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ISOLATION OF NATURAL NRF2 ACTIVATORS FROM AMERICAN GINSENG

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For the Degree of Master of Science in

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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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ACKNOWLEDGEMENTS

First, I would like to thank my mentor, Dr. Taixing Cui for his advice, direction, and providing me all the resources and material to complete my project. Also, I would like to thank Bin Li and all of my colleagues in the lab for their help and support. Finally, a special thanks to Dr. Joseph Janicki for his time, comments, and instructions as a member of my thesis committee.

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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 fractioned 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 chemotactic protein-1 (MCP-1), but it has no effects on regulate expression of interleulin-6 (IL-6), interleulin-1β (IL-1β) and tumor necrosis factor alpha

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(TNF-α), this results suggested activation of specific pathway; that is Panaxynol potently activated Nrf2 signaling; but, it did not affect NF-κB 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-κB, 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

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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-/- 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

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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-κB 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

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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 β-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 α-OH at carbon-6 and/or β-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-la, 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

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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-diyn-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.,

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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 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

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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 (Mulkahy 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-κB and the NF-E2-related factors (Nrfs) 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 Nrfs 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.

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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 Nrfs, 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

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elements also affect the ARE-mediated induction of antioxidant genes (Li et al., 1992; Prestera et al., 1993; Wasserman et al., 1997). Nrf2 is more potent than Nrf1in activation of ARE-mediated gene expression (Venugopal et al., 1996). The exact mechanisms by which Nrfs 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 at 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 regulate activity of Nrf2. Keap1binds 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.

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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-κB) pathway

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

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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-κB comes from that description (Sen et al., 1986). NF-κB is retained in the cytoplasm by a family of inhibitory proteins known as inhibitors of NF-κB (IκBs). Phosphorylation of IκB by the IκB kinase (IKK) leads to IκB degradation and release of NF-κB. Unbound NF-κB translocated to the nucleus and bind to κB elements (Hayden et al., 2004). NF-κB regulates gens responsible for apoptosis, cell adhesion, proliferation, the innate- and adaptive-immune responses, inflammation and tissue remodeling (Bonizzi at 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-κB family members, RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2), and different NF-κB 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-κB 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 IκB 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-κB 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

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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).

IκB binds NF-κB and masks its nuclear localization signal that is found in the RHD region of the NF-κB. Thus, it retains NF-κB in the cytoplasm (Verma et al., 1995; Baldwin, 1996). In eukaryotic cells, there are five IκB family members; that include IκBα, IκBβ, IκBε, Bcl-3and IκBγ. IκBα, IκBβ and IκBε are the three principal IκBs. All members of IκB 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 IκB (Hayden et al., 2004). IκBα is the most studied member of IκB, 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 IκBα and NF-κB; that means activation of NF-κB causes up-regulation of transcription of IκBα, 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-κB to be maintained in the nucleus despite the up-regulation of IκB synthesis in the cytoplasm (Thompson et al., 1995). IκBβ is a 45-kDa; it has three domains and binds the same rel subunits as IκBα (Thompson et al.,

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1995). Both IκBα, IκBβ are degraded after cells are treated with the inducing agents (e.g. LPS); however, IκBα, level is elevated later, because of the auto-regulatory feedback loop between IκBα and NF-κB, whereas IκBβ level remains low. IκBε is a specific inhibitor of p65 and c-Rel complexes. Bcl-3 has structure similar to other IκBs (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). IκBγ is a 70-kDa molecule detected only in lymphoid cells (Inoue et al., 1992). Very little is known about the IκBγ.

IκB kinase (IKK) contains IKKα (IKK1) and IKKβ (IKK2), which are kinase subunits and a regulatory subunit, NEMO (NF-κB essential modifier) or IKKγ. In the classical NF-κB signaling pathway, IKKβ phosphorylates both IκBα on Serine 32 and Serine 36, and IκBβ on Serine 19 and Serine 23. In the alternative pathway, IKKα 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-κB. Stimulation of cells with inducers of NF-κB results in phosphorylation of IκB by IκB kinase (IKK) (Brockman et al., 1995; Brown et al., 1995; Traenckner et al., 1995; Whiteside et al., 1995); Phosphorylated IκB 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-κB (Beg et al., 1993; DiDonato et al., 1995; Traenckner et al., 1994; Chen et al., 1995). NF-κB plays an important role in activation of immune cells by up regulating many cytokines, particularly; IL-1, IL-6, TNFα and IFN-γ. All of these cytokines have multiple effects that contribute to inflammation (Kishimoto et al., 1994). NF-κB is important in the regulation of the acute phase response of inflammation and

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production of complement factor B, serum amyloid A protein 1, angiotensinogen, α1 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-κB activation which suggesting that NF-κB 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-κB-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-α (TNFα), interleukin-1 (IL-1), lipopolysaccharide (LPS) or engagement of the T-cell receptor (TCR). On this pathway there is rapid phosphorylation of IκBα and IκBβ by IKKβ 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-β 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 IKKα by the NF-κB 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

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phosphorylated tyrosines act as binding sites for STAT proteins. Jak kinases then phosphorylate STAT proteins, which then dissociated form 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-α and IFN-β) and type II interferon (IFN-γ) 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 el 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

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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

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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 (Dérijard et al., 1994), both c-Jun (Devary et al., 1992) and ATF2 (Gupta et al., 1995) are phosphorylated. Phosphorylation

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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

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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 rt 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 (PGG2), followed by peroxidase cleavage of the PGG2 peroxide to the endoperoxide (PGH2) which is converted by specific isomerases into prostaglandins (PGE2, PGF2, TxA2, PGI2) (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).

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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-α (TNF-α), 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 secret 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 κB (NF-κB) 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

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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 κB kinase (IKK) complex which in turn translocate NF-κB 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-α/β 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-kB pathway in RAW264.7 macrophages

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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 ◦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_1 3.5 (0.1), Re 21.2 (0.4), Rb_1 45.1 (1.6), Rc 15.5 (0.7), Rb_2 2.2 (0.1), Rd 17.8 (0.4) mg/g± 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

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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.

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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% O2: 5% CO2 humidified atmosphere at 37º 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, $Nrf2^{+/+}$) and Nrf2 knockout ($Nrf2^{-/-}$) mice were housed under standard conditions. Nrf2 knockout ($Nrf2^{-/-}$) mice were generated by breeding heterozygous Nrf2 ($Nrf2^{+/-}$) mice. Male $Nrf2^{-/-}$ mice grow normally and did not exhibit any clinical manifestations of illness, therefore, male $Nrf2^{-/-}$ 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 $Nrf2^{-/-}$ littermates. Peritoneal macrophages were elicited by intra-peritoneal injection of 1ml starch (6%) in distilled water. After 72h, cells were

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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% O2: 5% CO2 humidified atmosphere at 37º 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 by1x 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 $1x10^5$/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 $OD_{supernatant,}$ 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_{lysate}, 500 µl of 1% triton was added to Raw 264.7 cells, and incubate for 30 min at 37º 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

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

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 VaSO4, 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

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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 the by mixing the following reagents for 2 gels (depend on protein size). For gel 8%; H2O 4.7ml, 30% Acrylamide/Bis 2.7ml, 1.5M Tris-HCl (pH8.8) 2.5ml, 10% SDS 0.1ml. For gel 10%; H2O 4.1ml, 30% Acrylamide/Bis 3.3ml, 1.5M Tris-HCl (pH8.8) 2.5ml, 10% SDS 0.1ml. For gel 12%; H2O 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 H2O and 4% Stacking gels were prepared with mixing H2O 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/H2O 450ml) was filled to top of cartridge. The 10X running

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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 5.0µl 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/ H2O 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-IκBα (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-β-actin (Sigma A1978, Sigma–Aldrich). Primary antibodies were diluted in 5% milk and stored overnight at 4ºC 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

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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^2) 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

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kept on ice for use or stored at -80º C. Quantification of RNA was performed at 260vnm using a Biophotometer (Eppendorf). The dilutions were made for cDNA synthesis, and then stored at -80º 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 (Effendorf, Hauppauge, NY). The cycle consisted of 25º C for 5 minutes, 42º C for 30 minutes, and 85 º C for 5 minutes, and 4º C hold. After cDNA synthesis, each sample of the cDNA was diluted with DNase/RNase-free water and stored at -20º 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, 95º C for 1.5 min, followed by 40 cycles of denaturation for 15 seconds, 95º C, annealing for 30 seconds,

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

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 37º 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 4º C. Rinsed3 times with PBS and incubated with goat anti-rabbit delight 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.).

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2.8. Statistics

Values are expressed as mean ± 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.

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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'-GGTCCGCAAGAGAGTGCTGT-3'	5' -GCACGCTGAGTACCTCATTGG -3'	119
MCP-1	NM_011333.3	5'-GCCCCACTCACCTGCTGCTA-3'	5'-TTACGGGTCAACTTCACATTCAAA-3'	278
MIP-1β	NM_013652.2	5'-CCAGCTCTGTGCAAACCTAACC-3'	5'-GCCGGGAGGTGTAAGAGAAAC-3'	158
TNF-α	NM_013693.3	5' -GCCACCACGCTCTTCTGTCTAC-3'	5' -GGGTCTGGGCCATAGAACTGAT-3'	103
IL-1β	NM_008361.3	5' -ACCTTCCAGGATGAGGACATGA-3'	5' -CTAATGGGAACGTCACACACCA-3'	116
IL-10	NM_010548.2	5' -GTTGCCAAGCCTTATCGGAA-3'	5'-GCTCCACTGCCTTGCTCTTAT-3'	116
IL-6	NM_031168.1	5' -CACATGTTCTCTGGGAAATCG-3'	5' -TTGTATCTCTGGAAGTTTCAGATTGTT-3'	116
NQO1	NM_008706.5	5'-CGGTATTACGATCCTCCCTCAACA-3'	5' -AGCCTCTACAGCAGCCTCCTTCAT-3'	120
TGF-βR1	NM_001277255.1	5'-GCATTGGCAAAGGTCGGTTT-3'	5'-TGCCTCTCGGAACCATGAAC-3'	103
HO-1	NM_010442.2	5' -AGGAGATAGAGCGCAACAAGCAGA-3'	5' -CCAGTGAGGCCCATACCAGAAG-3'	116
GAPDH	NM_008084.2	5'-ATGTTCCAGTATGACTCCACTCACG-3'	5'-GAAGACACCAGTAGACTCCACGACA-3'	171

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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, dichloremethane fraction, ethyl acetate fraction and maltodextrin at 0, 10, 50,100,500, 1000 μg/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 μg/ml. For dichloremethane, ethyl acetate and maltodextrin, 80% of RAW264.7 cells survived up to the dose 1000 μg/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 μg/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 μg/ml for crude, hexane, water, butanol, dichloremethane 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

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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-1β 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-1β 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 dichloremethane 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, dichloremethane 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

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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-1β, RAW 264.7 macrophages were stimulated with LPS 1μg/ml and ginseng crude 100μg/ml or hexane fraction 100μg/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-1β mRNA in in unstimulated RAW 264.7 cells and maximal expression in cells treated with LPS for 4 h. Ginseng crude and hexane fraction inhibited MCP-1β expression at mRNA level in LPS-induced RAW 264.7 macrophages (figure 3.5). American ginseng crude alone slightly increased the expression of MCP-1β mRNA. We conclude that, Ginseng crude and hexane potently suppresses LPS-induced iNOS expression at both the mRNA and protein levels and MCP-1β expression at mRNA levels which indicates that hexane is the most potent inflammatory suppressor of the ginseng sub-fractions.

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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 μM 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 1μM (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-1β 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-1β 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 1μg/ml and ginseng crude 100μg/ml or

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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-1β mRNA in unstimulated RAW 264.7 cells and maximal expression in cells treated with LPS for 4 h. Panaxynol inhibited iNOS and MCP-1β 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-1β 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-1β, we investigated crude, hexane and panaxynol on other pro-inflammatory cytokines namely; TGF-βR1, IL-1β, IL-6, and TNF-α in LPS-inflamed RAW264.7 macrophages. LPS suppressed TGF-βR1 at the mRNA level, but crude, hexane and panaxynol, all stimulate the expression of TGF-βR1 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-1β, IL-6, and TNF-α 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 my indicate activation of specific pathway.

3.7 Effect of American ginseng crude, hexane and panaxynol on NF-κB pathway in RAW 264.7 macrophages

LPS activated the NF-κB pathway by enhancing IκB degradation and the subsequent release and activation of NF-κB transcriptional activity in RAW264.7

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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

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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 $Nrf2^{-/-}$ 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 $Nrf2^{-/-}$mice. All of the above results collectively clearly indicate that Nrf2 is an essential mediator for panaxynol to suppress inflammatory responses in inflamed macrophages.

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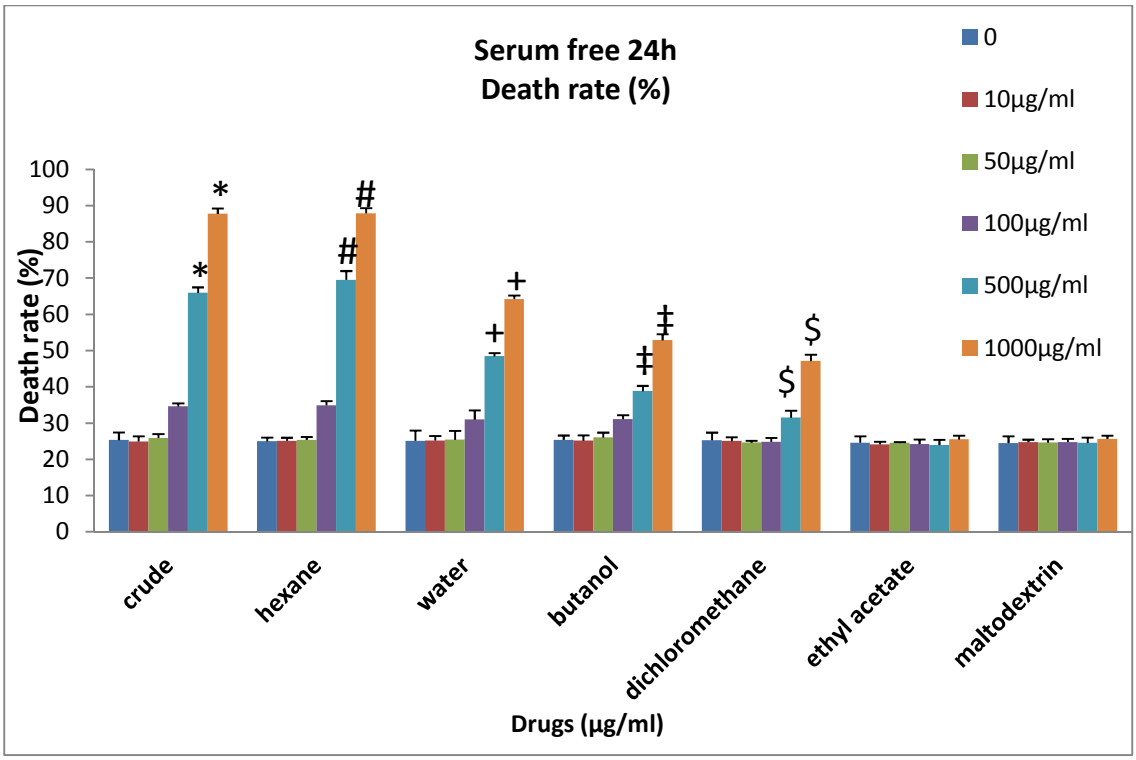

Figure 3.1. Cytotoxicity of ginseng crude, hexane fraction, water fraction, butanol fraction, dichloremethane fraction, ethyl acetate fraction and maltodextrin in RAW264.7 macrophages cultured in serum free DMEM. Raw 264.7 cells were cultured in 24-well plate in 10% FBS DMEM medium for 24h, changed to 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 (dichloremethane 0μg).

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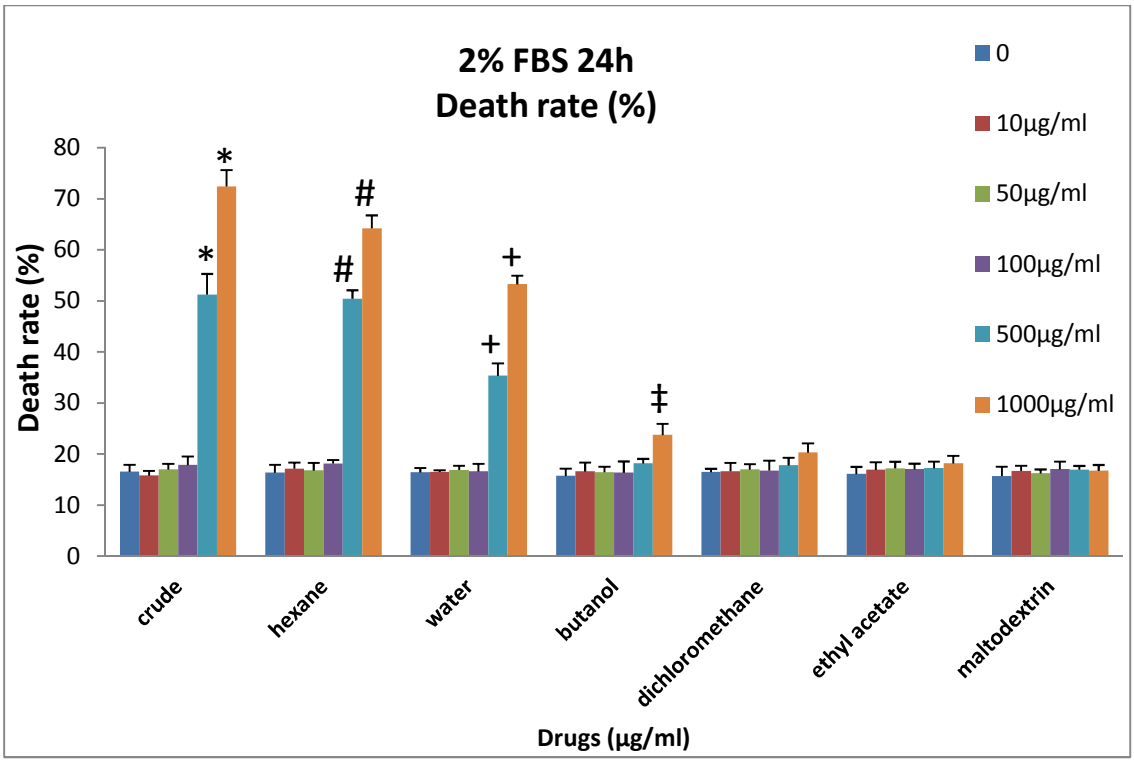

Figure 3.2. Cytotoxicity of ginseng crude, hexane fraction, water fraction, butanol fraction, dichloremethane fraction, ethyl acetate fraction and maltodextrin in RAW264.7 macrophages cultured in 2% FBS DMEM. Raw 264.7 cells were cultured in 24-well plate in 10% FBS DMEM medium for 24h, changed to 2% 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).

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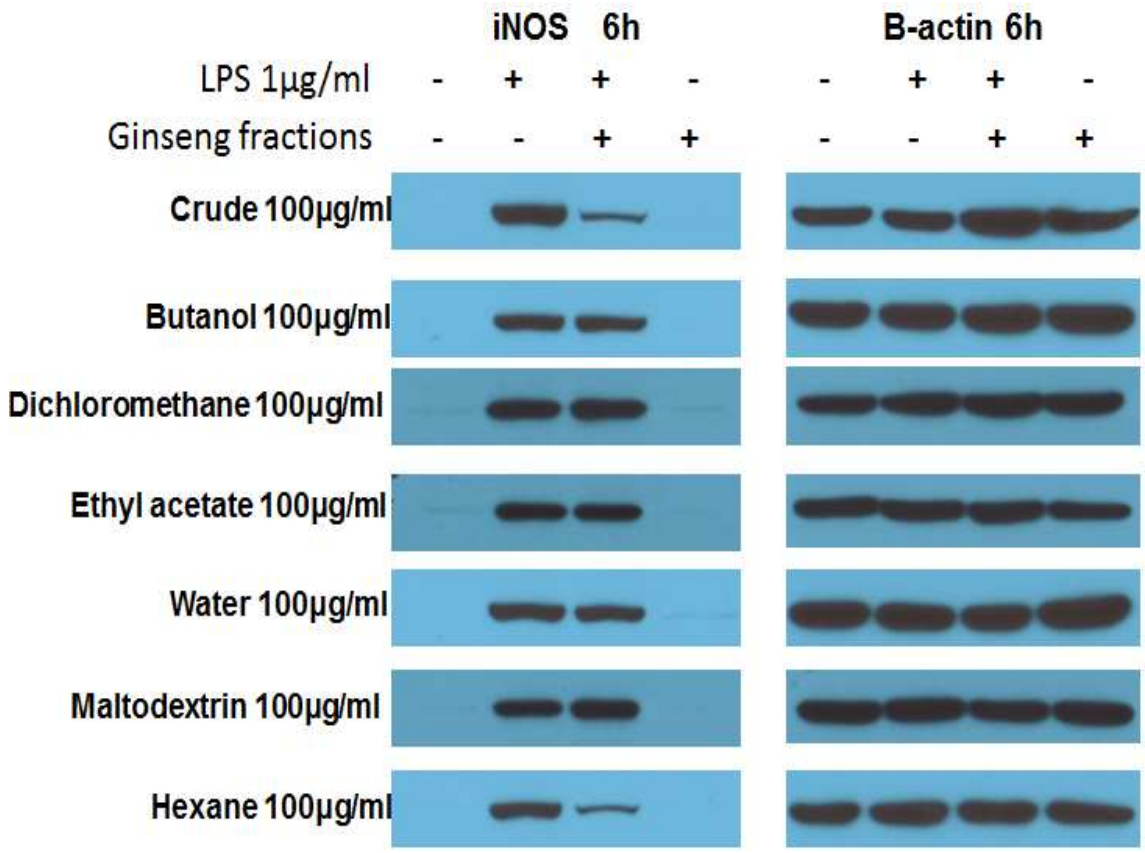

Figure 3.3. Western blot analyses of iNOS and β-actin proteins. Effect of ginseng crude, butanol fraction, hexane fraction, water fraction, dichloremethane fraction, ethyl acetate fraction and maltodextrin on expression of iNOS protein in inflamed RAW264.7 macrophages. RAW 264.7 cells were cultured in 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 dichloremethane or ethyl acetate or maltodextrin (dose100µg/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.

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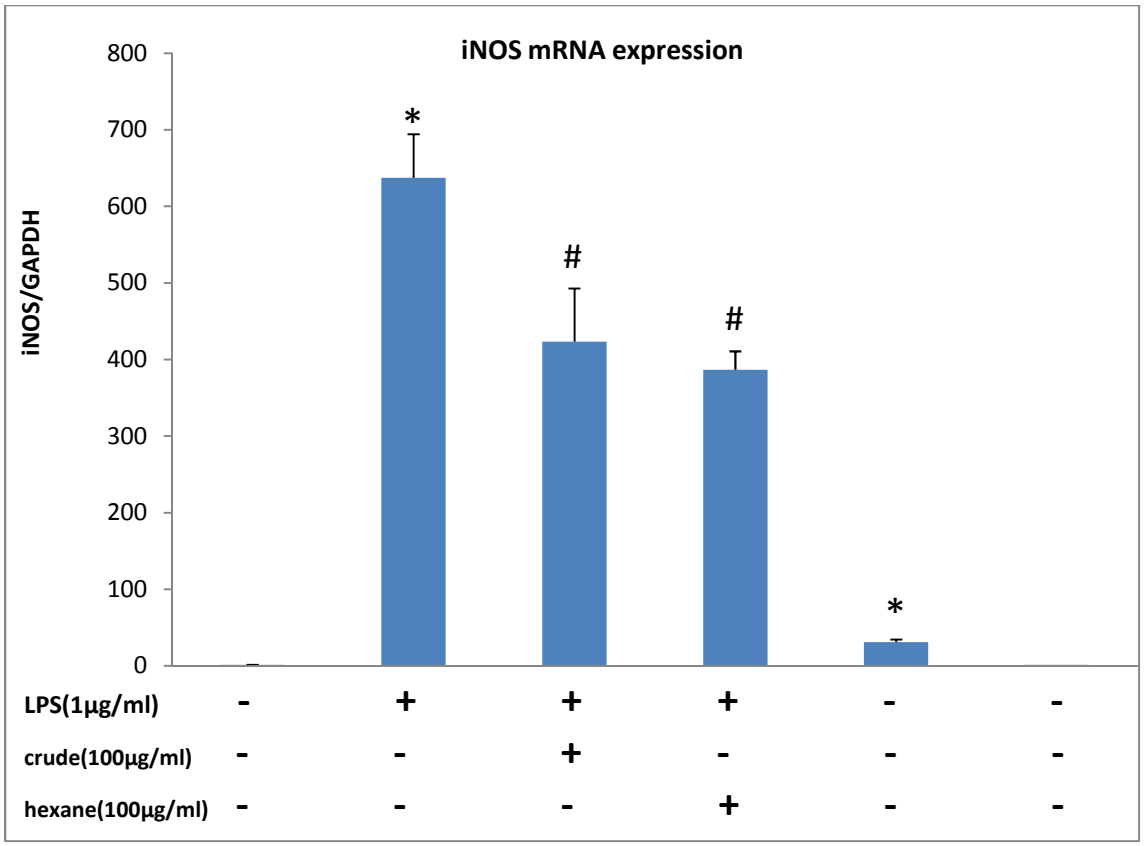

Figure 3.4. Quantitative analysis of mRNA levels of iNOS by Q-PCR . Effect of ginseng crude and hexan on LPS-induced expression of iNOS in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1μg/ml, ginseng crude and hexane (dose100μg/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).

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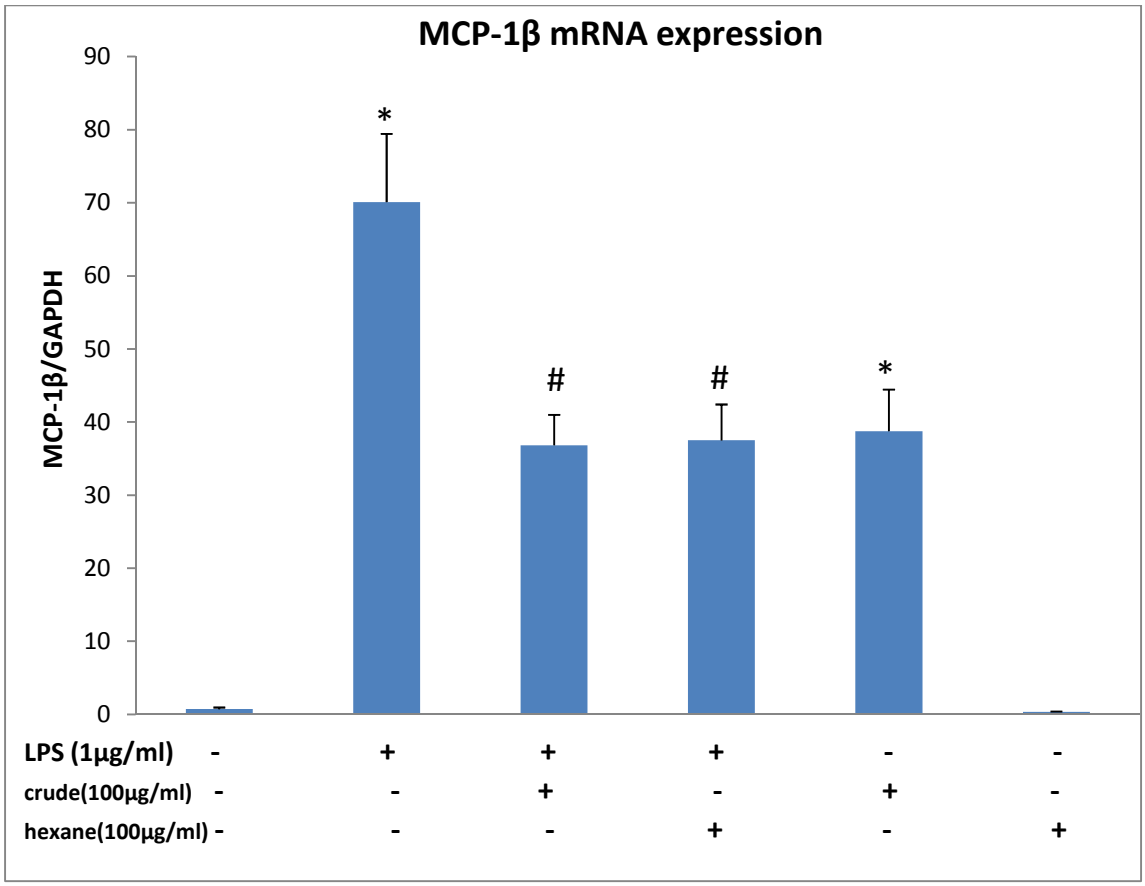

Figure 3.5. Quantitative analysis of mRNA levels of MCP-1β by Q-PCR . Effect of ginseng crude, hexane and panaxynol on LPS-induced expression of MCP-1β in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1μg/ml, ginseng crude, hexane (dose100μg/ml) and panaxynol 500nM as indicated for 4h. mRNA levels of MCP-1β were determined by Q-PCR. n=4, *$p < 0.05$ vs. control (−), #$p < 0.05$ vs. LPS (1μg/ml).

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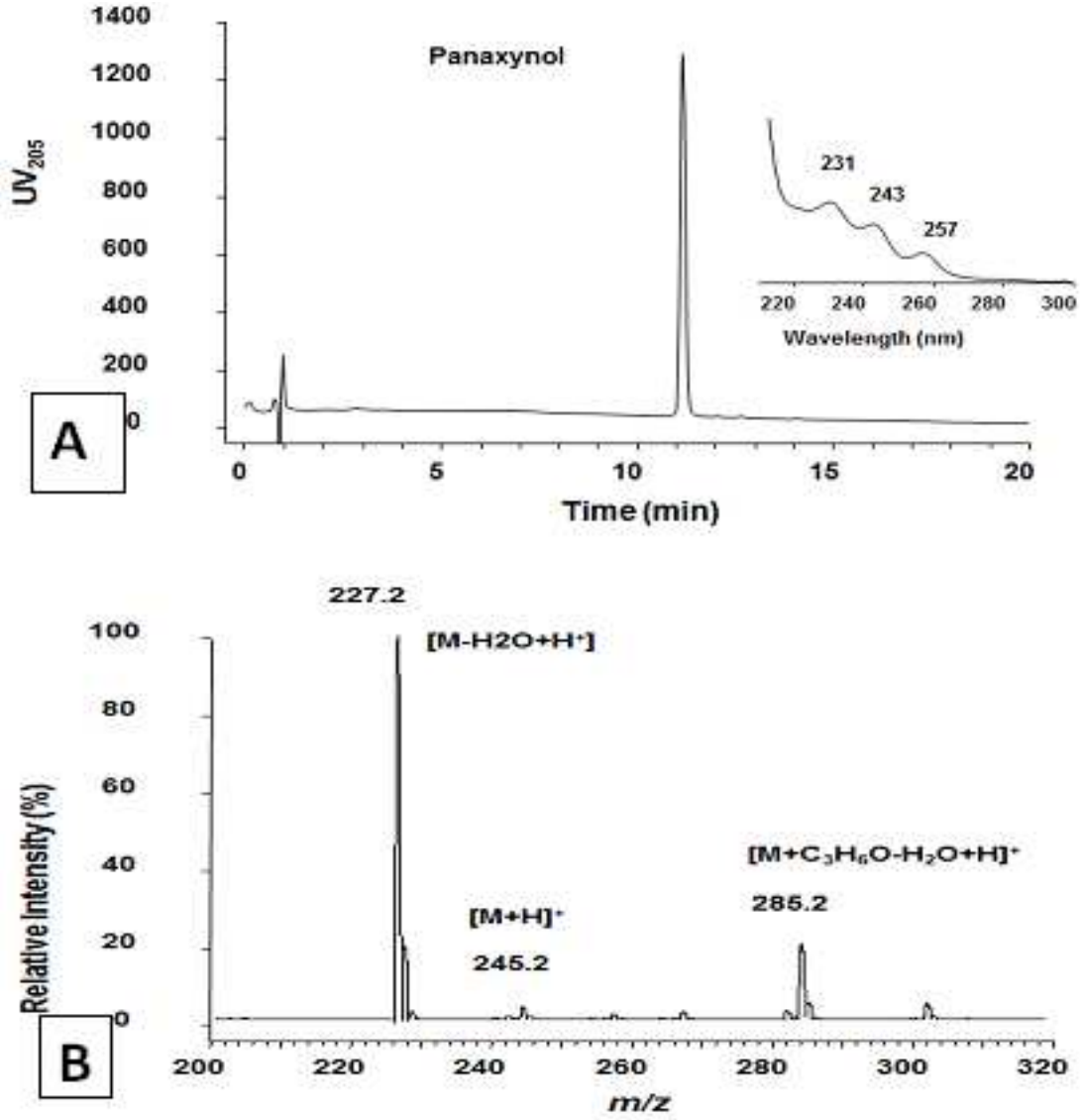

Figure 3.6. Isolation of panaxynol from American ginseng hexane sub-fraction. (A). Ultra-violet liquid chromatography (LC-UV) DAD analysis of purified panaxynol. (B). liquid chromatography-mass spectrometry (LC-MS) analysis of purified panaxynol.

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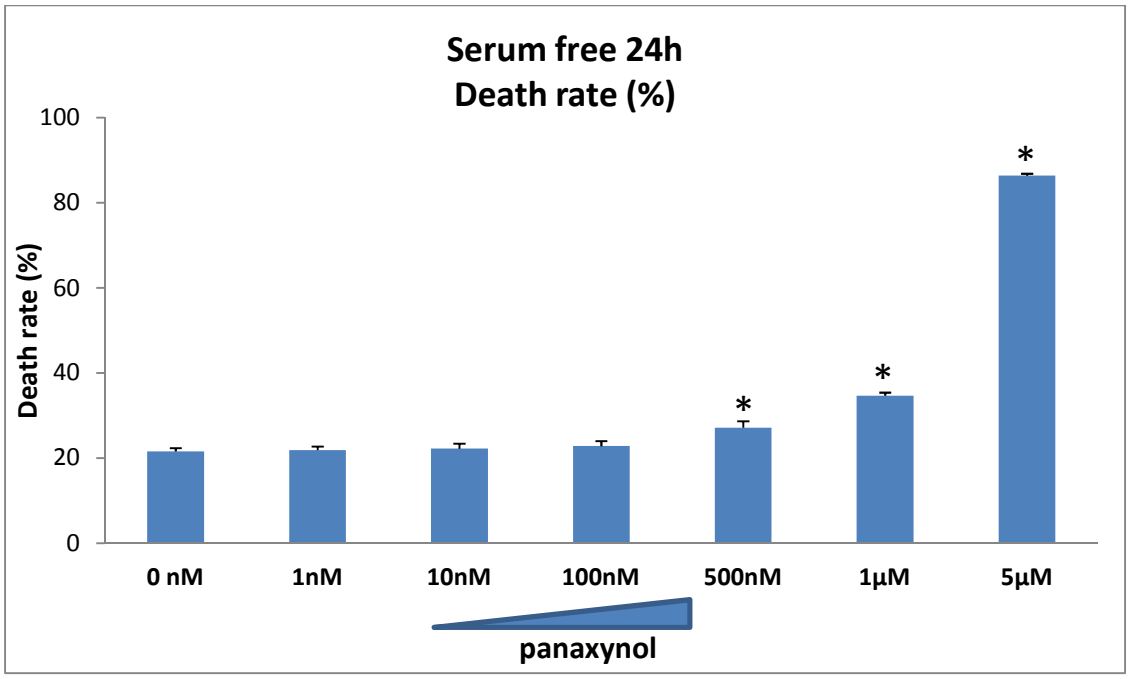

Figure 3.7. Cytotoxicity of panaxynol in RAW264.7 macrophages were cultured in serum free DMEM medium. Raw 264.7 cells were cultured in 24-well plate in 10% FBS DMEM medium for 24h, changed to serum free DMEM medium and stimulated with different doses of panaxynol (0, 1, 10, 100, 500 nM and 1, 5 µM)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

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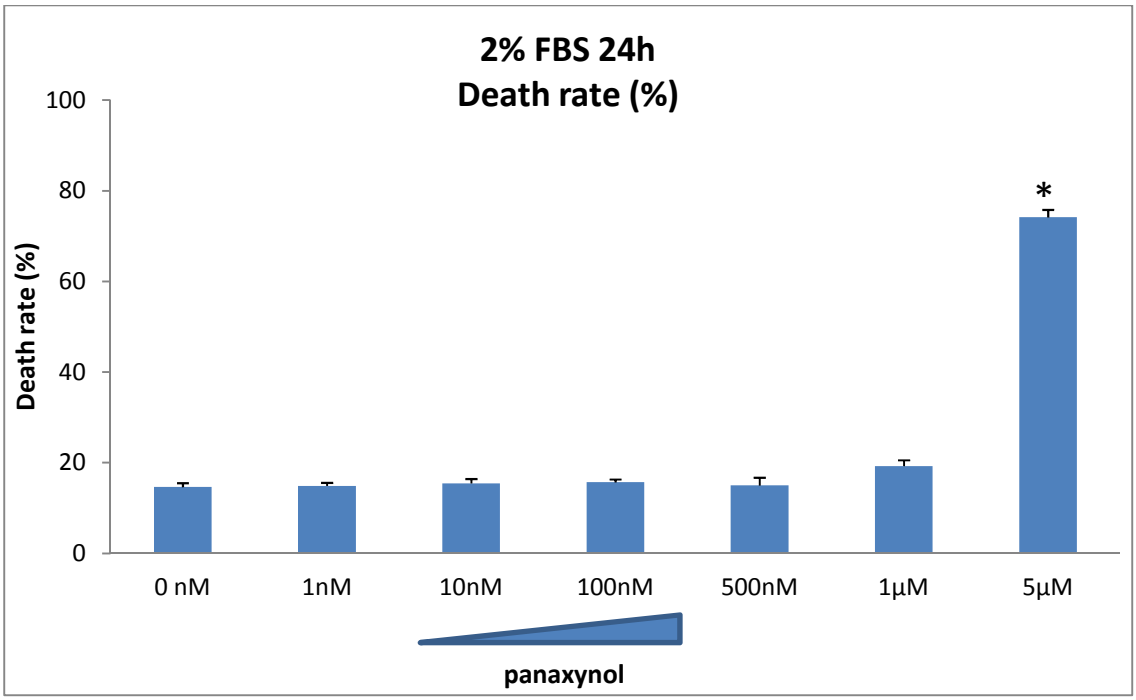

Figure 3.8. Cytotoxicity of panaxynol in RAW264.7 macrophages cultured in 2% FBS DMEM. Raw 264.7 cells were cultured in 24-well plate in 10% FBS DMEM medium for 24h, changed to 2% FBS DMEM medium and stimulated with different doses of panaxynol (0, 1, 10, 100, 500 nM and 1, 5 µM) for 24h. Cell viability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

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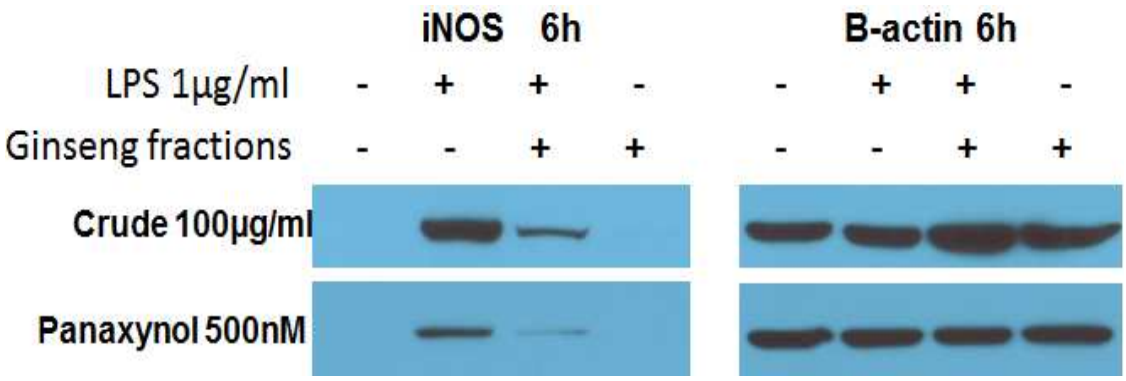

Figure 3.9. Western blot analyses of iNOS and β-actin proteins. Effect of panaxynol on expression of iNOS protein in inflamed RAW264.7 macrophages. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM and stimulated with or without LPS 1µg/ml, ginseng crude 100µg/ml and panaxynol 500nM 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.

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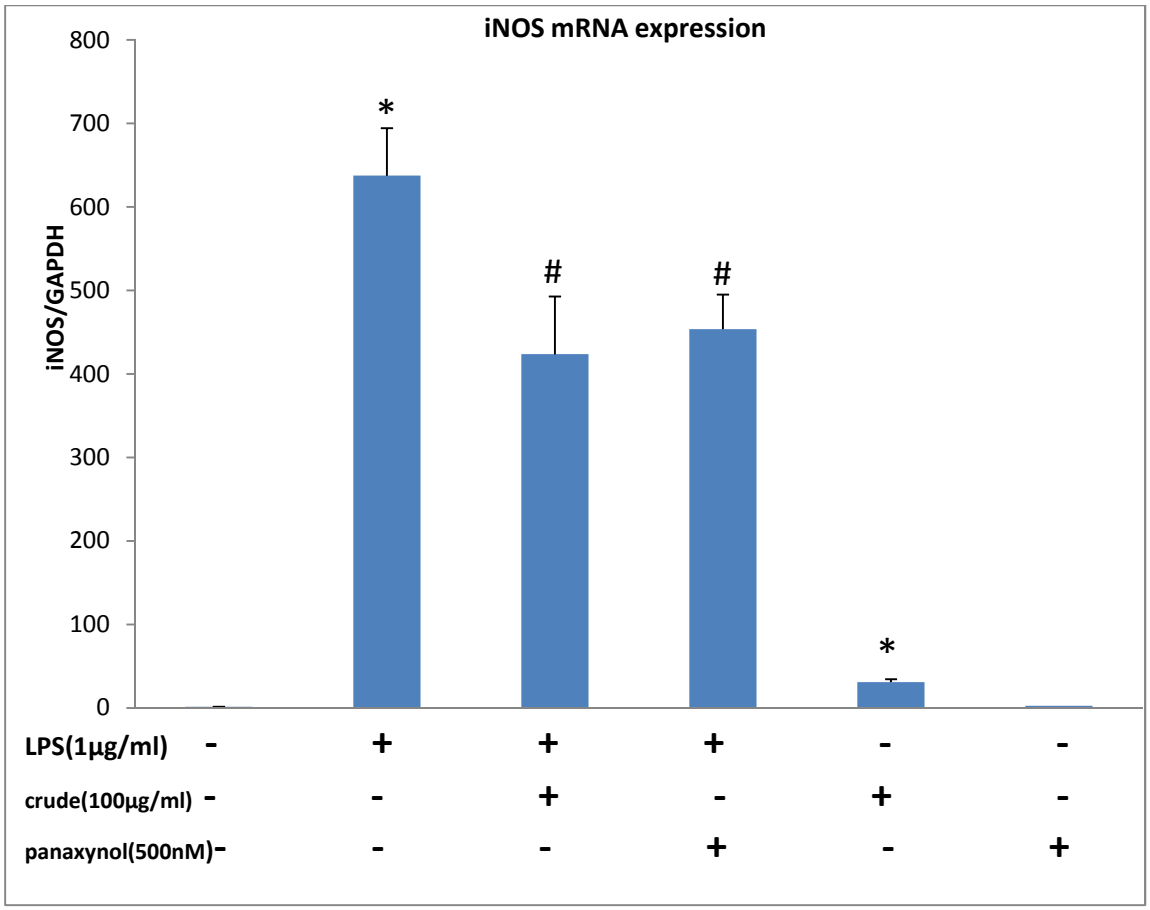

Figure 3.10. Quantitative analysis of mRNA levels of iNOS by Q-PCR . Effect of ginseng crude and panaxynol on LPS-induced expression of iNOS in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1μg/ml, ginseng crude 100μg/ml 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).

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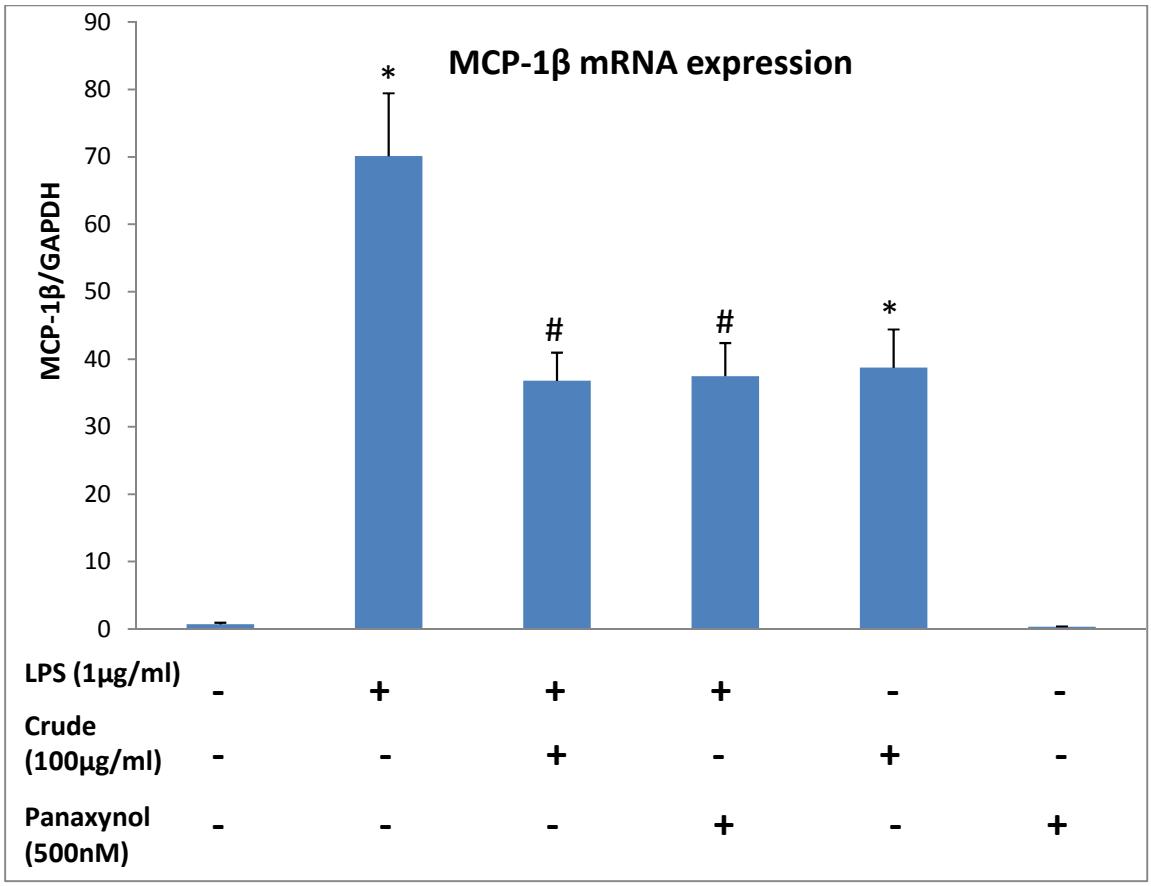

Figure 3.11. Quantitative analysis of mRNA levels of MCP-1β by Q-PCR . Effect of ginseng crude and panaxynol on LPS-induced expression of MCP-1β in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1μg/ml, ginseng crude 100μg/ml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

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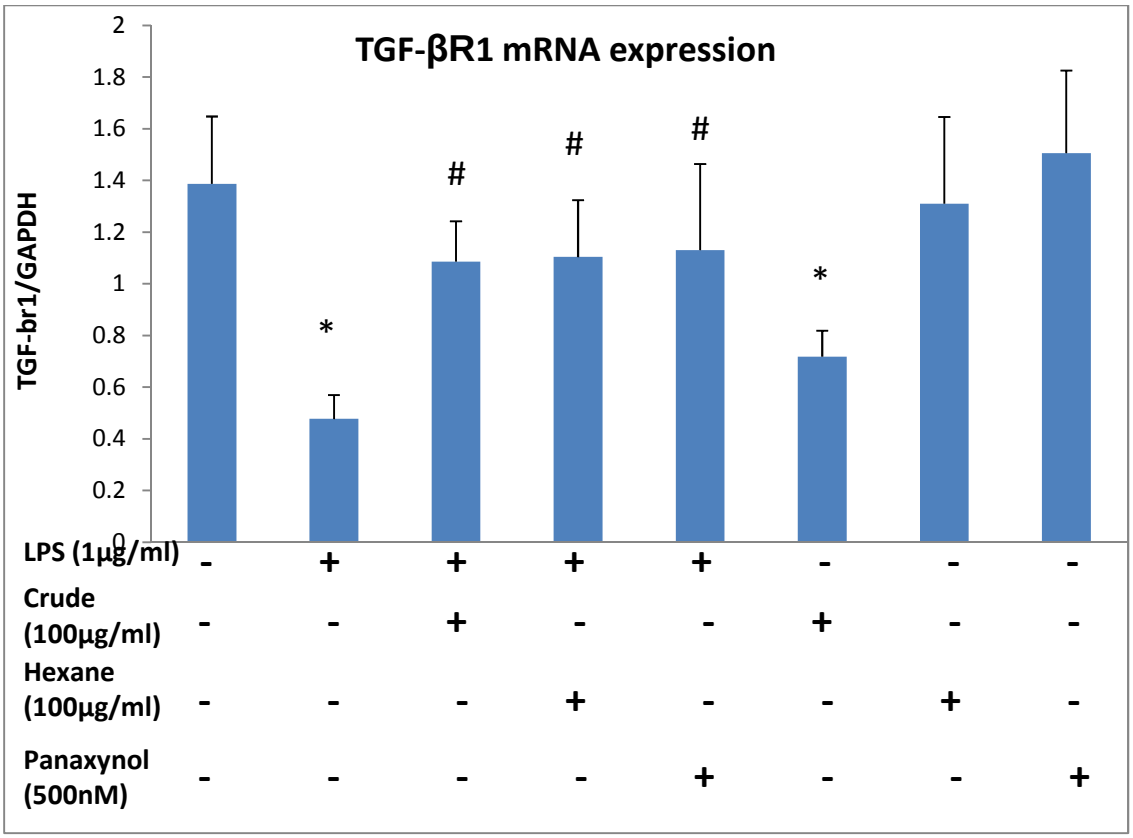

Figure 3.12. Quantitative analysis of mRNA levels of TGF-βR1 by Q-PCR . Effect of ginseng crude, hexane and panaxynol on LPS-induced expression of TGF-βR1 in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1μg/ml, ginseng 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).

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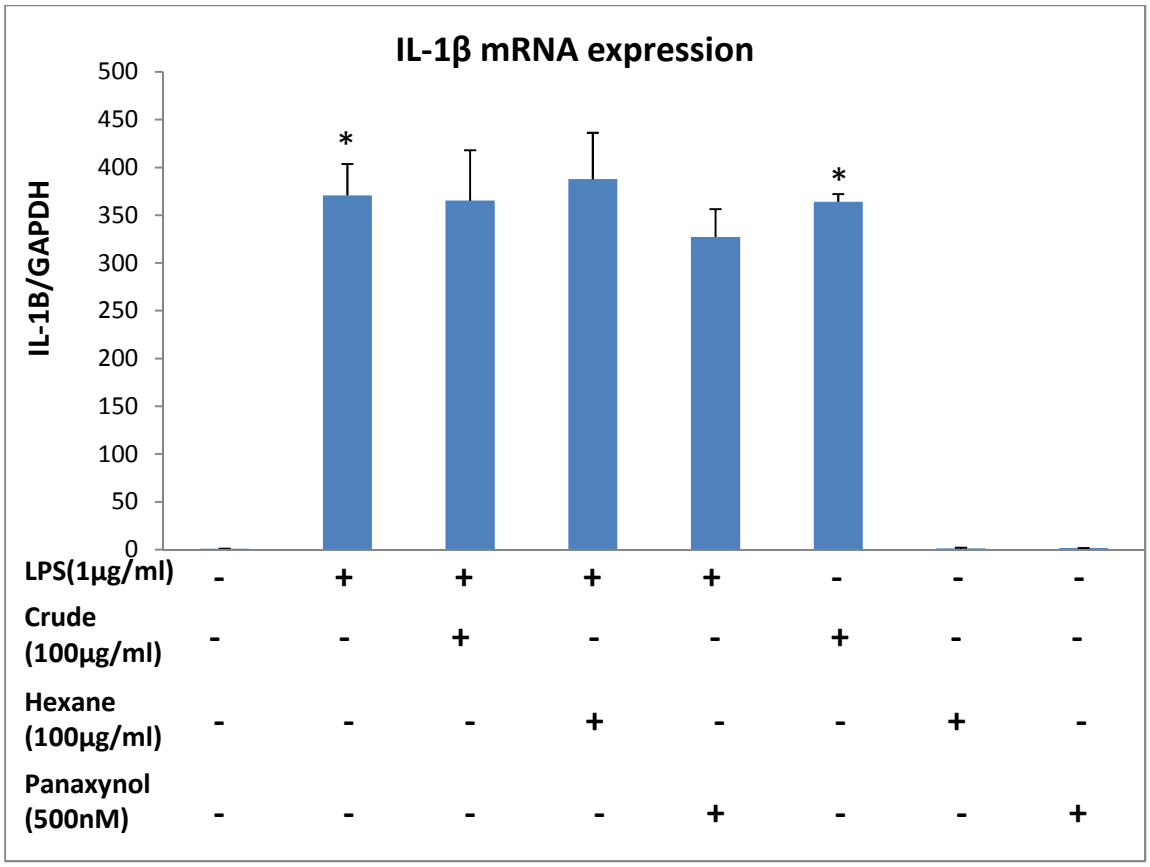

Figure 3.13. Quantitative analysis of mRNA levels of IL-1β by Q-PCR . Effect of ginseng crude, hexane and panaxynol on LPS-induced expression of IL-1β in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1μg/ml, ginseng crude, hexane (dose100μg/ml) and panaxynol 500nM as indicated for 4h. mRNA levels of IL-1β were determined by Q-PCR. n=4, *p < 0.05 vs. control (−), #p < 0.05 vs. LPS (1μg/ml).

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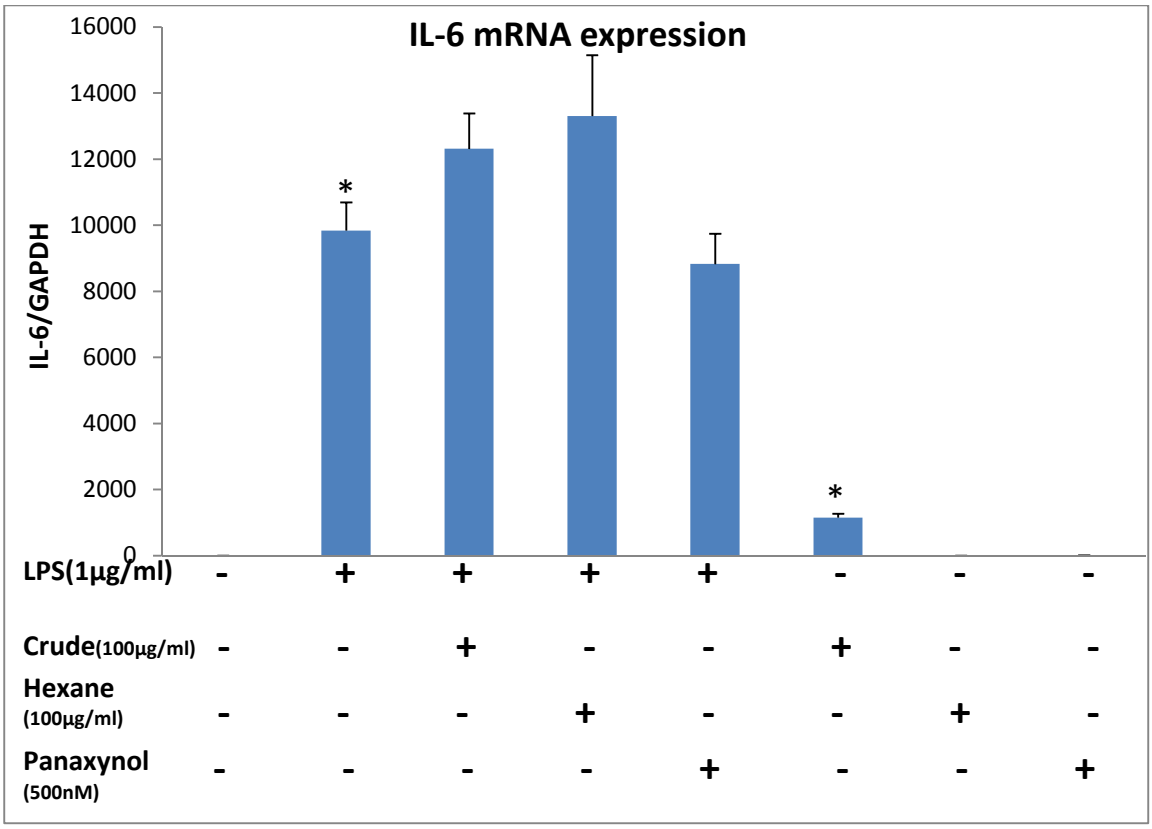

Figure 3.14. Quantitative analysis of mRNA levels of IL-6 by Q-PCR . Effect of ginseng crude, hexane and panaxynol on LPS-induced expression of IL-6 in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1μg/ml, ginseng crude, 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).

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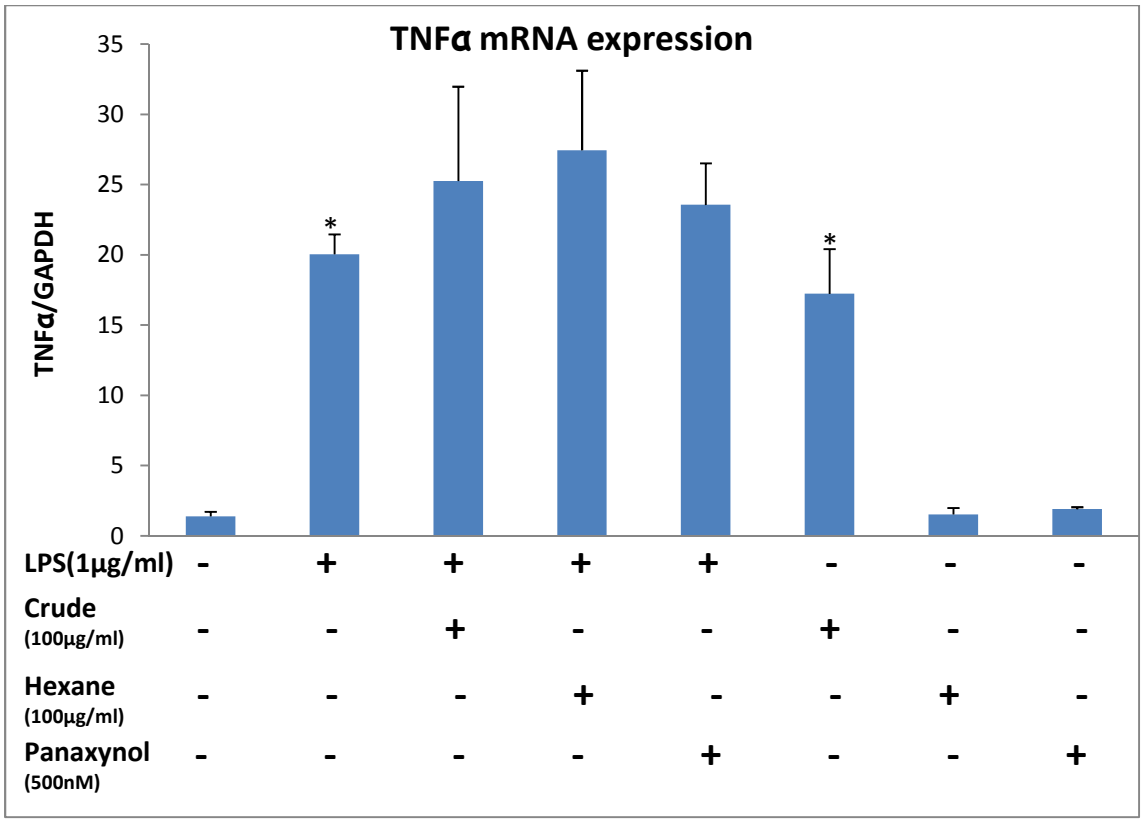

Figure 3.15. Quantitative analysis of mRNA levels of TNFα by Q-PCR . Effect of ginseng crude, hexane and panaxynol on LPS-induced expression of TNFα in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with or without LPS 1μg/ml, ginseng crude,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

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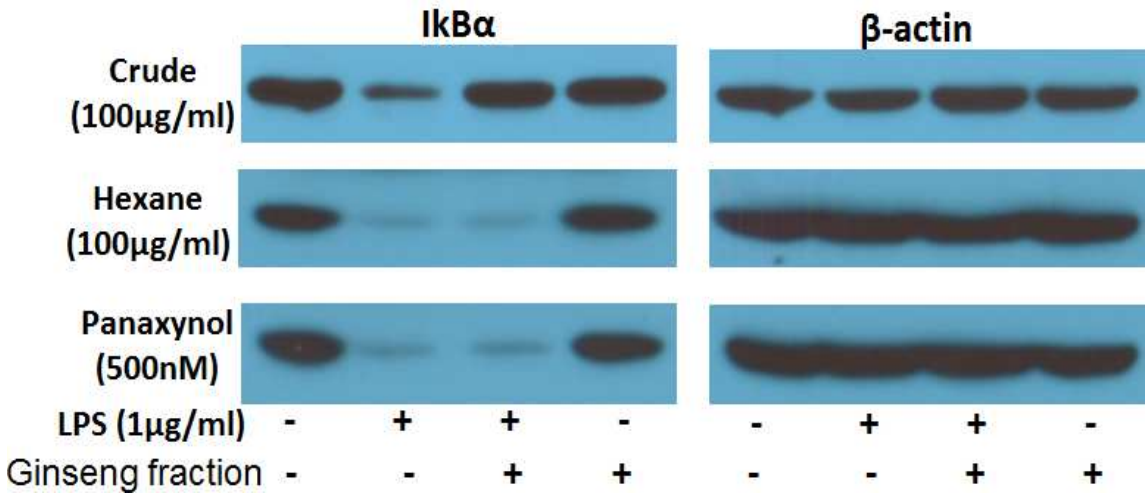

Figure 3.16. Western blot analyses of IKBα and β-actin proteins. Effect of ginseng crude, hexane and panaxynol on expression of IKBα protein in inflamed RAW264.7 macrophages. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM and stimulated with or without LPS 1µg/ml and ginseng crude or hexane (dose100µg/ml) or panaxynol 500nM as indicated for 30 minutes. Protein levels of IKBα were determined by Western blot. Load protein is 20µg/well. Results are representatives of three independent experiments.

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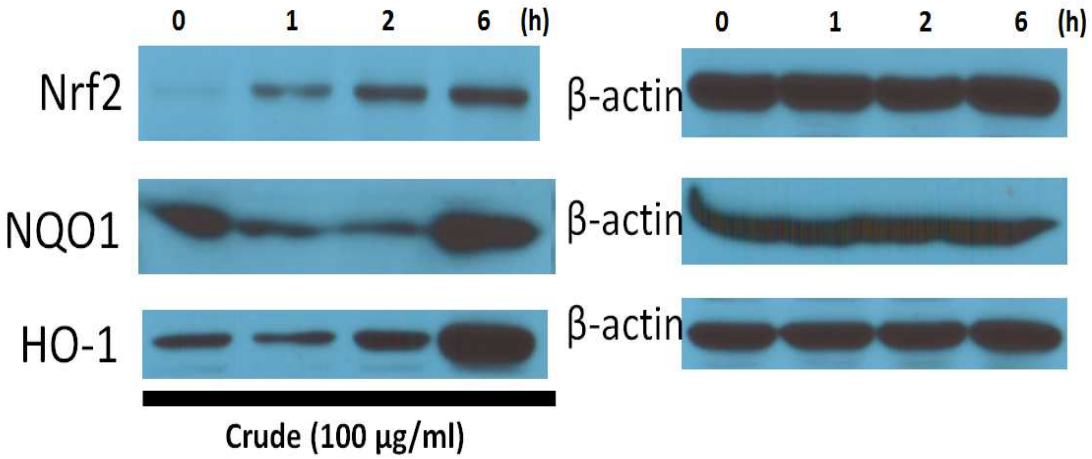

Figure 3.17. Western blot analyses of Nrf2, NQO1, HO-1 and β-actin proteins. Effect of ginseng crude on the activation of Nrf2 signaling in RAW264.7 macrophages. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM and stimulated with ginseng crude 100µg/ml for 1, 2, 6 h. Protein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

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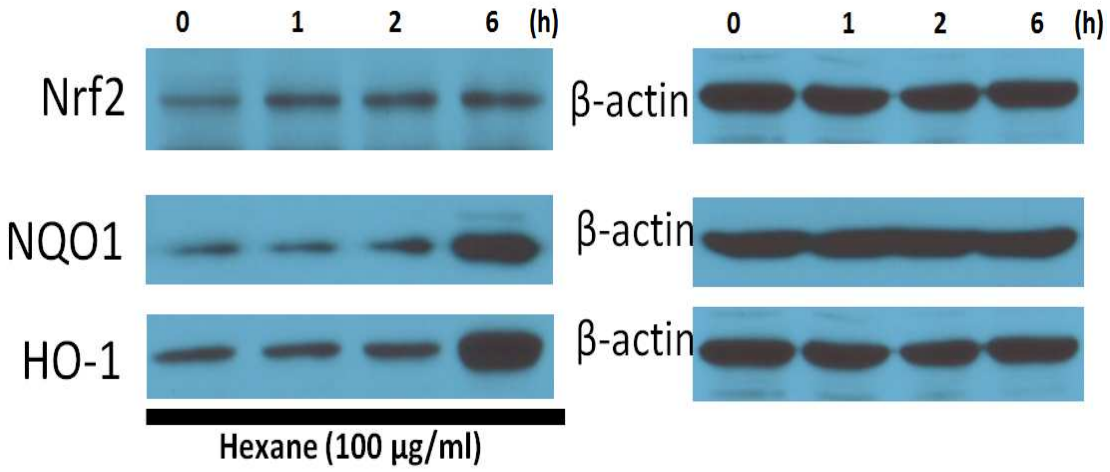

Figure 3.18. Western blot analyses of Nrf2, NQO1, HO-1 and β-actin proteins. Effect of hexane on the activation of Nrf2 signaling in RAW264.7 macrophages. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM and stimulated with hexane 100µg/ml for 1, 2, 6 h. Protein levels of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

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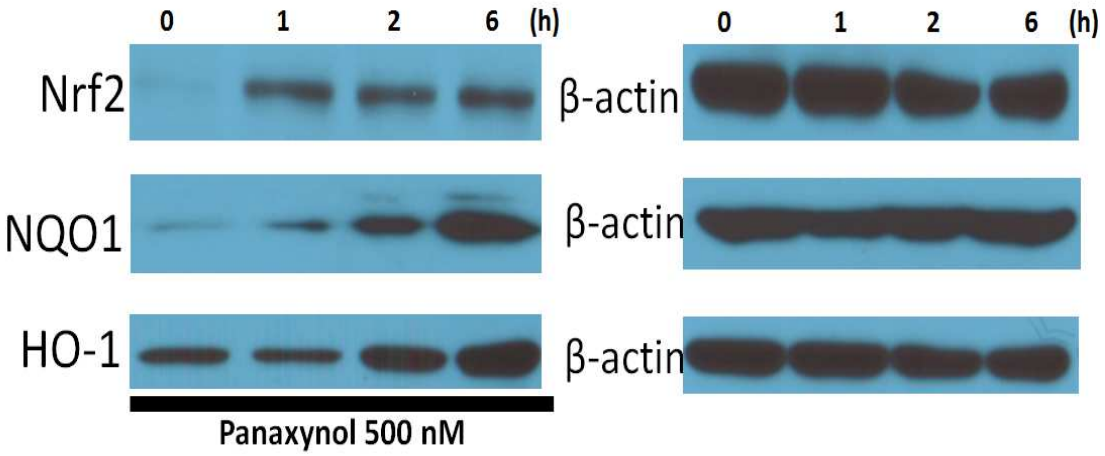

Figure 3.19. Western blot analyses of Nrf2, NQO1, HO-1 and β-actin proteins. Effect of Panaxynol on the activation of Nrf2 signaling in RAW264.7 macrophages. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM and stimulated with Panaxynol 500nM for 1, 2, 6 h. Protein levels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

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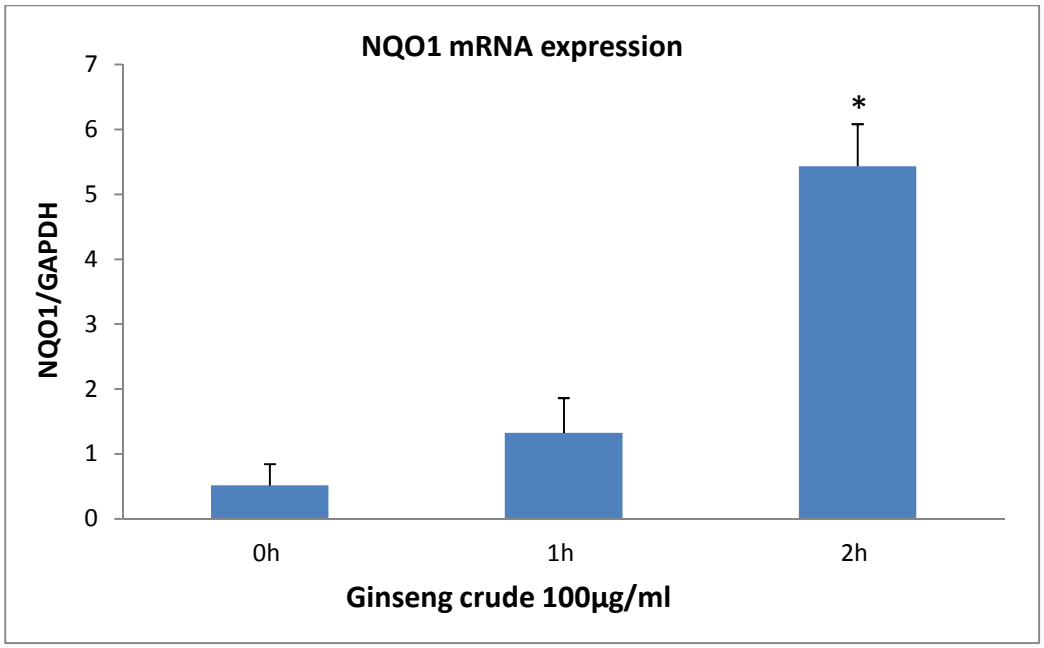

Figure 3.20. Quantitative analysis of mRNA levels of NQO1 by Q-PCR. Effect of ginseng crude on expression of NQO1 mRNA in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with ginseng crude 100μg/ml for 0, 1, 2 h. mRNA levels of NQO1 were determined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

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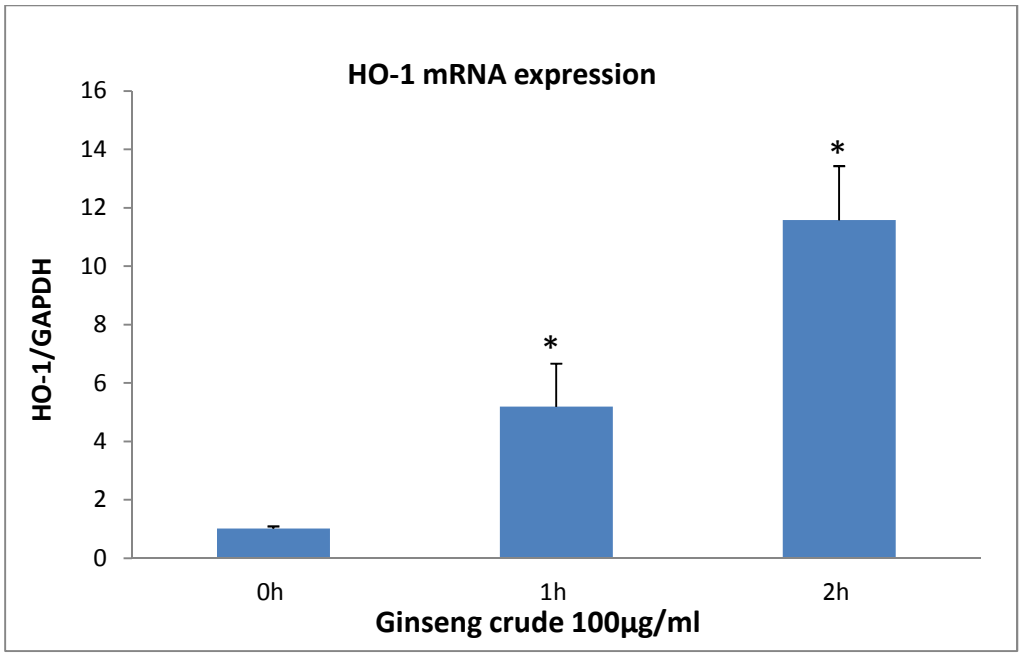

Figure 3.21. Quantitative analysis of mRNA levels of HO-1 by Q-PCR. Effect of ginseng crude on expression of HO-1 mRNA in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with ginseng crude 100µg/ml for 0, 1, 2 h. mRNA levels of HO-1 were determined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

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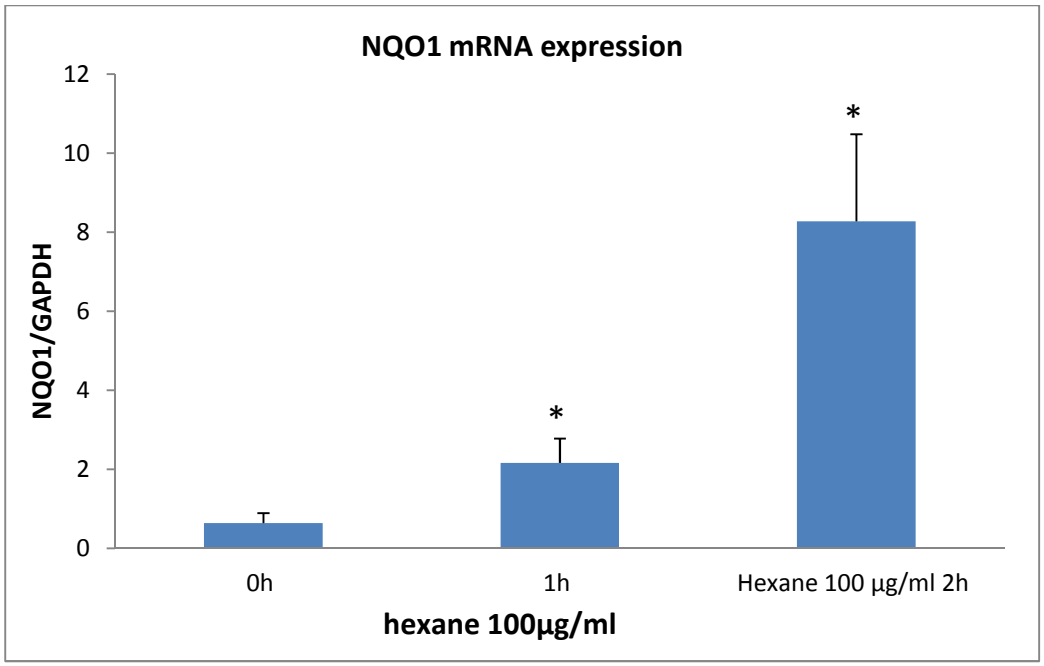

Figure 3.22. Quantitative analysis of mRNA levels of NQO1 by Q-PCR. Effect of hexane on expression of NQO1 mRNA in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with hexane 100µg/ml for 0, 1, 2 h. mRNA levels of NQO1 were determined by Q-PCR.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

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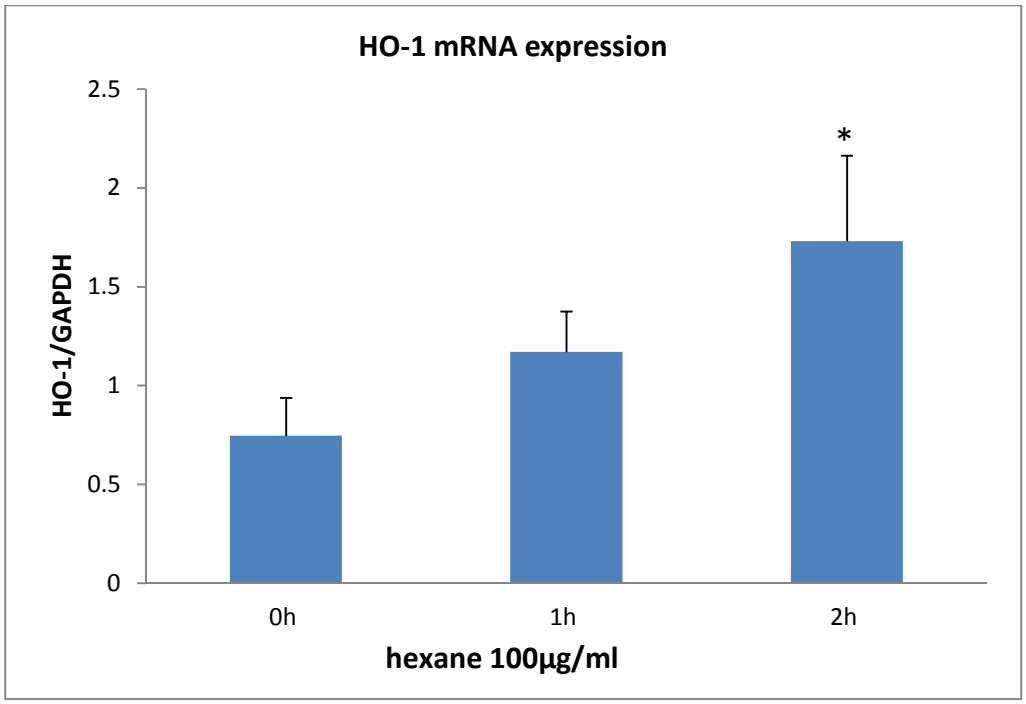

Figure 3.23. Quantitative analysis of mRNA levels of HO-1 by Q-PCR. Effect of hexane on expression of HO-1 mRNA in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with hexane 100µg/ml for 0, 1, 2 h. mRNA levels of HO-1 were determined by Q-PCR.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

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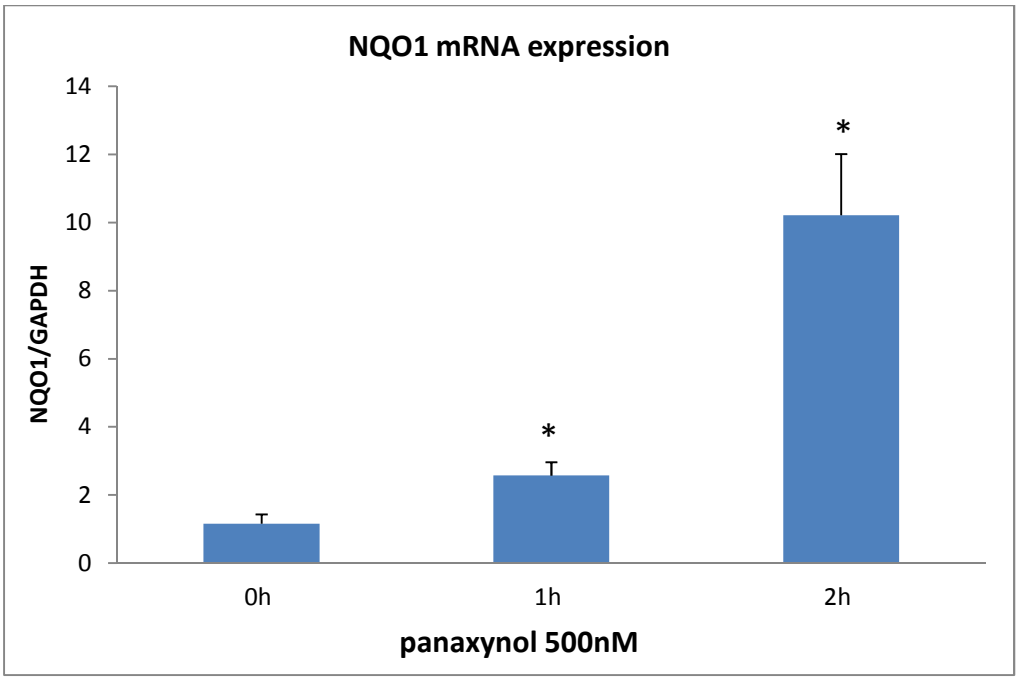

Figure 3.24. Quantitative analysis of mRNA levels of NQO1 by Q-PCR. Effect of panaxynol on expression of NQO1 mRNA in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with panaxynol 500 nM for 0, 1, 2 h. mRNA levels of NQO1 were determined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

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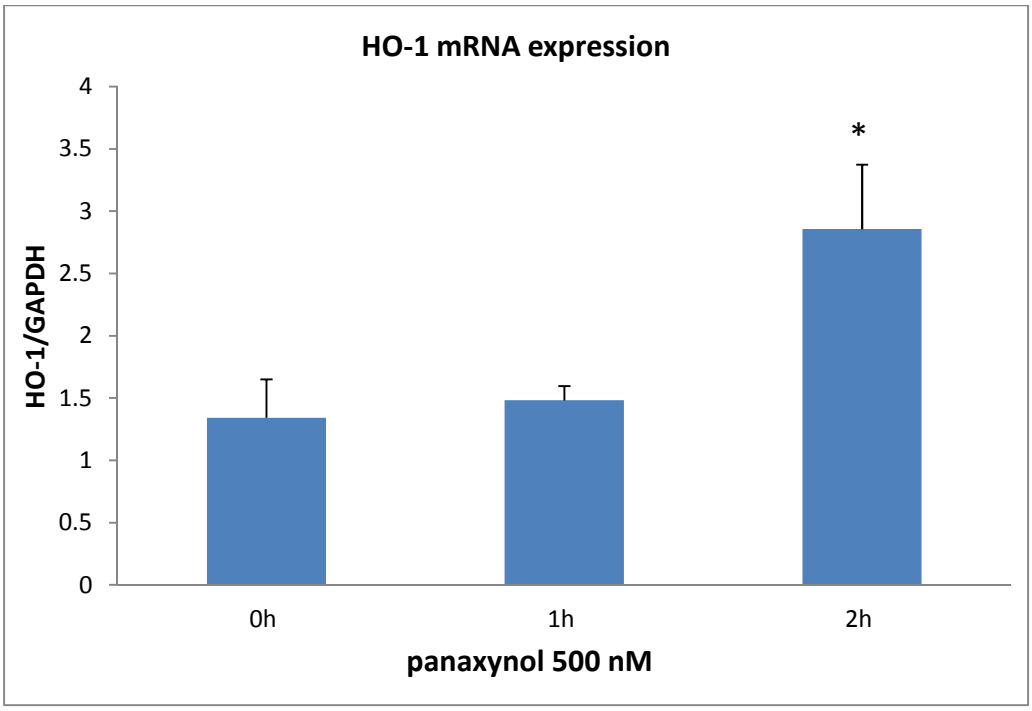

Figure 3.25. Quantitative analysis of mRNA levels of HO-1 by Q-PCR. Effect of panaxynol on expression of HO-1 mRNA in RAW264.7 cells. RAW 264.7 cells were cultured in 10% FBS DMEM for 48h, changed to 2% FBS DMEM, and stimulated with panaxynol 500 nM for 0, 1, 2 h. mRNA levels of HO-1 were determined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

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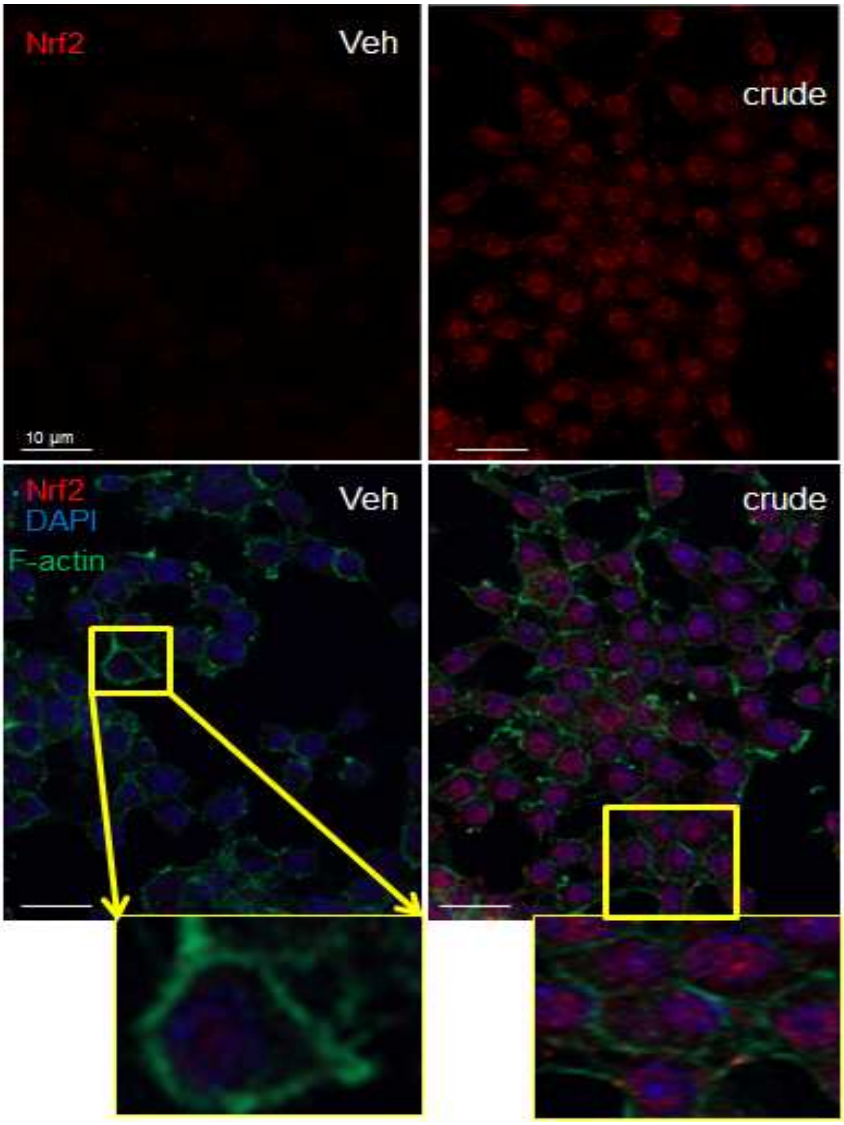

Figure 3.26. Immunofluorescence staining of Nrf2. Effect of ginseng crude on activation of Nrf2 signaling in RAW264.7 macrophages. RAW264.7 cells were treated with or without ginseng crude (100µg/ml) for 1 h. Nrf2 (red); F-action (Green); Nuclei (Blue).

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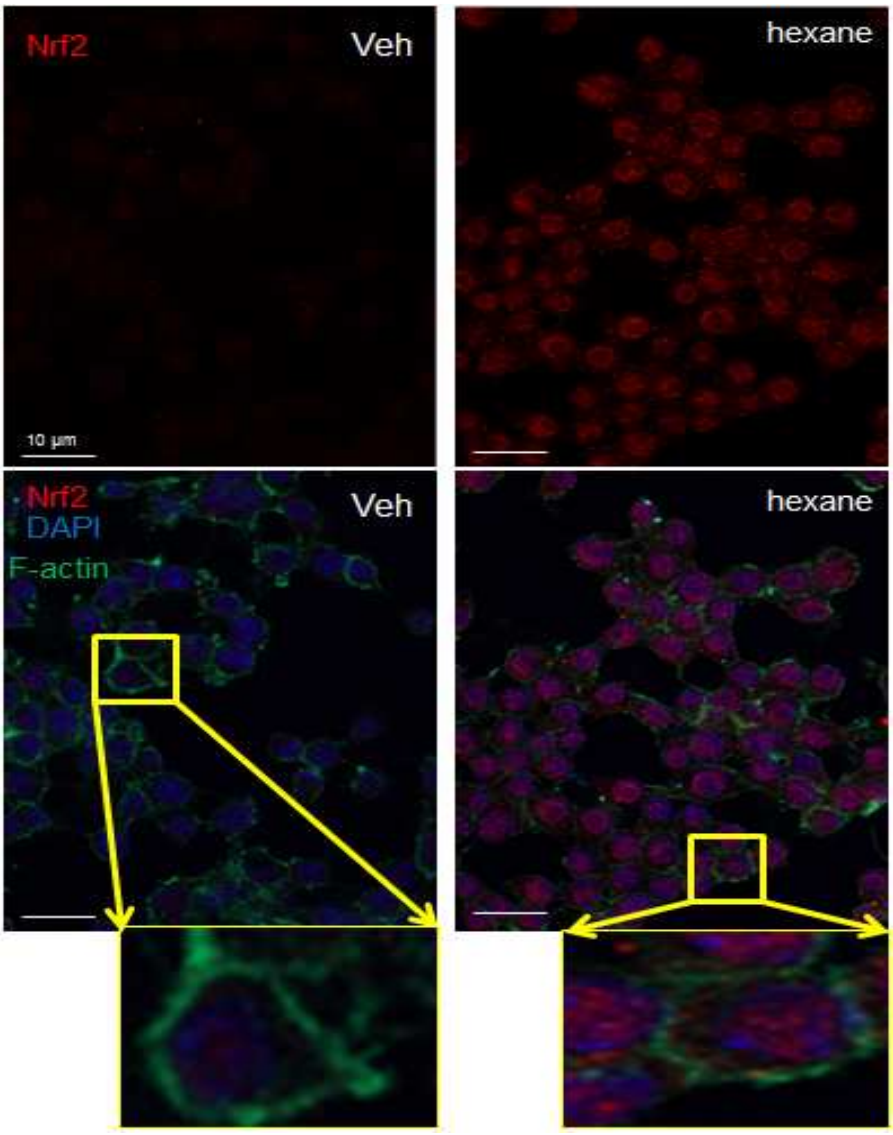

Figure 3.27. Immunofluorescence staining of Nrf2. Effect of hexane on activation of Nrf2 signaling in RAW264.7 macrophages. RAW264.7 cells were treated with or without hexane (100µg/ml) for 1 h. Nrf2 (red); F-action (Green); Nuclei (Blue).

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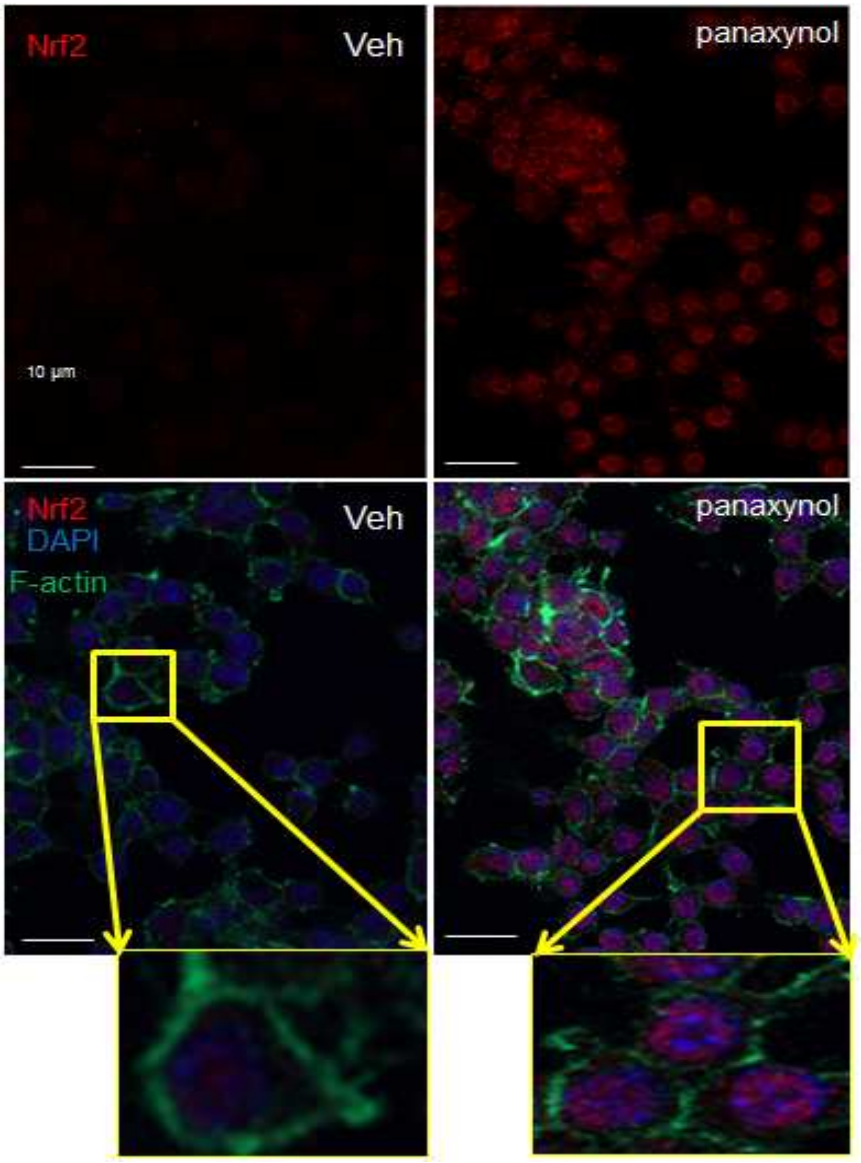

Figure 3.28. Immunofluorescence staining of Nrf2. Effect of panaxynol on activation of Nrf2 signaling in RAW264.7 macrophages. RAW264.7 cells were treated with or without panaxynol (500nM) for 1 h. Nrf2 (red); F-action (Green); Nuclei (Blue).

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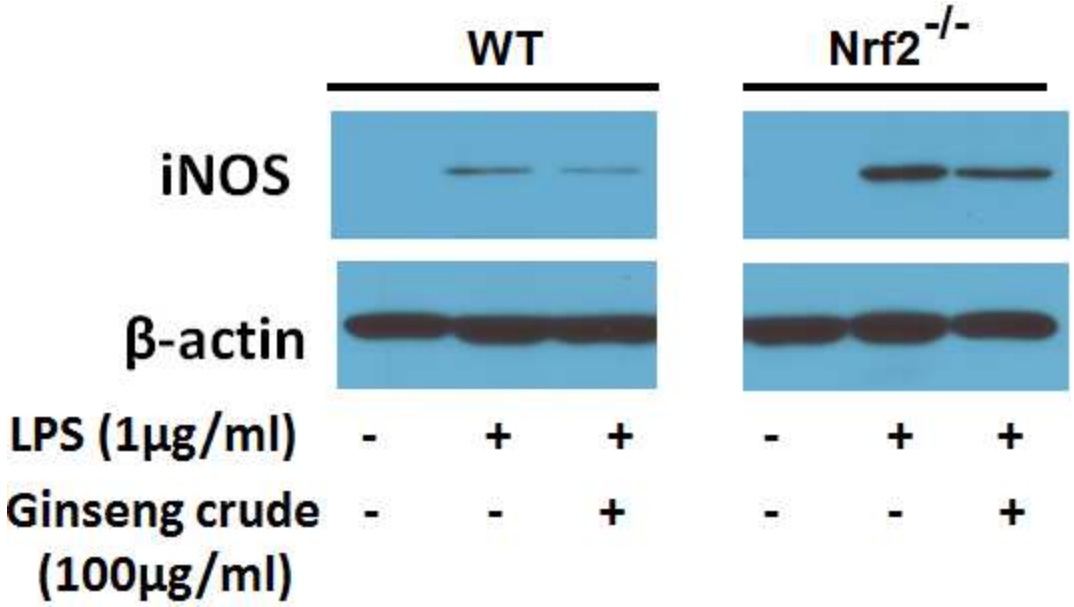

Figure 3.29. Western blot analyses of iNOS and β-actin proteins. Effect of ginseng crude on iNOS expression in inflamed peritoneal-derived macrophages isolated from WT and Nrf2$^{-/-}$ mice. Peritoneal macrophages were elicited by intraperitoneal injection of 1ml starch (6%) in distilled water. After 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 by1x PBS and changed to frsh 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.

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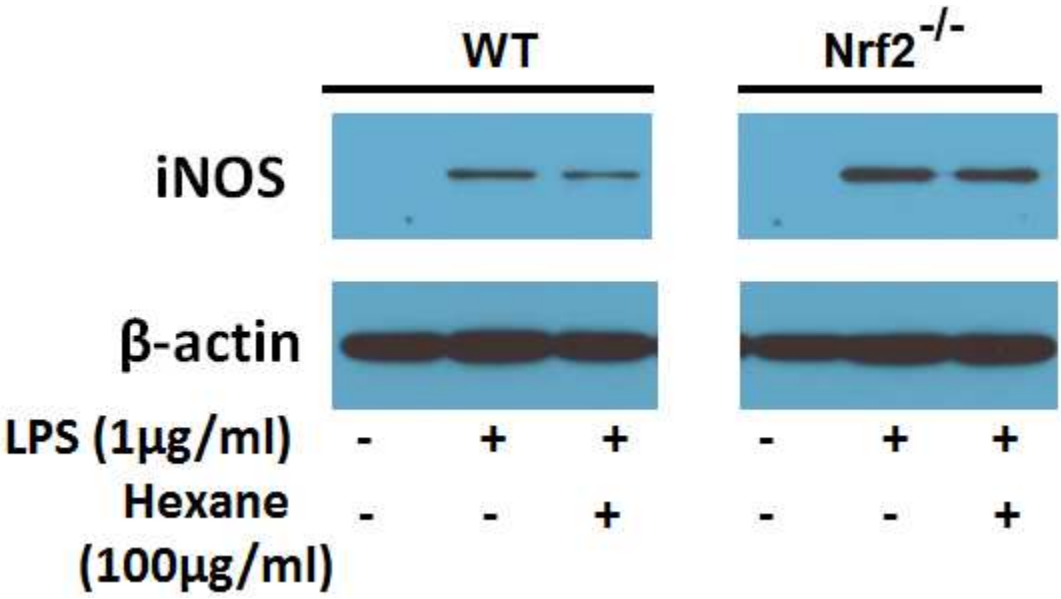

Figure 3.30. Western blot analyses of iNOS and β-actin proteins. Effect of hexane on iNOS expression in inflamed peritoneal-derived macrophages isolated from WT and Nrf2$^{-/-}$ mice. Peritoneal macrophages were elicited by intraperitoneal injection of 1ml starch (6%) in distilled water. After 72h cells were harvested and plated in 10% FBS DMEM medium with 100U/ml penicillin and streptomycin and 2mM glutamine. After 2 hours cells were washed by1x PBS and changed to frsh 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.

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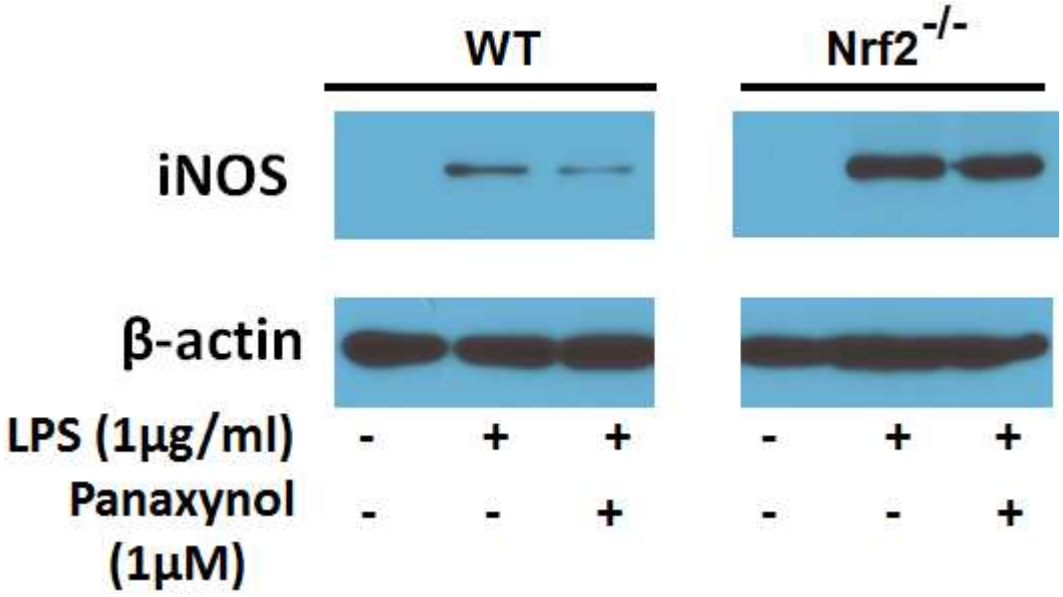

Figure 3.31. Western blot analyses of iNOS and β-actin proteins. Effect of panaxynol on iNOS expression in inflamed peritoneal-derived macrophages isolated from WT and Nrf2$^{-/-}$ mice. Peritoneal macrophages were elicited by intraperitoneal injection of 1ml starch (6%) in distilled water. After 72h 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 by1x PBS and changed to frsh 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.

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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-1β 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

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demonstrate that Panaxynol suppresses the inflammatory response in LPS-inflamed macrophages by specifically activating Nrf2 signaling independent of NF-κB, 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-/-mice. But, the precise mechanism by which Panaxynol activates Nrf2 in macrophages remains unknown.

NF-κB pathway also plays a critical role in the pro-inflammatory response in macrophage. We observed that Panaxynol cannot regulate the NF-κB pathway. Panaxynol was unable to prevent degradation of IκB and hence, cannot active the NF-κB pathway in the inflamed macrophages. Also, panaxynol is unable to inhibit the cytokines that are produced by NF-κB pathway activation, namely, IL-1β, IL-6 and TNFα. 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-1β, IL-1β, IL-6, and TNF-α mRNA level, panaxynol alone does not affect iNOS, MCP-1β, IL-1β, IL-6, and TNF-α. 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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REFERENCES

Adams, J., Kelso, R., Cooley, L. (2000). The kelch repeat superfamily of proteins: propellers of cell function. Trends Cell Biol., 10, 17–24.

Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, M. K., Cook, J. L. (1999). Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J. Biol. Chem., 274, 26071–26078.

Alexander, W. S., Hilton, D. J. (2004). The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. Annu Rev Immunol., 22, 503-529.

Angel, P., Hattori, K., Smeal, T., Karin, M. (1988). The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell, 55, 875–885.

Angel, P., Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim. Biophys. Acta., 1072, 129–157.

Argetsinger, L. S., Campbell, G. S.,Yang, X., Witthuhn, B., Silvennoinen, O., Ihle, N., Carter-Su, C. (1993). Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell, 74, 237-244.

Assinewe, V.A., Baum, B.R., Gagnon, D., Arnason, J.T. (2003). Phytochemistry of wild populations of Panax quinquefolius L. (North American ginseng). J. Agric. Food Chem., 51, 4549–4553.

Attele, A.S., Wu, J.A., Yuan, C.S. (1999). Ginseng pharmacology: multiple constituents and multiple actions. Biochem. Pharmacol., 58, 1685–1693.

Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A., Karin, M. (1995). Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis. Science, 270, 286–290.

Baldwin, A. S. (1996). The NF-κB and IκB proteins: new discoveries and insights. Annu. Rev. Immunol., 14, 649–681.

Bao, H.Y., Zhang, J., Yeo, S.J., Myung, C.S., Kim, H.M., Kim, J.M., Park, J.H., Cho, J., Kang, J.S. (2005). Memory enhancing and neuroprotective effects of selected ginsenosides. Arch. Pharm. Res., 28, 335–342.

www.manaraa.com

Bardwell, V. J., Treisman, R. (1994). The POZ domain: a conserved protein-protein interaction motif. Genes Dev., 8, 1664–1677.

Beg, A. A., Finco, T. S., Nantermet, P. V., Baldwin, A. S. Jr. (1993). Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκB: a mechanism for NFκB activation. Mol. Cell. Biol., 13, 3301– 3310.

Beg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S., Baltimore, D., 1995. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-κB. Nature, 376, 167–170.

Blackwell, T. S., Christman, J. W. (1997). The Role of Nuclear Factor-kB in Cytokine Gene Regulation Am J Respir Cell Mol Biol., 17, 3-9.

Blank, V., Kourilsky, P., Israel, A. (1991). Cytoplasmic retention, DNA binding and processing of the NF-κB p50 precursor are controlled by a small region in its C-terminus. EMBO J., 10, 4159–4167.

Bloom, D. A., Jaiswal, A. K. (2003). Phosphorylation of Nrf2 at Ser(40) by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/ accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. J. Biol. Chem., 278, 44675–44682.

Bonizzi, G., Karin, M., 2004. The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol. 25, 280–288.

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H., Yancopoulos, G. D. (1991). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell, 65, 663–675.

Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slaughter, C., Moomaw, C., Hsu, J., Cobb, M. H. (1990). An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science, 249, 64–67.

Bours, V., Burd, P. R., Brown, K., Villalobos, J., Park, S., Ryseck, R. P., Bravo, R., Kelly, K., Siebenlist, U. (1992). A novel mitogen inducible gene product related to p50/ p105-NF-κB participates in transactivation through a κB site. Mol. Cell. Biol., 12, 685–695.

Breen, A. P., Murphy, J. A. (1995). Reactions of oxyl radicals with DNA. Free Radic. Biol. Med., 18, 1033–1077.

Breimer, L. H. (1990). Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: the role of DNA base damage. Mol. Carcinog., 3, 188–197.

www.manaraa.com

Brockman, J. A., Scherer, D. C., McKinsey, T. A., Hall, S. M., Qi, X., Lee, W. Y., Ballard, D. W. (1995). Coupling of a signal response domain in IκB to multiple pathways for NF-κB activation. Mol. Cell Biol., 15, 2809–2818.

Brown, A., Linhoff, M., Stein, B., Wright, K., Baldwin, A., Basta, P., Ting, J. (1994). Function of NF-κB/Rel binding sites in the major histocompatibility complex class II invariant chain promoter is dependent on cell-specific binding of different NF-κB/Rel subunits. Mol. Cell. Biol., 14, 2926–2935.

Brown, K., Gerstberger, S., Carlson, L., Granzoso, G., Siebenlist, U. (1995). Control of IκB-α proteolysis by site-specific, signal-induced phosphorylation. Science, 267, 1485–1488.

Brown, K., Park, S., Kanno, T., Franzoso, G., Siebenlist, U. (1993). Mutual regulation of the transcriptional activator NF-κB and its inhibitor, IκB-α. Proc. Natl. Acad. Sci., 90, 2532–2536.

Burns, K., Martinon, F., Esslinger, C., Pahl, H., Schneider, P., Bodmer, J. L., Di Marco, F., French, L., Tschopp, J. (1998). MyD88, an adaptor protein involved in interleukin-1 signaling. J Biol Chem., 273, 12203–12209.

Cao, Z., Henzel, W. J., Gao, X. (1996). IRAK: a kinase associated with the interleukin-1 receptor. Science, 271, 1128–1131.

Chan, J. Y., Han, X., Kan, Y. W. (1993). Cloning of Nrf1, an NF-E2- related transcription factor, by genetic selection in yeast. Proc. Natl. Acad., Sci. 90, 11371–11375.

Chen, J., Zhao, R., Zeng, Y.M., Meng, H., Zuo, W.J., Li, X., Wang, J.H. (2009). Three new triterpenoid saponins from the leaves and stems of Panax quinquefolium. J. Asian Nat. Prod. Res., 11, 195–201.

Chen, Z., Hagler, J., Palombello, V., Melandri, F., Scherer, D., Ballard, D., Maniatis, T. (1995). Signal-induced site-specific phosphorylation targets IκB to the ubiquitin proteasome pathway. Genes Dev., 9, 1586–1597.

Chiao, P. J., Miyamoto, S., Verma, I. M. (1994). Autoregulation of IκB-α activity. Proc. Natl. Acad. Sci., 91, 28–32.

Choi, A. M., Alam, J. (1996). Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidantinduced lung injury. Am. J. Respir. Cell. Mol. Biol., 15, 9–19.

Christensen, L.P. (2009). Ginsenosides chemistry, biosynthesis, analysis, and potential health effects. Adv. Food Nutr. Res., 55, 1–99.

www.manaraa.com

Chung, C. D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P., Shuai, K. (1997). Specific inhibition of Stat3 signal transduction by PIAS3. Science, 278, 1803-1805.

Clancy, R. M., Abramson, S. B. (1995). Nitric oxide: a novel mediator of inflammation. Proc Soc Exp Biol Med., 210, 93–101.

Cohen, J. (2002). The immunopathogenesis of sepsis. Nature, 420, 885–891.

Cooney, R. N. (2002). Suppressors of cytokine signaling (SOCS): inhibitors of the JAK/STAT pathway. Shock, 17, 83-90.

Cullinan, SB., Gordan, JD., Jin, J., Harper, J, W., Diehl, J. A. (2004). The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. Mol Cell Biol., 24, 8477–8486.

Curran, T., Franza B. R. Jr. (1988). Fos and Jun: the AP-1 connection. Cell, 55, 395–397.

Darnell, J. E. Jr. (1997). STATs and gene regulation. Science, 277, 1630-1635.

Dérijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., Karin, M., Davis, R. J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell, 76, 1–20.

Devary, Y., Gottlieb, R., Smeal, T., Bauskin, A. R., Ben-Neriah, Y., Karin, M. (1992). The mammalian ultraviolet response is triggered by activation of Src tyrosine kinases. Cell, 71, 1081–1091.

Dhakshinamoorthy, S., Jaiswal, A. K. (2001). Functional characterization and role of INrf2 in antioxidant response element-mediated expression and antioxidant induction of NAD(P)H:quinone oxidoreductase1 gene. Oncogene, 20, 3906–3917.

Dhakshinamoorthy, S., Long, D. J. II., Jaiswal, A. K. (2000). Antioxidant regulation of genes encoding enzymes that detoxify xenobiotics and carcinogens. Curr. Top. Cell. Regul., 36, 201–206.

DiDonato, J., Mercurio, F., Karin, M. (1995). Phosphorylation of IκB precedes but is not sufficient for its dissociation from NF-κB. Mol. Cell. Biol., 15, 1302–1311.

Domann, F. E., Levy, J. P., Birrer, M. J., Bowden, G. T. (1994). Stable expression of a c-JUN deletion mutant in two malignant mouse epidermal cell lines blocks tumor formation in nude mice. Cell Growth Differ., 5, 9–16.

Dong, Z., Birrer, M. J., Watts, R. G., Matrisian, L. M., Colburn, N. H. (1994). Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. Proc. Natl. Acad. Sci., 91, 609–613.

www.manaraa.com

Dong, Z., Crawford, H. C., Lavrovsky, V., Taub, D., Watts, R., Matrisian, L. M., Colburn, N. H. (1997). A dominant negative mutant of Jun blocking 12-O-tetradecanoylphorbol-13-acetate-induced invasion in mouse keratinocytes. Mol. Carcinog., 19, 204–212.

Doni, M. G., Whittle, B. J., Palmer, R. M., Moncada, S. (1988). Actions of nitric oxide on the release of prostacyclin from bovine endothelial cells in culture. EurJ Pharmacol., 151, 19–25.

Du, X.W., Wills, R.B. H., Stuart, D.L. (2004). Changes in neutral and malonyl ginsenosides in American ginseng (Panax quinquefolium) during drying, storage and ethanolic extraction. Food Chem., 86, 155–159.

Franzoso, G., Bours, V., Azarenko, V., Park, S., Tomita-Vamaguchi, M., Kanno, T., Brown, K., Siebenlist, U. (1993). The oncoprotein Bcl-3 can facilitate NF-κB-mediated transactivation by removing inhibiting p50 homodimers from select kB sites. EMBO J., 12, 3893–3901.

Franzoso, G., Bours, V., Park, S., Tomita-Yamaguchi, M., Kelly, K., Siebenlist, U. (1992). The candidate oncoprotein Bcl-3 is an antagonist of p50/NF-κB-mediated inhibition. Nature, 359, 339–342.

Frost, J. A., Geppert, T. D., Cobb, M. H., Feramisco, J. R. (1994). A requirement for extracellular signal-regulated kinase (ERK) function in the activation of AP-1 by Ha-Ras, phorbol 12-myristate 13-acetate, and serum. Proc. Natl. Acad. Sci., 91, 3844–3848.

Fujihara, M., Connolly, N., Ito, N., Suzuki, T. (1994a). Properties of protein kinase C isoforms in a macrophage cell line and their roles in LPS-induced nitric oxide production. J Immunol., 152, 1898–1906.

Fujihara, M., Ito, N., Pace, J. L., Watanabe, Y., Russell, S. W., Suzuki, T. (1994b). Role of endogenous interferon-h in lipopolysaccharide-triggered activation of the inducible nitric-oxide synthase gene in a mouse macrophage cell line. J Biol Chem., 269, 12773–12778.

Fujihara, M., Muroi, M., Muroi, Y., Ito, N., Suzuki, T. (1993). Mechanism of lipopolysaccharide-triggered junB activation in a mouse macrophage-like cell line. J Biol Chem., 268, 14898–14905.

Fujihara, M., Muroib, M., Tanamotob, K., Suzukic, T., Azumaa, H., Ikedaa, H. (2003). Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacology & Therapeutics, 100, 171–194.

Fuzzati, N. (2004). Analysis methods of ginsenosides. J. Chromatogr. B., 812, 119–133.

www.manaraa.com

Giustarini, D., Dalle-Donne, I., Tsikas, D., Rossi, R. (2009). Oxidative stress and human diseases: origin, link, measurement, mechanisms, and biomarkers. Crit. Rev. Clin. Lab. Sci., 46, 241–281.

Gao, J. J., Filla, M. B., Fultz, M. J., Vogel, S. N., Russell, S. W., Murphy, W. J. (1998). Autocrine/paracrine IFN-αβ mediates the lipopolysaccharide-induced activation of transcription factor Stat1α in mouse macrophages: pivotal role of Stat1α in induction of the inducible nitric oxide synthase gene. J Immunol., 161, 4803–4810.

Garavito, R. M., Mulichak, A. M. (2003).The structure of mammalian cyclooxygenases. Annu Rev Biophys Biomol Struct., 32, 183–206.

Gentz, R., Rauscher III, F. J., Abate, C., Curran, T. (1989). Parallel association of Fos and Jun leucine zippers juxtaposes DNA binding domains. Science, 243, 1695–1699.

Gerondakis, S., Grossmann, M., Nakamura, Y., Pohl, T. & Grumont, R. (1999). Genetic approaches in mice to understand Rel/NF-κB and IκB function: transgenics and knockouts. Oncogene, 18, 6888–6895.

Ghosh, S., Gifford, A. M., Rivere, L. R., Tempest, P., Nolan, G. P., Baltimore, D. (1990). Cloning of the p50 DNA binding subunit of NF-κB: homology to rel and dorsal. Cell, 62, 1019–1029.

Ghosh, S., Karin, M. (2002). Missing pieces in the NF-κB puzzle. Cell, 109, 81–96.

Gille, H., Sharrocks, A., Shaw, P. (1992). Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. Nature, 358, 414–417.

Gillis, C.N., (1997). Panax ginseng pharmacology: a nitric oxide link? Biochemical Pharmacology, 54, 1–8.

Goodwin, D. C., Gunther, M. R., His, L. C., Crews, B. C., Eling, T. E., Mason, R. P., Marnett, L. J. (1998). Nitric oxide trapping of tyrosyl radicals generated during prostaglandin endoperoxide synthase turnover. Detection of the radical derivative of tyrosine 385. J BiolChem., 273, 8903–8909.

Grilli, M., Chiu, J. J-S., Lenardo, M. (1993). NF-κB and Rel: participants in a multiform transcriptional regulatory system. Int. Rev. Cytol., 143, 1–62.

Guha, M., Mackman, N. (2001). LPS induction of gene expression in human monocytes. Cell Signal, 13, 85–94.

Gupta, S., Campbell, D., Derijard, B., Davis, R. J. (1995). Transcription factor ATF2 regulation by the JNK signal transduction pathway. Science, 267, 389–393.

www.manaraa.com

Hambleton, J., Weinstein, S. L., Lem, L., DeFranco, A. L. (1996). Activation of c-Jun N-terminal kinase in bacterial lipopolysaccharidestimulated macrophages. Proc Natl Acad Sci., 93, 2774-2778.

Han, J., Lee, J. D., Bibbs, L., Ulevitch, R. J. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science, 265, 808–811.

Hansen, S,. Baeuerle, P. A,. Blasi, F. (1994a). Purification, reconstitution, and IκB association of the c-Rel-p65 (RelA) complex, a strong activator of transcription. Mol. Cell Biol., 14, 2593–2603.

Hansen, S., Guerrini, L., Blasi, F. (1994b). Differential DNA sequence specificity and regulation of HIV-1 enhancer activity by cRel-RelA transcription factor. J. Biol.Chem., 269, 22230–22237.

Hayden, M. S., Ghosh, S., 2004. Signaling to NF-κB. Genes Dev. 18, 2195–2224.

Hayes, J. D., McMahon, M., Chowdhry, S., Dinkova-Kostova, A. T. (2010). Cancer chemoprevention mechanisms mediated through the Keap1-Nrf2 pathway. Antioxid Redox Signal, 13, 1713–1748.

Herrera-Velit, P., Reiner, N. E. (1996). Bacterial lipopolysaccharide induces the association and coordinate activation of p53/56lyn and phosphatidylinositol 3-kinase in human monocytes. J Immunol., 156, 1157–1165.

Hirakura, K., Morita, M., Niitsu, K., Ikeya, Y., Maruno, M. (1994). Constituents of panax ginseng; linoleoylated polyacetylenes from the root of p. ginseng. Phytochemistry, 35, 963-967.

Hoffmann, A., Leung, T. H., Baltimore, D. (2003). Genetic analysis of NF-κB/Rel transcription factors defines functional specificities. EMBO J., 22, 5530–5539.

Hofseth, L. J., Ying, L. (2006). Identifying and defusing weapons of mass inflammation in carcinogenesis. Biochim Biophys Acta., 1765, 74–84.

Hofseth, L.J.,Wargovich, M.J. (2007). Inflammation, cancer and targets of ginseng. The Journal of Nutrition, 137, 183–185.

Huang, C., Ma, W. Y., Young, M. R., Colburn, N., Dong, Z. (1998). Shortage of mitogen-activated protein kinase is responsible for resistance to AP-1 transactivation and transformation in mouse JB6 cells. Proc. Natl. Acad. Sci., 95, 156–161.

Ichikawa, T., Li, J.Q., Nagarkatti, P., Nagarkatti, M., Hofseth, L.J., Windust, A., Cui, T. X. (2009). American ginseng preferentially suppresses STAT/iNOS signaling in activated macrophages. J. Ethnopharmacol., 125, 145–150.

www.manaraa.com

Inoue, J-i., Derr, L., Kakizuka, A., Verma, I. (1992). IκBγ, a 70 kd protein identical to the C-terminal half of p110 NF-κB: a new member of the IκB family. Cell, 68, 1109–1120.

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, K., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M., Nabeshima, Y. (1997). An Nrf2/small maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem. Biophys. Res. Commun., 236, 313–322.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D., Yamamoto, M. (1999). Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev., 13, 76–86.

Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., O'Connor, T., Yamamoto, M. (2003). Keap1 regulates both cytoplasmic–nuclear shuttling and degradation of Nrf2 in response to electrophiles. Genes Cells, 8, 379–391.

Jaiswal, A. K. (2000). Regulation of genes encoding NAD(P)H:quinone oxdoreductases. Free Radic. Biol. Med., 29, 254–262.

Jakway, J. P., DeFranco, A. L. (1986). Pertussis toxin inhibition of B cell and macrophage responses to bacterial lipopolysaccharide. Science, 234, 743– 746.

Jia, L., Zhao, Y.Q. (2009). Current evaluation of the millennium phytomedicineginseng (I): etymology, pharmacognosy, phytochemistry, market and regulations. Curr. Med. Chem., 16, 2475–2484.

Jiang, H.P., Qiu, Y.K., Cheng, D.R., Kang, T.G., Dou, D.Q. (2008). Structure elucidation and complete NMR spectral assignments of two new dammarane-type tetraglycosides from Panax quinquefolium. Magn. Reson. Chem., 46, 786–790.

John, S.,Vinkemeier, U., Soldaini, E., Darnell, J. E. Jr., Leonard, W. J. (1999). The significance of tetramerization in promoter recruitment by Stat5. Mol Cell Biol., 19, 1910-1918.

Joseph, P., Jaiswal, A. K. (1994). NAD(P)H:quinone oxidoreductase1 (DT diaphorase) specifically prevents the formation of benzo(a)- pyrene quinone–DNA adducts generated by cytochrome P4501A1 and P450 reductase. Proc. Natl. Acad. Sci., 91, 8413–8417.

Karin, M. (1995). The Regulation of AP-1 Activity by Mitogen-activated Protein Kinases. J Biol Chem., 270, 16483-16486.

Katoh, Y., Iida, K., Kang, M. I., Kobayashi, A., Mizukami, M., Tong, K. I., McMahon, M., Hayes, J. D., Itoh, K., Yamamoto, M. (2005). Evolutionary conserved N-terminal

www.manaraa.com

domain of Nrf2 is essential for the Keap1-mediated degradation of the protein by proteasome. Arch. Biochem. Biophys., 433, 342–350.

Katoh, Y., Itoh, K., Yoshida, E., Miyagishi, M., Fukamizu, A., Yamamoto, M. (2001). Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. Genes Cells, 6, 857–868.

Kawai, T., Adachi, O., Ogawa, T., Takeda, K., Akira, S. (1999). Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity, 11, 115–122.

Kerr, M. E., Bender, C. M.; Monti, E. (1996). J. An introduction to oxygen free radicals. Heart Lung, 25, 200–209.

Kieran, M., Blank, V., Logeat, F., Vandekerckhove, J., Lottspeich, F., Le Bail, O., Urban, M. B., Kourilsky, P., Baeuerle, P. A., Israil, A. (1990). The DNA binding subunit of NF-κB is identical to factorKBF1 and homologous to the rel oncogene product. Cell, 62, 1007–1018.

Kishimoto, T., Taga, T., Akira, S. (1994). Cytokine signal transduction. Cell, 76, 253–262.

Kobayashi, A., Kang, MI., Okawa, H., Ohtsuji, M., Zenke, Y., Chiba, T., Igarashi, K., Yamamoto, M. (2004). Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol., 24, 7130–7139.

Kobayashi, M., Itoh, K., Suzuki, T., Osanai, H., Nishikawa, K., Katoh, Y., Takagi, Y., Yamamoto, M. (2002). Identification of the interactive interface and phylogenic conservation of the Nrf2-Keap1 system. Genes Cells, 7, 807–820.

Kong, X., Thimmulappa, R., Kombairaju, P., Biswal, S. (2010). NADPH oxidase-dependent reactive oxygen species mediate amplified TLR4 signaling and sepsis-induced mortality in Nrf2-deficient mice. J Immunol., 185, 569–577.

Kopp E, Ghosh S. (1995). NF-κB and rel proteins in innate immunity. Adv. Immunol., 58, 1–27.

Kretzschmar, M., Reinhardt, D., Schlechtweg, J., Machnik, G., Klinger, W., Schirrmeister, W. (1992).Glutathione homeostasis in rats chronically treated with ethanol: evidence for an increased hepatic GSH export in vivo. Exp. Toxicol. Pathol., 44, 344–348.

Kroncke, K. D., Fehsel, K., Kolb-Bachofen, V. (1995). Inducible nitric oxide synthase and its product nitric oxide, a small molecule with complex biological activities. Biol Chem., 376, 327–343.

www.manaraa.com

Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., Woodgett, J. R. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. Nature, 369, 156–160.

Lee, J. M., Hanson, J. M., Chu, W. A., Johnson, J. A. (2001). Phosphatidylinositol 3-kinase, not extracellular signal-regulated kinase, regulates activation of the antioxidant-responsive element in IMR-32 human neuroblastoma cells. J. Biol. Chem., 276, 20011–20016.

Lee, O. H., Jain, A. K., Papusha, V., Jaiswal, A. K. (2007). An autoregulatory loop between stress sensors INrf2 and Nrf2 controls their cellular abundance. J. Biol. Chem., 282, 36412–36420.

Leonard, W. J. (1999). Type I cytokines and interferons and their receptors. In: Paul WE, ed. Fundamental Immunology. 4th ed. Philadelphia, PA: Lippincott Raven, 741-774.

Leonard, W. J., O'Shea, J. J. (1998). Jaks and STATs: biological implications. Annu Rev Immunol., 16, 293-322.

Li, G.Y., Zeng, Y.M., Meng, H., Li, X., Wang, J.H. (2009). A new triterpenoid saponin from the leaves and stems of Panax quinquefolium L. Chinese Chem. Lett., 20, 1207–1210.

Li, J.Q., Ichikawa, T., Jin, Y., Hofseth, L.J., Nagarkatti, P., Nagarkatti, M., Windust, A., Cui, T.X. (2009a). An essential role of Nrf2 in American ginseng-mediated antioxidative actions in cardiomyocytes. J. Ethnopharmacol., 130, 222–230.

Li, X.L., Wang, C.Z., Sun, S., Mehendale, S.R., Du, W., He, T.C., Yuan, C.S. (2009b). American ginseng berry enhances chemopreventive effect of 5-FU on human colorectal cancer cells. Oncol. Rep., 22, 943–952.

Li, Y., Jaiswal, A. K. (1992). Regulation of human NAD(P)H:quinone oxidoreductase gene: role of AP1 binding site contained within human antioxidant response element. J. Biol. Chem., 267, 15097–15104.

Maicas, N., Ferrandiz, M. L., Brines, R., Ibanez, L., Cuadrado, A., Koenders, M., van den Berg, W., Alcaraz, M. (2011). Deficiency of Nrf2 accelerates the effector phase of arthritis and aggravates joint disease. Antioxid Redox Signal, 4, 889–901.

Marais, R., Wynne, J., Treisman, R. (1993). The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell, 73, 381–393.

Marks-Konczalik, J., Chu, S. C., Moss, J. (1998). Cytokine-mediated Transcriptional Induction of the Human Inducible Nitric Oxide Synthase Gene Requires Both Activator Protein 1 and Nuclear Factor kB-binding Sites. J Biol Chem., 273, 22201-22208.

www.manaraa.com

McMahon, M., Thomas, N., Itoh, K., Yamamoto, M., Hayes, J. D. (2004). Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron. J. Biol. Chem., 279, 31556–31567.

Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S., Janeway Jr., C. A. (1998). MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol. Cell, 2, 253–258.

Mercurio, F., DiDonato, J. A., Rosette, C., Karin, M. (1993). p105 and p98 precursor proteins play an active role in NF-κB mediated signal transduction. Genes Dev., 7, 705–718.

Minghetti, L., Polazzi, E., Nicolini, A., Creminon, C., Levi, G. (1996). Interferon-γ and nitric oxide down-regulate lipopolysaccharide-induced prostanoid production in cultured rat microglial cells by inhibiting cyclooxygenase-2 expression. J Neurochem., 66, 1963–1970.

Moi, P., Chan, K., Asunis, I., Cao, A., Kan, Y. W. (1994). Isolation of NFE2- related factor 2 (Nrf2), a NF-E2 like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of h-globin locus control region. Proc. Natl. Acad. Sci., 91, 9926–9930.

Moncada, S., Higgs, E. A. (1995). Molecular mechanisms and therapeutic strategies related to nitric oxide. FASEB J., 9, 1319–1330.

Mulkahy, R. T., Wartman, M. A., Bailey, H., Gipp, J. J. (1997). Constitutive and β-naphthoflavone-induced expression of the human γ-glutamylcysteine synthetase heavy subunit gene is regulated by a distal antioxidant response element/TRE sequence. J. Biol. Chem., 272, 7445–7454.

Muroi, M., Suzuki, T. (1993). Role of protein kinase A in LPS-induced activation of NF-κB proteins of a mouse macrophage-like cell line. Cell Signal, 5, 289–298.

Muzio, M., Natoli, G., Saccani, S., Levrero, M., Mantovani, A. (1998). The human toll signaling pathway: divergence of nuclear factor κB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). J. Exp. Med., 187, 2097–2101.

Muzio, M., Ni, J., Feng, P., Dixit, V. M. (1997). IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science, 278, 1612–1615.

Nakamura, S., Sugimoto, S., Matsuda, H., Yoshikawa, M. (2007). Medicinal flowers. XVII. New dammarane-type triterpene glycosides from flower buds of American ginseng, Panax quinquefolium L. Chem. Pharm. Bull., 55, 1342–1348.

www.manaraa.com

Navarro, L., David, M. (1999). p38-dependent activation of interferon regulatory factor 3 by lipopolysaccharide. J Biol Chem., 274, 35535–35538.

Needleman, P., Turk, J., Jakschik, B. A. (1986). Morrison, A. R., Lefkowith, J. B., Arachidonic acid metabolism. Annu Rev Biochem., 55, 69–102.

Neri, A., Chang, C. C., Lombardi, L., Salina, M., Corradini, P., Maiolo, A. T., Chaganti, R. S., Dalla-Favera, R. (1991). B cell lymphoma aassociated chromosomal translocation involves candidate oncogene lyt-10, homologous to NF-κB p50. Cell, 67, 1075–1087.

Nguyen, T., Huang, H. C., Pickett, C. B. (2000). Transcriptional regulation of the antioxidant response element: activation by Nrf2 and repression by MafK. J. Biol. Chem., 275, 15466–15473.

Nolan, G. P., Fujita, T., Bhatia, K., Huppi, C., Liou, H. C., Scott, M. L., Baltimore, D. (1993). The bcl-3 proto-oncogene encodes a nuclear IκB-like molecule that preferentially interacts with NF-κB p50 and p52 in a phosphorylation-dependent manner. Mol. Cell Biol., 13, 3557–3566.

Ohno, H., Takimoto, G., McKeithan, T. W. (1990). The candidate proto-oncogene bcl-3 is related to genes implicated in cell lineage determination and cell cycle control. Cell, 60, 991–997.

Pahl, H. L. (1999). Activators and target genes of Rel/NF-κB transcription factors. Oncogene, 18, 6853–6866.

Pasparakis, M., Luedde, T. & Schmidt-Supprian, M. (2006). Dissection of the NF-κB signaling cascade in transgenic and knockout mice. Cell Death Differ., 13, 861–872.

Perkins, N. D. (2003).Oncogenes, tumor suppressors and p52 NF-κB. Oncogene, 22, 7553–7556.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res., 29, 2002-2007.

Pickett, C. B., Lu, A. Y. H. (1989). Glutathione S-transferases: gene structure, regulation and biological function. Annu. Rev. Biochem., 58, 743–764.

Poindexter, B. J., Allison, A. W., Bick, R. J., Dasgupta, A. (2006). Ginseng: Cardiotonic in adult rat cardiomyocytes, cardiotoxic in neonatal rat cardiomyocytes. Life Sci., 79, 2337-2344.

Poudyal, D., Le, P., M., Davis, T., Hofseth, A., Chumanevich, A., Wargovich, M., Nagarkatti, M., Nagarkatti, P., Windust, A., Hofseth. L. (2012). A Hexane fraction of

www.manaraa.com

American Ginseng suppresses mouse colitis and associated colon cancer: anti-inflammatory and proapoptotic echanisms. Cancer Prev. Res., 5, 685-696.

Prestera, T., Holtzclaw, W. D., Zhang, Y., Talalay, P. (1993). Chemical and molecular regulation of enzymes that detoxify carcinogens. Proc. Natl. Acad. Sci., 90, 2965–2969.

Qi, L. W., Wang, C. Z., Yuan, C. S. (2011). Ginsenosides from American ginseng: Chemical and pharmacological diversity. Phytochemistry, 72, 689–699.

Qu, C.L., Bai, Y.P., Jin, X.Q., Wang, Y.T., Zhang, K., You, J.Y., Zhang, H.Q. (2009). Study on ginsenosides in different parts and ages of Panax quinquefolius L. Food Chem., 115, 340–346.

Raetz, C. R. (1990). Biochemistry of endotoxins. Annu Rev Biochem., 59, 129–170.

Rauscher, F. J. D., Sambucetti, L. C., Curran, T., Distel, R. J., Spiegelman, B. M. (1988). Common DNA binding site for Fos protein complexes and transcription factor AP-1. Cell, 52, 471–480.

Rogers, R. S., Horvath, C. M., Matunis, M. J. (2003). SUMO modification of STAT1 and its role in PIAS-mediated inhibition of gene activation. J Biol. Chem., 278, 30091-30097.

Rosen, G. M., Pou, S., Ramos, C. L., Cohen, M. S., Britigan, B. E. (1995). Free radicals and phagocytic cells. FASEB J., 9, 200–209.

Rushmore, T. H., Morton, M. R., Pickett, C. B. (1991). The antioxidant responsive element: activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J. Biol. Chem., 266, 11632–11639.

Saklatvala, J., Davis, W., Guesdon, F. (1996). Interleukin 1 (IL1) and Tumour Necrosis Factor (TNF) Signal Transduction Philos Trans R Soc Lond B Biol. Sci., 351, 151-157.

Salvemini, D., Misko, T. P., Masferrer, J. L., Seibert, K., Currie, M. G., Needleman, P. (1993). Nitric oxide activates cyclooxygenase enzymes. Proc. Natl. Acad. Sci., 90, 7240–7244.

Scheinman, R. I., Cogswell, P. C., Lofuist, A. K., Baldwin, A. S. (1995). Role of transcriptional activation of IκB-α in mediation of immunosuppression by glucocorticoids. Science, 270, 283–286.

Schindler, C., Darnell, J. E. Jr. (1995). Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu. Rev. Biochem., 64, 621-651.

www.manaraa.com

Schmid, R. M., Perkins, N. D., Duckett, C. S., Andrews P. C, Nabel G. J. (1991). Cloning of an NF-κB subunit which stimulates HIV transcription in synergy with p65. Nature, 352,733–736.

Schmidt, D., Muller, S. (2003). PIAS/SUMO: new partners in transcriptional regulation. Cell Mol. Life Sci., 60, 2561-2574.

Schulze-Osthoff, K., Bauer, M., Vogt, M., Weesselborg, S., Baeuerle, P. A. (1997). Reactive oxygen intermediates as primary signals and second messengers in the activation of transcription factors. New York: Chapman & Hall, 239–259.

Scott, M. L., Fujita, T., Liou, H. C., Nolan, G. P., Baltimore, D. (1993). The p65 subunit of NF-κB regulates IκB by two distinct mechanisms. Genes Dev., 7, 1266–1276.

Sekhar, K. R., Yan, X. X., Freeman, M. L. (2002). Nrf2 degradation by the ubiquitin proteasome pathway is inhibited by KIAA0132, the human homolog to INrf2. Oncogene, 21, 6829–6834.

Sen, R., Baltimore, D., 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell. 46, 705–16.

Shin, H. R., Kim, J. Y., Yun, T. K., Morgan, G., Vainio, H. (2000). The cancer-preventive potential of Panax ginseng: a review of human and experimental evidence. Cancer Causes Control, 11, 565–576.

Simmons, D. L., Botting, R. M., Hla, T. (2004).Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol Rev., 56, 387–437.

Smeal, T., Angel, P., Meek, J., Karin, M. (1989). Different requirements for formation of Jun: Jun and Jun: Fos complexes. Genes & Dev., 3, 2091–2100.

Soldati, F., Sticher, O. (1980). HPLC separation and quantitative determination of ginsenosides from Panax ginseng, Panax quinquefolium and from ginseng drug preparations. Planta. Med., 39: 348-357.

Spelman, K., Burns, J., Nichols, D., Winters, N., Ottersberg, S., Tenborg, M. (2006). Modulation of cytokine expression by traditional medicines: a review of herbal immunomodulators. Alternative Medicine Review, 11, 128–150.

Stavro, P.M., Woo, M., Heim, T.F., Leiter, L.A., Vuksan, V. (2005). North American ginseng exerts a neutral effect on blood pressure in individuals with hypertension. Hypertension, 46, 406–411.

Stewart, D., Killeen, E., Naquin, R., Alam, S., Alam, J. (2003). Degradation of transcription factor Nrf2 via the ubiquitin–proteasome pathway and stabilization by cadmium. J. Biol. Chem., 278, 2396– 2402.

www.manaraa.com

Sun, S., Ganchi, P, Ballard, D., Greene, W. (1993). NF-κB controls expression of inhibitor IκB-α: evidence for an inducible autoregulatory pathway. Science, 259, 1912–1915.

Sweet, M. J., Hume, D. A. (1996). Endotoxin signal transduction in macrophages. J. Leucoc. Biol., 60, 8-26.

Swierkosz, T. A., Mitchell, J. A., Warner, T. D., Botting, R. M., Vane, J. R. (1995). Co-induction of nitric oxide synthase and cyclo-oxygenase: interactions between nitric oxide and prostanoids. Br. J. Pharmacol, 114, 1335–1342.

Taylor, B. S., de Vera, M. E., Ganster, R. W., Wang, Q., Shapiro, R. A., Morris, S. M., Jr., Billiar, T. R., Geller, D. A. (1998). Multiple NF-kB Enhancer Elements Regulate Cytokine Induction of the Human Inducible Nitric Oxide Synthase Gene. J. Biol. Chem., 273, 15148-15156.

Thelen, M., Dewald, B. (1993). Baggiolini, M. Neutrophil signal transduction and activation of the respiratory burst. Physiol. Rev., 73, 797–821.

Thompson, J., Phillips, R., Erdjument-Bromage, H., Tempst, P., Ghosh, S. (1995). IκBβ regulates the persistent response in a biphasic activation of NF-κB. Cell, 80, 573–582.

Traenckner, B-M., Pahl, H., Henkel, T., Schmidt, K., Wilk, S., Baeuerle, P. (1995). Phosphorylation of human IκB-α on serines 32 and 36 controls IκB-α proteolysis and NF-κB activation in response to diverse stimuli. EMBO J., 14:2876–2883.

Traenckner, E.,Wilk, S., Baeuerle, P. (1994). A proteasome inhibitor prevents activation of NF-κB and stabilizes a newly phosphorylated form of IκB-α that is still bound to NF-κB. EMBO J., 13:5433–5441.

Treisman, R. (1992). The serum response element. Trends Biochem. Sci., 17, 423–426.

Treisman, R. (1994). Ternary complex factors: growth factor regulated transcriptional activators. Curr. Opin. Genet. & Dev., 4, 96–101.

Tsuchida, S., Sato, K. (1992). Glutathione transferases and cancer. Crit. Rev. Biochem. Mol. Biol., 27, 337–384.

Van Dam, H., Duyndam, M., Rottier, R., Bosch, A., de Vries-Smits, L., Herrlich, P., Zantema, A., Angel, P., van der Eb, A. J. (1993). Heterodimer formation of cJun and ATF-2 is responsible for induction of c-jun by the 243 amino acid adenovirus E1A protein. EMBO J., 12, 479–487.

Venugopal, R., Jaiswal, A. K. (1996). Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H: quinone oxidoreductase1 gene. Proc. Natl. Acad. Sci., 93, 14960–14965.

www.manaraa.com

Verma, I. M., Stevenson, J. K., Schwarz, E. M., Van Antwerp, D., Miyamoto, S. (1995). Rel/NF-κB/IκB family: intimate tales of association and dissociation. Genes Dev., 9, 2723–2735.

Von Knethen, A., Brune, B. (1997). Cyclooxygenase-2: an essential regulator of NO-mediated apoptosis. FASEB J., 11, 887–895.

Vuksan, V., Stavro, M.P., Sievenpiper, J.L., Beljan-Zdravkovic, U., Leiter, L.A., Josse, R.G., Xu, Z. (2000). Similar postprandial glycemic reductions with escalation of dose and administration time of American ginseng in type 2 diabetes. Diabetes Care, 23, 1221–1226.

Wang, A.B., Wang, C.Z., Wu, J.A., Osinski, J., Yuan, C.S. (2005). Determination of major ginsenosides in Panax quinquefolius (American ginseng) using, highperformance liquid chromatography. Phytochem. Anal., 16, 272–277.

Wang, C.Z., Mehendale, S.R., Yuan, C.S. (2007). Commonly used antioxidant botanicals: active constituents and their potential role in cardiovascular illness. Am. J. Chin. Med., 35, 543–558.

Wang, J.H., Li, W., Sha, Y., Tezuka, Y., Kadota, S., Li, X. (2001). Triterpenoid saponins from leaves and stems of Panax quinquefolium L. J. Asian Nat. Prod. Res., 3, 123–130.

Wang, Y.Z., Chen, J., Chu, S.F., Wang, Y.S., Wang, X.Y., Chen, N.H., Zhang, J.T. (2009). Improvement of memory in mice and increase of hippocampal excitability in rats by ginsenoside Rg1's metabolites ginsenoside Rh1 and protopanaxatriol. J. Pharmacol. Sci., 109, 504–510.

Ward, J. F. (1994). The complexity of DNA damage: relevance to biological consequences. Int. J. Radic. Biol., 66, 427–432.

Wasserman, W., Fahl, W. E. (1997). Functional antioxidant responsive elements. Proc. Natl. Acad. Sci., 94, 5361–5366.

Watkins, D. N., Garlepp, M. J., Thompson, P. J. (1997). Regulation of the inducible cyclo-oxygenase pathway in human cultured airway epithelial (A549) cells by nitric oxide. Br. J. Pharmacol., 121, 1482–1488.

Watts, R. G., Huang, C., Young, M. R., Li, J. J., Dong, Z., Pennie, W. D., Colburn, N. H. (1998). Expression of dominant negative Erk2 inhibits AP-1 transactivation and neoplastic transformation. Oncogene, 17, 3493–3498.

Weinstein, S. L., Sanghera, J. S., Lemke, K., DeFranco, A. L., Pelech, S. L. (1992). Bacterial lipopolysaccharide induces tyrosine phosphorylation and activation of

www.manaraa.com

mitogen-activated protein kinases in macrophages. J. Biol. Chem., 267, 14955–14962.

Wesche, H., Henzel, W. J., Shillinglaw, W., Li, S., Cao, Z. (1997). MyD88: an adaptor that recruits IRAK to the IL-1 receptor complex. Immunity, 7, 837– 847.

Whiteside, S. T., Ernst, M. K., LeBail, O., Laurent-Winter, C., Rice, N., Israel, A. (1995). N- and C-terminal sequences control degradation of MAD3/IκBα in response to inducers of NF-κB activity. Mol. Cell Biol., 15, 5339–5345.

Whitmarsh, A. J., Davis, R. J. (1996). Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J. Mol. Med., 74, 589–607.

Wild, A. C., Moinova, H. R., Mulcahy, R. T. (1999). Regulation of γ-glutamyl cysteine synthetase subunit gene expression by transcription factor Nrf2. J. Biol. Chem., 274, 33627–33636.

Williams, M. A., Rangasamy, T., Bauer, S. M., Killedar, S., Karp, M., Kensler, T. W., Yamamoto, M., Breysse, P., Biswal, S., Georas, N. (2008). Disruption of the transcription factor Nrf2 promotes pro-oxidative dendritic cells that stimulate Th2-like immunoresponsiveness upon activation by ambient particulate matter. J. Immunol., 181, 4545–4559.

Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., Ihle, N. (1993). JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell, 74, 227-236.

Wulczyn, F. G., Naumann, M., Scheidereit, C. (1992). Candidate proto-oncogene bcl- 3 encodes a subunit-specific inhibitor of transcription factor NF-κB. Nature, 358, 597–599.

Xie, T., Belinsky, M., Xu, Y., Jaiswal, A. K. (1995). ARE- and TRE-mediated regulation of gene expression: response to xenobiotics and antioxidants. J. Biol. Chem., 270, 6894–6900.

Xu, B.B., Liu, C.Q., Gao, X., Zhang, W.Q., Wang, S.W., Cao, Y.L. (2005). Possible mechanisms of the protection of ginsenoside Re against MPTP-induced apoptosis in substantia nigra neurons of Parkinson's disease mouse model. J. Asian Nat. Prod. Res., 7, 215–224.

Xu, L., Chen, W.F., Wong, M.S. (2009). Ginsenoside Rg1 protects dopaminergic neurons in a rat model of Parkinson's disease through the IGF-I receptor signalling pathway. Br. J. Pharmacol., 158, 738–748.

www.manaraa.com

Xue, F., Cooley, L. (1993). kelch encodes a component of intercellular bridges in Drosophila egg chambers. Cell, 72, 681–693.

Yoshikawa, M., Murakami, T., Yashiro, K., Yamahara, J., Matsuda, H., Saijoh, R., Tanaka, O. (1998). Bioactive saponins and glycosides, XI. Structures of new dammarane-type triterpene oligoglycosides, quinquenosides I, II, III, IV, and V, from American ginseng, the roots of Panax quinquefolium L. Chem. Pharm. Bull., 46, 647–654.

Young, M. R., Li, J. J., Rincon, M., Flavell, R. A., Sathyanarayana, B. K., Hunziker, R., Colburn, N. (1999). Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. Proc. Natl. Acad. Sci., 96, 9827–9832.

Yu, R., Chen, C., Mo, Y. Y., Hebbar, V., Owuor, E. D., Tann, T. H., Kong, A. N. T. (2000). Activation of mitogen-activated protein kinase pathways induces antioxidant response element-mediated gene expression via a Nrf2-dependent mechanism. J. Biol. Chem., 275, 39907–39913.

Yuan, C.S., Dey, L. (2001). Multiple effects of American ginseng in clinical medicine. Am. J. Chin. Med., 29, 567–569.

Zhang, D. D., Hannink, M. (2003). Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol. Cell Biol., 23, 8137–8151.

Zhang, D. D., Lo, S. C., Cross, J. V., Templeton, D. J., Hannink, M. (2004). Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. Mol. Cell Biol., 24, 10941–10953.

Zhang, D.D. (2006). Mechanistic studies of the Nrf2-Keap1 signaling pathway. Drug Metab. Rev., 38, 769–789.

Zhang, G.Z., Liu, A.L., Zhou, Y.B., San, X., Jin, T.W., Jin, Y. (2008). Panax ginseng ginsenoside-Rg(2) protects memory impairment via anti-apoptosis in a rat model with vascular dementia. J. Ethnopharmacol., 115, 441–448.

Zhang, Q., Didonato, J., Karin, M., McKeithan, T. (1994). BCL3 encodes a nuclear protein which can alter the subcellular location of NF-κB proteins. Mol. Cell Biol., 14, 3915–3926.

Zhao, W.J., Li, P.Y. (2004). Ginsenoside Re improved memory impairment in aged rats and mice. Neurobiol. Aging, 25, S582–S583.

Zidorn, C., Johrer, K., Ganzera, M., Schubert, B., Sigmund, E. M., Mader, J., Greil, R., Ellmerer, E. P., Stuppner, H. (2005). Polyacetylenes from the Apiaceae vegetables,

www.manaraa.com

carrot, celery, fennel, parsley, and parsnip and their cytotoxic activities. J. Agr. Food Chem., 53, 2518-2523.

Zipper, L. M., Mulcahy, R. T. (2000). Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes. Biochem. Biophys. Res. Commun., 278, 484–492.

Zipper, L. M., Mulcahy, R. T. (2002). The Keap1 BTB/POZ dimerization function is required to sequester Nrf2 in cytoplasm. J. Biol. Chem., 277, 36544–36552.

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