中國醫藥大學 藥學院 藥物安全研究所 碩士論文

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異柴胡內酯異構物作用於巨噬細胞的抗發炎活性

Structural and biological evaluation of isochaihulactone as a anti-inflammatory agent in macrophages

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中華民國 98 年 7 月

Catalog

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Abstract

Isochaihulactone is a lignan isolated from Nan-Chai-Hu, the root of *Bupleurum scorzonerifolium*, an important Chinese herb in the treatment of influenza, fever, malaria, cancer, and menstrual disorders in China, Japan, and many other parts of Asia. Isochaihulactone has been reported to exhibit cytotoxicity against human lung cancers; however, its immune modulation properties have never been reported. Natural isolated isochaihulactone is composed E-form and Z-form isomers, and it is not clear which isomer responses for the bioactivities of isochaihulactone. Using organic synthesis, the pure E-form and Z-form isomers of isochaihulactone were obtained. We found that Z-form, but not E-form isochaihulactone is toxic to murine macrophages, indicating that Z-form isomer responses for the anti-cancer activity of isochaihulactone. Further, we found that E-form isochaihulactone reduced the expression of pro-inflammatory mediators, including TNF-α, IL-6, IL-1β, NO, and iNOS in LPS-stimulated macrophages. In addition, E-form isochaihulactone inhibited the phosphorylation of ERK1/2, p38, and JNK1/2 in LPS-stimulated macrophages. Our current results demonstrated the safety and anti-inflammatory properties of E-form isochaihulactone that could provide the possibility for its future pharmaceutical application in the realm of immune-modulation.

Keywords：Isochaihulactone, Lipopolysaccharide, Macrophages, Inflammation

中文摘要

異柴胡內酯源自南柴胡根部的萃取物之木酚素結構化合物，南柴胡在亞洲地區的國家常用於治療流感、發燒、瘧疾、腫瘤、女性經期不順等疾病。南柴胡根部萃取出的異柴胡內酯具有肺癌細胞毒殺性，然而在免疫調節相關的生物活性則少有文獻發表過。萃取出的異柴胡內酯包含近 1:1 的 E 和 Z 構型的立體易構物。經由化學合成的方式分別取得 E 和 Z 的異柴胡內酯純異構物，經由實驗結果發現到 Z 構形的異柴胡內酯對小鼠巨噬細胞具有毒殺性，即 Z 構形是主要的抗癌活性的成份。然而沒有毒性的 E 構形異柴胡內酯用來測定抑制由活化巨噬細胞而誘導發炎的活性。實驗數據顯示 E 構形異柴胡內酯能夠抑制脂多醣刺激巨噬細胞產生的發炎因子，包括 IL-6、TNF-α、IL-1β、NO 與 iNOS。在訊息傳遞途徑方面，E 構形異柴胡內酯抑制了 ERK1/2、p38 以及 JNK1/2 之磷酸化。本實驗的數據表示 E 構形異柴胡內酯對細胞安全無毒性，且具有抗發炎活性，未來 E 構形異柴胡內酯可以在免疫調節的藥理研究上做更多的運用。

Chapter 1 Introduction

Ch 1.1 Inflammation

Inflammation is a complex bio-response process during the injury and infection. It usually appeared the sign of red, swollen, heat and pain[1]. When inflammation occurs, a lot of the immune-response will be induced to react in the target site or defeat the microbes' invasion, including the cytokines secretion[2], white blood cells infiltration, immuno-cellular response[3] (example: T- and B- lymphocyte) and etc (Figure 01). Inflammation is like a double-edged effect. Adequate inflammation presented immune-response and defense for microbes' invasion, in other word, acute inflammation[1]; while chronic inflammation brought a lot of risk factor for health, including diabetes, cardiovascular disease, neurological disorder, cancer[3], and etc. Acute inflammation proceeds vascular change obviously, including vasodilation[4], increased permeability, slow blood flow and other vascular response induced by a lot of acute inflammatory mediators[5]. For examples: cytokines (IL-1β, IL-6, TNF-α) derived primarily from macrophages, nitrous oxide[6] released from macrophages (Figure 02) and endothelial cells for cytotoxic to invasive microbes, histamine[7] from mast cells and platelets, prostaglandins[8] as an eicosanoid from mast cells, and others important for production and accumulation during inflammation. Those inflammatory markers mentioned above were widely used for tests in variant research about inflammation, even for other diseases concerned about inflammation,

like cancer, cardiovascular disease atherosclerosis[9], asthma[10], glomerulonephritis[11], rheumatoid arthritis[12], and so on. Severe diseases were often relevant to chronic inflammation with many risks, including systemic inflammation due to obesity[13], insulin resistance[14], and etc**.**

Ch 1.2 Lipopolysaccharide (LPS)

All microbes have their own pathogen-associated molecular pattern (PAMP)[15]. The major component of microbes' cell wall was peptidoglycan. With the more specific elements composed in the cell wall, they were divided into Gram-positive with teichoic acid, and Gram-negative with lipoprotein and lipopolysaccharide (Figure 03), respectively. LPS acts as a role of prototypical endotoxin[16] because of the binding for the CD14/TLR4/MD2 receptor complex (Figure 04). The CD14/TLR4/MD2 receptor complex[17] represents the process of secretion of pro-inflammatory cytokines in a variety of cells, especially in macrophages with the signal transduction into nucleus for complex biological immune-response. That is, LPS was widely used in a lot of researches concerned about inflammation in immunology.

Ch 1.3 Macrophages

Macrophages were derived from monocytes of white blood cells. They are the first-line defense for the microbes' infection and invasion[18]. They will prosecute phagocytosis first when encountering pathogens, that is, the innate immunity. Later they will secrete lysozyme to lyze the contents. Despite endocytosis[19], macrophages could release cytokines and chemokines to attract lymphocytes infiltration and present the microbes to T cells with for adaptive immunity[20]. All in all, macrophage plays an important roll in biological immunity, both for innate and adaptive immunity (Figure 05). Besides, what were more about researches of macrophages is the surface receptor expression, including CD14, CD80, CD86[21], MHC class I[22], MHC class II[23], and the toll-like receptor on them.

Ch 1.4 Toll-like receptor

Toll-like receptors are a family of pathogen recognition receptors (PRR) and exist in a variety of cell surface[24], including macrophage, dendritic cell, mast cell, liver cell, neuron, and etc. They were found and divided fifteen kinds (from TLR1 to TLR15) and distributed in a variety of different kinds of cells (Figure 06). Each kind of toll-like receptor has its own ligands. Take lipopolysaccharide (LPS) for instance, LPS is the specific ligand for toll-like receptor 4[25], especially TLR4 on macrophages.

With the ligation of toll-like receptor and specific ligand, the cell would transduce signals into nucleus to operate complex biochemical responses[26]. All in all, toll-like receptors are the key molecules for the immune system to microbes' infection.

Ch 1.5 Signal transduction

Signal transduction is a mechanism that converts a cell into a specific biological response with the ligation between receptor and signaling molecules, including cytokine[28], chemokine, growth factor[27], environmental stimuli, and so on. The most common pathways are cAMP dependent pathway[29], IP3/DAG pathway[30], and mitogen-activated protein kinase (MAPKs)[31], which is relevant to ligation of LPS and TLR4 on macrophages during the inflammation (Figure 04). The common downstream of MAPKs include extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNKs), and p38 isoforms. According to reported studies[31], ERK1/2 is activated by growth factor (for example: endothelial growth factor, and platelet-derived growth factor[32]) and modulates the cell proliferation and differentiation. The other JNKs and p38[33] has the property of response to stress stimuli, such as cytokines, to regulate cell proliferation.

Ch 1.6 Chai-Hu

Chai-Hu is the plant of Umbelliferae and divided into *Buplerum chinenseDC* (Figure 07) and *Buplernm scorzonerifolium Wild.* (Figure 08), with their own common name for Bei Chai-Hu and Nan Chai-Hu, repectively. Chai-Hu was often harvested during the season of spring and autumn, and got rid of the stem, leaves, and mud. And the final step is to dehumidify. There are several points to distinguish the Nan and Bei Chai-Hu, for example of their appearance, Bei Chai-Hu has the larger tip of root with more lower branches, and the harder body for barely broken; while Nan Chai-Hu is smaller roots and non or fewer branches, and fragile. The more important for the difference between them is the components: Bei Chai-Hu (*Buplerum chinense DC*) has α-Spinasterol, Adonitol, Saikosaponin A B C D, and volatile oil[34]; and Nan Chai-Hu (*Buplerum scorzonerifolium Wild*) has Saikosaponin[35], chaihunaphthone, flavones, lignans[36], and isochaihulactone[37] found. Bei Chai-Hu (*Buplerum chinense DC*) had been reported for macrophages immunomodulatory activity[38], saikosaponin from Bei Chai-Hu has the antioxidative protection[39], and others. Nan Chai Hu is a traditionally used Chinese herbal medicine, which usually for treatment in influenza, fever, malaria, cancer and menstrual disorders in China, Japan, and many other parts of Asia. It had been reported about the components flavones and lignans for immunosuppression[40], acetone extracts for suppressing telomerase activity of cancer

cells[41], isochaihulactone for anti-lung cancer activity[37], and others. Isochaihulactone is one of the extracts from the roots of Nan Chai Hu (*Bupleurum scorzonerifolium*), while there is little research for anti-inflammatory effects of this compound. According to the chemical structure, isochaihulactone could be distinguished the stereoisomeric characteristic for E- and Z-form structure in the double bond of carbon number 2 and 5 site (Figure 09). The nature product is composed with the mixture of the isomers for E- and Z-form isochaihulactone. The pure compounds of E- and Z-form isochaihulactone were obtained with chemically synthesis from Prof. Yulin Lam, department of Chemistry, National University of Singpore (Figure 10, Appendix), thus it could be investigated the different effects of the isomers. What is more specific trait for isochaihulactone is the white needle crystals; mp 137–138^{0}C; IR (KBr) νmax cm^{-1}: 1745, 1635, 1581, 1335, 1153; UV ($CHCl_3$) λmax nm (log ε): 247 (4.08), 298 (4.17), 327 (4.08); for ^{1}H and ^{13}C NMR ($CDCl_3$); HREIMS m/z 398.1374 (calcd for $C_{22}H_{22}O_7$ $[M]^+$ 398.1365); EIMS, 70 eV, m/z (rel. int.): 398 ($[M]^+$, 18), 263 (100), 207 (16), 135 (35)[37].

Ch 1.7 Stereoisomerism

Stereoisomerism is that isomeric molecules have the same molecular formula in the bonded atoms, which they are different from the group distribution in the three

dimensional space, including terms of enantiomers, diastereomers, Cis-trans and E-Z isomerism, and others. Cis-trans and E-Z isomerism is similar. For example of the molecule formula $C_2H_2Cl_2$, between the double bond the larger radius atom Chlorine in the same side is the cis-form (Figure 11, A), while in the counter side is the trans-form (Figure 11, B), while E-Z isomerism has the other definition: between the double bond, two groups connected to the single bond at the same part are different from each other. Take the fluoromethylpentene for instance (Figure11, C), the left part is fluorine is the priority to methyl group, and the right part is the ethyl group. Both the priority part in the same direction is the Z-form, and vice versa for E-form (Figure11, D).

Ch 1.8 Experimental aims

Plants or materials for Chinese herbal medicines were usually the natural products. If their components have the isomeric compounds, they consists the mixture of isomers in different percentage. Nan Chai-Hu were widely used for several disease mentioned above. Isochaihulactone from Nan Chai-Hu has approved the anti-lung cancer activity[37] for its cytotoxicity. When the Nan Chai-Hu was taken by patients, there may be risks for cell damage due to the reported cancer cell cytotoxicity, not to mention about the more vulnerablly normal cells than cancer ones. Isochaihulactone

was supposed that the SAR (structure and activity relationship) of isochaihulactone has something to do with it. When the pure isomers were obtained form chemical synthesis (Appendix), both the E- and Z-form could be tried to find out the possibility for different isomers resulting in different effects respectively. If the supposition was approved, the natural product isn't always better than artificial one because of the cytotoxic isomer existed in the natural product. The safer isomer could be obtained with chemical synthesis for drug-safety use instead of taking natural product.

Chapter 2 Materials and methods

Ch 2.1 Chemicals

Natural extracts of isochaihulactone (E- and Z-form mixture) was from National I-Lan University. Pured E- and Z-form of isochaihulactone were synthesized from Prof. Yulin Lam, department of Chemistry, National University of Singapore. Molecular weight is 398.41 g/mole. Steroids, prednisolone, was chosen for positive control (SIGMA-ALODRICH^{TM}). Both they were dissolved in DMSO dimethyl sulfoxide (BIOSHOP®).

Ch 2.2 Devices

CO_2 incubator (Thermo Scientific Revco Elite II)

Vortex Mixer (VWR Vortexer 2)

Inverted microscopy (Optima)

Centrifuge (Aron laboratory instruments inc)

Microcentrifuge (Thermo Scientific)

Microplate reader (Bio-Tek instrument, INC.)

Electrophoresis power supply (Bio-Rad Scientific)

Mini-PROTEIN® 3 Cell (Bio-Rad Scientific)

Electrophoretic transfer cell (Bio-Rad Scientific)

pH meter (Thermo Scientific)

Autoclaver (EZ medica company)

Electronic balance (OHAUS)

Kodak Medical X-ray Processor 101

Ch 2.3 Cell culture

The murine macrophage cell line, J774A.1 obtained from ATCC (Rockville, MD, USA), and RAW264.7, were cultured in RPMI 1640 medium (HyClone®, Thermo) supplemented with 10% fetal bovine serum, which composed of FBS (bioind®) and 1%, 25 mM HEPES and 1% L-glutamine. Cells were incubated at 37^{o}C in a humidified atmosphere containing 5% CO_2 / 95% air. And the subculture is to use the suitable medium for them, both macrophages J774A.1 and RAW264.7 with RPMI medium in 100mm cell culture dish. Till their growth to about 80% area distribution of 100mm cell culture dish, the cultured medium should be demediumed and added the PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) in order to wash out clearly at least twice. And then the dish is added new medium and gathering all available cells for different experimental purposes, including keeping the cells growth (meidium changed only when cells are few), and seeding cells of the same counts with cell-count meter into 60 mm cell culture dish. When cells have grown for almost over 80% area of 100 mm cell culture dish, they could be

freezed with several steps. First part of step is the same as to change the medium and gather cells, FBS with 10% DMSO is added and moved into tube. And the tube full of cells and FBS with 10% DMSO is put in 4^0C, -20^0C, for one hour respectively. After that, the tube should be stored in -80^0C refrigerator for over-night. After the day, the tube is moved into the liquid nitrogen device. When we want to defrost cells, the tube with cells and FBS with 10% DMSO could be got from the liquid nitrogen device, and warm the tube in the water bath with the temperature 37^0C, and then pour the liquid into a sterile tube and centrifuge in 1000 rpm for 5 minute. The supernatant medium is taken out and added new one for cell culture.

Ch 2.4 Cell viability

Macrophages were seeded in $5*10^5$/ml, 100 μl/well for 24 hours. The chemical was added with specific concentration in it for 24 hours. After that, Alamar Blue® (AbD Serotec) was added 10 μL in. Read the plate with ELISA reader in wavelength 570 nm and 600 nm after 4 hours at least. The numbers of absorption were counted with an equation to show the cell survival percentage compared to the control, which is added with dimethyl sulfoxide (DMSO).

Ch 2.5 Nitric Oxide NO release test

NO, an inflammatory marker, was released after macrophages stimulated by lipolysaccharides (LPS). The Griess Reagent System (Promega®) is composed of Sulfanilamide Solution (1% sulfanilamide in 5% phosphoric acid) and NED Solution (0.1% N-1-napthylethylenediamine dihydrochloride in water). Fist step is to 100 μl of sample was mixed with 50 μl Sulfanilamide Solution and shaking the plate for 30 minutes for well-mixed. Later, 50 μl NED solution was added into the 150 μl solution and shaking for 10 minutes (light protection with aluminum foil coverage). The three react with the nitrite group to form colored azo group and then tested with ELISA reader in the wavelength 520 nm.

Ch 2.6 Western Blotting analysis

For measurement of iNOS, proIL-1, and MAPKs phosphorylation at the protein level, the lysate of macrophages is harvest and de-pellet to be separated by 8~10% sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred electrophoretically onto a 0.2 μm microporous polyvinylidene fluoride (PVDF) membrane (MILLIPORE, Immobilon^{TM}). The membranes were blocked with 5% skim milk (Anchor®) in phosphate buffer saline (PBS). The membrane was connected with specific primary antibody and incubated with horseradish peroxidase

(HRP) Opti-ECL reagent 1 and reagent 2 mixture (Millipore) to conjugate the secondary antibody. The fluorescent emission from the membrane was sensed with X-ray film (BIOMAN) inside the radiographic cassette and wash it with film machine. The membrane was re-blotted with stripping buffer for 30 minutes and conjugated the α-actin or the protein total form as the internal control.

Ch 2.7 Enzyme-linked immunosorbent assay (ELISA)

ELISA kits were purchased from R & D system DuoSet®, including The KIT for mouse IL-6, TNF-α, and IL-1β. 96-wells plate (Costar, tower tear®) was coated the capture antibody for 24 hours at the room temperature and then blocking it with reagent diluent for 2 hours at least, and the plate was well-prepared. The standard line was determined with standard solution in indicated concentration, and the samples and standard ones were added 100 μl into the well for 2 hours for first step reaction. Later is the aspiration step with wash buffer for about 3~5 times. The second step is the detection antibody 100 μl added into the well for 2 hours reaction. Aspiration is necessary as the same the end of first step. The third step is the streptavidine solution 50 μl added into the well for 20 minutes and then aspiration step. The fourth step is the substrate solution for the dye 50 μl added into the well for within 30 minutes because of the color should be well-control. The fifth step is stop solution (2M

sulfonic acid) 50 μl added to cut the reaction of substrate and samples. Finally the plate was read with the ELISA reader (μ-Quant®) in wavelength 450 nm.

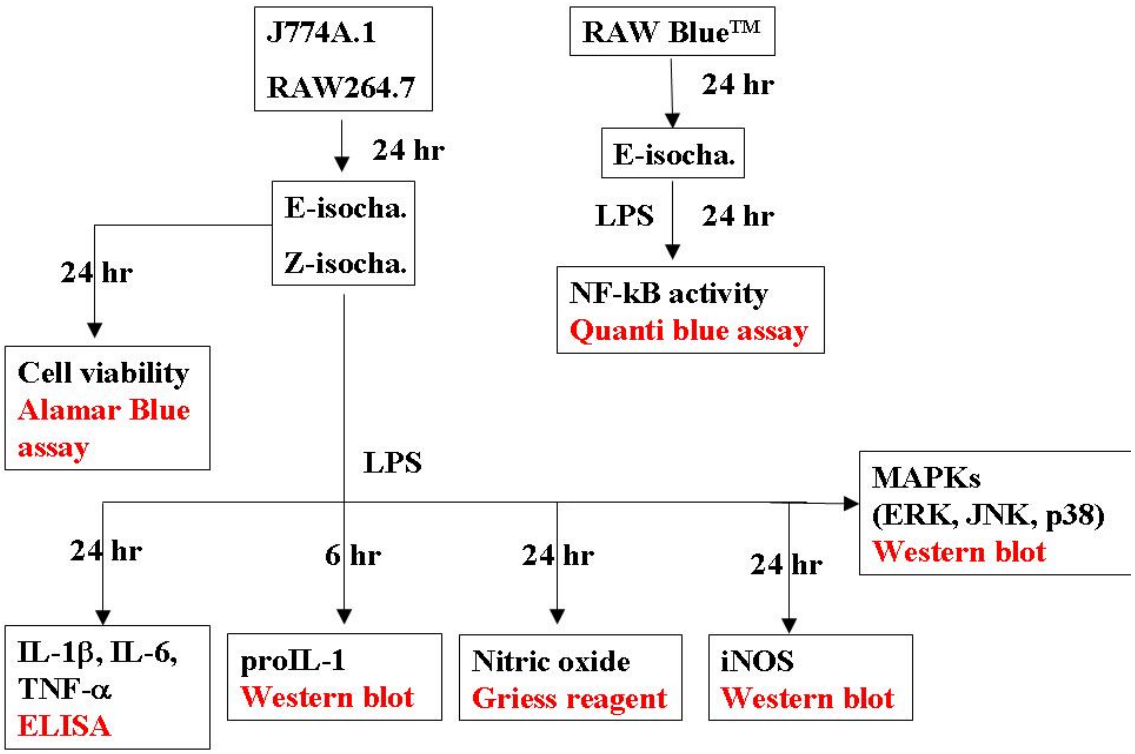
Ch 2.8 NF-κB assay

RAW-BLUE^{TM} Cells (InvivoGen), as a mouse macrophages cell line with transfected reporter gene of secreted embryonic alkaline phosphatase (SEAP), were tested for the production of NF-κB with the QUANTI-Blue^{TM} reagent (InvivoGen), as a medium for detection and quantification of SEAP. SEAP is a gene inducible by NF-κB transcription factor (Figure 12). Cells were resistant to the selectable marker Zeocin^{TM}, a research used antibiotic. The 200μl QUANTI-Blue^{TM} reagent and 20μl medium are mixed and stored in 37^{0}C for light protection in 30 minutes. If the color didn't change significantly, check it every 15 minutes with the ELISA reader to test the optical density for the NF-κB activity test.

Ch 2.9 statistical analysis

The study use the statistical software of Sigmastat version 2.1 for analysis all experimental data. The expression of all results is mean ± standard deviation, and uses the one-way ANOVA analysis. $P<0.05$ expresses the significant difference.

Ch 2.10 Experimental flow chart



Chapter 3 Results

Ch 3.1 Cell viability investigation

To investigate the survival of murine macrophages,both J774A.1 and RAW 264.7 (concentration 0, 0.6, 1.2, 2.5, 5, 10, 20, 40 μM) were treated with E or Z-form Isochaihulactone in different doses for 24 hours and cell viability was assayed by AlarmarBlue assay.

Ch 3.1.a Effect of isochaihulactone on the viability of J774A.1 macrophages

The Z-form of isochaihulactone showed the obvious cytotoxicity when they were exposed to the concentration exceeding 5μM (Z-isochaihulactone 0 μM for 100 ± 4.92, 0.6 μM for 119.64 ± 2.86, 1.2 μM for 113.63 ± 8.62, 2.5 μM for 124.98 ± 0.57, 5 μM for 107.87 ± 2.35, 10 μM for 60.04 ± 4.52, 20 μM for 61.44 ± 3.69, 40 μM for 50.80 ± 4.77), while E-form of isochaihulactone didn't effect the cell viability (E-isochaihulactone 0 μM for 100 ± 4.92, 0.6 μM for 114.38 ± 8.51, 1.2 μM for132.54 ± 2.09, 2.5 μM for 118.58 ± 11.12, 5 μM for 129.84 ± 8.82, 10 μM for 123.50 ± 10.74, 20 μM for 126.77 ± 3.55, 40 μM for 112.27 ± 6.28) (Figure 13)

Ch 3.1 b Effect of isochaihulactone on the viability of RAW264.7 macrophages

This result is similar to J774A.1 that the Z-form of isochaihulactone showed the obvious cytotoxicity when they were exposed to the concentration exceeding 5 μM (Z-isochaihulactone 0 μM for 100±1.39, 0.6 μM for 104.80 ± 9.30, 1.2 μM for 104.46 ± 12.65, 2.5 μM for 105.59 ± 3.65, 5 μM for 92.93 ± 7.35, 10 μM for 22.19 ± 4.90, 20 μM for 26.92 ± 5.33, 40 μM for 29.92 ± 4.45), while E-form of isochaihulactone didn't effect the cell viability (E-isochaihulactone 0 μM for 100 ± 1.39, 0.6 μM for 107.37 ± 9.68, 1.2 μM for 109.15 ± 2.37, 2.5 μM for 105.55 ± 8.15, 5 μM for 103.27 ± 8.64, 10 μM for 106.78 ± 2.69, 20 μM for 98.18 ± 3.64, 40 μM for 96.41 ± 7.25) (Figure 14)

Ch 3.2 Anti-inflammatory activity

There's a lot of inflammatory markers, including proIL-1, Nitric oxide (NO), inducible nitric oxide synthase (iNOS), cytokine IL-6, TNF-α, and IL-1β. The experiment was to investigate the expression of those inflammatory markers with E- and Z-form, from different concentration range 6~40 μM.

Ch 3.2.a E-isocha. inhibited the IL-1β secretion and proIL-1β expression in LPS-stimulated J774A.1 macrophages

Macrophages J774A.1 was treated Z-isochihulactone (0.6, 1.2, 2.5, 5 μM)

and E-isochaihulactone (0.6, 1.2, 2.5, 5, 10, 20 μM) for investigation on the IL-1β production after LPS stimulation for 24 hours. Z-isochaihulactone has little effect for IL-1β production (control for 10.5 ± 5.20 pg/ml, LPS for 330.68 ± 20.45pg/ml, 0.6 μM for 351.93 ± 30.1 pg/ml, 1.2 μM for 353.81 ± 25.86 pg/ml, 2.5 μM for 311.93 ± 27.43 pg/ml, 5 μM for 306.31 ± 24.63 pg/ml) (Figure 15), while E-isochaihulactone appears obvious efficiency when concentration > 10 μM, even more better inhibition for 20 μM (0.6 μM for 357.56 ± 26.1 pg/ml, 1.2 μM for 346.31 ± 24.46 pg/ml, 2.5 μM for 289.43 ± 25.33 pg/ml, 5 μM for 265.06 ± 23.43 pg/ml, 10 μM for 134.43 ± 27.03 pg/ml, 20 μM for 55.68 ± 10.24 pg/ml) (Figure15). With higher concentration of E-isochaihulactone, the former of IL-1β, pro IL-1 is inhibited in dose denpendence. (Figure 16)

Ch 3.2.b E-isocha. inhibited NO release and iNOS expression in LPS-stimulated RAW264.7 macrophages

To find out that nitrous oxide was produced by amino acid L-arginine through the enzyme inducible Nitric Oxide synthase (iNOS) and released during the inflammation occurrence. NO concentration (control for 0.5 ± 1.00 μM, LPS for 19.20 ± 0.57 μM, 2.5 μM for 18.93 ± 0.73 μM, 5 μM for 17.98 ± 1.53 μM,

10 μM for 14.87 ± 2.03 μM, 20 μM for 9.13 ± 1.24 μM) (Figure 17) and iNOS (Figure 18) were both inhibited effectively compared to LPS stimulation by E-isochaihulactone in dose-dependence. The steroids, prednisolone, as a positive control, did inhibit the NO release, too. (1 μM for 6.56 ± 2.03 μM, 10 μM for 3.18 ± 1.03 μM) (Figure 17)

Ch 3.2.c E-isocha. inhibited cytokines production in LPS-stimulated J774A.1 macrophages

To investigate if the cytokine released from murine macrophages when they encountered the microbes' infection, E- and Z-isochihulactone was treated to find out the effect of released cytokines, including IL-6 (control for 0.1 ± 0.32 ng/ml, LPS for 25.10 ± 1.15 ng/ml, Z-isochaihulactone 0.6 μM for 23.78 ± 1.21 ng/ml, 1.2 μM for 22.99 ± 1.36 ng/ml, 2.5 μM for 21.85 ± 1.43 ng/ml, 5 μM for 18.13 ± 1.16 ng/ml, E-isochaihulactone 0.6 μM for 23.49 ± 1.21 ng/ml, 1.2 μM for 21.92 ± 1.36 ng/ml, 2.5 μM for 21.38 ± 1.43 ng/ml, 5 μM for 18.72 ± 1.13 ng/ml, 10 μM for 16.03 ± 1.23 ng/ml, 20 μM for 9.56 ± 1.34 ng/ml) (Figure 19), and TNF-α (control for 0.1 ± 0.27 ng/ml, LPS for 9.50 ± 0.27 ng/ml, Z-isochaihulactone 0.6 μM for 9.97 ± 0.31 ng/ml, 1.2 μM for 9.16 ± 0.32 ng/ml, 2.5 μM for 8.78 ± 0.35 ng/ml, 5 μM for 7.00 ± 0.27 ng/ml, E-isochaihulactone

0.6 μM for 9.78 ± 0.31 ng/ml, 1.2 μM for 10.41 ± 0.32 ng/ml, 2.5 μM for 9.78 ± 0.35 ng/ml, 5 μM for 7.55 ± 0.27 ng/ml, 10 μM for 6.03 ± 0.33 ng/ml, 20 μM for 4.53 ± 0.32 ng/ml)(Figure 20). It resulted that E-form of isochaihulactone did effectively inhibit the cytokine release from murine macrophages J774A.1, while Z-form of isochaihulactone did little effectively.

Ch 3.2.d E-isocha. inhibited cytokines production in LPS-stimulated RAW264.7 macrophages

In order to seek if E-isochaihulactone has the same effect on other macrophages, RAW264.7 was used for cytokine evaluation. It is design for E-isochaihulactone treated without Z-form, and steroid, prednisolone, as a anti-inflammatory positive control plus. After LPS stimulation for 24 hours, IL-6 and TNF-α were released from RAW264.7. That results in that E-isochaihulactone inhibits IL6 (control for 0.1 ± 0.37 ng/ml, LPS for 5.82 ± 0.24 ng/ml, 2.5 μM for 5.41 ± 0.23 ng/ml, 5 μM for 5.14 ± 0.11 ng/ml, 10 μM for 4.33 ± 0.12 ng/ml, 20 μM for 1.53 ± 0.05 ng/ml) (Figure 21) and TNF-α (control for 0.5 ± 0.22 ng/ml, LPS for 3.68 ± 0.47 ng/ml, 2.5 μM for 3.51 ± 0.35 ng/ml, 5 μM for 3.30 ± 0.58 ng/ml, 10 μM for 3.17 ± 0.70 ng/ml, 20 μM for 2.72 ± 0.18 ng/ml) in dose-dependence (Figure 22)

Ch 3.3 Mechanism of signal transduction pathway

According to the results above, it's approve that E-isochaihulactone has the anti-inflammatory activity for inhibition on cytokines and NO. To investigate the mechanism for anti-inflammatory activity, the signal transduction pathways could be important marker. With the ligation of LPS and TLR4 receptor complex, there are several pathways for inflammation, including the activation of NF-κB and LPS induced phosphorylation.

Ch 3.3.a E-isocha. had no effect on NF-κB activity in LPS-stimulated RAW-BLUE™ cells

NF-κB is test with macrophages RAW BLUE. E-isochaihulactone is added in different concentration and steroid, prednisolone, as a positive control. E-isochaihulactone slightly inhibits NF-κB activation compared to positive control. (relative optical density, control for 0.001 ± 0.02, LPS for 1.75 ± 0.04, 2.5 μM for 1.66 ± 0.03, 5 μM for 1.69 ± 0.03, 10 μM for 1.61 ± 0.01, 20 μM for 1.53 ± 0.01, prednisolone 1 μM for 1.00 ± 0.02, 10 μM for 0.93 ± 0.01) (Figure 23)

Ch 3.3.b E-isocha. inhibited phosphorylation of ERK1/2 in LPS-stimulated RAW264.7 macrophages (time course study)

To find out the MAPKs activation, ERK is chosen to test the time course. The experiment is divided into two subset for E-isochaihulactone 20 μM untreated and treated after LPS stimulation for different point of time, from 0, 10 ,20 , 30, 60 minutes. E-isochaihulactone inhibits the ERK phosphrylation for blocking the signal transduced into nucleus. (Figure 24)

Ch 3.3.c E-isocha. inhibited phosphorylation of ERK1/2 in LPS-stimulated RAW264.7 macrophages (dosage study)

In addition to time course study investigation, dosage study could be used to make sure the effects. As expected, E-isochaihulactone shows the dose-dependence inhibition for phosphorylation of ERK in concentration 5 μM, 10 μM, 20 μM, and 40 μM. (Figure 25)

Ch 3.3.d E-isocha. inhibited phosphorylation of p38 in LPS-stimulated RAW264.7 macrophages

E-isochaihulactone inhibited phosphorylation of p38 after LPS-stimulated in 30 minutes in concentration 5 μM, 10 μM, 20 μM, and 40 μM. (Figure 26).

What is more, the inhibition on p-p38 is obviously stronger than p-ERK.

Ch 3.3.e E-isocha. inhibited phosphorylation of JNK1/2 in LPS-stimulated RAW264.7 macrophages

E-isochaihulactone inhibited phosphorylation of JNK1/2 after LPS-stimulated for 30 minutes in concentration 5 μM, 10 μM, 20 μM, and 40 μM (Figure 27).

Chapter 4 Discussion and Conclusion

Ch 4 Discussion and Conclusion

Clinically prescriptive medicine of anti-inflammation has a lot of side effects. Widely used medicine for anti-inflammation included steroids[42], non-steroid anti-inflammatory drug (NSAID)[43], some other herbal medicine, and etc. Most of them have the side effects, for examples of NSAID, acetylsalicylic acid (Aspirin®) for Reye's syndrome in children[44], diclofenac for gastrointestinal discomfort and tissue necrosis[45], indomethacin for peptic ulcer[46], and others. In addition, for examples of steroids, prednisolone for Cushing's syndrome and increase of blood glucose[47], dexamethasone for increased appetite and osteoporosis[48], methylprednisolone for glaucoma[49], and so on. Those anti-inflammatory agents mentioned above are common used in clinical therapy in a lot of cases of inflammation in the clinical prescription. Because of little found new indication about anti-inflammation, for safety of drug use, it will take more and more researches about new compounds for anti-inflammatory effect. These new compounds usually origin from synthesis to partition techniques, including extraction, purification, and other techniques for isolation, especially for Chinese herbal medicine or natural products which might have unknown chemicals.

There were a lot of researches about the Chai-Hu with different medical effects, and the ingredients derived with different techniques, even for the branches of species.

The common medical-used part for Chai-Hu is the root part. The ingredients included Saikosaponin, chaihunaphthone, flavones, lignans, and isochaihulactone[35-37] had been found. Besides, polysaccharides derived from the root of *Bupleurum smithii var. parvifolium* has the macrophage immunomodulatory activity, like phagocytosis[50]. Saikosaponin from *Bupleurum chinense DC.* has the protections on the antioxidative for those ROS (reactive oxygen species), like hydrogen peroxide (H_2O_2), superoxide radicals ($O2^-$), and malondialdehyde (MDA) which had high risks for health. There were more about Nan Chai-Hu (*Bupleurum scorzonerifolium*) for the ingredients found and the effects. For examples, isochaihulactone and chaihunaphthone inhibits the CD28 costimulation, IL-2 concentration, and CD28-costimulated T cells[51]; With acetone-extracted chemicals has the anti-A549 cells activity for arrest the cell cycle and telomerase activity for cell viability assay[52]. In addition, saikosaponin has the anti virus effects of coronavirus[53]. There was also has anti-inflammatory activity for Chai-Hu, especially for the inhibitions on the proliferation and activity of T-cell[54].

Chai-Hu has been used for a lot of disease since two thousands years ago. Most of prescribed for medical use is the natural products, even though with different technology for obtaining the target compound, which means, once the compound has the isomeric character. They are mixed for components in percentage. In this study, we found that isochihulactone, which has the E- and Z-form isomer, has the diverge

for the isomers with different effects on macrophage, especially for the cell cytotoxicity. Z-form, but not E-form, is toxic to cells that results E-isocha. has less limit for the higher dose to try to investigate for other medical effects because of the cell safety. But the mechanism of the anti-inflammatory effects is still not clear, and needs more experiments for more approval for that. If the effects are approved, it could proceed for animal model study, even for clinical trial. For the drug use safety, if the compound has few or free side effects to patients, it may be for prescribed medical use instead of currently used anti-inflammatory drugs mentioned above. And the pure E-isocha. could be obtained with chemical synthesis, and taken by patients in a safe condition for Z-isocha. free anti-inflammatory drug. It would break the myth for populations about the natural product is better than artificial events.

Chapter 5. Figures

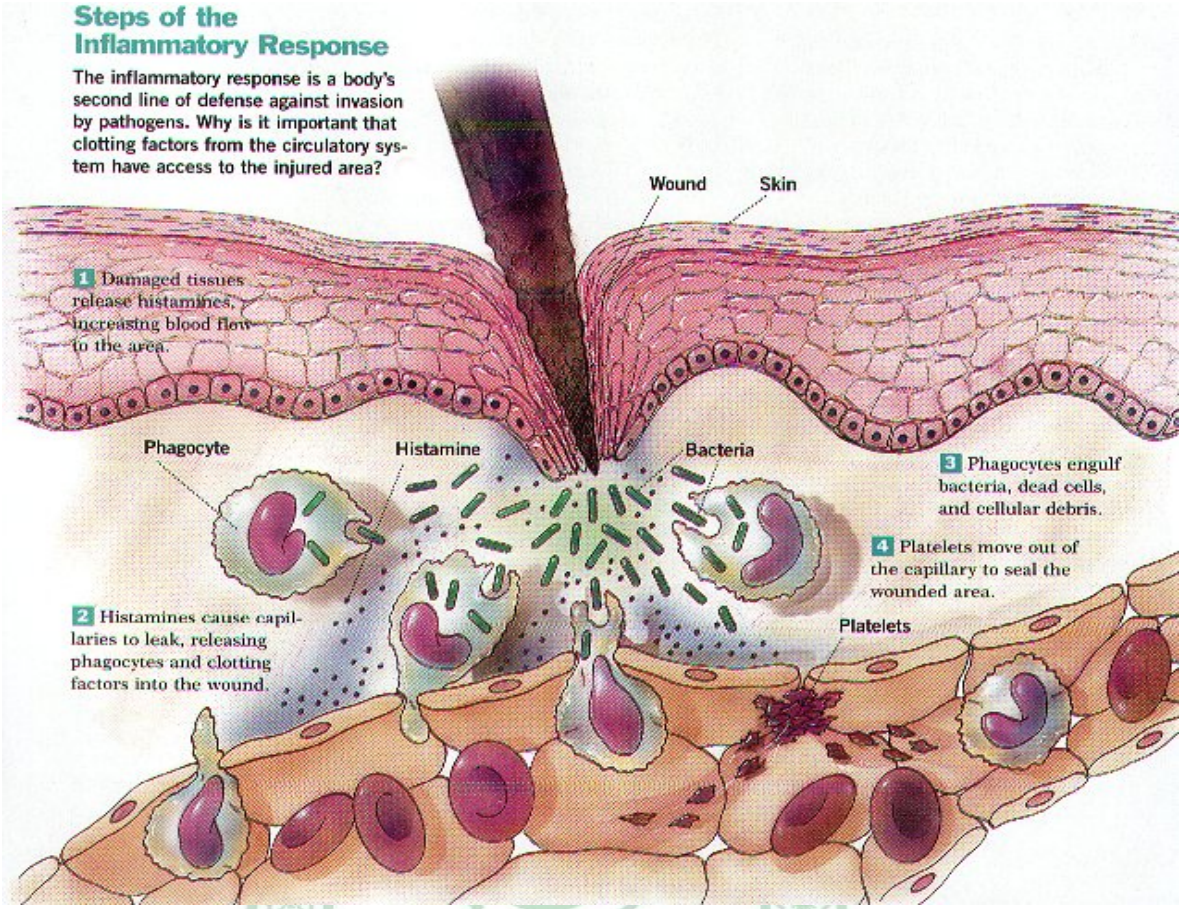


Figure 1 the process of inflammation

http://www.goodpsych.com/storage/inflammation.jpg

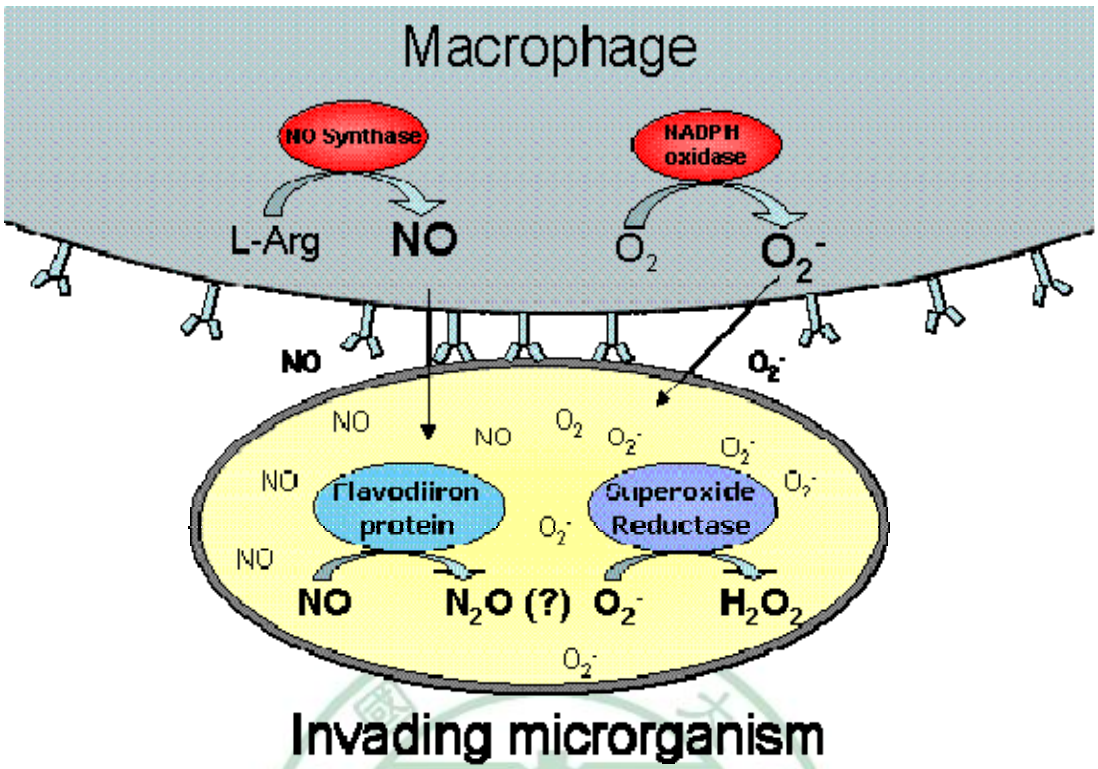


Figure 2 nitric oxide released from macrophage

http://www.itqb.unl.pt/~Metalloproteins_Bioenergetics/macrophage.gif

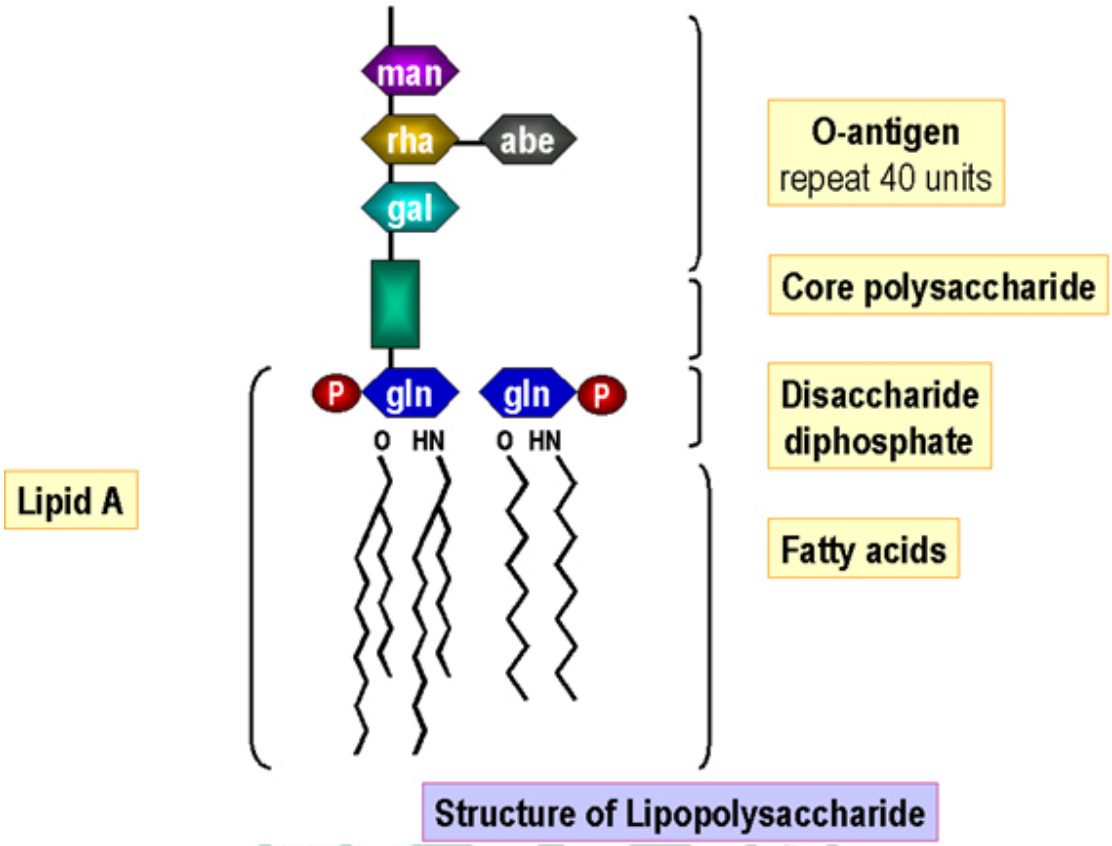


Figure 3 the structure of lipopolysaccharide (LPS)
http://pathmicro.med.sc.edu/fox/lps.jpg

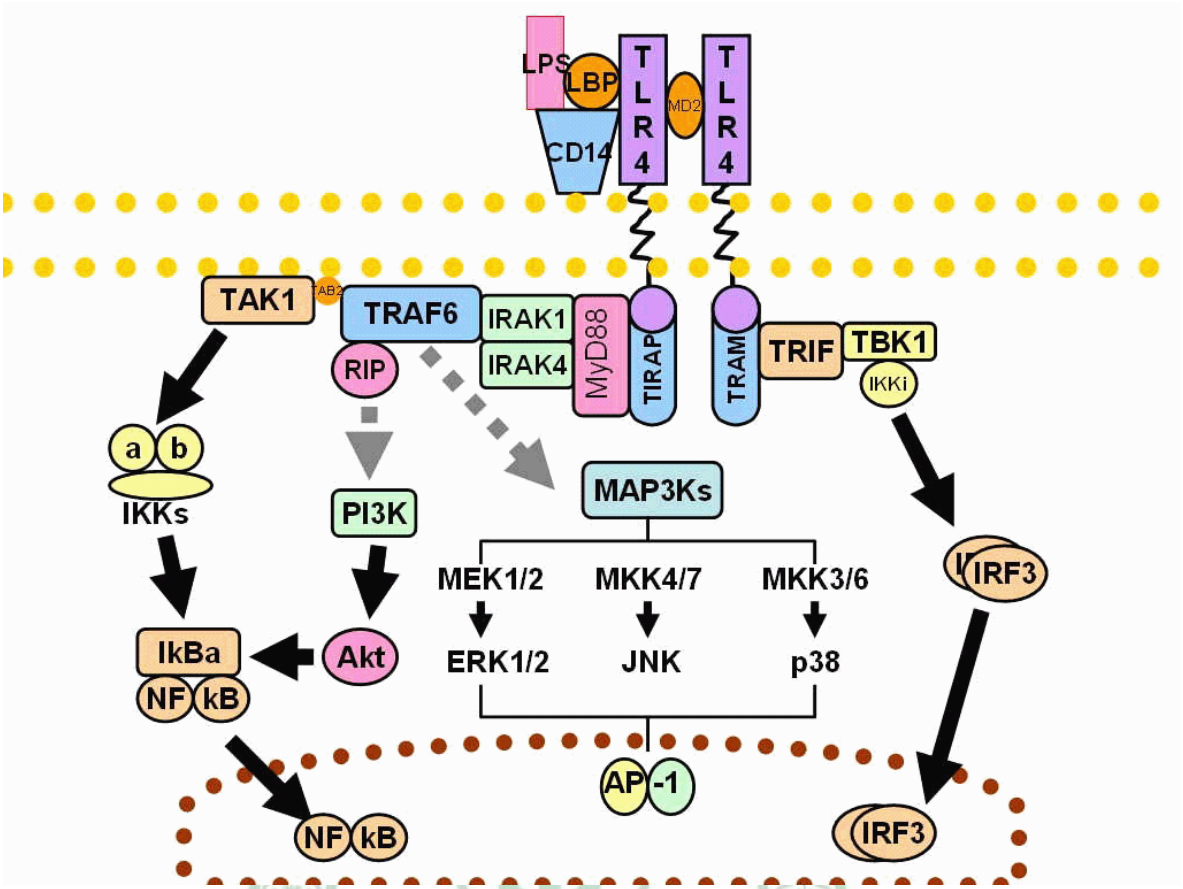
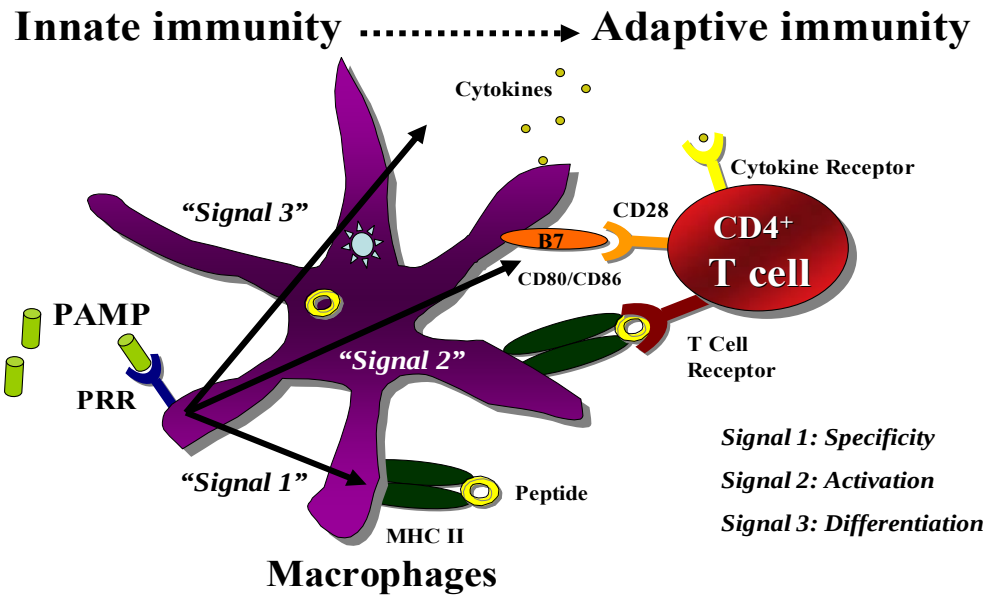


Figure 4 The CD14/TLR4/MD2 receptor complex and signals

http://www.pharmaprojects.com/contentimg/0908/TLRdiag1.gif



PAMP: pathogen associated molecular pattern

PRR: pattern recognition receptor

Figure 5 macrophage plays a role in immunity

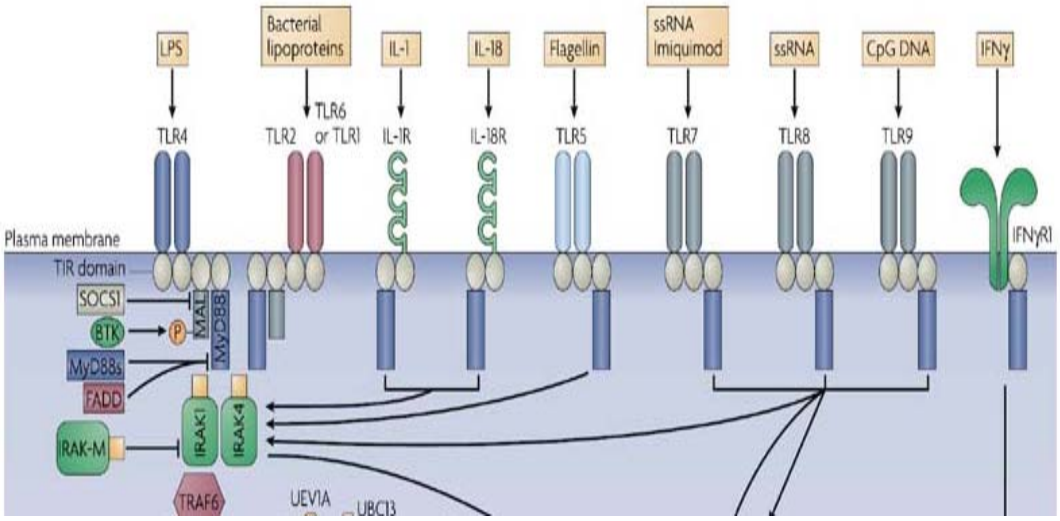


Figure 6 pathogen recognition receptors toll-like receptor

Modified from: http://www.nature.com/nri/journal/v7/n5/images/nri2079-f3.jpg

Figure 7 Bei Chai-Hu (*Buplerum chinense DC*)
http://www.db5318.com/fengye/beicaihu2.jpg

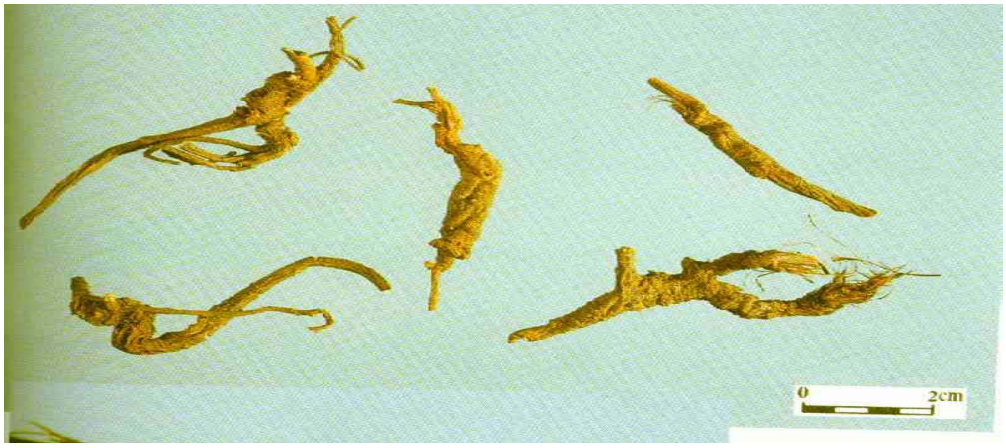


Figure 8 Nan Chai-Hu (*Buplernm scorzonerifolium Wild*)
http://www.sanqi.com.tw/original-chinese-herb/chai-hu/chaihu-03.jpg

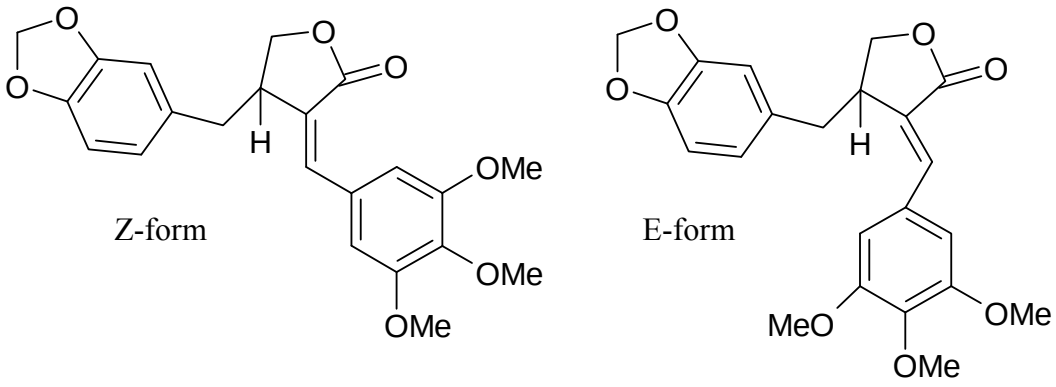
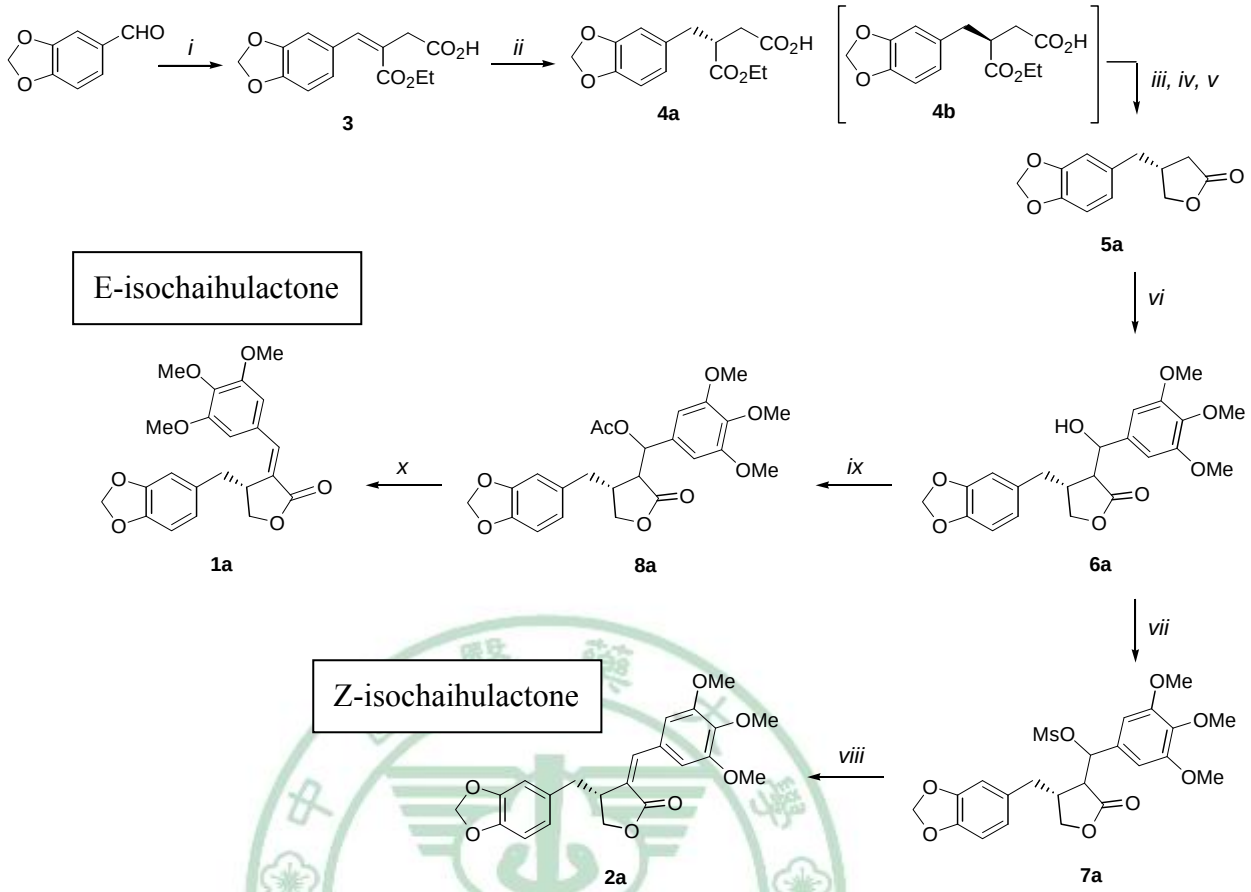


Figure 9 the structure of E- and Z-form isochaihulactone

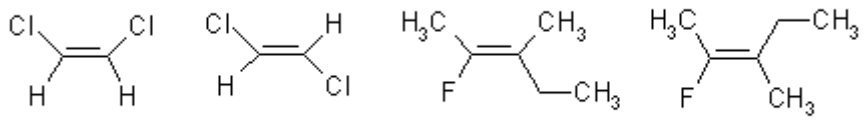


Reaction conditions: (*i*) diethyl succinate, EtONa; (*ii*) catASium® M(S)Rh or catASium® M(R)Rh, H_2; (*iii*) KOH, $CaCl_2$; (*iv*) $NaBH_4$; (*v*) 3 M HCl; (*vi*) LDA, 3,4,5-trimethoxybenzaldehyde; (*vii*) MsCl, TEA; (*viii*) DBU; (*ix*) Ac_2O, TEA; (*x*) DBU.

Figure 10 the chemical synthesis for E- and Z-isochaihulactone

by Yulin Lam,

Ddepartment of Cchemistry, National University of Singapore



A B C D

Figure 11 definition of structure of cis-trans and E-Z stereoisomerism The chlorine is the priority element for dichloroethene cis-form (A) and trans-form (B); the fluorine and ethyl group are the priority elements for fluoromethylpentene Z-form (C) and E-form (D)

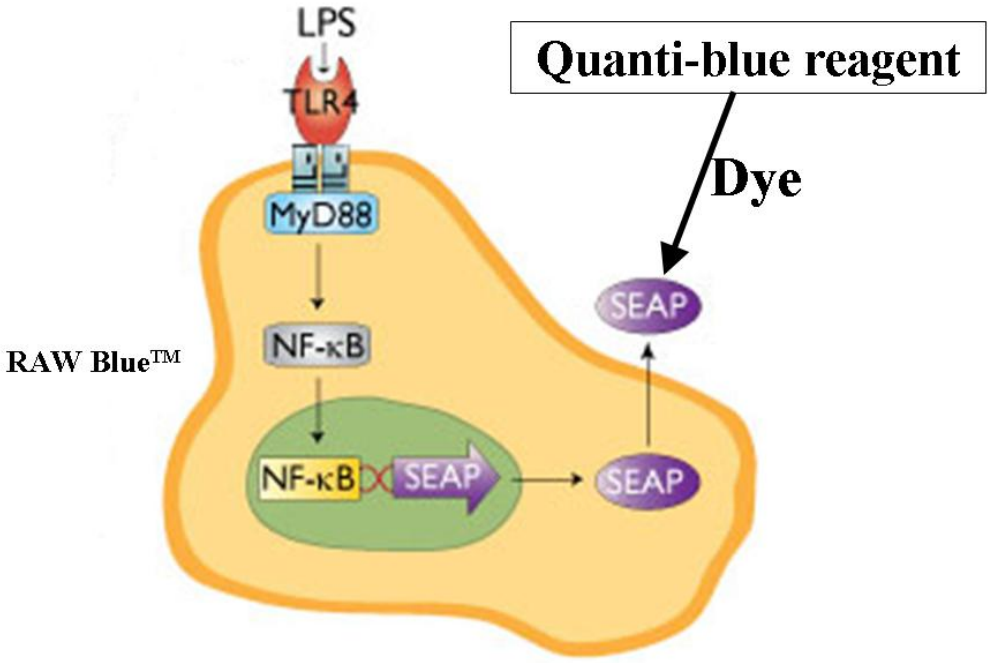


Figure 12 RAW BLUE cells with reporter gene for NF-κB assay
Modified from: http://www.autogenbioclear.com/D/pics/HekblueIL1b_assay.jpg

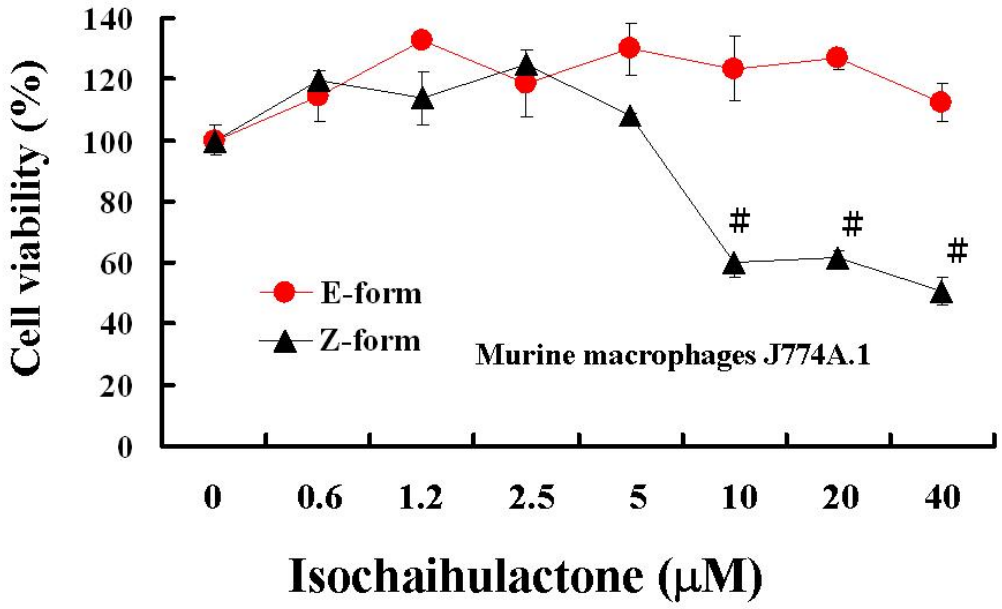


Figure 13 Effect of isochaihulactone on the viability of J774A.1 macrophages

Murine macrophages J774A.1 were seeding in $5*10^5$/ml in 96-well plate, well/100 μl and treated with isochaihulactone E-form (●) and Z-form (▲), respectively, in 24 hours with the Alamar Blue reagent at least 4 hours to show the cell proliferation. # $p < 0.05$ v.s isochai. 0 μM.

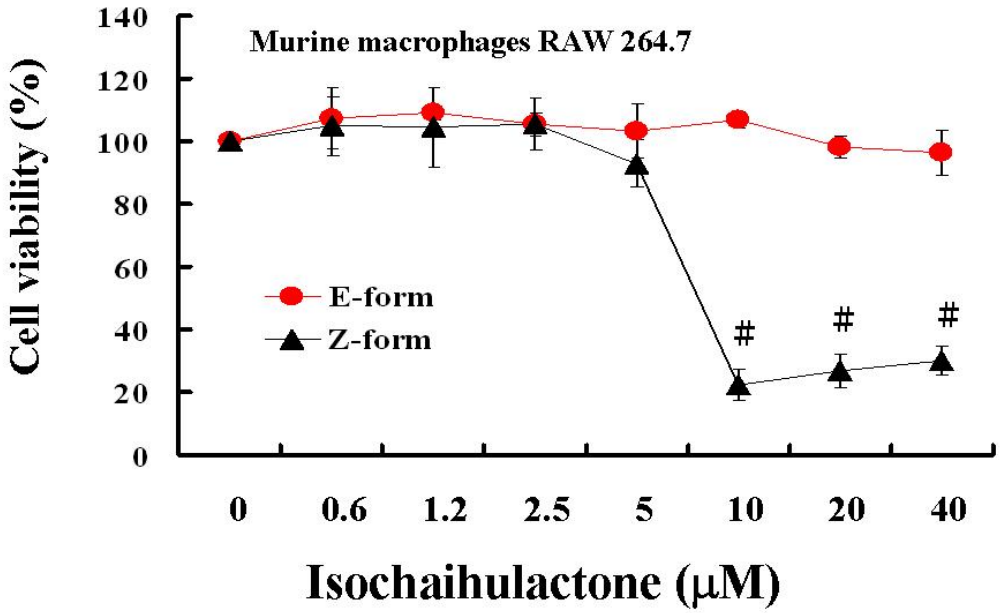


Figure 14 Effect of isochaihulactone on the viability of RAW264.7 macrophages

Murine macrophages RAW264.7 were seeding in $5*10^5$/ml in 96-well plate, well/100 μl and treated with isochaihulactone E-form (●) and Z-form (▲), respectively, in 24 hours. With the Alamar Blue reagent at least 4 hours to show the cell proliferation. # $p < 0.05$ v.s isochai. 0 μM.

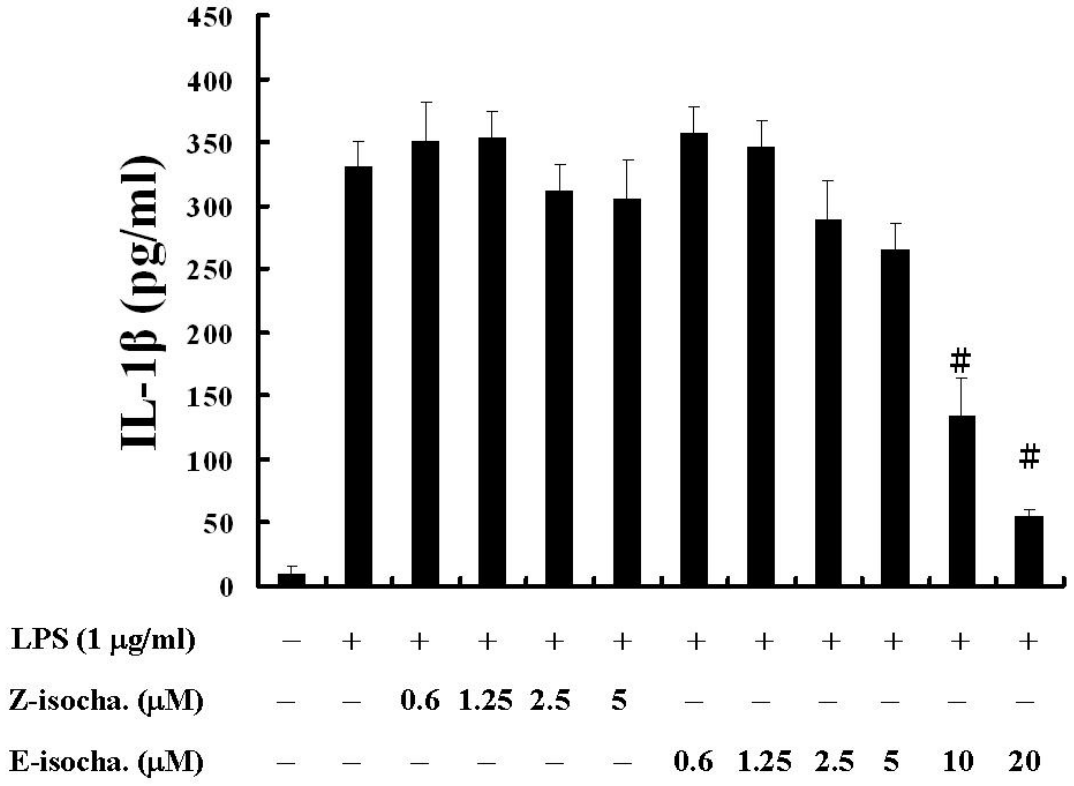


Figure 15 E-isocha. inhibited the IL-1β secretion in LPS-stimulated J774A.1 macrophages

Murine Macrophages J774A.1 were seeding in $5*10^5$/ml in 96-well plate, well/100μl and pre-treated for 30 minutes with E-form and Z-form of isochaihulactone, respectively. And then LPS was treated for 24 hours. The cell medium was harvested and tested the IL-1β production with ELISA. # $p < 0.05$ v.s LPS stimulated only.

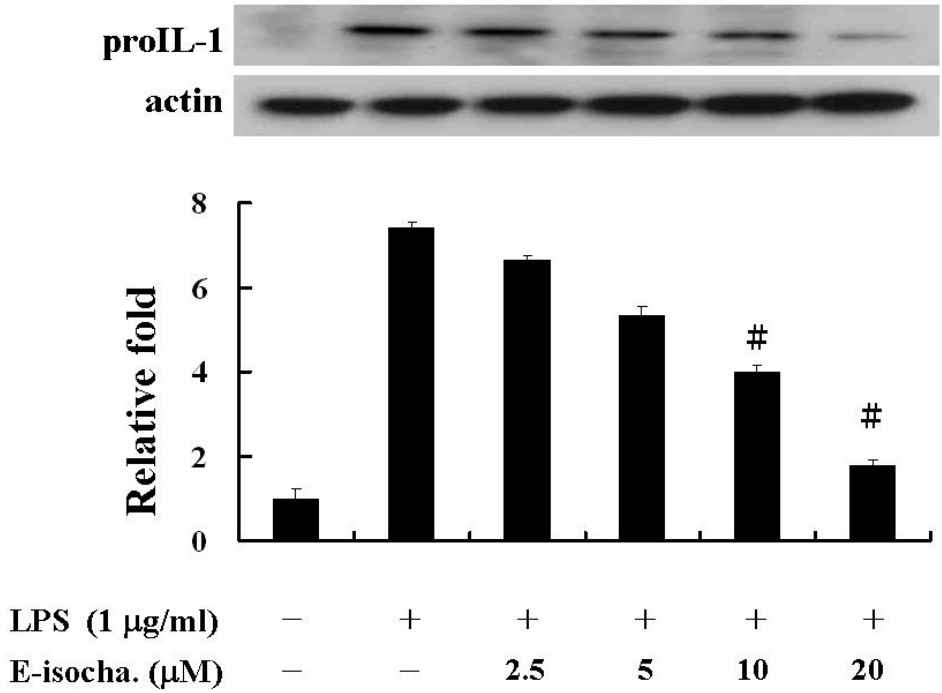
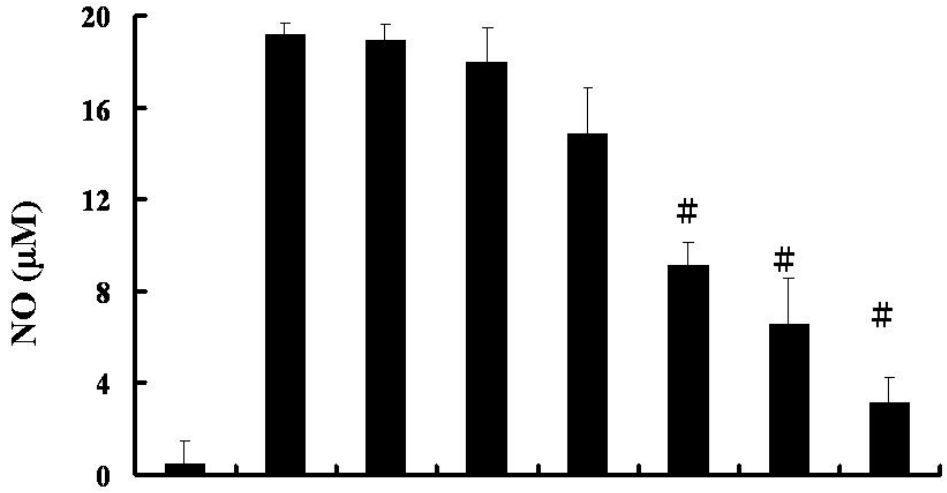


Figure 16 E-isocha. inhibited proIL-1β expression in LPS-stimulated J774A.1 macrophages

Murine macrophages J774A.1 were seeding pre-treated with E-form of Isochaihulactone from 2.5μM~20μM for 30 minutes, respectively, and then treated with lipopolysaccharide (LPS) 1μg/ml for 6 hours. Harvest the cell lysate to test the expression of pro IL-1 with western blotting. # $p < 0.05$ v.s LPS stimulated only.



LPS (1 μg/ml)	−	+	+	+	+	+	+	+
E-isocha. (μM)	−	−	2.5	5	10	20	−	−
Prednisolone (μM)	−	−	−	−	−	−	1	10

Figure 17 E-isocha. inhibited NO release from RAW264.7 macrophages

Murine macrophages RAW264.7 were pre-treated with E-form of Isochaihulactone and Prednisolone for 30 minutes, respectively. LPS was treated in 24 hours and the medium was harvested to test NO concentration with Griess Reagent. # $p < 0.05$ v.s LPS stimulated only.

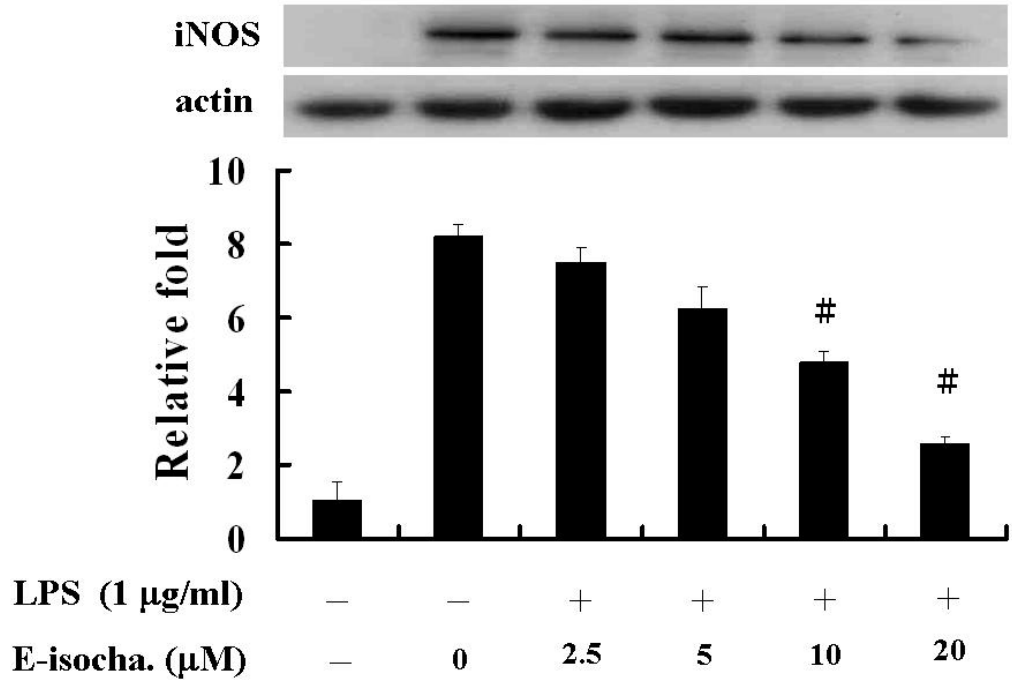


Figure 18 E-isocha. inhibited iNOS expression in LPS-stimulated RAW264.7 macrophages

Murine macrophages RAW264.7 were pre-treated with E-form of Isochaihulactone for 30 minutes, respectively. LPS was treated in 24 hours and the cell lysate was harvested to test the expression of iNOS and actin, as an internal control with Western blotting. # $p < 0.05$ v.s LPS stimulated only.

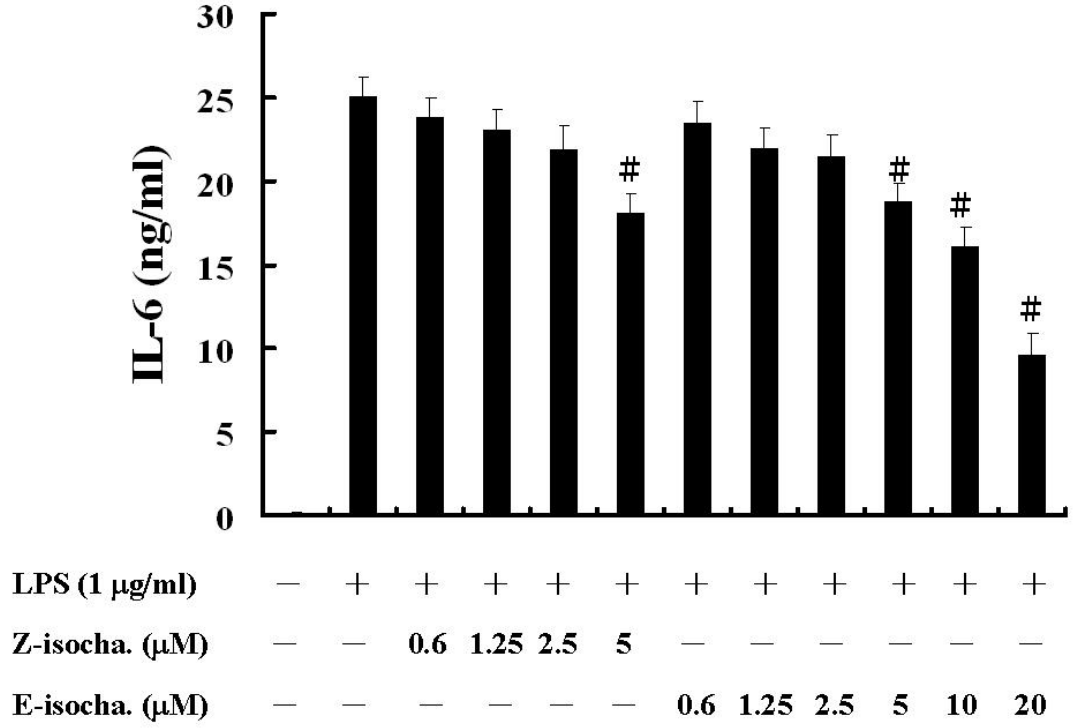


Figure 19 E-isocha. inhibited IL-6 production in LPS-stimulated J774A.1 macrophages

Murine Macrophages J774A.1 were pre-treated for 30 minutes with E-form and Z-form of Isochaihulactone, respectively. And then LPS was treated for 24 hours. The cell medium was harvested and tested the IL-6 production with ELISA. # $p < 0.05$ v.s LPS stimulated only.

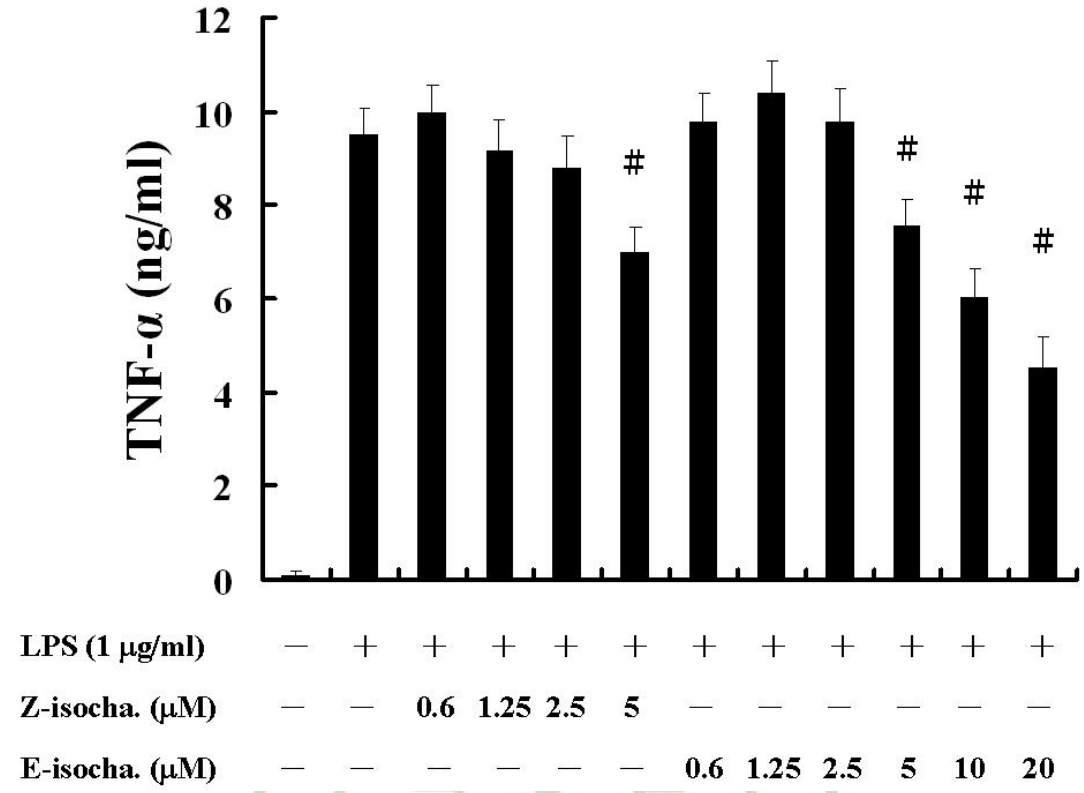


Figure 20 E-isocha. inhibited TNF-α production in LPS-stimulated J774A.1 macrophages

Murine Macrophages J774A.1 were pre-treated for 30 minutes with E-form and Z-form of Isochaihulactone, respectively. And then LPS was treated for 24 hours. The cell medium was harvested and tested the TNF-α production with ELISA. # $p < 0.05$ v.s LPS stimulated only.

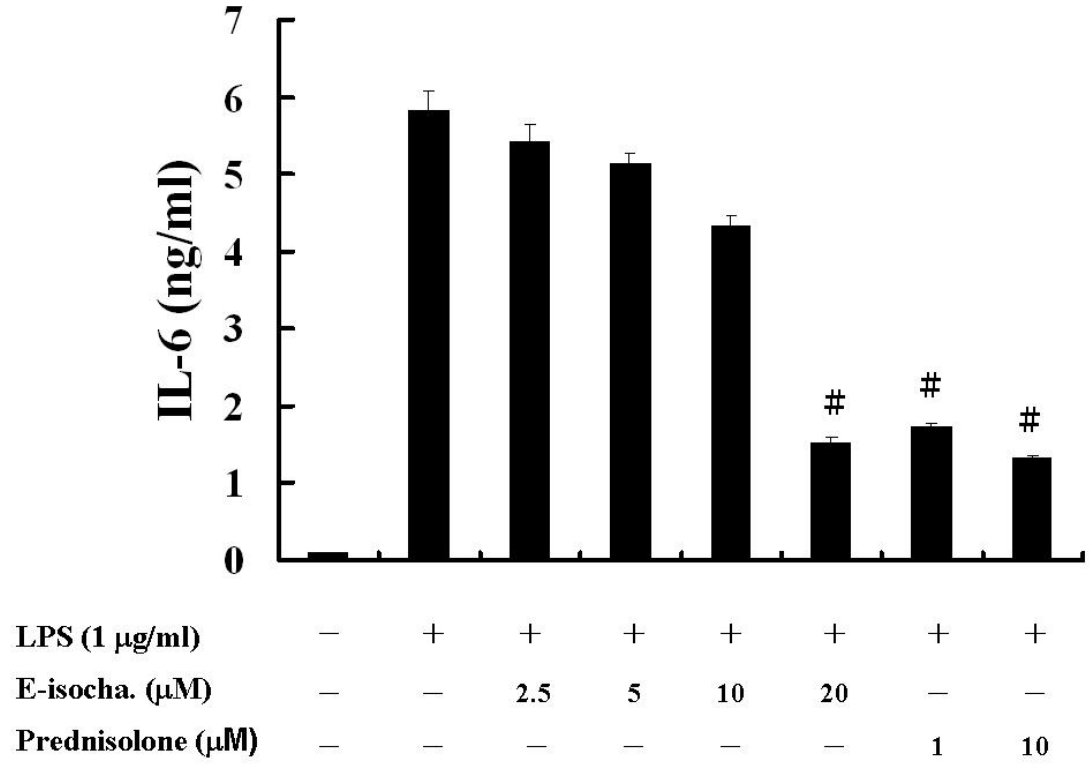


Figure 21 E-isocha. inhibited IL-6 production in LPS-stimulated RAW264.7 macrophages

Murine Macrophages RAW264.7 were pre-treated for 30 minutes with E-form of Isochaihulactone and Prednisolone, as a positive control, respectively. And then LPS was treated for 24 hours. The cell medium was harvested and tested the IL-6 production with ELISA. # $p < 0.05$ v.s LPS stimulated only.

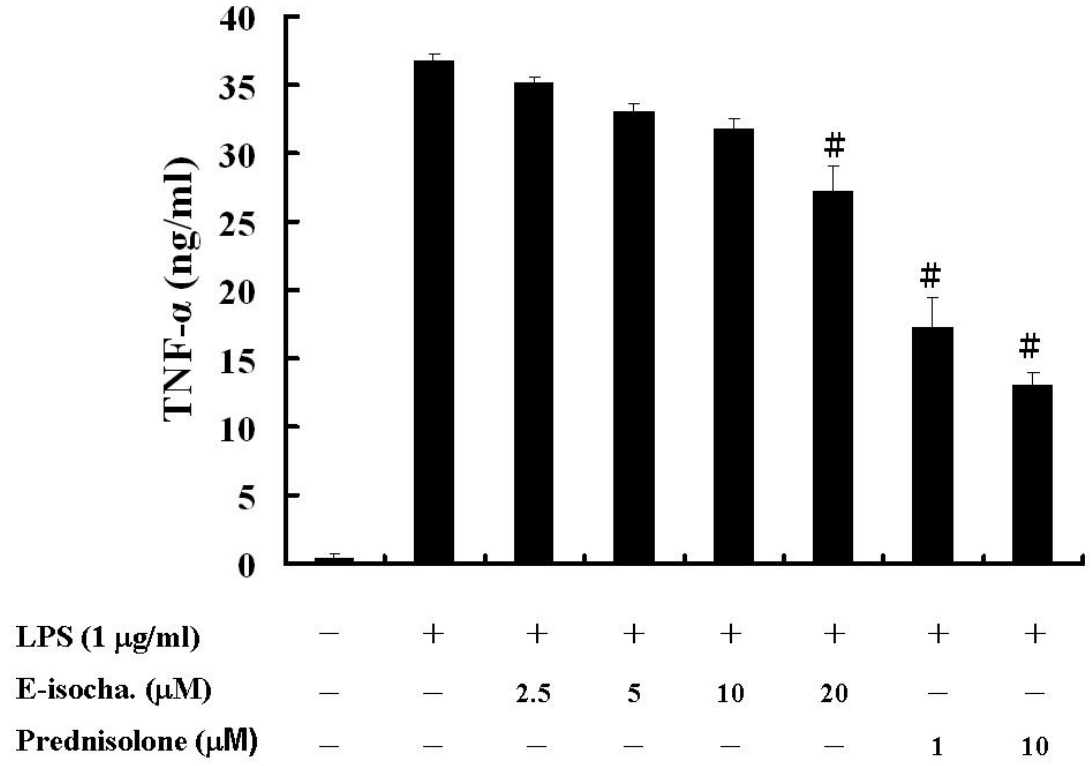


Figure 22 E-isocha. inhibited TNF-α production in LPS-stimulated RAW264.7 macrophages

Murine Macrophages RAW264.7 were pre-treated for 30 minutes with E-form of Isochaihulactone and Prednisolone, as a positive control, respectively. And then LPS was treated for 24 hours. The cell medium was harvested and tested the TNF-α production with ELISA. # $p < 0.05$ v.s LPS stimulated only.

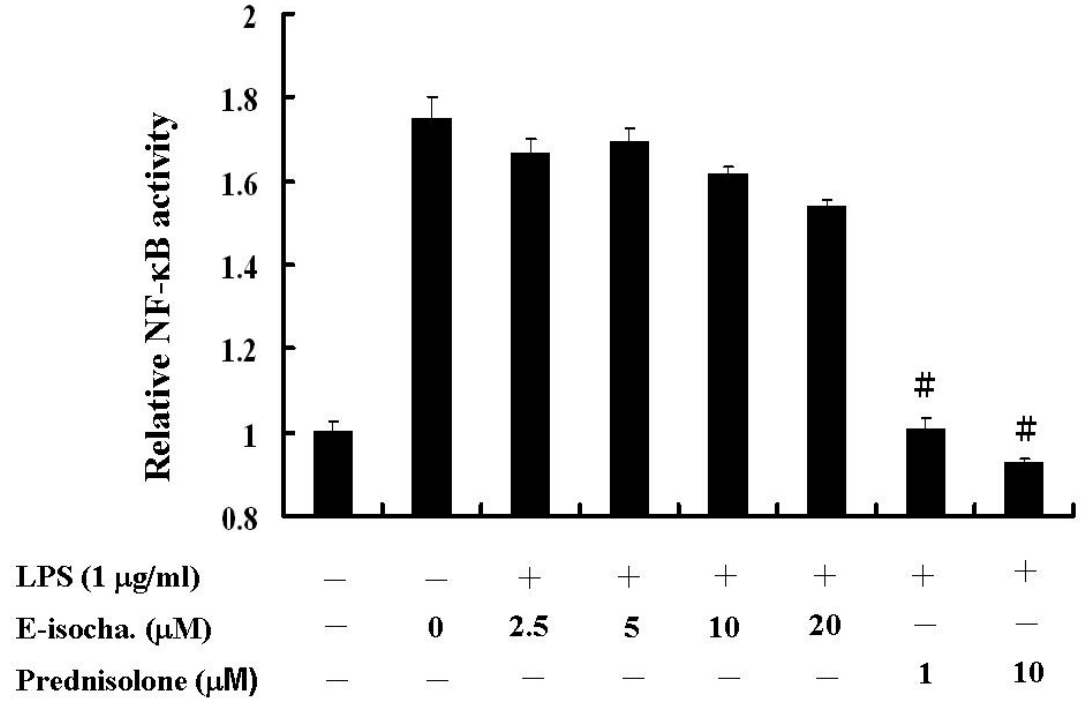


Figure 23 E-isocha. had no effect on NF-κB activity in LPS-stimulated RAW-BLUE™ cells

Murine Macrophages RAW-Blue were pre-treated for 30 minutes with E-form of isochaihulactone and prednisolone as a positive control, respectively. And then LPS was treated for 24 hours. The cell medium was harvested and tested the optical density (OD) value in relative activity of NF-κB production with Quanti-Blue reagent. # $p < 0.05$ v.s LPS stimulated only

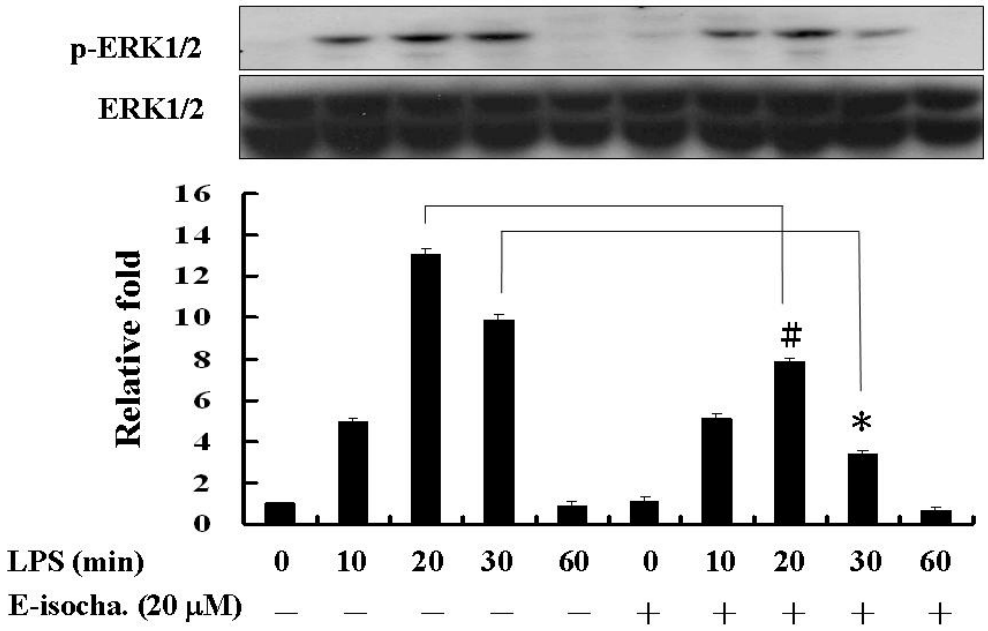


Figure 24 E-isocha. inhibited phosphorylation of ERK1/2 in LPS-stimulated RAW264.7 macrophages (time course study)

Murine Macrophages RAW264.7 were pre-treated E-isocha. (20 μM) And DMSO for 0 minutes, and LPS added. The cell lysate was harvested in 0, 10, 20, 30, and 60 minutes. The p-ERK was tested with Western Blot. # $p < 0.05$ v.s E-isocha. free in 20 minutes; * $p < 0.05$ v.s E-isocha. free in 30 minutes.

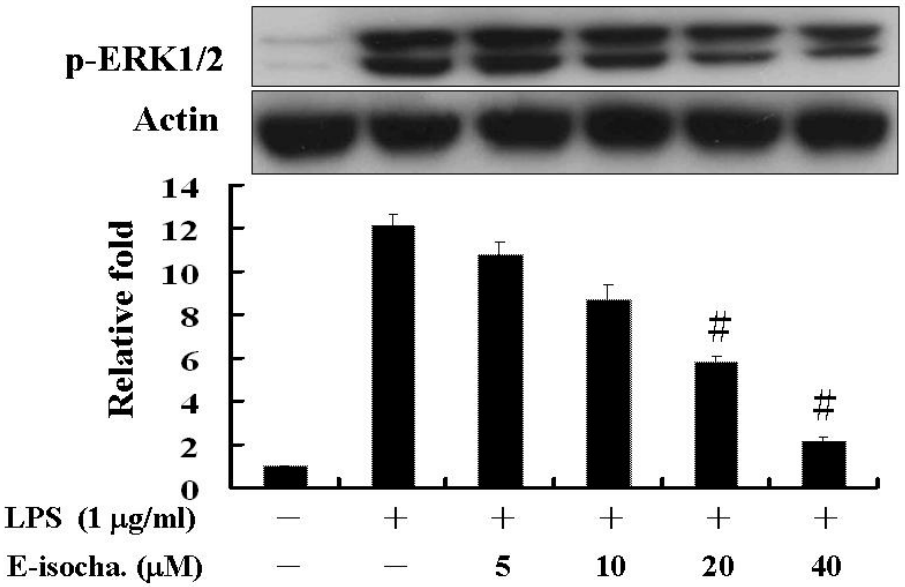


Figure 25 E-isocha. inhibited phosphorylation of ERK1/2 in LPS-stimulated RAW264.7 macrophages (dosage study)

Murine Macrophages RAW264.7 were pre-treated E-isocha. 5, 10, 20, and 40 μM for 30 minutes, and LPS added. The cell lysate was harvested in 20 minutes. p-ERK was tested with Western Blot. # $p < 0.05$ v.s LPS stimulated only.

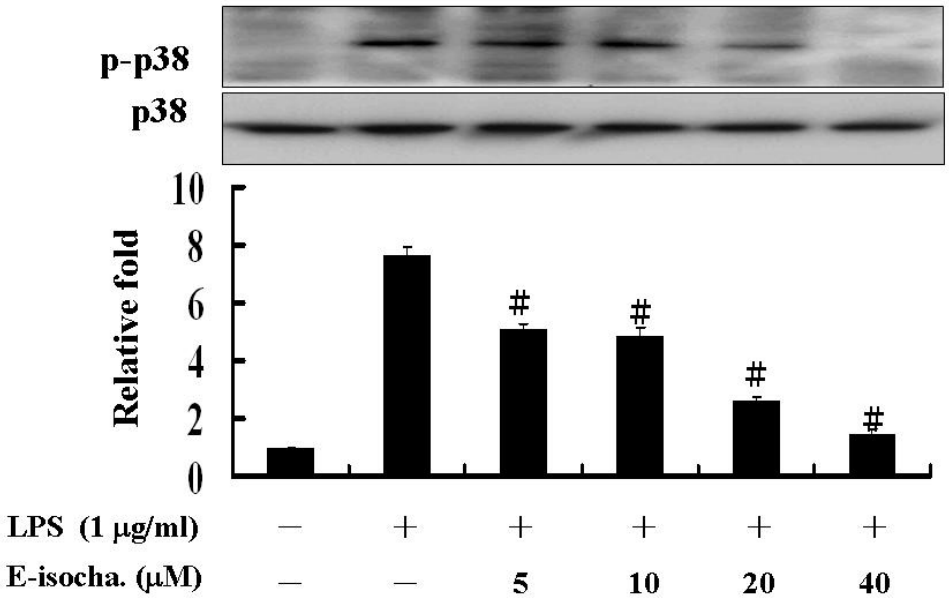


Figure 26 E-isocha. inhibited phosphorylation of p38 in LPS-stimulated RAW264.7 macrophages

Murine Macrophages RAW264.7 were pre-treated E-isocha. 5, 10, 20, and 40 μM for 30 minutes, and LPS added. The cell lysate was harvested in 20 minutes. p-p38 was tested with Western Blot. # $p < 0.05$ v.s LPS stimulated only

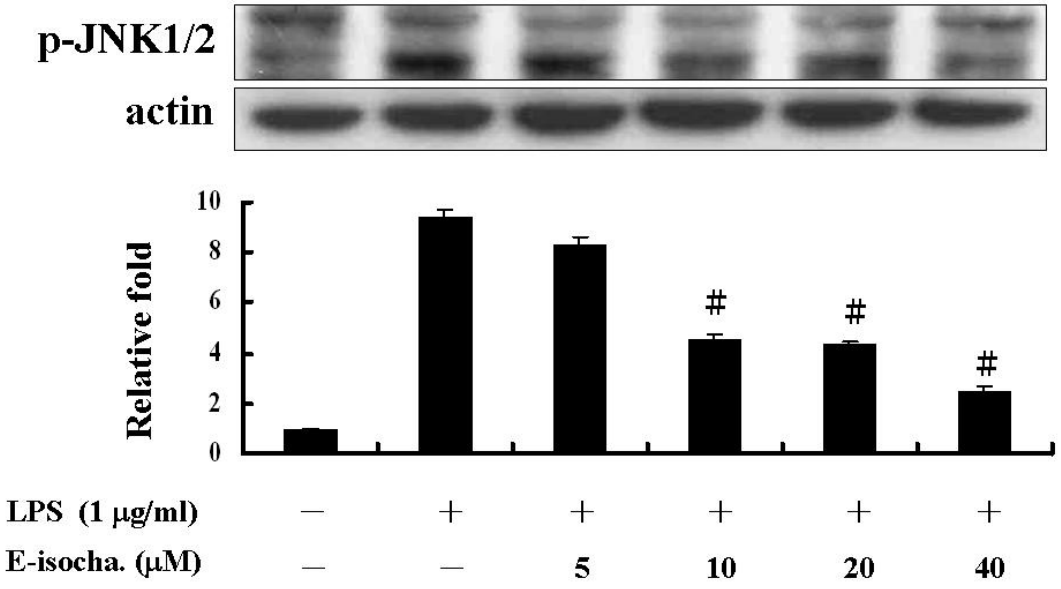


Figure 27 E-isocha. inhibited phosphorylation of JNK1/2 in LPS-stimulated RAW264.7 macrophages

Murine Macrophages RAW264.7 were pre-treated E-isocha. 5, 10, 20, and 40 μM for 30 minutes, and LPS added. The cell lysate was harvested in 20 minutes. p-JNK was tested with Western Blot. # $p < 0.05$ v.s LPS stimulated only

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Appendix

Chemistry

The synthesis of racemic anhydropodorhizol **1** was previously described by Minami *et al*[1] while the asymmetric synthesis of (-)-anhydropodorhizol **1a** was reported by Koga and coworkers[2]**.** To the best of our knowledge, the synthesis of isochaihulactone **2** has never been reported. In this proposal, we aim to synthesize the four compounds shown in Figure 1**.**

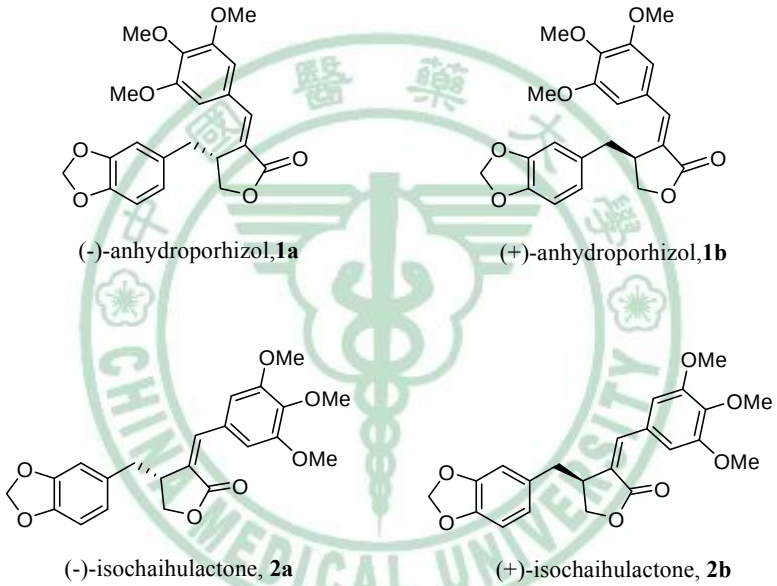


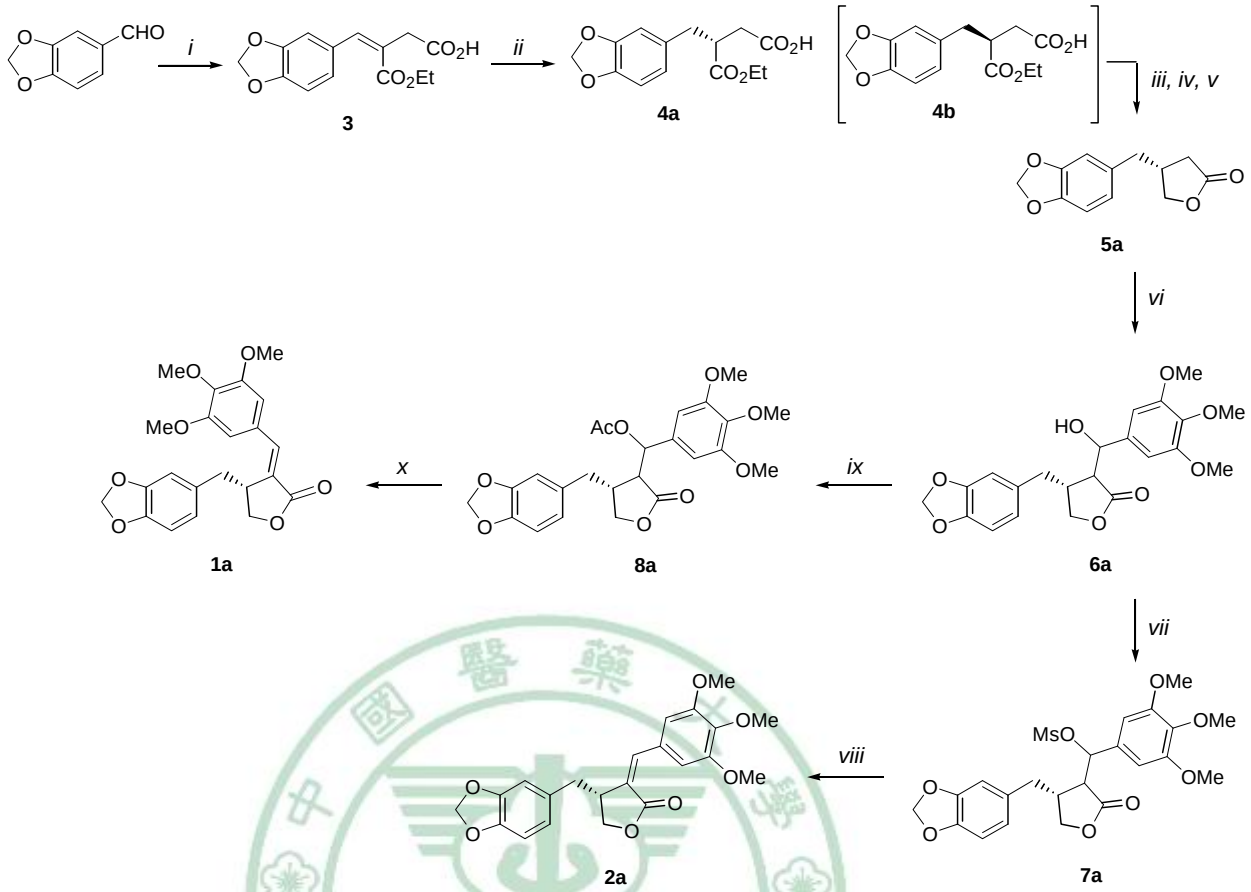
Figure 1. List of compounds to be synthesized.

Our proposed synthetic strategy for the preparation of target compounds **1a**, **1b**, **2a** and **2b** is shown in Scheme 1. Compound **3** can be readily obtained via Stobbe condensation of piperonal and diethyl succinate. Subsequent asymmetric hydrogenation of **3** would yield either **4a** or **4b**. There are several known catalysts available for the asymmetric hydrogenation of itaconic acid derivatives[3-5] which could be applied to the hydrogenation of **3**. In the preparation of compounds **4a** and **4b**, we

propose to use catASium® M(S)Rh and catASium® M(R)Rh respectively as the chiral catalysts as these catalysts are commercially available, relatively low costs and have been shown to demonstrate excellent enantioselectivity in the hydrogenation of itaconic acid derivatives.[4]

Chemoselective reduction of the potassium salts of **4a** and **4b** with $CaCl_2/NaBH_4$ in ethanol could provide the desired lactones **5a** and **5b** respectively. Aldol condensation of **5a** and **5b** with 3,4,5-trimethoxybenzaldehyde would afford the alcohol **6a** and **6b** respectively which could be treated with either acetic anhydride or methanesulfonyl chloride to provide the corresponding acetate or mesylate respectively. Elimination of the mesylate moiety from **7a** and **7b** would yield **2a** and **2b** respectively. On the other hand, base promoted elimination of the formed acetate **8a** and **8b** will afford **1a** and **1b** respectively.

Scheme 1[a,b]



Reaction conditions: (*i*) diethyl succinate, EtONa; (*ii*) catASium® M(S)Rh or catASium® M(R)Rh, H_2; (*iii*) KOH, $CaCl_2$; (*iv*) $NaBH_4$; (*v*) 3 M HCl; (*vi*) LDA, 3,4,5-trimethoxybenzaldehyde; (*vii*) MsCl, TEA; (*viii*) DBU; (*ix*) Ac_2O, TEA; (*x*) DBU.

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