ANTI-INFLAMMATORY EFFECT OF RESVERATROL METABOLITE, δ-VINIFERIN, ON LIPOPOLYSACCHARIDE-STIMULATED MURINE MACROPHAGE

By

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ABSTRACT OF THE THESIS

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Inflammation is a complicated physiological and pathological process in response to stimuli. In general, a controlled inflammatory response is a beneficial defense system, which provides protection against infection. However, it can become detrimental when the response is prolonged and dysregulated called chronic inflammation. Over the past several years, chronic inflammation has been recognized to be the root of various human diseases, including neurological disorders, metabolic disorders, obesity, cardiovascular diseases and cancers.

Resveratrol is a well-known natural antioxidative and anti-inflammatory compound

from grapes. In the previous study, the antioxidative properties of resveratrol metabolites have been suggested to be more active than resveratrol. δ-Viniferin is a main resveratrol dehydrodimer metabolite and has been identified in grape cell cultures and wines. It possesses two resorcinol skeletons, which are determinants of radical scavenging and antioxidative potential. Hence, we researched in the anti-inflammatory effect of δ-viniferin. We prepared this compound *in vitro* by the oxidative dimerization of resveratrol with horseradish peroxidase in the presence of H_2O_2.

In this study, the cytotoxic and anti-inflammatory effects on LPS-stimulated murine macrophage were examined. According to the data, we found that δ-viniferin suppressed the levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and phosphorylated inhibitor κBα (p-IκBα) protein expressions, as well as the down-stream product, nitric oxide, in murine RAW 264.7 cells induced with lipopolysaccharide (LPS). Moreover, the inhibitory effects of δ-viniferin on inflammation associated signaling pathways, such as the blockade of LPS-induced IκBα phosphorylation and degradation, the decrease of PI3K and Akt phosphorylation, and the reduction of LPS-induced transcriptional activity of NF-κB. According to the results, δ-viniferin might have potential to be developed into an effective anti-inflammatory agent.

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Chapter 1. Introduction

1.1. Inflammation

Inflammation is a physiological and pathological process in response to stimuli, like tissue injury, chemical irritation or microbial pathogen infection (*1, 2*). It plays a crucial role in the immune response to combat foreign invaders of the body. There are two types of inflammatory conditions, acute inflammation and chronic inflammation. Acute inflammation is a short-term resolved process and self-limiting response. Redness, heat, pain and swelling are signal transductions of inflammation (*3*). In general, a controlled inflammatory response is a beneficial defense system, which provides protection against infection as well as tissue repair process. Under this condition, mast cells secrete chemokines and pro-inflammatory cytokines that cause angiectasis, increase permeability, and subsequently recruit leukocytes, macrophages and a wide range of immune cells to invade the affected area. Leukocytes and macrophages infiltrate the damaged regions, remove the irritation and repair the tissue (*4*). As inflammation progresses, certain "stop signals" such as lipoxins at appropriate checkpoints prevent further immune cells into traffic tissue (*5*).

However, when the response becomes detrimental prolonged and dysregulated

called chronic inflammation. It includes active inflammation, tissue destruction and attempts at tissue repair (*1, 4*). Over the past few decades, chronic inflammation has been recognized as the root causes of various human diseases. Common chronic inflammatory diseases include neurological disorders, chronic inflammatory diseases, obesity, bone, muscular and skeletal diseases, metabolic disorder, cardiovascular diseases and cancers (*1*).

1.2. Inflammation and Oxidative Stress

The inflammatory processes may induce DNA mutations in cells *via* oxidative/nitrosative stress. They occur when the generation of free radicals and active intermediates exceeds in the system, which means the system is unable to neutralize and eliminate the excessive products (*6*). About reactive oxygen species (ROS), it is the main factor of tissue damage and leads to many diseases. The extreme productions of ROS and reactive nitrogen species (RNS) by immune cells, such as superoxide anion ($\cdot O_2$-), hydroxyl radical (·OH), hydrogen peroxide (H_2O_2), nitric oxide (NO) and singlet oxygen ($^1O_2$) will attack normal tissue around the infected area. In addition, they cause oxidative DNA damage and gene mutation, which may influence normal cell functions and consequently lead to inflammatory diseases and cancer (*7*).

1.3. Inflammation and Carcinogenesis

The links between inflammation and cancer were first indicated in the nineteenth century (*8*). At first, the underlying cause of cancer researchers had focused primarily on genetic changes (*9*). Nevertheless, researchers find out that tumors often arise at sites of chronic inflammation, and inflammatory cells presented in biopsied samples from tumors as well (*10*). The developing tumor can commandeer the inflammatory component (wound-healing process) of immune system to foster carcinogenesis.

As shown in Figure 1.1, the innate immune system converges on bacteria, eliminates invaders and forms a clot in normal wound healing. Whereas, the innate cells and the acute immune response signals are commandeered by chronically inflamed precancerous tissue to develop a blood supply and the tumor grow (*9*).

For the serious concern about chronic inflammation, approximately 20% of all human cancers in adults are caused by chronic inflammatory states and/or chronic inflammation. It is implicated in the development of a diverse range of human cancers, linking to cancer of the liver, stomach, large intestine, biliary tree and urinary bladder, and significant evidence to link it to cancer of the oesophagus, lung and pancreas (*11, 12*).

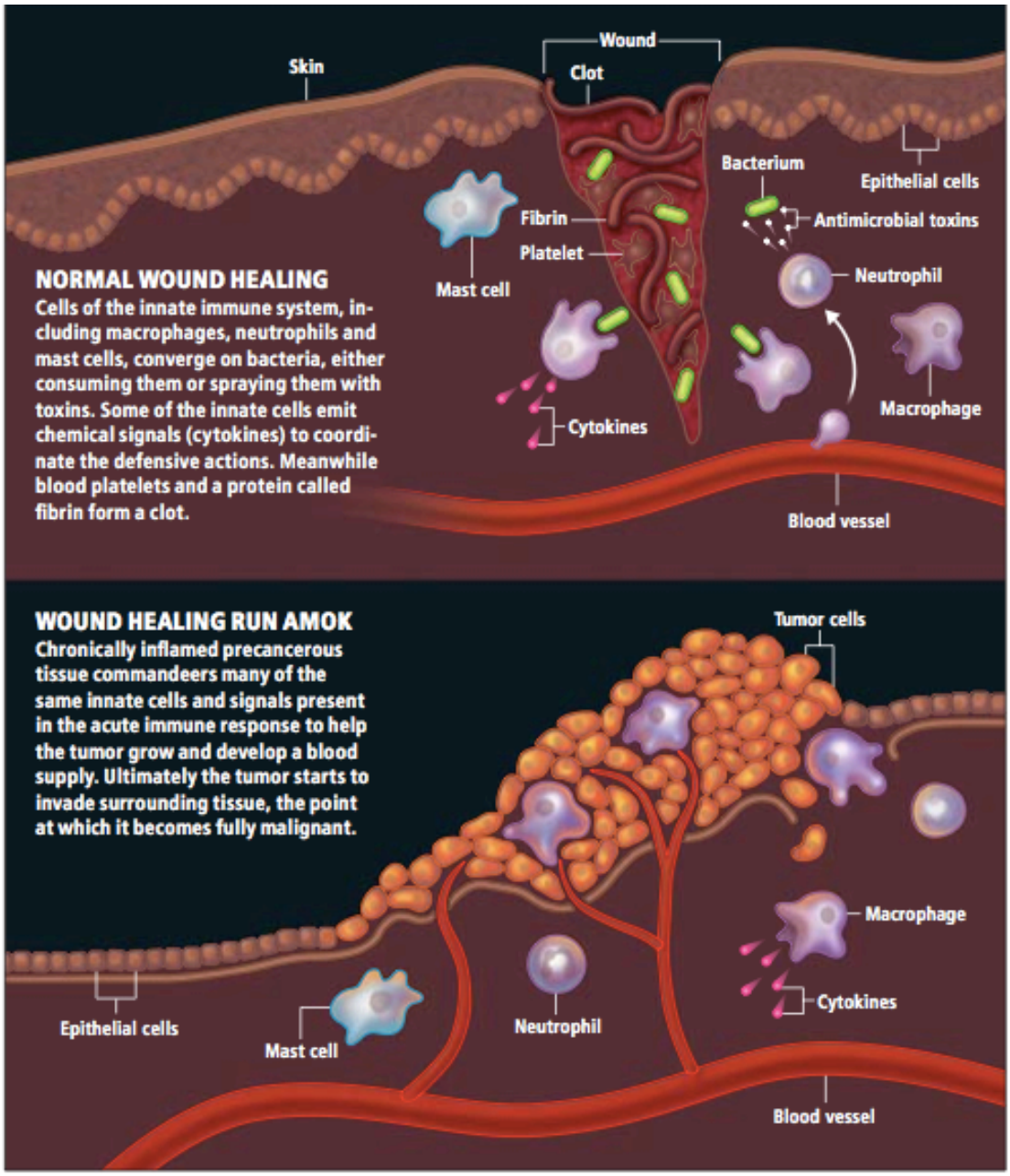


Figure 1.1. Normal wound healing and wound healing run amok (*9*).

1.4. Inflammatory Cell Mechanism and Mediators

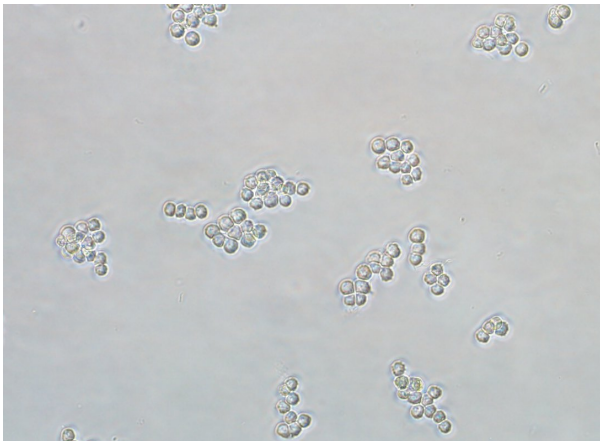
1.4.1. Macrophage and Lipopolysaccharide

The immune system defends us against the infection as a front barrier. The system consists innate and adaptive systems. The innate system is the first line of defense against infection, including macrophages, mast cells, granulocytes, dendritic cells and natural killer cells. About the adaptive system, B cells and T cells are involved and target invaders with greater specificity (*9*). In the system, macrophages play a central role in inflammatory responses. Macrophages are immune defenders engulf and consume pathogen invaders (*13*). They are produced by the differentiation and maturation of monocytes in tissue, and next to migrate to the region of tissue injury, guided by chemotactic factors. Macrophages are considered as the crucial roles in the body's response to immunogenic challenges as well. (*14*).

Since murine macrophages, RAW 264.7 cell lines, are one of the common cell line models in inflammatory studies, we applied them as a model cell line in our study. They were isolated from the tissue of Abelson murine leukemia virus-induced tumor in male adult BALB/c *Mus musculus* (mice). The culture property of the cells is adherent (*15*). In the research, a classical model of macrophage stimulation is bacterial endotoxin, lipopolysaccharide (LPS). After the treatment of LPS, the morphology of RAW 264.7

changed from roundness to spindle shape as shown in Figure 1.2. As shown in Figure 1.3, LPS is a kind of bacterial endotoxin and the primary component of the outer membrane of Gram-negative bacteria. It is a classical and well-known model of macrophage stimulation (*14*). After LPS binds to the receptor, the signaling pathways are induced involved in modulating activation Nuclear Factor-κB (NF-κB).

(A)



(B)

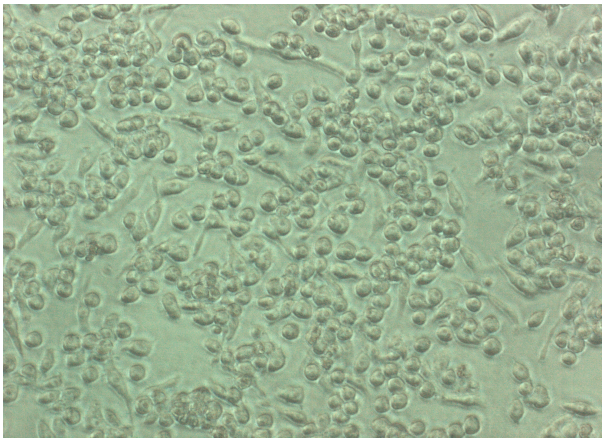


Figure 1.2. Morphology changes of RAW 264.7 cells.
(A) Unstimulated RAW 264.7 cells (B) RAW 264.7 cells stimulated with 100 ng/mL LPS for 12 h.

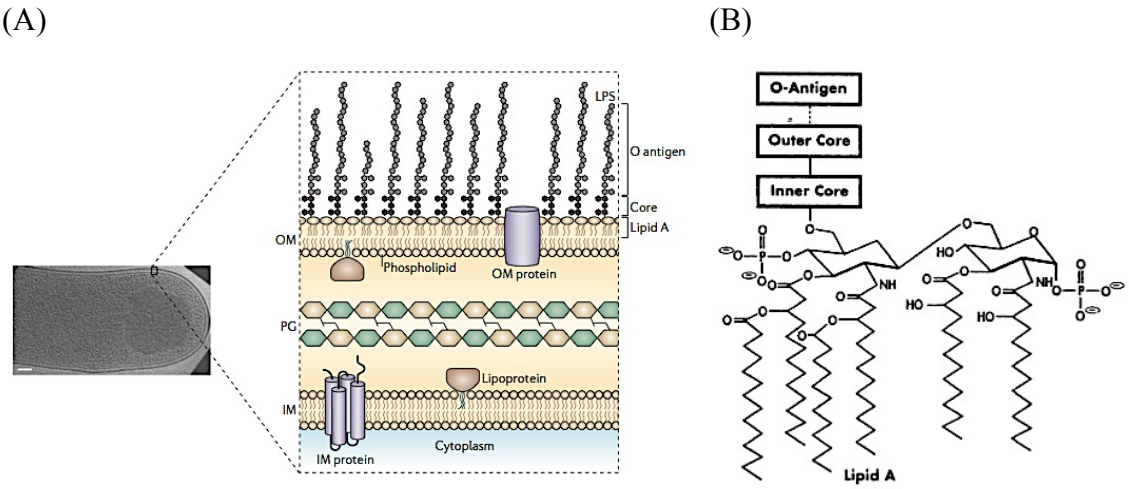


Figure 1.3. Lipopolysaccharide (LPS).
(A) An *Escherichia coli* cell, showing the characteristic inner membrane (IM) and outer membrane (OM) (scale bar of 200 nm). The outer membrane of the Gram-negative cell contains LPS, which is anchored to the membrane by the LPS lipid A domain (*16*). (B) Structure of LPS (*17*).

1.4.2. Nuclear Factor-κB (NF-κB)

Nuclear Factor-κB (NF-κB) has long been considered as a target for new anti-inflammatory drugs. It is an important family of transcription factor that play critical roles in inflammation, immunity, cell proliferation, differentiation, and survival (*18*). NF-κB complex in mammals contains p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), p100/p52 (NF-κB2) proteins. There are two common kinds of NF-κB activating pathways, canonical and non-canonical pathways, and they both can be induced by LPS. In this study, we focused on canonical NF-κB activating pathways.

In most cells, NF-κB signaling pathway has long been considered a prototypical pro-inflammatory signaling. It can be activated by diverse pro-inflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor α (TNF-α). Additionally, many studies have implied that the activation of NF-κB can be modulated through inhibitor of κB (IκB)-kinase (IKK), phosphoinositide 3-Kinase (PI3K)/Protein Kinase B (Akt) and IκB signaling pathways (*19-22*).

IKKα and IKKβ form an IKK multiprotein complex with a scaffold protein called NEMO stands for IKKγ (*23*). IKKα and IKKβ are responsible for phosphorylating IκBs (*24*). IKK protein will be phosphorylated, and then phosphorylated IKK will activate IκB protein. The family of IκB sequesters NF-κB complex in the cytosol. After that phosphorylated IκB is ubiquitylated and degraded by proteasome. p65 is phosphorylated at serine 276 in the N-terminal Rel homology domain, bringing about an intensive transcriptional activity (*17*). Subsequently the liberated NF-κB can translocate to the nucleus and regulate the target genes. They next trigger the level of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions and produce downstream productions, which causes inflammation (*25*) (Figure 1.4).

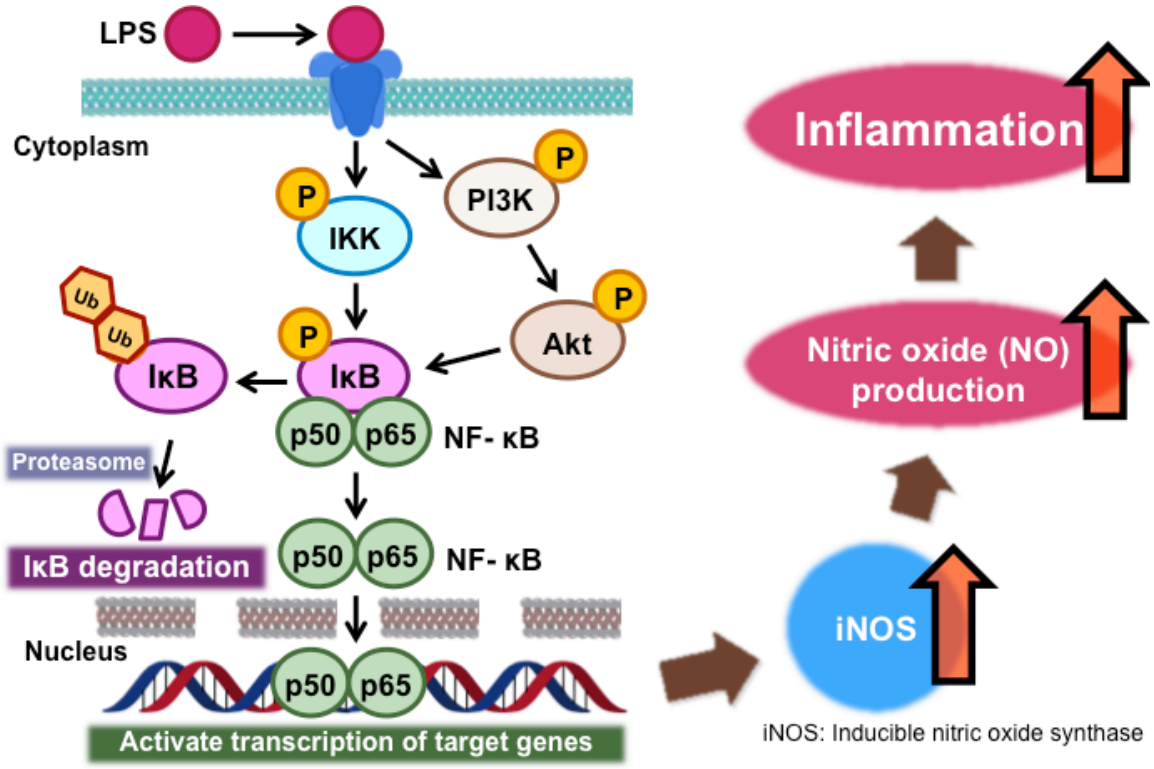


Figure 1.4. Simplified scheme of inflammatory cellular mechanism.

1.4.3. Inducible Nitric Oxide Synthase (iNOS)

Nitric oxide synthase (NOS) is a family of enzymes which consists with diverse classes of NOS (neuronal NOS, endothelial NOS, inducible NOS and mitochondria NOS) (*26*), depending on the location of expression and manner of expression.

Usually, iNOS is normally absent from macrophages and hepatocytes, but when specific cytokines induce these cells, iNOS enzyme is produced. Once produced, the enzyme synthesizes large amounts of nitric oxide (NO). NO plays essential roles in animals. It is a key physiological messenger and effector molecule in many biological

systems. For instance, DNA synthesis can be suppressed by nitric oxide, which can inactivate ribonucleotide reductase. Also, DNA can be damaged directly by deamination (*27*). iNOS is transcriptionally regulated. The level of iNOS expression enhances then triggers NO production, which causes inflammation.

1.5. Chemoprevention

Chemoprevention means the usage of natural or synthetic compounds to prevent, attenuate or reverse the occurrence of diseases. About the chemoprevention of anti-inflammation, Pan *et al.* and Bisht *et al.* have demonstrated that many phytochemicals have potential on anti-inflammatory activities, such as flavonoids, curcumin and resveratrol, they act through a variety mechanisms to prevent and attenuate inflammatory responses (*1, 28*).

In our study, the metabolite of resveratrol, δ-viniferin, was used as an agent and endotoxin, LPS, was used as a stimulus. We treated stimulated murine macrophages, RAW 264.7 cells, with δ-viniferin to see if it had chemopreventive property to reduce inflammatory response.

1.6. Peroxidase

Horseradish peroxidase (EC 1.11.1.7) is mixture of isoenzymes and isolated from horseradish root (*29*) (Figure 1.5). The total molecular weight of horseradish peroxidase is 42 kDa. It is heme-containing enzyme (metalloenzyme) and the most abundant in C type (*30*). Peroxidases may act as oxygenases, catalases and oscillating reactors, and play an essential role in almost all living systems and distribute throughout the plant and animal kingdoms (*31*).

Horseradish peroxidase has been most thoroughly studied and is frequently used to illustrate the peroxidase reaction cycle (*32*):

$$\text{heme[Fe(III)]} + H_2O_2 \rightarrow \text{heme[O = Fe(IV) } - R^+]_{(\text{Comp I})} + H_2O \quad (1)$$

$$\text{heme[O = Fe(IV) } - R^+]_{(\text{Comp I})} + S \rightarrow \text{heme[O = Fe(IV)]}_{(\text{Comp II})} + S(2) \quad (2)$$

$$\text{heme[O = Fe(IV)]}_{(\text{Comp II})} + S \rightarrow \text{Heme[Fe(III)]} + S(3) \quad (3)$$

In our study, horseradish peroxidase presented as a hydrogen donor (H_2O_2 oxidoreductase) and resveratrol was used as a substrate for peroxidase.

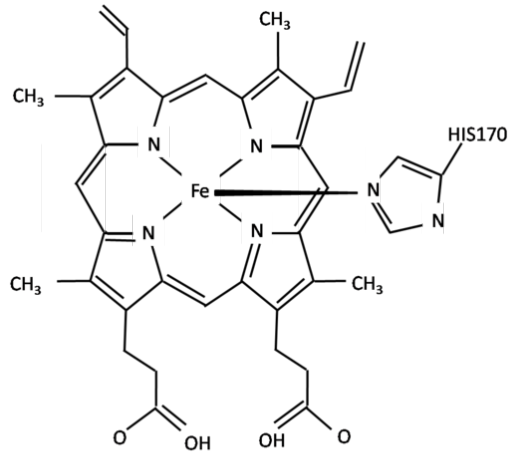


Figure 1.5. Chemical structure of ion heme group in horseradish peroxidase.

1.7. Stilbenes

Stilbenes are small molecules with ~200-300 g/mol molecular weight, a subclass of polyphenolic compounds (*33*). They are naturally occurred in a wide range of plant sources, aromatherapy products, and dietary supplements, but grapes and wine are considered the most important dietary sources (*34*). Family members of the stilbene have C6-C2-C6 basic skeleton and consist of two phenol rings linked by a styrene double bond (Figure 1.6). There are several well-known stilbenes; for instance, resveratrol, pterostilbene and piceatannol. Stilbenes are considered to play a central role in the human diet due to their anti-oxidation, anti-mutagenic and anti-carcinogenic potencies (*35*).

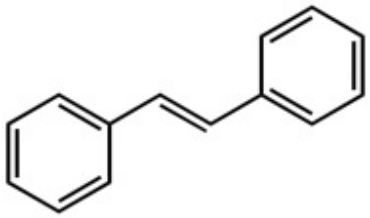


Figure 1.6. Chemical structure of stilbene.

1.7.1. Resveratrol

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a natural polyphenolic, non-flavonoid phytoalexin compound found in various plants, including berries, grapes, and peanuts (Figure 1.7). This polyphenol is synthesized in the leaf epidermis, the pericarp and the lignified plant tissue (stalks and kernels) of the berries, but not in the flesh. Resveratrol exists in two isomeric forms, *trans*- and *cis*- configurations, and *trans*- is isomerized to *cis*- by ultraviolet radiation (UV) exposure (*14*). The concentration ranges of free *trans*- and *cis*- resveratrols are 0.2-13 mg/L in red wine and 0.1-0.8 mg/L in white wines. In contrast, grape juice offers a content of *trans*-resveratrol in a range of 0.09-0.18 mg/L (*36*).

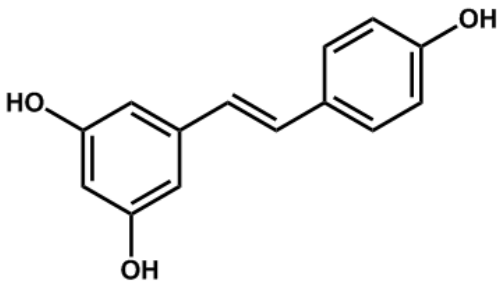


Figure 1.7. Chemical structure of *trans*-resveratrol.

Resveratrol is one of the well-known members in stilbene family. Many studies showed that resveratrol has a lot of biological functions, including antioxidation, anti-aging, anti-inflammation, anti-cardiovascular diseases and anti-cancer (*37*). Focusing on its anti-inflammatory bioactivity, a number of published papers had explored the anti-inflammatory effects of resveratrol (Table 1).

In the late 1990s, Tsai *et al.* (*38*) indicated that resveratrol suppressed the inflammatory response of LPS-stimulated RAW 264.7 macrophages through down-regulation the basal level of *iNOS* gene and iNOS protein expressions, resulting in decreased NO generation; moreover, the decline of IκBα phosphorylation repressed the binding activity of NF-κB and the level of Nuclear NF-κB. Similar data were found by Matsuda *et al.* (*39*) in LPS-activated mice peritoneal exudate macrophages, which demonstrated that resveratrol blocked NO generation in this model. Resveratrol was also

shown to be potent dual suppressant of both iNOS and COX-2 inductions (*40*). Another experiment performed in LPS-induced N9 mouse microglial cells was manifested by Bi *et al*. (*41*) who investigated the effects of resveratrol on significant inhibition of the phosphorylated p38 MAPKs, which interrupted the degradation of IκBα. In another study, Qureshi *et al.* (*42*) revealed that resveratrol decreased the pro-inflammatory cytokines and gene expressions, such as *TNF-α*, *IL-1β*, *IL-6* and *iNOS* genes, in LPS-stimulated RAW 264.7 cells and thioglycolate-elicited peritoneal macrophages from C57BL/6 or BALB/c mice. The scientists also found that resveratrol could act as a proteasome inhibitor which had an ability to decrease the enzymatic activities of proteasomal chymotrypsin-like, trypsin-like, and post-acidic (post-glutamase) sites, thereby suppressing NF-κB activity. A recent study had reported that resveratrol had an ability to attenuate the release of TNF-α and IL-6 in U-937 human macrophages (*43*). According to these studies, potent suppressive effects of resveratrol on inflammatory responses were reported, suggesting a therapeutic potential research of anti-inflammatory effects on its metabolite, δ-viniferin.

Table 1. Mechanism and efficacious concentrations of resveratrol related to anti-inflammatory activity

Model	Stimulus	Mechanism Signaling pathways	Efficacious concentrations	Reference
RAW 264.7 macrophages	LPS	↓ NO generation ↓ iNOS protein induction ↓ *iNOS* gene expression ↓ NF-κB binding activity ↓ IκBα phosphorylation ↑ Cytosolic IκBα protein ↓ Nuclear NF-κB level	3–30 μM	Tsai *et al.*, 1999 (*38*)
ddY mice peritoneal exudate macrophages	LPS	↓ iNOS protein induction	100 μM	Matsuda *et al.*, 2000 (*39*)
RAW 264.7 macrophages	LPS/ IFN-γ	↓ iNOS ↓ COX-2	50 μM	Murakami *et al.*, 2003 (*40*)
N9 mouse microglial cells	LPS	↓ Phosphorylation of p38 MAPK	0.01–10 μg/mL	Bi *et al.*, 2005 (*41*)
RAW 264.7 macrophages		Proteasomal enzymatic activity: ↓ Chymotrypsin-like ↓ Trypsin-like ↓ Post-glutamase	20 μM	Qureshi *et al.*, 2012 (*42*)
RAW 264.7 macrophages	LPS	↓ NO production ↓ TNF-α secretion ↓ *TNF-α*, *IL-1β*, *IL-6* and *iNOS* gene expressions	16 μM	
HEK293T human embryonic kidney cells	LPS	↓ NF-κB activation	40 μM	
C57BL/6 or BALB/c mice thioglycolate-elicited peritoneal macrophages	LPS	↓ *TNF-α*, *IL-1β*, *IL-6* and *iNOS* gene expressions	40 μM	
U-937 human macrophages	LPS	↓ TNF-α release ↓ IL-6 release	10 μM	Walker *et al.*, 2014 (*43*)

1.7.2. δ-Viniferin

Stilbenes occur in oligomeric and polymeric forms, so-called viniferin (*34*). δ-Viniferin is phytoalexin polyphenol as the main dehydrodimer metabolite in resveratrol, which can be found in natural sources such as grapevine leaves cell cultures and grape wines (Figure 1.8). In the experiment, δ-viniferin was prepared by the oxidative oligomerization of resveratrol with horseradish peroxidase (*44*). The possible participation of horseradish peroxidase in the formation of δ-viniferin has been firstly suggested by Langcake and Pryce (*45*). It was also shown in a previous study that horseradish peroxidase would oxidize resveratrol into δ-viniferin in the presence of H_2O_2 (*46*).

About the bioactivity of δ-viniferin, researchers suggested that δ-viniferin has the antifungal compounds produced in grapevine (*Vitis vinifera*) leaves in response to fungal infection or U.V. irradiation (*45*). δ-Viniferin also has the potential to inhibit 5α-reductase, which can prevent benign prostatic hyperplasia (*47*). In addition, Nicotra *et al.* manifested that the antioxidant property of δ-viniferin is comparable with resveratrol and pterostilbene (*48*).

These researches disclosed the preliminary information on the bioactivity of δ-viniferin, but still remain not very clear. Better understanding of the molecular

mechanisms by which anti-inflammation effects of δ-viniferin may lead to further development of new strategies for dysregulated inflammatory prevention at many sites. Therefore, we used δ-viniferin to study the cytotoxicity and anti-inflammatory mechanism.

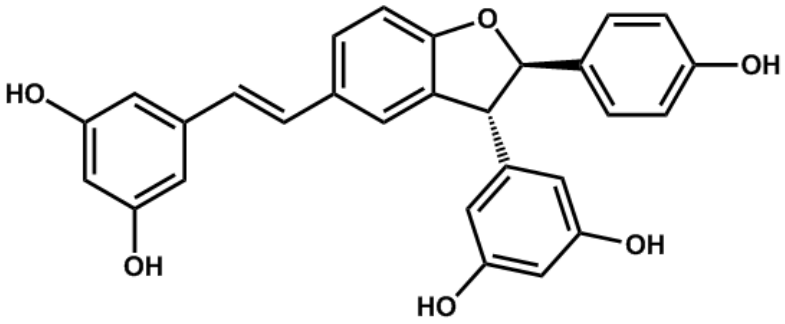


Figure 1.8. Chemical structure of δ-viniferin.

Chapter 2. Hypothesis and Research Objectives

2.1. Hypothesis

Inflammation is the risk of numerous human diseases, and resveratrol is an effective anti-inflammatory agent. Since δ-viniferin is the peroxidase-catalyzed metabolite of resveratrol, we are interested in the anti-inflammatory activity of δ-viniferin as well. Thereby, we hypothesized that δ-viniferin has the potential to be developed into an effective anti-inflammatory agent.

2.2. Research Objectives

2.2.1. Phase I: Chemical Experiment

※ To synthesize and isolate δ-viniferin from peroxidase-mediated formation of resveratrol.

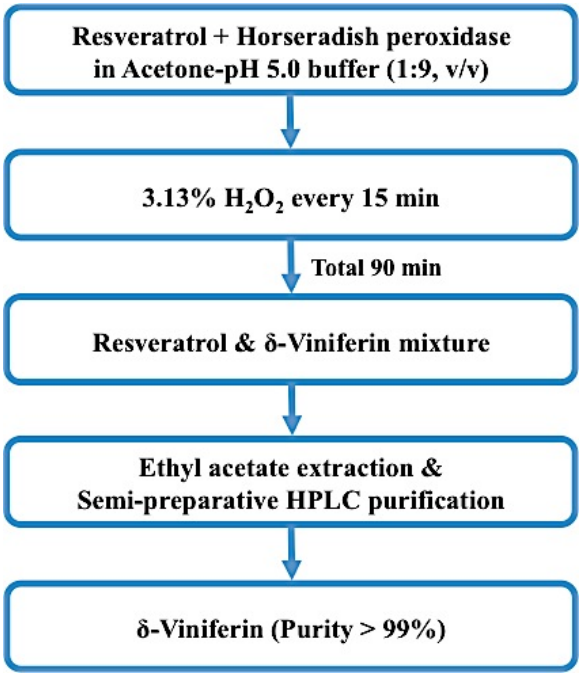
2.2.2. Phase II: Biological Experiment

※ To determine the cytotoxic effects and nitric oxide production of resveratrol and δ-viniferin.

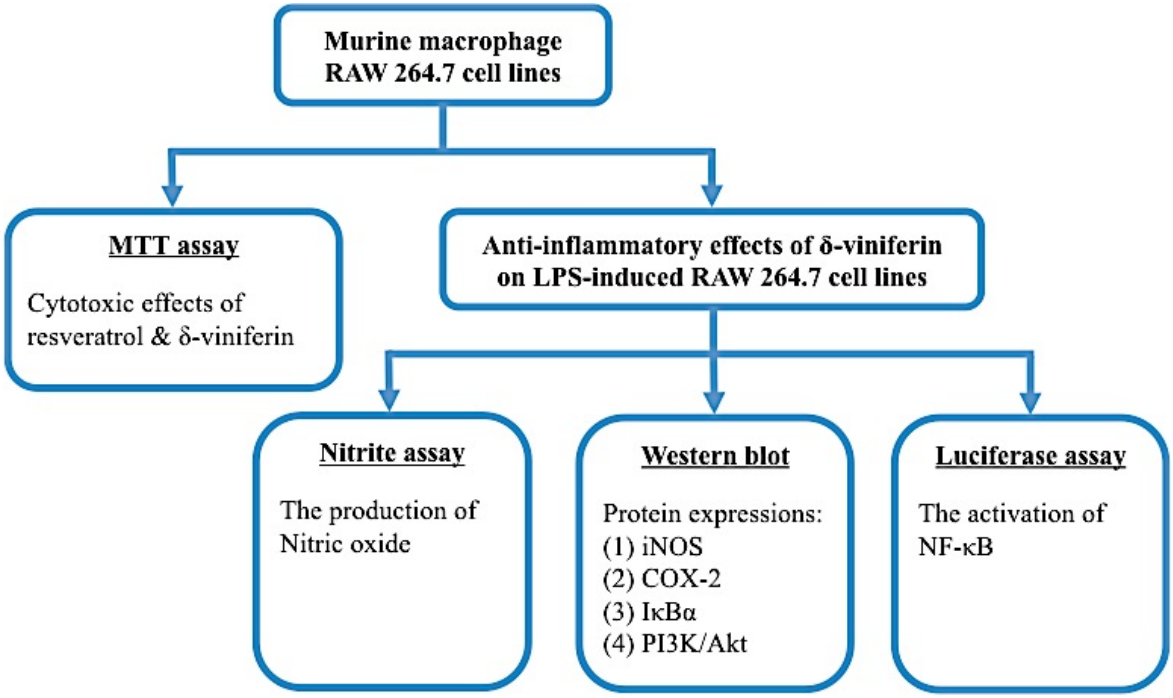
※ To evaluate the expressions of major inflammatory protein markers.

Chapter 3. Experimental Design

3.1. Chemical Experiment



3.2. Biological Experiment



Chapter 4. Materials and Methods

4.1. Preparation and Identification of δ-Viniferin

500 mg of resveratrol (ChromaDex, Irvine, CA) was added in the 37 °C water-bath heated acetone-pH 5.0 phosphate buffer mixture (1:9 v/v, 500 mL), which contained 5 mg horseradish peroxidase (Sigma-Aldrich, St. Louis, MO). While being stirred, 8 mL of 3.13% hydrogen peroxide (H_2O_2) (Thermo Fisher Scientific, Waltham, MA) was added every 15 min for 90 min. After last addition of H_2O_2, stirring was continued for an additional 15 min. Then removed acetone by rotavapor (Büchi, Flawil, Switzerland). Ethyl estate was used for extracting resveratrol and δ-viniferin mixture. Finally, δ-viniferin was isolated by semi-preparative HPLC system (Gilson, Middleton, WI), using a C18 reversed-phase column (150 x 21.20 mm, 5 μm) with a mobile phase of 40% acetonitrile / 60% water at a flow rate 1 mL/min. Detection was at 306 nm.

4.1.1. High-Performance Liquid Chromatography (HPLC)

The HPLC system equipped with the Dionex UltiMate 3000 SD pump and UltiMate 3000 UV-vis detector (Thermo Fisher Scientific, Waltham, MA). The data were acquired and processed using Chromeleon Chromatography Data System (CDS) software (Thermo

Fisher Scientific, Waltham, MA). The purity of δ-viniferin was > 99% determined by HPLC using an ODS Hypersil (RP C-18), 250 x 4.6 mm, 5 μm column (Thermo Fisher Scientific, Waltham, MA) with UV-vis detector at 306 nm.

4.1.2. Liquid Chromatography-Electron Spray Ionization Mass Spectrometry (LC-MS) and Nuclear Magnetic Resonance (NMR)

The Mass spectra were acquired with a combined HPLC particle beam MS-engine. The LC-ESI-MS was operated in a positive mode using Water ZQ mass detector and Water LC system. Ionization was generally achieved *via* ES. The NMR spectra recorded on a VARIAN spectrometer (Varian, Palo Alto, CA). The inspection of ^{1}H NMR spectrum of δ-viniferin was dissolved in DMSO-d_6 and recorded at 400 MHz.

4.2. Anti-inflammatory Effects of δ-Viniferin

4.2.1. Cell Culture

Murine macrophage cell lines, RAW264.7 cells, were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% endotoxin-free, heat-inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY), 100 units/mL penicillin, and 100 μg/mL

streptomycin. Cells were incubated in a humidified incubator with 5% CO_2 at 37 °C, and split every 2~3 days. We split the cells when they were cultivated at a density of 70~80% full of the dish. Medium was removed and 1× Phosphate buffered saline (PBS) was used to wash the dish. We subsequently tapped the dish and used fresh medium to wash down the cells. The fallen cells were collected in centrifuge tube, and we centrifuged the tube with 1000 rpm for 5 minute. After that, the supernatant was sucked up and the pellet was dispersed by fresh medium. Finally, the cells were seeded in dishes or wells with a proper density.

After the cells grew stably and reached at a density of $2\sim3\times10^6$ cells/mL, the medium was removed and 1× PBS was used to gently wash the dish. The medium was replaced serum-free DMEM without phenol red. The cells were induced by incubation in medium containing 100 ng/mL LPS (*E. coli* 0127: E8). Resveratrol and δ-viniferin were dissolved in dimethyl sulfoxide (DMSO, Kenilworth, NJ), respectively. These dissolution compounds were treated cells together with LPS.

4.2.2. Cell Viability Assay

RAW 264.7 cells were seeded at a density of 1×10^6 cells/mL (100 μL/well) into 96-well plates and cultivated overnight in a humidified incubator with 5% CO_2 at 37 °C.

Then, the cells were treated with 5, 10, 15, 20 and 50 μM resveratrol or δ-viniferin solution for 24 h. The cells of control group were treated with DMSO to yield a final concentration of 0.05% v/v. After 24 h incubation, the numbers of cell proliferation were evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylte- trazolium bromide) assay as follows: the 96-well plate was centrifuged with 3000 rpm for 10 minutes, and then medium was removed. 1× PBS (100 μL/well) was used to gently wash the wells and centrifuged with 3000 rpm for 10 minutes to remove PBS. Then, we mixed 2% MTT solution (Sigma-Aldrich, St. Louis, MO) with medium in 1 to 9 ratios, and 100 μL mixture was added in each well. After incubated the cells for 2~3 h at 37 °C, the supernatant was removed, and the MTT-formazan crystals formed by viable cells were dissolved in 150~200 μL of DMSO. Finally, the absorbance of the mixture at a wavelength of 570 nm was monitored with an enzyme-linked immunosorbent assay (ELISA) microplate reader (Thermo Fisher Scientific, Waltham, MA).

4.2.3. Nitrite Assay

RAW 264.7 cells were seeded at a density of 1×10^6 cells/mL (1 mL/well) into 24-well plates and cultivated overnight in a humidified incubator with 5% CO_2 at 37 °C. Then, the medium was removed, and 1× PBS (1 mL/well) was used to gently wash the

wells. The medium was replaced with 300 μL/well serum-free DMEM without phenol red. The cells were treated with 1, 5, 10 and 15 μM resveratrol or δ-viniferin solution and 100 ng/mL LPS or LPS only for 24 h.

After incubated for 24 h, 100 μL conditional medium was taken and mixed with 100 μL Griess reagent (50 μL 1% sulfanilamide in 5% phosphoric acid and 50 μL 0.1% naphthylethylenediamine dihydrochloride in water). Nitrite production was evaluated by the ELISA microplate reader (Thermo Fisher Scientific, Waltham, MA) at a wavelength of 550 nm was monitored. A standard curve was generated with $NaNO_2$.

4.2.4. Total Protein Extraction and Determination of Protein Concentration

For the extraction of total protein (prepared for β-actin, iNOS, COX-2, IκBα, phosphorylated IκBα, PI3K, phosphorylated PI3K, Akt and phosphorylated Akt), the compounds treated and stimulated RAW 264.7 cells were washed with 1× PBS and lysed in a cold gold lysis for 1 h at 4 °C (vortexed eppendorf tubes every 10 min for 1 h). The eppendorf tubes were centrifuged 12,000 rpm for 30 min at 4 °C. Then, we collected supernatant for the determination of protein concentrations by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). 0.4 μL total protein extraction was added in 200 μL Bio-Rad protein assay dye reagent. After 15 min reaction in dark place, the

absorbance of the mixture at a wavelength of 595 nm was monitored with an ELISA microplate reader. A standard curve was generated with 2 mg/mL BSA solution.

※ Gold lysis buffer:

Glycerol	10% (v/v)
Triton X-100	1% (v/v)
EDTA	5 mM
EGTA	1 mM
NaF	10 mM
NaCl	137 mM
Tris (pH 7.9)	20 mM
Sodium orthoranadate (Na_3VO_4)	1 mM
β-glycerl phosphate	100 mM
Sodium pyrophosphate ($Na_4P_2O_7$)	1 mM
Note: After confected these chemicals, one protease inhibitor cocktail tablet (Roche, Indianapolis, IN) was added in 50 mL gold lysis buffer.	

4.2.5. Western Blot Analysis

Total proteins from (50 μg) whole-cell lysates were resolved by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (PVDF, EMD Millipore, Billerica, MA). The membranes were then blocked at room temperature for 1 h with blocking solution and incubated with the primary antibody diluentovermnight at 4 °C. After a night, the membranes were washed with 0.2% TPBS, subsequently probed with the secondary antibody diluent (anti-mouse, anti-rabbit or anti-goat IgG antibody) conjugated to horseradish peroxidase (Becton, Dickinson and Company, Franklin Lakes,

NJ). The immune complexes were visualized with enhanced chemiluminescence kits (Amersham, UK). Primary antibodies of specific protein were purchased from diverse locations as below: Anti-β-actin antibody was from Sigma-Aldrich; anti-iNOS and anti-IκBα antibodies were from Santa Cruz Biotechnology (Dallas, TX); anti-COX-2 antibody was from BD Company; anti-phosphorylated IκBα was from Upstate Biotechnology (subsidiary of EMD Millipore, Billerica, MA); anti-PI3K, anti-phosphorylated PI3K and anti-Akt were from Cell Signaling (Danvers, MA); anti-phosphorylated Akt was from Imgenex (subsidiary of Novus Biologicals, Littleton, CO).

※ Tris-glycine electrophoresis buffer (1× SDS-PAGE running buffer):

Tris-base	25 mM
Glycine	250 mM
SDS	0.1%

※ Transfer buffer (for Western blotting):

Tris-base	60 g
Glycine	288 g
Note: Tris-base and glycine were dissolved in 4 L ddH_2O for preparing 5× transfer buffer stock solution. 1 L 5× transfer buffer stock solution, 1 L methanol and 3 L ddH_2O were used to prepare transfer buffer working solution.	

※ 5× Sample loading buffer:

SDS	12%
Glycerol	35%
Bromophenol blue	0.02%
Tris-HCl (pH 6.8)	350 mM
2-mercaptoethanol	30%

※ Blocking solution (for Western blotting):

Sodium acide	0.1%
Tris-base	20 mM
Tween-20	0.2%
NaCl	125 mM
BSA	1%

※ 0.2% TPBS: 0.2 % Tween-20/1× PBS.

※ Primary antibody diluent:

Primary antibody was diluted to one thousandth with blocking solution.

※ Secondary antibody diluent:

Secondary antibody was diluted to one in five thousandth with blocking solution.

※ Resolving gels (15 mL):

Concentration of gel / Unit: mL	8%	10%	12%	15%
H_2O	7.9	7.15	6.4	5.27
40% acrylamide (mix)	3.0	3.75	4.5	5.63
1.5 M Tris-HCl (pH 8.8)	3.8	3.8	3.8	3.8
10% SDS	0.15	0.15	0.15	0.15
10% ammonium persulfate	0.15	0.15	0.15	0.15
TEMED	0.009	0.006	0.006	0.006

4.2.6. Transient Transfection and Luciferase Assay

RAW 264.7 cells were seeded in a 6 cm dish with antibiotics-free DMEM. The medium was replaced with serum-free Opti-MEM (Gibco, Grand Island, NY) when the cells reached 70~90% confluence. Subsequently, the cells were transiently transfected with the pNF-κB-Luc plasmid reporter gene (Stratagene, La Jalla, CA) using Lipofectamine 2000 in accordance with the instructions of the manufacturer (Invitrogen, subsidiary of Thermo Fisher Scientific, Carlsbad, CA). After 24 h incubation, 2×10^6 cells/mL cells (1 mL/well) were incubated in a 24-well plate replacing with DMEM for 4~6 h and co-incubated with 5 and 15 μM δ-viniferin solution and 100 ng/mL LPS or LPS only for 3 h. Each well was washed with cold 1× PBS and collected by 100 μL serum-free DMEM without phenol red. Luciferase activity was evaluated by means of the britelite^{TM} plus Ultra-High Sensitivity Luminescence Reporter Gene Assay System (PerkinElmer, Waltham, MA). Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence Counter (Packard 9912V) in single-photon counting mode for 0.1min/well.

4.2.7. Statistical Analysis

Data are expressed in terms of mean ± SD. The statistical significance between LPS-

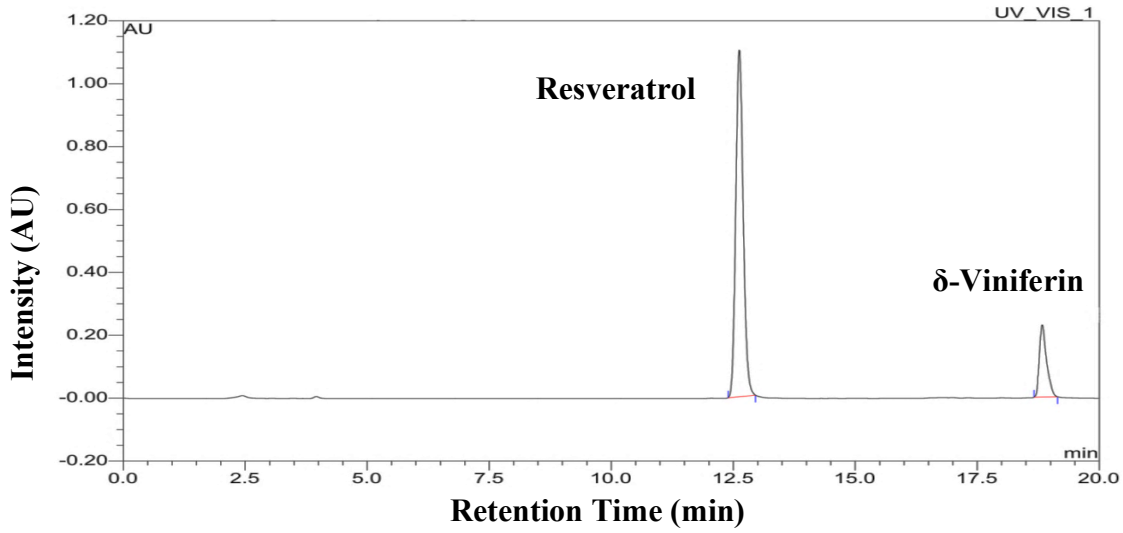
and resveratrol plus LPS-treated or LPS- and δ-viniferin plus LPS-treated cells were accessed by one-way ANOVA. Additionally, one-way Student's *t*-test was used to access the statistical significance between the LPS only and δ-viniferin plus LPS-treated cells. A p-value < 0.05 was considered statistically significant.

Chapter 5. Results and Discussion

5.1. HPLC Chromatograms of Resveratrol and δ-Viniferin Mixture and Pure δ-Viniferin

The HPLC profiles of resveratrol and/or δ-viniferin mixture are shown at Figure 5.1. These compounds were run under 40% acetonitrile and 60% water, and used a C18 reversed-phase column and UV detector at 306 nm. Figure 5.1A is the chromatogram of resveratrol and δ-viniferin mixture. As shown in the graph, resveratrol is at retention time (t_R) = 12.7 min and δ-viniferin at t_R = 18.9 min. Since δ-viniferin is the dehydrodimer of resveratrol, the elution time of δ-viniferin in the chromatography is longer than resveratrol. The other chromatogram (Figure 5.1B) is the graph of pure δ-viniferin after isolated by semi-preparative HPLC system. In order to identify the compound, we used LC-MS and NMR.

(A)



(B)

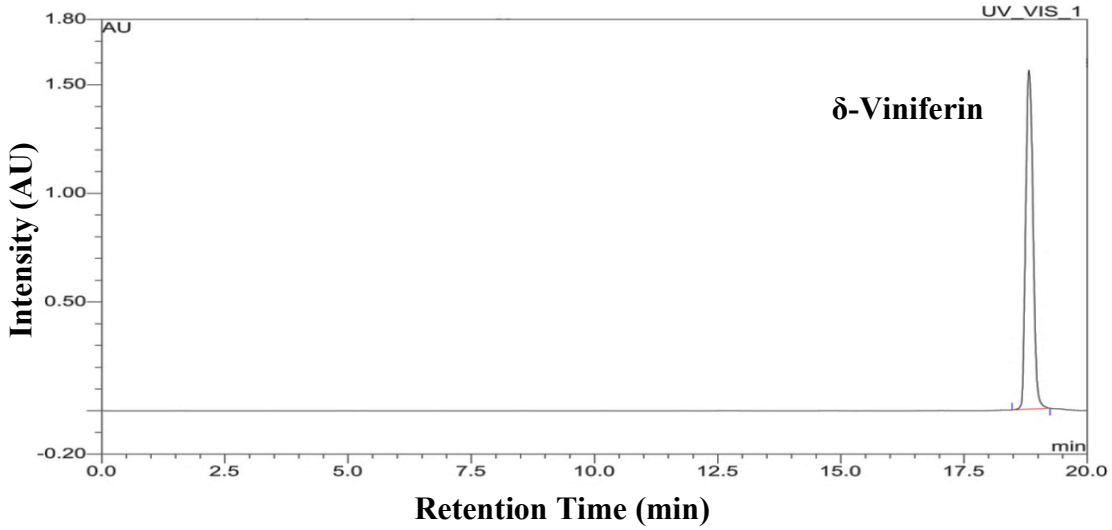


Figure 5.1. HPLC chromatograms of (A) resveratrol and δ-viniferin mixture (B) pure δ-viniferin.

The resveratrol and δ-viniferin mixture was prepared by the oxidative dimerization of resveratrol with horseradish peroxidase.

5.2. Identification of δ-Viniferin

LC-ESI-MS and ^{1}H NMR were used to confirm δ-viniferin identity. As presented in Figure 5.2, the LC fraction detection had purity greater than 99% and a molecular ion peak at *m/z* 455.2 $[MH]^{+}$, corresponding to the structure of δ-viniferin (*m/z* calculated for $C_{28}H_{22}O_6$ is 454), while ^{1}H NMR spectrum of δ-viniferin in DMSO-d_6 (δ ppm 2.5), reported in Figure 5.3. The ^{1}H NMR spectrum showed the presence of one 4-hydroxybenzene moiety (multiplet at 7.25 ppm and 6.87 ppm), and two aliphatic protons (doublets at 4.50 and 5.45 ppm) (*47-49*). Based on the results, we verified that resveratrol could further form dehydrodimer, δ-viniferin, in horseradish peroxidase/H_2O_2 system (Figure 5.4).

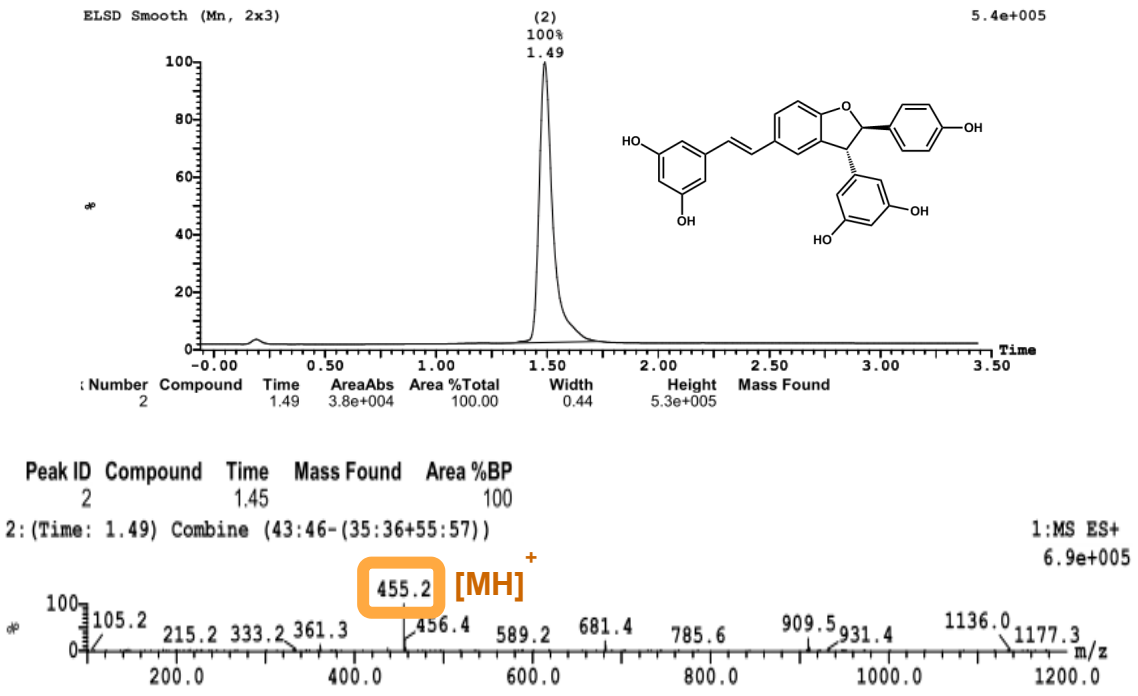


Figure 5.2. LC chromatogram and positive ion ESI-MS spectrum of δ-viniferin.

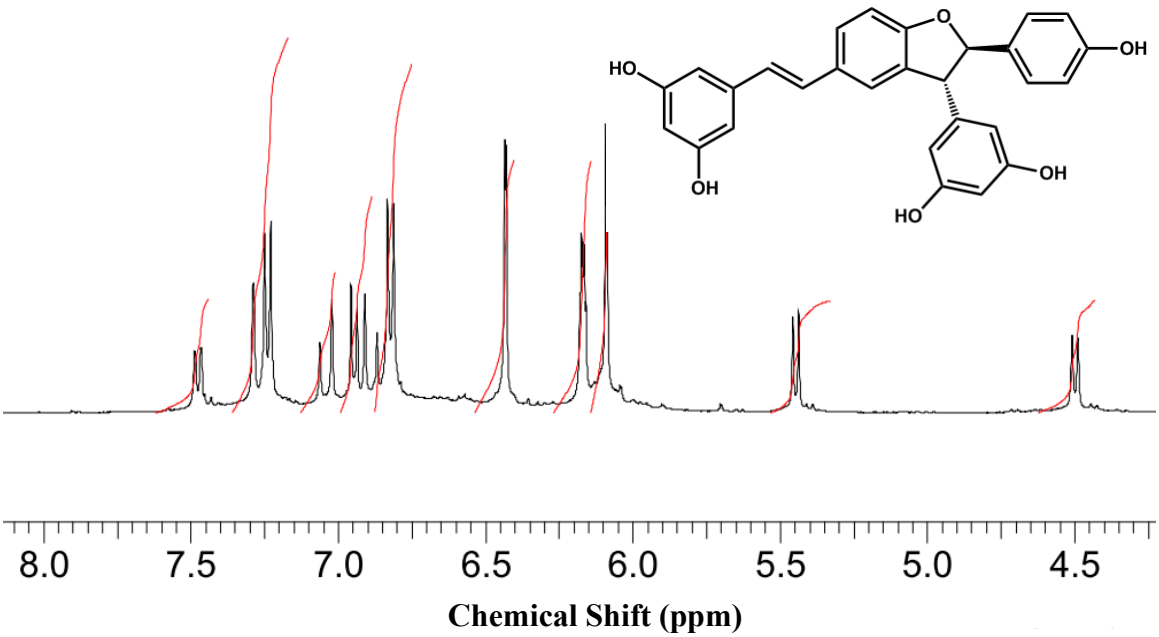


Figure 5.3. The ^{1}H NMR spectrum of δ-viniferin.

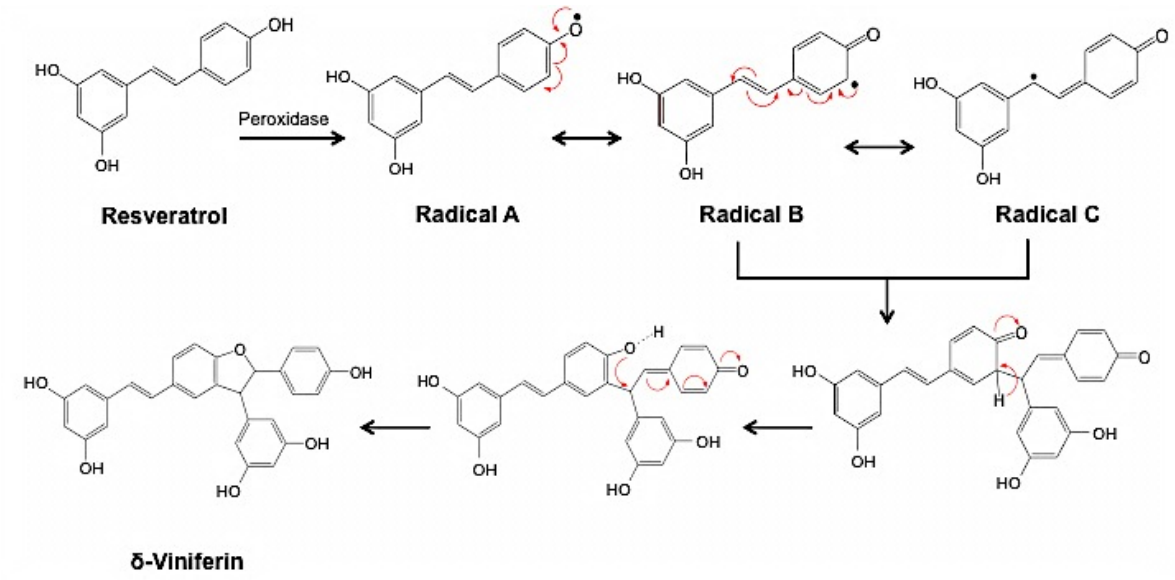


Figure 5.4. Mechanism of δ-viniferin oxidative process.

5.3. Cytotoxic Effects of Resveratrol and δ-Viniferin on RAW 264.7 Cells Based on MTT Assay

MTT assay was used to determine the cytotoxic effects of resveratrol & δ-viniferin. The cytotoxic effect of resveratrol and δ-viniferin on RAW 264.7 cells was represented as a percentage of growth of control group, and the concentration of sample required to inhibit 50% of cell growth (IC_{50}) was determined by interpolation from the dose response. Figure 5.5 shows the IC_{50} of resveratrol is 20 μM and δ-viniferin is > 50 μM. There was no significant difference between the group of δ-viniferin. The higher cell viability means lower cytotoxic effect; hence, this result comes out that δ-viniferin had low cytotoxic effect.

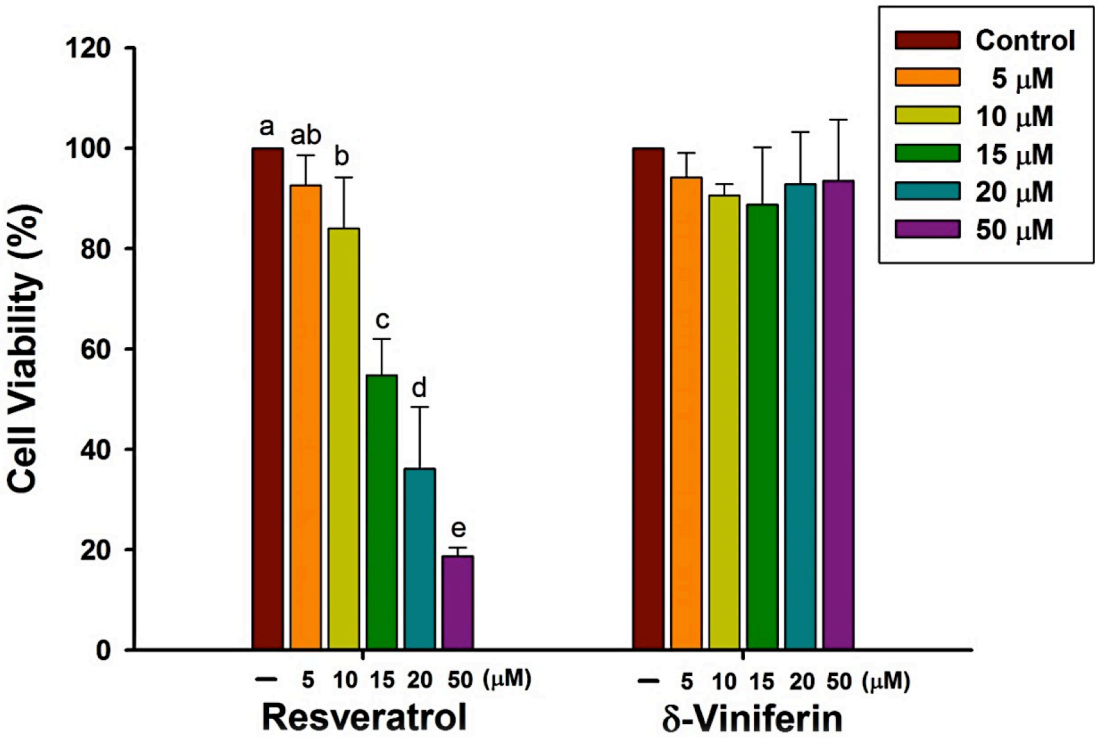


Figure 5.5. Cytotoxic effects of resveratrol and δ-viniferin in RAW 264.7 cells based on MTT assay.

Cells were treated with different concentrations of resveratrol or δ-viniferin for 24 h. The data are expressed as means ± SD of triplicate tests. $P < 0.05$.

5.4. Effects of Resveratrol and δ-Viniferin on LPS-induced Nitrite Production in RAW 264.7 Cells.

Nitrite is a primary, stable and nonvolatile breakdown product of nitric oxide. Nitrite assay was based on the Griess reaction (*26*) and used to determine the nitric oxide production, which is an inflammatory mediator to evaluate the condition of inflammatory response. To investigate the anti-inflammatory effects of resveratrol and δ-viniferin, the concentrations of nitrite in culture media were determined after incubated for 24 h. As shown in Figure 5.6, there was a significant difference and dose dependent trend between the LPS-treated group and the δ-viniferin group, but the resveratrol group was not in a dose dependent manner. It means δ-viniferin inhibited the production of nitric oxide, which was the down-stream product of the inflammatory cellular mechanism.

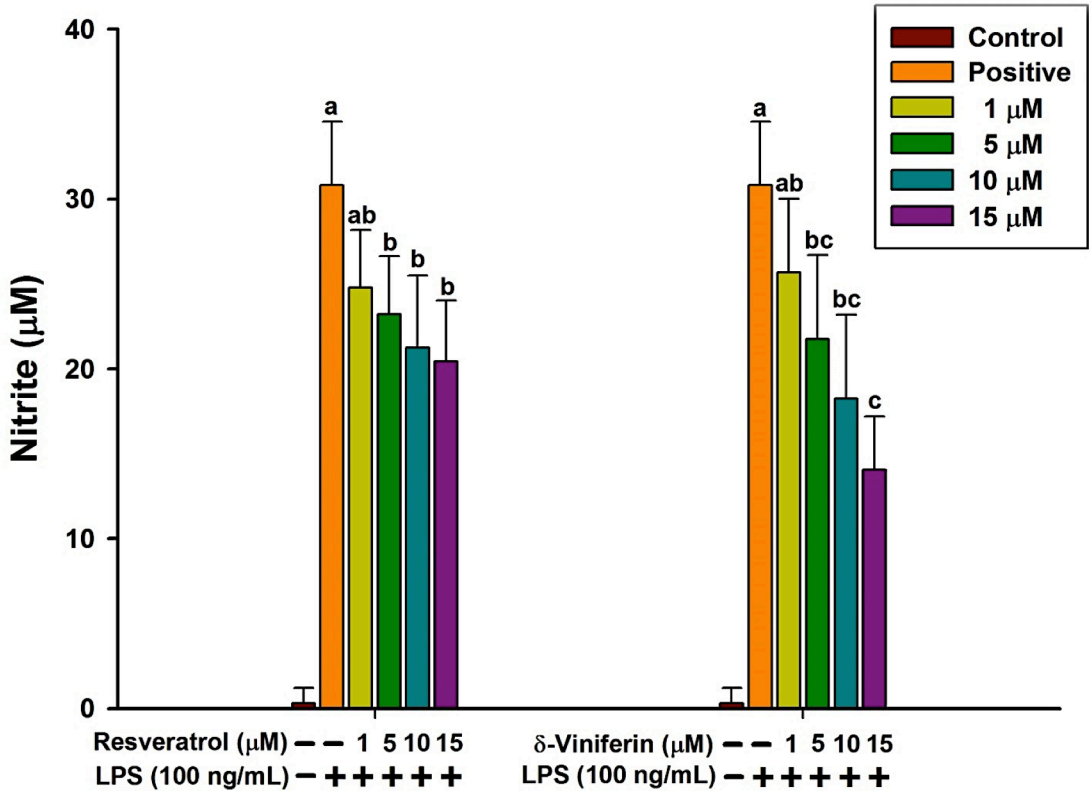


Figure 5.6. Effects of resveratrol and δ-viniferin on LPS-induced nitrite production in RAW 264.7 cells.

Cells were treated with 100 ng/mL LPS only or with different concentrations of resveratrol or δ-viniferin. After incubation for 24 h, the concentration of nitrite in culture media was determined by the Griess assay. The data are expressed as means ± SD of triplicate tests. $P < 0.05$.

5.5. Effects of Resveratrol and δ-Viniferin on LPS-induced iNOS and COX-2 Protein Expressions in RAW 264.7 Cells.

iNOS & COX-2 are key protein markers and pro-inflammatory mediators in inflammatory response. The effects of resveratrol and δ-viniferin on LPS-stimulated iNOS and COX-2 protein expressions were analyzed by western blot. The outcome showed that LPS treatment significantly increased iNOS and COX-2 protein expression, whereas 15 μM δ-viniferin markedly inhibited the level of iNOS and COX-2 levels (Figure 5.7). This result was accorded with the reduction of nitrite.

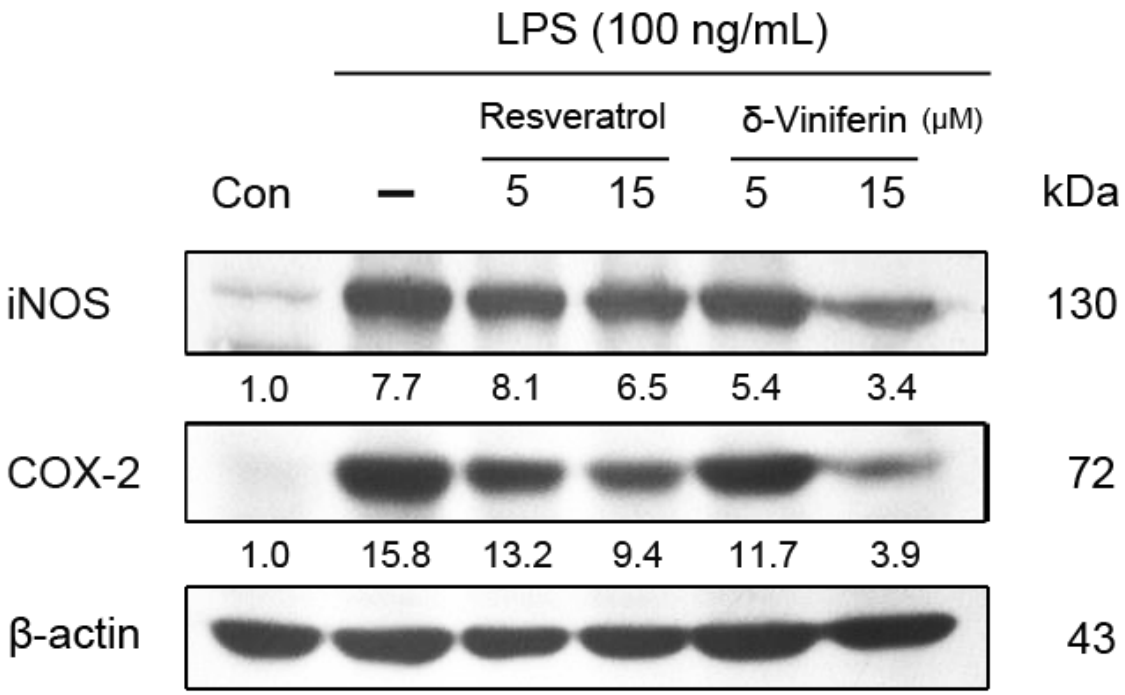


Figure 5.7. Effects of resveratrol and δ-viniferin on LPS-induced iNOS and COX-2 protein expressions in RAW 264.7 cells.

The cells were treated with 100 ng/mL LPS only or with various concentrations of resveratrol or δ-viniferin (5 and 15 μM) for 24 h. The levels of iNOS and COX-2 were analyzed by Western blotting. β-actin was used as a loading control.

5.6. δ-Viniferin Blocked NF-κB Activation in LPS-stimulated RAW 264.7 Cells.

The activation of NF-κB has been demonstrated as a crucial role to regulate gene expressions in LPS-stimulated inflammatory responses, including iNOS and COX-2. Therefore, we conducted a luciferase activity to confirm the suppressive effect of δ-viniferin on NF-κB activation. RAW 264.7 cells were transiently transfect with a NF-κB-dependent luciferase reporter plasmid (p-NF-κB-Luc) and subsequently treated with LPS alone or with δ-viniferin. From Figure 5.8, LPS stimulated NF-κB transcriptional activity to increase 4-fold compared with control group. However, the activity strongly reduced by δ-viniferin in a dose dependent manner.

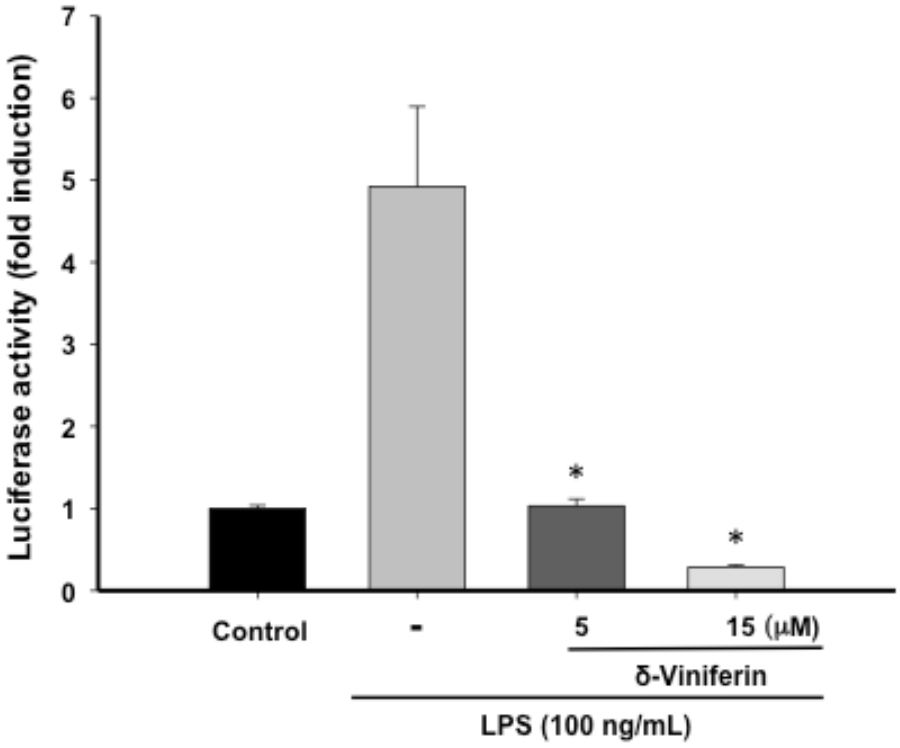


Figure 5.8. Effects of δ-viniferin on LPS-stimulated activation of NF-κB in RAW 264.7 cells.

The cells were transiently transfected with p-NF-κB-Luc reporter gene and then treated with 100 ng/mL LPS alone or with 5 and 15 μM δ-viniferin for 3 h. The data are expressed as means ± SD of triplicate tests. **P* < 0.05.

5.7. Inhibitory Effects of δ-Viniferin on LPS-induced Phosphorylation and Degradation of IκBα.

NF-κB is located in cytoplasm with its inhibitor, IκBα, in unstimulated cells. After the induction of LPS, IKK phosphorylates IκBα. Phosphorylated IκBα is then ubiquitylated and degraded by proteasome. Subsequently, NF-κB translocates from cytoplasm to nucleus. Hence, the effects of δ-viniferin on LPS-induced phosphorylation and degradation of IκBα were explored as a time course study by Western Blot analysis. As can be seen in Figure 5.9A, the level of phosphorylated IκBα increased after 15 min in LPS group. However, the group of δ-viniferin blocked the level of phosphorylated IκBα. Figure 5.9B demonstrated that the expression of phosphorylated IκBα was decreased, so the treatment of δ-viniferin suppressed the activation and translocation of NF-κB to the nucleus.

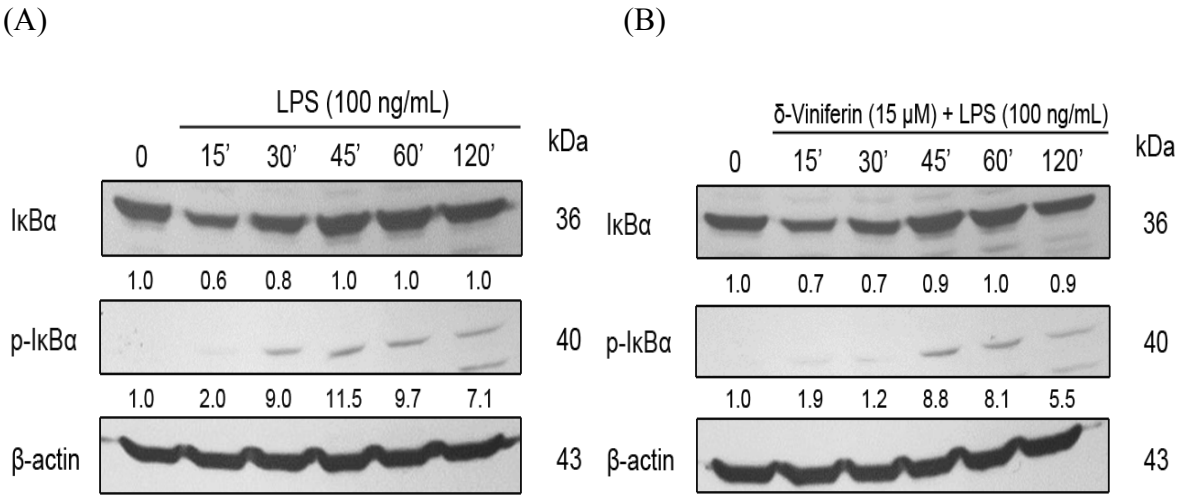


Figure 5.9. Inhibitory effects of δ-viniferin on LPS-stimulated phosphorylation and degradation of IκBα in RAW 264.7.

The cells were treated with (A) 100 ng/mL LPS only or (B) co-treatment with LPS and δ-viniferin (15 μM) in time course study. Total cell lysates were prepared for Western blot analysis. These experiments were repeated three times with similar results.

5.8. Blockade of δ-Viniferin on Phosphorylation of PI3K/Akt in LPS-stimulated RAW 264.7 Cells.

LPS triggers PI3K/Akt signaling is another major upstream molecules for NF-κB activation. We next examined whether δ-viniferin can modulate this upstream signaling pathways by carrying out Western blot analysis to detect the protein levels of phosphorylated PI3K/Akt. When cells treated with LPS alone, the phosphorylation of PI3K/Akt were increased in cells treated with LPS alone at 30 min (Figure 5.10). When cells co-treated with δ-viniferin, the phosphorylation of PI3K/Akt was decreased with 5 and 15 μM δ-viniferin in a dose dependent manner.

These results of Western Blot analysis indicated that δ-viniferin suppressed the LPS-stimulated NF-κB activation by attenuation of IKK and PI3K/Akt pathways, which interrupted the degradation of IκBα and further downstream iNOS and COX- 2 protein expressions (Figure 5.11).

Since the excessive generation of free radical has been suggested as a link between inflammation and cancer (*6*), the potency of anti-oxidation and free radical scavenging may be a factor to influence the results that is associated with the structure-activity relationship. From chemical structure viewpoint, δ-viniferin has one phenolic OH group and two resorcinol groups, and they are determinants of radical scavenging and/or

anti-oxidative potential (*50*). δ-Viniferin possesses a phenylethene side chain, and the orientation and configuration of the side chain play an important part in bioactivity property as indicated by Hirano *et al.* (*47*).

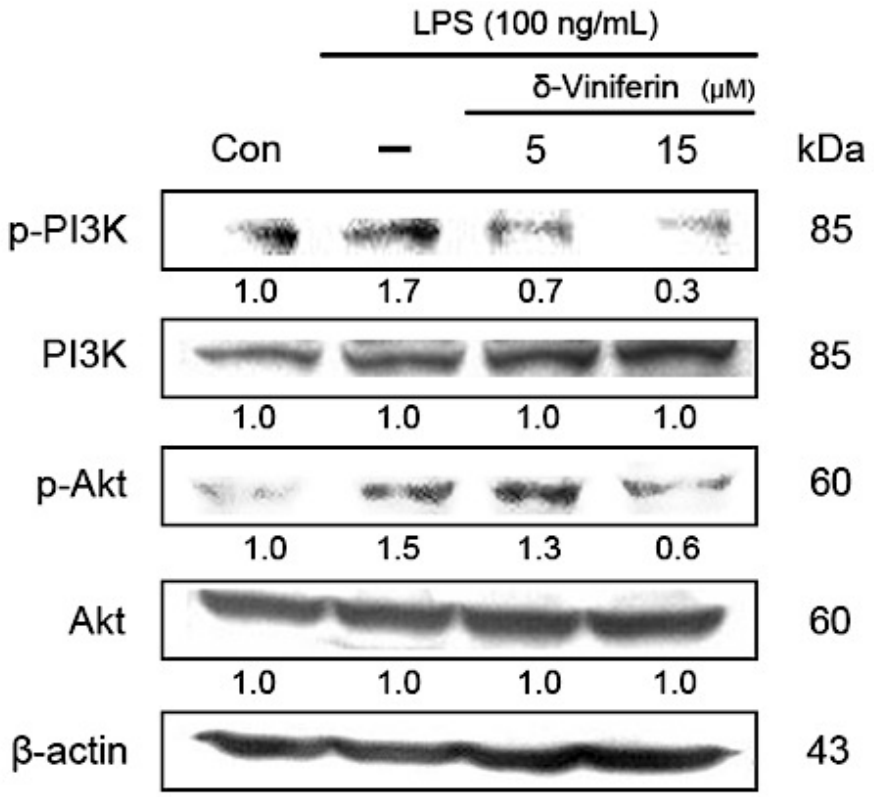


Figure 5.10. Effects of δ-viniferin on LPS-induced PI3K/Akt protein expressions in RAW 264.7 cells.

The cells were treated with 100 ng/mL LPS only or with various concentrations of δ-viniferin (5 and 15 μM) for 30 min. The levels of PI3K/Akt were analyzed by Western blotting. β-actin was used as a loading control.

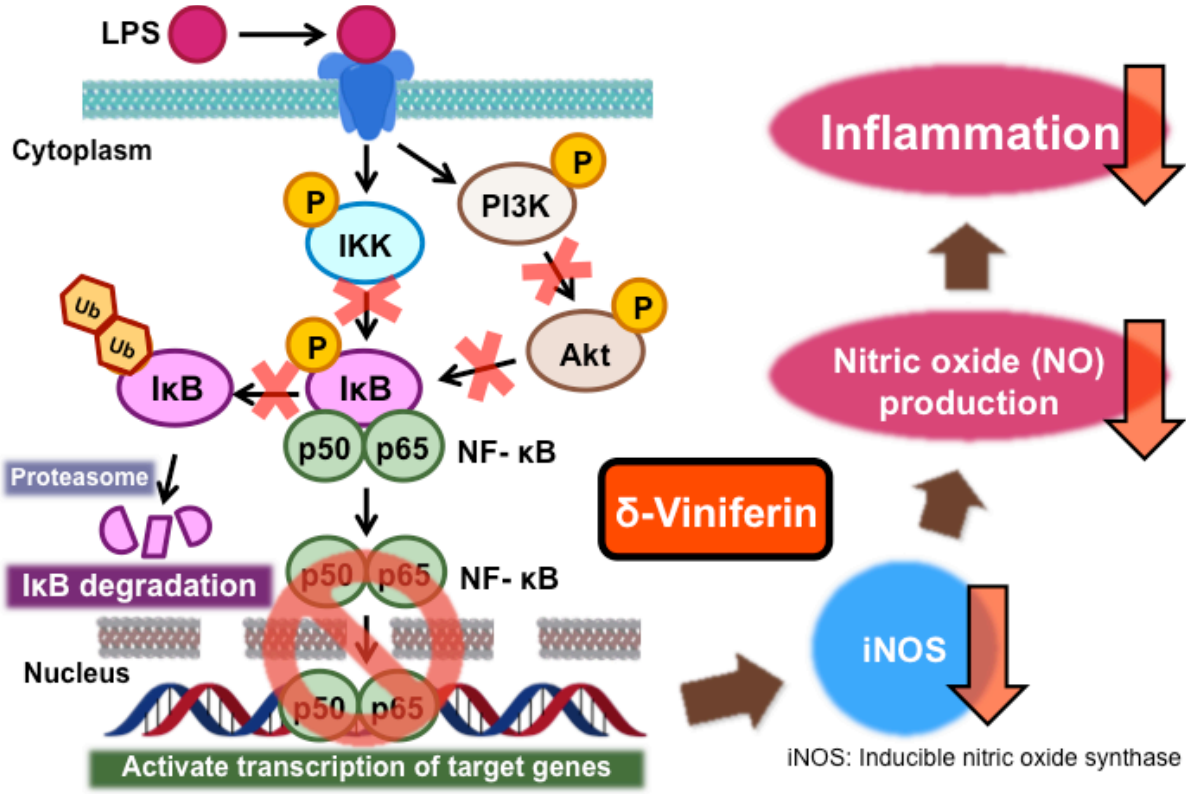


Figure 5.11. Anti-inflammatory effects of δ-viniferin.

Chapter 6. Conclusion

In our data, they demonstrated that δ-viniferin was successfully synthesized and isolated from peroxidase reaction of resveratrol in the presence of H_2O_2. The pure compound has been identified as δ-viniferin by LC-MS and NMR. About the anti-inflammatory effects, δ-viniferin suppressed LPS-stimulated iNOS and COX-2 expressions in murine macrophage through the blockage of intracellular signaling pathways, particularly affecting the activation of IKK pathway, PI3K/Akt pathway, the degradation and phosphorylation of IκBα as well as translocation of NF-κB.

Clear evidence and molecular basis for the anti-inflammatory mechanism of δ-viniferin have provided, and they reveal further potential of δ-viniferin as a novel agent for the treatment of inflammatory diseases. A new generation of anti-inflammatory compound may join traditional chemotherapies.

On the other hand, the response of immune cells can contribute pro- and anti-inflammatory consequences, depending on the disease stages and the signals they receive. Thus, the inflammatory response must be controlled precisely, because excessive amounts could damage the host and too little could lead to immunodeficiency. The

inflammatory balance in the microenvironment has been turn into a very crucial viewpoint and concern in new chemotherapy research.

APPENDIX

List Of Abbreviations

Akt	Protein kinase B
COX	Cyclooxygenase
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionization
IκB	Inhibitor of κB
IKK	IκB kinase
IL-1	Interleukin 1
HPLC	High-performance liquid chromatography
iNOS	Inducible Nitric Oxide Synthase
LC-MS	Liquid chromatography–mass spectrometry
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
m/*z*	Mass/charge
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NOS	Nitric oxide synthase
PG	Prostaglandin
PI3K	Phosphoinositide 3-kinase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TNF-α	Tumor necrosis factor α

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