CHAPTER 1

Introduction

1.1 Historical Background

Inflammation is the primary physiological reaction in the body, which purpose of inflammation is a protective process to infection by viruses, bacteria, parasites, fungi or tissue injury (1). The inflammatory process is need to remove the initial cause of cell damage and clean out tissues injury from inflammatory sites and also to restore tissue homeostasis. Then, inflammation is a beneficial response for the body. However, if inflammation is un-regulated, it can become chronic, inducing pathogenesis and inflammatory disorder.

Inflammatory process can be classified as acute and chronic inflammation. Acute inflammation is an early response to tissue injury or infection that result in the movement of inflammatory cells include that neutrophils and leukocytes, plasma proteins and inflammatory mediators to the injured sites and their activation to eliminate the harmful stimuli. However, if inflammatory processes continues, which may cause protracted inflammation, called as chronic inflammation is a continuous stage at the site of inflammation. Chronic inflammation is characterized by the exceedingly produce of cytokines, mediators, reactive oxygen and nitrogen species by macrophages. Prolonged inflammation leading to excess destruction of extracellular matrix and healing of the tissue lead to fibrosis and angiogenesis (2). Several studies have shown that prolonged inflammation performs a critical duty in the beginning and development of several inflammatory disorders including cardiovascular disease, arthritis, diabetes and cancer (3-5). Moreover, chronic inflammation can also result in mutations and involve with tumorigenesis.

Macrophages is the major inflammatory cells that participate in inflammation processes. Once macrophages is stimulated by inflammatory inducer including cytokines and

lipopolysaccharide (LPS). The activated macrophages are excessive produce inflammatory mediators including prostaglandin E_2 (PGE_2), nitric oxide (NO) and pro-inflammatory cytokines including interleukin-1 (IL-1), IL-6 and tumor necrosis factor-alpha (TNF-α) (6,7). Moreover, the expression of inducible nitric oxide synthases (iNOS) and cyclooxygenase-2 (COX-2) are up-regulated in activated macrophages (8). Many reports indicated that the abnormal up-regulation of inflammatory mediators have been involved with the pathogenicity of inflammatory disorder. Several signaling pathways and transcription factors are related with the expression of inflammatory genes in macrophages. The nuclear factor kappa B (NF-κB) and activating protein-1 (AP-1) are the transcription factors that play a significant function in regulate the expression of inflammatory genes (9). The activation of NF-κB and AP-1 are regulated by cellular kinase such as mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3′-kinase/Akt (PI3-K/Akt). The MAPKs signaling molecules such as extracellular signal regulated kinase1/2 (ERK1/2), p38 MAPK and c-Jun NH_2-terminal kinase (JNK) have been implicated in the expression of inflammatory gene during inflammation process. In addition, the PI3-K/Akt signaling molecules mediated inflammatory signals that trigger the transcription of inflammatory gene by AP-1 and NF-κB (10).

Macrophage activation and the release of inflammatory mediators are necessary for the progression of numerous disease. LPS-stimulated macrophages are generally used as a model system since LPS is a pathogen which trigger the activation of toll-like receptor 4 (TLR4). The TLR4 signaling can lead to the activation of MAPKs, or Akt, which in turn activates NF-κB and AP-1 activities and finally initiates inflammatory responses (11). Therefore, the agent capable of suppressing these signaling molecules may be potentially useful in the therapeutic method for inflammatory disorder.

A variety of phytochemical compounds have been applied to anti-inflammatory agent, Crebanine is an aporphine alkaloid that has been separated from the tube of *Stephania venosa*, which is also well known as a rich source of alkaloids.

Crebanine has pharmacological safety and exhibits a variety of biological activities, including, an anti-microbial, anti-arrhythmic, anti-invasion and anti-proliferation activity

in cancer cells (12-14). From previous studied indicated that crebanine decreased TNF-α induces lung cancer cells proliferation and cell invasion through reducing NF-κB activation (15). However, anti-inflammatory activity and molecular mechanisms of crebanine have not been elucidated.

In the current research, we evaluated the effect of crebanine on LPS-stimulated the production of inflammatory cytokines and mediators include that IL-6, TNF-α, PGE_2, and NO. The effect of crebanine on the level of COX-2 and iNOS protein expression were determined. Additionally, we also determined the molecular inflammatory mechanisms throughout the activation of inflammatory transcription factors including NF-κB and AP-1 and MAPKs, PI3-K/Akt signaling pathways.

1.2 Literature review

1.2.1 Process of inflammation

Inflammation is a primary component of the immune system, which is an intrinsically beneficial response. Inflammation process leads to removal of inflammatory stimuli and restore the tissue structure and physiological function to homeostasis. Inflammation processes are involved with immune cells, blood vessels, and molecular mediators. However, un-regulated inflammation has harmful effects, these processes can interfere or damage healthy cells, and causing the pathogenesis of many inflammatory disorders.

At basic knowledge, types of inflammation can be categorized as acute and chronic inflammation. Acute inflammation is a rapid response to tissue injury or infection. The tissue damaging or infection that lead to rapid recruitment of granulocytes typically neutrophils and basophils to the inflammatory site. The alterations in vascular size by vasodilation process lead to increase blood flow and enhances vascular permeability. Vasodilation permit blood-derived products, such as plasma proteins, fluid and immune cells to accumulate in the inflammatory sites. Acute inflammatory processes are triggered by a several of stimuli including inflammable material; bacterial, viral, parasitic infections or microbial toxins, physical and chemical agents, thermal injury, irradiation, environmental chemicals, tissue necrosis and hypersensitivity reactions (16).

When triggered by a variety of physical, chemical, biological stimuli or tissue injury, inflammation is a cumulative result of several reactions. That response to the alteration of the circulation in the blood vessel, changes in vascular caliber. Vasodilation process was occurred in the earliest manifestations of acute inflammation then results in exposing of new capillary in the inflammatory site, thus comes about increased blood flow. Followed by increasing of the vascular permeability of the microvascular with the overflow of protein-rich fluid called exudate into the extravascular tissues.

The loss of these protein from the plasma decreases the intravascular osmotic pressure and increases the osmotic pressure of the interstitial fluid. At the early stage of inflammation, neutrophils are the first leukocyte to accumulate along the endothelial wall for migrate to the inflammatory sites via extravasation.

The process of leukocyte movement to the sites of inflammation through the blood vessels is known as extravasation. As shown in Figure 1, when leukocyte was activated, that bind to specific G protein-coupled receptors on the endothelial wall. Then, signal transduction induces the expression of cell adhesion molecules include that P-selectin, E-selectin and immunoglobulin ligands such as ICAM-1 and VCAM-1 on endothelial surfaces. This receptor binds softly to carbohydrate ligands on leukocyte surfaces and lead to rolling along the endothelial wall surface called endothelial adhesion. Next, the leukocyte can be move between endothelial cells and pass the basement membrane into the tissue known as transmigration (17). And then, movement of leukocytes to the inflammatory sites depend on chemotactic gradient towards the injured site. The type of migrating leukocyte depend on the stage of the inflammatory response. In practically patterns of acute inflammation, neutrophils take action in the inflammatory infiltrate through the first 6 to 24 h, then are substituted by monocytes or macrophages in 24 to 48 h. At the inflammatory site, membrane receptors for complement proteins and immunoglobulins recognize and bind the pathogen leading to the phagocytosis process of the stimuli and destruction within the intracellular phagosome.

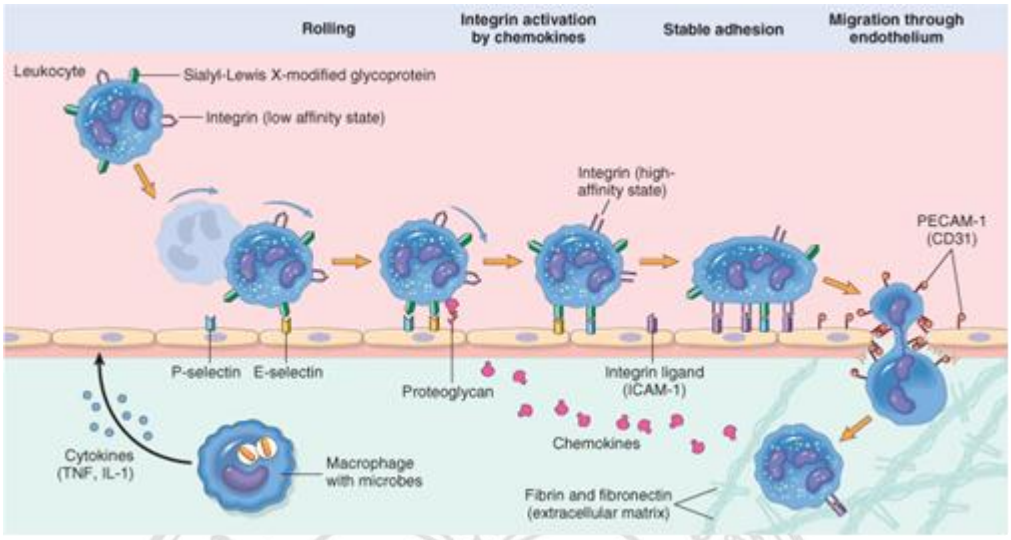

Figure 1. The process of leukocyte movement to the inflammatory sites (2)

Prolonged inflammatory stimulation was occurred that results in chronic inflammation. In states of chronic inflammation is characterized by leukocyte recruitment especially monocytes or macrophages and lipoprotein accumulation. Macrophages and other inflammatory cells produce high levels of pro-inflammatory cytokines including IL-1β, IL-6, IL-8 and TNF-α. These macrophages also generate ROS and RNS that may cause DNA damage (18). In addition, when macrophages are activated continuously that resulting in the destruction of extracellular matrix (ECM). The chronically tissue damage promotes the level of inflammatory mediators leading to increased collagen production, releasing of growth factors and other ECM modulators. Chronic inflammation microenvironment established by all the exceeding cell proliferation rate. Moreover, inflammatory site is often hypoxia condition that is an important angiogenic signal. These signal activates the hypoxia-inducible factor signaling cascades, which induces the production of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). This factor lead to associated with angiogenesis process (19).

1.2.2 Mediators of inflammation.

Pro-inflammatory cytokines

Cytokines are small glycoproteins produced by many cell types including macrophages, or leukocytes, neutrophils, fibroblasts, and vascular endothelial cells. They are a regulators of various physiological and pathological functions including immunity, trauma and inflammatory process. In inflammatory process, activated macrophages generate a large amount of cytokines including interferons (IFNs), TNF-α, IL-1 and IL-6, which are potently pro-inflammatory cytokines. IL-6 mediates its results via binding to receptors on the cell surface, called IL-6R, which function in both membrane-bound and soluble forms, resulting in the inflammatory responses. For example, the alteration of the body temperature (fever), recruiting more immune cells to the inflammatory site and increasing vascular permeability. TNF-α is another pro-inflammatory cytokines of acquired immunity. Increasing of TNF-α level cause the classical sign of inflammation such as heat, swelling, redness and pain. As reported in Figure 2, TNF-α caused induce other inflammatory mediators that lead to tissue damage and loss of function. Moreover, TNF-α can also stimulates the level of IL-1, IL-6, and TNF-α production itself by the macrophages stimulation (20).

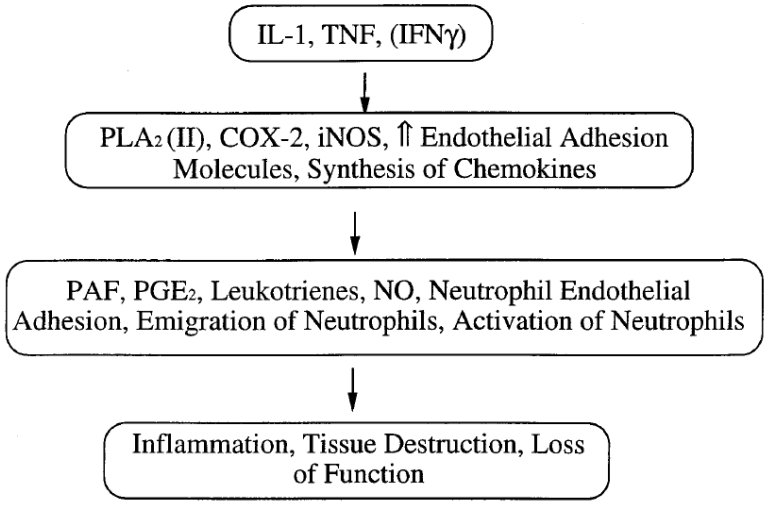

Figure 2. The inflammatory cascade triggered by IL-1 and TNF-α (21)

Nitric oxide (NO)

NO is a biological molecule which acts a role in the inflammatory condition. In normal physiological conditions, NO gives an anti-inflammatory effect. Whereas, it is considered as an inflammatory mediator when overproduction in abnormal condition. As shown in Figure 3, NO was generated from L-arginine and molecular oxygen by nitric oxide synthase (NOS). NOS is generated by three isoforms of NOS including nNOS, iNOS and eNOS. In the cell, nNOS and iNOS are predominantly found in the cytosol, iNOS catalyzes the oxidation of L-arginine to NO and L-citrulline. One molecule of L-arginine generates one molecule of NO, the nitrogen atom of the latter deriving from a terminal guanidino group of the arginine side chain. In platelets and also vascular smooth muscle, NO is result to the increasing levels of cGMP initiate the activation of cGMP-dependent protein kinases cascade, which regulate smooth muscle relaxation and inhibit the platelet adhesion. In inflammatory condition, NO is associated with vasodilatation and involved in immune reactions via cytokines-activated macrophages, which release NO in high concentrations (22). Recently study have reported that the transcriptional activity of the iNOS gene is induced by pro-inflammatory cytokines include that IL-1, TNF-α, γIFN and endotoxin. Moreover, the iNOS expression was also upregulated in LPS-induced macrophages.

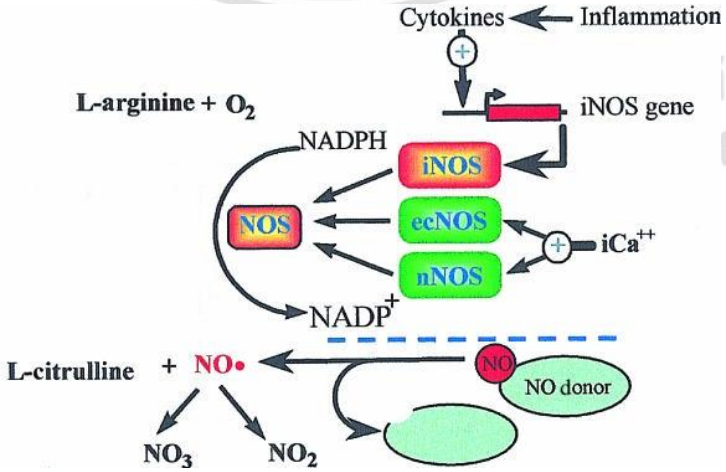

Figure 3. The L-arginine-nitric oxide pathway (23)

Cyclooxygenase (COX)

COX is the enzyme which catalyzes the modification of arachidonic acid (AA) and other polyunsaturated fatty acids to prostaglandins (PGs) and thromboxanes (TX) that presented in Figure 4. These two isoforms of COX are main enzymes in PG production pathways, COX-1 is constitutively expressed in most tissues and COX-2 is an enzyme that is expressed only triggered by pro-inflammatory cytokines and mitogens (24). Many study showed that COX-2 is stimulated with inflammatory stimuli, which is an essential resource of prostaglandins formation in inflammatory process, as well as degenerative disorders include that arthritis, autoimmune diseases and cancer. Prostaglandins are members of the eicosanoid family. The most abundant prostaglandins in the human body is prostaglandin E_2 (PGE_2), which present in the initiation of inflammatory reaction. Their biosynthesis is markedly increased in inflammatory sites, resulting in the classical signs of inflammation including, redness and edema have been caused from raised blood stream into the inflammatory sites via PGE_2-mediated the extension of arterial dilatation and raised vascular permeability, and pain have been effected from the activity of PGE_2 on peripheral sensory neurons and on central sites in the spinal cord and the brain. Therefore, PGE_2 is an essential mediator of immunopathology in chronic inflammatory disorder such as rheumatoid arthritis and osteoarthritis.

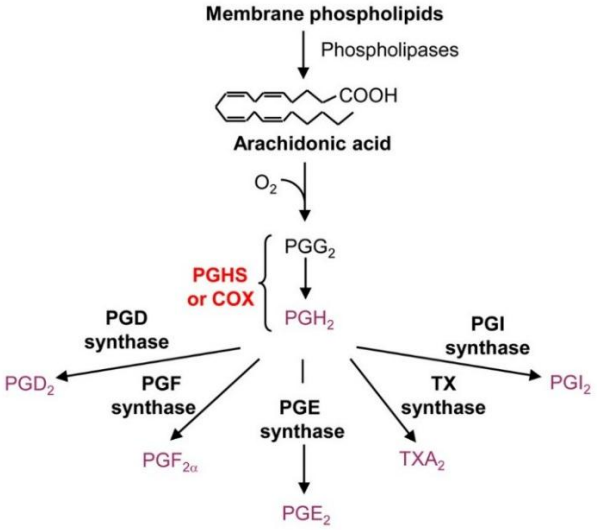

Figure 4. Biosynthesis of prostaglandins (25)

1.2.3 Diseases-associated with chronic inflammation.

In chronic inflammation, macrophages and other inflammatory cells produce an excessive amount of growth factors, pro-inflammatory cytokines, ROS and RNS that may cause continuous tissue damage and contributes to the pathogenesis. Chronic inflammation was found to mediate a many of inflammatory diseases, including cardiovascular diseases, cancer, arthritis, diabetes and neurodegenerative disorders.

Atherosclerosis

Atherosclerosis is a chronic cardiovascular disease results in the development of plaques and progressive stenosis of blood vessels. Inflammation has been found to associate in every stage of atherosclerosis, since initiation to progression process. As shown in Figure 5, the atherosclerosis lesion is started by inflammation involved with retained low-density lipoprotein (LDL) particles in the endothelial cells of vessel wall. This LDLs are made up totally of monocyte-derived macrophages. Inflammation in the vessel wall proceeds as cascades, which begin with endothelial cell activation, resulting in the expression of adhesion molecules on the cell surface, vascular cell adhesion molecules, selectins, integrins, and the production and releasing of pro-inflammatory cytokines and chemokines including IFNs, IL-1β, IL-6, TNF-α, monocyte chemoattractant protein-1, macrophage inflammatory protein-1. Monocytes subsequently mature into macrophages and engulf lipids, becoming foam cells that subsequently contribute to eventual atherosclerotic plaque formation. These formation reduce the elasticity of the vessel walls leading to increase pulse pressure and continuous stenosis of blood vessels. The atheroma further develops with the recruitment of T-cells, mast cells, and macrophages to the intima. Recruited inflammatory cells elaborate various pro-inflammatory cytokines, which promote the expression of matrix-degrading proteases, alteration of smooth muscle cells and remodeling of the extracellular matrix, causing the lesion to increase in vascular size resulting in thinning of the fibrous cap and therefore instability (26).

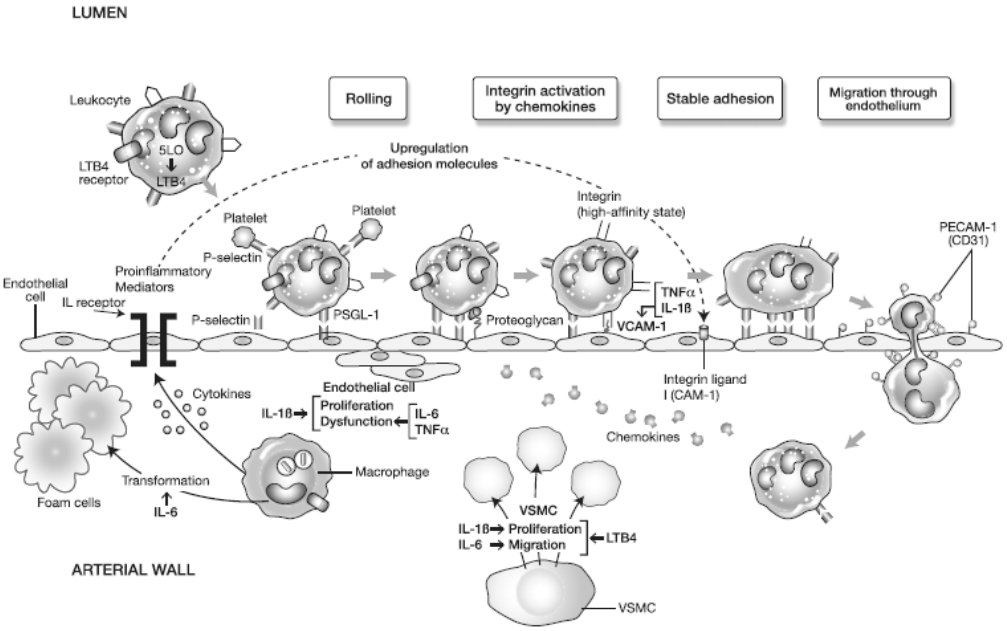

Figure 5. The outline of inflammatory pathways involved in atherosclerosis (27)

Cancer

Cancer is a consequence of genomic damage and mutation, which can rise from many causes including infection, exposure to genotoxic agents, such as ultraviolet light, carcinogens and inflammation. Inflammatory responses, which play considerable roles in multiple platforms of tumor growth or tumorigenesis, including proliferation, initiation, promotion, malignant invasion, metastasis and angiogenesis as shown in Figure 6. Chronic inflammation is a risk cause for most type of cancers is well identified. Many evidence has demonstrated that an inflammatory surrounding is an important component of all tumors. In initiation stage of tumorigenesis, an inflammatory microenvironment can enhance the proliferation of mutated cells. Activated macrophages is produce the ROS and RNI which to ability of inducing DNA damage and genomic instability. In promotion stage, an inflammatory cells act as a source of a many pro-inflammatory cytokines including IL-6, IL-11, TNF-α and IL-1β. These cytokines has many tumors-promoting effects in cancer-related inflammation (28).

Cancer and inflammation are related by two pathways, the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is activated by genetic events that cause neoplasia. These events include the activation of many types of oncogene by mutation, chromosomal rearrangement or amplification, and the inactivation of tumor-suppressor genes. Cells that are transformed in this manner produce inflammatory mediators, thereby generating an inflammatory microenvironment in tumors for which there is no underlying inflammatory condition. By contrast, in the extrinsic pathway, inflammatory or infectious conditions augment the risk of developing cancer at certain anatomical sites. The two pathways converge, resulting in the activation of transcription factors, mainly NF-κB, signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor 1α (HIF1α), in tumor cells. These transcription factors coordinate the production of inflammatory mediators, including cytokines and chemokines, as well as the production of COX-2. These factors recruit and activate various leukocytes, most notably cells of the myelomonocytic lineage. The cytokines activate the same key transcription

factors in inflammatory cells, stromal cells and tumor cells, resulting in more inflammatory mediators being produced and a cancer-related inflammatory microenvironment being generated.

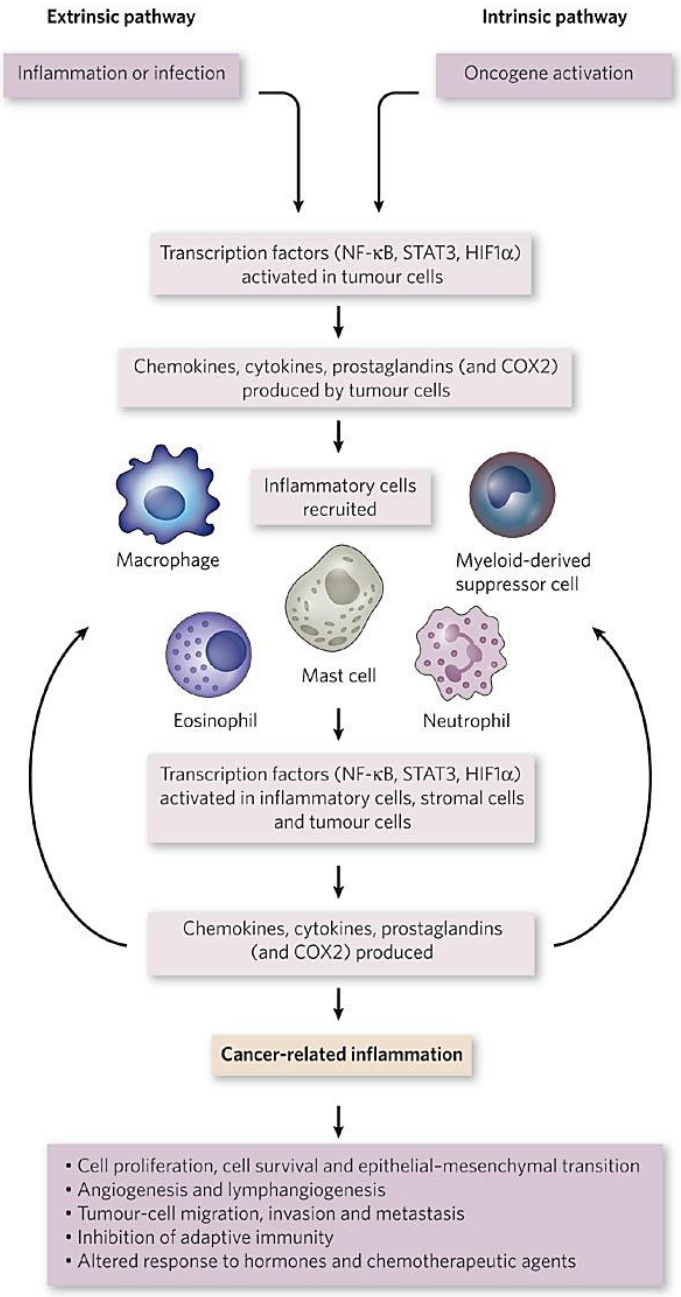

Figure 6. Cancer-related inflammation (29)

Rheumatoid arthritis (RA)

RA is a chronic inflammatory disease of the joints that is characterized by pain, swelling, and destruction of synovial joints, which can lead to loss of function. As shown in Figure 7, the inflammation of the synovial membrane within joints, resulting in the recruitment and activation of inflammatory cells, the synovial tissue is infiltrated with B and T lymphocytes, plasma cells, mast cells fibroblasts and especially macrophages. Macrophages are critically involved in the pathogenesis of RA. Not only do they produce a variety of pro-inflammatory cytokines and chemokines including TNF-α, IL-6, IL-1, and C-reactive protein (CRP), nitric oxide, matrix-degrading enzymes including MMPs and also excessively generated and intracellular accumulated the reactive oxygen species (ROS), but they also contribute to the cartilage and bone destruction in RA via NF-κB mechanisms (30).

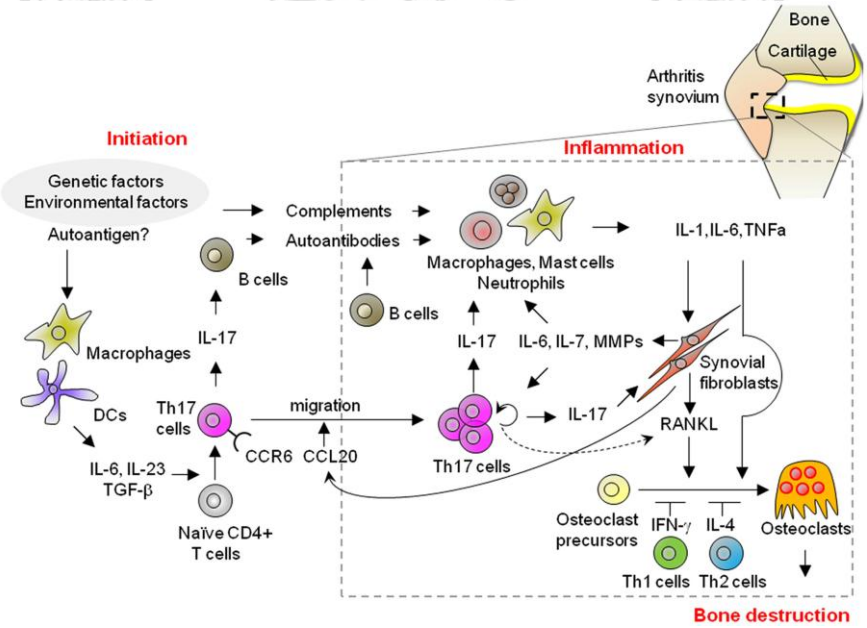

Figure 7. The interaction of immune cells and inflammatory mediators play a role in rheumatoid arthritis (31)

1.2.4 Molecular signaling pathways of inflammation in macrophages

A several transcription factors and cellular signaling pathways are associated with the development of inflammation, this process stimulates a series of signaling molecules via the pattern of inflammatory recognition receptors including TNFR and TLR4. These receptors can activates a several of intracellular signal transduction cascade such as MAPKs, PI3-K/Akt and subsequently transcription factors including NF-κB and AP-1.

Mitogen-activated protein kinases (MAPKs)

MAPKs is a intracellular signaling pathway, which is a family of serine/threonine protein kinases that mediate various cellular activities, including gene expression, cell differentiation, cell proliferation, cell survival and cell death and involve in cellular responses to external stress stimuli. There are three major groups of MAPKs pathway that lead to altered gene expression including, ERK1/2, JNK and p38 MAPKs. In inflammatory conditions, MAPKs signaling pathways are stimulated by inflammatory inducer, including UV radiation, inflammatory cytokines and infection that transmit the signal required to stimulate the production of inflammatory mediators. The activation of MAPKs lead to product of inflammatory cytokines including TNF-α, IL-1 and IL-12 (32).

Phosphatidylinositol 3′-kinase/Akt (PI3-K/Akt)

PI3-K is a heterodimeric complex comprising of a p85 regulatory subunit and a p110 catalytic subunit. Many reports shown that PI3-K regulate cellular signaling induced by cytokines, growth factors and tumor antigens. When the activated receptor binds PI3-K, then the phosphorylates phosphatidylinositol catalyzing the production of phosphatidylinositol 3,4,5-triphosphate as a potent second messenger. This product can activate downstream signaling molecules including Akt. Akt is a serine/threonine protein kinase also called as protein kinase B (PKB). The activated Akt separates from the membrane to act on its targets in the cytosol and the nucleus. The PI3-K/Akt signaling pathway has been shown to perform on a physiological processes such as cell survival, cell proliferation cell migration and

apoptosis (33). Likewise these signaling has been regulated in LPS-induced inflammatory reactions.

Nuclear factor kappa B (NF-κB)

NF-κB is one of the most transcription factors that regulates gene expressions associated with cell proliferation, cell adhesion and especially inflammatory responses. Which is activated by numerous stimuli that include pro-inflammatory cytokines such as IL-1, TNF-α, epidermal growth factor (EGF), bacteria, viruses, physical and chemical stresses. NF-κB is a dimeric transcription factor formed by the heterodimer of proteins in the Rel family, including p50 and p65 subunits and makes complex with IκB subunit that prevents movement of p50/p65 into the nucleus. As demonstrated in Figure 8, NF-κB is considered transcription factor that play an important function in developing of inflammatory process. Two major signaling pathways lead to translocation of NF-κB dimers from the cytoplasm to the nucleus. The principle pathway of NF-κB activation is called the canonical or classical pathway. This canonical pathway is activated by stress, pro-inflammatory cytokines and LPS through the TLR4, which act through the activation of the inhibitory factor IκB kinase (IKK). IKK is a repressor protein kinases. IKK activation induces the inhibitor of NF-κB (IκB) phosphorylation. Then, the phosphorylated IκB is degraded by the ubiquitin-proteasome system, consequently the free NF-κB dimers from the cytoplasmic. NF-κB/IκB complex able to translocate into the nucleus, where it binds to specific enhancer elements of inflammatory genes. Which encoding pro-inflammatory cytokines, chemokines, growth factors, adhesion molecules, and inducible enzymes (34).

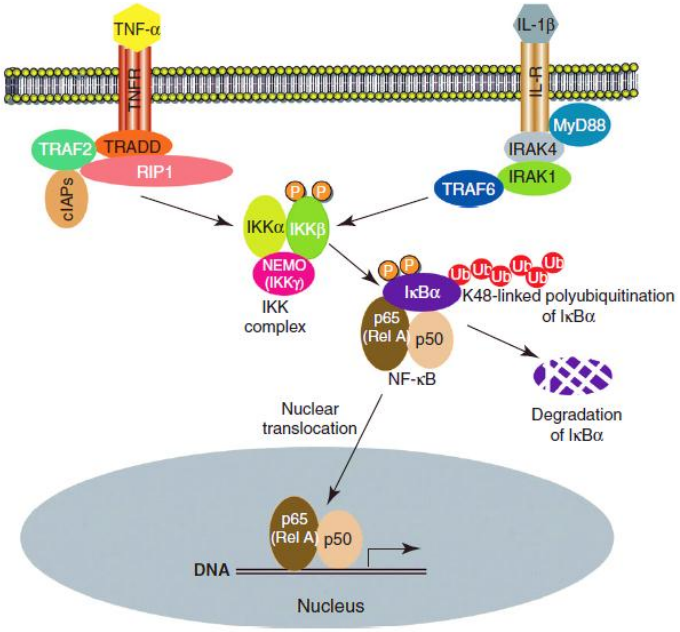

Figure 8. NF-κB signaling pathway (35)

Activating protein-1 (AP-1)

AP-1 has a function in cell proliferation, differentiation and cell transformation. AP-1 acts in homodimeric or heterdimeric protein complex that mainly composed of Jun protein family (c-Jun, JunB, and JunD) and Fos protein family (c-Fos, FosB, Fra-1 and Fra-2). The activity of AP-1 was shown to regulated by a several of physiological and pathological stimuli, include that growth factors, cytokines, stress signals and microbial infection with bacteria and viruses. Similar to NF-κB, AP-1 transcription factor can translocate into the nucleus, where it responsible for inflammatory immune responses, which bind the promoters of inflammatory mediators including IL-6, IL-8, TNF-α and CD38 during inflammation (36).

LPS-induce inflammatory mechanisms

LPS, an important structural component of the outer membrane of Gram-negative bacteria, LPS consisting of three parts, Lipid A that is the main PAMPs of LPS, A core oligosaccharide and O side chain, as shown in Figure 9. Monocytes or macrophages coordinate the innate immune response to LPS by inducing an

inflammatory cytokines expression. The over-reaction of LPS is bring to a sepsis, septic shock, or inflammatory reaction.

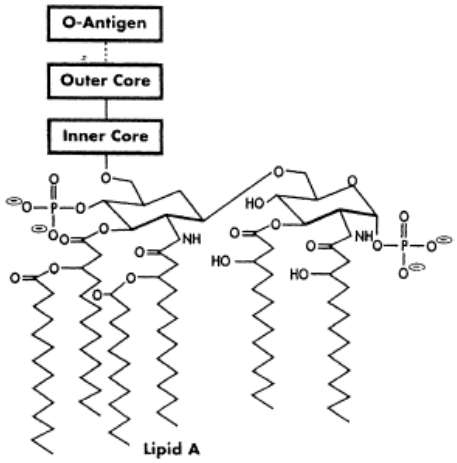

Figure 9. Structure of lipopolysaccharide (9)

The stimulation of LPS in mammalian cells happens via a cascade of interaction with several proteins including LBP, CD14, MD-2 and TLR4. LPS binding protein or LBP is a soluble shuttle protein, where it recognizes and forms a high-affinity complex with the lipid A moiety of LPS. Then forming a ternary complex with CD14, CD14 is a glycosylphosphatidylinositol-anchored protein. The parts of CD14 are necessary required for LPS binding, transfer to TLR4/MD-2 receptor complex and regulates LPS recognition. MD-2 is an adaptor protein in the initiation of TLR4 and necessary for LPS-induced inflammatory signaling. LPS acts certainly connect to MD-2, that in turn connects with TLR4, Then inducing TLR4 aggregation and signal transduction (9).

All TLRs are type 1 transmembrane receptors, an extracellular leucine-rich repeat (LRR) domain and conserved intracellular Toll/Interleukin-1 receptor (TIR) domain. It is supposed that the LRRs are involved in the recognition of the specific ligands of the TLRs, also such as PAMPs. Many evidence indicating that, TLR4 is the LPS signaling receptor. On LPS stimulation, TLR4 undergoes oligomerization and recruits its downstream adaptor proteins through interactions with the TIR domains, there are five TIR domain-containing adaptor proteins including, MyD88,

TIRAP, TRIF and TRAM. The TLR4 signaling response to LPS has been divided into MyD88-dependent and MyD88-independent signaling pathways, as shown in Figure 10.

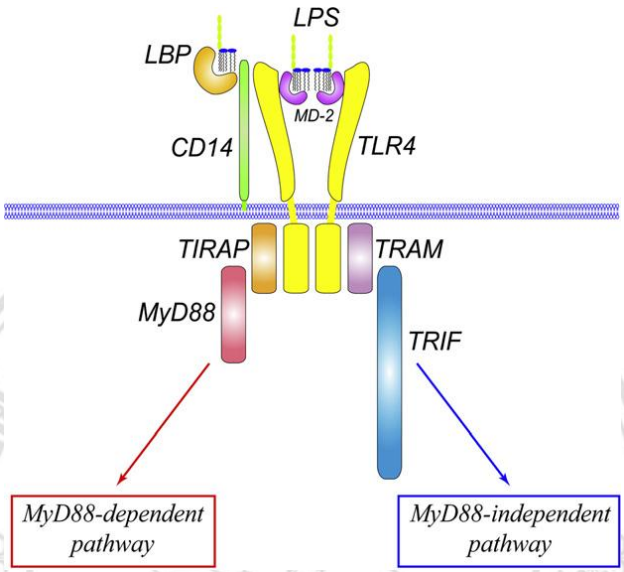

Figure 10. Overview of LPS/TLR4 signaling pathways (11)

MyD88-dependent signaling pathway is responsible for pro-inflammatory cytokines expression, Upon LPS stimulation, MyD88 recruits and activated IRAKs to TLR4 and then associates with TRAF6, is a critical adaptor protein for the MyD88-dependent pathway downstream of IRAKs, leading to the activation of TAK1. At this point, TAK1 by turn triggers downstream IKK complex, resulting in the degradation of IκB-α and subsequent the p65 translocation into the nucleus as shown in Figure 11 (11). Moreover, downstream events in the activation of the MyD88-dependent pathway by LPS, TAK-1 also play as an activator of p38 MAPK as well as JNK in MAPKs pathway, leading to the induction of AP-1 transcription factor. Both pathways eventually result in the regulation of pro-inflammatory cytokines expression including, TNF-α, IL-1β, and IL-6. While MyD88-independent pathway, LPS inducement leads to activation of the transcription factor IRF-3. Whereas, TRIF and TRAM are weighty TIR-containing adaptor protein that regulates MyD88-independent signaling.

TRIF recruits TRAF3 to activate the IRF3 via TANK-binding protein (TBK-1), and then the phosphorylated IRF3 can attach to the ISRE, such as IFN-β. IFN-β can in turn initiates the STAT-1, resulting in the stimulation of several IFN-inducible genes. The PI3-K/Akt pathway is another signaling plays an important role as a regulator of excessive innate immune and the TLR-mediated LPS-induced acute inflammatory responses. Recently studies have suggested that the PI3-K/Akt pathway is the downstream signaling constituent of the MyD88-dependent TLR4 signaling pathway. Which associated with the level of inflammatory mediator expression in activated macrophages via the NF-κB activation. Because the PI3-K/Akt signaling cascade can regulate the transactivation of NF-κB via NF-κB p65 phosphorylation on serine 536. Furthermore, the phosphorylation and translocation of AP-1 transcription factor was also activated by PI3-K/Akt signals (37). Therefore, the suppression of MAPKs and PI3-K/Akt, NF-κB and AP-1 signaling pathway can be considered for possible therapeutic treatment for the inhibition of inflammatory process.

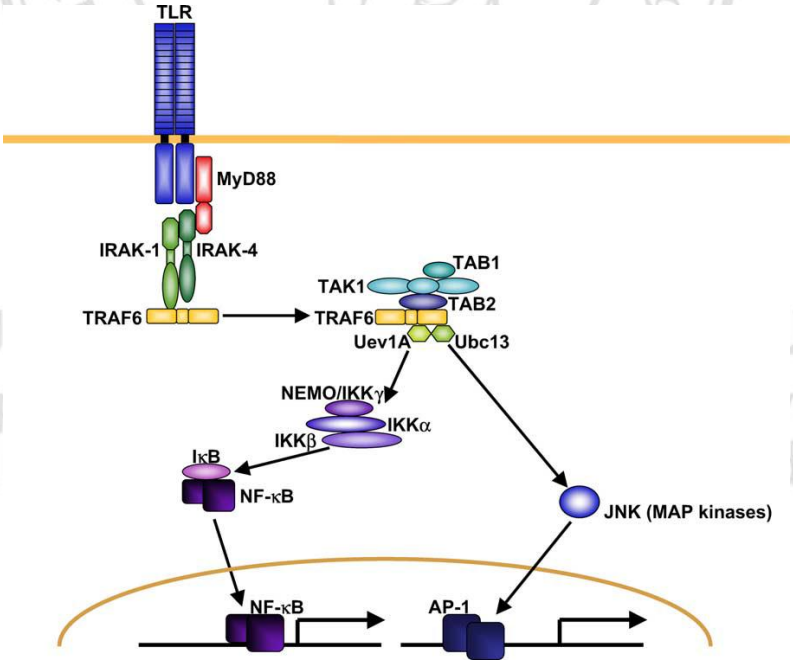

Figure 11. The involvement of TLR-mediated MyD88-dependent pathway (38)

1.2.5 Inhibition of inflammation from natural product

It is widely believed that natural products derived from herbs, have been used as a potential source of new molecules or phytochemical agents to treats for inflammatory disorders. There are numerous natural products that have been reported as showing cancer chemoprevention, antioxidant, immunomodulatory and anti-inflammation.

For example, Curcumin is a lipophilic polyphenol natural product from the turmeric, which has been isolated from the ground rhizome of the *Curcuma Longa* which is belonging to Zingiberaceae family. In traditional medicine, curcumin has been used to treat various common illnesses that include stomach upset, ulcers, jaundice, arthritis, wounds, acnes and skin infections. Curcumin has been attributed numerous pharmacological activities include that anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, anti-proliferative activities and a potent anti-oxidant and free radical scavenger. Many studies have revealed that curcumin modulates the inflammatory reaction via down-regulating the activity of COX-2 and iNOS enzymes; suppresses the production of the pro-inflammatory cytokines including TNF-α, IL-1, -2, -6, -8, -12, and monocyte chemoattractant protein (MCP) by down-regulates via mitogen-activated and Janus kinases. Moreover, curcumin also inhibits the activation of NF-κB by blocking the IκB phosphorylation. Inhibition of NF-κB activation consequently down-regulates the level of COX-2 and iNOS expression, resulting in the suppression of inflammatory gene expression (39,40).

Resveratrol (trans-3,5,4′-trihydroxystilbene) is other natural polyphenolic compound, that found in red grapes, berry fruits and peanuts, which has been pharmacological activities including anti-oxidant and anti-inflammatory effects. Various research has exhibited that resveratrol could be an applicant for inflammatory disease treatment because resveratrol was inhibited NF-κB activation induced by TLR4-mediated signaling, which down-regulates the inflammatory gene products including TNF-α. In addition, resveratrol shown the protective

effects by inhibiting cell death and preventing inflammation, which might be interrelated with the TLR4/ NF-κB signaling pathway (41).

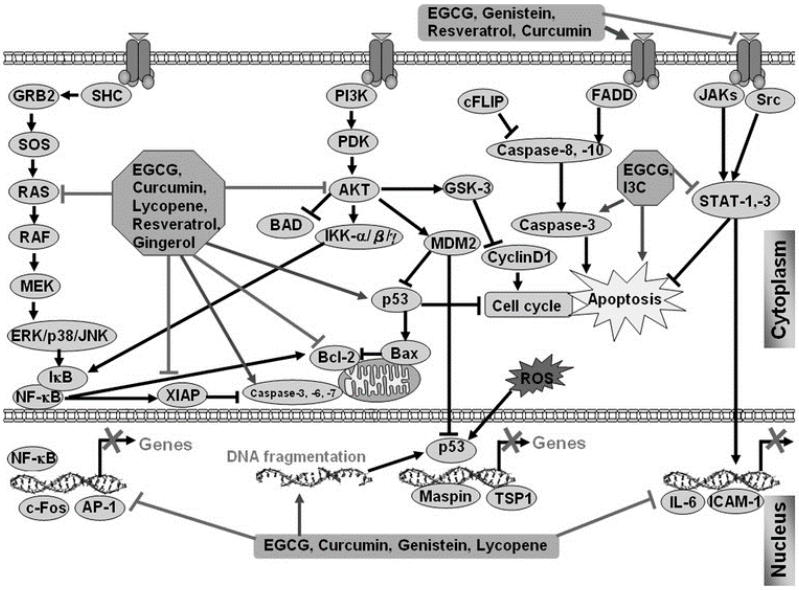

Figure 12. Effects of anti-inflammatory plant natural products on activation and suppression of multiple cell signaling pathways (42)

Berberine is a natural isoquinoline derivative alkaloid isolated from plants in a member of *Berberidaceae* family such as Rhizoma coptidis. In traditional medicine in China, berberine has been used for the treatment of many cases including gastrointestinal disorders and also diarrhea. Many researches have established that to display pharmacological safety and many biological activities including decreased insulin resistance, probable immune-modulatory activities, suppression of adipogenesis, anti-tumor and also anti-inflammatory effect (43). Berberine have shown anti-inflammatory activities because it is able to inhibited cell cycle progression, induced apoptosis, and inhibited angiogenesis. Recent studies shown that berberine reduced the expression of VEGF, TNF-α, IL-1β, IL-6, and IL-17 since berberine considerably suppresses MAPKs protein activation in arthritis rat model. Moreover, berberine shown to suppress the nuclear translocation and DNA binding activity of NF-κB, leading to inhibit the releasing of IL-6, TNF-α and their gene expression in LPS-activated PBMCs (44).

1.2.6 Biological activities of crebanine

Alkaloids are the main bioactive ingredients that has been separated from the *Stephania venosa* (Menispermaceae family) consisting of crebanine, O-methylbulbocapnine, tetrahydropalmatine and N-methyltetrahydropalmatine. *S.venosa* is a phytochemical plant has been used as a folk treatment in Thai traditional medicine, including nerve tonic, aphrodisiac, appetizer, asthma, microbial infection, hyperglycemia and cancer. Crebanine has been proven the pharmacological safety and its several biological activities including anti-arrhythmia, anti-microbial, improvement of neurodegenerative diseases, as well as anti-invasion and anti-proliferation activities in cancer cells (13,14). In addition, crebanine has been studies in in vivo model such as blocked Na^+ channels in guinea-pig ventricular myocytes and improved the memory impairments stimulated by scopolamine in mice (45,46). Our previous study suggested that crebanine decreased TNF-α induce lung cancer cells proliferation and also activated cell apoptosis by regulated cell cycle proteins and induced the intrinsic and extrinsic pathways in program cell death (15). Furthermore, crebanine suppressed cancer cell invasion via inhibition of constitutive NF-κB activation. However, the effect of crebanine on the anti-inflammatory property and mechanisms has not been reported.

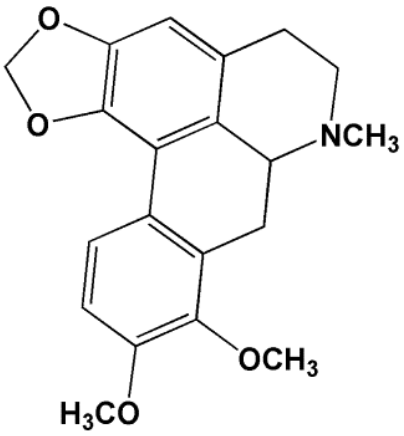

Figure 13. The chemical structure of crebanine (14)

1.3 Objectives

1. To investigate the effect of crebanine on the production of pro-inflammatory cytokines (IL-6 and TNF-α) in LPS-induced RAW 264.7 cells.

2. To investigate the effect of crebanine on the expression of enzyme and inflammatory mediators such as COX-2, iNOS, PGE_2 and NO in LPS-induced RAW 264.7 cells.

3. To investigate the effect of crebanine on the inflammatory intracellular signaling including PI3-K/Akt, MAPKs signaling pathway and NF-κB and AP-1 transcription factors in LPS-induced RAW 264.7 cells.