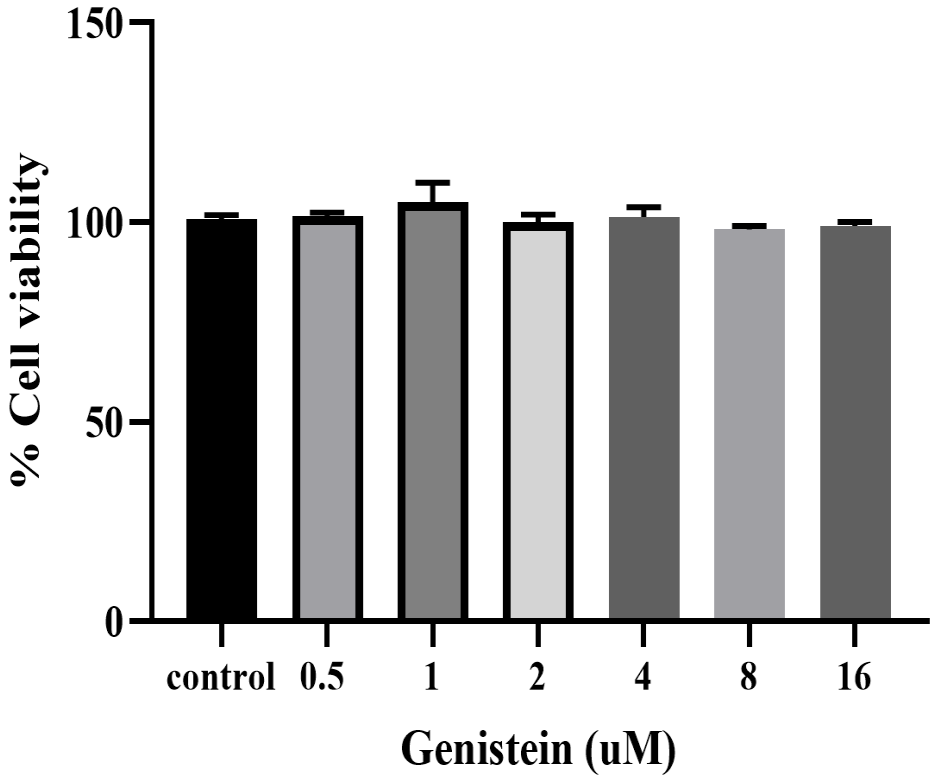


FIGURE 29 Effect of genistein on RAW 264.7 macrophage cell viability. Cells were treated with genistein at the concentrations of 0.5, 1, 2, 4, 8 and 16 μM and detected by MTT assay. Data are the mean ± SEM (n= 4). As the result showed genistein has no cytotoxicity with Raw 264.7 cells.

4.9 Morphology of Macrophage RAW264.7 Cells.

The cell morphology of the macrophage RAW 264.7 cells under DS extract and lupalbigenin treatment in the presence or absence of LPS (1 μg/mL). The cells were monitored under optical microscopy (100×) for 18 hours; see Figure12-17. In control, untreated cells, the cell morphology generally showed a round form whereas LPS-activated RAW 264.7. Cells had changed to an irregular form with accelerated spreading and forming pseudopodia. The co-treatment of LPS with lupalbigenin reduced the level of cell spreading and pseudopodia formation by suppressing cell differentiation. However, the results doesn't not clear lupalbigenin can be able to protect LPS-induced cell change because after treated with lupalbigenin and LPS , Raw cell doesn't look the same as control that could be the effect of cells stress.

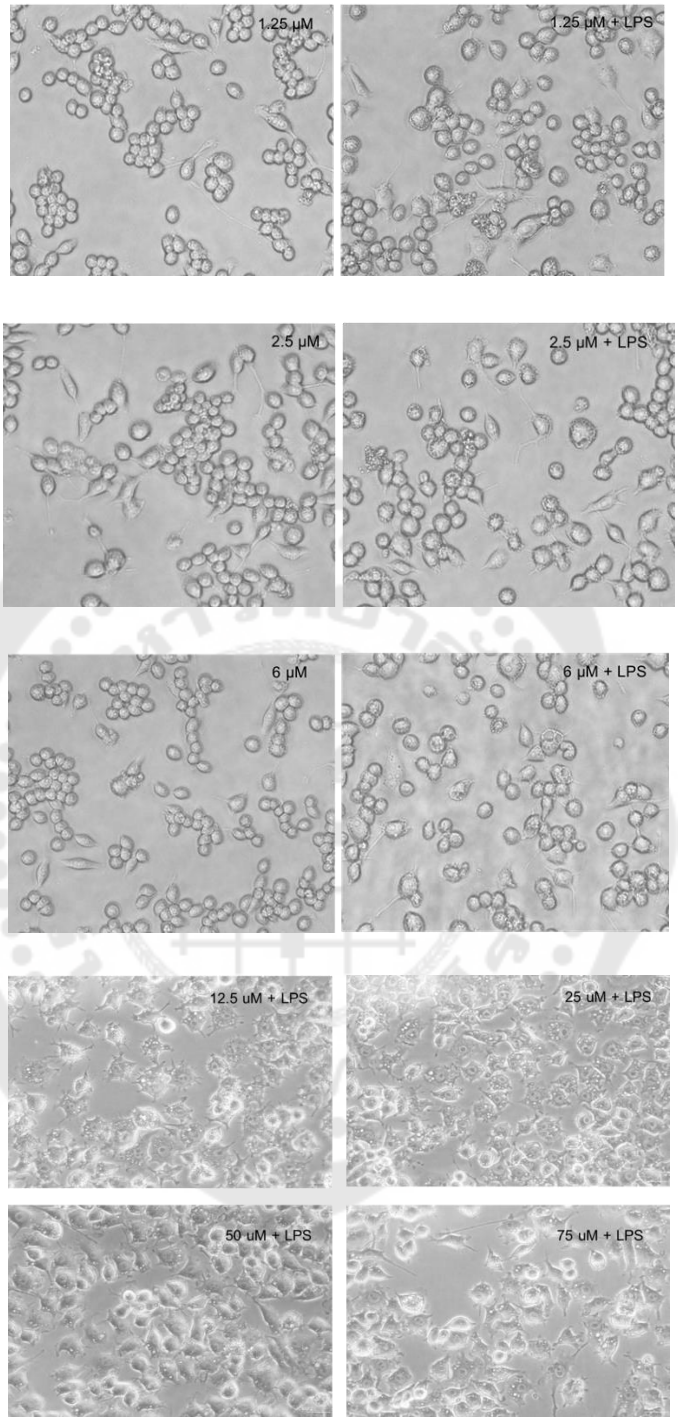


FIGURE 30 Morphology of macrophage RAW 264.7 cell with lupalbigenin at 1.25, 2.5 and 6 µM with and without LPS and 12.5 -75 µM with 1 µg/mL of LPS. visualized by optical microscopy (×100).

The cells were pretreated with lupalbigenin at 1.25, 2.5 and 6 μM for 1 h before exposed with 1 μg/mL LPS for 18 hours. (a) Control (b) 1 μg/mL LPS treatment only, (c-e) combination of LPS and 1.25 μM, 2.5 μM and 6 μM lupalbigenin. On the result of Raw 264.7 cells were treated with 1.25-6 μM of lupalbigenin incubated with 1 μg/mL had clearly protective activity of cell damage from the inflammatory inducing agent by lipopolysaccharide. (Figure. 30)

Cell morphology of macrophage RAW 264.7 cells upon genistein treatment in the presence of LPS (1 μg/mL) was determined. The cells were monitored under optical microscopy (100×) for 18 hours. The co-treatment of LPS with genistein obviously reduced cell damaged comparing with cell were treated with LPS only. (Figure.31)

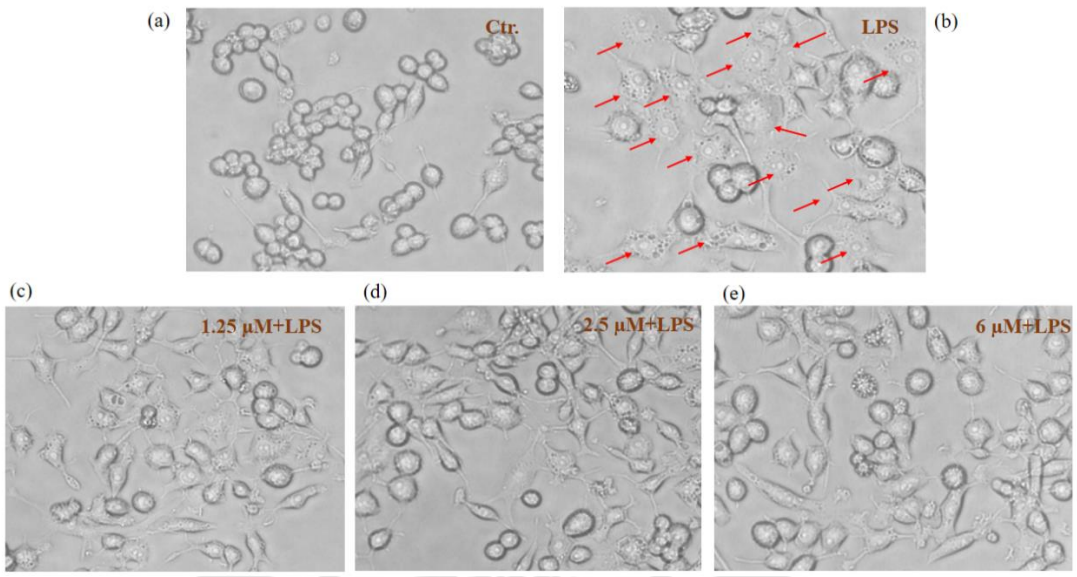


FIGURE 31 Morphological changes in RAW 264.7 macrophage cell visualized by optical microscopy (200x).

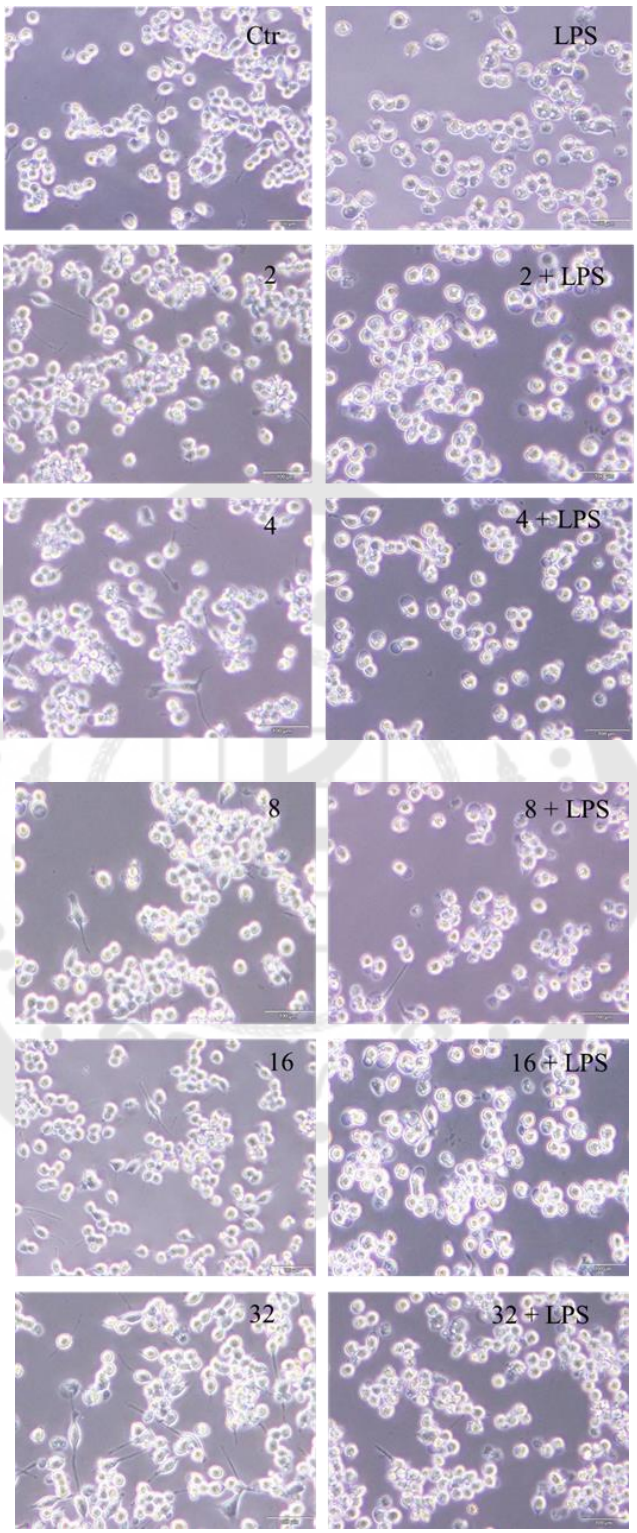


FIGURE 32 Morphology cell change in Raw 264.7 cell of 2-32 uM of Genistein with and without 1 ug/ml of LPS 18 h. cell visualized by optical microscopy (×100).

4.10 Effect of lupalbigenin on Nitrite Production

NO is a major inflammatory mediator during the immune-inflammatory response and could be measured in culture medium after incubation with the stimuli in Griess reaction. The result showed that lupalbigenin inhibited nitric oxide production as not dose-dependent and non-significantly. However, 12.5 – 75 μM lupalbigenin are toxic in RAW 264.7 macrophages, therefore lupalbigenin at concentrations of 1.25, 2.5 and 6 μM were chosen for further study(Fig 32).

Genistein, isoflavone can be found in *Derris scandens* extract[72] is using to compare the result with lupalbigenin, prenylated of genistein. The results showed genistein inhibited nitric oxide production as a dose-dependent manner but no cytotoxicity between 0.5 – 16 μM.

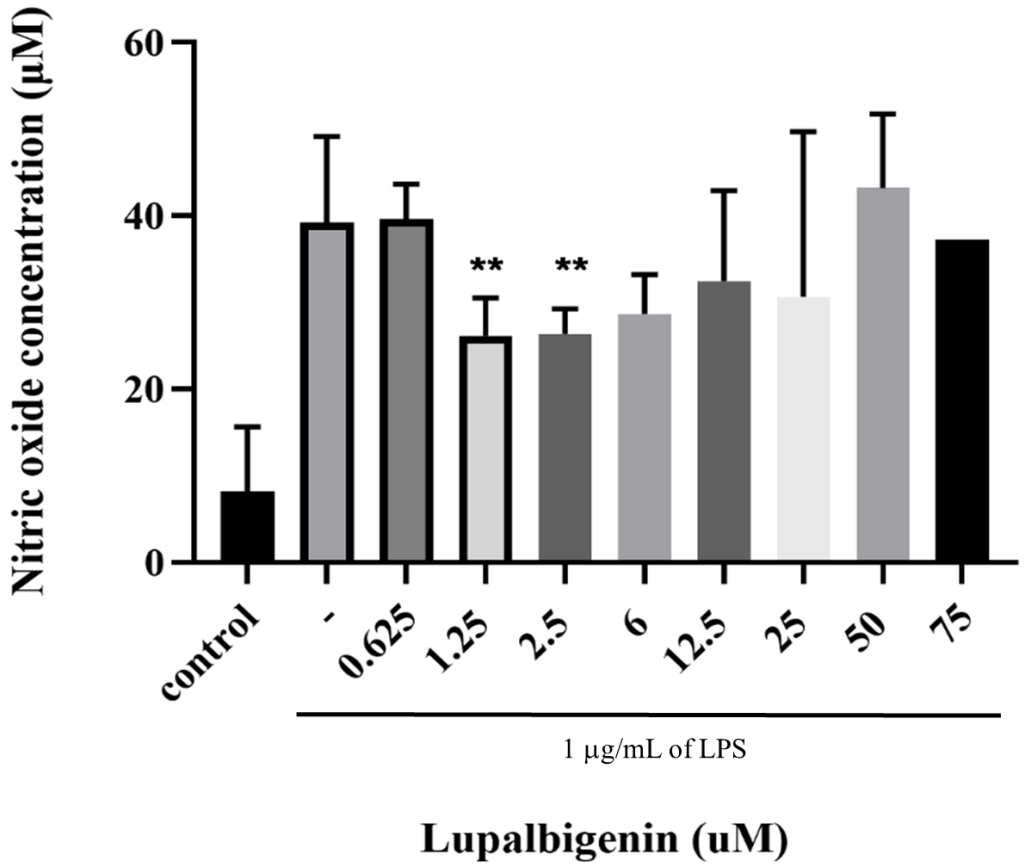


FIGURE 33 Effect of lupalbigenin with 1 µg/mL of LPS induced RAW 264.7 macrophage cells on nitric oxide induction. After 24 h treatment, NO production was measured by the Griess reaction assay. Value is the mean ±SD (n=6).

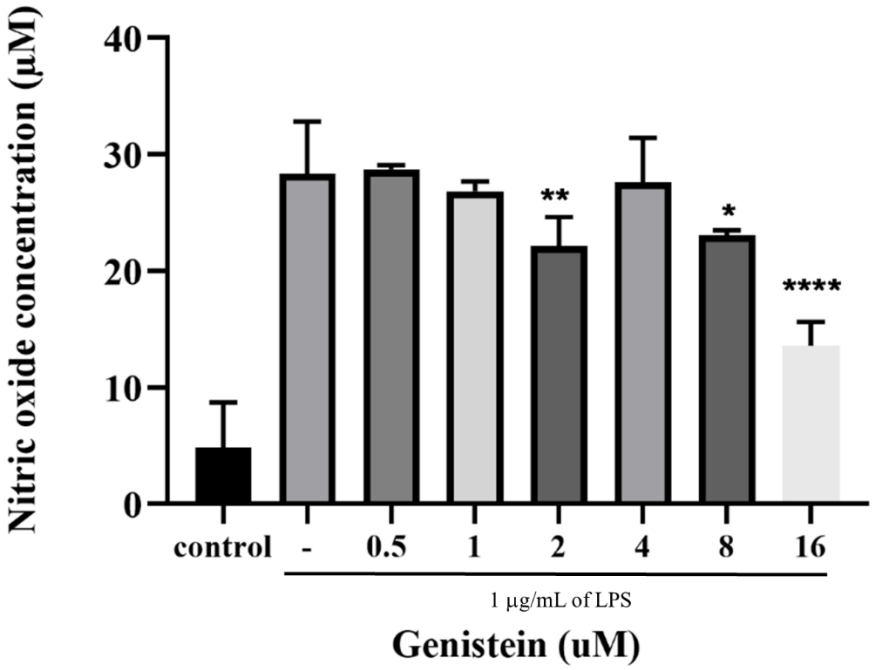


FIGURE 34 Nitric oxide production by Griess reagent of pretreated with Genistein. Value is the mean ±SD (n=4).

4.11 Inhibitory effect of lupalbigenin on iNOS, IL-6 and TNF-α gene expressions

The results showed LPS-treatment obviously increased iNOS, IL-6 and TNF-α gene expression compared to the control group while the effect of lupalbigenin concentration at 6, 2.5 and 1.25 μM demonstrated decrease IL-6 and TNF-α. A lower concentration at 1.25 μM resulted in a significant reduction of IL-6 and TNF-α whereas 2.5 μM of lupalbigenin potentially inhibited iNOS than the other.

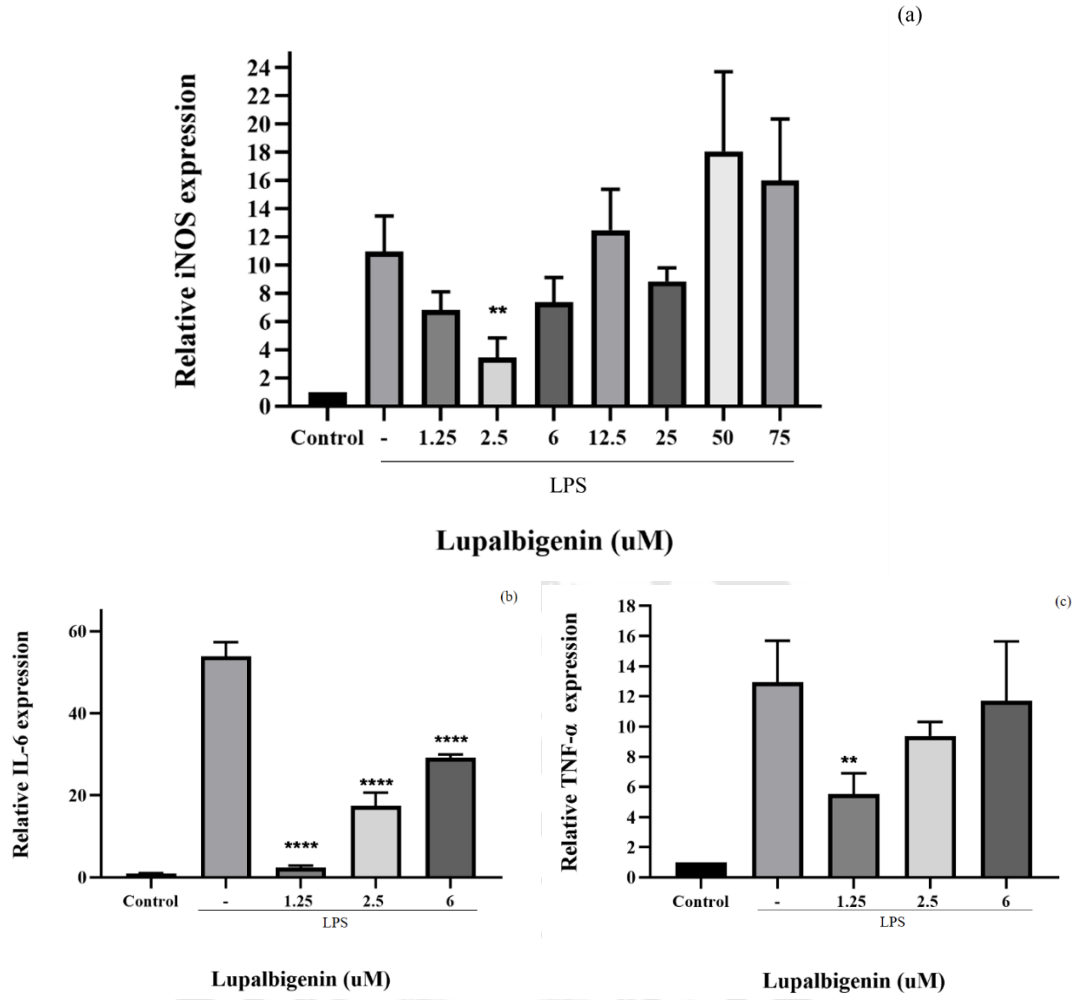


*FIGURE 35 Effects of lupalbigenin on pro-inflammatory cytokine mRNA expression in LPS-induced RAW 264.7 cells. Cells were treated with different concentrations of lupalbigenin 1.25 – 75 μM of lupalbigenin for iNOS and 1.25, 2.5 and 6 μM of lupalbigenin for IL-6 and TNF-α and stimulated with LPS (1 μg/mL) for 24 h. The mRNA levels of (a) iNOS, (b) IL-6 and (c) TNF-α were determined by real-time RT-PCR analysis. Values represent the means ± SDs of three independent experiments. * $p < 0.05$, ** $p < 0.005$, *** $p \leq 0.001$ and **** $p < 0.001$ vs the LPS-induced only group.*

4.12 Inhibition of iNOS, COX-2 and TNF-α protein expressions by western blotting

Lupalbigenin at 6, 2.5 and 1.25 μM of having been chosen to study the possess of inflammatory protein by LPS-induced. The LPS-stimulated Raw 264.7 cells showed significant iNOS, COX-2 and TNF-α protein expression. At three concentrations as we used showed effective inhibited of iNOS protein level expression but at 6 μM doesn't have any effect with COX-2 and TNF-α. For 2.5 and 1.25 μM had shown highly effective to decrease both COX-2 and TNF-α level especially 1.25 μM demonstrated significant decreased inflammatory protein expression whether compared with LPS treated only or in treated group with lupalbigenin.

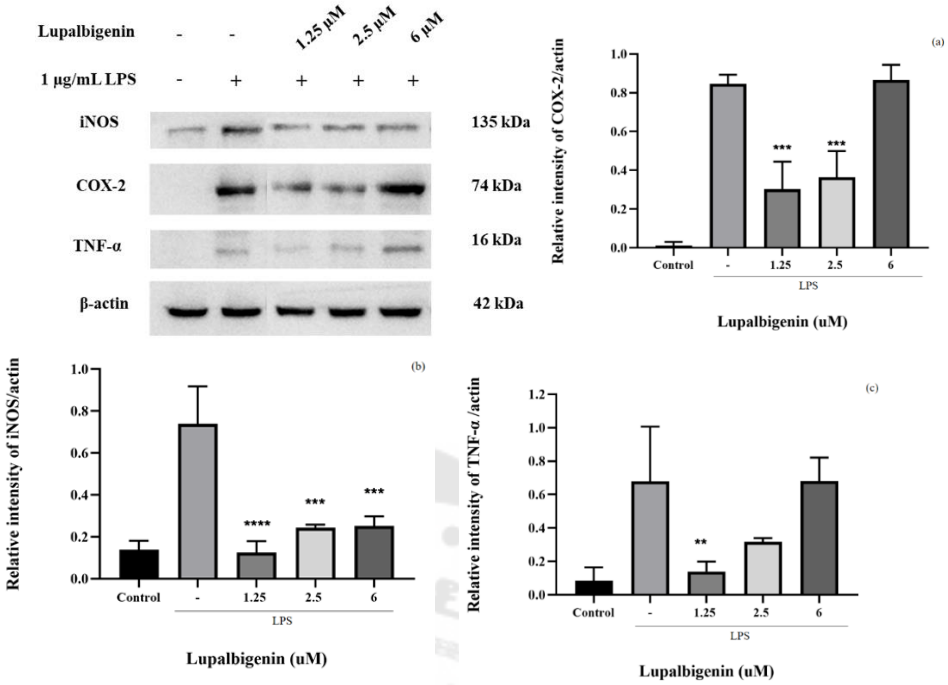


*FIGURE 36 Effects of lupalbigenin on LPS-induced COX-2, iNOS and TNF-α protein expression in RAW 264.7 cells. Cells were treated with lupalbigenin 1.25, 2.5 and 6 μM for 1 h and then treated with LPS for 24 h. The total protein was then analyzed by Western blot. All data were normalized by β-actin and bars indicate mean ± standard error of three independent experiments. ** p <0.005, *** p ≤0.001 and **** p < 0.001 vs the LPS-induced only group.*

4.13 Effect of Lupalbigenin on NF-κB translocation in LPS-induced Raw 264.7 cells

NF-κB proteins are heterodimers represented by two monomers p50 and p65. Their activation is prevented by IκB-α. The activation of by IκB-α phosphorylation induces the translocation of phosphorylated NF-κB from the cytoplasm into the nucleus for the regulation of specific transcription factors. In this study, to investigate the effect of Lupalbigenin on the regulation of the NF-κB pathway, the p-NF-κB protein was examined using western blotting analysis as shown in Figure 37. The result showed lupalbigenin at 2.5 and 1.25 μM were negatively regulated phosphorylation of P-p65 NF-κB.

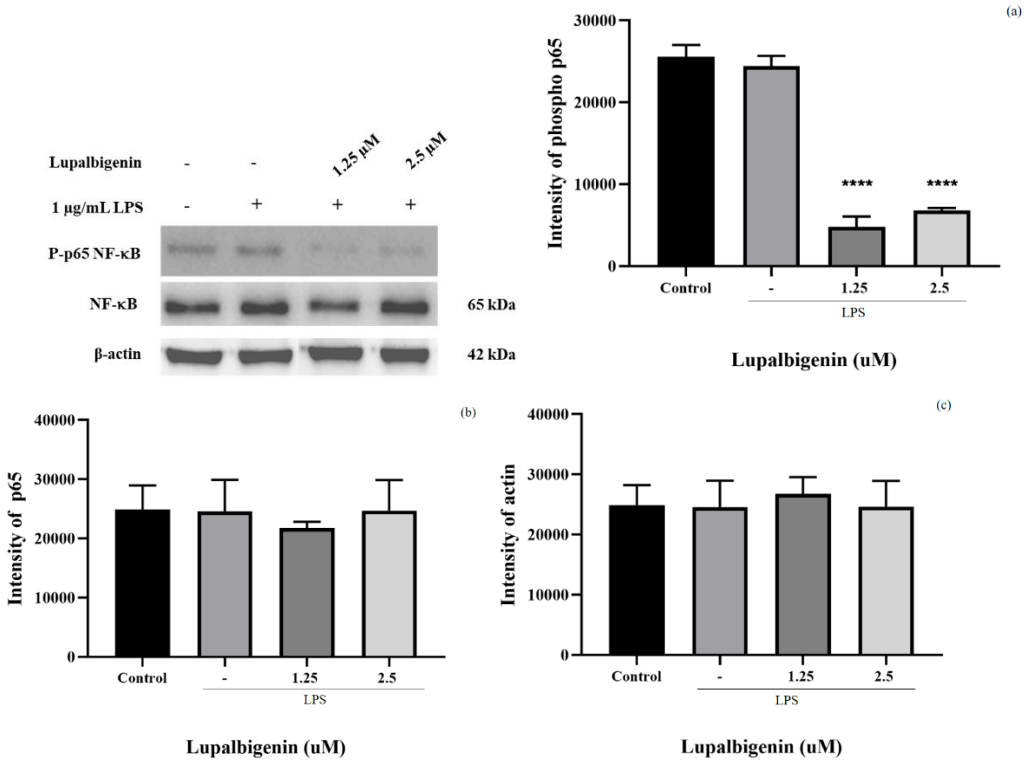


*FIGURE 37 Effect of lupalbigenin on NF-κB in LPS-stimulated in RAW 264.7 cells. Cells were pretreated with 1.25 and 2.5 µM of lupalbigenin for 1 h and then incubated with 1µg/mL LPS for 30 min. The total protein was then analyzed by Western blot. Protein intensity was quantified by Image J, (a) phosphorylated p65 (b) p65 and (c) β-actin. Bars indicate mean ± standard error of three independent experiments. **** $p < 0.001$ vs the LPS-induced only group.*

We also performed NF-κB visualized by using immunofluorescence to evaluate the effect of Lupalbigenin on its translocation. The result showed that Lupalbigenin inhibited p- NF-κB translocation from cytoplasm to nucleus. These results reveal that Lupalbigenin regulates the inflammatory response of Raw 264.7 cells by inhibiting the phosphorylation of p- NF-κB.

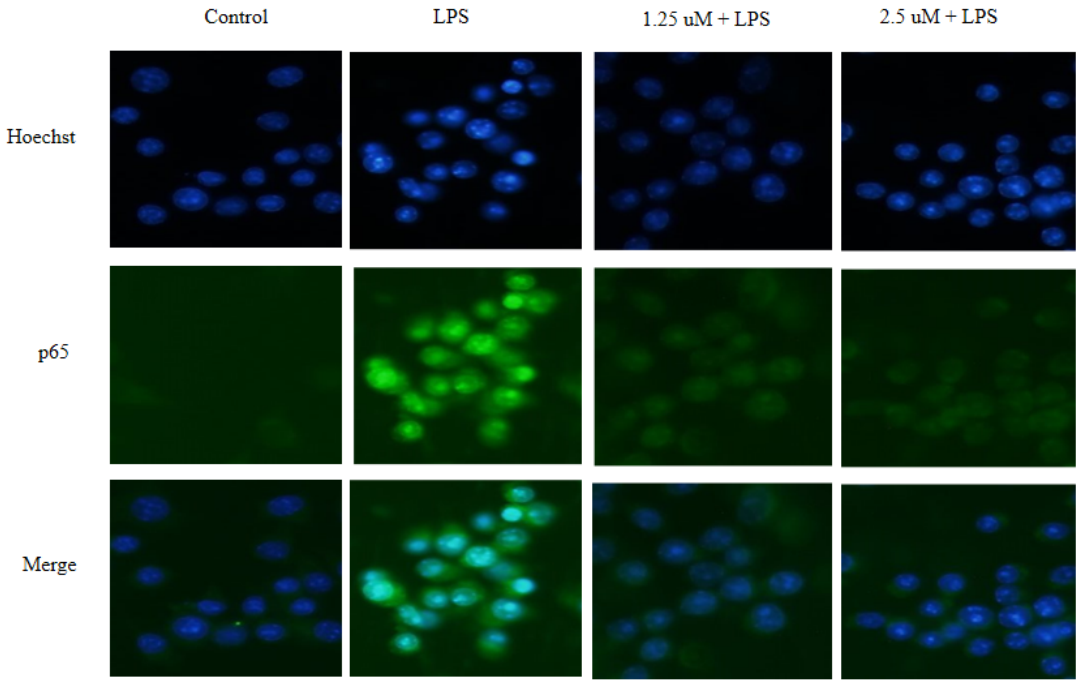


FIGURE 38 Lupalbigenin effectively inhibited NF-κB p65 translocation from cytoplasm to nucleus.NF-κB, p65 localization was visualized (green color) and Hoechst was used for nuclear staining (blue color) by immunofluorescence confocal microscopy (200x). The nuclear translocation of p65 in untreated (a), Raw cell with LPS only (b), pretreated 1 h with lupalbigenin 1.25 μM (c) and 2.5 μM (d) and then induced with LPS for 3 h. In condition with lupalbigenin treated cells were less appearances than in Raw cells with LPS-treated only.

4.14 Lupalbigenin inhibited P38 phosphorylation on MAKP pathway activation.

As MAPK protein is also crucial to Nitric oxide and pro-inflammatory cytokine production,therefore, a potential efficient target of Lupalbigenin, Raw cells were initially treated with Lupalbigenin for 1 h and later on stimulated with 1 ug/mL of LPS for 30 min. The phosphorylation form of ERK1/2 was increased at 1.25μM of Lupalbigenin treated whereas SAP/JNK phosphorylation slightly decreased when treated with 1.25 μM and 2.5 μM Lupalbigenin. Moreover lupalbigenin were markedly inhibited p38 phosphorylation in Raw 264.7 cells by LPS-induced as shown in figure 39.

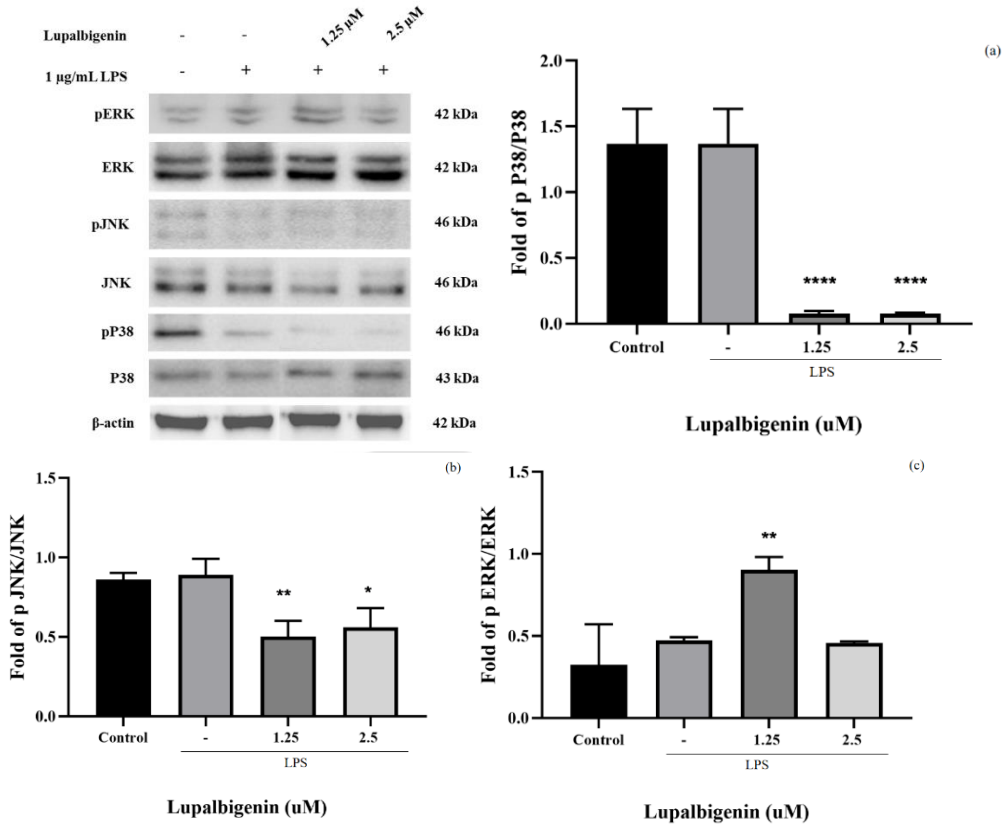


*FIGURE 39 Effect of lupalbigenin on LPS-induced phosphorylation of MAPKs pathway in RAW 264.7 cells. The cells were pretreated with 1.25 μM and 2.5 μM of lupalbigenin and then incubated with 1μg/mL LPS for 30 min. The whole-cell lysate was prepared for measurement of a phosphorylated and non-phosphorylated form of P-38, JNK and ERK1/2 by western blot analysis. The relative protein level was quantified by Image J and normalized to β-actin level (a), (b) and (c). Bars indicate mean ± standard error of three independent experiments. * p <0.05** p <0.005 , *** p ≤0.001 and **** p < 0.001 vs the LPS-induced only group.*

4.15 The effect of Genistein, lupalbigenin and 17β-estradiol against Phenylephrine, KCl, and U46619

Inhibitory Effect of genistein and lupalbigenin on to determine whether isoflavones effect on porcine splenic artery contraction. The tissues were pretreat contracted with vehicle control (DMSO), 30 μM genistein, 30 μM lupalbigenin and 30 μM 17β-estradiol. Tissues were then exposed to a cumulative concentration of phenylephrine (100nM-10μM), KCl (6mM-60mM) and U46619 (1nM-0.1μM). As a comparison, genistein has significantly inhibited contraction in the presence of phenylephrine and completely suppress tissue contraction with U46619 while 17β-estradiol has more potent in KCl present. Lupalbigenin, genistein derivative with 2 prenyl groups has slightly suppressed tissue contraction in the presence of U46619.

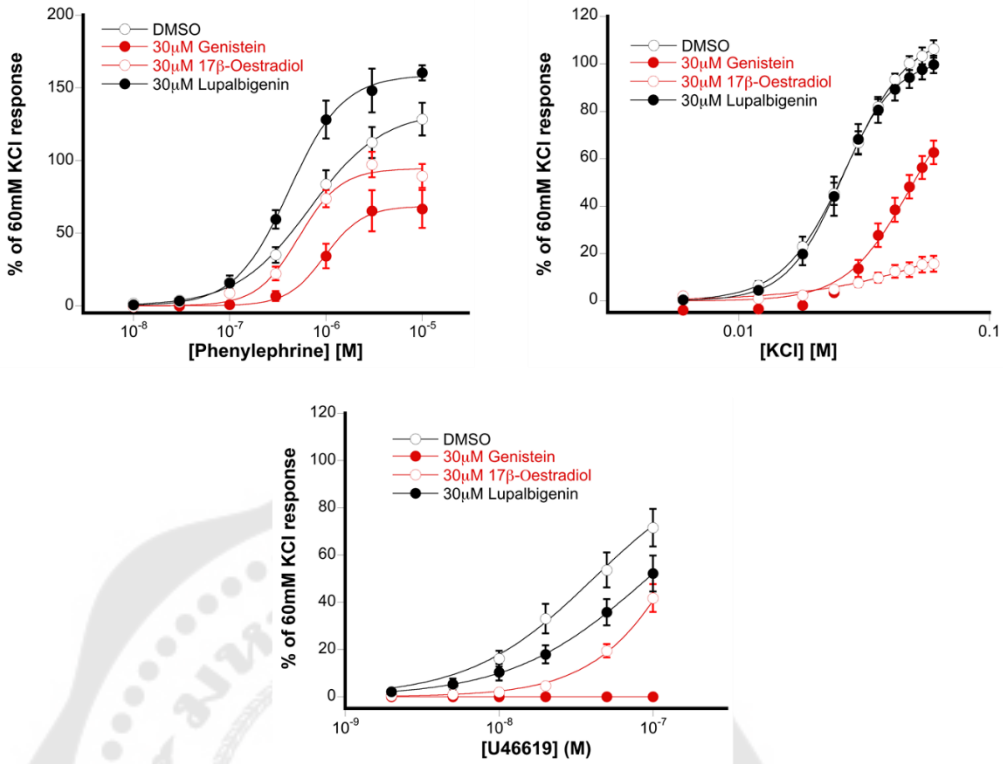


FIGURE 40 Contractile responses to phenylephrine, KCl, and U46619 in the porcine splenic artery. Contractions are expressed as a percentage of the response to 60 mM KCl and are means ± SEM of six experiments.

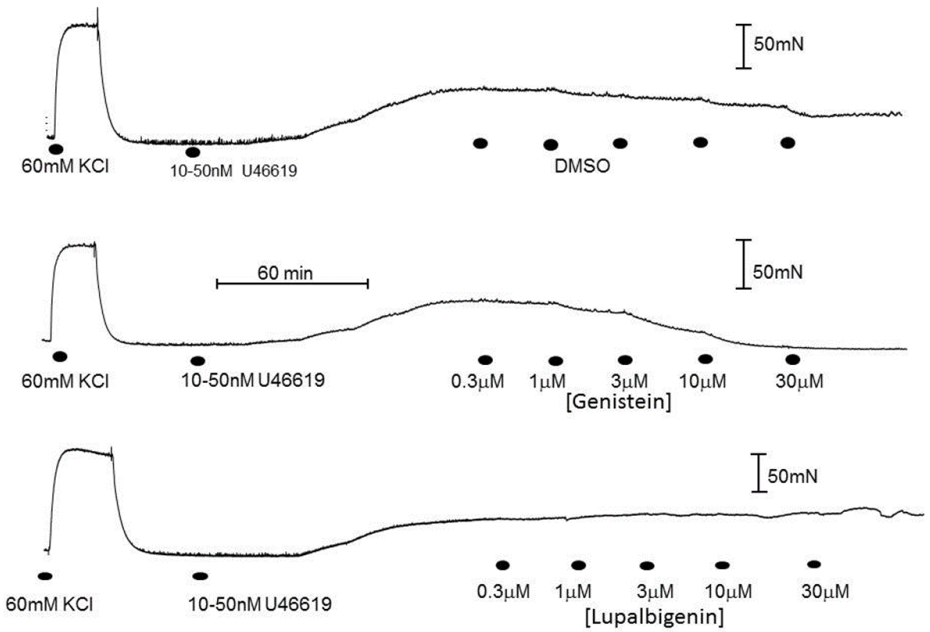


FIGURE 41 Representative trace recording of the effect of vehicle, DMSO, genistein and lupalbigenin on U46619-induced contractions of the porcine isolated splenic artery.

4.16 Relaxation effect of polyphenol with U46619-indued tone

Tissues were induce contracted with the thromboxane-mimetic U46619 (1-10nM) under these conditions was between 10% and 20% of the response to 60 mM KCl. Tissues were then exposed with a cumulative concentration of lupalbigenin, oestradiol, genistein and vehicle (DMSO). We also performed the study of fulvestrant , an estrogen receptor antagonist that can inhibit receptor dimerization[75], As results showed genistein has more efficiency to relaxed tone than oestradiol and lupalbigenin while fuvestrant has no effect with tissues contraction in porcine splenic artery.

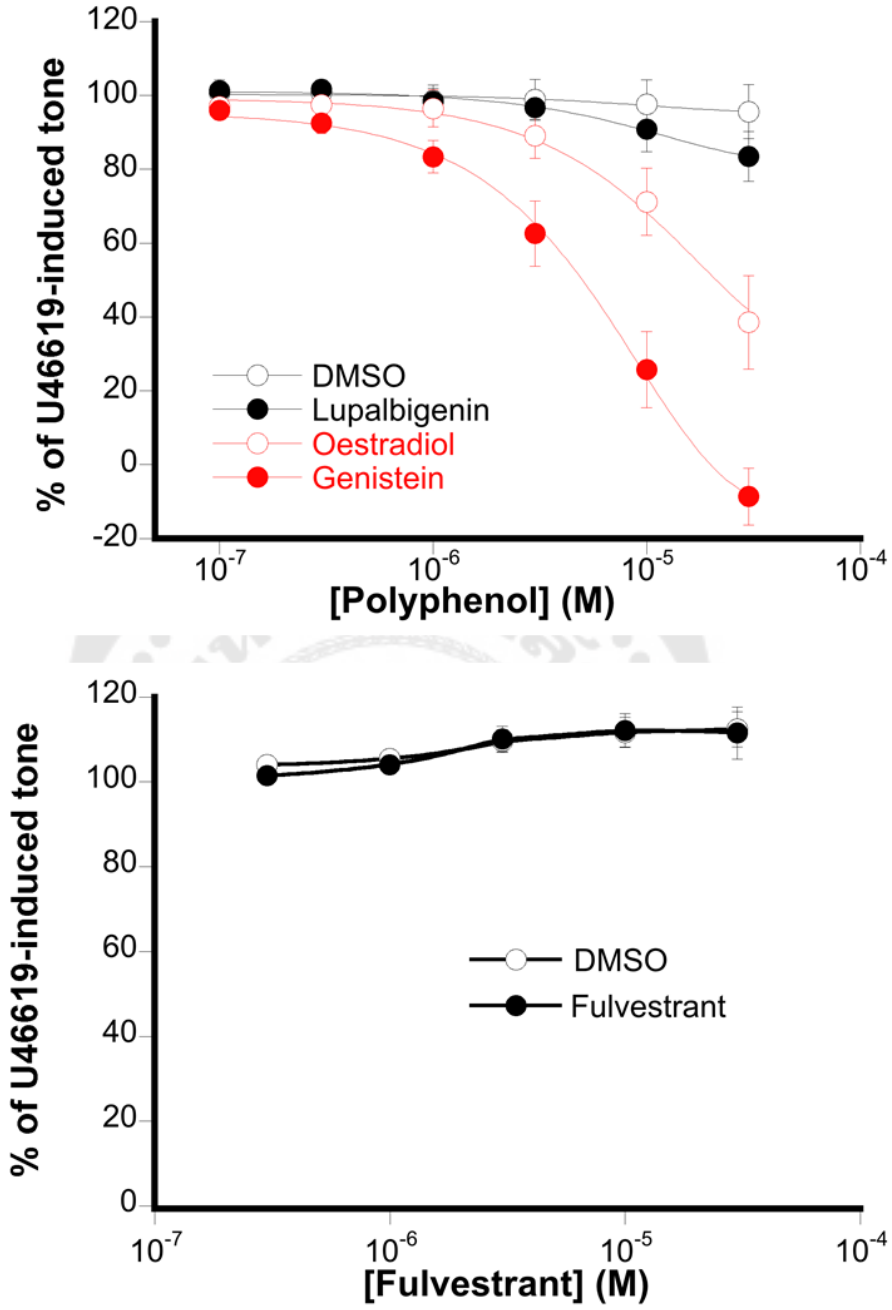


FIGURE 42 Characterization of relaxation with polyphenols in porcine splenic artery and relaxation of DMSO and Fulvestrant after pre-contracted with U46619. Means ± SEM of seventh experiments.

4.17 Effect of Fulvestrant against 17β-estradiol and lupalbigenin against genistein

Tissues were induce contracted with the thromboxane-mimetic U46619 (1-10nM) under these conditions was between 10% and 20% of the response to 60 mM KCl. Tissues were then exposed with a cumulative concentration of 17β-estradiol in with and without 10 μM fulvestrant and the other tissue was exposed a cumulative concentration of genistein with and without 30 μM lupalbigenin. The results showed fuvestrant doesn't have any effect with 17β-estradiol in porcine splenic artery as same as lupalbigenin and genistein.

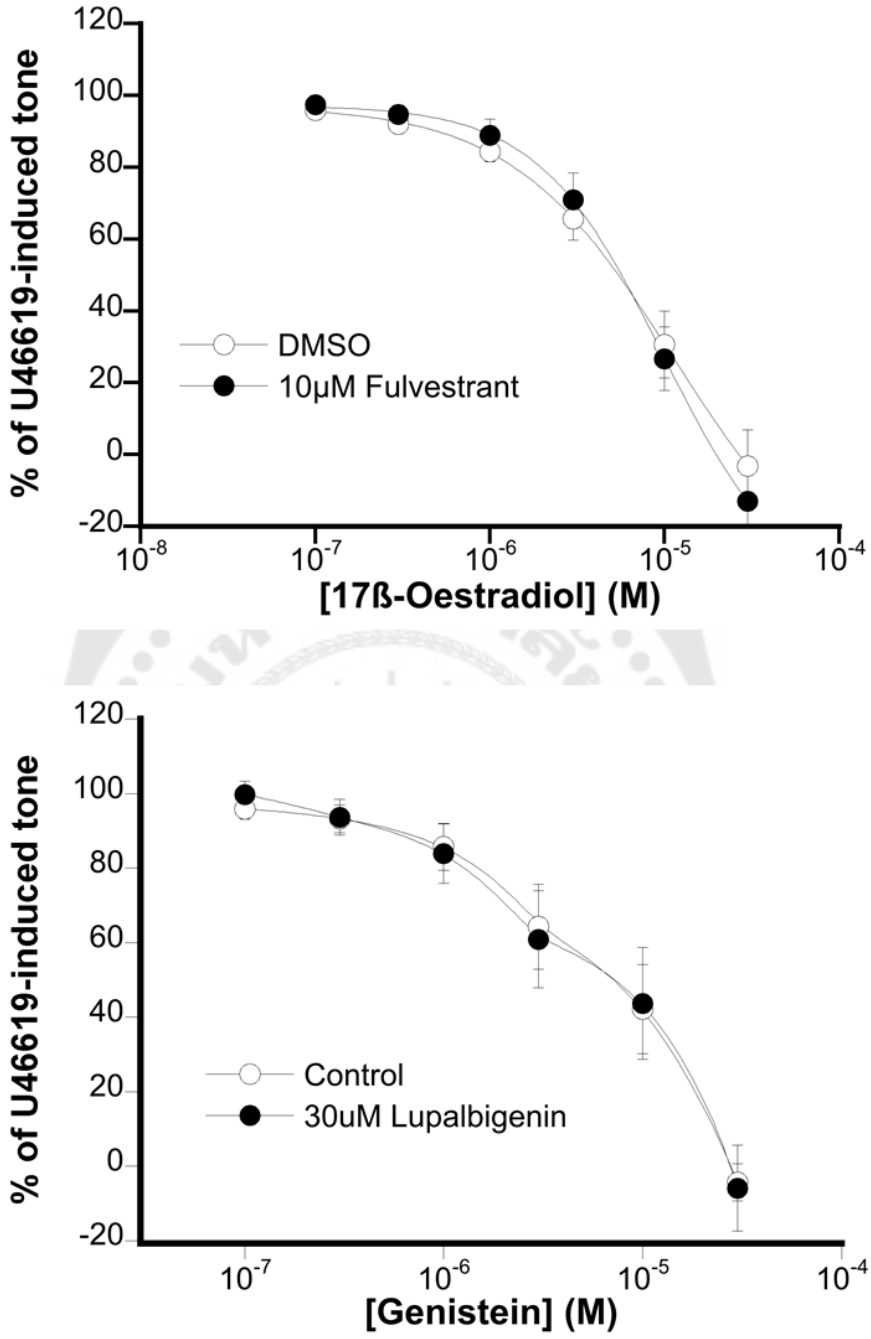


FIGURE 43 Relaxation of 17β-estradiol in the present of 10 μM fulvestrant and genistein in the present of 30 μM of lupalbigenin after pre-contracted with U46619. Means ± SEM of seventh experiments.

4.18 Effect of Ca^{2+} free and single concentration of genistein, oestradiol, fulvestrant, and lupalbigenin in porcine splenic artery contraction by over long period.

Before each experiment, the tissues were contracted at least three times with 60 mM KCl, until the final two responses to KCl differed by less than 10%. Contractile responses were expressed as a percentage of the final KCl response. Each tissue were then exposed with normal K-H, Ca^{2+} free K-H, DMSO, 30 μM genistein and 30 μM lupalbigenin suspended in a bath containing Krebs-Henseleit buffer (no Ca^{2+} in Ca^{2+} free condition) maintained at 37°C and constantly gassed with 95% O_2/5% CO_2 for 14 h. The results showed that no contraction of tissue in the condition of without Ca^{2+} similarly at the tissue exposed with the presence of 30 μM genistein. Surprisingly, fulvestrant in the first period of incubation had suppressed contraction but after 5 hours increased contraction instead while 30 μM lupalbigenin slightly induced contraction after 5 hours of incubation time.

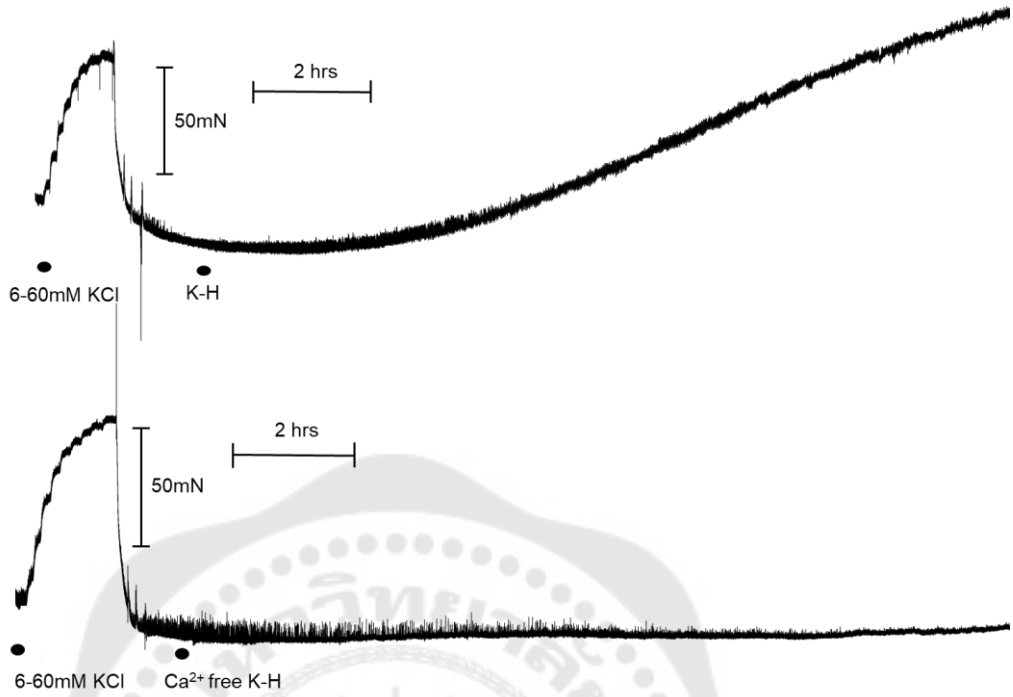


FIGURE 44 Representative trace recording of the effect of Ca^{2+} fee K-H in porcine splenic artery in 14 h. period

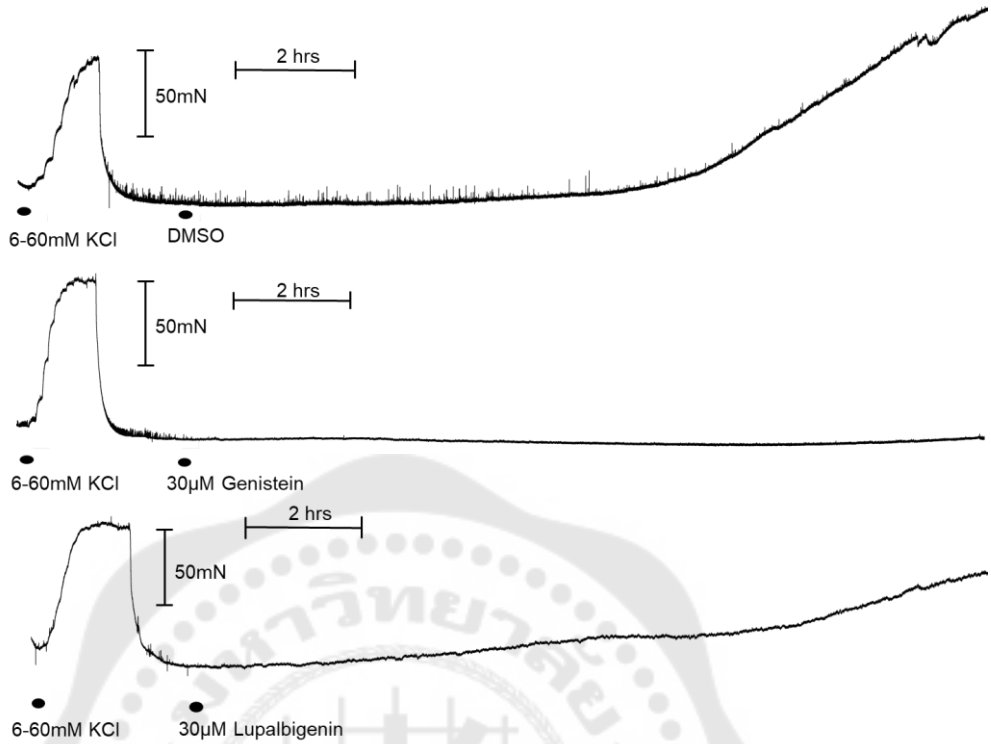


FIGURE 45 Representative trace recording of the effect of genistein, oestradiol and lupalbigenin in porcine splenic artery in 14 h. period

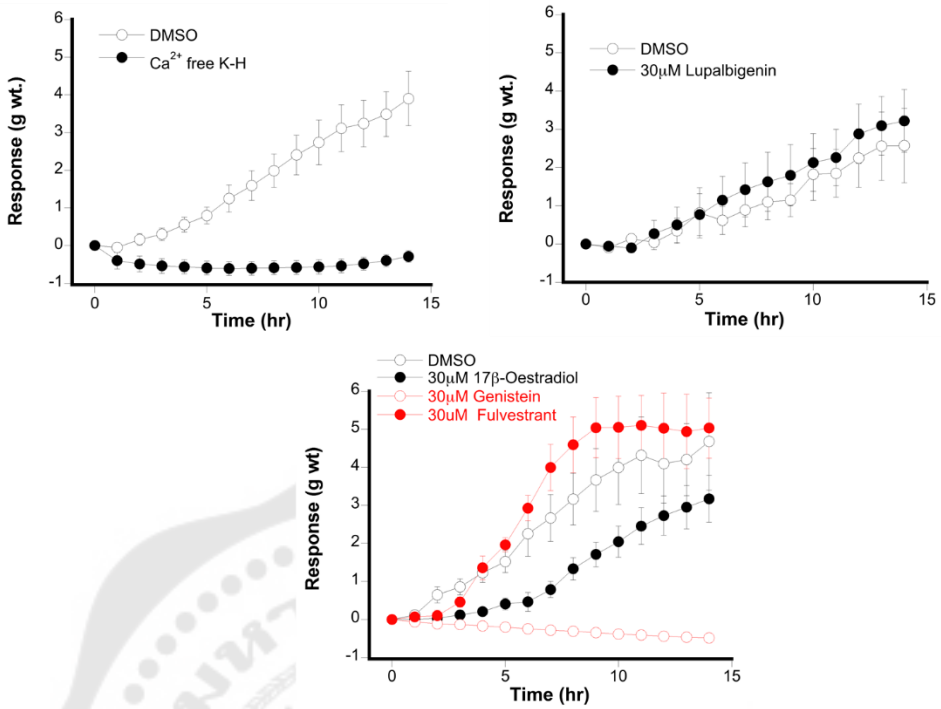
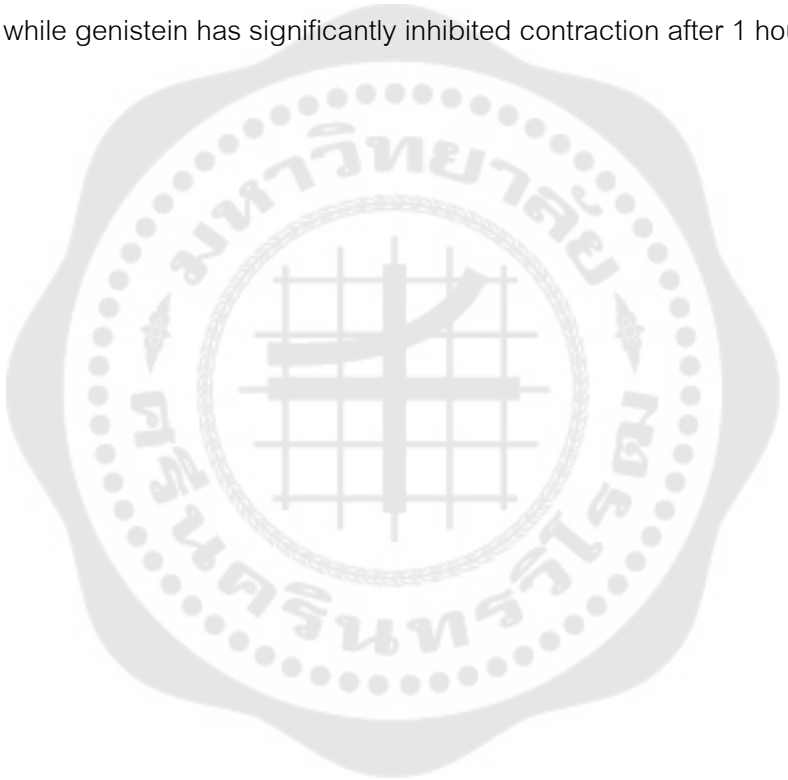


FIGURE 46 Contraction response curves of K-H, K-H Ca^{2+} free, genistein, oestradiol and lupalbigenin for 14 h incubation. The Contractions are expressed as (g.wt) against time incubation (h) and are means ± SEM of 4-8 observations depend on the experiment.

4.19 Effect of differences concentration of genistein and 17β-estradiol in porcine splenic artery contraction by over long period.

We performed a study of contraction response in porcine splenic artery with 1 μM, 3 μM, and 10 μM for genistein and with 3 μM, 10 μM, and 30 μM for 17β-estradiol to clarify how effect of tissue contraction related with concentration. As the results, after 7 h of incubation 17β-estradiol showed suppressed contraction by dose-dependent until 14 hours while genistein has significantly inhibited contraction after 1 hour of incubation.



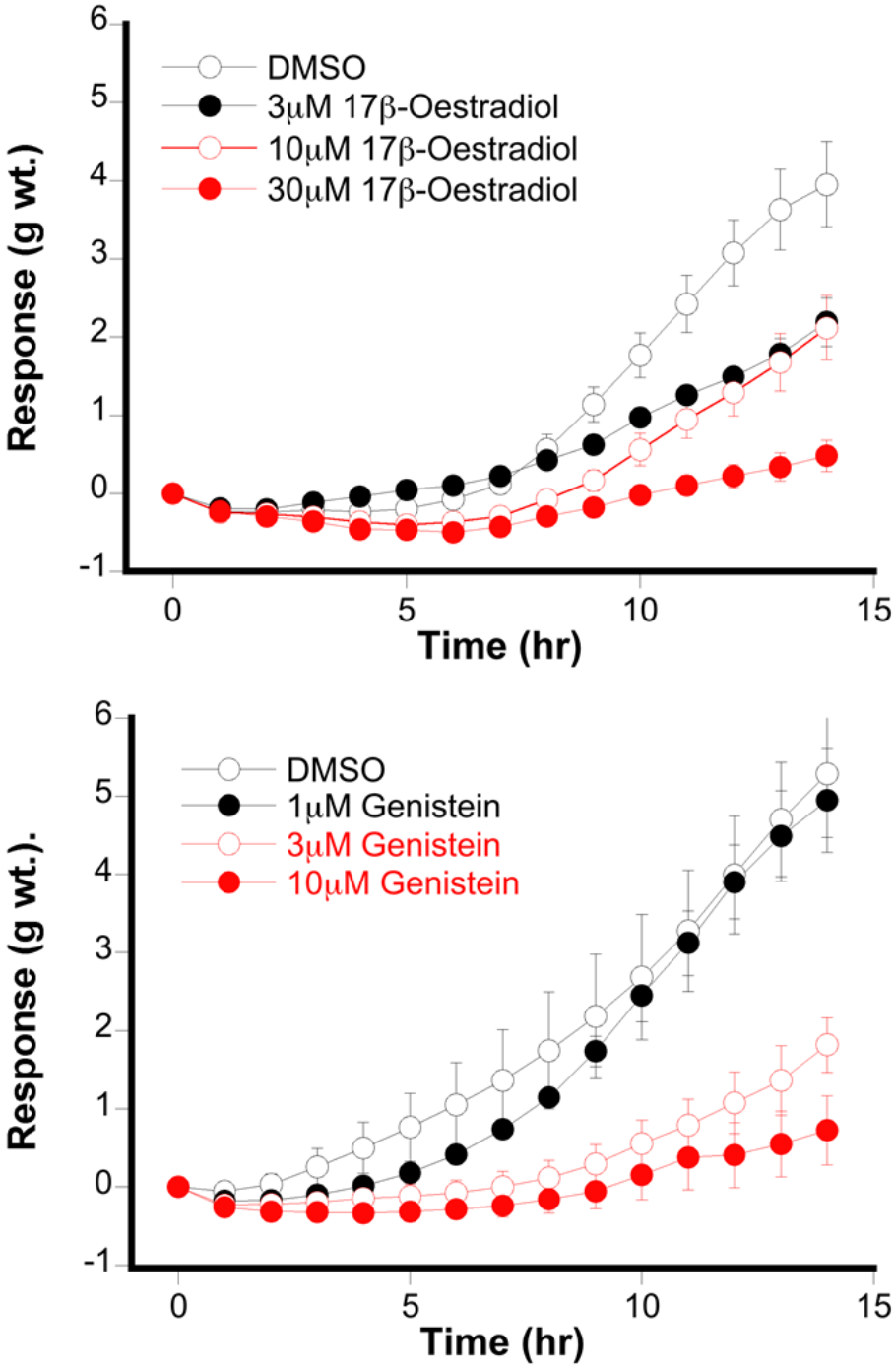


FIGURE 47 Effects of dependent contraction curves of 17β-estradiol and genistein on 14 h incubation period in porcine splenic artery. n = 6. Contractions are expressed as (g.wt) againt time incubation (h) and are means ± SEM of six experiments.

4.20 Effect of genistein and 17β-estradiol against fulvestrant and co treated genistein & lupalbigenin in porcine splenic artery contraction by over long period.

As summarized in Figure 48, 10 μM of 17β-estradiol and 3 μM of genistein were chosen to study with and without fulvestrant. The presence of 1 μM fulvestrant with 3 μM of genistein showed constantly interfered genistein suppressed contraction from 4 hours of incubation while 1 μM fulvestrant with 10 μM of 17β-estradiol had effected from 4 hours until 11 hours.

As the results of 30 μM of lupalbigenin don't have any effect of vascular smooth muscle in porcine splenic artery we are wondering lupalbigenin may act as an estrogen receptor antagonist. Therefore, we performed the tissue incubated with 3 μM of genistein with and without 30 μM lupalbigenin. Surprisingly, we found lupalbigenin encouraged genistein suppressed tissue contraction constantly from 3 hours of incubation.

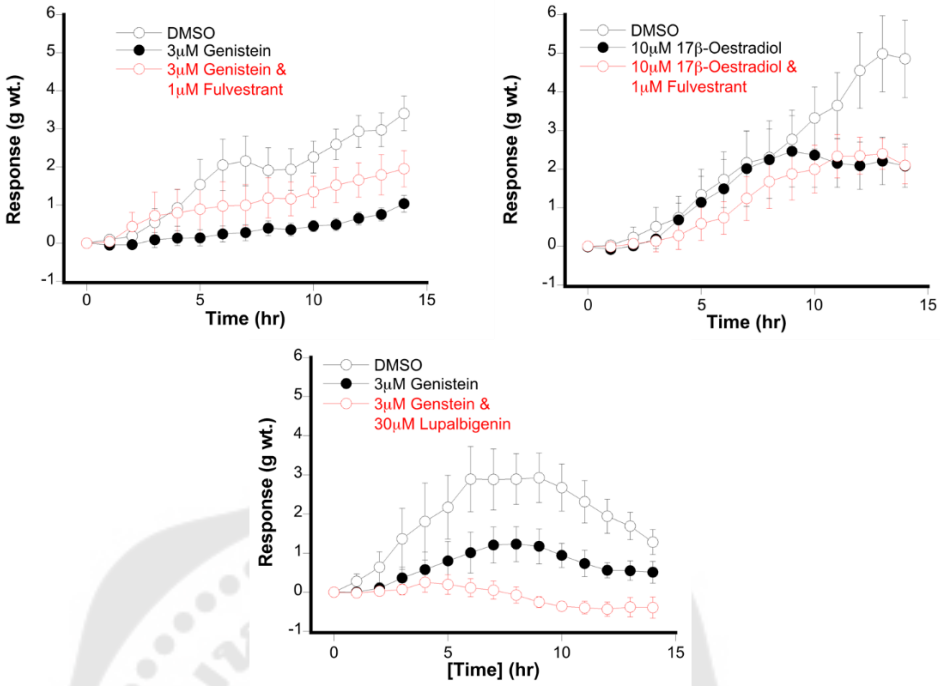


FIGURE 48 Effect of 14 h incubation of genistein and 17β-estradiol with and without fulvestrant and lupalbigenin encouraged genistein suppressed tissue contraction. Contractions are expressed as (g.wt) againt time incubation (h) and are means ± SEM of 6-10 observations depend on the experiment.

Chapter 5

Discussion

According to previous research, as we know *Derris Scandens* extract in the organic solvent has demonstrated anti-cancer and oxidative stress in vitro[70, 71]and some research showed anti-inflammatory activity in the patient by oral administrating used[76]. However, this is the first study we performed in *vitro* model by using LPS induced inflammation with Raw 264.7 macrophages cell, moreover, the three different solvents we were used are sunflower oil, light mineral oil, and rice bran oil, general solvent formative to producing lotion and cream, to extract the stem of *Derris Scanden* Benth. All samples were provided from Bangkok Lab and Cosmetics, Co. Ltd., who supported part of my Ph.D. scholarship.

The objective of the study is to investigate the anti-inflammatory activity of *DS* in three different solvents in molecular biology and choosing the best one to be part of product formative to allowed the customer to be confident and evaluate traditional herbal research turn to economical approaching.

Cell viability results showed 10, 50 and 100 µg/mL of DS extract and their solvent don't have any cytotoxicity with RAW 264.7 cell after 24 h incubation. An anti-oxidative stress agent was determined by DPPH assay which can represent by the percentage of scavenging to normalize oxidative agent. *DS* in sunflower oil (DSF) seems to be a good anti-oxidant as L-ascorbic acid which showed increased % scavenging by dose-dependent manner whereas *DS* in light mineral oil (DSL) also shown antioxidant activity. The % scavenging of DS in rice bran oil (DSR) showed the negative value that could be mean rice bran oil can extract lots of oxidative unknown compounds, unfortunately we can't identify with using our lab equipments. However, to confirm a good representation of antioxidant of *DS* in sunflower oil should perform specific internal antioxidant cell detection such as ROS to convince the resulting weather *DS* in sunflower oil dose and may be useful for further study.

Nitric oxide (NO) determination by using Griess reagent is a well-known anti-inflammatory screening process that has been used to identify whether the compound has activity or not. We collected media of cells after treated with LPS for 24 h to measure NO in 96-well Elisa plate at 540 nm absorbance versus $NaNO_2$ standard. The result of macrophages cell was pretreated with *DS* extract and their solvent only founded that at 100 µg/mL were increased NO level more than 50 µg/mL. On the other hand, the result of *DS* extracts with LPS shown NO inhibitory activity in DS in sunflower oil (DSF), sunflower oil, and DS in rice bran oil (DSR). These results allowed us to work continued in a molecular pathway with DS extract.

We decided to continued work with *DS* in sunflower oil (DSF), *DS* in rice bran oil (DSR) and their solvent because the result from NO detection was clearly inhibited. *DS* in sunflower oil and sunflower oil [(74)] only showed high effectiveness inhibit iNOS and TNF-α inhibition this might be indicated both of them could act as a potential anti-inflammatory agent. Additionally, *DS* in rice bran oil also demonstrated partial of iNOS and TNF-α and clearly that might from the effect of *Derris Scanden* extract in while doesn't have any inhibited in rice bran oil. Unfortunately, we couldn't provide the experiment to distinguish the work compound from *DS* extracts in sunflower oil and rice bran oil.

As we mentioned from the literature review, another inflammatory stimulus to detect inducible inflame cell change and work wide is COX-2. We detected the COX-2 protein expression level by western blotting. At 100 µg/mL of DS in sunflower oil, DS in rice bran oil and their solvent were pretreated with cells before incubated with 1 µg/mL of LPS were added. The result showed DS extract slightly decreased COX-2 expression comparing with LPS only.

The second part of the thesis was about purified compounds from 50% Ethanol aqueous stem of *Derris Scandense* Benth extract until we were able to achieve a pure form of lupalbigenin for use in this research which took nearly 50% of the time period of my thesis dissertation. Since lupalbigenin has only been found in the last ten years,

there is not a great deal of research involving molecular signaling pathway whether in a cancer approach or anti-inflammatory activity. In this study, we initiated our research.

We first started to optimize a non-toxic concentration of lupalbigenin using cell viability assay by MTT and also with cell morphology change by microscopic determination. Both methods showed that lupalbigeine with concentrations lower than 6 μM did not show any cytotoxicity activity with Raw 264.7 cells. Therefore we focused further study on the investigation of anti-inflammatory activity of lupalbigenin in lower concentrations which had not been investigated before.

Nitric oxide (NO) determination by using Griess reagent is a well-known anti-inflammatory screening process that has been used to identify whether the compound has activity or not. The principal to detect NO secretion in medium from LPS-induced cell change demonstrated a significant increase between untreated cells and cells with LPS-induced. As we see from the results lupalbigenin has an effect on nitric oxide induction as a dose-dependent manner. However, the result from cell viability indicated that from 12.5 μM of lupalbigenin and above are cytotoxic in Raw 264.7 cells which is similar to a previous study[77].

According to the results of NO inhibition to investigate on inflammatory gene and protein expression, we performed the real-time RT-PCR to study the expression level of iNOS, IL-6, and TNF-α, those are inducible cytokine and secretion when the cells are stimulated by infectious or pain. LPS can be markedly induced the expression of iNOS, IL-6 and TNF-α in Raw cells compared with control or untreated whereas lupalbigenin had shown the inhibition differently upon the concentration. iNOS gene expression results of lupalbigenin 1.25-75 μM showed that non-significant inhibition whereas 1.25 μM has significantly inhibited either IL-6 and TNF-α gene expression. COX-2, iNOS and TNF-α protein expression by western blotting were performed next stage, the expression of TNF-α and COX-2 with 6 μM of lupalbigenin doesn't seem has any

inhibited in the other hand could be slightly induced the expression of them whereas 1.25 and 2.5 μM showed significantly negative regulation of inflammatory protein expression.

In term of the high concentration results of lupalbigenin seem to increased nitric oxide, COX-2 and TNF-α that may cause from increasing of lipophilic in cell regarding to its has 2 prenyl group while genistein doesn't so that while lupalbigenin showed anti-inflammatory activity at low concentration rather than high.

To confirm the up steam of pro-inflammatory expression, Nuclear factor-Kappa B (NF-κB) is a crucial pathway to regulate the transcription of inflammatory cytokines and mediators during the inflammatory process activated by LPS stimulation. Once activated, NF-κB can induce collaboratively the expression of pro-inflammatory cytokine genes and the release of cytokines under the inflammatory process. Therefore, molecule or chemical targeting of NF-κB signaling pathways are considered to be a potential. The results from western blotting showed 1.25 μM and 2.5 μM of lupalbigenin significantly inhibited the phosphorylation of p65 which cannot continue the process of inflammatory gene transcription and protein translation eventually. Moreover the visualizing of NF-κB to detect the translocation from cytoplasm to nuclease has been performed in the first time to study with lupalbigenin. As a result, the LPS-stimulated cells shown the high capacity of NF-κB translocation while lupalbigenin can be an obvious decrease of the translocation.

Mitogen-activated protein kinase (MAPK) pathway containing three sequentially activated protein kinases are key components of a series of vital signal transduction pathways that regulate processes such as cell proliferation, cell differentiation, and cell death in eukaryotes from yeast to humans). MAPK regulates inflammatory response via p38 is involved in regulating the expression of iNOS and TNF-α gene in the macrophages whereas ERK and JNK are involved in regulations of pro-

inflammatory cytokine and iNOS. Results of the present study showed that low concentration of lupalbigenin inhibited the LPS-induced phosphorylation of JNK and especially in marked on phosphorylation of p38 from these may also suggest lupalbigenin could be effective to protecting cell death from LPS infected.

This study also performed the experiments with vascular smooth muscle contraction in porcine splenic artery of lupalbigenin compared with genistein and oestradiol ,17β-estradiol . The results of the short term incubation period showed lupalbigenin had slightly decreased with U46619 constrictor agent while contracts the tissue by phenylephrine and KCl has not seen any effect. For the over long period experiments, lupalbigenin demonstrated no vasodilation activity while genistein showed strongly suppressed vascular smooth muscle contraction similar to the results from incubated tissue 14 hours with Ca^{2+} K-H. It seems obvious lupalbigenin doesn't have the effect with nitric oxide production in Raw cell indued with LPS and no activity for vasodilator in vascular smooth muscle in porcine splenic artery, these may be because lupalbigenin act as an estrogens receptor antagonist, therefore, we performed the co-treatment the tissue with genistein and lupalbigenin. Surprisingly, we found lupalbigenin encouraged genistein suppressed tissue contraction in overlong period incubation. However, to predicted the activity of genistein inhibited the vascular smooth muscle in porcine splenic artery can not be confirmed because the result from 17β-estradiol against fulvestrant doesn't significantly inhibit.

As the previous study about lupalbigenin or diphenyl genistein has more effect with α- receptor in estrogenic affect our result may support the activity of lupalbigenin could interfere vascular smooth muscle via estrogen receptor and moreover, the of genistein suppressed vascular contraction may be possible involving either decreased Ca^{2+} influx in the tissue or by inhibited estrogen receptor. To identify both activities of genistein and lupalbigenin should preceded the experiment more specific in cell culture receptor antagonist or study with the other prenylated group of the other isoflavone.

Conclusion

Derris Scanden Benth. extract in three different solvents including in sunflower oil [74] , light mineral oil (LMO), and rice bran oil (RBO) all of them is a well-known cosmetic industry to use as a major ingredient to produce lotion and cream. This is the first time to study DS in a cosmetic solvent to investigate anti-inflammatory activity in the molecular study.

The high potential to be an anti-inflammation agent and should be a candidate to mixed with cream for topping is *DS* in Sunflower oil because it showed clearly inhibited NO and COX-2 expression which is involved cell signaling inflammation. However, only sunflower oil also showed similarly, this might be referred to there are promoted each other and seem interesting in defining this hypothesis by performing a technique to separate compound from them.

DS in rice bran oil (DSR) had shown anti-inflammatory activity less than as DS in sunflower oil (DSF) but the results were clearly the inhibition was from *DS* extract by using rice bran oil.

Lupalbigenin has been purified from the 50% Ethanol aqueous stem of *Derris Scandense* Benth. In the past over ten years do not have much research about the anti-inflammatory activity of *Derris Scandense* for *in vitro* whether purified form or crude extract, this is a first letter to start form purify compound until getting the hits and continually worked through the anti-inflammatory activity on the molecular and signaling pathway in Raw 264.7 macrophages cells. The result from cell viability, cell morphology change protection, and NO inhibition was indicated at the use of 6 μM and below had a potential to protecting harmed cells form LPS stimuli and have an effect to be a nitric oxide inhibitory agents. At 1.25 and 2.5 μM of Lupalbigenin could be work as a strong drug to control down-regulation of COX-2, and TNF-α protein expression and IL-6 and TNF-α gene expression level. Lupalbigenin could be the potential to inhibit the upstream of inflammatory activity control via NF-κB translocation and MAPKs by down-regulate the phosphorylation of p38 and JNK in LPS-induced Raw 264.7 macrophages cells. Lupalbigenin doesn't inhibit nitric oxide production but could be inhibiting the other gene

and protein of inflammation. This is the first time to study the effect of lupalbigenin with vascular smooth muscle in porcine splenic artery and demonstrated interesting results between lupalbigenin co-treated with genistein in over long period incubation. The study about estrogen receptor more specific with lupalbigenin, genistein and 17β-estradiol should be performed in the future.

REFERENCES

1. Chavalittumrong P. Chronic toxicity study of crude extract of Derris scandens Benth. JSciTechnol. 1999(21):425-33.

2. Chuthaputti A. Immunomodulating activity of Derris scandens Benth. . Thai J Pharm Sci. 1998(22):137-48.

3. Jansakul C. Some pharmacological studies of a hypothensive fraction from Derris scandens J Sci Soc Thailand. 1997(23):323-34.

4. Chuankamnerdkarn M, Sutthivaiyakit S, Thasana N, Pisutjaroenpong S. Prenylated Isoflavones from Derris scandens2002. 1901-6 p.

5. Rukachaisirikul V, Sukpondma Y, Jansakul C, Taylor WC. Isoflavone glycosides from Derris scandens. Phytochemistry. 2002;60(8):827-34.

6. Sekine T, Inagaki M, Ikegami F, Fujii Y, Ruangrungsi N. Six Diprenylisoflavones, Derrisisoflavones A—F, from Derris scandens1999. 87-94 p.

7. Rao MN. Four isolfavones and two 3-aryl coumarins from stems of Derris scandens. Phytochemistry. 1994;37(1):267-69.

8. P. Falshaw C, A. Harmer R, D. Ollis W, E. Wheeler R, R. Lalitha V, V. Subba Rao N. Natural occurrence of 3-aryl-4-hydroxycoumarins. Part II. Phytochemical examination of Derris scandens (Roxb.) Benth1969.

9. Johnson AP, Pelter A, Stainton P. Extractives from Derris scandens. Part I. The structures of scandenin and lonchocarpic acid. Journal of the Chemical Society C: Organic. 1966(0):192-203.

10. Pelter A, Stainton P. The extractives from Derris scandens. Part II. The isolation of osajin and two new isoflavones, scandenone and scandinone. Journal of the Chemical Society C: Organic. 1966(0):701-4.

11. P. Johnson A, Pelter A. The structure of robustic acid, a new 4-hydroxy-3-phenylcoumarin1966.

12. Ganapaty S, Sumitra Josaphine J, Steve Thomas P. Anti-Inflammatory Activity of Derris scandens. Journal of Natural Remedies; Volume 6, Issue 1, January 2006. 2006.

13. Libby P. Inflammatory mechanisms: the molecular basis of inflammation and disease. Nutr Rev. 2007;65(12 Pt 2):S140-6.

14. Bakewell L, Burdge GC, Calder PC. Polyunsaturated fatty acid concentrations in young men and women consuming their habitual diets. The British journal of nutrition. 2006;96(1):93-9.

15. Soehnlein O. Direct and alternative antimicrobial mechanisms of neutrophil-derived granule proteins. Journal of molecular medicine (Berlin, Germany). 2009;87(12):1157-64.

16. Feghali CA, Wright TM. Cytokines in acute and chronic inflammation. Frontiers in bioscience : a journal and virtual library. 1997;2:d12-26.

17. Iwalewa EO, McGaw, L. J., Naidoo, V., & Eloff, J. N. Inflammation: the foundation of diseases and disorders. A review of phytomedicines of South African origin used to treat pain and inflammatory conditions. African Journal of Biotechnology. 2007;6(25):2868-85.

18. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. Annual review of biochemistry. 2000;69:145-82.

19. Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, et al. Cyclooxygenase in biology and disease. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 1998;12(12):1063-73.

20. Smyth EM, Grosser T, Wang M, Yu Y, FitzGerald GA. Prostanoids in health and disease. Journal of lipid research. 2009;50 Suppl:S423-8.

21. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. Arterioscler Thromb Vasc Biol. 2011;31(5):986-1000.

22. Zhang J-M, An J. Cytokines, inflammation, and pain. Int Anesthesiol Clin. 2007;45(2):27-37.

23. Arvin B, Neville LF, Barone FC, Feuerstein GZ. The Role of Inflammation and Cytokines in Brain Injury. Neuroscience & Biobehavioral Reviews. 1996;20(3):445-52.

24. Spranger J, Kroke A, Möhlig M, Hoffmann K, Bergmann MM, Ristow M, et al. Inflammatory Cytokines and the Risk to Develop Type 2 Diabetes. Diabetes. 2003;52(3):812.

25. Lotz M. The role of nitric oxide in articular cartilage damage. Rheumatic diseases

clinics of North America. 1999;25(2):269-82.

26. Baldwin AS, Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annual review of immunology. 1996;14:649-83.

27. Nomura Y. NF-kappaB activation and IkappaB alpha dynamism involved in iNOS and chemokine induction in astroglial cells. Life sciences. 2001;68(15):1695-701.

28. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nature immunology. 2001;2(8):675-80.

29. Zandi E, Chen Y, Karin M. Direct phosphorylation of IkappaB by IKKalpha and IKKbeta: discrimination between free and NF-kappaB-bound substrate. Science (New York, NY). 1998;281(5381):1360-3.

30. Lewis TS, Shapiro PS, Ahn NG. Signal transduction through MAP kinase cascades. Advances in cancer research. 1998;74:49-139.

31. Fukunaga R, Hunter T. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. The EMBO journal. 1997;16(8):1921-33.

32. Waskiewicz AJ, Flynn A, Proud CG, Cooper JA. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. The EMBO journal. 1997;16(8):1909-20.

33. Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, et al. ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell. 1991;65(4):663-75.

34. Todd JL, Tanner KG, Denu JM. Extracellular regulated kinases (ERK) 1 and ERK2 are authentic substrates for the dual-specificity protein-tyrosine phosphatase VHR. A novel role in down-regulating the ERK pathway. The Journal of biological chemistry. 1999;274(19):13271-80.

35. Weston CR, Davis RJ. The JNK signal transduction pathway. Current opinion in genetics & development. 2002;12(1):14-21.

36. Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, et al. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science

(New York, NY). 2000;288(5467):870-4.

37. Ono K, Han J. The p38 signal transduction pathway: activation and function. Cellular signalling. 2000;12(1):1-13.

38. Bergmann M, Hart L, Lindsay M, Barnes PJ, Newton R. IkappaBalpha degradation and nuclear factor-kappaB DNA binding are insufficient for interleukin-1beta and tumor necrosis factor-alpha-induced kappaB-dependent transcription. Requirement for an additional activation pathway. The Journal of biological chemistry. 1998;273(12):6607-10.

39. Nick JA, Avdi NJ, Young SK, Lehman LA, McDonald PP, Frasch SC, et al. Selective activation and functional significance of p38alpha mitogen-activated protein kinase in lipopolysaccharide-stimulated neutrophils. J Clin Invest. 1999;103(6):851-8.

40. Kotlyarov A, Neininger A, Schubert C, Eckert R, Birchmeier C, Volk HD, et al. MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. Nature cell biology. 1999;1(2):94-7.

41. Wang G, Jacquet L, Karamariti E, Xu Q. Origin and differentiation of vascular smooth muscle cells. The Journal of physiology. 2015;593(14):3013-30.

42. Webb RC. SMOOTH MUSCLE CONTRACTION AND RELAXATION. Advances in Physiology Education. 2003;27(4):201-6.

43. Dehaini H, Fardon M, Abou-Saleh H, Elyazbi A, Eid A, Eid A. Estrogen in vascular smooth muscle cells: A friend or a foe? Vascular Pharmacology. 2018.

44. Gencel VB, Benjamin MM, Bahou SN, Khalil RA. Vascular effects of phytoestrogens and alternative menopausal hormone therapy in cardiovascular disease. Mini Rev Med Chem. 2012;12(2):149-74.

45. Morito K, Hirose T, Kinjo J, Hirakawa T, Okawa M, Nohara T, et al. Interaction of phytoestrogens with estrogen receptors alpha and beta. Biological & pharmaceutical bulletin. 2001;24(4):351-6.

46. Patisaul HB. Endocrine disruption by dietary phyto-oestrogens: impact on dimorphic sexual systems and behaviours. Proc Nutr Soc. 2017;76(2):130-44.

47. Patisaul HB, Jefferson W. The pros and cons of phytoestrogens. Front Neuroendocrinol. 2010;31(4):400-19.

48. Rincón-León F. FUNCTIONAL FOODS. In: Caballero B, editor. Encyclopedia of Food Sciences and Nutrition (Second Edition). Oxford: Academic Press; 2003. p. 2827-32.

49. Cross J. The genetics of pre-eclampsia: A feto-placental or maternal problem? Clinical genetics. 2003;64:96-103.

50. Murphy JG, Khalil RA. Decreased [Ca(2+)](i) during inhibition of coronary smooth muscle contraction by 17beta-estradiol, progesterone, and testosterone. The Journal of pharmacology and experimental therapeutics. 1999;291(1):44-52.

51. Ma Y, Cheng WT, Wu S, Wong TM. Oestrogen confers cardioprotection by suppressing Ca2+/calmodulin-dependent protein kinase II. Br J Pharmacol. 2009;157(5):705-15.

52. Speroni F, Rebolledo A, Salemme S, Roldan-Palomo R, Rimorini L, Anon MC, et al. Genistein effects on Ca2+ handling in human umbilical artery: inhibition of sarcoplasmic reticulum Ca2+ release and of voltage-operated Ca2+ channels. Journal of physiology and biochemistry. 2009;65(2):113-24.

53. Torregrosa G, Burguete M, x, a C, Pérez-Asensio FJ, Salom JB, et al. Pharmacological profile of phytoestrogens in cerebral vessels: in vitro study with rabbit basilar artery. European Journal of Pharmacology. 2003;482(1):227-34.

54. Wang L-D, Qiu X-Q, Tian Z-F, Zhang Y-F, Li H-F. Inhibitory effects of genistein and resveratrol on guinea pig gallbladder contractility in vitro. World J Gastroenterol. 2008;14(31):4955-60.

55. Gao H, Liang M, Bergdahl A, Hamren A, Lindholm MW, Dahlman-Wright K, et al. Estrogen attenuates vascular expression of inflammation associated genes and adhesion of monocytes to endothelial cells. Inflammation research : official journal of the European Histamine Research Society [et al]. 2006;55(8):349-53.

56. Xue Q, Xiao D, Zhang L. Estrogen Regulates Angiotensin II Receptor Expression Patterns and Protects the Heart from Ischemic Injury in Female Rats. Biol Reprod. 2015;93(1):6-.

57. Dharmappa KK, Mohamed R, Shivaprasad HV, Vishwanath BS. Genistein, a potent inhibitor of secretory phospholipase A2: a new insight in down regulation of inflammation.

Inflammopharmacology. 2010;18(1):25-31.

58. Trompezinski S, Denis A, Schmitt D, Viac J. Comparative effects of polyphenols from green tea (EGCG) and soybean (genistein) on VEGF and IL-8 release from normal human keratinocytes stimulated with the proinflammatory cytokine TNFalpha. Archives of dermatological research. 2003;295(3):112-6.

59. Maskarinec G, Steude JS, Franke AA, Cooney RV. Inflammatory markers in a 2-year soy intervention among premenopausal women. J Inflamm (Lond). 2009;6:9-.

60. Sandoval MJ, Cutini PH, Rauschemberger MB, Massheimer VL. The soyabean isoflavone genistein modulates endothelial cell behaviour. British Journal of Nutrition. 2010;104(2):171-9.

61. Mahabusarakam W, Deachathai S, Phongpaichit S, Jansakul C, Taylor WC. A benzil and isoflavone derivatives from Derris scandens Benth. Phytochemistry. 2004;65(8):1185-91.

62. Sreelatha T, Hymavathi A, Rama Subba Rao V, Devanand P, Usha Rani P, Madhusudana Rao J, et al. A new benzil derivative from Derris scandens: Structure-insecticidal activity study. Bioorg Med Chem Lett. 2010;20(2):549-53.

63. Sittiwet C. Antimicrobial Properties of Derris scandens Aqueous Extract. Journal of Biological Sciences. 2009(9):607-11.

64. Rani PU. Bioactivity evaluation of prenylated isoflavones derived from Derris scandens Benth against two stored pest larvae. Journal of Biopesticides 6(1):14-21.

65. Sriwanthana B, Chavalittumrong P. In vitro effect of Derris scandens on normal lymphocyte proliferation and its activities on natural killer cells in normals and HIV-1 infected patients. Journal of ethnopharmacology. 2001;76(1):125-9.

66. Jansakul C. Some pharmacological studies of a hypotensive fraction from derris scandens. J Sci Soc Thailand. 1997(23):323-34.

67. Botta B, Vitali A, Menendez P, Misiti D, Delle Monache G. Prenylated flavonoids: pharmacology and biotechnology. Current medicinal chemistry. 2005;12(6):717-39.

68. Kretzschmar G, Zierau O, Wober J, Tischer S, Metz P, Vollmer G. Prenylation has a compound specific effect on the estrogenicity of naringenin and genistein. The Journal of

steroid biochemistry and molecular biology. 2010;118(1-2):1-6.

69. Ming L-G, Lv X, Ma X-N, Ge B-F, Zhen P, Song P, et al. The Prenyl Group Contributes to Activities of Phytoestrogen 8-Prenynaringenin in Enhancing Bone Formation and Inhibiting Bone Resorption In Vitro2013.

70. Kuljittichanok D, Diskul-Na-Ayudthaya P, Weeraphan C, Chokchaichamnankit D, Chiablaem K, Lirdprapamongkol K, et al. Effect of Derris scandens extract on a human hepatocellular carcinoma cell line. Oncol Lett. 2018;16(2):1943-52.

71. Hematulin A, Meethang S, Ingkaninan K, Sagan D. Derris scandens Benth extract potentiates radioresistance of Hep-2 laryngeal cancer cells. Asian Pacific journal of cancer prevention : APJCP. 2012;13(4):1289-95.

72. Laupattarakasem P, Houghton PJ, Hoult JR. Anti-inflammatory isoflavonoids from the stems of Derris scandens. Planta medica. 2004;70(6):496-501.

73. Flower RJ. The development of COX2 inhibitors. Nature reviews Drug discovery. 2003;2(3):179-91.

74. Rainsford KD. Anti-inflammatory drugs in the 21st century. Sub-cellular biochemistry. 2007;42:3-27.

75. Carlson RW. The history and mechanism of action of fulvestrant. Clinical breast cancer. 2005;6 Suppl 1:S5-8.

76. Kuptniratsaikul V, Pinthong T, Bunjob M, Thanakhumtorn S, Chinswangwatanakul P, Thamlikitkul V. Efficacy and Safety of Derris scandens Benth Extracts in Patients with Knee Osteoarthritis. The Journal of Alternative and Complementary Medicine. 2011;17(2):147-53.

77. Mahabusarakama W. A benzil and isoflavone derivatives from

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