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# Isolation of Natural Nrf2 Activators from American Ginseng

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ISOLATION OF NATURAL NRF2 ACTIVATORS FROM AMERICAN GINSENG

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## DEDICATION

I dedicate my thesis to my family. A special feeling of gratitude to my loving parents, Saleh Abdalkader and Fathia Ramadan, who taught me how to write the alphabet. To my wife Huda, who has never left my side, for being there for me throughout the entire master program. To my brothers and my children who encourage me to continue whenever I face tough situations.

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## ABSTRACT

Nuclear factor erythroid-2 related factor 2 (Nrf2), a major transcription factor of the endogenous antioxidant defense system, has been proposed as a potential therapeutic target for the treatment of various diseases. However, a small molecule-driven Nrf2 signaling therapeutic approach remains to be established. Herein, we report one such possibility discovered by the bioassay-based fractionation of American ginseng. Since crude extract of American ginseng was found to suppress the expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-inflamed macrophages via activating Nrf2, we further fractionated the crude extract using solvents including hexane, dichloromethane, ethyl acetate, butanol, and water, and found that the hexane fraction was the most effective fraction in activating Nrf2-mediated suppression of iNOS expression in macrophages. By utilizing preparative, reverse-phase HPLC and a comparative analysis by analytical scale LC-UV, we found that hexane fraction contains predominantly polyacetylenes and linolenic acid, and we identified panaxynol as the major compound in polyacetylenes. We report a unique and Nrf2-dependent anti-inflammatory profile of panaxynol in inflamed macrophages. In lipopolysaccharide (LPS)-inflamed RAW264.7 macrophages, panaxynol dramatically suppressed the expression of pro-inflammatory cytokines including inducible nitric oxide synthase (iNOS) and monocyte chemoattractant protein-1 (MCP-1), but it has no effects on regulate expression of interleukin-6 (IL-6), interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor alpha

(TNF- $\alpha$ ), this results suggested activation of specific pathway; that is Panaxynol potentially activated Nrf2 signaling; but, it did not affect NF- $\kappa$ B activity in LPS inflamed macrophages. Moreover, knockout of Nrf2 blocked the panaxynol induced anti-inflammatory responses in LPS-inflamed macrophages. These results demonstrated that panaxynol suppresses pro-inflammatory responses in macrophages via an activation of Nrf2 independently of NF- $\kappa$ B, suggesting a potential mechanism responsible for the American ginseng-mediated health benefits and a unique therapeutic potential of panaxynol for specific targeting Nrf2-mediated resolution of inflammation.

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## CHAPTER 1

### INTRODUCTION

#### **1.1. introduction overview**

Ginseng has been one of the most commonly used herbal medicines in Asia. Recently, it has become a popular herbal medicine in United States. It is one of the top best-selling herbal medicines in the world (Yun, 2001). In general, chemical ingredients obtained from ginseng species can be classified into; ginsenosides, polysaccharides, polyynes, flavonoids, and volatile oils (Poudyal et al., 2012). Ginsenosides (also called triterpenoid saponins) are the major active ingredients in American ginseng and the most studied compound (Attele et al., 1999; Jia and Zhao, 2009). However, other additional bioactive products are contributed to the overall effect of ginseng. Among these bioactive metabolites is the C17-polyacetylenes (Qi et al., 2011). Polyynes have been less studied and therefore less is known about their medicinal properties (Poudyal et al., 2012). By using bioassay-guided fractionation American ginseng can be further fractionated into; water, butanol, ethyl acetate, dichloromethan, and hexane sub-fractions. We found that the hexane fraction was the most effective fraction in suppression of iNOS expression in macrophages. By utilizing preparative, reverse-phase HPLC and a comparative analysis by analytical scale LC-UV, we found the hexane fraction contains predominantly polyacetylenes and linolenic acid. Also, we identified the major component of the polyacetylenes is panaxynol, which could be a potential promising anti-inflammatory drug. Chronic inflammation has bad effects on human health in general. Research

evidence shows that chronic inflammation contributes to cancer (Hofseth et al., 2006) and pathogenesis of many diseases such as autoimmune diseases and atherosclerosis. Many molecules and pathways are involved in inflammation. In the cardiovascular system, several anti-ischemic, anti-arrhythmic and anti-hypertensive effects of ginseng are linked to the antioxidant properties of the ginseng (Wang et al., 2007). Several studies show that ginseng regulates inflammation (Hofseth et al., 2007; Spelman et al., 2006). It can selectively inhibit expression of the inducible nitric oxide synthase via activating the Nrf2 pathway (Li et al., 2009a). Some reports show that ginseng exerts toxic effects in the heart (Poindexter et al., 2006). This contradiction, may be because of the bioactive contents of ginseng extract can differ, depending on the method of extraction, subsequent treatment, age and part of the plant extracted, season of its collection, or even the geographical location (Soldati F et al., 1980). However, the mechanisms of action of American ginseng at the cellular and molecular level are not fully understood.

Nuclear factor erythroid-2 related factors (NF-E2-related factors) includes; Nrf1, Nrf2, and Nrf3. Nrf2 belong to Cap “n” Collar the family of basic leucine zipper (bZip) transcription factors. Nrf2 genes are available in liver, lung, small intestine and many other tissues (Hayes et al., 2010). Nrf2 control the basal and inducible expression of a battery of antioxidant genes and other cytoprotective phase II detoxifying enzymes in response to oxidative stress and inflammatory responses (Li et al., 2009a). Macrophages from Nrf2<sup>-/-</sup> mice generate 2-fold higher reactive oxygen species levels after lipopolysaccharide stimulation compared with cells from wild-type animals (Kong, et al., 2010). Nrf2 regulates an antioxidant defense system and its deficiency leads to augmented oxidative stress and cytokine production (Williams, et al., 2008). Nrf2 has a

protective role against joint inflammation and degeneration in arthritis (Maicas, et al., 2011). All these data make Nrf2 an attractive target for drugs to treat diseases.

Herein, we report a unique anti-inflammatory profile of hexane and panaxynol by activating Nrf2 signaling without effecting NF- $\kappa$ B pathway in LPS inflamed macrophages, suggesting a novel potential of panaxynol as therapeutic agent to resolve inflammation in macrophages by specifically targeting Nrf2 signaling.

## **1.2. American Ginseng**

American Ginseng is one of the species of genus *Panax*; which includes; *Panax ginseng* (Chinese and Korean ginseng), *Panax notoginseng* (Chinese Sanqi ginseng), and *Panax quinquefolius* (American ginseng). Genus *Panax* is named by German-Russian botanist Carl Meyer in 1842. The name is derived from the Greek language, “pan” means all and “akos” means healing, exactly means all-healing (Gillis, 1997). American ginseng mainly grows in Wisconsin, British Columbia, southern Quebec and Minnesota (Assinewe et al., 2003). Nowadays, in United States, ginseng preparations are sold as dietary supplements, rather than medications and ginseng preparations do not require approval as a drug by the Food and Drug Administration (FDA) (Gillis, 1997). Ginseng has long been of the most commonly used herbal medicines in Asia. Recently, it has become a popular herbal medicine in United States. It is one of the top best-selling herbal medicines in the world (Yun, 2001). Ginseng preparations are extracted from roots, leaves, berries and flower buds of ginseng, and in our experiments we will use root preparations.

Ginseng is believed to be most potent when harvested after 4–5 y of growth (Shin et al., 2000). Ginsenosides (also called triterpenoid saponins) are the major active

ingredients in American ginseng (Attele et al., 1999; Jia and Zhao, 2009). Ginsenosides are members of a steroid family with a four trans-ring rigid steroid skeleton (Attele et al., 1999; Wang et al., 2005). About 60 ginsenosides have been isolated from different parts of *P. quinquefolius* (Christensen, 2009; Jia and Zhao, 2009; Jiang et al., 2008; Nakamura et al., 2007; Qu et al., 2009). This wide diversity of the ginsenosides is related to differences in sugar quantities, types and attachment positions (Fuzzati, 2004; Jia and Zhao, 2009). In addition, stereoisomerism and changeable C-20 side-chain further increase the structural diversity of ginsenosides (Christensen, 2009; Nakamura et al., 2007). Ginsenosides isolated from *P. quinquefolius* are divided into two main groups; protopanaxadiol (PPD) and protopanaxatriol (PPT) (Qu et al., 2009). In the PPD group, sugar residues are attached to the  $\beta$ -OH at C-3 and/or C-20. This group includes the following compounds; G-Rb1, G-Rc, G-Rb2, G-Rb3, G-Rd, 20(*S*)-G-Rg3, G-F2, Gypenoside XVII, G-Rs1, pseudo-G-RC1, pseudo-G-RFs, Q-I, Q-II, Q-III, Q-V, Q-L10 and Q-L14 (Chen et al., 2009; Jiang et al., 2008; Li et al., 2009; Nakamura et al., 2007; Wang et al., 2001). In the PPT group, sugar residues are attached to the  $\alpha$ -OH at carbon-6 and/or  $\beta$ -OH at C-20. This group includes the following compounds; G-Rg1, 6-*O*-acetyl-G-Rg1, G-Re, 20(*S*)-G-Rg2, 20(*S*)-acetyl-G-Rg2, 20(*R*)-acetyl-G-Rg2, G-F3, G-Ia, F-E, G-F1 and Q-L17 (Jia et al., 2008; Nakamura et al., 2007). Other minor ginsenosides include; malonyl derivatives (acidic ginsenosides) (Du et al., 2004), ocotillol-type ginsenosides, oleanane-type ginsenosides, and dammarane saponins with a modified steroid skeleton (Nakamura et al., 2007; Yoshikawa et al., 1998). Nomenclature derives from the mobility of these ginsenosides in a one-dimensional thin layer chromatographic system. But Ginsenoside content of ginseng extracts can differ, depending on the method



of extraction, subsequent treatment, age and part of the plant extracted, season of its collection, or even the geographical location (Soldati F et al., 1980). Bioactivity of *P. ginseng* is mainly assigned to the presence of ginsenosides. However, additional bioactive products are contributed to the overall effect of ginseng. Among these bioactive metabolites is the C17-polyacetylenes, which include panaxynol and panaxydol (Qi et al., 2011). Recently, more than 10 polyacetylenes were isolated from white ginseng and *P. quinquefolium* (Hirakura et al., 1994). Panaxynol (PNN, falcarinol, 3(R)-(9Z)-heptadeca-1, 9-dien-4, 6-diyne-3-ol) has been identified not only in *Panax ginseng*, *P. quinquefolium*, *P. notoginseng* and *P. japonicus*, but also in common dietary plants (Zidorn et al., 2005). Other minor components include amino acids, peptides, and minerals.

Ginseng has been used in traditional Chinese medicine to enhance stamina and capacity to overcome physical and psychological stress, also, many recent researches show that ginseng is useful in treatment of some diseases. American ginseng enhanced cognitive performance, memory function and mood of mice (Bao et al., 2005; Wang et al., 2009; Zhang et al., 2008; Zhao and Li, 2004); also, it shows beneficial effect on neurodegenerative disease models of Parkinson's and Alzheimer's diseases (Xu et al., 2005, 2009). In cardiovascular system, several anti-ischemic, anti-arrhythmic and anti-hypertensive effects of ginseng are linked to the antioxidant properties of ginseng (Wang et al., 2007), and it shows antioxidant and protective effects in cultured cardiomyocytes by activating Nrf2 pathway (Li et al., 2009a). It enhances the effect of 5-fluorouracil in human colon cells (Li et al., 2009b). It is well known that chronic inflammation is the pathogenesis of many diseases such as autoimmune diseases and atherosclerosis. Several studies show that ginseng regulates inflammation (Hofseth et al., 2007; Spelman et al.,

2006). It can selectively inhibit expression of the inducible nitric oxide synthase via suppression of signal transducer and activator of transcription cascade in inflamed macrophages (Ichikawa et al., 2009). Few reports are contradictory; American ginseng exerted a neutral acute effect on blood pressure (Stavro et al., 2005), and a clinical study conducted to evaluate the efficacy of American ginseng in lowering postprandial glycaemia in patients with type 2 diabetes reported that American ginseng had no added benefit in people with diabetes (Vuksan et al., 2000). Some reports show that ginseng exerts toxic effects in the heart (Poindexter et al., 2006). This contradiction may be related to the fact that the ginsenoside content of ginseng extracts are different. Therefore, use of standardized, authentic ginseng in research is critical. American ginseng has drawn attention for their valuable potential use in medicine. However, the mechanisms of action of American ginseng at the cellular and molecular level are not fully understood.

### **1.3. Reactive Oxygen Species (ROS)**

Reactive oxygen species (ROS) are generated by cells in response to exposure to heavy metals, drugs, xenobiotics and ionizing radiation. ROS have a huge impact on the survival of cells and organs (Breimer, 1990). ROS are also produced as by-products in physiological responses, such as the activation of neutrophils during inflammation and infection (Thelen et al., 1993) and protective cytotoxic processes (Kerret et al., 1996). ROS include both free radicals, such as the superoxide anion and the hydroxyl radical, and oxidants such as hydrogen peroxide. Cells respond to oxidative stress by activation of a battery of defensive genes encoding antioxidant proteins (Dhakshinamoorthy et al., 2000; Jaiswal et al., 2000). The antioxidant proteins and cytoprotective phase II detoxifying

enzymes provide the necessary protection against oxidative stress. These include NAD(P)H:quinone oxidoreductase 1 (NQO1), NRH:quinone oxidoreductase 2 (NQO2), glutathione S-transferase Ya subunit (GST Ya subunit), heme oxygenase 1 (HO-1), g-glutamylcysteine synthetase (g-GCS), also known as glutamate cysteine ligase (GCL), ferritin, Cu/Zn superoxide dismutase and glutathione peroxidase. NQO1 and NQO2 catalyze metabolic reductive detoxification of redox cycling quinones (Joseph et al., 1994). GST Ya catalyzes conjugation of hydrophobic electrophiles and ROS with glutathione, leading to their excretion (Pickett et al., 1989; Tsuchida et al., 1992; Kretzschmar et al., 1992). HO-1 catalyzes the first and rate-limiting step in heme catabolism (Choi et al., 1996). g-GCS plays a key role in the regulation of glutathione metabolism (Mulcahy et al., 1997). The antioxidant proteins protect cells against oxidative stress. However, accumulations of ROS cause membrane and DNA damage, mutagenicity, apoptotic cell death, cellular transformation, and cancer (Ward, 1994; Breen et al., 1995; Rosen et al., 1995). The signal transduction pathways responsible for sensing oxidative stress and activating the defensive genes are still not fully understood. Factors activator protein 1(AP-1), NF- $\kappa$ B and the NF-E2-related factors (Nrf) are the most studied transcription factors, and it is known if they are activated by ROS (Dhakshinamoorthy et al., 2000; Jaiswal et al., 2000; Schulze-Osthoff et al., 1997).

#### **1.4. Nuclear factor erythroid-2 related factors (NF-E2-related factors) pathway**

NF-E2-related factors includes; Nrf1, Nrf2, and Nrf3. The Nrf) belong to Cap “n” Collar the family of basic leucine zipper (bZip) transcription factors, which have a conserved basic region-leucine zipper structure. They have nearly five similar regions; which are; leucine zipper, basic, cap’n collar (CNC), acidic, and hydrophobic regions.

Nrf2 has an additional region, INrf2 binding site; which binds to an inhibitor of Nrf2 (also called KEAP1). The basic region, just upstream of the leucine zipper region, is responsible for DNA binding. The acidic region is required for transcriptional activation. The cap'n'collar region is highly conserved among the Nrf2s, but the function of this region remains unknown. Nrf1 and Nrf2 are both 66-68 kDa proteins; however, they run at 100 kDa in SDS-PAGE (Chan et al., 1993; Moi et al., 1994). Six domains have been identified in Nrf2 (Neh 1-6 domains) (Itoh et al., 1999). In the C-terminal half of Nrf2, there are three domains, Neh1, Neh3, and Neh6. Neh1 contains a CNC-type basic-leucine zipper DNA binding motif, and Neh6 contains a serine-rich conserved region. In the N-terminal half, there are three other domains, Neh2, Neh4 and Neh5. Neh4 and Neh5 have been shown to interact with CBP (CREB Binding Protein, CREB: cAMP Responsive Element Binding protein) for transactivation (Katoh et al., 2001). Neh2 acts as the regulatory domain for cellular stress responses, and it interacts with a cytoplasmic protein Keap1 (Itoh et al., 1999). Neh2 domain contains two motifs; DLG and ETGE. The DLG motif is important for ubiquitination and degradation of Nrf2 (Katoh et al., 2005; McMahon et al., 2004), while the ETGE motif is essential for interacting with Keap1 (Kobayashi et al., 2002). In addition, seven lysine residues of the Neh2 domain have been shown to be essential, for Keap1-dependent polyubiquitination and degradation of Nrf2 (Zhang et al., 2004; Cullinan et al., 2004; Kobayashi et al., 2004).

Antioxidant response element (ARE) is a cis-acting enhancer sequence that regulates induction of genes encoding antioxidant enzymes (Dhakshinamoorthy et al., 2000; Jaiswal et al., 2000). The core nucleotide sequence of ARE is 5'-RTGACNNNGC-3' (Xie et al., 1995; Rushmore et al., 1991). However, other nearby sequences and

elements also affect the ARE-mediated induction of antioxidant genes (Li et al., 1992; Prester et al., 1993; Wasserman et al., 1997). Nrf2 is more potent than Nrf1 in activation of ARE-mediated gene expression (Venugopal et al., 1996). The exact mechanisms by which Nrf1 and Nrf2 bind to ARE are still under investigation. Some reports show that Nrf1 and Nrf2 require another protein to heterodimerize with it, before binding to ARE. Jun (c-Jun, Jun-D, and Jun-B) and small Maf (MafG, MafK, and MafF) proteins have been shown to heterodimerize with Nrf1 and Nrf2, and the complexes are able to bind to ARE, and mediate expression of NQO1 and GST (Alam et al., 1999; Wild et al., 1999; Nguyen et al., 2000; Itoh et al., 1997). Nrf2 cascade is the major antioxidant defense system in the cell.

The inhibitor of Nrf2 (INrf2) or Keap1 (Kelch-like ECH-associated protein 1) is a protein which regulates activity of Nrf2. Keap1 binds to Nrf2 and retains it in the cytoplasm. The treatment of cells with antioxidants leads to the release of Nrf2 from INrf2, and translocate Nrf2 into the nucleus where it binds to ARE (Dhakshinamoorthy et al., 2001; Itoh et al., 1999). Binding of INrf2 to Nrf2 leads to degradation of Nrf2. One suggested mechanism is that INrf2 functions as a substrate adaptor of the Cullin 3 (Cul3)-based E3 ligase machinery which leads to ubiquitylation and thereby targets Nrf2 for degradation by the 26S proteasome (Sekhar et al., 2002; Stewart et al., 2003; Zhang et al., 2003). Keap1 has four functional domains: BTB (Broad complex, Tram-track, and Bric-a-brac) (Bardwell et al., 1994), IVR (intervening region), DGR (double glycine repeat) (Adams et al., 2000; Xue et al., 1993), and CTR (C-terminal region). The BTB domain acts as a dimerization domain, and dimerization of Keap1 is important for its function (Zipper et al., 2002). DGR and CTR domains interact with the Neh2 domain of Nrf2.

This intermolecular interaction allows Keap1 to regulate the rate of Nrf2 protein turnover through ubiquitin signaling and proteasomal proteolysis. It has also been reported that Nrf2 regulates Keap1 by controlling its transcription (Lee et al., 2007).

The cytosolic factors that modified Nrf2 and/or INrf2 remain largely uncharacterized. Some cytoplasmic kinases, including protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and Phosphatidylinositol 3-kinase (PI3K), have been suggested to modify Nrf2 and participate in the mechanism of signal transduction from antioxidants to the ARE (Bloom et al., 2003; Zipper et al., 2000; Yu et al., 2000; Lee et al., 2001). Protein kinase C is the most extensively studied factor, and it is demonstrated that antioxidant-induced PKC phosphorylation of serine 40 in Nrf2, leading to dissociation of Nrf2 from INrf2 (Bloom et al., 2003; Lee et al., 2001). The mechanism of release of Nrf2 by PKC is still under investigation, and there are two hypotheses. In the first hypothesis, antioxidants induce the expression of, or activate PKC, which in turn phosphorylates Nrf2 that is bound to INrf2. The phosphorylation releases Nrf2 from INrf2 and leads to nuclear accumulation of Nrf2. In the second hypothesis, the INrf2 binds only to unphosphorylated Nrf2. Antioxidants induce/activate PKC, which phosphorylates free Nrf2. The phosphorylated Nrf2 escapes INrf2-mediated degradation, and accumulates in the nucleus (Sekhar et al., 2002; Stewart et al., 2003; Itoh et al., 2003; Bloom et al., 2003). However, it is still under investigation, whether Nrf2 is completely released from Keap1 or just partially dissociated (Zhang, 2006).

### **1.5. Nuclear factor kappa B (NF- $\kappa$ B) pathway**

NF- $\kappa$ B is a transcription factor that can induce and repress gene expression by binding to specific sequences known as  $\kappa$ B elements, in promoters and enhancers, and it

exists in most of cell types (Hayden et al., 2004). It was described in 1986 as a nuclear factor necessary for transcription of immunoglobulin kappa light chain in B cells, and the name NF- $\kappa$ B comes from that description (Sen et al., 1986). NF- $\kappa$ B is retained in the cytoplasm by a family of inhibitory proteins known as inhibitors of NF- $\kappa$ B (I $\kappa$ Bs). Phosphorylation of I $\kappa$ B by the I $\kappa$ B kinase (IKK) leads to I $\kappa$ B degradation and release of NF- $\kappa$ B. Unbound NF- $\kappa$ B translocated to the nucleus and bind to  $\kappa$ B elements (Hayden et al., 2004). NF- $\kappa$ B regulates genes responsible for apoptosis, cell adhesion, proliferation, the innate- and adaptive-immune responses, inflammation and tissue remodeling (Bonizzi et al., 2004; Gerondakis et al., 1999; Pasparakis et al., 2006; Pahl, 1999; Beg et al., 1995). But, the expression of these genes is tightly controlled by other signaling and transcription-factor pathways.

In eukaryotic cells, there are five NF- $\kappa$ B family members, RelA (p65), RelB, c-Rel, p50/p105 (NF- $\kappa$ B1) and p52/p100 (NF- $\kappa$ B2), and different NF- $\kappa$ B complexes are formed from their homo and heterodimers. Because the dimer is made up by two proteins, each protein contacts one half of the DNA binding site. All NF- $\kappa$ B family members contain about 300 amino acids at the N-terminal domain called the Rel-homology domain (RHD); this region is responsible for DNA-binding, dimerization and interaction with I $\kappa$ B family members and it contains a nuclear localization sequence. Furthermore, all Rel subfamily members (RelA, RelB and c-Rel) contain C-terminal transcriptional activation domains. Despite structural similarities; all NF- $\kappa$ B subunits have distinct and non-overlapping functions (Gerondakis et al., 1999; Hoffmann et al., 2003). P50/p65, p50/c-rel, p65/p65, and p65/c-rel are all transcriptionally active, whereas p50 homodimer and p52 homodimer are transcriptionally repressive (Hansen et al., 1994

a,b; Brown et al., 1994). The mRNA from the cloning of p50 was found to encode for a much larger protein of approximately 105 kDa (Ghosh et al., 1990; Kieran et al., 1990). Similarly, the mRNA for p52 also encodes a protein of 100 kDa (Bours et al., 1992; Neri et al., 1991; Schmid et al., 1991). The N-terminal of p105 and p100 are identical to the p50 and p52 proteins, respectively. But the C-terminal of p105 and p100 shows several ankyrin repeats. P100 and p105 are post-translationally cleaved to produce p50 and p52 (Blank et al., 1991; Mercurio et al., 1993).

I $\kappa$ B binds NF- $\kappa$ B and masks its nuclear localization signal that is found in the RHD region of the NF- $\kappa$ B. Thus, it retains NF- $\kappa$ B in the cytoplasm (Verma et al., 1995; Baldwin, 1996). In eukaryotic cells, there are five I $\kappa$ B family members; that include I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\epsilon$ , Bcl-3 and I $\kappa$ B $\gamma$ . I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$  are the three principal I $\kappa$ Bs. All members of I $\kappa$ B contain the ankyrin-repeat motifs which are regions of protein/protein interaction. As mentioned before, p100 and p105 contain ankyrin repeats and are sometimes act as I $\kappa$ B (Hayden et al., 2004). I $\kappa$ B $\alpha$  is the most studied member of I $\kappa$ B, it is a 37-kDa protein that has three domains; an N-terminal domain that is phosphorylated in response to signals, a central ankyrin repeat domain, and a C-terminal PEST domain that is controlled the basal turnover of the protein (Verma et al., 1995). There is an auto-regulatory feedback loop between I $\kappa$ B $\alpha$  and NF- $\kappa$ B; that means activation of NF- $\kappa$ B causes up-regulation of transcription of I $\kappa$ B $\alpha$ , which serves to shut off the signal (Brown et al., 1993; Chiao et al., 1994; Sun et al., 1993; Scott et al., 1993). Continuous presence of inducing agents (e.g. LPS) causes NF- $\kappa$ B to be maintained in the nucleus despite the up-regulation of I $\kappa$ B synthesis in the cytoplasm (Thompson et al., 1995). I $\kappa$ B $\beta$  is a 45-kDa; it has three domains and binds the same rel subunits as I $\kappa$ B $\alpha$  (Thompson et al.,



1995). Both I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  are degraded after cells are treated with the inducing agents (e.g. LPS); however, I $\kappa$ B $\alpha$ , level is elevated later, because of the auto-regulatory feedback loop between I $\kappa$ B $\alpha$  and NF- $\kappa$ B, whereas I $\kappa$ B $\beta$  level remains low. I $\kappa$ B $\epsilon$  is a specific inhibitor of p65 and c-Rel complexes. Bcl-3 has structure similar to other I $\kappa$ Bs (Ohno et al., 1990). Bcl-3 is located in the nucleus and binds specifically to p50 and p52 homodimers (Franzoso et al., 1992; Franzoso et al., 1993; Nolan et al., 1993; Wulczyn et al., 1992; Zhang et al., 1994). I $\kappa$ B $\gamma$  is a 70-kDa molecule detected only in lymphoid cells (Inoue et al., 1992). Very little is known about the I $\kappa$ B $\gamma$ .

I $\kappa$ B kinase (IKK) contains IKK $\alpha$  (IKK1) and IKK $\beta$  (IKK2), which are kinase subunits and a regulatory subunit, NEMO (NF- $\kappa$ B essential modifier) or IKK $\gamma$ . In the classical NF- $\kappa$ B signaling pathway, IKK $\beta$  phosphorylates both I $\kappa$ B $\alpha$  on Serine 32 and Serine 36, and I $\kappa$ B $\beta$  on Serine 19 and Serine 23. In the alternative pathway, IKK $\alpha$  phosphorylates p100 and causing its inducible processing to p52 (Ghosh et al., 2002). Many agents like UV light, ROS, LPS and double-stranded RNA can activate NF- $\kappa$ B. Stimulation of cells with inducers of NF- $\kappa$ B results in phosphorylation of I $\kappa$ B by I $\kappa$ B kinase (IKK) (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995; Whiteside et al., 1995); Phosphorylated I $\kappa$ B is then polyubiquitinated on lysine 21 and 22 by ubiquitin ligases; and this triggers the rapid degradation by the 26S proteasome, and finally, release of NF- $\kappa$ B (Beg et al., 1993; DiDonato et al., 1995; Traenckner et al., 1994; Chen et al., 1995). NF- $\kappa$ B plays an important role in activation of immune cells by up regulating many cytokines, particularly; IL-1, IL-6, TNF $\alpha$  and IFN- $\gamma$ . All of these cytokines have multiple effects that contribute to inflammation (Kishimoto et al., 1994). NF- $\kappa$ B is important in the regulation of the acute phase response of inflammation and

production of complement factor B, serum amyloid A protein 1, angiotensinogen,  $\alpha$ 1 acid glycoprotein and the C3 component of complement (Grilli et al., 1993; Kopp et al., 1995). Anti-inflammatory drugs such as the salicylates and dexamethasone inhibit NF- $\kappa$ B activation which suggesting that NF- $\kappa$ B may be a good target for potential therapies against chronic inflammatory diseases (Auphan et al., 1995; Scheinman et al., 1995).

There are two main NF- $\kappa$ B-activation pathways; the canonical or classical pathway and the non-canonical or alternative pathway. The classical pathway is induced by various inflammatory stimuli, like tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), interleukin-1 (IL-1), lipopolysaccharide (LPS) or engagement of the T-cell receptor (TCR). On this pathway there is rapid phosphorylation of I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  by IKK $\beta$  and subsequent ubiquitin-induced degradation by the 26S proteasome (Hayden et al., 2004). The alternative pathway is induced by stimulation of the CD40 and lymphotoxin- $\beta$  receptors, B-cell-activating factor of the TNF family (BAFF), LPS and latent membrane protein-1 (LMP1) of Epstein–Barr virus (Bonizzi et al., 2004; Perkins, 2003). Here, activation of IKK $\alpha$  by the NF- $\kappa$ B inducing kinase (NIK) results in the formation of p52 from p100 (Bonizzi et al., 2004).

#### **1.6. The Janus family tyrosine kinase–signal transducer and activator of transcription (Jak-STAT) pathway**

Jak-STAT signaling pathway is activated mainly by type I cytokines and interferons (also called type II cytokines). Ligand binding induces the oligomerization of the appropriate receptor components. Janus family tyrosine kinases (Jak kinases) are associated with or recruited to cytoplasmic end of the receptors. Jak kinases phosphorylate tyrosine residues in the receptor cytoplasmic domains, and these

phosphorylated tyrosines act as binding sites for STAT proteins. Jak kinases then phosphorylate STAT proteins, which then dissociated from the receptors and translocate to the nucleus, where they act as transcription factors by binding to consensus DNA-recognition motifs, called gamma-activated sites (GAS) (Darnell, 1997; Leonard et al., 1998). Cytokines are classified into type I and type II. Type I cytokines include many interleukins, such as IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-12, IL-13, IL-15, and IL-21, thymic stromal lymphopoietin (TSLP) and several other hematopoietic molecules, including stem cell factor (SCF), granulocyte colony stimulating factor (G)-CSF, granulocyte-macrophage (GM)-CSF, erythropoietin (EPO), and thrombopoietin (TPO). Type II cytokines refers to the type I interferon (IFN- $\alpha$  and IFN- $\beta$ ) and type II interferon (IFN- $\gamma$ ) as well as IL-10 and IL-22 (Leonard, 1999).

There are 4 Jak kinases; Jak1, Jak2, Jak3, and tyrosine kinase 2 (Tyk2). Three of these kinases, Jak1, Jak2, and Tyk2, are present in most cell types, whereas Jak3 is found in lympho-hematopoietic cells. Structurally, Jak kinases are subdivided into Jak kinase homology (JH) domains. The JH1 domain is the tyrosine kinase catalytic domain. JH2 domain has no catalytic action but it is required for normal function of Jak kinases (Leonard, 1999; Leonard et al., 1998). N-terminal region of the Jak kinases is responsible for interaction of Jak kinases with cytokine receptors (Witthuhn et al., 1993; Argetsinger et al., 1993). There are 7 STAT proteins, Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, and Stat6. STAT proteins contain an N-terminal oligomerization domain, coiled-coil domain, DNA binding domain, SH2 domain, a conserved tyrosine residue, and a C-terminal transactivation domain (Leonard, 1999; Leonard et al., 1998; Darnell, 1997). STAT dimerization is based on a bivalent interaction in which the SH2 of each of 2 STAT

protein monomers associates with the phosphorylated tyrosine of the other monomer. The N-terminal oligomerization domain can mediate STAT-STAT oligomerization (John et al., 1999; Schindler et al., 1995).

The duration and magnitude of STAT activation is critically regulated by a number of different mechanisms to ensure proper function of normal cells. Activated STAT can be inhibited by protein inhibitors of activated STAT proteins (PIAS) by direct association with STAT. PIAS family members include PIAS1, PIAS3, PIASx and PIASy (Schmidt et al., 2003; Chung et al., 1997; Rogers et al., 2003). Suppressors of cytokine signaling (SOCS) proteins inhibit STAT signaling by either directly binding to JAK, binding to the receptor, or by competing with STATs for the docking sites at the receptor (Cooney, 2002). There is an auto-regulatory negative feedback loop between STAT and SOCS. Upon activation, STATs bind to the promoter region of SOCS genes and activate the transcription of SOCS genes. SOCS proteins include; CIS (cytokine-inducible SH2 domain protein) and SOCS1–7 (Alexander et al., 2004).

### **1.7. Activator protein 1 pathway (AP-1)**

Activator protein 1 (AP-1) is a transcription factor consists of members of the Jun and Fos family of proteins (Angel et al., 1991; Curran et al., 1988; Rauscher et al., 1988). AP-1 binds DNA target sites as Jun/Jun homodimers and Jun/Fos heterodimers, and it regulates cellular proliferation, differentiation, and cell death (Domann et al., 1994; Dong et al., 1994; Dong et al., 1997; Young et al., 1999). AP-1 can be activated by cytokines, growth factors, and UV light (Whitmarsh et al., 1996; Angel et al., 1991). AP-1 proteins are a family of basic leucine zipper (bZip) proteins. The leucine zipper domains directs dimerization of Jun and Fos. The transactivation domain of AP-1 is essential for the

protein activity (Gentz et al., 1989). AP-1 transcription factor expression is activated by mitogen-activated protein kinase family (MAPK) (Frost et al., 1994; Huang et al., 1998; Watts et al., 1998). The MAPK family includes the extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase/stress-activated kinases (JNK/SAPK), and p38 kinase (Boulton et al., 1990; Boulton et al., 1991; Kyriakis et al., 1994). ERKs (ERK 1 and ERK 2) are activated by mitogen stimulation through a cascade of kinases, including Ras, Raf, and MAPK kinase (MEK) (Watts et al., 1998; Huang et al., 1998).

The serum response factor and the ternary complex factor (TCF), both bind to the serum response element (SRE) and increased c-fos production (Treisman, 1992).

Mitogenic stimulation activates ERK group of MAPKs, which phosphorylate Elk-1; Elk-1 is one of the member of TCFs (Treisman, 1994; Gille, 1992; Marais et al., 1993).

Phosphorylation of Elk-1 facilitates formation of the ternary complex composed of itself, the serum response factor, and the SRE and causes c-fos induction (Gille, 1992). The synthesis and translocation of c-Fos to the nucleus is increased. c-Fos combines with pre-existing Jun proteins to form stable AP-1 dimers (Smeal et al., 1989). Increased stability of AP-1 results in higher levels of binding activity between AP-1 and DNA.

c-jun promoter is simple and most of its inducers operate through the c-jun TPA response element (TRE) (Angel et al., 1988). c-jun TRE is more efficiently recognized by c-Jun/ activating transcription factor 2 (ATF2) heterodimers (Van Dam et al., 1993).

ATF2 is a constitutively expressed protein while c-jun is mostly inducible. Following exposure to stimuli that activate JNK group of MAPKs (Dérjard et al., 1994), both c-Jun (Devary et al., 1992) and ATF2 (Gupta et al., 1995) are phosphorylated. Phosphorylation

of c-Jun and ATF2 stimulates their ability to activate transcription, thereby leading to c-jun induction and increase in AP-1 activity.

### **1.8. Nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2)**

There are three major isoforms of Nitric oxide synthase (NOS); two are classified together as constitutive NOS isoforms (cNOS) which are endothelial-derived NOS (eNOS) and neuronal-derived NOS (nNOS). The third isoform is inducible NOS (iNOS). Nitric oxide is generated via the oxidation of the terminal guanidino nitrogen of L-arginine by NOS (Moncada et al., 1995). cNOS isoforms are expressed constitutively, and calcium dependent. It releases Nitric oxide in small amounts in response to agonist/receptor interaction for a short period of time. This Nitric oxide is important for control of organ blood flow by vasodilation, inhibition of aggregation and adhesion of platelets to the vascular wall and smooth muscle cell proliferation (Moncada et al., 1995). iNOS is a cytokine-inducible and calcium independent. It is expressed in many cells in response to inflammatory stimuli, such as cytokines and lipopolysaccharide (LPS). NO released from iNOS is large in amount and it has pro-inflammatory action (Clancy et al., 1995; Kroncke et al., 1995). Expression of iNOS gene is controlled by NF-kB and AP-1. 7.2-kb and 8.3-kb 5'-flanking regions of the human iNOS gene were found to contain NF-kB- and AP-1 binding regulatory regions respectively. JunD and Fra-2 heterodimers are bound to upstream and downstream AP-1 sites. RelA/RelA and RelA/p50 are bound to upstream and downstream NF-kB sites respectively (Taylor et al., 1998; Marks-Konczalik et al., 1998). Both LPS and cytokines can activate AP-1 and NF-kB, and they are able to induce expression of iNOS (Sweet et al., 1996; Blackwell et al., 1997; Saklatvala et al., 1996). Extracellular signal-regulated kinases (ERKs), c-jun N-terminal

kinase/stress-activated protein kinases (JNK/SAPKs), and p38 MAPK pathways have been implicated in the activation of AP-1 which in turn induces expression of iNOS (Marks-Konczalik et al., 1998; Karin, 1995).

There are two COX isoforms; COX-1 (constitutive enzyme) and COX-2 (inducible enzyme). COX-1 is present in tissues such as the stomach, gut, or kidney, where PGs play a cytoprotective role in maintaining normal physiological processes. COX-2 is present in stimulated host defense cells and produce pro-inflammatory PGs (Simmons et al., 2004). Both isoforms are composed of three domains; an epidermal growth factor binding domain, the membrane binding domain, and the COX catalytic domain containing the cyclooxygenase and peroxygenase active sites (Garavito et al., 2003). COX enzyme converts arachidonic acid (AA) to a cyclic endoperoxide (PGG<sub>2</sub>), followed by peroxidase cleavage of the PGG<sub>2</sub> peroxide to the endoperoxide (PGH<sub>2</sub>) which is converted by specific isomerases into prostaglandins (PGE<sub>2</sub>, PGF<sub>2</sub>, TxA<sub>2</sub>, PGI<sub>2</sub>) (Needleman et al., 1986).

It has been reported that NO Regulate Cyclooxygenase Pathway (Salvemini et al., 1993; von Knethen et al., 1997; Watkins et al., 1997), but the mechanism is still not fully understood. It is not clear whether COX regulation is mediated by NO or iNOS. Contrary, other reports showed that LPS-induced COX-2 expression in microglial cells and their subsequent PG release are enhanced by co-incubation with NOS inhibitors (Minghetti et al., 1996), and NO donors applied exogenously to LPS-treated microglial cells inhibit their PG release and reduce expression of COX-2 (Goodwin et al., 1998). This has also been found in other cell types, including vascular endothelial cells (Doni et al., 1988) and the J774 macrophage cell line (Swierkosz et al., 1995).

## 1.9. Mechanisms of activation of macrophage by lipopolysaccharide

Lipopolysaccharide (LPS) is a complex glycolipid composed of a hydrophilic polysaccharide and a hydrophobic lipid A. LPS is a component of the outer membrane of Gram-negative bacteria and one of the most potent initiators of inflammation by producing pro-inflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), monocyte chemo-attractant protein (MCP), macrophage inflammatory protein-1 beta (MIP-1b) and interleukin (IL-1, IL-6, IL-8, and IL-12). In addition; macrophages treated with LPS secrete platelet-activating factor, prostaglandins, enzymes, and free radicals, such as nitric oxide (Raetz, 1990; Cohen, 2002). LPS activates macrophage by various mechanisms, which include protein kinase A (Muroi et al., 1993), protein kinase C (Fujihara et al., 1994a), Src-related kinases (Herrera-Velit et al., 1996), the 3 classes of mitogen-activated protein kinases (MAPK): extracellular signal-regulated kinase 1 (ERK1) and ERK2 (Weinstein et al., 1992), p38 MAPK (Han et al., 1994), c-Jun N-terminal kinases (JNK) (Hambleton et al., 1996), G-proteins (Jakway et al., 1986) activation of transcription factors including nuclear factor  $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1) (Fujihara, et al., 1993; Guha et al., 2001). LPS binds to specific receptors to form complex, the LPS complex is composed of 3 proteins; CD14, Toll-like receptor 4 (TLR4), and myeloid differentiation protein-2 (MD-2) (Fujihara, et al., 2003).

TLRs have cytoplasmic regions called TIR domain, and the downstream signals are mediated by the adaptor protein myeloid differentiation factor 88 (MyD88), a family of IL-1 receptor-associated kinases (IRAK), and TNF receptor-activated factor 6 (TRAF6). The human MyD88 contains 2 domains; an N-terminal death domain and a C-terminal TIR domain. The TIR domain of MyD88 associates with the TIR domain of



TLR, and the death domain interacts with the N-terminal death domain of IRAK (Muzio et al., 1997; Wesche et al., 1997; Burns et al., 1998; Medzhitov et al., 1998). IRAKs (mainly IRAK-1 and 2) are subsequently phosphorylated and are dissociated from the receptor complex and interact with TRAF6 (Cao et al., 1996; Muzio et al., 1998). TRAF6 activates MAPK kinases, which activate AP-1. TRAF6 also activates the inhibitors of  $\kappa$ B kinase (IKK) complex which in turn translocate NF- $\kappa$ B to the nucleus (Fujihara, et al., 2003). LPS can induce the nuclear translocation of interferon regulatory factor 3 (IRF3), and binding of IRF3 to IFN stimulated response element can produce interferons (Navarro, et al., 1999; Kawai et al., 2001). Interferon- $\alpha/\beta$  through autocrine and paracrine function can activate Jak-STAT pathway which induces expression of inducible nitric oxide synthase (iNOS) (Fujihara et al., 1994b; Gao et al., 1998).

#### **1.10. Aims of the study**

American ginseng has drawn attention for their valuable potential use in medicine as a potential target of therapy. However, the mechanisms of action of American ginseng at the cellular and molecular level are not fully understood. On this project, we have two main aims. First aim: to isolate a novel single natural compound from American ginseng extract that exerts the strongest anti-inflammatory effects with minimum cytotoxicity on macrophages. Second aim: to investigate the effects of the natural compound isolated from American ginseng in suppression of inflammatory responses focusing on Nrf2 and NF- $\kappa$ B pathway in RAW264.7 macrophages

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1. American ginseng extracts

A standardized American ginseng extract was supplied by the National Research Council of Canada, Institute for National Measurement Standards (NRCC-INMS). This extract was derived from four year old, cultivated, ginseng roots and processed by Canadian Phytopharmaceuticals Corporation (Richmond, British Columbia, Canada). The identity of the roots was independently confirmed by Agriculture and Agri-Foods Canada and a voucher specimen of the *P. quinquefolius* used in this study deposited with the University of Ottawa herbarium (UO 19908). Plant morphology conformed to that for *P. quinquefolius* and the presence of a marker compound ginsenoside F11, unique to *P. quinquefolius*, confirmed by liquid chromatography-mass spectrometry. Following thorough homogenization, 4000 1 g lots of the extract were bottled under argon, irradiated (5 kGy), and stored under cryogenic conditions ( $-80\text{ }^{\circ}\text{C}$ ). Periodic analyses of the extract over 4 years have shown no significant change in ginsenoside content which is 10.5% (w/w) measured as the sum of: Rg<sub>1</sub> 3.5 (0.1), Re 21.2 (0.4), Rb<sub>1</sub> 45.1 (1.6), Rc 15.5 (0.7), Rb<sub>2</sub> 2.2 (0.1), Rd 17.8 (0.4) mg/g $\pm$  one standard deviation, respectively. The American ginseng extract comprises an aqueous ethanolic extract of ginseng root that has been spray dried on a maltodextrin support (comprising 40% by weight of the material) to yield a free flowing powder. This material was re-dissolved in aqueous ethanol with the ethanol content low enough (< 40%) that the solution was immiscible with a series of

solvents (hexane, dichloromethane, ethyl acetate and butanol) used to partition the extract based on polarity. Using a separatory funnel, the aqueous solution was shaken with a volume of solvent (e.g. hexane) and the layers (aqueous and organic) allowed separating into two phases and the organic phase removed; this was repeated three times for each solvent from hexane to butanol. The three organic layers for each solvent were combined and reduced to dryness by vacuum centrifugation. The residual aqueous layer was reduced by vacuum centrifugation and freeze drying. The result was 5 fractions: water/aqueous (80% of whole material by weight), butanol (17%) ethyl acetate (1%), dichloromethane (1%), and hexane (1%). Aliquots of these fractions were re-deposited on maltodextrin to yield final weights equivalent to the starting material (e.g., if 10g of material is fractionated, the resulting 1.7g of butanol soluble material would be re-deposited on 8.3 g of maltodextrin to give 10 g final weight) and used in bioassay using whole American ginseng and plain maltodextrin as positive and negative controls respectively. By utilizing preparative, reverse-phase HPLC and a comparative analysis by analytical scale LC-UV, we found that hexane fraction contains predominantly polyacetylenes and linolenic acid. Moreover, we identified panaxynol as a major compound of polyacetylenes. Panaxynol was isolated and prepared by National Research Council of Canada, Institute for National Measurement Standards. Solutions of American ginseng crude and fractions, at different concentrations, were freshly prepared before each experiment. Briefly, American ginseng extract was weighed and dissolved (DMEM) and then the solution was passed through a syringe filter and then diluted by DMEM to get the working solutions with different concentrations for each experiment.

## 2.2. Cell cultures and animals

RAW264.7 cells are a murine macrophage cell line (American Type Culture Collection, Rockville, MD; cat # TIB-71). RAW 264.7 cells are a macrophage-like, Abelson leukemia virus transformed cell line derived from BALB/c mice. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Cocalico Biologicals, Reamstown, PA) for 24h and then RAW264.7 cells changed to 2% FBS DMEM or serum free DMEM for 24h for cell toxicity experiments. For other experiments, RAW264.7 cells cultured in 10% FBS DMEM for 48h and then changed to 2% FBS DMEM for a period of time indicated in each experiment. The culture condition is a 95% O<sub>2</sub>: 5% CO<sub>2</sub> humidified atmosphere at 37° C. For routine maintenance in culture (passage), cells are seeded at a confluence of approximately 10% and grown to a confluence of approximately 80%. This procedure requires the cells to be passaged every two days. Cultures are not maintained beyond three months and we used cells in passage 3 to 10. All of the animal procedures were conducted in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the University of South Carolina Institutional Animal Care and Use Committee. Littermate wild-type (WT, Nrf2<sup>+/+</sup>) and Nrf2 knockout (Nrf2<sup>-/-</sup>) mice were housed under standard conditions. Nrf2 knockout (Nrf2<sup>-/-</sup>) mice were generated by breeding heterozygous Nrf2 (Nrf2<sup>+/-</sup>) mice. Male Nrf2<sup>-/-</sup> mice grow normally and did not exhibit any clinical manifestations of illness, therefore, male Nrf2<sup>-/-</sup> at 8-16 weeks of age were used in our study. Peritoneal derived macrophages were generated from adult male 8–16 weeks old WT and Nrf2<sup>-/-</sup> littermates. Peritoneal macrophages were elicited by intra-peritoneal injection of 1ml starch (6%) in distilled water. After 72h, cells were

harvested and cultured in 10% FBS DMEM medium with penicillin (100U/ml), streptomycin (100U/ml), 2mM glutamine and 20% mouse L929 (American Type Culture Collection, ATCC) cell supernatant as a source of colony-stimulating factor (CSF), in a 95% O<sub>2</sub>: 5% CO<sub>2</sub> humidified atmosphere at 37° C. Eight days after the culture, flow cytometer (BD Accuri™ C6, BD Biosciences) analysis confirmed that almost 100% of these cells are double positive with macrophage specific biomarkers CD11b and F4/80 using anti-CD11b (cat# 14-0112, eBioscience) and anti-F4/80 (cat# 123109, Biolegend). Cells washed by 1x PBS and change to new 10% FBS DMEM medium for 12h then stimulate cells with drugs.

### **2.3. Cell viability assays**

Cell viability assays were determined by monitoring the release of the cytoplasmic enzyme; lactate dehydrogenase (LDH), utilizing a cytotoxicity detection kit (Clontech Laboratories, Inc.). Briefly; cell viability was calculated as follows; Raw 264.7 cells  $1 \times 10^5$ /well in 24 well plate cultured in 10% FBS DMEM medium for 24h, then changed to SF DMEM medium and stimulate with drugs for 24h. To measure the OD<sub>supernatant</sub>, the supernatant was removed and only 100 µl kept which will be used for measurement and placed in the optically clear, flat-bottom 96-well plate. 100 µl of freshly prepared Reaction Mixture was added to each well that contains the supernatant and incubated for up to 30 min at room temperature, and protected from light. For the OD<sub>lysate</sub>, 500 µl of 1% triton was added to Raw 264.7 cells, and incubate for 30 min at 37° C; then 100 µl of the solution was used for measurement and incubated with 100 µl Reaction Mixture 30 min at room temperature, and protected from light. The absorbance

was measured at 490 or 492 nm (reference wavelength 690 nm). Percent cytotoxicity calculated by:  $OD_{\text{supernatant}} / OD_{\text{(supernatant+lysate)}}$ .

#### **2.4. Cells lysis, protein extraction and protein quantification**

For Cells lysis and protein extraction we used NP40 or RIPA buffer and all the procedure were done on ice. The NP40 lysis buffer contained 1% Nonidet P-40, 25 mM Hepes (pH7.5), 50 mM NaCl and 5 mM EDTA and the RIPA lysis buffer contained sodium chloride 150mM, NP-40 1%, Tris (pH8.0) 50mM, Sodium deoxycholate 0.5% and SDS 0.1%. Briefly, lysis buffer was Prepared by use of 100µl of NP40 or 100µl RIPA buffer per well and 100x Protease Inhibitor Complex (sigma, P8340) and Phosphatase Inhibitor (sigma, P5726) 100x diluted to 1x. We removed the DMEM medium and washed the wells twice with 500µl VaSO<sub>4</sub>, then lysis buffer cocktail was added (100µl/well) and kept on ice 10 min, and then we used a scraper to scrape the wells and place the liquid in Shaker for 30 min to 1 hour at 4°C followed by centrifugation at 16100xg, 4°C for 15 min. Finally, we transferred the supernatant to new tubes on ice being careful not to transfer of white the pellets and then tubes stored at -80°C. Protein was quantified by Bradford Assay (if NP40 used) or Lowry assay (if RIPA used). For Bradford Assay, we used Bio-Rad dye reagent and a spectrophotometer (Eppendorf Biophotometer) set to 595 nm. A standard curve with known amounts of bovine serum albumin (BSA) was generated in each assay. The linear range of the assay for BSA is 0.2-0.9 mg/ml. Protein Assay Dye Reagent (DR) Concentrate (Catalog no. 500-0006, Bio-Rad) was diluted (Dye Reagent 25% and distilled, deionized (DDI) water 75%) and then 10 µl of protein sample was added to 1ml DR and incubate at room temperature for at least 5 minutes before measuring the absorbance at 595 nm. For Lowry assay, we used

bio-rad DC protein assay kit (Catalog no. 500-0112). A standard curve with known amounts of bovine serum albumin (BSA) was generated in each assay. Protein samples were diluted with 0.1 N NaOH, 40µl /well, and Dye Reagent was added and kept for 15 minutes in the dark. Protein concentration was detected at 750 nm within 1 hour. Sample protein was boiled at 95°C for 10 minutes. Protein was aliquoted for SDS-PAGE and Western blotting.

## **2.5. Western blot analysis**

### **2.5.1. SDS-polyacrylamide gel electrophoresis**

After assembling the glass plates (Mini-PROTEAN Tetra ell, Bio-Rad, 165-8001), resolving gels were prepared by mixing the following reagents for 2 gels (depend on protein size). For gel 8%; H<sub>2</sub>O 4.7ml, 30% Acrylamide/Bis 2.7ml, 1.5M Tris-HCl (pH8.8) 2.5ml, 10% SDS 0.1ml. For gel 10%; H<sub>2</sub>O 4.1ml, 30% Acrylamide/Bis 3.3ml, 1.5M Tris-HCl (pH8.8) 2.5ml, 10% SDS 0.1ml. For gel 12%; H<sub>2</sub>O 3.4ml, 30% Acrylamide/Bis 4.0ml, 1.5M Tris-HCl (pH8.8) 2.5ml, 10% SDS 0.1ml. Immediately afterwards, we added 10% APS 50µl and TEMED 5µl prior to pouring the gel and we poured 5ml of the acrylamide solution into the gel holder, then the top of the gel was covered by 70% ethanol to the top of the plate, and at least 30 minutes was allowed for polymerization (30min-1h). The top of the gels were rinsed with H<sub>2</sub>O and 4% Stacking gels were prepared with mixing H<sub>2</sub>O 3.05ml, 30% Acrylamide/Bis 0.65ml, 1.5M Tris-HCl (pH8.8) 1.25ml, 10% SDS 0.05ml, 10% APS 25 µl and TEMED 5 µl. Stacking gel loaded to the top of the glass plates and the comb inserted and kept at room temperature for 30 min to 1 hour. After assembling the gel apparatus, 500 ml of 1X running buffer (10x running buffer 50ml/H<sub>2</sub>O 450ml) was filled to top of cartridge. The 10X running

buffer was made of 57.3 g glycine, 12.1 g Trizma Base, and 4 g SDS, and filled up to 500 ml with nanopure water. Wells were flushed prior to loading. Protein samples were combined with a 5X bromophenol blue loading dye containing 5% 2-mercaptoethanol and then loaded into wells. For the first well comb, we loaded 5.0 $\mu$ l of protein molecular weight markers. Gel was run at constant voltage 100V for 60 to 90 min.

### **2.5.2. Immunoblotting**

Protein in gels was transferred to PVDF membrane after wetting the membrane for 30 seconds in methanol. Transfer buffer was constituted with 2.93 g glycine, 5.82 g Trizma Base, 200 ml methanol, and 1 liter nanopure water. We soaked the membrane and filter papers in 1x transfer buffer for 5-10min (10x transfer buffer 100 ml /methanol 200ml/ H<sub>2</sub>O 700 ml), and assembled the cassette in the following order; fiber pad, filter paper, gel, membrane, filter paper and fiber pad and we added the transfer buffer, and transfer at 100V for 1-2h on ice.

### **2.5.3. Western blot**

The membrane was blocked in 5% skim milk /1X tris buffered saline-Tween (TBS-T) for 1h and the membrane was washed with 1X TBST for 3 times, 10 minutes for each time. The primary antibodies included: anti-iNOS (BD610431, BD Biosciences); anti-I $\kappa$ B $\alpha$  (sc-371, Santa Cruz Biotechnology, Inc.); anti-Nrf2 (sc-722, Santa Cruz Biotechnology, Inc.); anti-NQO1 (ab34173, Abcam Inc.); anti-HO-1 (SPA-896, Stressgen Biotechnologies); and anti- $\beta$ -actin (Sigma A1978, Sigma-Aldrich). Primary antibodies were diluted in 5% milk and stored overnight at 4°C on a shaker (membrane /3-5ml). The membrane was washed with 1X TTBS for 3 times, 10 minutes for each time. HRP-conjugated secondary antibody was incubated with the membrane for 1h at



the room temperature on a shaker then washed three times for 10min each with TBS-T. ECL solution was prepared by adding 1:1 Reagent 1 and Reagent 2 from the ECL kit. The ECL solution was mixed well, and added onto the well-drained membrane for 1 minute. The membrane was drained extremely well without touching the protein lanes and then the membrane was placed inside two pieces of transparency plastic inside a film cassette. Film exposures for an appropriate time was performed and developed.

## **2.6. Quantitative real-time PCR (Q-PCR)**

### **2.6.1. RNA isolation**

RNA was isolated by using TRIzol reagent (cat#15596-026, life technologies). DMEM media was aspirated from each well containing cells. Trizol (1 ml/10 cm<sup>2</sup>) was pipetted to each well and incubated 5 minutes at room temperature to lyse the cells. 0.2 mL of chloroform was added to 1 ml of TRIzol used for homogenization and tube vigorously agitated by hand for 15 second and incubated for 3 minutes at room temperature and then centrifuged at 12,000 × g for 15 minutes at 4°C. The mixture was separated into a lower red interphase, and a colorless upper aqueous phase which contains the RNA. The aqueous phase was removed by pipetting the solution out and placing it into a new tube. 0.5 mL of 100% isopropanol was added to the aqueous phase, per 1 mL of TRIzol and incubated at room temperature for 10 minutes and then centrifuged at 12,000 × g for 10 minutes at 4°C. After removing the supernatant from the tube, only the RNA pellet remained which was washed with 1 ml of 75% ethanol per 1 ml of TRIzol. The tube was centrifuged at 7500 × g for 5 minutes at 4°C. The wash was then removed and the RNA pellet air dried for 5–10 minutes. The RNA pellet was re-suspended in 50 µL of RNase-free water and incubated in a water bath at 60°C for 15 minutes and then

kept on ice for use or stored at -80° C. Quantification of RNA was performed at 260nm using a Biophotometer (Eppendorf). The dilutions were made for cDNA synthesis, and then stored at -80° C.

### **2.6.2. cDNA synthesis**

cDNA was synthesis by using iScript Select cDNA Synthesis Kit (cat# 170-8897, Bio-Rad). The cDNA synthesis reaction consisted of 1µg of RNA, 4 µl of 5X iScript reaction mix, random primer 2 µl and 1 µl of iScript Reverse Transcriptase enzyme. cDNA synthesis reaction was added to a 0.2 ml to each well of a 96-well PCR reaction plate on ice. cDNA synthesis was carried out in a thermal cycler (Eppendorf, Hauppauge, NY). The cycle consisted of 25° C for 5 minutes, 42° C for 30 minutes, and 85 ° C for 5 minutes, and 4° C hold. After cDNA synthesis, each sample of the cDNA was diluted with DNase/RNase-free water and stored at -20° C for later usage for real time PCR.

### **2.6.3. Real time PCR**

All primers used are listed in table 2.1. Expression levels of target genes were normalized by concurrent measurement of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Quantification was performed by real-time PCR as described before (Pfaffl, 2001). Briefly, cDNA samples were amplified in 4 well replicates and a water negative control was included in each PCR run. Each PCR reaction was composed of cDNA, 1X SSoAdvanced SYBR Green Supermix of upstream primer, and the same amount of downstream primer. The real-time PCR was performed in Mastercycler EP Realplex (Eppendorf, Westbury, NY) with the initial denaturation, 1X, 95° C for 1.5 min, followed by 40 cycles of denaturation for 15 seconds, 95° C, annealing for 30 seconds,

60° C, elongation for 30 seconds, 72° C, followed by 10 min final extension at 72° C, and a melt curve using 80 cycles of 0.5° C increments starting at 60°C.

## **2.7. Immunofluorescence and confocal microscopic analysis**

Raw 264.7 cells cultured on Lab-Tek Chamber Slides (Thermo Scientific) were treated by ginseng crude, hexane or panaxynol for 1 h, rinsed with PBS at 37° C to remove the media, and then fixed with 4% paraformaldehyde at room temperature for 15 min. Immunofluorescence staining was performed with rabbit anti-Nrf2 (sc-13032, Santa Cruz Biotechnology, Inc.) antibodies. Nuclei were labeled using blue dye of 40, 6-diamidino-2-phenylindole (DAPI, Sigma–Aldrich). F-actin was labeled using green dye of Alexa Fluor 488 phalloidin (Invitrogen) as previously described (Li et al., 2009a). Briefly, after fixation the samples were rinsed in buffer. Permeabilize the cells in 0.2% Triton X-100 in buffer for 5 minutes, and rinsed again. The slides were then incubated with 10% Goat serum blocking solution for 1 hour to block non-specific binding of immunoglobulin. Slides were incubated with primary antibody (as specified above) in Goat serum blocking solution overnight at 4° C. Rinsed 3 times with PBS and incubated with goat anti-rabbit deligt 549 (secondary antibody) in Goat serum blocking solution for 1h at room temperature. They were then rinsed in PBS for 3min. and protected from light by covering slides with aluminum foil. Nuclei were stained with 1ml of 5 µg/ml 4, 6-diamidino-2-phenylindole (DAPI) per well for 15 minutes. Cells were washed twice in PBS per well for 15 minutes. One drop of 1, 4-diazabicyclo [2.2.2] octane (DABCO) solution was dropped on the middle of the slide, and slowly pulling the coverslip down to the slide to avoid bubbles. Images were acquired using a confocal microscope (LSM510META, Carl Zeiss Inc.).

## **2.8. Statistics**

Values are expressed as mean  $\pm$  SD in the text and figures. The data were analyzed using ANOVA with the Newman-Keuls' test unless specified. Values of P  $<0.05$  were considered to be statistically significant.

**Table 2.1. A summary of primers for Q-PCR analysis.**

Gene	Gene access #	Sense primer	Antisense primer	PCR product size (bp)
iNOS	NM_010927.3	5'- GGTCCGCAAGAG AGTGCTGT-3'	5' - GCACGCTGAGTA CCTCATTGG -3'	119
MCP-1	NM_011333.3	5'- GCCCCACTCACC TGCTGCTA-3'	5'- TTACGGGTCAAC TTCACATTCAAA- 3'	278
MIP-1 $\beta$	NM_013652.2	5'- CCAGCTCTGTGC AAACCTAACC-3'	5'- GCCGGGAGGTGT AAGAGAAAC-3'	158
TNF- $\alpha$	NM_013693.3	5' - GCCACCACGCTC TTCTGTCTAC-3'	5' - GGGTCTGGGCCA TAGAACTGAT-3'	103
IL-1 $\beta$	NM_008361.3	5' - ACCTTCCAGGAT GAGGACATGA-3'	5' - CTAATGGGAACG TCACACACCA-3'	116
IL-10	NM_010548.2	5' - GTTGCCAAGCCT TATCGGAA-3'	5'- GCTCCACTGCCT TGCTCTTAT-3'	116
IL-6	NM_031168.1	5' - CACATGTTCTCT GGGAAATCG-3'	5' - TTGTATCTCTGG AAGTTTCAGATT GTT-3'	116
NQO1	NM_008706.5	5'- CGGTATTACGAT CCTCCCTCAACA- 3'	5' - AGCCTCTACAGC AGCCTCCTTCAT- 3'	120
TGF- $\beta$ R1	NM_00127725 5.1	5'- GCATTGGCAAAG GTCGGTTT-3'	5'- TGCCTCTCGGAA CCATGAAC-3'	103
HO-1	NM_010442.2	5' - AGGAGATAGAG CGCAACAAGCAG A-3'	5' - CCAGTGAGGCC ATACCAGAAG-3'	116
GAPDH	NM_008084.2	5'- ATGTTCCAGTAT GACTCCACTCAC G-3'	5'- GAAGACACCAGT AGACTCCACGAC A-3'	171

## CHAPTER 3

### RESULTS

#### **3.1. Cell viability of RAW264.7 macrophages in response to American ginseng crude, ginseng fractions**

To examine cell viability of RAW264.7 macrophages in response to American ginseng crude and ginseng fractions, cells were cultured in serum free or 2% FBS DMEM medium and treated with different doses of ginseng crude, hexane fraction, water fraction, butanol fraction, dichloromethane fraction, ethyl acetate fraction and maltodextrin at 0, 10, 50, 100, 500, 1000  $\mu\text{g/ml}$ . Cell viability was determined by a LDH assay kit. We found that about 80% of RAW264.7 macrophages survived at 2% FBS DMEM medium of crude, hexane and water. For butanol, 80% of RAW264.7 cells survived up to the dose 500  $\mu\text{g/ml}$ . For dichloromethane, ethyl acetate and maltodextrin, 80% of RAW264.7 cells survived up to the dose 1000  $\mu\text{g/ml}$  (figure 3.2). However, cells viability was only 75% when cultured in serum free DMEM medium, and cells viability was decreased to about 65% at dose of 100  $\mu\text{g/ml}$  of crude, hexane, water and butanol when cells were cultured in serum free DMEM medium (figure 3.1). Thus, we used ginseng crude and fractions within a range of non-cytotoxic doses (100  $\mu\text{g/ml}$  for crude, hexane, water, butanol, dichloromethane and ethyl acetate) and 2% FBS DMEM medium in our experiments. Maltodextrin was used during the process of fractionation of ginseng, and we tested it to ensure that it had no toxic effects on RAW264.7 macrophages. We

found that 80% of RAW264.7 macrophages survived at 2% FBS DMEM medium when treated with maltodextrin up to the dose of 1000 µg/ml.

### **3.2. Effect of American ginseng crude and ginseng fractions on LPS-induced iNOS and MCP-1 $\beta$ expression in RAW 264.7 macrophages**

We hypothesized that some of Ginseng sub-fractions could suppress the inflammatory response in macrophages and we used the expression of iNOS and MCP-1 $\beta$  to reflect the pro-inflammatory status of LPS-inflamed RAW 264.7 macrophages as described above in the introduction. RAW 264.7 macrophages were stimulated with LPS 1µg/ml and ginseng crude 100µg/ml or butanol fraction 100µg/ml or hexane fraction 100µg/ml or water fraction 100µg/ml or dichloromethane fraction 100µg/ml or ethyl acetate fraction 100µg/ml or maltodextrin 100µg/ml for 6 hours as indicated and protein levels of iNOS were determined by Western blot as in figure 3.3. We choose the time point 6 hours to extract the iNOS protein because previous experiments in our laboratory indicated; LPS stimulated RAW 264.7 macrophages produced maximum level of iNOS at this time point (data not shown). Maltodextrin was used during the process of fractionation of ginseng, and we tested it to ensure that it had no effects on iNOS. There was a minimal expression of iNOS protein in LPS-unstimulated RAW 264.7 cells and maximal expression in cells treated with LPS appeared at 6 h. Ginseng crude and hexane fraction can inhibit LPS-induced iNOS protein expression, while butanol fraction, water fraction, dichloromethane fraction and ethyl acetate fraction have no effects on LPS-induced iNOS protein expression in RAW 264.7 macrophages as shown in figure 3.3. For Q-PCR we performed mRNA level analysis only for cells treated with Ginseng crude and hexane as they showed effects on iNOS at the protein level. RAW 264.7 macrophages

were stimulated with LPS, Ginseng crude and hexane for 4h as indicated in figure 3.4 and then mRNA levels of iNOS were determined by Q-PCR. There was minimal expression of iNOS mRNA in unstimulated RAW 264.7 cells and maximal expression in cells treated with LPS for 4 h. Ginseng crude and hexane fraction can inhibit iNOS expression at mRNA level in LPS-induced RAW 264.7 macrophages (figure 3.4), which confirm western blot results. American ginseng crude alone slightly increased the expression of iNOS mRNA as shown in figure 3.6. We conclude that, Ginseng crude and hexane potently suppress LPS-induced iNOS expression at both the mRNA and protein levels.

We focused on hexane as a potential novel compound from American ginseng that potently suppresses inflammation. To determine the effects of crude and hexane on expression of MCP-1 $\beta$ , RAW 264.7 macrophages were stimulated with LPS 1 $\mu$ g/ml and ginseng crude 100 $\mu$ g/ml or hexane fraction 100 $\mu$ g/ml for 4 hours as indicated in figure 3.5 and then mRNA levels of iNOS were determined by Q-PCR. There was minimal expression of MCP-1 $\beta$  mRNA in unstimulated RAW 264.7 cells and maximal expression in cells treated with LPS for 4 h. Ginseng crude and hexane fraction inhibited MCP-1 $\beta$  expression at mRNA level in LPS-induced RAW 264.7 macrophages (figure 3.5). American ginseng crude alone slightly increased the expression of MCP-1 $\beta$  mRNA. We conclude that, Ginseng crude and hexane potently suppresses LPS-induced iNOS expression at both the mRNA and protein levels and MCP-1 $\beta$  expression at mRNA levels which indicates that hexane is the most potent inflammatory suppressor of the ginseng sub-fractions.



### **3.3. Isolation of panaxynol from American ginseng hexane sub-fraction.**

To further purify hexane and isolate a single compound, by utilizing preparative, reverse-phase High-performance liquid chromatography (HPLC) and a comparative analysis by analytical scale Ultra-violet liquid chromatography (LC-UV), we found that hexane fraction contains predominantly polyacetylenes and linolenic acid. Moreover, we identified panaxynol as a major compound of polyacetylenes (fig. 3.6). Panaxynol was isolated and prepared by National Research Council of Canada, Institute for National Measurement Standards.

### **3.4. Cell viability of RAW264.7 macrophages in response to panaxynol**

To examine cell viability of RAW264.7 macrophages in response to panaxynol, cells were cultured in serum free or 2% FBS DMEM medium and treated with different doses of panaxynol at 0, 1, 10, 100, 500 nM and 1, 5  $\mu$ M for 24h. Cells viability was determined by LDH assay kit. We find that 80% of RAW264.7 macrophages survived at 2% FBS DMEM medium up to the dose of 1 $\mu$ M (figure 3.8). The cells viability was decreased to about 70% at dose of 500 nM of panaxynol when cells were cultured in serum free DMEM medium (figure 3.7). Thus, we used panaxynol within a range of non-cytotoxic doses (500 nM) and 2% FBS DMEM medium in our experiments.

### **3.5. Effect of panaxynol on LPS-induced iNOS and MCP-1 $\beta$ expression in RAW 264.7 macrophages**

To determine if panaxynol is the active anti-inflammatory compound in hexane, we used the expression of iNOS and MCP-1 $\beta$  as indicators of the pro-inflammatory status of LPS-inflamed RAW 264.7 macrophages as described above in the introduction. RAW 264.7 macrophages were stimulated with LPS 1 $\mu$ g/ml and ginseng crude 100 $\mu$ g/ml or

panaxynol 500nM for 6 hours and protein levels of iNOS were determined by Western blot as in figure 3.9. There was minimal expression of iNOS protein in unstimulated RAW 264.7 cells and maximal expression in cells treated with LPS for 6 h. Panaxynol inhibited LPS-induced iNOS protein expression. For Q-PCR, there was minimal expression of iNOS and MCP-1 $\beta$  mRNA in unstimulated RAW 264.7 cells and maximal expression in cells treated with LPS for 4 h. Panaxynol inhibited iNOS and MCP-1 $\beta$  expression at the mRNA level in LPS-induced RAW 264.7 macrophages (figure 3.10. and 3.11.). We conclude that panaxynol potently suppresses LPS-induced iNOS and MCP-1 $\beta$  expression.

### **3.6. Effect of American ginseng crude, hexane and panaxynol on a selected set of pro-inflammatory factors in RAW 264.7 macrophages.**

In addition to iNOS and MCP-1 $\beta$ , we investigated crude, hexane and panaxynol on other pro-inflammatory cytokines namely; TGF- $\beta$ R1, IL-1 $\beta$ , IL-6, and TNF- $\alpha$  in LPS-inflamed RAW264.7 macrophages. LPS suppressed TGF- $\beta$ R1 at the mRNA level, but crude, hexane and panaxynol, all stimulate the expression of TGF- $\beta$ R1 at the mRNA level, and reverse the suppression induced by LPS(FIG. 2.12.). But crude, hexane and panaxynol had no effect on the expression of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  at the mRNA level (figs. 3.13, 3.14 and 3.15). These results show a unique anti-inflammatory profile of hexane and panaxynol in macrophages which may indicate activation of specific pathway.

### **3.7 Effect of American ginseng crude, hexane and panaxynol on NF- $\kappa$ B pathway in RAW 264.7 macrophages**

LPS activated the NF- $\kappa$ B pathway by enhancing I $\kappa$ B degradation and the subsequent release and activation of NF- $\kappa$ B transcriptional activity in RAW264.7

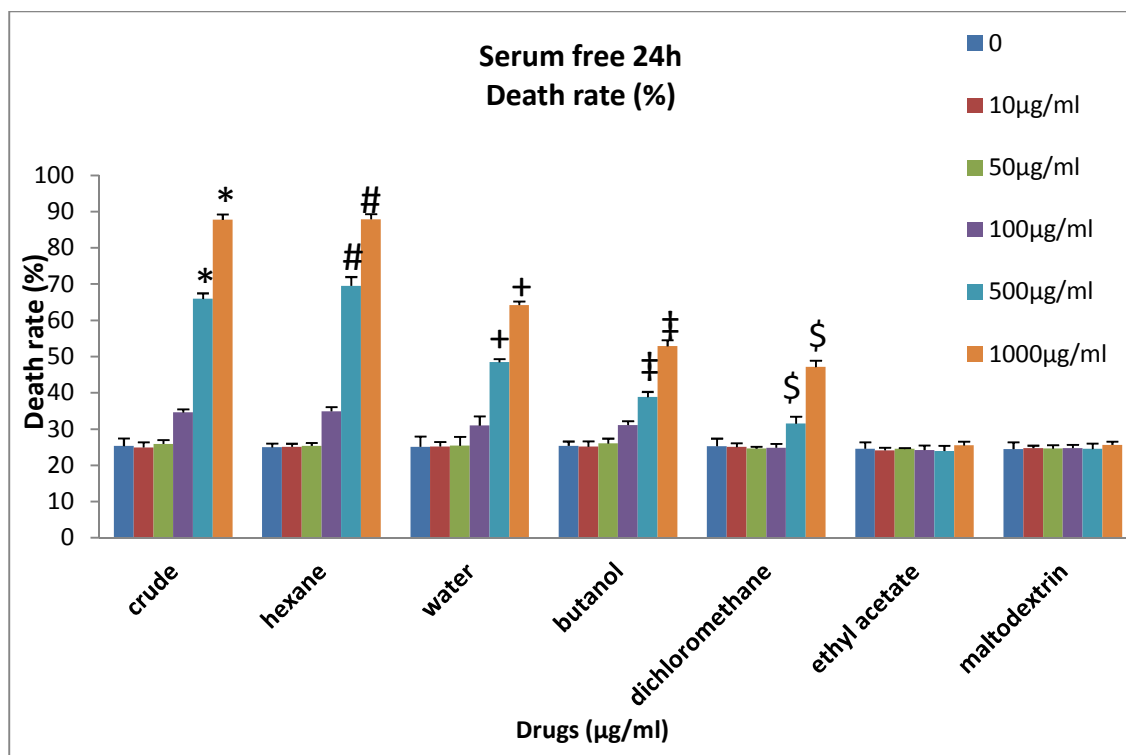
macrophages. Ginseng crude (100µg/ml), hexane (100µg/ml) and panaxynol (500nM) were unable to prevent degradation of IκB and hence, the activation of the NF-κB pathway in the inflamed macrophages as seen by Western blot analysis in figure 3.16. Also, Ginseng crude, hexane and panaxynol were unable to inhibit the cytokines that are produced by NF-κB pathway activation, namely, IL-1β, IL-6 and TNFα (figs. 3.13, 3.14 and 3.15). These results indicate that Ginseng crude, hexane and panaxynol inhibits pro-inflammatory responses in macrophages by mechanisms other than the NF-κB signaling pathway.

### **3.8 Effect of American ginseng crude, hexane and panaxynol on Nrf2 pathway in RAW 264.7 macrophages**

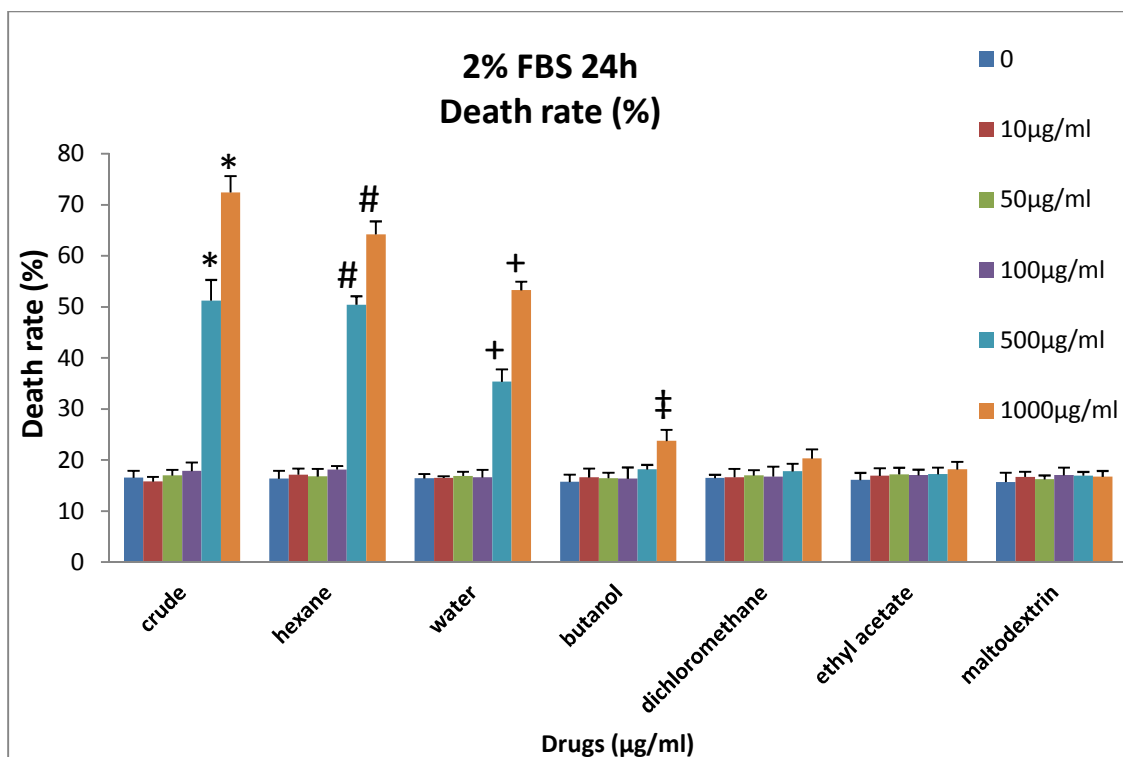
Western blot analysis indicated that treatment of RAW264.7 macrophages with ginseng crude 100µg/ml rapidly up-regulated Nrf2 protein expression that was sustained for at least 6 h (Fig. 3.17 upper row). Also, Nrf2 downstream genes, namely NAD(P)H:quinone oxidoreductase (NQO-1) and heme oxygenase 1 (HO-1) were induced by ginseng crude as a consequence of Nrf2 activation. Protein levels of NQO-1 and HO-1 were dramatically up-regulated by ginseng crude (fig 3.17 middle and lower rows). The increase of NQO-1 and HO-1 at the mRNA levels were also confirmed by Q-PCR (Fig. 3.20 and 3.21). This result suggested that American ginseng activates Nrf2 in macrophages. Hexane 100µg/ml and panaxynol 500 nM showed the same effects as ginseng crude on up-regulation of Nrf2, NQO-1 and HO-1 protein expression (fig. 3.18 and 3.19 respectively). Also, hexane and panaxynol increase expression of NQO-1 and HO-1 at the mRNA level (fig. 3.22 and 3.23 for hexane; fig. 3.24 and 3.25 for panaxynol). Immunofluorescence staining of Nrf2 and confocal microscopic analysis

revealed that crude, hexane and panaxynol up regulated Nrf2 protein translocation into nuclei (fig. 3.26, 3.27 and 3.28). Taken together, these results clearly demonstrate that panaxynol up-regulate Nrf2 protein levels, Nrf2 translocation into nuclei and activate Nrf2-driven expression of a group of anti-oxidative genes namely; HO-1 and NQO-1 in RAW264.7 macrophages.

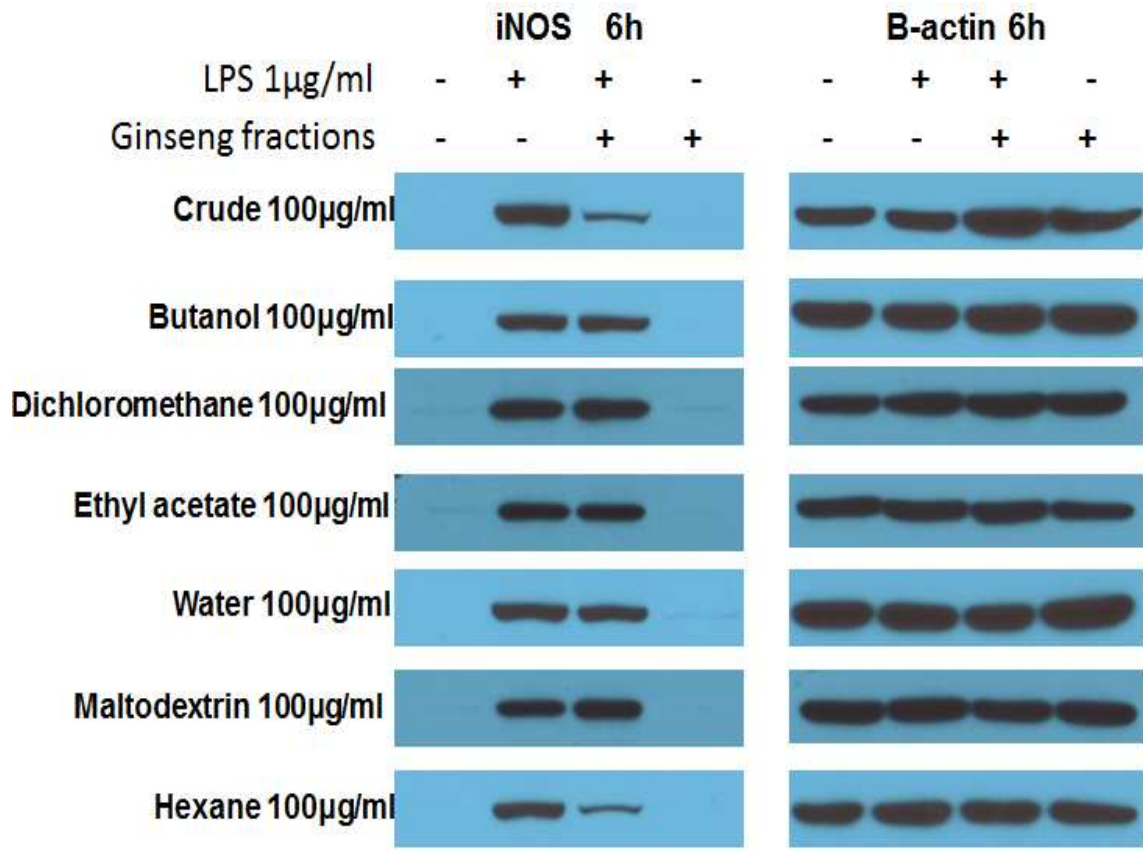
Finally, we explored if Nrf2 is the mediator by which ginseng crude, hexane and panaxynol induce anti-inflammation effects in macrophages by using the primary cultures of macrophages from wild type (WT) and Nrf2<sup>-/-</sup> mice. Figure 3.29, 3.30 and 3.31 show that ginseng crude, hexane and panaxynol dramatically inhibited LPS-induced expression of iNOS in primary macrophages from WT at 6h. However, ginseng crude, hexane and panaxynol were unable to block iNOS expression in LPS- inflamed macrophages from Nrf2<sup>-/-</sup> mice. All of the above results collectively clearly indicate that Nrf2 is an essential mediator for panaxynol to suppress inflammatory responses in inflamed macrophages.



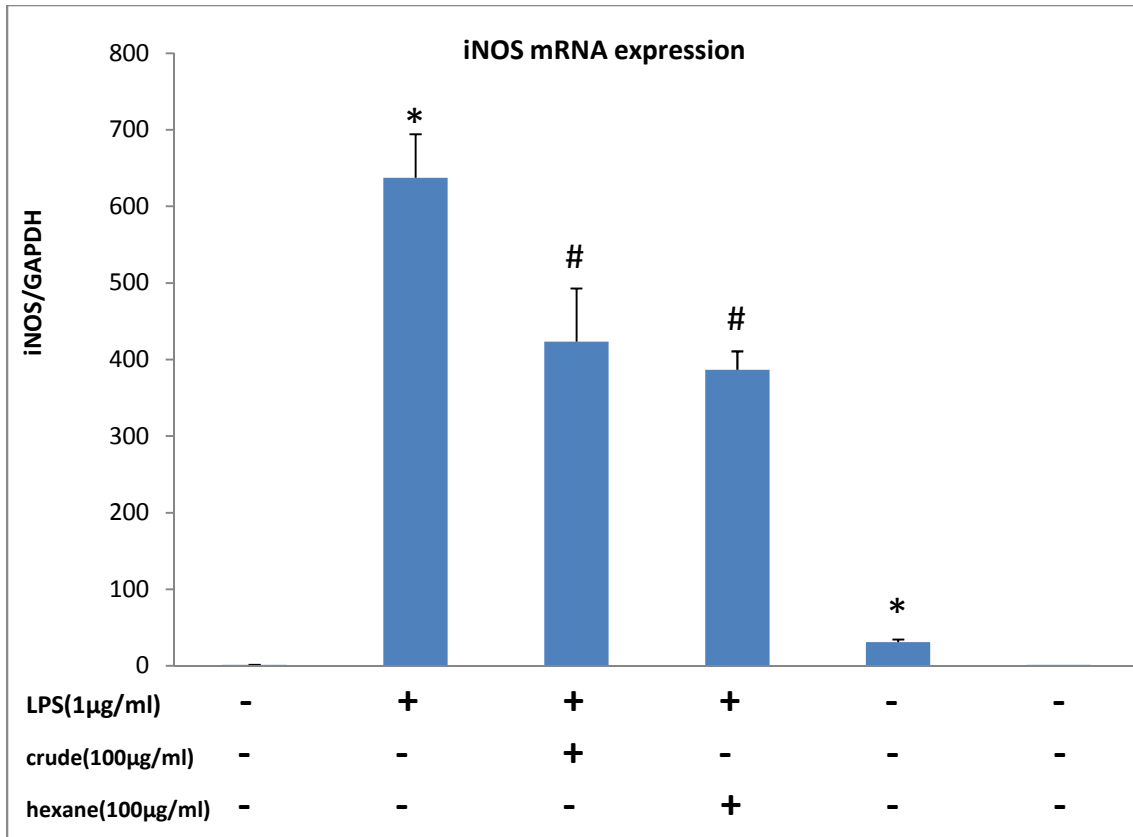
**Figure 3.1. Cytotoxicity of ginseng crude, hexane fraction, water fraction, butanol fraction, dichloromethane fraction, ethyl acetate fraction and maltodextrin in RAW264.7 macrophages cultured in serum free DMEM.** Raw 264.7 cells were cultured in 24-well plate in 10% FBS DMEM medium for 24h, changed to serum free DMEM medium and stimulated with different doses of drugs (0, 10, 50, 100, 500, 1000 µg/ml) for 24h. Cell viability was determined by LDH assay kit. n=4, \*p<0.05 vs. control (crude 0µg), #p<0.05 vs. control (hexane 0µg), + p<0.05 vs. control (water 0µg), ‡ p<0.05 vs. control (butanol 0µg), \$ <0.05 vs. control (dichloromethane 0µg).



**Figure 3.2. Cytotoxicity of ginseng crude, hexane fraction, water fraction, butanol fraction, dichloromethane fraction, ethyl acetate fraction and maltodextrin in RAW264.7 macrophages cultured in 2% FBS DMEM.** Raw 264.7 cells were cultured in 24-well plate in 10% FBS DMEM medium for 24h, changed to 2% FBS DMEM medium and stimulated with different doses of drugs (0, 10, 50, 100, 500, 1000 µg/ml) for 24h. Cell viability was determined by LDH assay kit. n=4, \*p<0.05 vs. control (crude 0µg), #p<0.05 vs. control (hexane 0µg), + p<0.05 vs. control (water 0µg), ‡ p<0.05 vs. control (butanol 0µg).

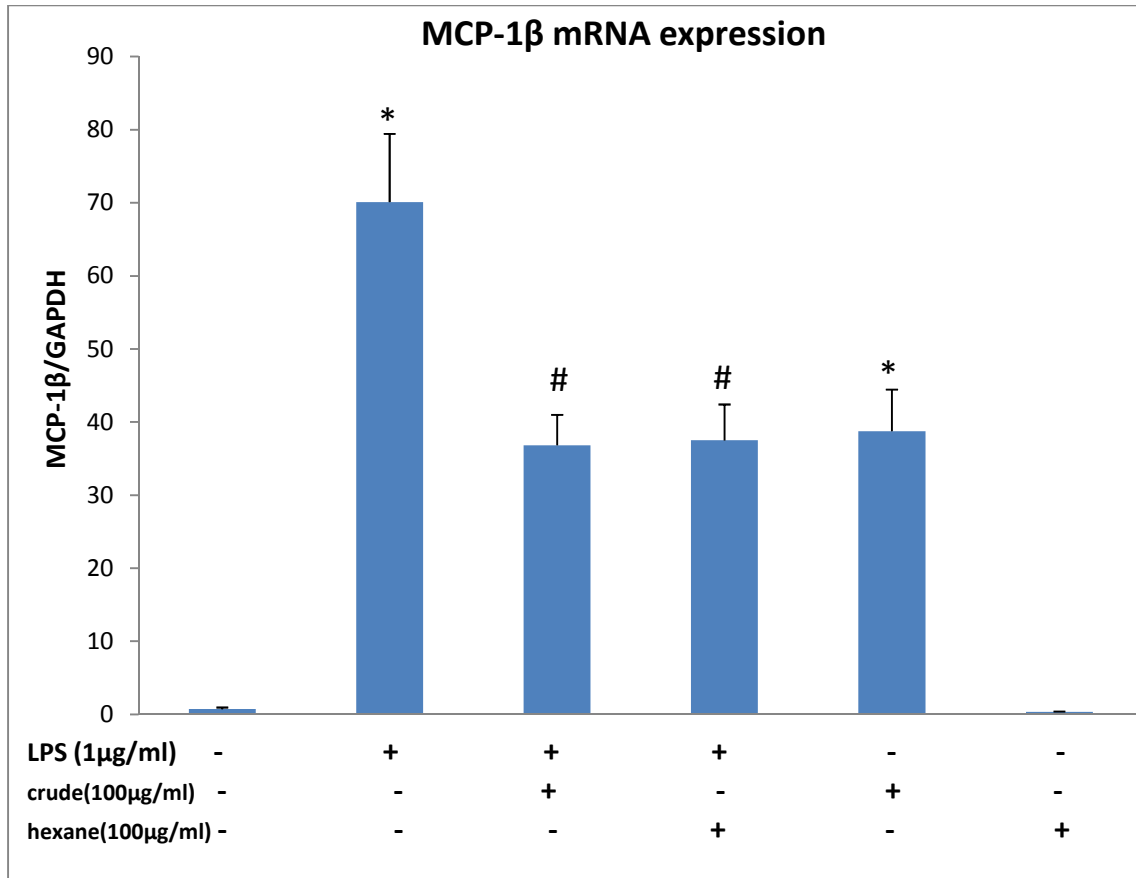


**Figure 3.3. Western blot analyses of iNOS and  $\beta$ -actin proteins. Effect of ginseng crude, butanol fraction, hexane fraction, water fraction, dichlore methane fraction, ethyl acetate fraction and maltodextrin on expression of iNOS protein in inflamed RAW264.7 macrophages.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM and stimulated with or without LPS 1µg/ml and ginseng crude or butanol or hexane or water or dichlore methane or ethyl acetate or maltodextrin (dose 100µg/ml) as indicated for 6h. Protein levels of iNOS were determined by Western blot. Load protein is 20µg/well. Results are representative of three independent experiments.

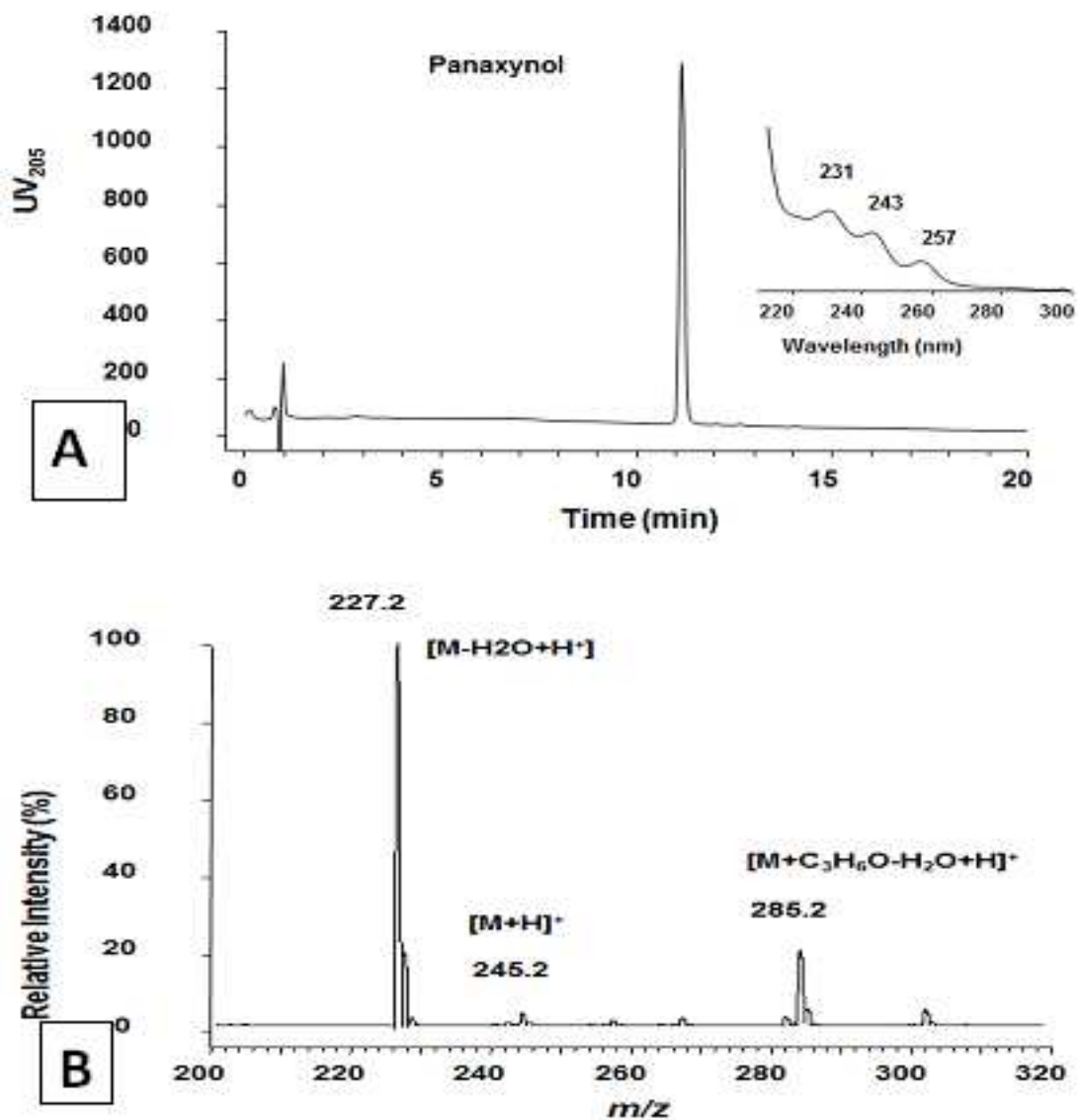


**Figure 3.4. Quantitative analysis of mRNA levels of iNOS by Q-PCR . Effect of ginseng crude and hexan on LPS-induced expression of iNOS in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1µg/ml, ginseng crude and hexane (dose 100µg/ml) as indicated for 4h. mRNA levels of iNOS were determined by Q-PCR. n=4, \*p < 0.05 vs. control (-), #p < 0.05 vs. LPS (1µg/ml).

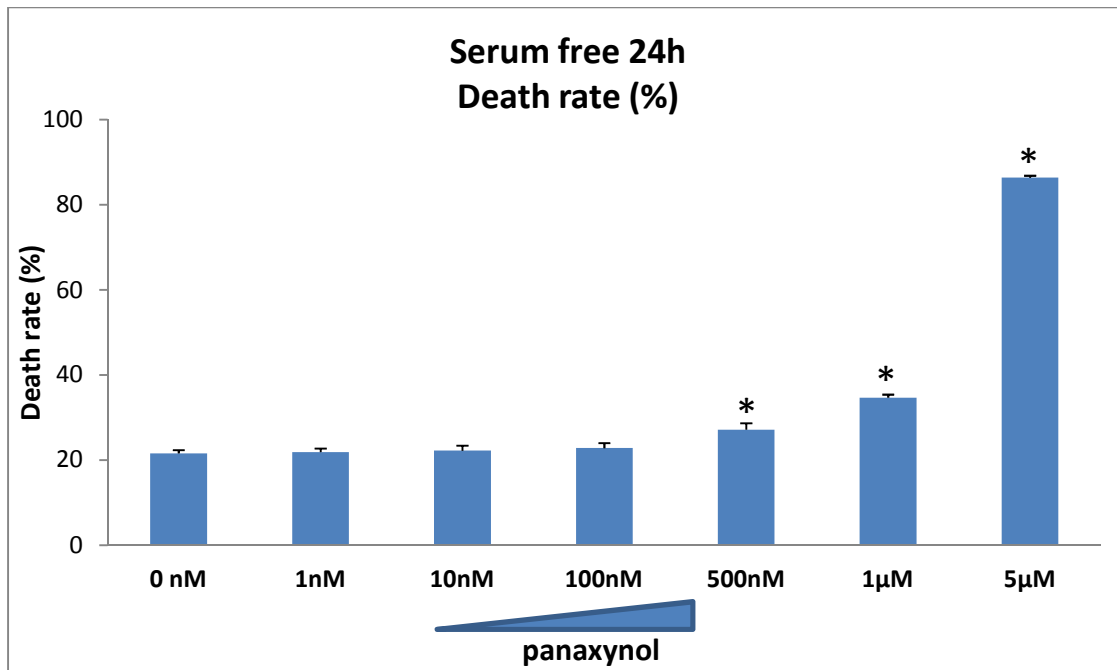




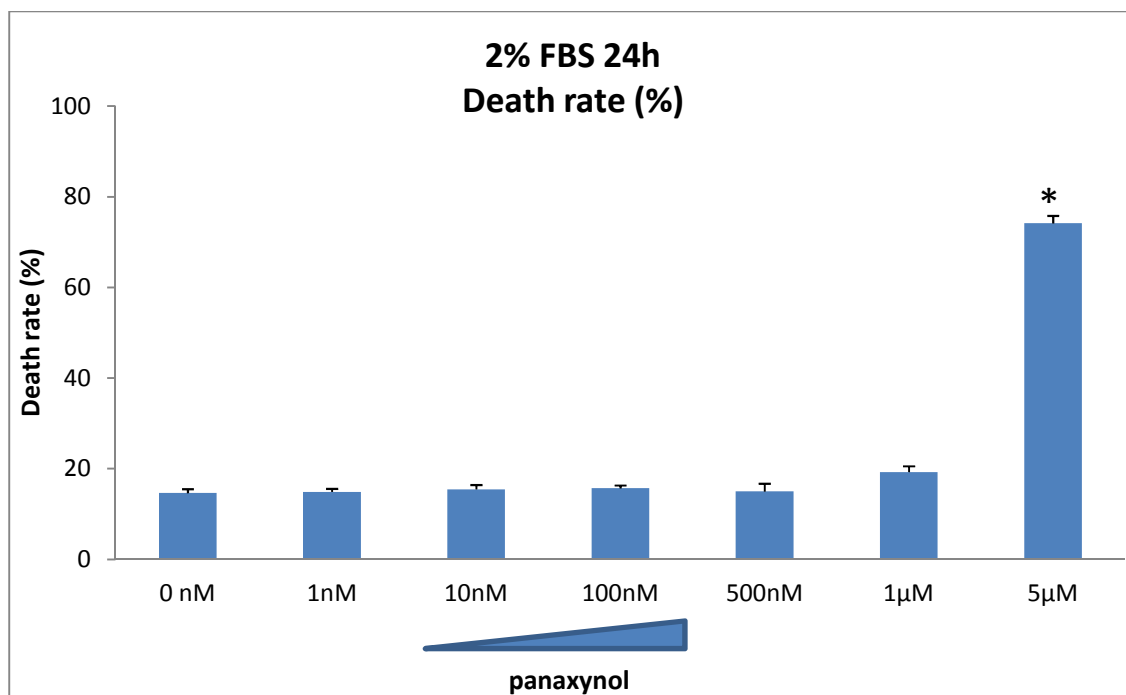
**Figure 3.5. Quantitative analysis of mRNA levels of MCP-1 $\beta$  by Q-PCR . Effect of ginseng crude, hexane and panaxynol on LPS-induced expression of MCP-1 $\beta$  in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1 $\mu$ g/ml, ginseng crude, hexane (dose 100 $\mu$ g/ml) and panaxynol 500nM as indicated for 4h. mRNA levels of MCP-1 $\beta$  were determined by Q-PCR. n=4, \*p < 0.05 vs. control (-), #p < 0.05 vs. LPS (1 $\mu$ g/ml).



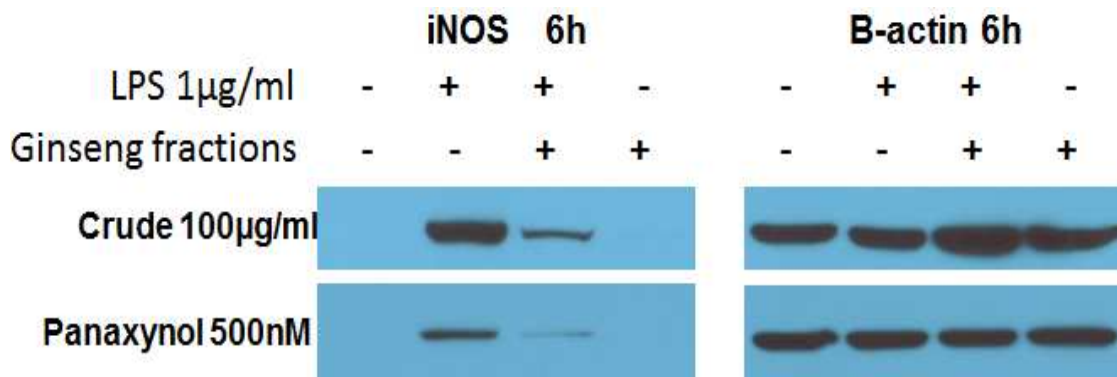
**Figure 3.6. Isolation of panaxynol from American ginseng hexane sub-fraction. (A).** Ultra-violet liquid chromatography (LC-UV) DAD analysis of purified panaxynol. **(B).** liquid chromatography-mass spectrometry (LC-MS) analysis of purified panaxynol.



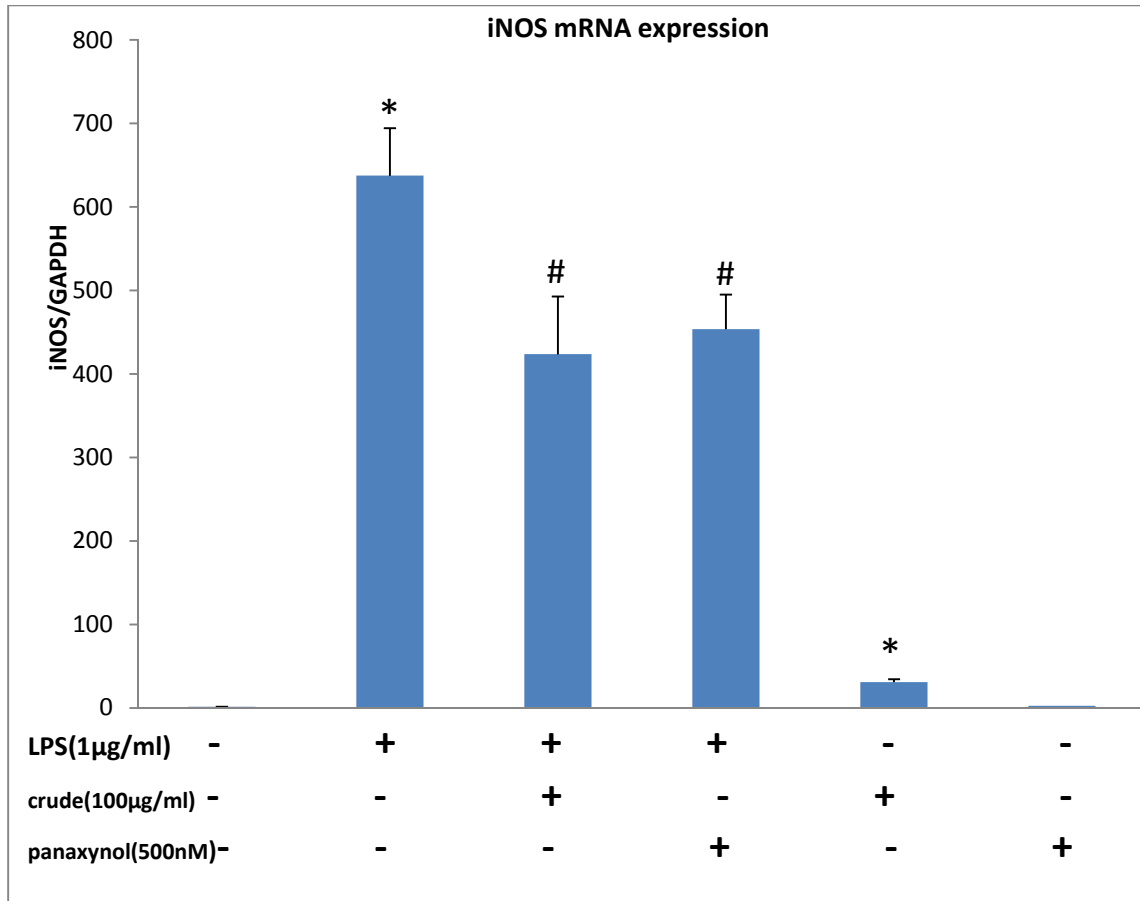
**Figure 3.7. Cytotoxicity of panaxynol in RAW264.7 macrophages were cultured in serum free DMEM medium.** Raw 264.7 cells were cultured in 24-well plate in 10% FBS DMEM medium for 24h, changed to serum free DMEM medium and stimulated with different doses of panaxynol (0, 1, 10, 100, 500 nM and 1, 5 μM) for 24h. Cell viability was determined by LDH assay kit. n=4, \*p<0.05 vs. control (0 nM).



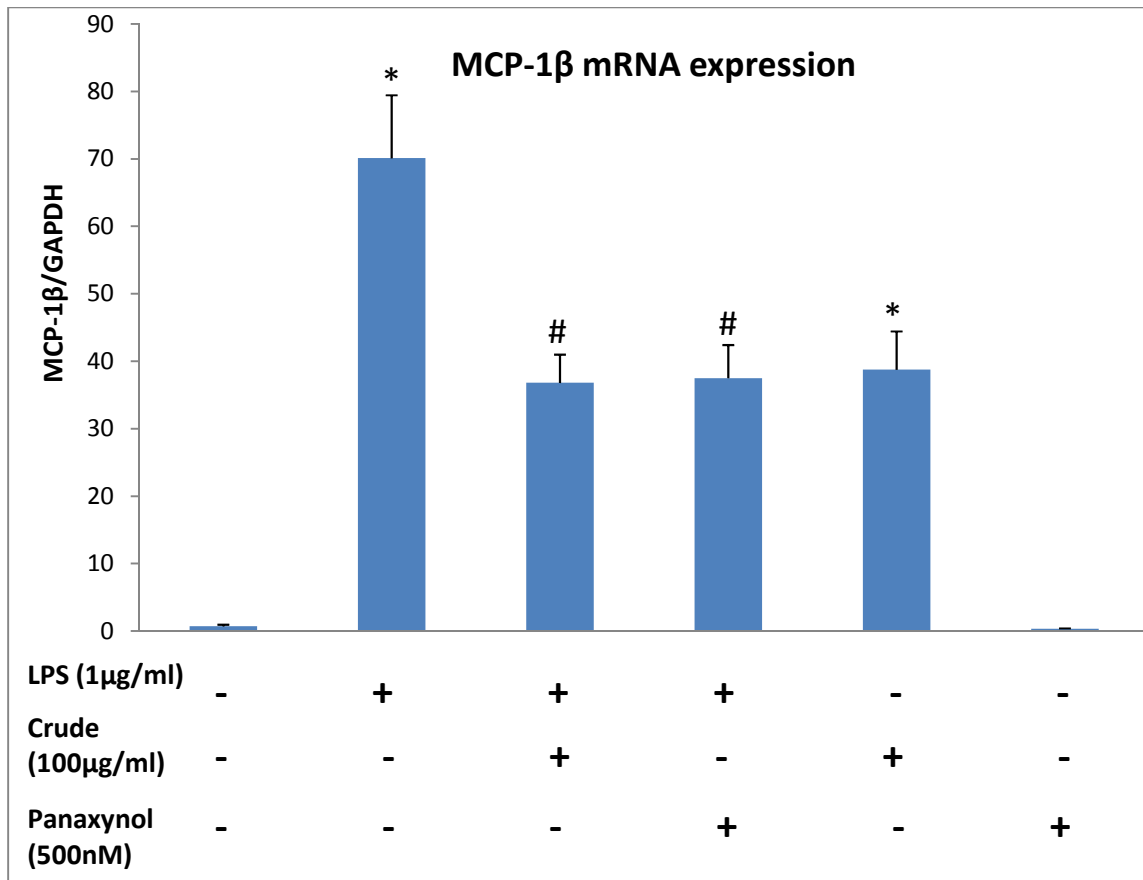
**Figure 3.8. Cytotoxicity of panaxynol in RAW264.7 macrophages cultured in 2% FBS DMEM.** Raw 264.7 cells were cultured in 24-well plate in 10% FBS DMEM medium for 24h, changed to 2% FBS DMEM medium and stimulated with different doses of panaxynol (0, 1, 10, 100, 500 nM and 1, 5 μM) for 24h. Cell viability was determined by LDH assay kit . n=4, \*p<0.05 vs. control (0 nM).



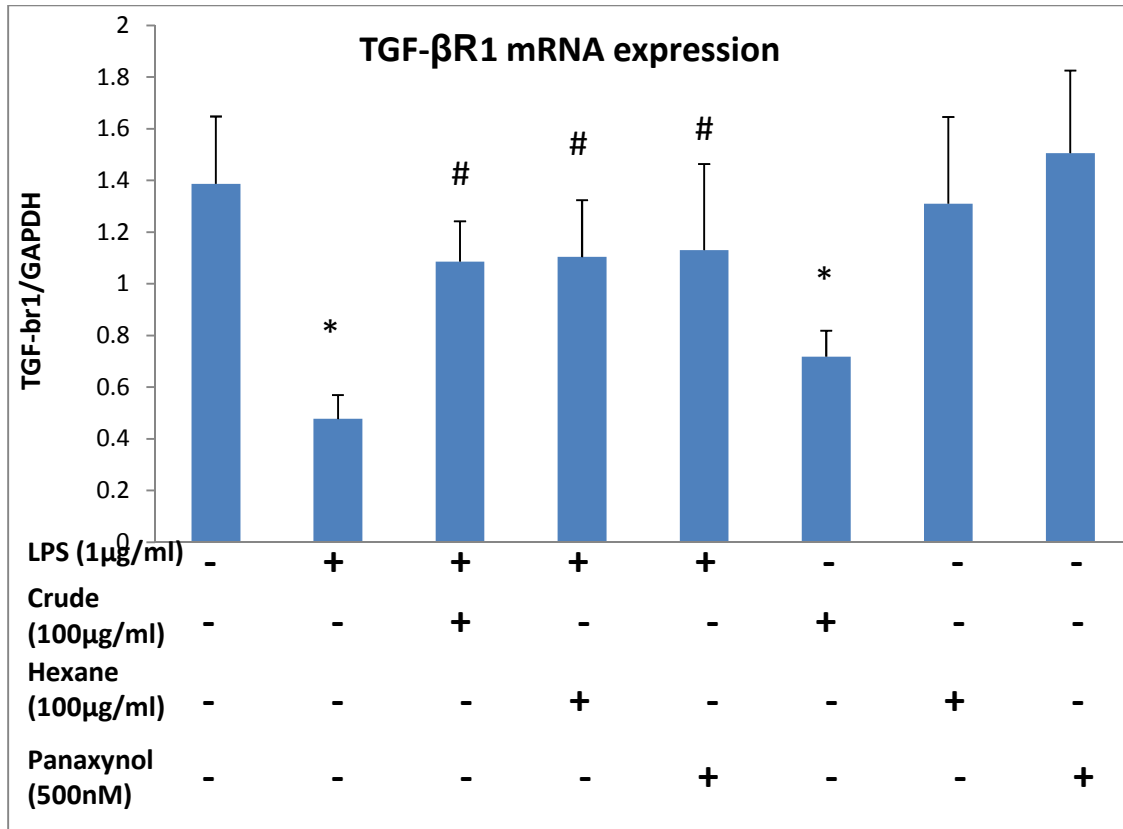
**Figure 3.9. Western blot analyses of iNOS and  $\beta$ -actin proteins. Effect of panaxynol on expression of iNOS protein in inflamed RAW264.7 macrophages.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM and stimulated with or without LPS 1 $\mu$ g/ml, ginseng crude 100 $\mu$ g/ml and panaxynol 500nM as indicated for 6h. Protein levels of iNOS were determined by Western blot. Load protein is 20 $\mu$ g/well. Results are representative of three independent experiments.



**Figure 3.10. Quantitative analysis of mRNA levels of iNOS by Q-PCR . Effect of ginseng crude and panaxynol on LPS-induced expression of iNOS in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1µg/ml, ginseng crude 100µg/ml and panaxynol 500nM as indicated for 4h. mRNA levels of iNOS were determined by Q-PCR. n=4, \*p < 0.05 vs. control (-), #p < 0.05 vs. LPS (1µg/ml).

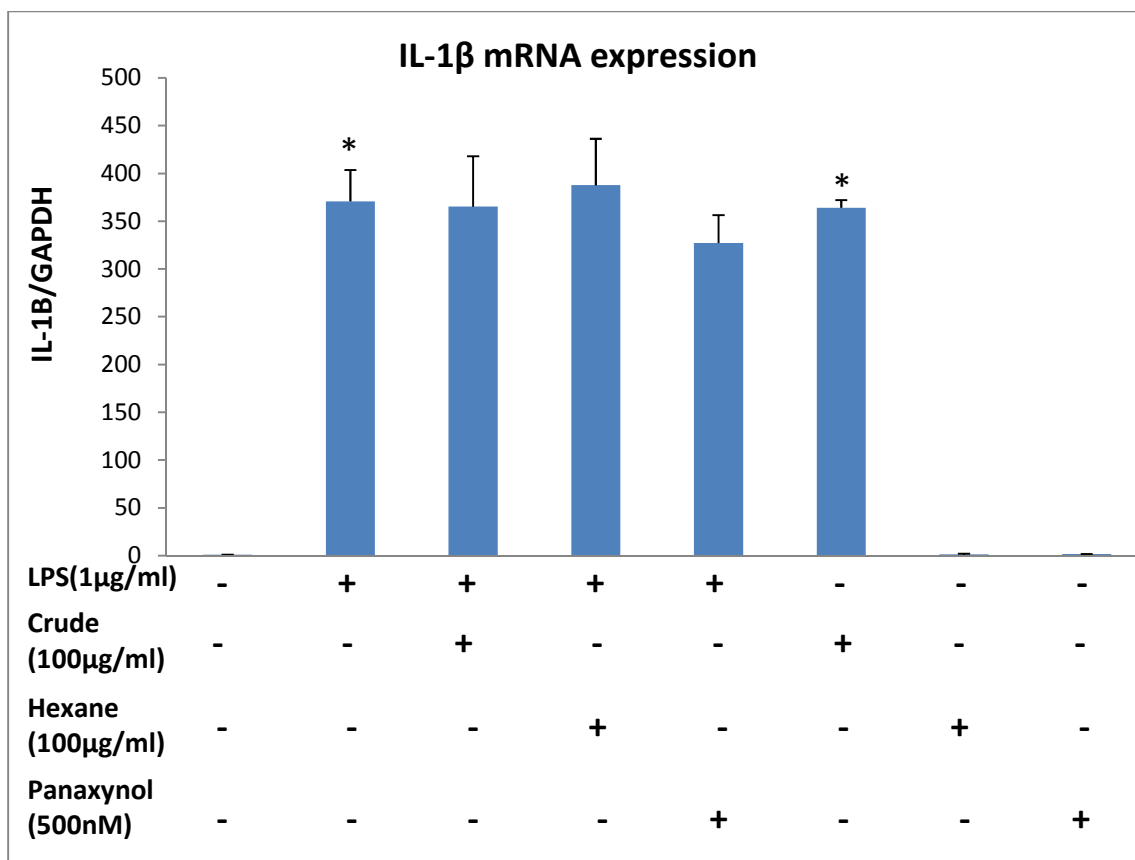


**Figure 3.11. Quantitative analysis of mRNA levels of MCP-1 $\beta$  by Q-PCR . Effect of ginseng crude and panaxynol on LPS-induced expression of MCP-1 $\beta$  in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1 $\mu$ g/ml, ginseng crude 100 $\mu$ g/ml and panaxynol 500nM as indicated for 4h. mRNA levels of MCP-1 $\beta$  were determined by Q-PCR. n=4, \*p < 0.05 vs. control (-), #p < 0.05 vs. LPS (1 $\mu$ g/ml).

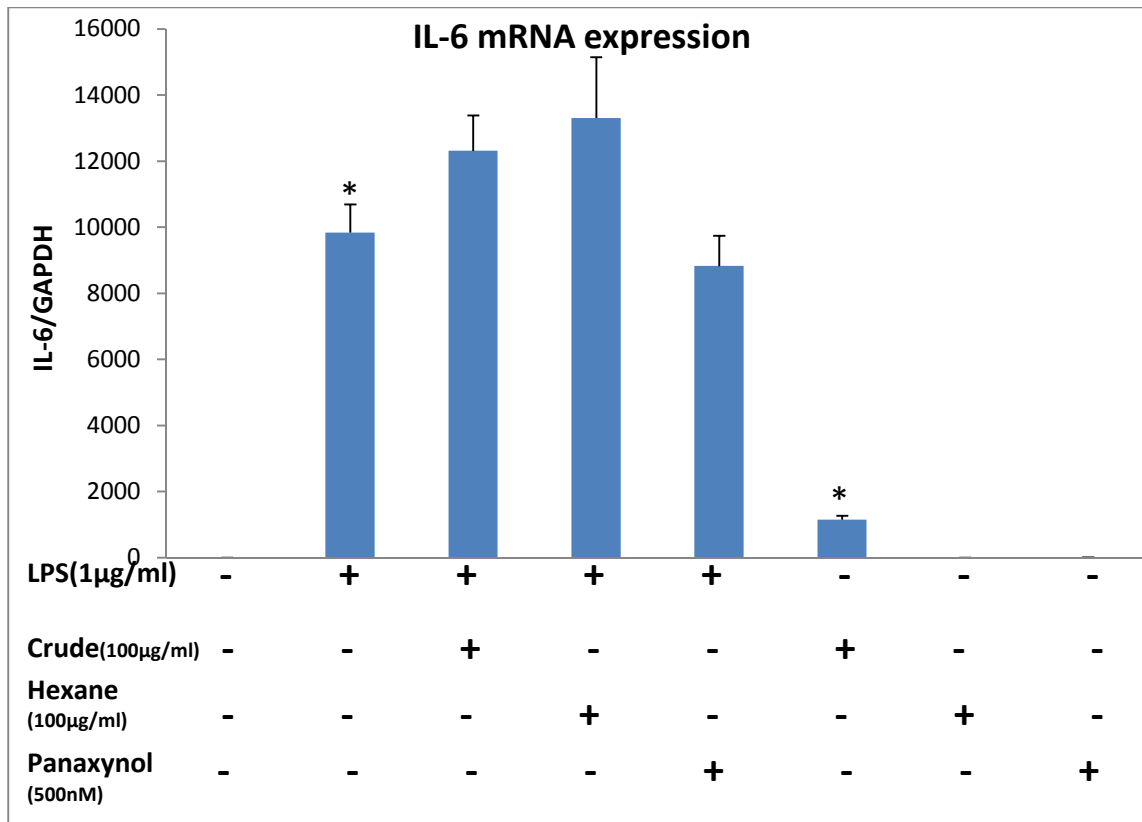


**Figure 3.12. Quantitative analysis of mRNA levels of TGF-βR1 by Q-PCR . Effect of ginseng crude, hexane and panaxynol on LPS-induced expression of TGF-βR1 in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1μg/ml, ginseng crude, hexane (dose100μg/ml) and panaxynol 500nM as indicated for 4h. mRNA levels of TGF-βR1 were determined by Q-PCR. n=4, \*p < 0.05 vs. control (-), #p < 0.05 vs. LPS (1μg/ml).

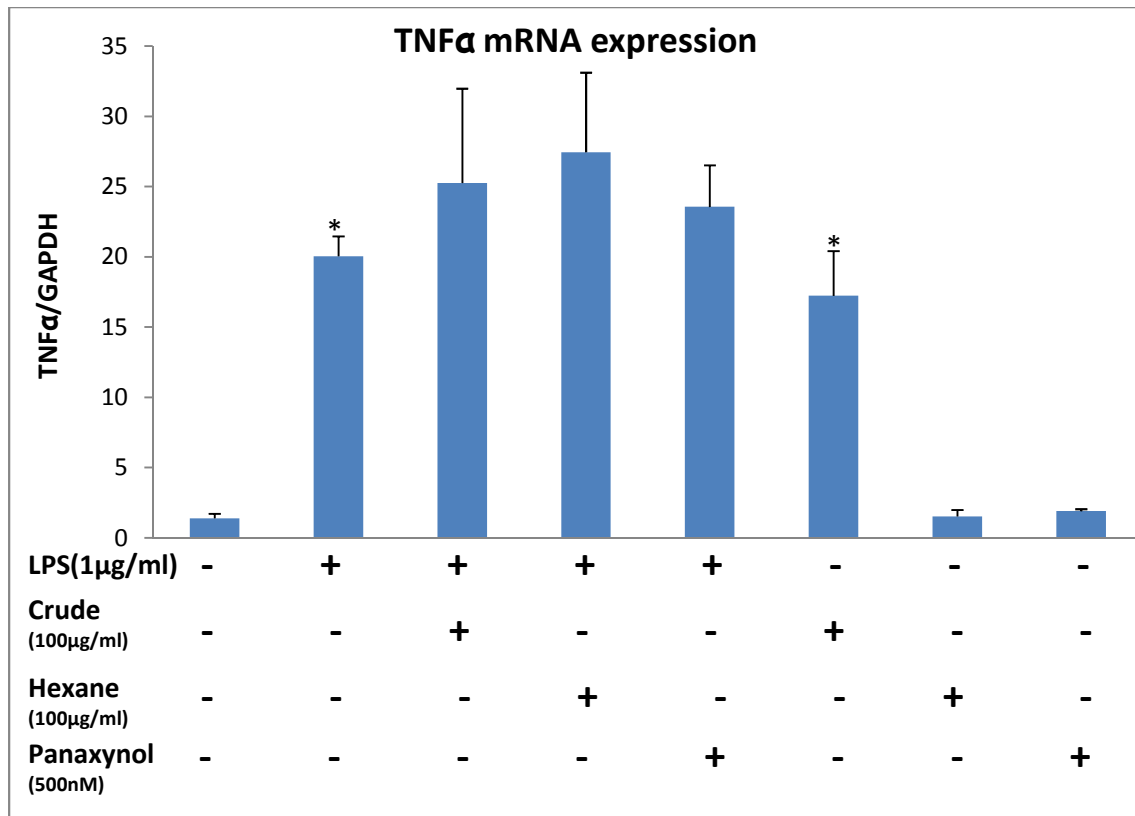




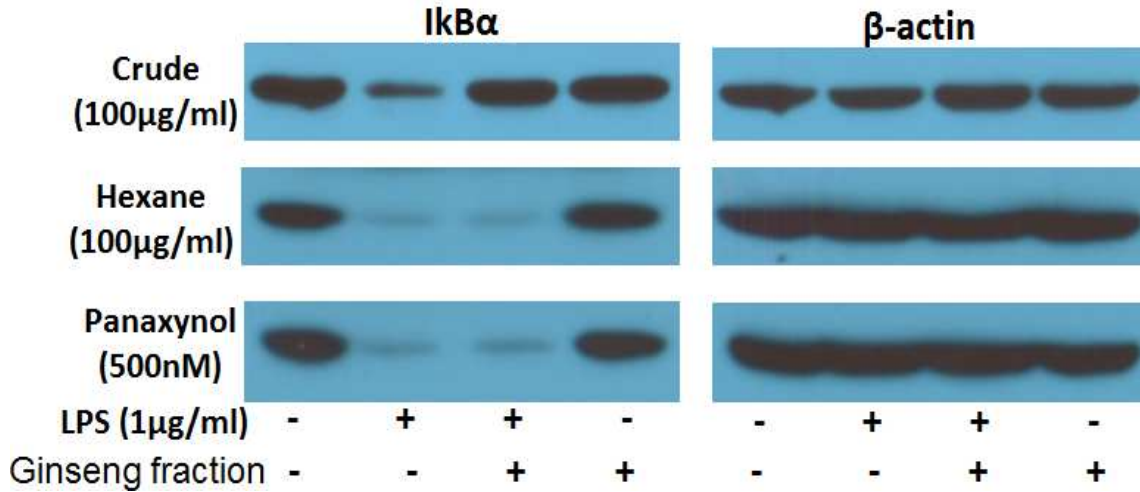
**Figure 3.13. Quantitative analysis of mRNA levels of IL-1 $\beta$  by Q-PCR . Effect of ginseng crude, hexane and panaxynol on LPS-induced expression of IL-1 $\beta$  in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1 $\mu$ g/ml, ginseng crude, hexane (dose100 $\mu$ g/ml) and panaxynol 500nM as indicated for 4h. mRNA levels of IL-1 $\beta$  were determined by Q-PCR. n=4, \*p < 0.05 vs. control (-), #p < 0.05 vs. LPS (1 $\mu$ g/ml).



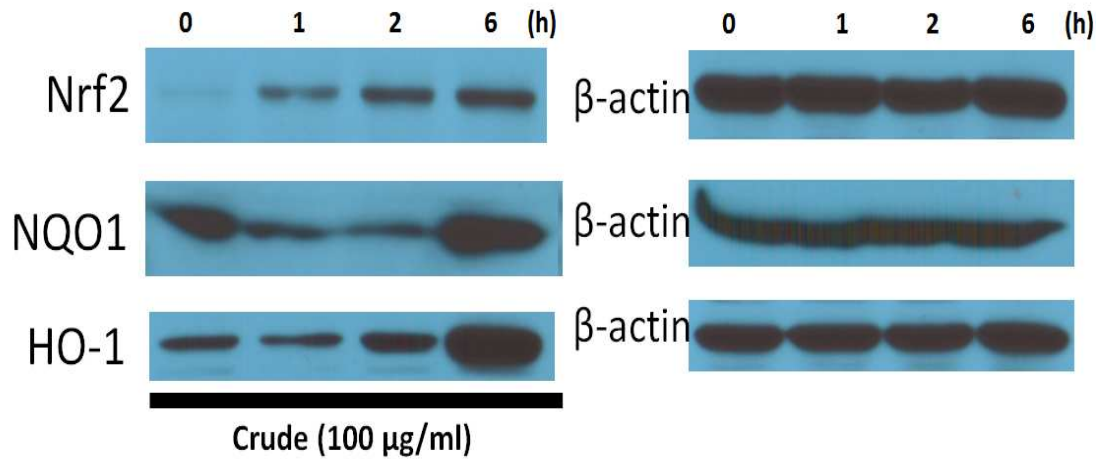
**Figure 3.14. Quantitative analysis of mRNA levels of IL-6 by Q-PCR . Effect of ginseng crude, hexane and panaxynol on LPS-induced expression of IL-6 in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1μg/ml, ginseng crude, hexane (100μg/ml) and panaxynol 500nM as indicated for 4h. mRNA levels of IL-6 were determined by Q-PCR. n=4, \*p < 0.05 vs. control (-), #p < 0.05 vs. LPS (1μg/ml).



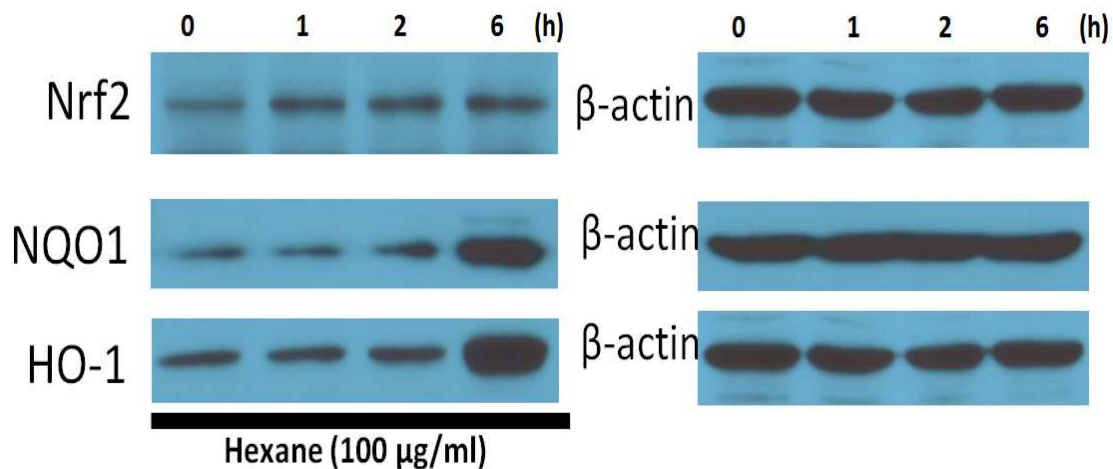
**Figure 3.15. Quantitative analysis of mRNA levels of TNF $\alpha$  by Q-PCR . Effect of ginseng crude, hexane and panaxynol on LPS-induced expression of TNF $\alpha$  in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1 $\mu$ g/ml, ginseng crude, hexane (100 $\mu$ g/ml) and panaxynol 500nM as indicated for 4h. mRNA levels of TNF $\alpha$  were determined by Q-PCR. n=4, \*p < 0.05 vs. control (-), #p < 0.05 vs. LPS (1 $\mu$ g/ml).



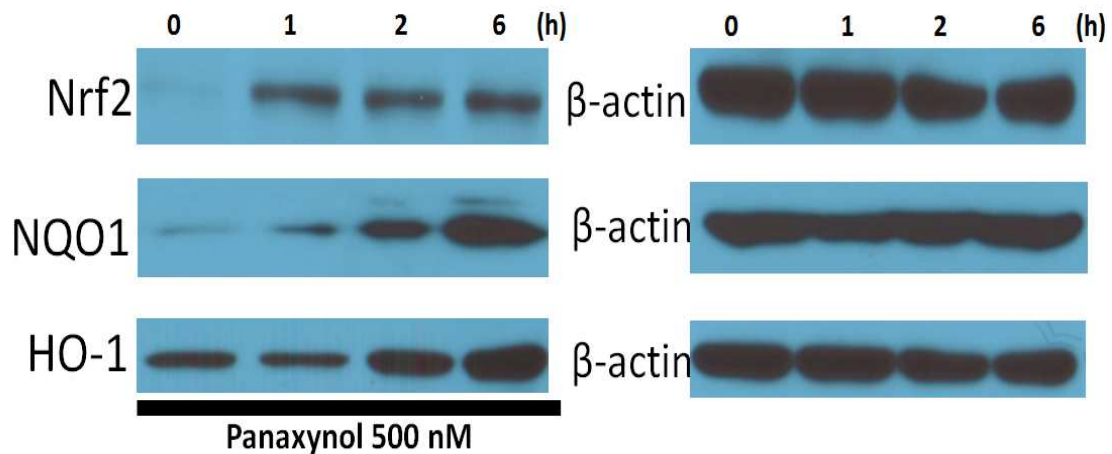
**Figure 3.16. Western blot analyses of IKB $\alpha$  and  $\beta$ -actin proteins. Effect of ginseng crude, hexane and panaxynol on expression of IKB $\alpha$  protein in inflamed RAW264.7 macrophages.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM and stimulated with or without LPS 1μg/ml and ginseng crude or hexane (dose 100μg/ml) or panaxynol 500nM as indicated for 30 minutes. Protein levels of IKB $\alpha$  were determined by Western blot. Load protein is 20μg/well. Results are representatives of three independent experiments.



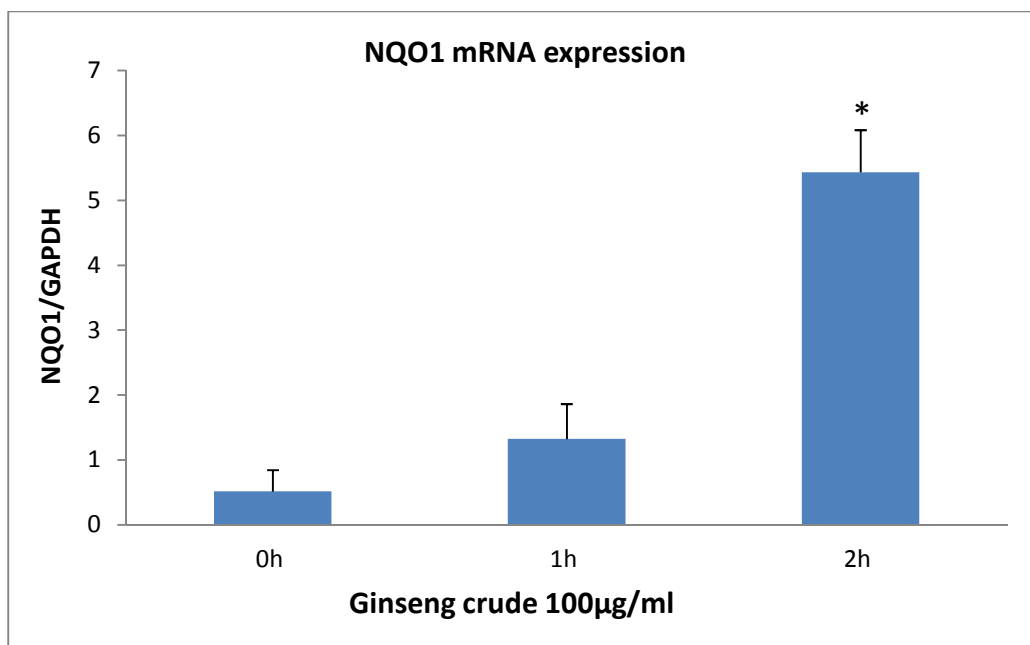
**Figure 3.17. Western blot analyses of Nrf2, NQO1, HO-1 and  $\beta$ -actin proteins. Effect of ginseng crude on the activation of Nrf2 signaling in RAW264.7 macrophages.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM and stimulated with ginseng crude 100µg/ml for 1, 2, 6 h. Protein levels of Nrf2, NQO1, HO-1 were determined by Western blot. Load protein is 30µg/well (n=3).



**Figure 3.18. Western blot analyses of Nrf2, NQO1, HO-1 and  $\beta$ -actin proteins. Effect of hexane on the activation of Nrf2 signaling in RAW264.7 macrophages.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM and stimulated with hexane 100 $\mu$ g/ml for 1, 2, 6 h. Protein levels of Nrf2, NQO1, HO-1 were determined by Western blot. Load protein is 30 $\mu$ g/well (n=3).

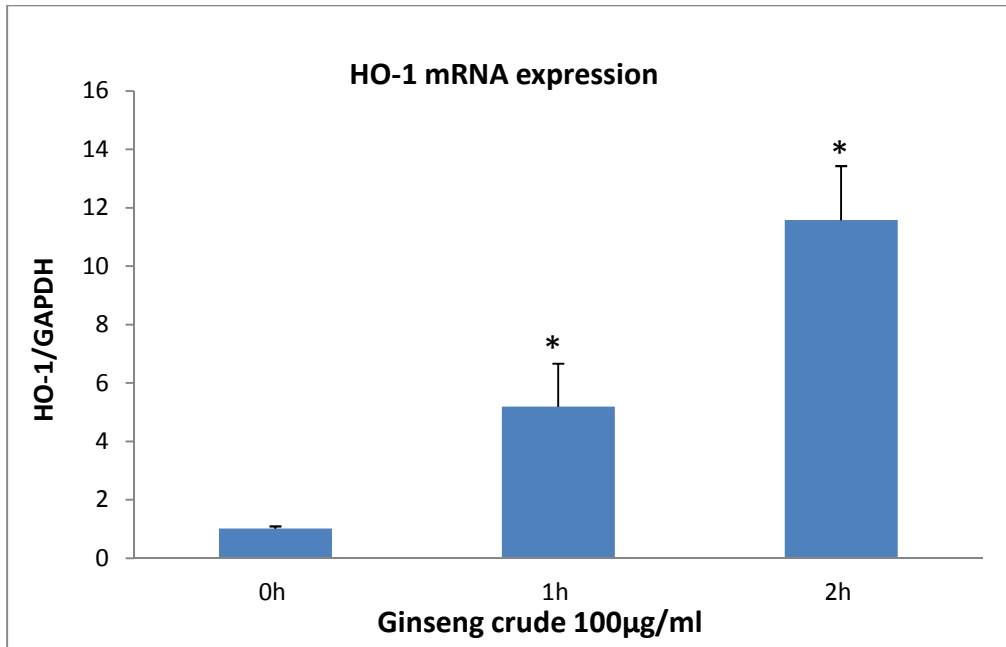


**Figure 3.19. Western blot analyses of Nrf2, NQO1, HO-1 and  $\beta$ -actin proteins. Effect of Panaxynol on the activation of Nrf2 signaling in RAW264.7 macrophages.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM and stimulated with Panaxynol 500nM for 1, 2, 6 h. Protein levels of Nrf2, NQO1, HO-1 were determined by Western blot. Load protein is 30 $\mu$ g/well (n=3).

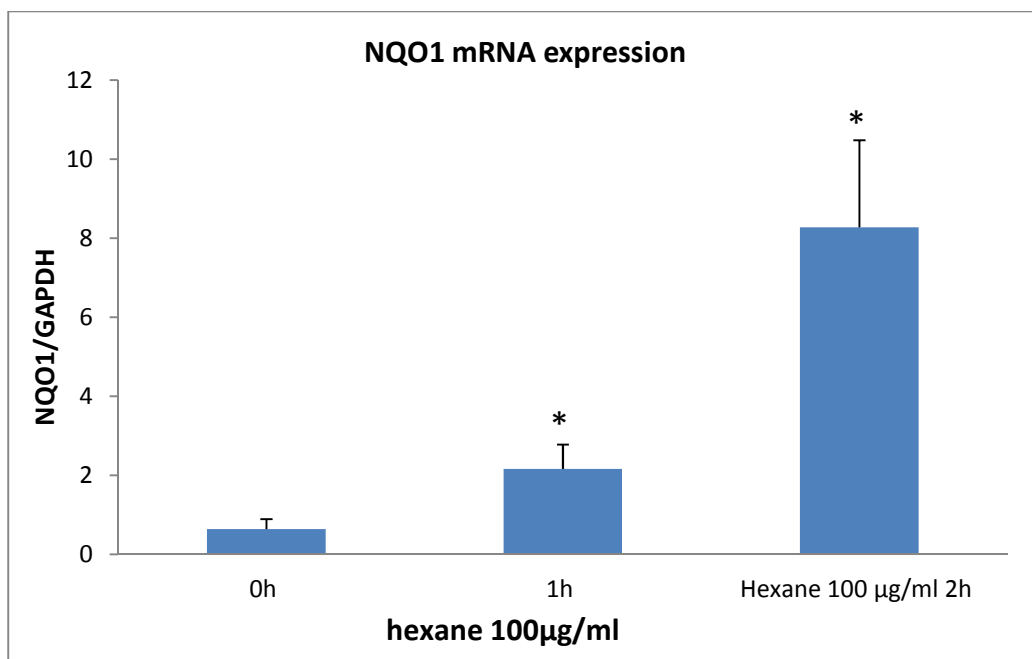


**Figure 3.20. Quantitative analysis of mRNA levels of NQO1 by Q-PCR. Effect of ginseng crude on expression of NQO1 mRNA in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with ginseng crude 100µg/ml for 0, 1, 2 h. mRNA levels of NQO1 were determined by Q-PCR. n=4, \*p < 0.05 vs. control (0h).

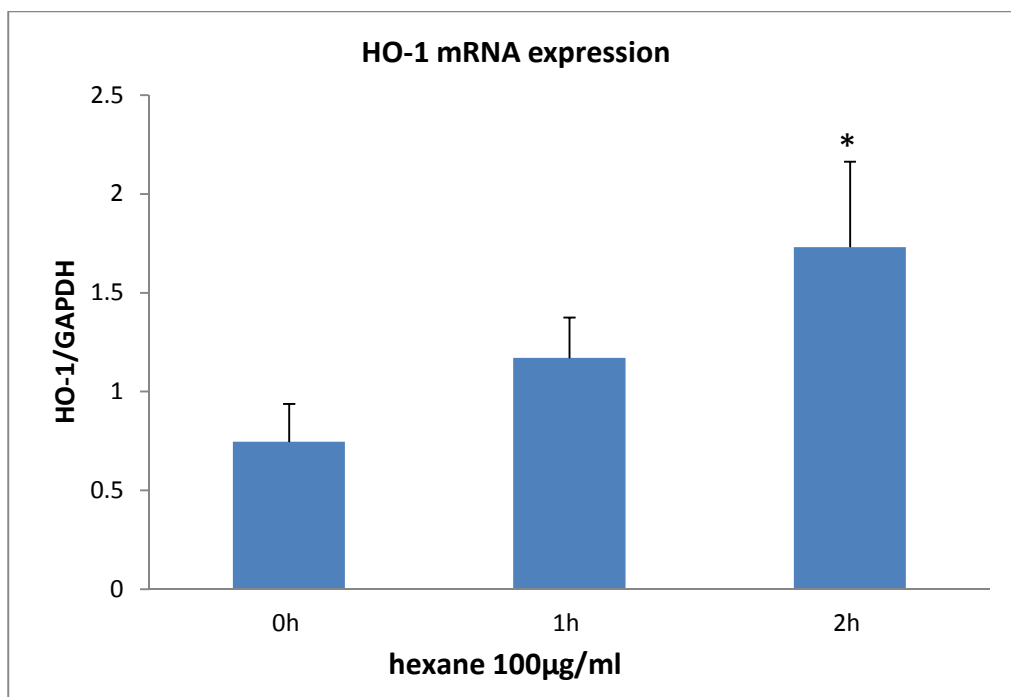




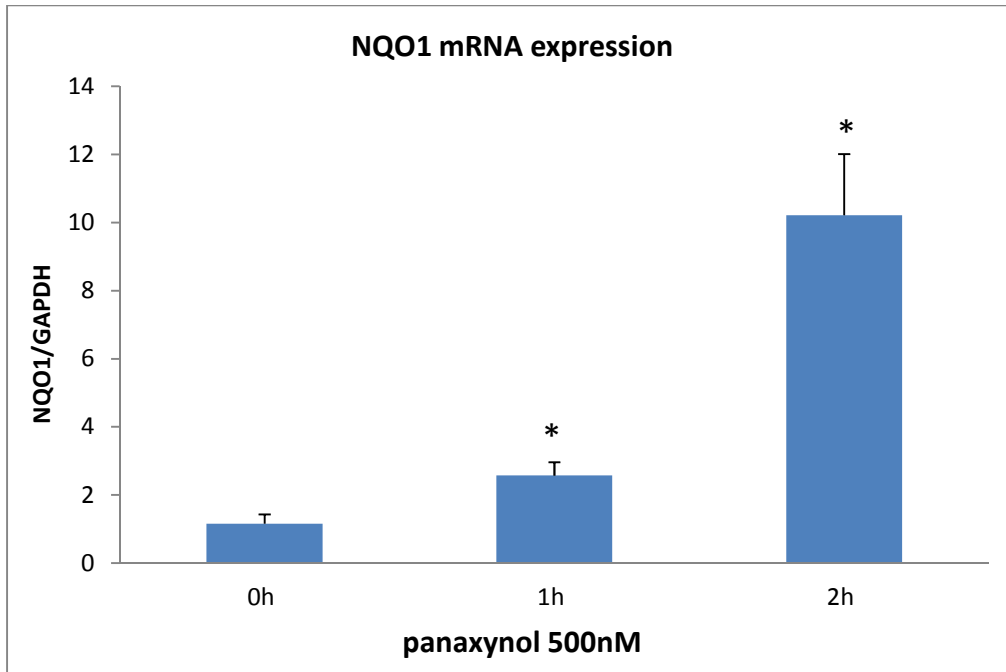
**Figure 3.21. Quantitative analysis of mRNA levels of HO-1 by Q-PCR. Effect of ginseng crude on expression of HO-1 mRNA in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with ginseng crude 100µg/ml for 0, 1, 2 h. mRNA levels of HO-1 were determined by Q-PCR. n=4, \*p < 0.05 vs. control (0h).



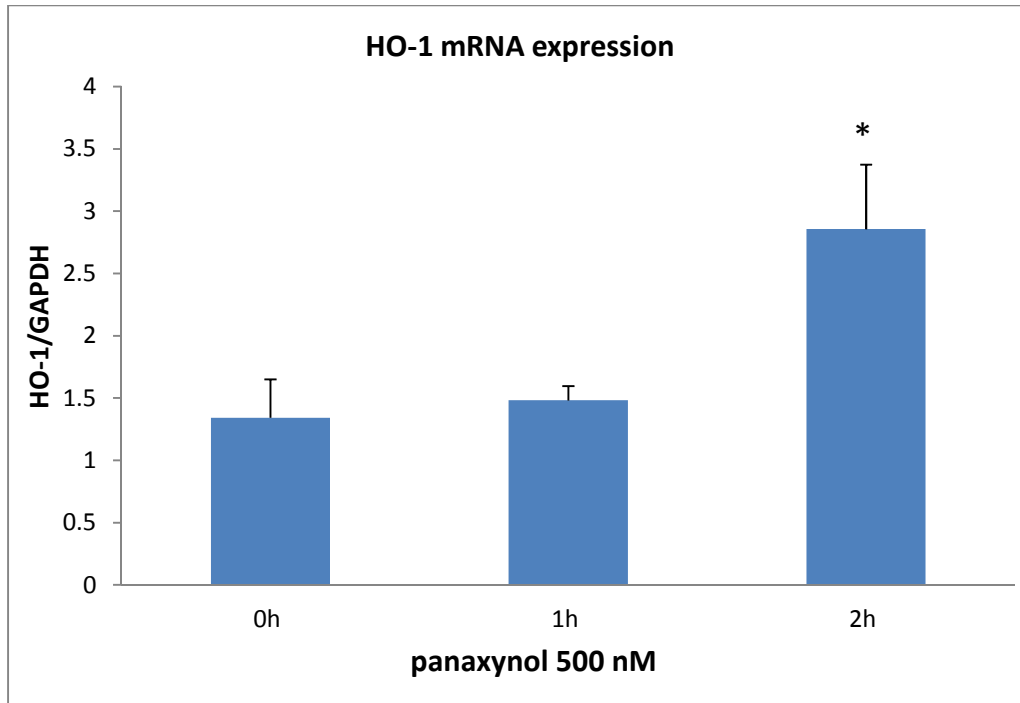
**Figure 3.22. Quantitative analysis of mRNA levels of NQO1 by Q-PCR. Effect of hexane on expression of NQO1 mRNA in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with hexane 100µg/ml for 0, 1, 2 h. mRNA levels of NQO1 were determined by Q-PCR. n=4, \*p < 0.05 vs. control (0h).



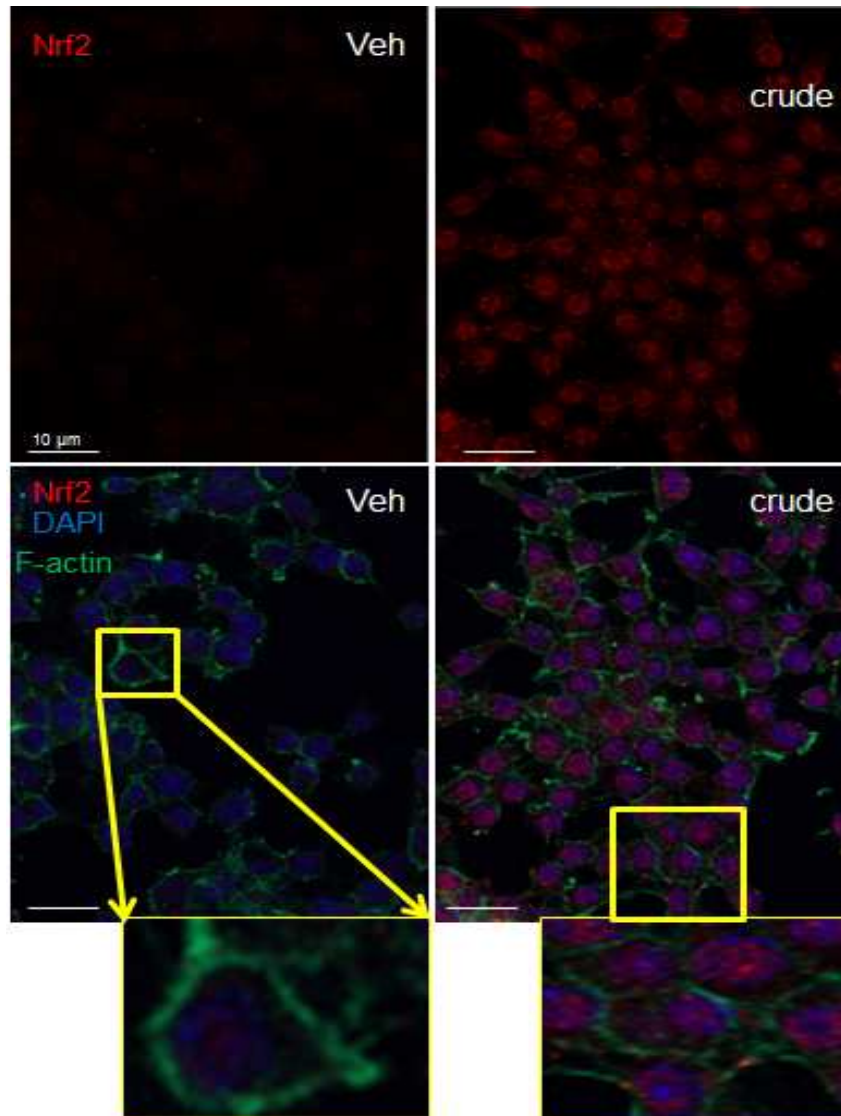
**Figure 3.23. Quantitative analysis of mRNA levels of HO-1 by Q-PCR. Effect of hexane on expression of HO-1 mRNA in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with hexane 100µg/ml for 0, 1, 2 h. mRNA levels of HO-1 were determined by Q-PCR. n=4, \*p < 0.05 vs. control (0h).



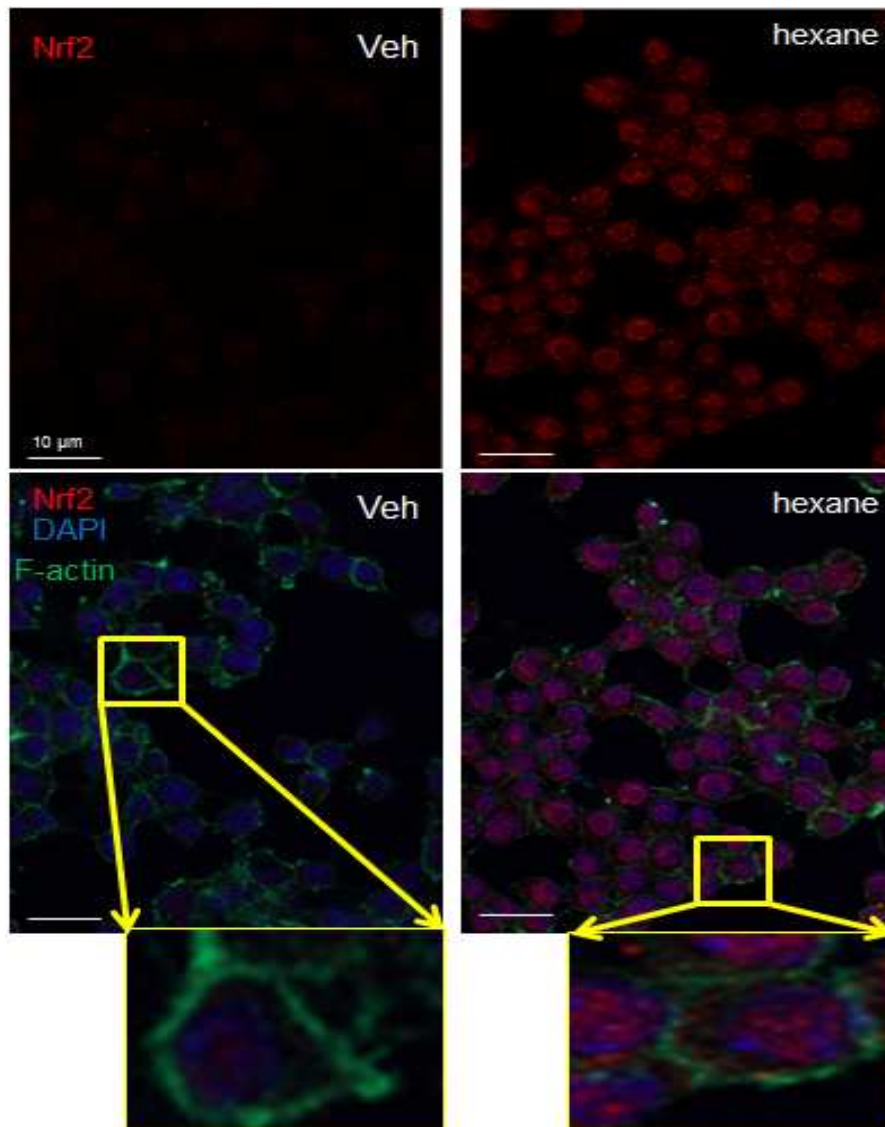
**Figure 3.24. Quantitative analysis of mRNA levels of NQO1 by Q-PCR. Effect of panaxynol on expression of NQO1 mRNA in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with panaxynol 500 nM for 0, 1, 2 h. mRNA levels of NQO1 were determined by Q-PCR. n=4, \*p < 0.05 vs. control (0h).



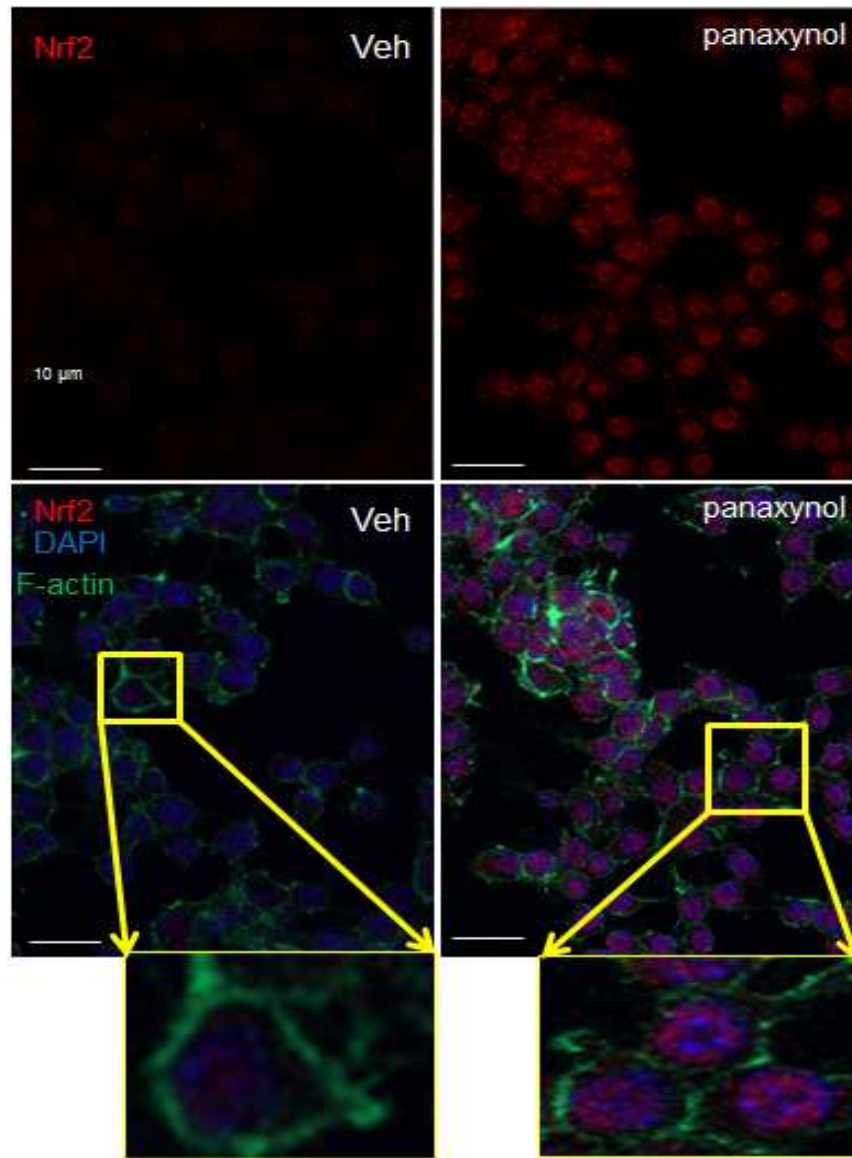
**Figure 3.25. Quantitative analysis of mRNA levels of HO-1 by Q-PCR. Effect of panaxynol on expression of HO-1 mRNA in RAW264.7 cells.** RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with panaxynol 500 nM for 0, 1, 2 h. mRNA levels of HO-1 were determined by Q-PCR. n=4, \*p < 0.05 vs. control (0h).



**Figure 3.26. Immunofluorescence staining of Nrf2. Effect of ginseng crude on activation of Nrf2 signaling in RAW264.7 macrophages.** RAW264.7 cells were treated with or without ginseng crude (100 $\mu$ g/ml) for 1 h. Nrf2 (red); F-actin (Green); Nuclei (Blue).

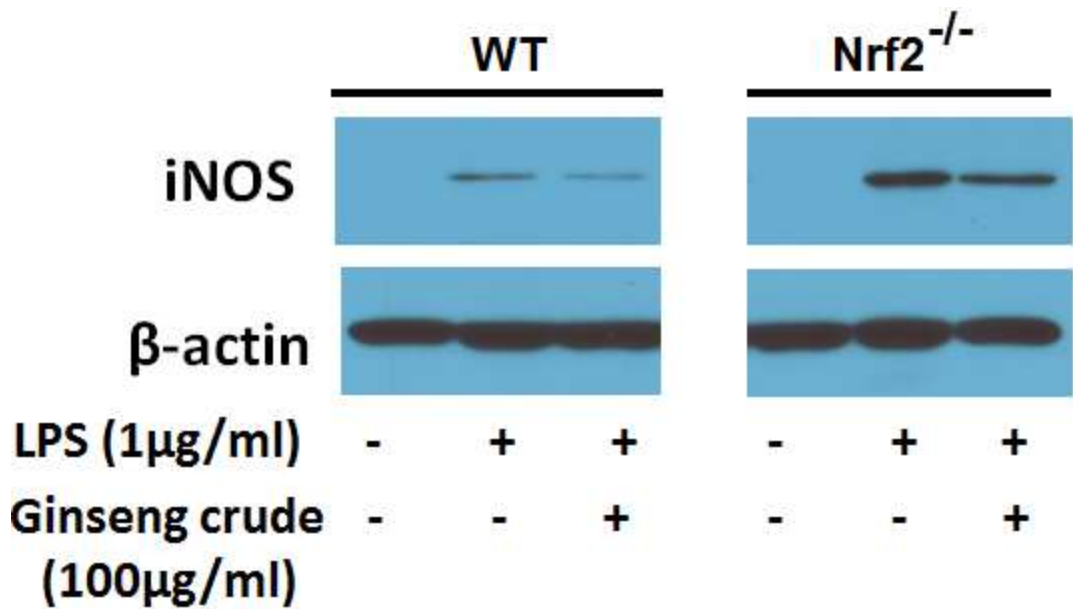


**Figure 3.27. Immunofluorescence staining of Nrf2. Effect of hexane on activation of Nrf2 signaling in RAW264.7 macrophages.** RAW264.7 cells were treated with or without hexane (100µg/ml) for 1 h. Nrf2 (red); F-actin (Green); Nuclei (Blue).

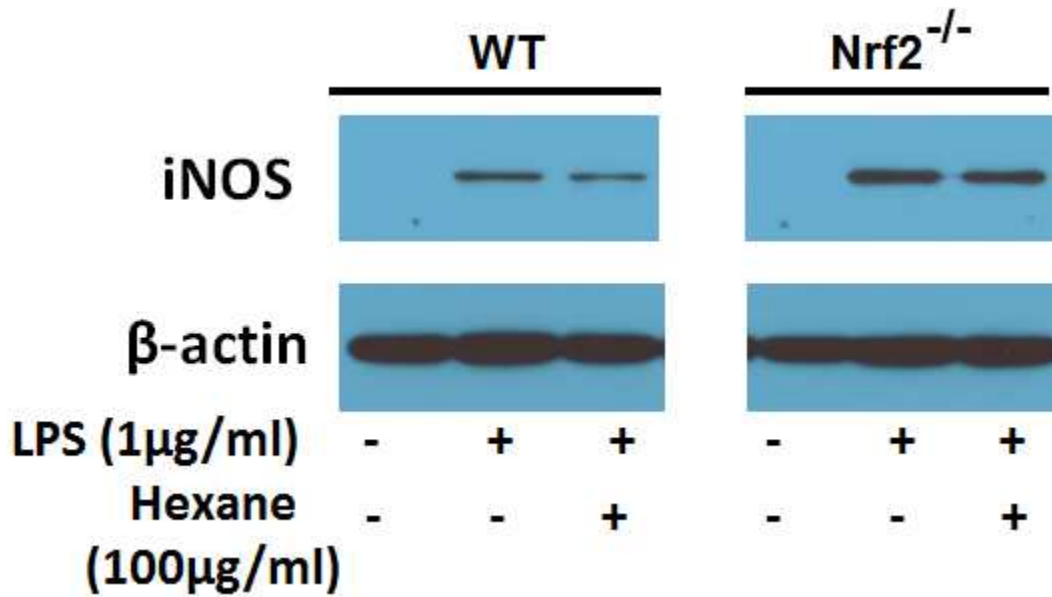


**Figure 3.28. Immunofluorescence staining of Nrf2. Effect of panaxynol on activation of Nrf2 signaling in RAW264.7 macrophages.** RAW264.7 cells were treated with or without panaxynol (500nM) for 1 h. Nrf2 (red); F-action (Green); Nuclei (Blue).

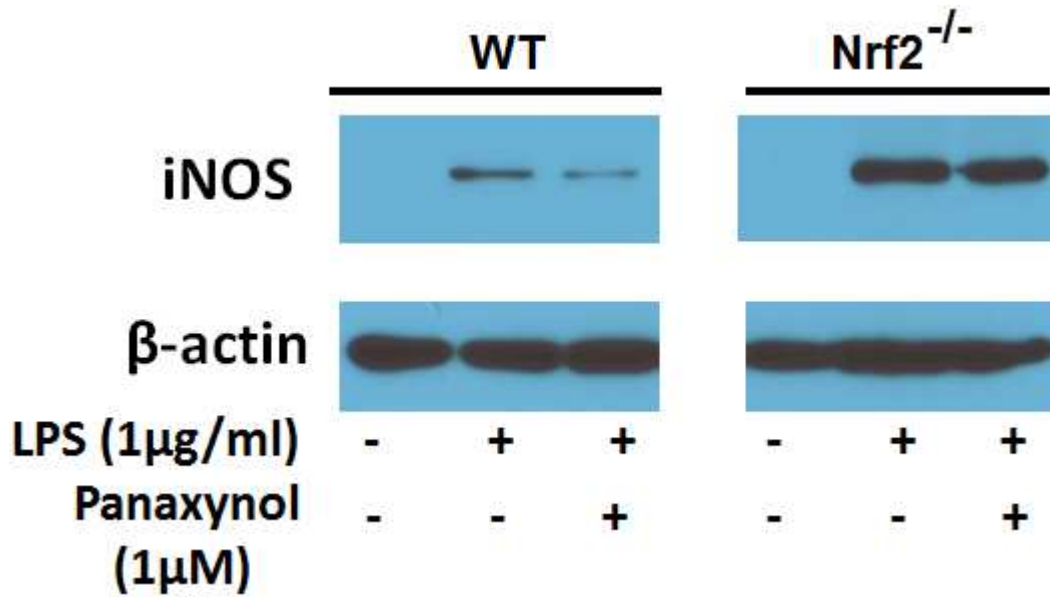




**Figure 3.29. Western blot analyses of iNOS and  $\beta$ -actin proteins. Effect of ginseng crude on iNOS expression in inflamed peritoneal-derived macrophages isolated from WT and Nrf2<sup>-/-</sup> mice.** Peritoneal macrophages were elicited by intraperitoneal injection of 1ml starch (6%) in distilled water. After 72h cells were harvested and plated in 10% FBS DMEM medium with 100U/ml penicillin and streptomycin and 2mM glutamine. After 2 hours cells were washed by 1x PBS and changed to fresh 10% FBS DMEM medium for 12h, and stimulated cells with ginseng crude for 6h. Protein levels of iNOS were determined by Western blot. Load protein is 10μg/well. Results are representative of three independent experiments.



**Figure 3.30. Western blot analyses of iNOS and  $\beta$ -actin proteins. Effect of hexane on iNOS expression in inflamed peritoneal-derived macrophages isolated from WT and Nrf2<sup>-/-</sup> mice.** Peritoneal macrophages were elicited by intraperitoneal injection of 1ml starch (6%) in distilled water. After 72h cells were harvested and plated in 10% FBS DMEM medium with 100U/ml penicillin and streptomycin and 2mM glutamine. After 2 hours cells were washed by 1x PBS and changed to fresh 10% FBS DMEM medium for 12h, and stimulated cells with hexane for 6h. Protein levels of iNOS were determined by Western blot. Load protein is 10μg/well. Results are representative of three independent experiments.



**Figure 3.31. Western blot analyses of iNOS and  $\beta$ -actin proteins. Effect of panaxynol on iNOS expression in inflamed peritoneal-derived macrophages isolated from WT and Nrf2<sup>-/-</sup> mice.** Peritoneal macrophages were elicited by intraperitoneal injection of 1ml starch (6%) in distilled water. After 72h cells were harvested and plated in 10% FBS DMEM medium with 100U/ml penicillin and streptomycin and 2mM glutamine. After 2 hours cells were washed by 1x PBS and changed to fresh 10% FBS DMEM medium for 12h, and stimulated cells with panaxynol for 6h. Protein levels of iNOS were determined by Western blot. Load protein is 10μg/well. Results are representative of three independent experiments.

## CHAPTER 4

### DISCUSSION

The public enthusiasm for ginseng to treat inflammatory diseases has been partly supported by evidence of ginseng-mediated antioxidant and anti-inflammatory effects (Wang et al., 2007; Hofseth et al., 2007; Spelman et al., 2006). Ginsenosides are the major active ingredients in American ginseng and the most studied compound (Attele et al., 1999; Jia and Zhao, 2009). However, other additional bioactive products like polyacetylenes are less studied.

Some reports show that ginseng exerts toxic effects or enhances the inflammatory response. This contradiction, may be due to the fact that the bioactive contents of ginseng extract can differ, depending on the method of extraction, subsequent treatment, age and part of the plant extracted, season of its collection, or even the geographical location (Soldati F et al., 1980). In the present project, we have demonstrated that American ginseng can be further fractionated into; water, butanol, ethyl acetate, dichloromethan, and hexane sub-fractions and the hexane fraction was the most effective fraction in suppressing iNOS and MCP-1 $\beta$  expression in macrophages. Then we found that hexane contains predominantly polyacetylenes from which we isolated Panaxynol which could be a potential promising anti-inflammatory drug.

Nrf2 up-regulates compensatory transcriptional genes in response to inflammatory or oxidative stress, which drives the induction of anti-oxidative pathways and detoxification enzymes, providing a defense against pathological damage. We

demonstrate that Panaxynol suppresses the inflammatory response in LPS-inflamed macrophages by specifically activating Nrf2 signaling independent of NF- $\kappa$ B, panaxynol up-regulation of Nrf2 protein levels, Nrf2 translocation into nuclei and activation of Nrf2-driven expression of a group of anti-oxidative genes namely; HO-1 and NQO-1. Also, panaxynol is unable to block iNOS expression in LPS- inflamed macrophages from Nrf2<sup>-/-</sup>mice. But, the precise mechanism by which Panaxynol activates Nrf2 in macrophages remains unknown.

NF- $\kappa$ B pathway also plays a critical role in the pro-inflammatory response in macrophage. We observed that Panaxynol cannot regulate the NF- $\kappa$ B pathway. Panaxynol was unable to prevent degradation of I $\kappa$ B and hence, cannot active the NF- $\kappa$ B pathway in the inflamed macrophages. Also, panaxynol is unable to inhibit the cytokines that are produced by NF- $\kappa$ B pathway activation, namely, IL-1 $\beta$ , IL-6 and TNF $\alpha$ . The results demonstrated in this project highlight a unique potential of Panaxynol to treat chronic inflammatory diseases.

While, Ginseng crude alone slightly, but significantly, increases the expression of iNOS, MCP-1 $\beta$ , IL-1 $\beta$ , IL-6, and TNF- $\alpha$  mRNA level, panaxynol alone does not affect iNOS, MCP-1 $\beta$ , IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . This finding could explain the controversial reports which show that Ginseng crude has pro-inflammatory effects and identifies Panaxynol to be a potential drug to treat chronic inflammatory diseases as it has only anti-inflammatory effects without showing pro-inflammatory effects and uniquely activates Nrf2. However, pharmacological efficacy and side effects of panaxynol need to be evaluated in future.

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