CHAPTER I

INTRODUCTION

1.1 Statement of the problems

Influenza viruses are continuous and harmful global threat to mankind and many animal species. The re-emerging disease in March and early April 2009 of influenza type A H1N1 gave rise to thousands of deaths and enormous economic losses each year. The current tools to prevent and control influenza virus include vaccines and antiviral drugs. Clinical trials with live, attenuated, cold-adapted influenza virus vaccines have shown a slightly higher protection rate. Phophylactically given antivirals, amantadine and rimantadine have been effective against influenza type A virus. Novel neuraminidase inhibitor which is sialic acid analogs, zanamavir and oseltamivir have prophylactic and therapeutic effects against all influenza types. Although some development in controlling influenza has taken place during the last few years, the disease is not under control. This fact provides much impetus to the scientific community for the discovery of new and less expensive anti-influenza drugs. Furthermore, drugs resistance and excessive inflammation due to overabundant production of proinflammatory cytokine called cytokine storm is considered an important factor in respiratory failure and disease pathogenesis. Therefore, this study was effort to inhibit cytokine storm focus on the effect of phytochemicals from herbs on IL-1β, TNF-α, and IL-2 release from influenza type A H1N1 induced peripheral blood mononuclear cells (PBMC) as a model.

1.2 Literature reviews

1.2.1 Influenza type A H1N1 pandemic

The emergence of highly contagious influenza type A H1N1 represents a serious threat to global human health. An influenza type A H1N1 pandemic was detected in March and early April 2009 at border between Mexico and United States (1, 2). During the first few weeks of surveillance, the virus spreaded worldwide by human-to-human transmission (3, 4), causing the World Health Organization (WHO) declared an influenza pandemic on June 11, 2009 (1, 3, 5). It has spread to over 74 countries around the world and over 440,000 cases, including 5,700 deaths, have been reported up to October 25, 2009 (Figure 1) (6), leading to the WHO to raise its pandemic alert to level 5 of 6 (3). In Thailand, 165 deaths have been found, and the ratio of spread 40.14 per 100,000 people has been reported up to October 7, 2009 by Ministry of Public Health (7).

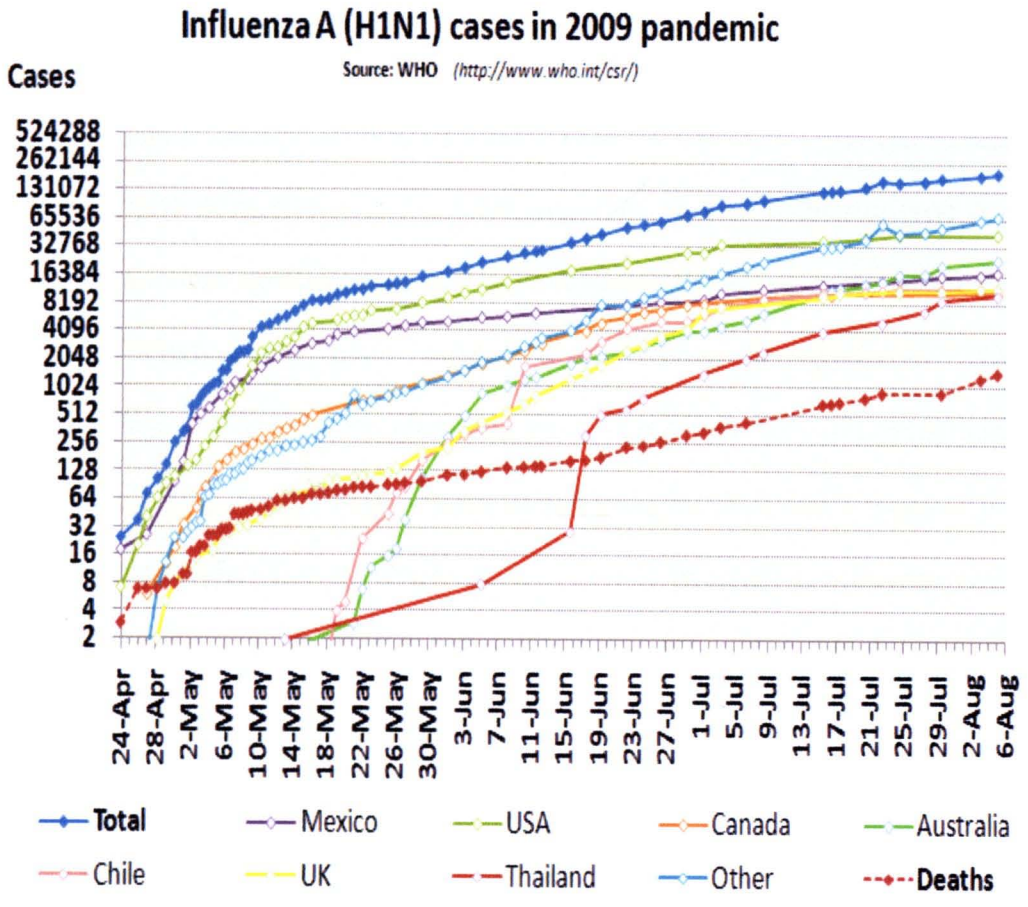


Figure 1. The influenza type A H1N1 cases in 2009 pandemic (6).

1.2.2 Biology of influenza viruses

Influenza viruses are member of the family Orthomyxoviridae, which enveloped and single-stranded RNA viruses classified in three types (A, B, and C), of which the type A is clinically the most important (8-10). Influenza type A viruses contain a genome composed of 8 single-stranded RNA segments that encode 11 proteins, including the main surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), they are classified into 16 HA subtypes (H1-H16) and 9 NA subtypes (N1-N9). So far, only 3 types of HA (H1, H2, H3) and 2 types of NA (N1, N2) have been widely prevalent in humans (11). The structure of influenza viruses has 3 parts: surface glycoprotein, including HA, and NA; RNA polymerase subunits, including polymerase A protein (PA), polymerase B1 protein (PB1), and polymerase B2 protein (PB2); and nucleocapsid, including nucleoprotein (NP), matrix proteins (M), and non-structural protein (NS) (Figure 2) (4, 8, 10, 12).

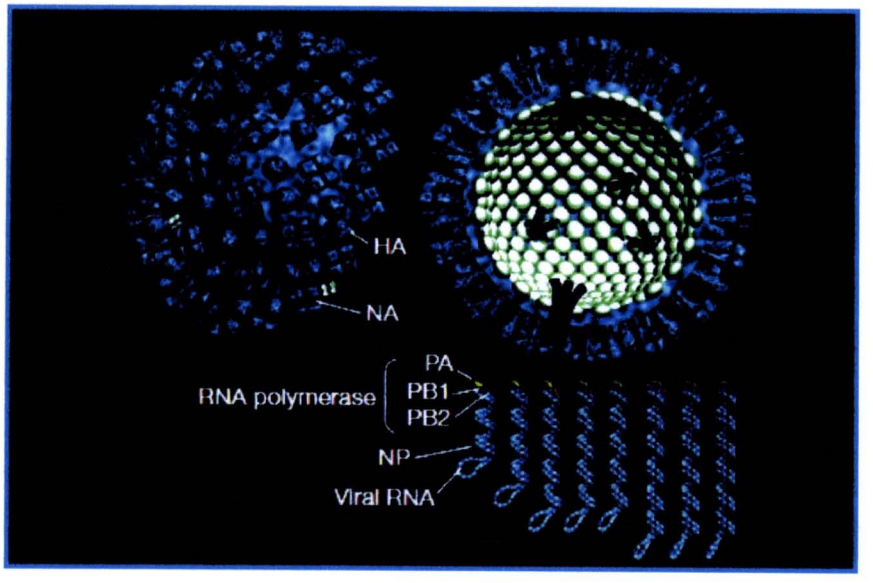


Figure 2. The structure of influenza viruses. Virions are decorated with two surface glycoproteins, HA and NA. The genome is composed of eight segments of single-stranded RNA that interact with the nucleoprotein and the components of polymerase complex (PB2, PB1 and PA) (10).

1.2.3 The mechanism of influenza type A virus infection

Influenza virus infection involves a series of steps: the virus attaches to host sialylated glycoproteins through HA and enters the cell by endocytosis, followed by pH-dependent fusion and release of viral genomic ribonucleoprotein (vRNP) complexes in the cytoplasm (13, 14). vRNP then translocate to the nucleus where transcription and replication of viral RNA occurs. Transcription and replication of viral RNA are carried out by RNA polymerase subunits (PA, PB1, and PB2) and nucleoprotein (NP). Newly synthesized vRNP complexes are exported from nucleus to the cytoplasm by nuclear export protein (NEP, formally called NS2), matrix protein M1, and are assembled into virions at the plasma membrane (4, 8, 10, 12). The neuraminidase facilitrates virus release from infected cells by removing sialic acid (10). The transcription and replication of influenza type A is shown in Figure 3.

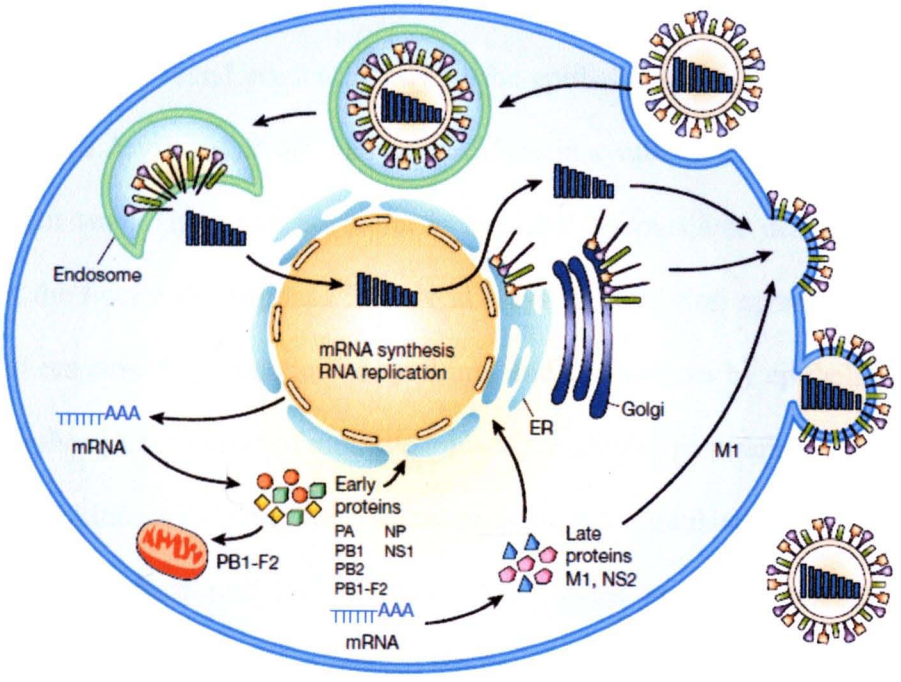


Figure 3. The transcription and replication of influenza type A virus. After receptor-mediated endocytosis, the viral ribonucleoprotein (vRNP) complexes are released into the cytoplasm and subsequently transported to the nucleus, where replication and transcription take place. Messenger RNAs are exported to the cytoplasm for translation. Early viral proteins, that is, those required for replication and transcription, are transported back to the nucleus. Late in the infection cycle, the M1 and NS2 proteins facilitate the nuclear export of newly synthesized vRNPs. PB1-F2 associates with mitochondria. The assembly and budding of progeny virions occurs at the plasma membrane (10).

In human, influenza A virus replicates throughout the respiratory tract, where the viral antigen is found predominantly in the epithelial cells (14). Once inside the cells, influenza A virus shut off the host cell protein synthesis and replicates in a fast and efficient way. This process results in host cell apoptosis or death by cytolysis. However, the host cells response in several ways to limit viral spreading. The most significant response is production of cytokines and chemokines by epithelial cells and leukocytes though activation of multiple transcriptional and posttranscriptional system (15, 16). Cytokines are extracellular signal proteins that stimulate adjacent and distant cells to activate host antiviral defense. Chemokines are low molecular weight chemoattractant cytokines which bind to their specific receptors in leukocytes, recruit inflammatory cells to site of infection, and activate innate immune responses (16). In addition, human respiratory epithelial cells respond to viral infections by mounting a cytokine response that contributes both to the innate and adaptive host defense (17). Human influenza A viruses have been previously reported to induce interleukin-1 (IL-1) α and β, tumor necrosis factor-α (TNF-α), interferon-α (IFN-α), interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte-attracting chemokines (18-19).

IL-1β and TNF-α are well known for their profound stimulating effects on neutrophil and macrophage functions (20). Both cytokines strongly up-regulate leukocyte adhesion molecules on the vascular endothelium, thereby mediating the first essential step for recruitment of neutrophils and/or macrophages into the respiratory tract. Many of these early response cytokines have overlapping and synergistic activities, and can even induce their own production or those of other cytokines. IL-1β and TNF-α, for example, stimulate the release of IL-6 and some chemokines (18, 21). Moreover, macrophages are antigen presenting cells to stimulate adaptive host defense T-cell by presenting influenza A viral peptide that production of interleukin-2 (IL-2) that send signaling to white blood cells to be ready against virus (22). The mechanism of cytokine storm evoked by influenza virus is shown in Figure 4.

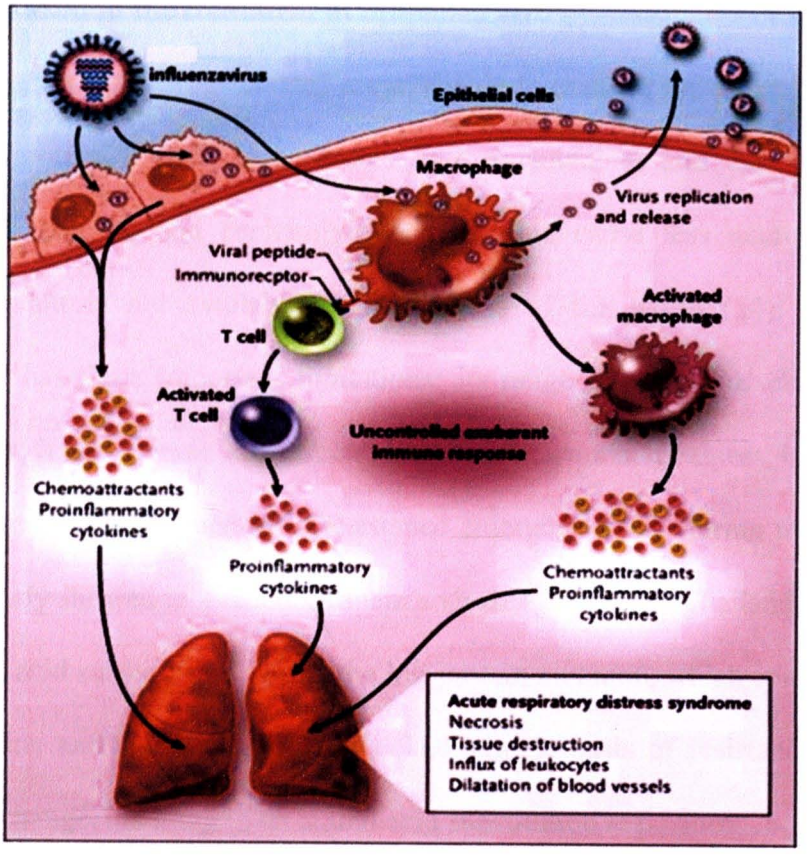


Figure 4. The mechanism of cytokine storm evoked by influenza virus. During the replication cycle innate immune response to the virus which is overproduction of proinflammatory cytokines and chemoattractants known as cytokine storm. As the result, many types of cytokine especially IL-1β, TNF-α and other immunity substances from white blood cells are produced to against the virus. In addition, immune cells (T-cells) produce IL-2 which sends signals to white blood cells to be ready to against virus (23).

1.2.4 Drugs used in the treatment of influenza virus

Currently, two drugs classes are available to manage influenza: the inhibitors of matrix protein 2 (M2), amantadine and rimantadine, and the neuraminidase inhibitors, zanamivir and oseltamivir. Rimantadine cause less neurotoxicity than amantadine but is not available in most parts of the world (24). Furthermore, amantadine has three important limitations: its range of activity exclude influenza type B virus; it has adverse side effects, including insomnia, dizziness, hallucinations, dizziness, and headache; and drug resistance emerges rapidly during treatment (24). Previous study showed that recent influenza viruses isolated in Thailand and Vietnam have amino acid substitutions within the M2 protein mutation, which confer resistance to amantadine and rimantadine (25-27). The genetic basis of resistance is a singer nucleotide change, resulting in an amino acid substitution at position 26, 27, 30, 31, or 34 in the membrane-spanning region of M2 (24). Neuraminidase inhibitor is a specific inhibitor of a wide range of influenza type A and B. Zanamivir is second-generation neuraminidase inhibitor whereas oseltamivir is third-generation neuraminidase inhibitor (24). Unfortunately, zanamivir is administered by inhalation, which decreases its usefulness for children, the elderly or those in an intensive care unit (27-28). Oseltamivir is an orally active prodrug of oseltamivir carboxylate (24) and has the frequency of nausea, vomiting, and gastrointestinal side effects which can be ameliorated if the drug is taken shortly after food (29-30). Neuraminidase inhibitory resistance involves either a mutation in the active site of the neuraminidase or mutation in the hemagglutinin. Three resistant variants with neuraminidase mutation (E119V, H274Y, and R292K) that have emerged in clinical trials show low infectivity and virulence in animal models, thus the relevance of these mutations in clinical

practice remains uncertain (24). The first report of emergence of neuraminidase inhibitor resistance (R152K) during treatment with zanamivir involved a recipient of bone marrow transplant (31). During clinical trials with oseltamivir, 1.3% (4 of 301) of post-treatment isolates from adults and adolescents and 8.6% (9 of 105) from children had low neuraminidase inhibitor susceptibility, indicating that viruses are likely to emerge in clinical practice (24). Despite comparable efficacies of the neuraminidase inhibitors in the therapy of human influenza, the WHO is preferentially recommend oseltamivir, probably because of the relatively lower serum level of zanamivir (32). The mechanism of antiviral drugs to inhibit influenza virus is shown in Figure 5.

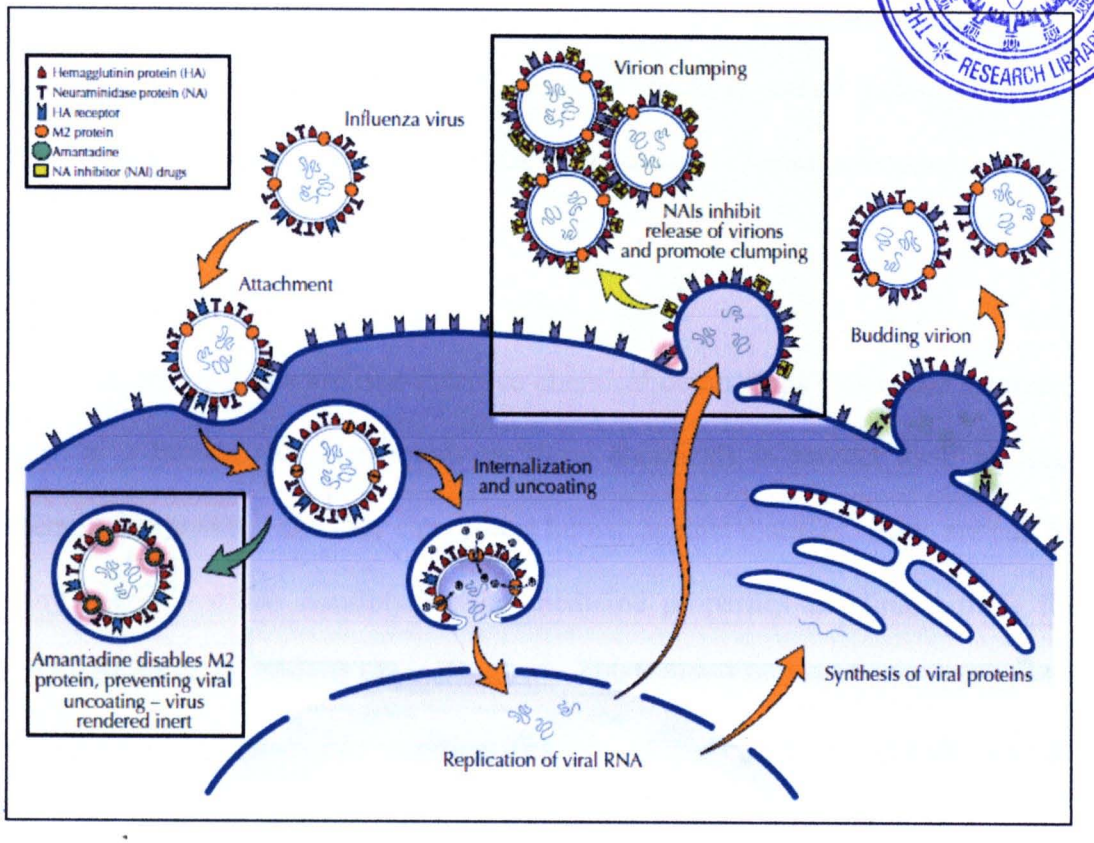


Figure 5. The mechanism of antiviral drugs to inhibit influenza virus. Influenza virus attachment, internalization, replication and exit from the host respiratory cell and steps inhibited by antiviral drugs. Amantadine blocks viral internalization and uncoating. Neuraminidase inhibitors prevent the neuraminadase from releasing budding viruses and dispersing virions (33).

Furthermore, drugs resistance and excessive inflammation due to overabundant production of proinflammatory cytokine called cytokine storm is considered an important factor in respiratory failure and disease pathogenesis (8).

1.2.5 Phytochemicals

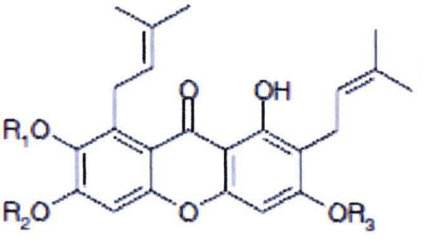
Phytochemicals are non-nutritive chemical compounds that occur naturally in plant. It is known that plant produces these chemicals to protect itself but recent research demonstrate that they can protect human against diseases. There are currently many phytochemicals possibly having medicine properties in clinical trials for a variety of diseases. Almost phytochemicals which found in Thai herbal sources have different anti-inflammatory properties. The study focus on phytochemicals; including xanthone from mangosteens, sesamin from sesame seeds, *Andrographis paniculata*, *Moringa oleifera*, *Houttuynia cordata*, *Herricium erinaceus*, and *p*-hydroxy cinnamaldehyde from *Alpinia galangal*, which have been reported to possess anti-inflammatory effect.

1.2.6 Xanthone

Mangosteen, *Garcinia mangostana* L. is used as a traditional medicine in Southeast Asia for inflammatory and septic ailments (Figure 6) (34). Xanthones are natural organic compounds isolated from mangosteen and among the xanthones, α-, β- and γ-mangostin are a class of polyphenolic compounds (Figure 7) (34-35).

Xanthone has various biochemical actions, a strong anti-inflammatory effect such as inhibition of allergy by decrease of histamine release and reduction of some postanoids synthesis though inhibition of cyclooxygenase (COX) activity and previous study is shown that 40% ethanol extract of mangosteeens has potent inhibitory activities on IgE-mediated histamine and prostaglandin E2 synthesis which chemical mediators of inflammation and/or allergy (36-38), antimicrobial effect by against methicilin resistant *Staphylococcus aureus* (37), antitumor effect by induced growth inhibition in human colon cancer DLD-1 cells associated with cell-cycle arrest by affecting the expression of cyclins, cdc2, and p27; G1 arrest, induced apoptosis though the activation of instrinsic pathway following the down-regulation of signaling cascades involving MAP kinases and the serine/threonine kinase Akt, antihepatotoxic effect and antioxidant effect (36).

Figure 6. Mangosteen, *Garcinia mangostana* L. (34).



α-Mangostin: R_1=CH_3, R_2=R_3=H

β-Mangostin: R_1=R_3=CH_3, R_2=H

γ-Mangostin: R_1=R_2=R_3=H

Figure 7. The chemical strucuture of xanthones (34-35). (Mw 359.4)

1.2.7 Sesamin

Sesame seeds (*Sesamum indicum* L.) have been the oldest condiment know to man for over 5000 years (Figure 8) (39). They are highly valued for their oil, which is exceptionally resistant to rancidity. Not only sesame seeds are a very good source of calcium, magnesium, copper zinc, iron, vitamin B6 and fiber, they are also a good source of essential amino acid, especially, methionine. In addition to these important nutrients, sesame seeds contain two unique substances: sesamin and sesamolin (40). Sesamin is one of the most abundant compounds and classified as a furanofuran-type lignin (Figure 9) (39). After consumption of sesamin, it changed into enterolactone, which is the major end-product of dietary lignan fermentation by mammalian interstinal microflora and absorbed though enterohepatic circulation, and excreated in urine (41).

Figure 8. *Sesamum indicum* L. and sesamine seeds (39).

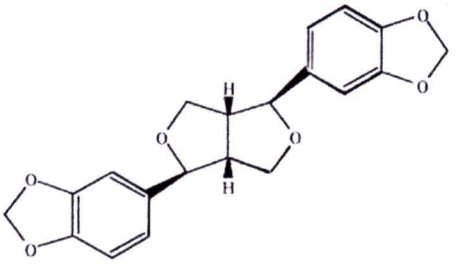


Figure 9. The chemical strucuture of sesamin (39). (Mw 354.4)

Sesamin has various biochemical actions, mainly related to lipid metabolism. Those actions include; anti-inflammatory effect that inhibits Δ^5-desaturase activity, resulting in accumulation of dihomo-γ-linolenic acid (DGLA), which displaces arachidonic acid (AA) and consequently decreases the formation of proinflammatory 2-series prostaglandins (PGE_2) (42), inhibition of cholesterol absorption and synthesis by reducing the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, which is the rate-limiting enzyme of cholesterol synthesis (43), anti-hypertensive effect by suppressing the enhancement of aortic superoxide (O_2^-) production in deoxy-corticosterone acetate (DOCA)-salt hypertensive rats (44), protection against ethanol and carbon tetrachloride-induced liver damage (45), synergy with γ-tocopherol to produce vitamin E activity (46), prevention of hypoxic or H_2O_2-stressed neuronal injury by suppressed reactive oxygen species (ROS) production, ERK1/2, JNK, p38 MAPKs and caspase-3 activation that correlated with reduction in lactate dehydrogenase (LDH) released from cells under hypoxia (47).

1.2.8 *Andrographis paniculata*

Andrographis paniculata Wall ex. Nees is a member of the family Acanthaceae and has a long history of therapeutic usage in Indian and Oriental medicine (Figure 10) (48). A number of active compounds are reported from the plant, which mainly include diterpene lactones, flavonoids, and polypherols (49). Diterpene lactones are including andrographolide, neoandrographolide, deoxyandrographolide, and deoxy-didehydroandrographolide (48). Andrographolide ($C_{20}H_{30}O_5$) is the major active compound isolated from *Andrographis paniculata*, which is mainly concentrated in leaves and can be easily isolated from the crude plants extracts as crystalline solid (49-50). The structure of andrographolide as (3-[2-[decahydro-6-hydroxy-5-(hydroxymethyl)-5, 8-adimethyl-2-methylene-1-napthalenyl] ethylidene] dihydro-4-hydroxy-2(3H)-furanone) is shown in Figure 11.

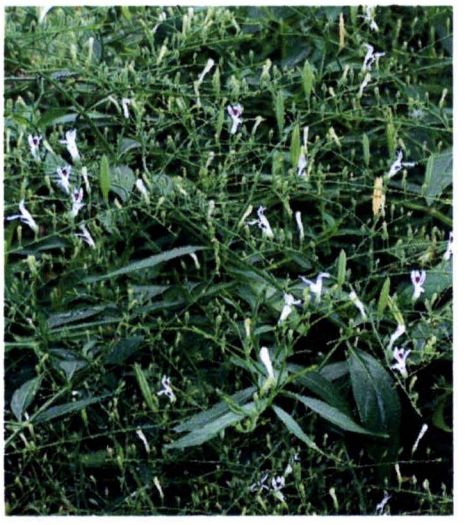


Figure 10. *Andrographis paniculata* Wall ex. Nees (49).

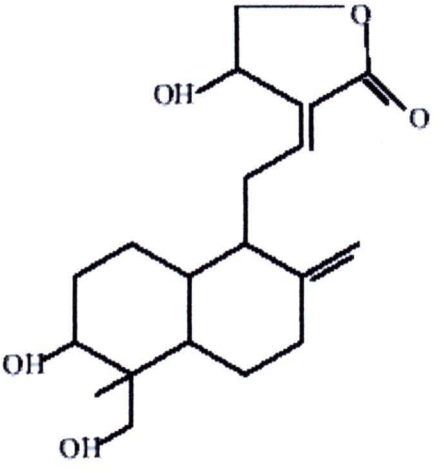


Figure 11. The chemical structure of andrographolide, which is a major active compound found in *Andrographis paniculata* (49).

Andrographolide has various biochemical actions, a strong anti-inflammatory effect that is active both *in vitro* and *in vivo*, and as effective as the steroid drug-dexamethasone, and like steroids, it appears to act primarily by inhibiting the expression of mRNA for inflammatory cytokines (50), anti-cancer effect by activates the extrinsic death receptor pathway (including caspase-3 and caspase-8), induces apoptotic cell death in certain human cancer cell types, and effectively induces cell cycle arrest in cancer cells at G0/G1 stage (49), prevent multiple organ (liver and kidney) dysfunction, which is a major cause of morbidity and mortality in sepsis, in rats suffering from endotoxaemia by inhibits the migration toward C5a, and that this effect is associated with inhibition of intracellular ERK1/2 and Akt signal transduction pathways (51).

1.2.9 *Moringa oleifera*

Moringa oleifera Lam is one of the best known and naturalized species of a monogeneric family Moringaceae (Figure 12). The leaves, fruits, fowers and immature pods of this tree are used as a highly nutritive vegetable. Moringa leaves have been reported to be a rich source of β-carotene, protein, vitamin C, calcium and potassium and act as a good source of natural antioxidant compounds such as ascorbic acid, flavonoids, phenolics and carotenoids (52). *Moringa oleifera* is rich in compounds containing the simple sugar rhamnose and a fairly unigue group of glucosinolates and isothiocyanates (53). The structures of phytochemicals from *Moringa oleifera* are shown in Figure 13.

Figure 12. *Moringa oleifera* Lam leaves (53).

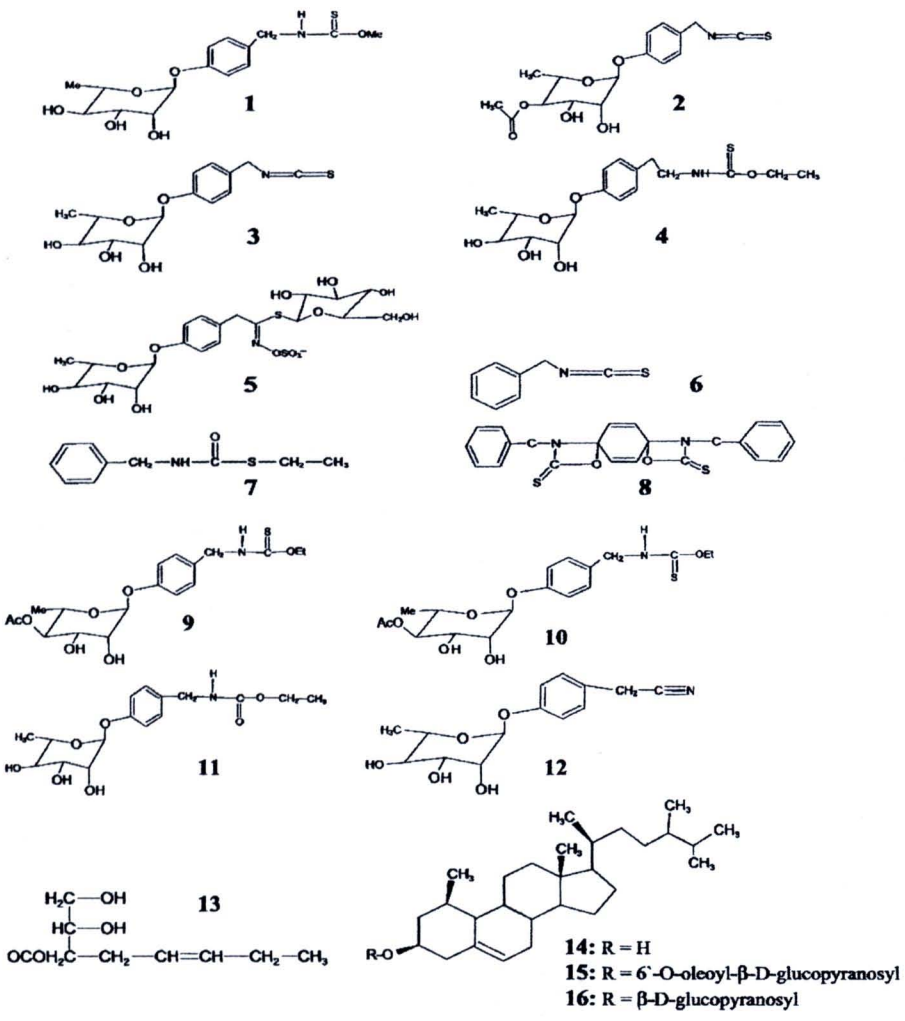


Figure 13. The structures of phytochemicals from *Moringa oleifera*; niazinin A [1], 4-(4′-O-acetyl-α-L-rhamnopyranosyloxy)benzyl isothiocyanate [2], 4-(-L rhamnopyrano syloxy) benzyl isothiocyanate [3], niazimicin [4], 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate[5], benzyl isothiocyanate [6], aglycon of deoxy-niazimicine (N-benzyl, S-ethylthioformate) [7], pterygospermin [8], niaziminin [9 + 10], O-ethyl-4-(α-L-rhamnosyl oxy) benzyl carbamate [11], niazirin [12], glycerol-1-(9-octadecanoate) [13], β-sitosterol [14], 3-O-(6′-O-oleoyl-β-D-glucopyranosyl)-β-sitosterol [15], β-sitosterol-3-O-β-D-glucopyranoside [16] (53).

Moringa oleifera leaves have various biochemical actions include; anti-inflammatory effect by significantly reduced carrageenin (a standard inflammatory agent) induced paw edema in mice after oral administration of *Moringa oleifera* (54), significant cholesterol lowering action in the serum of high fat diet fed rats which might be attributed to the presence of bioactive phytoconstituent such as β-sitosterol (55), antiulcerogenic and hepatoprotective effects in rats by the methabol fraction of *Moringa oleifera* leaves extract (56), inhibit the growth of microorganisms (*Psedomonas aeruginosa* and *Staphytococcus aureus*), pathogenic to man by the fresh leaf juice (57), significant inhibition of tumor promoter reduced Epstein-Barr virus activation by among the isothiocyanates naturally occurring 4-[4'-*O*-acetyl-α-i-rhamnosyloxy)benzyl] suggesting that the isothiocyano group is a critical structural factor for these effect (58).

1.2.10 *Houttuynia cordata*

Houttuynia cordata Thunb is member of the family Saururaceae. It is generally used in Chinese medicine therapy (59). *Houttuynia cordata* has biochemical actions include; anti-inflammatory effect of aqueous extracts by inhibiting NO production and/or TNF-α secretion and weaker free radical scavenging and xanthine oxidase inhibitory activity than vitamin E, anti-lipid peroxidation activity in rat liver homogenate which was close to that of vitamin E (60), significant decrease of the levels of superoxide dismutase, malondialdehyde, hydroxyproline, IFN-γ, and TNF-α in animal studies and antibacterial effect of essential oil against *Staphytococcus aureus* and *Sarcina ureae* (61).

Figure 14. *Houttuynia cordata* Thunb (59).

1.2.11 *Hericium erinaceus*

Hericium erinaceus (Bull. Ex Fr.) Pers (lion's mane) is an edible basidiomycetous fungus and a member of the family Hydnaceae (Figure 15). Its fruiting bodies are well known as a traditional Chinese medicine or food. This fungus contains polysaccharides, which exhibit immunomodulating activity and antiradiative effects (62). *Hericium erinaceus* has various biochemical actions include; anti-inflammatory effect by activating macrophages to produce cytokines, IL-1β, TNF-α, nitric oxide (NO), and other inflammatory mediators (63), antitumor effect by stimulating natural killer cells, T-cells, B-cells, and macrophage-dependent immune system responses (63), antioxidant effect of polysaccharides by reducing power, inhibition of lipid peroxidation, and 1,1-diphenyl-dipicrylhydrazyl radicals scavenging assays *in vitro* (64), antimicrobial effect, cytotoxic effect and promoting the synthesis of the neurogrowth factor (65).

Figure 15. *Hericium erinaceus* (Bull. Ex Fr.) Pers (lion's mane) (62).

1.2.12 *p*-hydroxycinnamaldehyde from *Alpinia galanga*

Alpinia galanga Linn. (B.L. Burtt) is Thai medicininal plant and one of the best known spices (Figure 16). Purification of acetone extract of *Alpinia galanga* afforded *p*-hydroxy- cinnamaldehyde has been reported (66). The chemical structure of *p*-hydroxy- cinnamaldehyde, as identified by nuclear magnetic resonance and mass spectrometry analyses, is shown in Figure 17.

The *p*-hydroxycinnamaldehyde has various biochemical actions include; anti-inflammatory effect by inhibiting extracellular matrix hyaluronan (HA), sulfated glycosaminoglycans (s-GAG), and matrix metalloproteinase-2 (MMP-2) released from IL-1β induced cartilage explants culture (66), inhibition of chemokine expression especially TNF-α, MCP-1, and IP-10 (67), antimicrobial effect, anti-rheumatic effect by suppression of prostaglandin synthesis though inhibition of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (68).

Figure 16. *Alpinia galanga* Linn. (B.L. Burtt) (66).

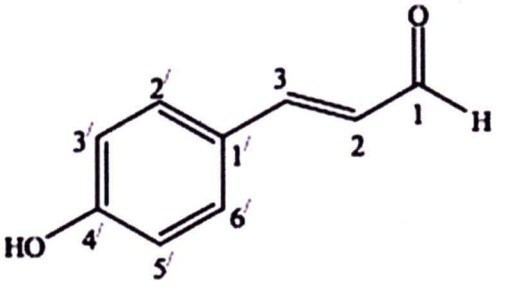


Figure 17. The chemical structure of *p*-hydroxycinnamaldehyde 1, 3-(4-hydroxy-phenyl)-propenal, the active compound of the acetone fraction of *Alpinia galanga* (66).

1.2.13 Objective

To investigate the effect and the mode of action of phytochemicals from herbs on IL-1β, TNF-α and IL-2 release as well as mRNA expression using influenza type A H1N1-induced peripheral blood mononuclear cells (PBMC).