IDENTIFICATION OF OXIDATIVE PRODUCTS OF PTEROSTILBENE AND 3´-HYDROXYPTEROSTILBENE IN *VITRO* AND EVALUATION OF ANTI-INFLAMMATORY AND ANTI-CANCER CELL PROLIFERATIVE ACTIVITY

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ABSTRACT OF THE THESIS

Identification of oxidative products of pterostilbene and 3′-hydroxypterostilbene in *vitro* and evaluation of anti-inflammatory and anti-cancer cell proliferative activity

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Pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene) is an important phenolic compound which is reported as a potential anti-oxidative, anti-inflammatory, and anti-cancer agent. It is a natural compound and belongs to the group of phytoalexins, agents produced by plants to fight infections like chemicals and fungal. In nature, pterostilbene can be oxidized by peroxidase from fungus, and peroxidase also exists in human body. So it may undergo oxidative process in human as well. 3′-hydroxypterostilbene is another natural phenolic compound and also is a metabolite of pterostilbene identified in mouse. The two adjacent hydroxyl groups in its molecule indicate that it may have a stronger bioactivity than pterostilbene.

To understand the possible oxidative reaction and major products in metabolic process, this research use peroxidase and phosphate-citrate buffer to simulate the peroxidation of pterostilbene and 3′-hydroxypterostilbene in *vitro*. The major oxidative products were

identified as pterostilbene *trans* dehydrodimer and 3′-hydroxypterostilbene *trans* dehydrodimer by LC/MS and NMR.

The anti-inflammatory and anti-cancer cell proliferative activity of three compounds, pterostilbene, 3′-hydroxypterostilbene, and pterostilbene *trans* dehydrodimer, was evaluated in this research. In anti-inflammatory evaluation, Griess assay was used to measure the nitric oxide production in RAW264.7 cells which were treated with the three compounds at different concentration. In anti-cancer cell proliferative study, MTT assay was used to measure the cell viability of two cancer cell lines: drug-sensitive HL-60 leukemia cell line and drug-resistant MDA-MB231 breast cancer cell line. According to our data, 3′-hydroxypterostilbene had a significant anti-inflammatory and anti-cancer cell proliferative activity.

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LIST OF ABBREVIATIONS

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
IC_{50}	Half maximal inhibitory concentration
CFU-GM	Colony forming unit granulocyte macrophage progenitor
eNOS	Endothelial Nitric Oxide Synthase
nNOS	Neuronal Nitric Oxide Synthase
iNOS	Inducible Nitric Oxide Synthase
LPS	Lipopolysaccharides
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
APCI	Atmospheric Pressure Chemical Ionization
ESI	Electrospray ionization
HPLC	High performance liquid chromatography
LC/MS	Liquid chromatography–mass spectrometry
NMR	Nuclear Magnetic Resonance
RPMI	Roswell Park Memorial Institute medium
DMEM	Dulbecco's Modified Eagle's medium

INTRODUCTION

1.1 Pterostilbene

1.1.1 Structure and natural sources

Pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxy-stilbene), a naturally occurring phytoalexin compound, is a dimethylated analog of resveratrol.

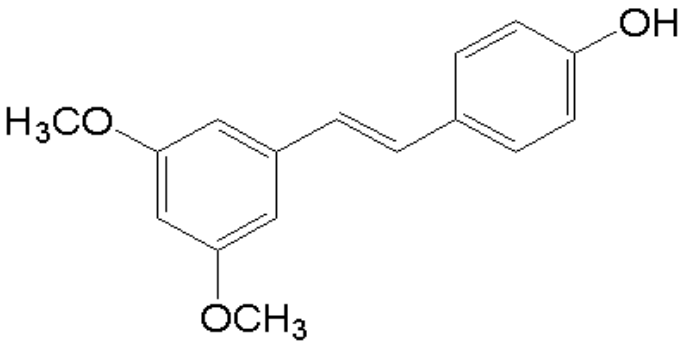


Figure 1. Chemical structure of pterostilbene

It is one of phenolic compounds named as stilbenes and is identified in many plant species as potent antifungal compound (Rodriguez-Bonilla, Mendez-Cazorla, Lopez-Nicolas, & Garcia-Carmona, 2011). The phytoalexin compounds are the secondary metabolites synthesized by plant's leaf tissues to fight environmental stress or fungal infections (Pont & Pezet, 1990). Over the years, pterostilbene attracted attention of many researchers because of its extensive biological activities. It displays anti-oxidant, anti-inflammatory, anti-cancer, anti-diabetes, and anti-cholesterol activity in cell culture and

animal studies (M. F. Lee, Liu, Cheng, Tsai, Ho, Liou, et al., 2013). Pterostilbene was first isolated from red sandalwood named as pterocarpus santalinus, which is a kind of plant widely growth in south India. Pterostilbene is also identified in grapes, blueberries and *Vitis vinifera* leaves (Pari & Satheesh, 2006). Blueberries contain about 15 μg pterostilbene per 100 g blueberries (McCormack & McFadden, 2012). Over the years, pterostilbene is used in traditional medicine or food to treat many kind of disease by human. Another kind of red sandalwood, pterocarpus marsupium, which also contains pterostilbene, is one of the traditional medicinal plants that have been used for many years in the treatment of diabetes. And darakchasava, which is a medicinal drink made primarily from dried grape berries, is used to treat cardiovascular and other ailments (Paul, Masih, Deopujari, & Charpentier, 1999).

1.1.2 Bioavailability, pharmacokinetics, and metabolism

Pterostilbene has similar pharmacologic properties as resveratrol. But pterostilbene has many advantages due to the difference of their structure. Pterostilbene contains two methoxy groups on the A ring and one hydroxyl group on the B ring. But resveratrol contain three hydroxyl groups. Resveratrol is photosensitive and unstable during metabolism (Goldberg, Ng, Karumanchiri, Yan, Diamandis, & Soleas, 1995). While the dimethoxyl modification makes pterostilbene more stable and able to generate more persistent biological effect than resveratrol (Meng, Yang, Chen, Wang, Zhang, Wang, et

al., 2008). The dimethyl ether structure of pterostilbene also makes it more lipophilic, resulting in a higher membrane permeability and cellular uptake. For stilbenoids, bioavailability is important for the essence of bioactivity. When administered orally, pterostilbene shows 12.5 +/- 4.7% absolute bioavailability in rat plasma. In human, less than 5 ng unchanged resveratrol was detected in plasma after a 25 mg oral dose in six volunteers. (Lin, Yue, & Ho, 2009). The half-life of pterosilbene is also seven times longer than resveratrol. The terminal elimination half-life of pterostilbene and resveratrol is 105 min and 14 min, respectively (Remsberg, Y áñez, Ohgami, Vega-Villa, Rimando, & Davies, 2008). Both of these indicate that pterostilbene has a better pharmacokinetic characteristic than resveratrol (Lin, Yue, & Ho, 2009).

The metabolism of pterostilbene in *vitro* has been studied in rat liver microsomes. It shows pterostilbene is metabolized by phase II enzyme and the major metabolite is pterostilbene glucuronide (Remsberg, Y áñez, Ohgami, Vega-Villa, Rimando, & Davies, 2008). Another research studies the metabolism of pterosilbene in *vivo*. In this research, the structure of urinary metabolites in mice were analyzed by LC/MS^n (n=1-3). Nine metabolic products are detected: pterostilbene glucuronide, pterostilbene sulfate, mono-demethylated pterostilbene, mono-demethylated pterostilbene glucuronide, mono-demethylated pterostilbene sulfate, mono-hydroxylated pterostilbene, mono-hydroxylated pterostilbene glucuronide, mono-hydroxylated pterostilbene sulfate, and mono-hydroxylated pterostilbene glucuronide sulfate. They are formed through hydroxylation

and demethylation of phase I biotransformation and glucuronidation and sulfation of phase II biotransformation (Shao, Chen, Badmaev, Ho, & Sang, 2010).

1.1.3 Cytotoxicity

Pterostilbene is cytotoxic to many kinds of cells, like various cancer cells, leukemia cells, and aortic vascular smooth muscle cells. A study tested the cytotoxicity of pterostilbene to prostate cancer cell line PC-3 and breast cancer cell line MCF-7. The cells were treated with pterostilbene at concentration ranging from 40 to 80 μM for 24 h. Then MTT assay was used to evaluate the anti-proliferative effect of pterostilbene. The IC_{50} values in MCF-7 and PC3 cell lines were 65.6 and 74.3 μM, respectively (Chakraborty, Gupta, Ghosh, & Roy, 2010).

Pterostilbene significantly inhibited platelet-derived growth factor (PDGF)-BB-induced abnormal proliferation of rat vascular smooth muscle cells. At 1, 3, and 5 μM, pterostilbene inhibited vascular smooth muscle cells proliferation by 68.5, 80.7, and 94.6%, respectively. At same concentration, pterostilbene also reduced DNA synthesis by 47.4, 76.7, and 100%, respectively. The molecular mechanism is that pterostilbene inhibited the PDGF-BB-induced phosphorylation of Akt kinase and down-regulated the cell cycle related proteins (Park, Lim, Hong, Yoo, Lee, Pyo, et al., 2010).

Pterostilbene exhibit a relatively low toxicity on normal cells. For example, the IC_{50} values of pterostilbene on sensitive leukemia cells HL60 and drug-resistant leukemia

cells HL60-R are 35 and 40 μM, respectively. And the AC_{50} values of them are 75 and 85 μM, respectively. While on normal hemopoietic cells, when the CFU-GM number is 50% lower than control after 7 days cell culture, the concentration of pterostilbene is about 150 μM. In addition, when the concentration of pterostilbene is 50 μM, there is no visible decrease of CFU-GM number on normal hemopoietic cells (Tolomeo, Grimaudo, Di Cristina, Roberti, Pizzirani, Meli, et al., 2005).

1.1.4 Anti-inflammatory activity

In previous study, pterostilbene showed anti-inflammatory activity in different cell lines, including macrophages, canine chondrocytes (CnC), and microglial cell line (Meng, et al., 2008; M.-H. Pan, Chang, Tsai, Lai, Ho, Badmaev, et al., 2008; Remsberg, Y áñez, Ohgami, Vega-Villa, Rimando, & Davies, 2008). Nitric oxide (NO) is one of inflammatory mediators in various cell types. It is an important cellular signaling molecule and produced by nitric oxide synthases (NOSs). But over-production of nitric oxide has a role in the pathogenesis of acute or chronic inflammation and causes tissue damage and vascular leakage in inflammatory organ. NOS enzymes have three groups. One is eNOS (Endothelial Nitric Oxide Synthase), one is nNOS (Neuronal Nitric Oxide Synthase), and the last one is iNOS (Inducible Nitric Oxide Synthase). The third group, iNOS, is an inducible isoform. The gene coding for iNOS is located on chromosome 17. iNOS produces large amount of NO stimulated by some factors, like proinflammatory

cytokines and bacterial lipopolysaccharide (LPS) (Knowles & Moncada, 1994). Cyclooxygenase-2 (COX-2) is an inducible enzyme and is induced by inflammatory mediators. It catalyzes free arachidonic acid to prostaglandins. Prostaglandins can stimulate proliferation, promote angiogenesis, and suppress apoptosis of tumor cells (Athar, Back, Kopelovich, Bickers, & Kim, 2009). In addition, COX-2-derived bioactive lipids, like prostaglandin E_2, are potential inflammatory mediators (M.-H. Pan, et al., 2008). NF-κB is a kind of protein which manages the transcription of DNA and is involved in cellular responses to cytokines, bacterial antigens, and free radicals. It plays an important role in the enhancement of iNOS and COX-2 gene expression. So iNOS, COX-2, and NF-κB are three important factors in the study of anti-inflammatory activity. Pterostilbene has been found to have strong inhibitory effect on lipopolysaccharide-induced iNOS and COX-2 over expression in murine RAW264.7 cells by significantly blocking the protein and mRNA expression of these two enzymes. The molecular mechanism is that pterostilbene reduces the transcriptional activity of NF-κB by blocking phosphorylation and degradation of IκBα and inhibiting the LPS-induced activation of PI3K/Akt, extracellular signal-regulated kinase 1/2 and p38 MAPK (M.-H. Pan, et al., 2008). In microglial cells, pterostilbene also reduces the LPS-induced protein and mRNA expression of iNOS. The pathway of this process is pterostilbene inhibits the production of proinflammatory cytokines, TNF-α, by blocking IκBα phosphorylation and degradation (Meng, et al., 2008). Subsequently, a decrease of NO production is observed.

1.1.5 Antioxidant activity

Because pterostilbene is a phenolic compound, the hydroxyl group on aromatic ring makes pterostilbene a strong oxygen scavenging agent. It shows antioxidant activity both in *vitro* and in *vivo*. The antioxidant activity of pterostilbene was first studied in *vitro* about its methyl linoleate oxidation inhibitory effect. In addition, the peroxyl-radical and DPPH free radical scavenging activity of pterostilbene is comparable to that of resveratrol. The antioxidant potential of pterostilbene and resveratrol is 237±58, and 253±53 μM, respectively. Both of them is stronger than Trolox as free radical scavengers (Rimando, Cuendet, Desmarchelier, Mehta, Pezzuto, & Duke, 2002). In breast cancer cell line MCF-7 and prostate cancer cell line PC3, which are treated with pterostilbene, the production of reduced formazone increased significantly and the level of intracellular reactive oxygen species was much lower than control (Chakraborty, Gupta, Ghosh, & Roy, 2010). In rat liver microsomes and human fibroblasts, pterostilbene could effectively inhibit lipid peroxidation and the oxidation of citronellal (Stivala, Savio, Carafoli, Perucca, Bianchi, Maga, et al., 2001).

The ability of pterostilbene to work as an antioxidant is important for its other health beneficial effect, such as anti-cancer and anti-inflammatory activity. For example, pterostilbene could inhibit dimethylnitrosamine-induced liver fibrosis in rats. One of the reason is that pterostilbene could ameliorate oxidative stress in the initiation and progression of chronic liver disease (M. F. Lee, et al., 2013).

1.1.6 Anti-cancer activity

Pterostilbene is an effective anti-cancer agent in many common malignancies by working as an anticarcinogenic compound. The 3,5-dimethoxy group on A-ring of pterostilbene provides pterostilbene a potential pro-apoptotic activities. This kind of structure makes pterostilbene to be more effective against several drug resistant cancer cells than resveratrol (Tolomeo, et al., 2005). It has been reported that pterostilbene induced apoptosis in breast, liver, lung, stomach, colon, leukemia, pancreas, and prostate cancers through intrinsic and extrinsic apoptotic pathways. Its antioxidant activity also facilitate its anti-cancer benefit in several cell lines. In *vitro*, pterostilbene could inhibit cancer cell growth by cell cycle alteration and apoptosis induction. In *vivo*, pterostilbene can inhibit tumorigenesis and cancer cell metastasis with negligible cytotoxicity and a relatively higher bioavailability comparing to resveratrol (McCormack & McFadden, 2012).

By regulation of mitochondrial proteins Bcl-2, Bax, Bad, and cytochrome *c*, pterostilbene induces intrinsic apoptosis in cancer cells. Pterostilbene could induce cell cycle phase arrest and change expression of cell cycle regulators such as Rb and p53. For example, the G0/G1 phase in bladder cancer cells treated with pterostilbene was increased, while G2/M phase was decreased at 48 and 72 h. Then S phase was arrested, ultimately result in decreased cell proliferation. In some cancer cell lines, like stomach and leukemia cells, Fas-receptor mediated mechanism, which is extrinsic apoptotic pathway, also involved in pterostilbene apoptosis-induced activity (McCormack & McFadden, 2012).

In breast cancer cell line MCF-7 and prostate cancer cell line PC3, the anti-proliferative activity of pterostilbene has been evaluated. At a concentration equivalent to IC_{50} value, pterostilbene introduced a significant apoptosis in both cell lines after 24 h of incubation. Also, a clear DNA fragmentation in these two cell lines was observed. The possible mechanism for pterostilbene's anti-proliferative characteristic in MCF-7 and PC3 cell lines was investigated in this study. In MCF-7 cells, after incubation with pterostilbene, the production of ROS increased by 8.5 times. This effect then influenced the intracellular mitochondrial membrane potential and ionic balance, which is needed for cancer cell growth. Pterostilbene also could inhibit cell proliferating factors, Akt and MMP9, the down regulation of which lead to activation of apoptosis (Chakraborty, Gupta, Ghosh, & Roy, 2010).

Another major mechanism of pterostilbene's anti-cancer property is pterostilbene could trigger the lysosomal membrane permeabilization pathway. Several cancer cells exhibited different grades of apoptosis under treatment of pterostilbene depending on their lysosomal heat shock protein 70 (HSP70) content, a stabilizer of lysosomal membrane. For example, A549 lung cancer cells which have low levels of HSP70 are more susceptible to pterostilbene, while MCF7 breast cancer cells are more susceptible due to a high level of HSP70 protein (Mena, Rodriguez, Ponsoda, Estrela, Jaattela, & Ortega, 2012).

1.1.7 Anti-diabetic activity

Pterostilbene has been reported to have anti-diabetic activity both in *vitro* and in *vivo*. It has been used in traditional medicine like *Pterocarpus marsupium* in the treatment of diabetes mellitus for many years (Pari & Satheesh, 2006). The aqueous extract of *Pterocarpus marsupium* hardwood, which contain pterostilbene, has been found to increase the insulin secretion and glucose uptake in mouse pancreatic and muscle tissues in a dose-dependent manner, respectively (Mohankumar, O'Shea, & McFarlane, 2012). In vivo, after orally administrated with pterostilbene (40 mg/kg), the plasma glucose level significantly decreased in streptozotocin- and nicotinamide-induced diabetic rats. In this study, a relatively long-term effect of pterostilbene on diabetic rats was examined, too. The normal and streptozotocin- and nicotinamide-induced diabetic rats were orally administrated with pterostilbene (40 mg/kg) for 6 weeks. A significant decrease of plasma glucose level and an enhancement of plasma insulin level were observed in both normal and diabetic rats. The glycosylated hemoglobin reduced and the total hemoglobin level increased after the treatment of pterostilbene. In diabetic rats, the increase activity of hepatic enzymes like hexokinase and a decrease activity of glucose-6-phosphatase and fructose-1,6-bisphosphatase were observed. While in normal rats, the activity of these enzymes were not significantly changed (Pari & Satheesh, 2006).

1.2 Resveratrol

1.2.1 Structure and natural sources

Resveratrol (*trans*-3,4',5-trihydroxystilbene) is a well-studied polyphenol compound present in grapes, berries, and peanuts. It is also found in various medicinal plants. For example, resveratrol was first identified in *Polygonum cuspidatum*, a traditional medicine used as an anti-inflammatory agents in China and Japan (Signorelli & Ghidoni, 2005). In plants, it mostly exists in glycosylated piceid forms (3-O-β-D-glucosides) (Athar, Back, Tang, Kim, Kopelovich, Bickers, et al., 2007).

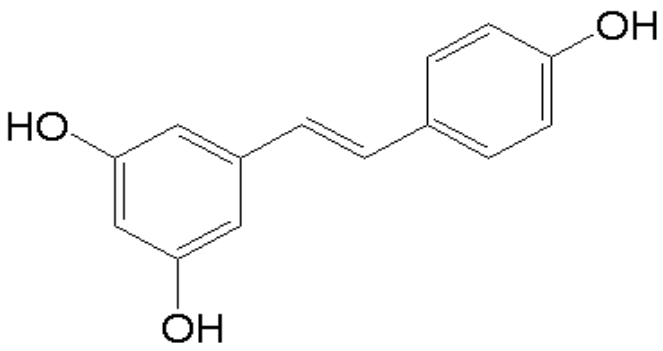


Figure 2. Chemical structure of resveratrol

As an antifungal agent, resveratrol is about 60-100 times weaker than pterostilbene. Red wine is a rich source of resveratrol and provides up to 10 times more resveratrol than white wine. Because in red wine making process, grape skin and often seeds are reserved during the fermentation process. The skin and seeds contain most of resveratrol in grapes (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012). One gram

fresh grape skin usually contains 50 to 100 μg of resveratrol by wet weight (Baliga, Meleth, & Katiyar, 2005). Red wine contains an average 1.5 to 3 mg resveratrol per liter (Rimando, Kalt, Magee, Dewey, & Ballington, 2004).

1.2.2 Bioavailability, pharmacokinetics, and metabolism

Resveratrol is photosensitive and unstable during metabolism, and its half-life is only 14 min. So it has a relative low bioavailability (Goldberg, Ng, Karumanchiri, Yan, Diamandis, & Soleas, 1995). But following oral administration to humans, resveratrol is much more efficiently absorbed comparing to other polyphenols, like quercetin and catechin (Soleas, Yan, & Goldberg, 2001). Glycosylation could prevent resveratrol from oxidative degradation, and glycosylated resveratrol is more stable and easier to be absorbed in the human gastrointestinal tract than resveratrol (Pezet, Gindro, Viret, & Spring, 2004). In humans, resveratrol is metabolized in liver by phase-2 drug-metabolizing enzymes to water-soluble *trans*-resveratrol-3-O-glucuronide and *trans*-resveratrol-3-O-sulfate in urine excretion (Walle, Hsieh, DeLegge, Oatis, & Walle, 2004). A research studied the pharmacokinetics of resveratrol and metabolites in human. They used two kind of resveratrol source: red wine and grape extract tablets. 17 kind of resveratrol conjugates as metabolites had been identified. Some of them formed in intestine and were catalyzed by microbiota, such as dihydroresveratrol-glucuronides. They found the resveratrol which administered as nutraceutical stayed longer in human's

gut than the resveratrol administered as red wine, because a higher concentration of microbial metabolites was detected in urine after grape extract tablets were consumed. This makes them easier to be metabolized (Rotches-Ribalta, Andres-Lacueva, Estruch, Escribano, & Urpi-Sarda, 2012). Although there are some reports indicate that in *vitro* the bioactivity of resveratrol metabolites is lower than that of resveratrol (Hoshino, Park, Kondratyuk, Marler, Pezzuto, van Breemen, et al., 2010), the conjugated resveratrol may exhibit higher health beneficial in *vivo*. Because the half-life of resveratrol metabolites is much longer than its precursor. The plasma half-life of resveratrol metabolites is about 9.2 h (Walle, Hsieh, DeLegge, Oatis, & Walle, 2004). Another reason is enzymes in human body may convert the metabolites back to resveratrol (Miksits, Wlcek, Svoboda, Kunert, Haslinger, Thalhammer, et al., 2009). In addition, *trans*-resveratrol-3-sulphate, *trans*-resveratrol-3,4'-disulphate, *trans*-resveratrol-3,5-disulphate, *trans*-resveratrol-3-glucuronide and *trans*-resveratrol-4'-O-glucuronide have been identified as main metabolites of resveratrol (Yu, Shin, Chow, Li, Kosmeder, Lee, et al., 2002).

1.2.3 Cytotoxicity

A research studies the cytotoxicity of resveratrol on human carcinoma cells and normal peripheral blood mononuclear cells. The result shows that resveratrol exhibits a higher cytotoxic activity against HeLa and MDA-MB-453 cells than unstimulated and stimulated human peripheral blood mononuclear cells (Matic, Zizak, Simonovic,

Simonovic, Godevac, Savikin, et al., 2010). Another research studies the toxicity of resveratrol on normal hemopoietic cells. In this research, bone marrow cells were treated with *trans*-resveratrol at 25, 50, 100, and 150μM, respectively. After 7 days of cell culture, the number of CFU-GM was calculated. The CFU-GM number of normal hemopoietic cells which are treated with 50 μM *trans*-resveratrol is about 30% lower than control. While for sensitive leukemia cell line HL60 and multidrug resistant leukemia cell line HL60-R, the concentration of *trans*-resveratrol which caused an approximate decrease of 30% in the CFU-GM number is about 2μM and 25μM, respectively (Tolomeo, et al., 2005). This result also indicates that resveratrol has a higher toxicity on leukemia cells than on normal hemopoietic cells.

1.2.4 Anti-inflammatory activity

The anti-inflammatory activity of resveratrol has been well studied. It can suppress lipopolysaccharide-induced macrophage activation by inhibiting NO generation. Resveratrol can reduce the amount of cytosolic iNOS protein and the activation of NF-κB (Tsai, Lin-Shiau, & Lin, 1999). The molecular mechanism is resveratrol specifically inhibits TRIF signaling in the TLR3 and TLR4 pathway by targeting TANK-binding kinase 1 and RIP1 in TRIF complex (Youn, Lee, Fitzgerald, Young, Akira, & Hwang, 2005). In addition, resveratrol can cross the blood-brain barrier and regulate some symptoms of debilitating neurological disorders (Meng, et al., 2008). Microglial are

macrophage-like cells and also mediators of inflammation in the central nervous system (Gonzalez-Scarano & Baltuch, 1999). Resveratrol can inhibit NO production and TNF-α in LPS-induced microglia by decreasing expression of iNOS and phosphorylation of p38 mitogen-activated protein kinases (MAPKs) (Bi, Yang, Dong, Wang, Cui, Ikeshima, et al., 2005).

1.2.5 Antioxidant activity

Resveratrol has an intrinsic antioxidant activity that could be related to its chemopreventive effects (Fernández-Mar, Mateos, García-Parrilla, Puertas, & Cantos-Villar, 2012).

Phenolic antioxidants could scavenge free radicals through two major reducing pathways: H-transfer as well as single electron transfer followed by proton transfer mechanisms (Mikulski & Molski, 2012). Recently, other mechanisms have been found: sequential proton loss electron transfer mechanism which is important in solution-phase (Litwinienko & Ingold, 2004). Resveratrol contains three active hydroxyl groups that could be potential hydrogen atom donor and have high potency to scavenge harmful free radicals in human body. It has reported that the antioxidant activity of resveratrol is higher than that of vitamin C and vitamin E (Soares, Andreazza, & Salvador, 2003). So resveratrol can help to prevent oxidative stress-induced cellular damage and apoptosis. The long-term moderate consumption of red wine which contains resveratrol is related to

the cardiovascular benefit. For example, the antioxidant activity of resveratrol could protect against progression of atherosclerosis (Mikulski & Molski, 2012). A research studied the antioxidant potency of several stilbenoids and demonstrated that this potency is mainly determined by number of hydroxyl groups, isopentenyl and isopentadienyl moiety (Chang, Lai, Djoko, Wu, Liu, Liu, et al., 2006). In *vitro*, resveratrol has been found to have effect DPPH•, $ABTS^{\cdot+}$, $DMPD^{\cdot+}$, $O2^{\cdot-}$, and H_2O_2 scavenging activities, reducing power, and Fe^{2+} chelating activities. Resveratrol inhibited 89.1% of lipid peroxidation for linoleic acid (Gülçin, 2010). In *vivo*, the total antioxidant activity of resveratrol was studies in rats. Resveratrol increases plasma antioxidant ability and reduces lipid peroxidation (Wenzel, Soldo, Erbersdobler, & Somoza, 2005; Whitehead, Robinson, Allaway, Syms, & Hale, 1995).

1.2.6 Antidiabetic activity

Resveratrol also shows antidiabetic activity. In *vivo*, resveratrol reduces the levels of blood glucose, glycosylated hemoglobin, blood urea, and pathophysiological enzyme activity in streptozotocin-nicotinamide induced diabetic rats. At the same time, resveratrol can enhance the levels of plasma insulin and hemoglobin (Palsamy & Subramanian, 2008). It can increase insulin sensitivity in liver and muscle. Resveratrol also upregulates some positive regulator of insulin production, such as pancreatic peroxisome proliferator activated-receptor-γ (PPARγ) and pancreatic-duodenal

homeobox-1 (PDX-1) (B.-H. Lee, Lee, Cheng, Chang, Hsu, & Wu, 2013). Additionally, resveratrol can activate nuclear factor-erythroid 2-related factor 2 (Nrf2) pathway which attenuate oxidative stress and avoid hyperglycemia then protect pancreatic function (Cheng, Cheng, Chiou, & Chang, 2012).

1.2.7 Anti-cancer activity

Resveratrol shows cancer chemopreventive activity in three stages of carcinogenesis: tumor initiation, promotion, and progression (Jang, Cai, Udeani, Slowing, Thomas, Beecher, et al., 1997). As an anti-initiation agent, resveratrol could act as an antioxidant and antimutagen to attenuate DNA damage from stimulating factors like carcinogens and free radicals. It can inhibit phase I enzymes like CYP450, which activate carcinogens, and induce phase II enzymes like UDP-glucuronyltransferase, which can make carcinogens being more hydrophilic and difficult to enter cells (Signorelli & Ghidoni, 2005). Resveratrol could slow tumor development through several mechanisms. It inhibits the enzymatic activity of cyclooxygenase-2 (COX-2), which is induced by inflammatory mediators and catalyzes the conversion of free arachidonic acid to prostaglandins. Prostaglandins can stimulate proliferation, promote angiogenesis, and suppress apoptosis of tumor cells. In the presence of COX-2, a significant oxidative DNA damage has been observed (Athar, Back, Kopelovich, Bickers, & Kim, 2009). In addition, resveratrol could induce cell cycle arrest and apoptosis in tumor cells.

Resveratrol modulates the major cell cycle mediators at micromole concentrations, arresting cancer cells at the G1/S, S, or G2/M phase of the cell cycle. Resveratrol induce apoptosis by activating p53, Fas/CD95/APO-1 death receptor, and other key proteins involved in DNA synthesis and cell cycle in various human cancer cells (Athar, Back, Kopelovich, Bickers, & Kim, 2009). Resveratrol influences the activity of transcriptional factors like NF-κB and AP1 (Signorelli & Ghidoni, 2005). Resveratrol can also inhibit adenosine monophosphate (AMP)-activated protein kinase (AMPK) which is a potential antitumor molecular target and associate in cancer development.

In *vitro*, resveratrol shows significant anti-proliferative activity in multiple cell lines. For example, resveratrol leads to a 70% growth inhibition in colon cancer CaCo-2 cells treated with 25μM of resveratrol. In *vivo*, it reported that rats induced by DMBA and administrated with resveratrol in diet (1g/kg) lifetime had a reduced susceptibility to mammary cancer (Athar, et al., 2007). In a clinical study, an inverse relationship was observed between resveratrol from grape consumption and human breast cancer (La Vecchia & Bosetti, 2006). However, some factors such as dosage, diet sources, and tumor origin may affect the efficiency of resveratrol treatment. For example, some in *vivo* research found resveratrol has no effect on specific cancer cases. Although there are some clinical studies which indicate resveratrol may be a potential anticancer agent, at present there is no convincing evidence that resveratrol can be used to cure cancer. On the other hand, the metabolism and safety of resveratrol still need to be investigated in the future.

1.3 3′-hydroxypterostilbene

1.3.1 Structure and natural sources

3′-hydroxypterostilbene (trans-3,5-dimethoxy-3′,4′-dihydroxystilbene) is a natural pterostilbene analogue. It has been isolated from a kind of shrub named *Sphaerophysa salsula*, which widely grows in central Asia and northwest China (Ma, Li, Li, & Wang, 2002). 3′-hydroxypterostilbene is colorless, needle-shaped crystals. The melting point of 3′-hydroxypterostilbene is 72 ℃ (Ma, Li, Li, & Wang, 2002). It has two hydroxyl groups on B ring.

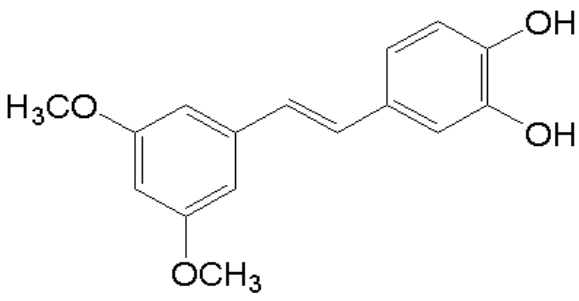


Figure 3. Chemical structure of 3′-hydroxypterostilbene

This structure indicates that 3′-hydroxypterostilbene may has a higher bioactivity than pterostilbene. At the same time, the two methoxy groups on A ring makes this compound more lipophilic than resveratrol and has a better cellular permeability. So 3′-hydroxypterostilbene is a potential bioactive agent for health benefit like resveratrol and pterostilbene.

Because of the additional hydroxyl group on B ring, 3′-hydroxypterostilbene is more chemically active. So it may undergo oxidative reaction and be catalyzed by peroxidase easier than pterostilbene in human. Therefore, this research will also study the oxidative process and major products of 3′-hydroxypterostilbene in *vitro*.

1.3.2 Pterostilbene metabolites

3′-hydroxypterostilbene is one of nine urinary metabolites of pterostilbene identified in mice (Shao, Chen, Badmaev, Ho, & Sang, 2010). In this research, female mice (24-30 g) were administered with pterostilbene in dimethyl sulfoxide by oral gavage (200mg/kg). After 24 hours, urine samples were collected. Then the structure of metabolites in urine were analyzed by LC/MS^n (n=1-3). Three kinds of detectors were used in analysis, including atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), and UV detector. 3′-hydroxypterostilbene was identified as one of nine major urinary pterostilbene metabolites in mice. It is formed through hydroxylation of phase I biotransformation in liver. In addition, three conjugated metabolites of mono-hydroxylated pterostilbene were identified: mono-hydroxylated pterostilbene glucuronide, mono-hydroxylated pterostilbene sulfate, and mono-hydroxylated pterostilbene glucuronide sulfate (Shao, Chen, Badmaev, Ho, & Sang, 2010).

1.3.3 Pharmacokinetics

A previous research studied the HPLC quantification method and pharmacokinetic characterization of 3′-hydroxypterostilbene in rats. In this study, 3′-hydroxypterostilbene glucuronide was identified as the major form of urinary metabolites. The mean half-life of 3′-hydroxypterostilbene in urine and serum is 1.02 h and 0.45 h, respectively. The mean rate of elimination was 0.69 h. The fraction of unchanged 3'-hydroxypterostilbene that excreted in urine was 17.15%. At last, the mean renal clearance was 2.23L/h/kg (Takemoto & Davies, 2009).

1.3.4 Cytotoxicity

The apoptotic activity of 3′-hydroxypterostilbene has been evaluated in different leukemia cell lines. In sensitive cell lines, HL60 and HUT78, 3′-hydroxypterostilbene is 50-97 times stronger than resveratrol as an apoptotic agent. The half maximal inhibitory concentration (IC_{50}) is 0.6 and 0.8 μM, respectively. 3'-hydroxypterostilbene could induce apoptosis in two Fas-ligand resistant lymphoma cell lines HUT78B1 and HUT78B3 and also the multi-drug-resistant leukemia cell lines HL60-R and K562-ADR while resveratrol could not. The half maximal inhibitory concentration (IC_{50}) of 3′-hydroxypterostilbene is between 0.8 and 1.2 μM. 3′-hydroxypterostilbene is found to be more active than other compounds including resveratrol, piceatannlo, and pterostilbene, especially for drug-resistant leukemia cell lines. The metabolism of apoptosis-induced

activity is that 3'-hydroxypterostilbene triggers the intrinsic apoptotic pathway in cells because it causes a disruption of the mitochondrial membrane potential ΔΨ and its apoptotic effects are inhibited by Z-VAD-fmk and the caspase-9-inhibitor Z-LEHD-fmk. At the same time, 3′-hydroxypterostilbene exhibits a low toxicity in normal hemopoietic stem cells. Again, bone marrow cells were treated with 3′-hydroxypterostilbene at 25, 50, 100, and 150 μM, respectively. After 7 days of cell culture, the number of CFU-GM was calculated. The CFU-GM number of normal hemopoietic cells which were treated with 50 μM 3′-hydroxypterostilbene was 50% lower than control. While in sensitive and drug-resistant leukemia cell lines, the IC_{50} and AC_{50} value of 3'-hydroxypterostilbene were between 0.6 and 3.5 μM (Tolomeo, et al., 2005).

1.4 Comparison of pterostilbene, resveratrol, and 3′-hydroxypterostilbene

Pterostilbene, resveratrol, and 3′-hydroxypterostilbene are all naturally-derived compounds. And 3′-hydroxypterostilbene is metabolite of pterostilbene identified in mice. Among these three compounds, resveratrol is most widely studied in the world. However, due to pterostilbene's better bioavailability and extensive health benefit, more and more scientists become interested in pterostilbene these years.

Comparing to resveratrol, pterostilbene contains two methoxy groups on the A ring and one hydroxyl group on the B ring. This dimethoxyl modification makes pterostilbene more stable and able to generate more persistent biological effect than resveratrol. The

dimethyl ether structure also makes it more lipophilic, resulting in a higher membrane permeability and cellular uptake than resveratrol. The third compound, 3′-hydroxypterostilbene also contains two methoxy groups on the A ring. However, it has two hydroxyl groups on B ring. This structure indicates that 3′-hydroxypterostilbene may have a similar bioavailability as pterostilbene, at the same time, possess an even higher bioactivity than its parent.

All of these three compounds exhibit a relatively low cytotoxicity in normal cells comparing to cancer cell line. Both pterostilbene and resveratrol could induce apoptosis in multiple cancer cells and inhibit tumorigenesis and cancer cell metastasis in *vivo*. They also have other health beneficial bioactivity, including anti-oxidant, anti-diabetic, and anti-inflammatory activity. Few reports which focus on the in *vitro* and in *vivo* bioactivity of 3′-hydroxypterostilbene has been published and these activities need to be further studied.

HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Both pterostilbene and 3′-hydroxypterostilbene are phenolic compounds and have been identified in multiple plant species as potent antifungal agents. In nature, pterostilbene in plants can be oxidized by peroxidase from fungus as model of detoxification process. For example, pterostilbene could undergo oxidative process by enzymes like peroxidase obtained from horseradish (Rodriguez-Bonilla, Mendez-Cazorla, Lopez-Nicolas, & Garcia-Carmona, 2011). In addition, other phytoalexin-type stilbenes like resveratrol can be oxidized and form a dimeric structure, too (Dercks & Creasy, 1989). As we know, phenols are one of the most suitable peroxidase substrates (Ponzoni, Beneventi, Cramarossa, Raimondi, Trevisi, Pagnoni, et al., 2007). Thus, our hypothesis is that pterostilbene and 3′-hydroxypterostilbene could undergo oxidative process in *vitro*. Pterostilbene and 3′-hydroxypterostilbene show anti-oxidative, anti-inflammatory, and anti-cancer activity. Our second hypothesis is that their metabolites from oxidative reaction may have strong bioactivity as well.

2.2 Research objectives

1. To purify and identify the major oxidative products of pterostilbene and 3′-hydroxypterostilbene in *vitro*.

2. To evaluate the anti-inflammatory and anti-cancer cell proliferative activity of the

oxidative products of pterostilbene in different cell lines.

MATERIALS AND METHODS

3.1 Materials

Pterostilbene was from PTerPure (Irvine, CA). 3´-hydroxypterostilbene was provided by Sabinsa Corp. (East Windsor, NJ). Both of them were used without further purification. The peroxidase from horseradish, pH 5.0 phosphate-citrate buffer, Lipopolysaccharide (LPS) (*Escherichia coli* 0127:E8), MTT, RPMI 1640 media, penicillin, dimethyl sulfoxide, sulfanilamide, and naphthylethylenediamine dihydrochloride were purchased from Sigma (St. Louis, MO). Pterostilbene, 3´-hydroxypterostilbene, and peroxidase are sensitive to light, so these samples were stored in darkness. The 3% hydrogen peroxide was obtained from Fisher Scientific (Fair Lawn, NJ). All of the solvents, including methanol, acetone, acetonitrile, and HPLC grade water (0.22 micron filtered) were purchased from Pharmco-AAPER (Brookfield, CT).

3.2 Enzymatic oxidation of pterostilbene and 3´-hydroxypterostilbene in *vitro*

3.2.1 HPLC analysis of reference standards

At first, 0.004g of each sample was totally dissolved in 1 mL of acetone. Then the purity of pterostilbene and 3´-hydroxypterostilbene was determined by HPLC system (Dionex®), including an Ultimate 3000 autosampler, an Ultimate 3000 pump, and an Ultimate 3000 variable wavelength UV-Vis detector. A Thermo Scientific C_{18} reversed-phase column (250 x 4.6 mm, 5 μm) was used for Pterostilbene analysis. A Phenomenex

Luna C18 (2) reversed-phase column (150 x 4.6 mm, 3 μm) was used for 3′-hydroxypterostilbene analysis. Guard column was applied for both columns in this study. The wavelength for both pterostilbene and 3′-hydroxypterostilbene was 306 nm. The HPLC system was performed using binary gradient condition. Mobile phase A (HPLC grade water), and mobile phase B (acetonitrile) were used for pterostilbene analysis. Mobile phase A (HPLC grade water), and mobile phase B (methanol) were used for 3′-hydroxypterostilbene analysis. The flow rate for both was 1 mL/min. For pterostilbene, the mobile phase started with 30% B. It increased linearly to 90% in 15min, and held for 5min. Then it decreased to 30% in 1min, and equilibrated for 3 min. For 3′-hydroxypterostilbene, the mobile phase started with 40% B. It increased linearly to 80% in 15 min, and held for 5 min. Then it decreased to 40% in 1 min, and equilibrated for 3 min. The injection volume for each sample is 10 μL. Pterostilbene and 3′-hydroxypterostilbene were eluted at 12.1 min and 10.2 min, respectively.

3.2.2 Enzyme assay

At first, 0.4 g of pterostilbene and 3′-hydroxypterostilbene were totally dissolved in 16mL of acetone, respectively. Then 6 mg of peroxidase and 144 mL of pH=5.0 phosphate-citrate buffer were added in these two solutions. After vortexing, the suspension liquids were incubated at 37 ℃. The oxidative reaction was started by adding 0.8 mL of 3% hydrogen peroxide. During the reaction, same amount of hydrogen peroxide was added in the

suspension liquids every 15 min. The total reaction time for pterostilbene and 3′-hydroxypterostilbene was 150 min and 18 h, respectively.

3.2.3 HPLC analysis of pterostilbene and 3′-hydroxypterostilbene oxidative products

After reaction, the suspension liquids were centrifuged at ca. 6000g for 10 min. The supernatant was removed. The residue of each was dissolved in about 150 mL of methanol. Then 10 μL of each oxidative products solution was injected for HPLC analysis using the same method in pterostilbene and 3′-hydroxypterostilbene standard analysis.

3.3 Purification of pterostilbene and 3′-hydroxypterostilbene oxidative products

Based on HPLC data, the pterostilbene oxidative product eluted at 16.34 min and 3′-hydroxypterostilbene oxidative product eluted at 16.42 min were purified by a Gilson® semi-preparative HPLC system (Gilson, Inc., Middleton, WI), including a 322 pump, a GX-271 liquid handler, a GX direct injection module, and a UV/VIS-155 detector. The column was Phenomenex® Luna C_{18} (2) column (150 x 21.20 mm, 5 μm). For pterostilbene, the mobile phase A is HPLC grade water, and mobile phase B is acetonitrile. The program started at 60% B. Then it increased linearly to 90% in 10min. For 3'-hydroxypterostilbene, the mobile phase A is HPLC grade water, and mobile phase B is methanol. The program started at 50% B. Then it increased linearly to 60% in 10min. The collected oxidative

products were analyzed by HPLC. Based on peak area, the purity for both purified pterostilbene and 3′-hydroxypterostilbene oxidative products were determined. The collected solution from semi-preparative HPLC was concentrated by rotary evaporator at 40 ℃. Then the samples were evaporated by vacuum freeze dryer to dryness. About 40mg of pterostilbene oxidative product and 30 mg of 3′-hydroxypterostilbene oxidative product were obtained for NMR analysis and bioactivity evaluation.

3.4 LC/ESI-MS analysis

An Hewlett-Packard® 1100 series LC/MSD System (Agilent Technologies, Waldbronn, Germany) was used for pterostilbene and 3'-hydroxypterostilbene oxidative products analysis. It consisted of a 5973Network mass selective detector, an autosampler, a quaternary pump system, a degasser, and an electrospray ionization (ESI) source. A Phenomenex® Luna PFP (2) column (150 x 4.6 mm, 3 μm) was used for HPLC and the flow rate was 0.7 mL/min. The HPLC system was performed using binary gradient condition. The mobile phase A was 0.1% formic acid in water, and mobile phase B was methanol. The method started with 75% B. It increased linearly to 80% in 10min, to 95% in 12min, to 100% in 1min, and held for 2min. Then it decreased to 75% in 1min, and equilibrate for 15min. The LC elute was introduced into the ESI source. We used positive mode for pterostilbene analysis and negative mode for 3′-hydroxypterostilbene analysis. The voltage of ESI ion source was 3500V. Helium gas was used as nebulizer gas at a flow

rate of 40 psi and nitrogen gas was used as auxiliary gas at a flow rate of 8.0 L/min. Full scan was acquired for pure pterostilbene and 3′-hydroxypterostilbene oxidative products analysis.

3.5 NMR analysis

The pterostilbene oxidative product was analyzed in deuterium methanol. The 3′-hydroxypterostilbene oxidative product was analyzed in deuterium dimethyl sulfoxide. ^{1}H (300 MHz) was acquired for pterostilbene oxidative product analysis on a Bruker 300 instrument (Bruker® BioSpin Corporation, Billerica, MA). ^{1}H (500 MHz) and ^{13}C (500 MHz) were acquired for 3'-hydroxypterostilbene oxidative product analysis on a Bruker 500 instrument (Bruker® BioSpin Corporation, Billerica, MA).

3.6 Bioactivity evaluation

3.6.1 Cell culture of Nitrite Assay

RAW 264.7 cells, which were derived from murine macrophages, were purchased from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI 1640 media (without phenol red) supplemented with 10% endotoxin-free, heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 100 units/mL penicillin, and 100 µg/mL streptomycin. The cells were cultured with medium containing *E. coli* LPS (100 ng/mL) and multiple concentrations of pterostilbene, 3′-hydroxypterostilbene, pterostilbene *trans*

dehydrodimer, and 3´-hydroxypterostilbene *trans* dimer that were dissolved in DMSO, when the density of cells was 1 x 10^6 cells/mL. Cells treated with 0.05% DMSO and 100ng/mL LPS only served as vehicle and positive control, respectively.

3.6.2 Nitrite Assay

The NO production was measured by measuring the nitrite concentration in the culture medium using the Griess assay. After centrifugation at ca. 1000g for 20min, 100 µL supernatant of each sample was mixed with same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Then a Dynatech MR-7000 enzyme-linked immunosorbent assay plate reader (Dynatech Laboratories, Chantilly, VA) was used to measure the absorbance of the mixture at 550 nm.

3.6.3 Cell culture of MTT Assay

Human drug-sensitive promyelocytic leukemia cells (HL-60) and drug-resistant breast cancer cells (MDA-MB-231) were obtained from American Type Culture Collection (Rockville, MD). The HL-60 cells were grown in 90% RPMI and 10% fetal bovine serum (GIBCO BRL, Grand Island, NY), supplemented with 2 mM glutamine (GIBCO BRL), 1% penicillin/streptomycin mix (100U/mL of penicillin and 100 µg/mL of streptomycin). The MDA-MB-231 cells were maintained in DMEM (high glucose) media, supplemented with

10% fetal bovine serum, 2 mM glutamine, 0.1 mM MEM Non-Essential Amino Acids, and 1% penicillin/streptomycin mix. All the cells were incubated at 37 ℃ in a humidified 5% CO_2 incubator.

3.6.4 MTT Assay

The different human cancer cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At first, the different human cancer cell lines were transferred into 24-well plates at density of 1 x 10^5 cells/mL. After overnight incubation, the cells were treated with different concentration of pterostilbene, 3′-hydroxypterostilbene, and pterostilbene oxidative product for 24h. Then 30 μL of MTT was added into each wells and incubated for 4h. At last, the cell proliferation was determined by scanning with an ELISA reader.

RESULTS

4.1 HPLC analysis

The HPLC spectra of pterostilbene and 3′-hydroxypterostilbene reference standards were shown in figure 5 and figure 6. The retention times of them were 12.1 min and 10.2 min, respectively. According to the peak areas, the purity of pterostilbene and 3′-hydroxypterostilbene were above 98%.

Then pterostilbene and 3′-hydroxypterostilbene reacted with hydrogen peroxide catalyzed by peroxidase for 150 min and 18 h, respectively. After enzymatic reaction, the HPLC spectra of pterostilbene and 3’-hydroxypterostilbene’s oxidative products which were dissolved in methanol were shown in figure 7 and figure 8. Both of pterostilbene and 3′-hydroxypterostilbene had two major oxidative products. All the oxidative products were eluted out later than their parents. These data indicated that the oxidative products were more lipophilic than pterostilbene and 3′-hydroxypterostilbene, respectively. The HPLC data also shown that 3′-hydroxypterostilbene was more stable than pterostilbene when oxidized by peroxidase under pH 5.0 environment.

For pterostilbene, the peak area percentage of the highest peak at 16.34 min was 75.24%. For 3′-hydroxypterostilbene, the peak area percentage of the highest peak at 16.42min was 68.84%. In this study, these two compounds were purified and then identified by NMR. According to peak areas, the purity of the purified compounds were above 98%. These samples were used in NMR analysis.

4.2 LC/ESI-MS analysis

The full scan of the purified pterostilbene and 3′-hydroxypterostilbene major oxidative products were shown in figure 9 and figure 10. Under positive mode, the mass of pterostilbene's major oxidative products was 511.4. Under negative mode, the mass of 3′-hydroxypterostilbene's major oxidative products was 541.0. This data indicated that both of them possibly were dimeric structure of their parents. In addition, we can see an obvious peak that is 271 Da in 3′-hydroxypterostilbene's MS spectra. This was source fragmentation from the 3′-hydroxypterostilbene's major oxidative product. This peak also verified the dimeric structure of the compound, because dimeric compounds were easily fragmented in ionization source. However, from the source fragmentation, we cannot elucidate the conjugated position of the two oxidative products. So NMR data was needed in order to identify their structures.

4.3 Structure elucidation of pterostilbene and 3′-hydroxypterostilbene major oxidative products

The ^{1}H NMR spectrum of pterostilbene major oxidative products was shown in figure 11. As we mentioned above, the molecular ion of this compound from LC/MS data was 511 Da under positive mode. The other evidence which can confirm the structure of this compound was the NMR data. Figure 11 was identical to pterostilbene *trans* dimer NMR data reported by Breuil et al. (Breuil, Jeandet, Adrian, Chopin, Pirio, Meunier, et al.,

1999) in the research of oxidation of pterostilbene by Laccase of *Botrytis Cinerea* in 1999. So the major oxidative product of pterostilbene in this study was identified as pterostilbene *trans* dehydrodimer.

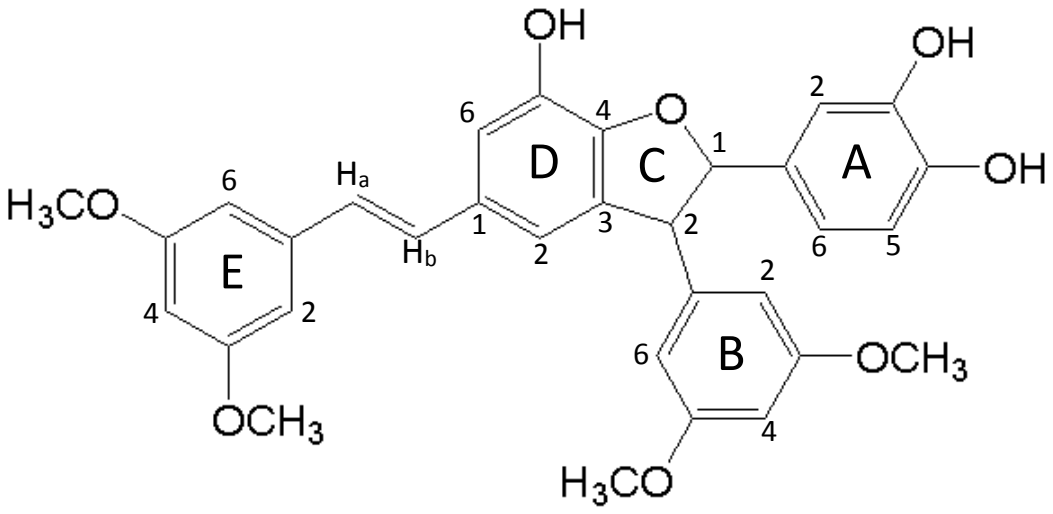


Figure 4. Labelled chemical structure of 3´-hydroxypterostilbene *trans* dehydrodimer

The ^{1}H NMR and ^{13}C NMR spectrums of 3´-hydroxypterostilbene major oxidative product were shown in figure 12 and figure 13. Its ^{1}H NMR spectrum showed similar patterns to those of pterostilbene oxidative product (Table 1). Assignments of protons Ha and Hb in the ethylenic group were determined by NMR at δ 7.04 ppm (*J*=16.5d, H-Ha) and δ 7.18 ppm (*J*=16.5d, H-Hb). The coupling constant indicated that the compound had *trans* structure and these result also corresponded to the ^{1}H NMR spectrum of pterostilbene *trans* dimer. Two groups of two equivalent methoxy groups were

determined by NMR at δ 3.65 ppm (singlet, 6H) and δ 3.76 ppm (singlet, 6H). Protons at δ 6.52 ppm (*J*=2.0-10.0dd, H-4E) and δ 7.13 ppm (*J*=2.0-10.5dd, H-6B) had doublet of doublets signals, which indicated that these two protons had correlations with protons at δ 6.66 ppm (*J*=2.0d, H-2E), δ 6.60 ppm (*J*=8.0d, H-6E), δ 7.22 ppm (*J*=2.0d, H-2B) and δ 6.97 ppm (*J*=8.5d, H-4B), respectively. So the structure of ring B and E were defined. The proton at δ 6.40 ppm (*J*=2.5d, H-6D) correlated to the proton at δ 6.73 ppm (*J*=2.0d, H-2D). So the structure of ring D was confirmed. Two nonaromatic protons were observed at δ 5.05 ppm (*J*=1.5d, H-1C2C), which confirmed the structure of ring C. The structure of ring A was determined by the chemical shift of aromatic protons, δ 6.38 ppm (*J*=2.0-4.5t, H-2A), δ 6.37 ppm (*J*=2.5-6.5t, H-5A) and δ 6.73 ppm (*J*=2.0d, H-6A). The proton assignments and NMR data was summarized in Table 1. The proposed structures of pterostilbene and 3´-hydroxypterostilbene *trans* dehydrodimer were shown in figure 14 and figure 15.

4.4 Bioactivity evaluation of pterostilbene, 3'-hydroxypterostilbene, and pterostilbene oxidative product

4.4.1 Anti-inflammatory activity

The anti-inflammatory activity was tested on RAW 264.7 cell line using Griess assay. The nitrite production data was shown in figure 16 and figure 17. The first column in figure 16 was the nitrite production of positive control, which was treated by LPS only.

The other three groups were pterostilbene, 3′-hydroxypterostilbene, and pterostilbene *trans* dimer's percentage nitrite production of positive control at different concentration. In figure 17, the first two groups were nitrite production of vehicle control and positive control, respectively. The other three groups were nitrite production of RAW264.7 cells treated with 3'-hydroxypterostilbene *trans* dimer in different concentration. In these figure, we can see at low concentration (5 μM), the cells which were treated with pterostilbene had the lowest nitrite production. This indicated that pterostilbene had the best anti-inflammatory activity among these three compounds at low concentration. But when the dosing concentration was higher than 10 μM, the anti-inflammatory effect of 3′-hydroxypterostilbene was significant. Even when the dosing concentration was at 20 μM, the nitrite production of the cells treated with 3′-hydroxypterostilbene was almost negligible. So in conclusion, both pterostilbene and 3′-hydroxypterostilbene were effective anti-inflammatory compound in *vitro*. While their dimeric products were more hydrophilic than their parents, so they have a lower bioavailability. This characteristic was a possible reason for their relatively lower anti-inflammatory activity than their parents.

4.4.2 Anti-cancer cell proliferative activity

The anti-cancer cell proliferative activity of pterostilbene, 3′-hydroxypterostilbene, and pterostilbene *trans* dimer were conducted using MTT assay on two cancer cell lines. The

first one was drug sensitive leukemia cell line HL-60, and the data of this cell line was shown in figure 18. All of the data were dose-dependent. The cells which were treated with 3'-hydroxypterostilbene at different concentration had the relatively lowest cell viability, especially at low dosing concentration (5 μM). This indicated that 3'-hydroxypterostilbene was a potential efficient anti-cancer agent. While for the other two compounds, the anti-proliferative effects were significant only at high dosing concentration.

The second cell line was breast cancer cell line MDA-MB231. The anti-proliferative effects of the three compounds were similar at different concentration. From figure 19 we can see the anti-proliferative effect of them on MDA-MB231 cell line was not as significant as the effect on HL-60 cell line. Because this kind of breast cancer cell line was drug resistant. But at high concentration (100 μM), the cell viability dropped down to about 40% when they were treated by these three compounds.

Table 1. NMR (500 MHz) spectra data of 3´-hydroxypterostilbene *trans* dehydrodimer (d in ppm)

Position	d (ppm)	*J* (Hz)	Signal
2B	7.22	2.0	d
Ha	7.04	16.5	d
Hb	7.18	16.5	d
6B	7.13	2.0-10.5	dd
4B	6.97	8.5	d
6A2D	6.73	2.0	d
2E	6.66	2.0	d
6E	6.60	8.0	d
4E	6.52	2.0-10.0	dd
6D	6.40	2.5	d
2A	6.38	2.0-4.5	t
5A	6.37	2.5-6.5	t
1C2C	5.05	1.5	d
OCH_3	3.76		s
OCH_3	3.65		s

s=singlet, d=doublet, t=triplet, dd=doublet of doublets

DISCUSSION

The mechanism of oxidative reaction of pterostilbene and 3′-hydroxypterostilbene by peroxidase was proposed as free radical reaction in this study. The mechanism for the synthetic process of pterostilbene and 3′-hydroxypterostilbene dimeric oxidative products were shown in figure 20. The dimeric structures indicated that the oxidative reaction took place at the 4′-OH position of pterostilbene and 3′-hydroxypterostilbene, and proceeded through the formation of radical cations and deprotonation of the phenolic hydroxyl groups to give phenoxy radicals. Then they will undergo a wide variety of coupling reactions. The radicals had the ability of electron-delocalization, so the coupling could take place at multiple sites. Therefore, depending on the structural characterization of parents, different dimeric structure formed in the oxidative biotransformation process. Pterostilbene and 3′-hydroxypterostilbene have been reported to have wide variety of health beneficial bioactivity in previous research. In this study, we compared the anti-inflammatory and anti-cancer cell proliferative activity of these two compounds as well as their *trans* dehydrodimers in multiple cell lines. We found that both pterostilbene and 3′-hydroxypterostilbene had significant anti-inflammatory and anti-cancer cell proliferative effect in *vitro*. Some metabolites may have a stronger bioactivity than their parents (M. H. Pan, Lai, Wu, Ho, & et al., 2012). While after oxidative dimerization, the major in *vitro* oxidative metabolites of pterostilbene and 3′-hydroxypterostilbene, did not show a significant inhibitory effect on cancer cell proliferation and NO production in

RAW264.7 cells. A possible reason was after dimerization, the compounds turned to be more hydrophilic so their bioavailability were lower than that of their parents. As we mentioned above, for stilbenoids, bioavailability is important for the essence of bioactivity. So pterostilbene and 3′-hydroxypterostilbene *trans* dehydrodimer showed relatively lower bioactivity than their parents.

According to our data, 3′-hydroxypterostilbene have the best bioactivity among the four compounds. However, the molecular mechanism is still not clear at this time. In the future, we need to investigate the inhibitory effect of 3′-hydroxypterostilbene on protein transcription and mRNA expression of iNOS and COX-2 enzyme, as well as nuclear translocation factor NFκB. And we will find out the metabolic fate of pterostilbene and 3′-hydroxypterostilbene in *vivo*, as well as their metabolic stability and bioactivity in animal study.

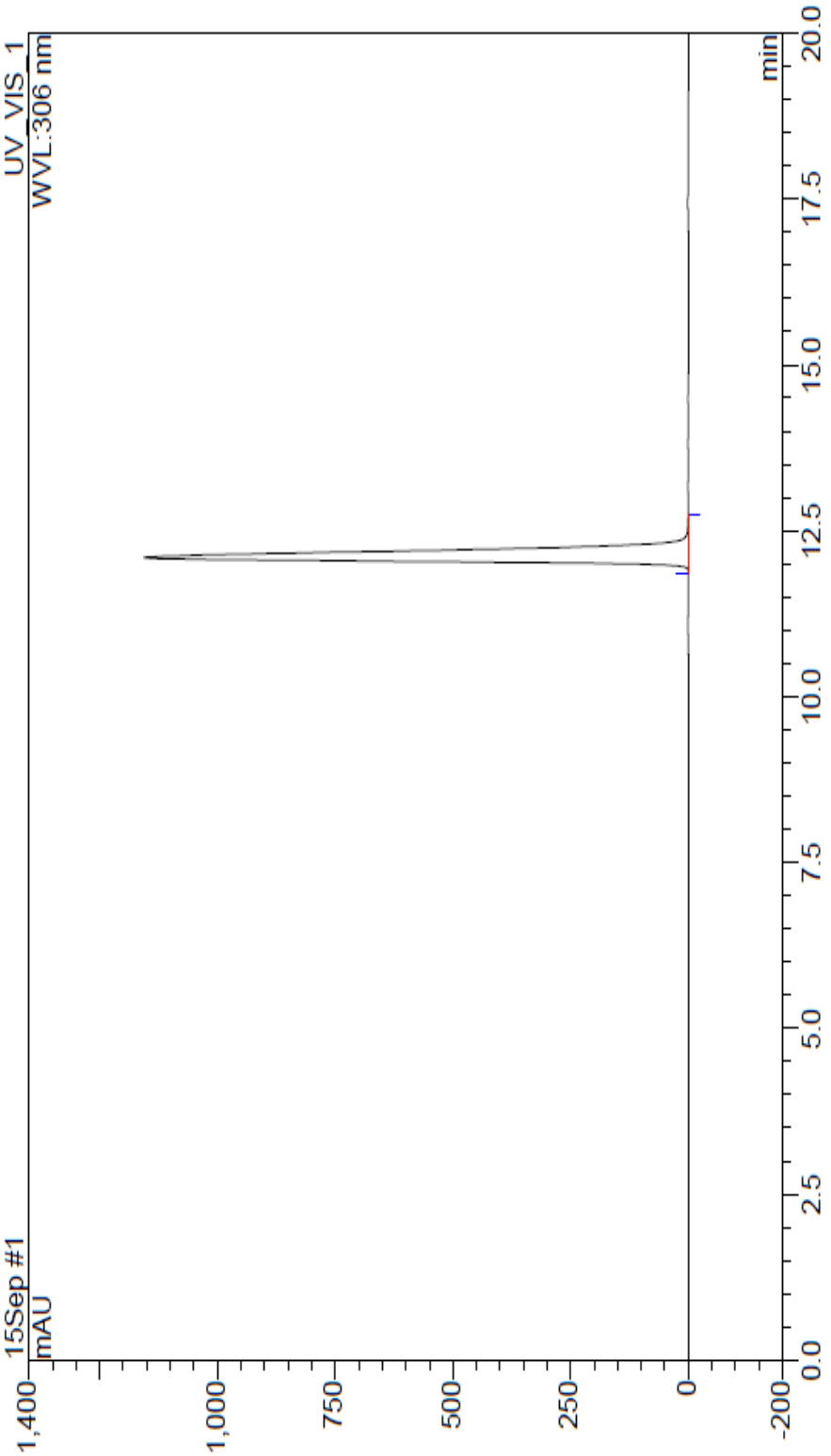


Figure 5. HPLC spectra of pterostilbene reference standad

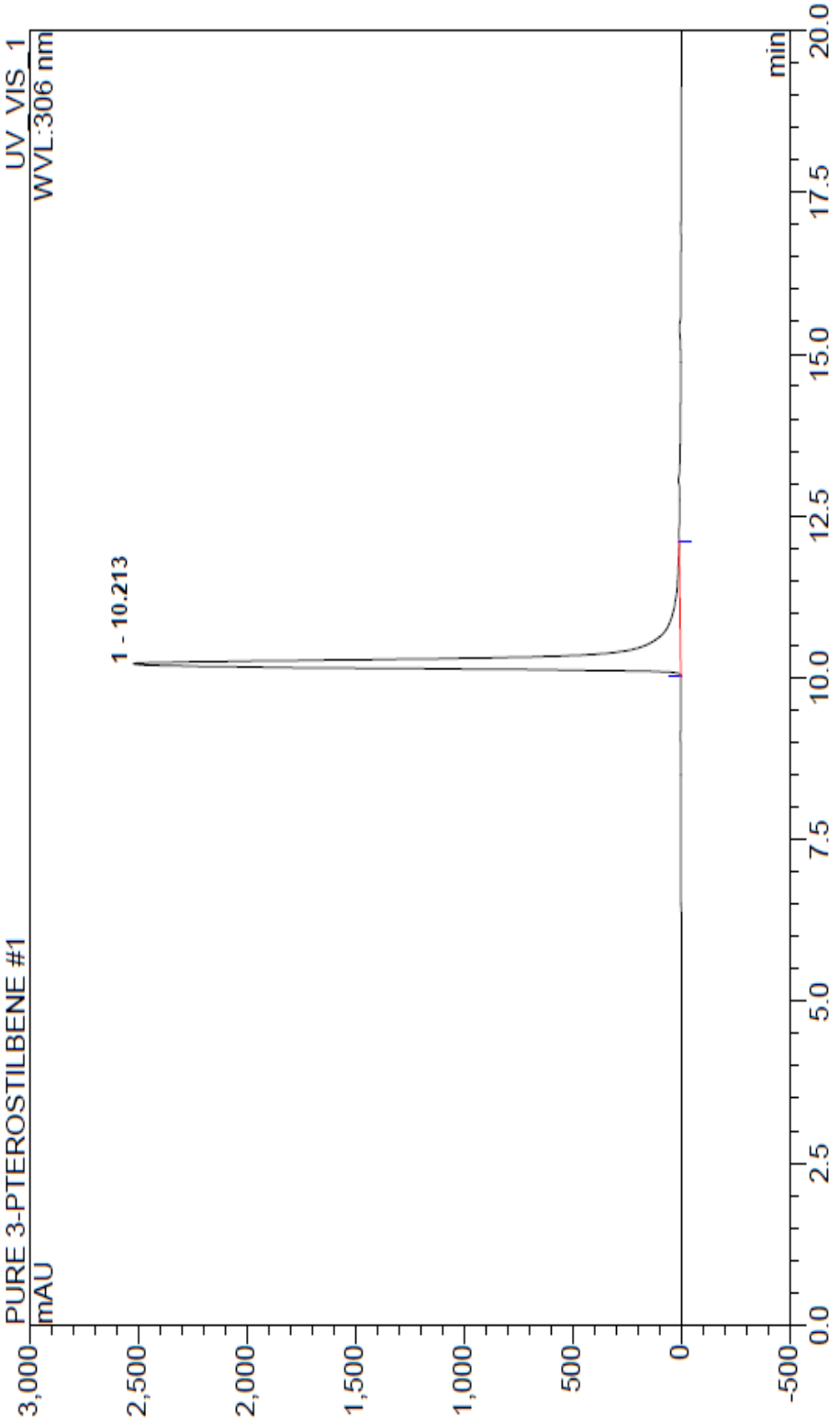


Figure 6. HPLC spectra of 3´-hydroxypterostilbene reference standard

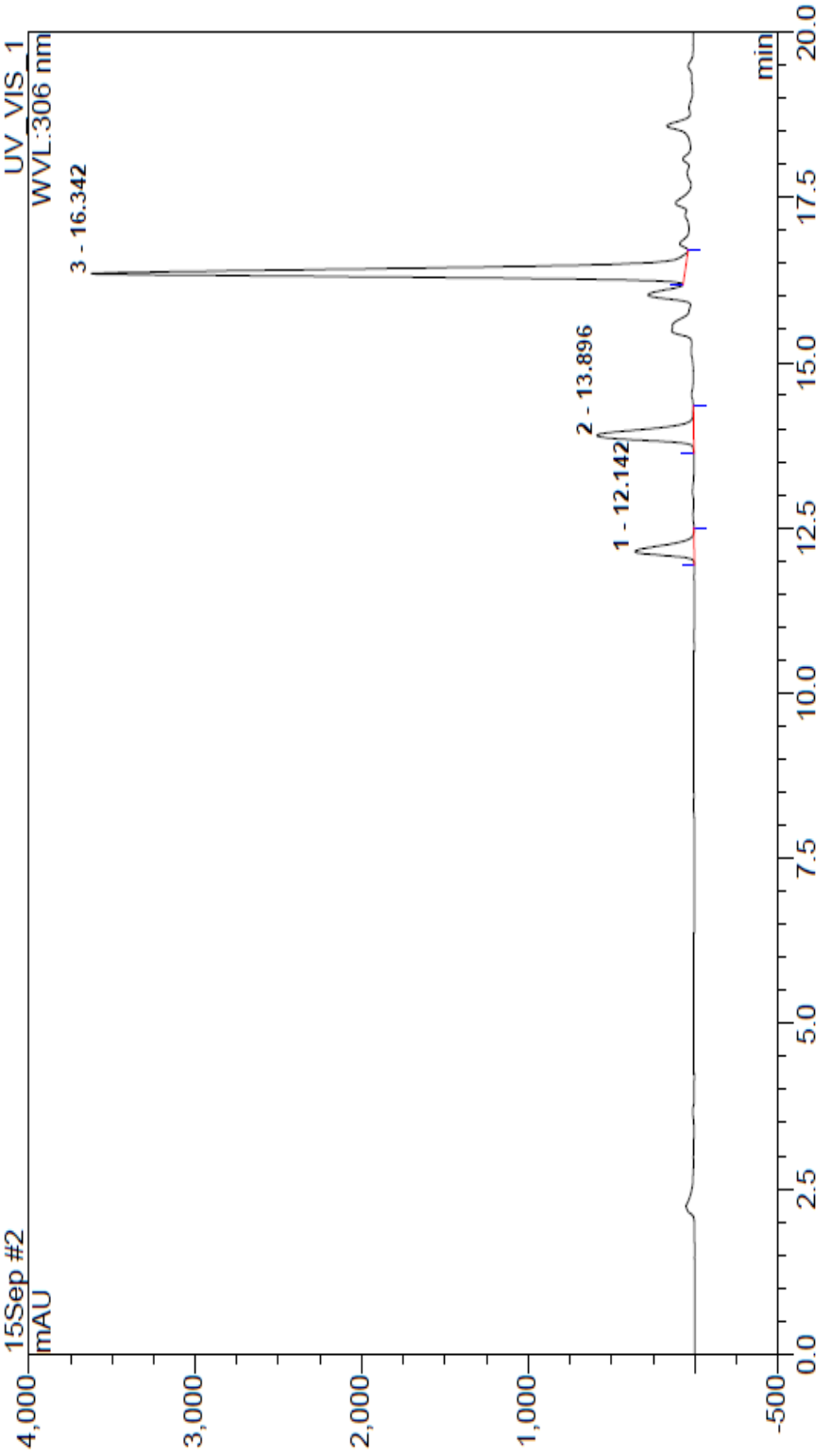


Figure 7. HPLC spectra of pterostilbene oxidative products

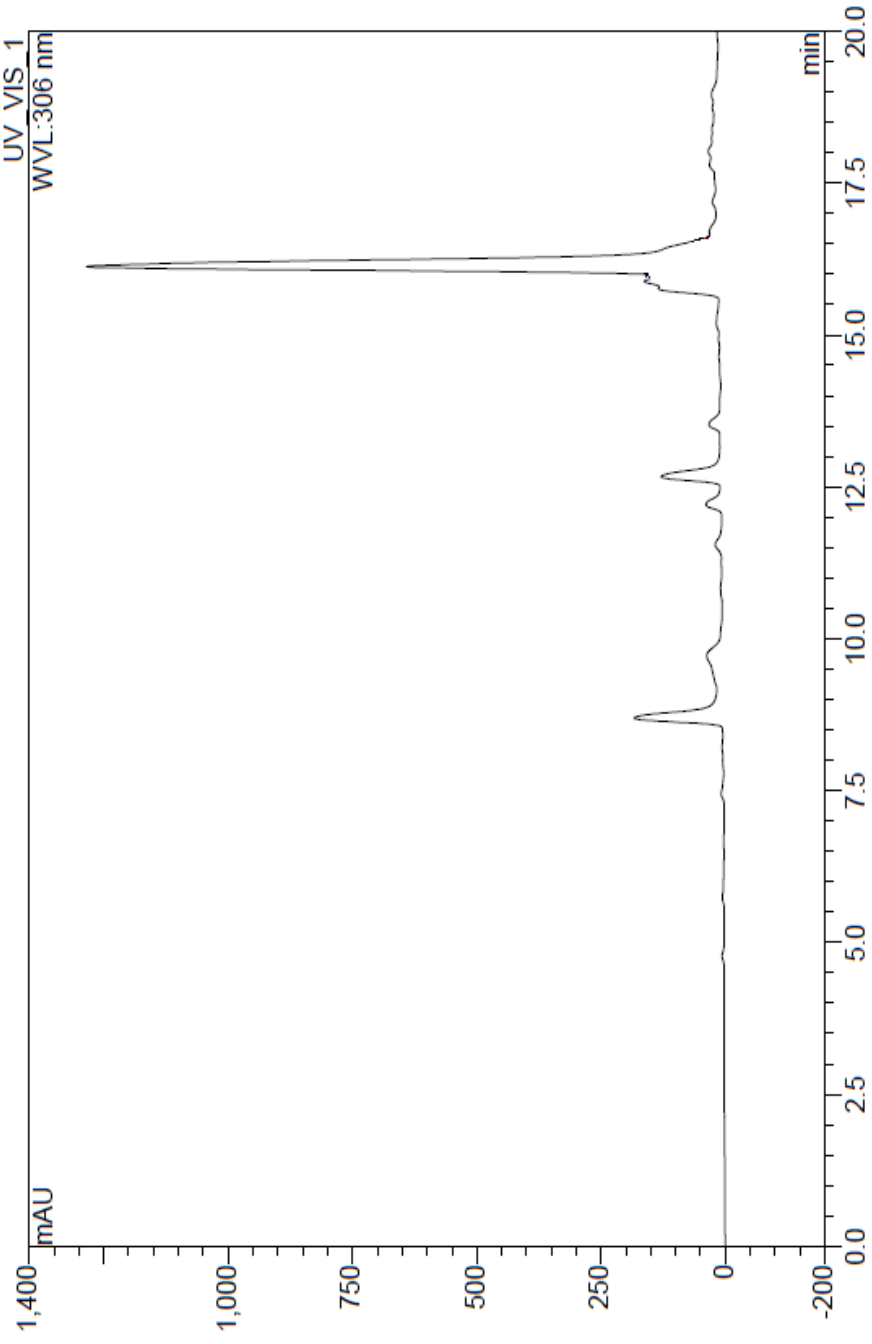


Figure 8. HPLC spectra of 3´-hydroxypterostilbene oxidative products

Figure 9. LC/(+)ESI-MS spectra of pterostilbene *trans* dehydrodimer

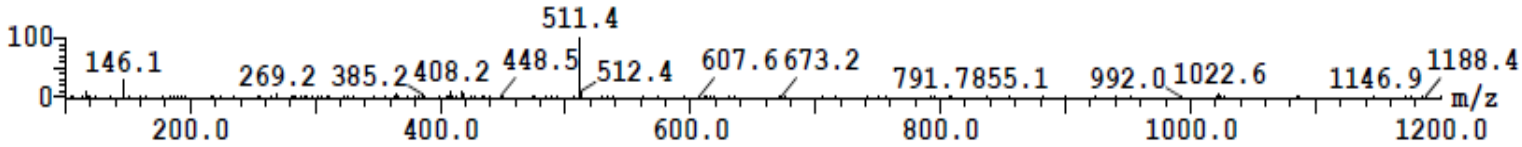


Figure 10. LC/(-)ESI-MS spectra of 3´-hydroxypterostilbene trans dehydrodimer

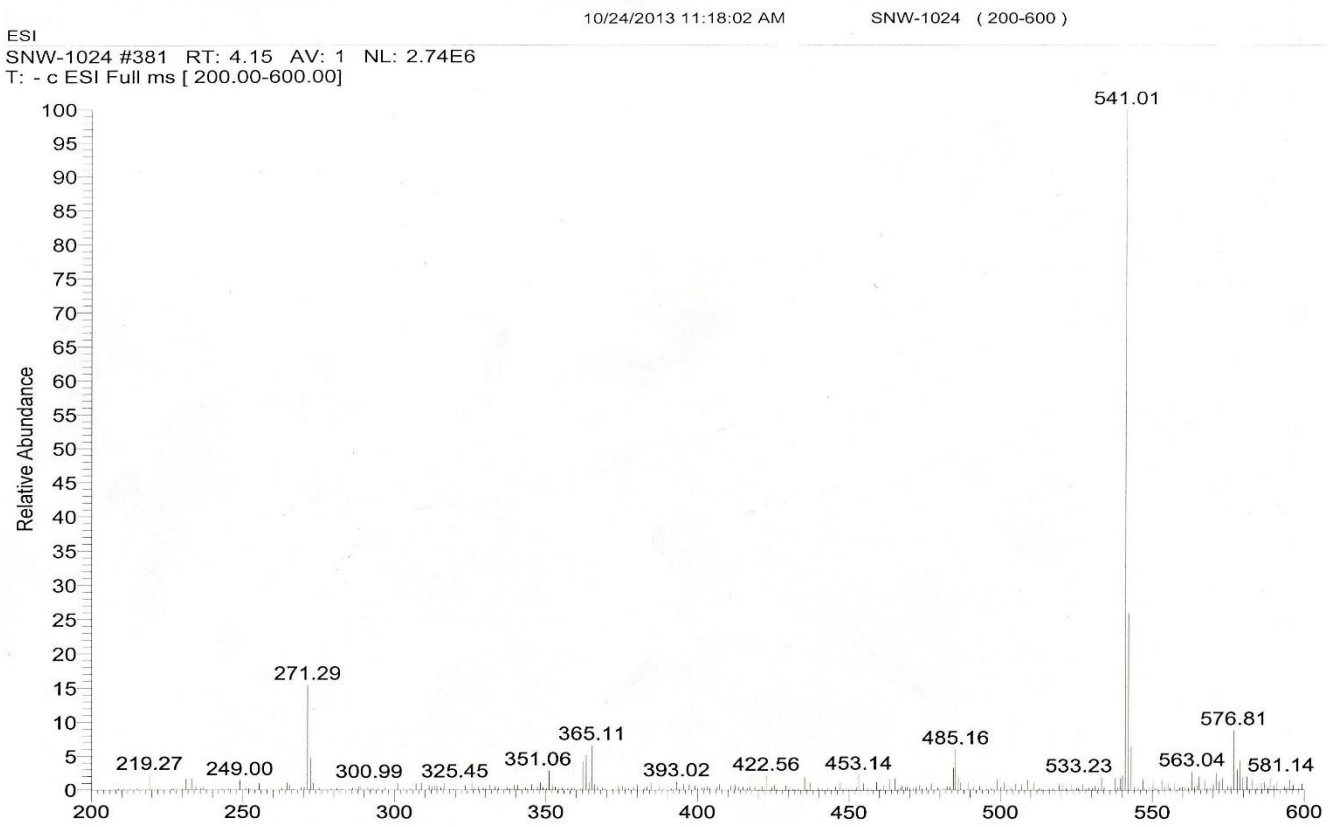


Figure 11. ^{1}H-NMR spectrum of pterostilbene *trans* dehydrodimer

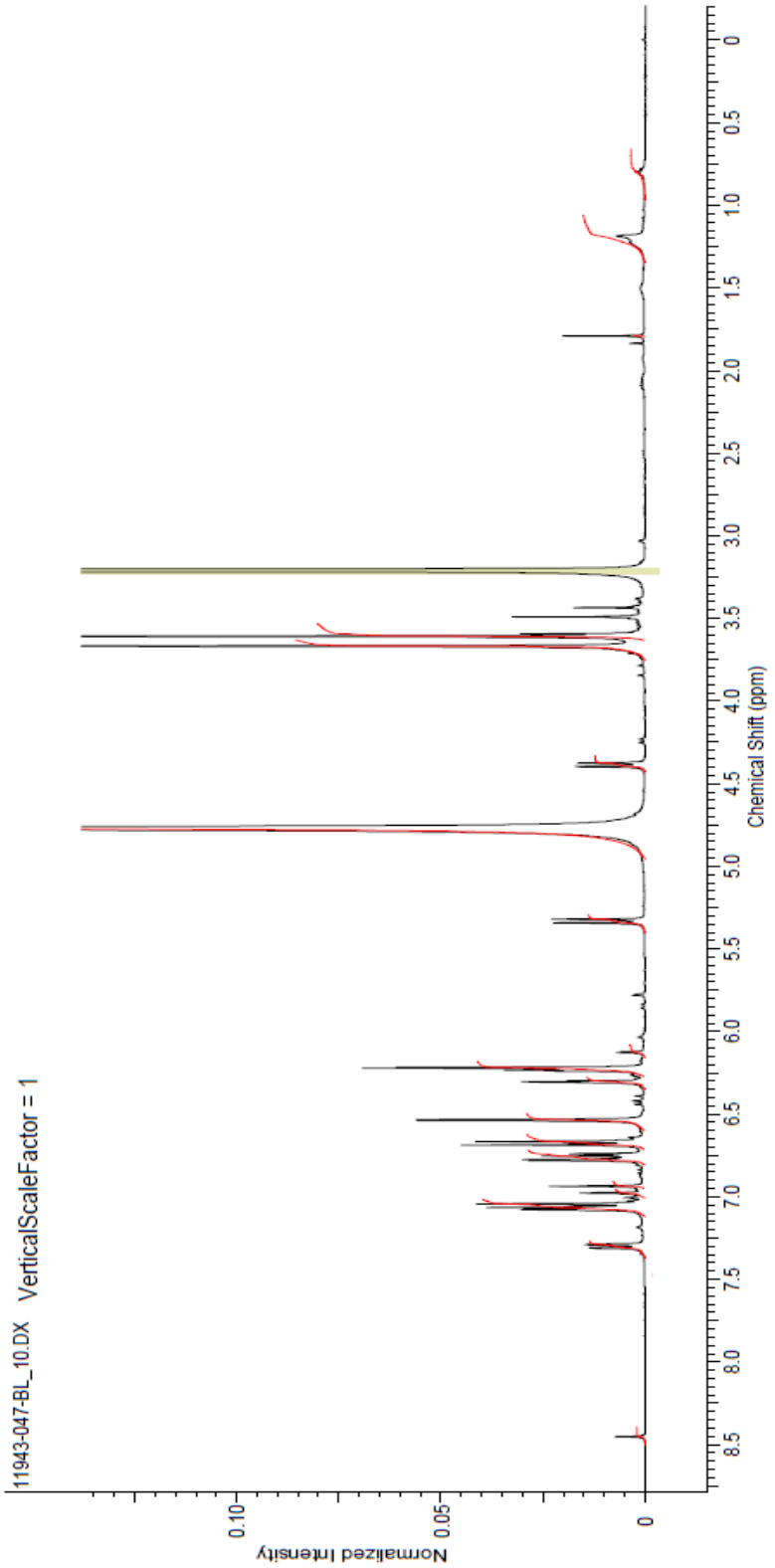


Figure 12. ^{1}H-NMR spectrum of 3′-hydroxypterostilbene *trans* dehydrodimer

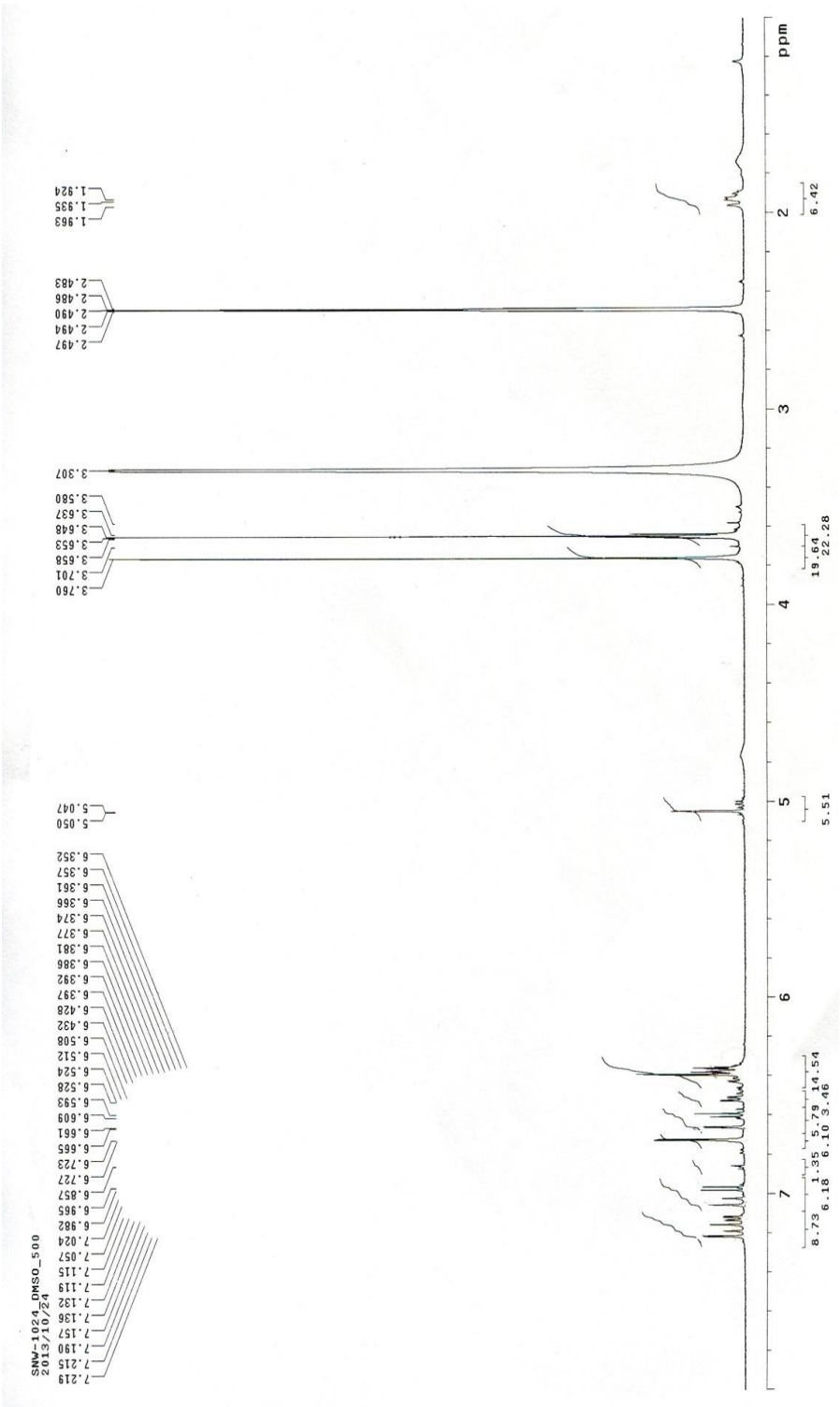
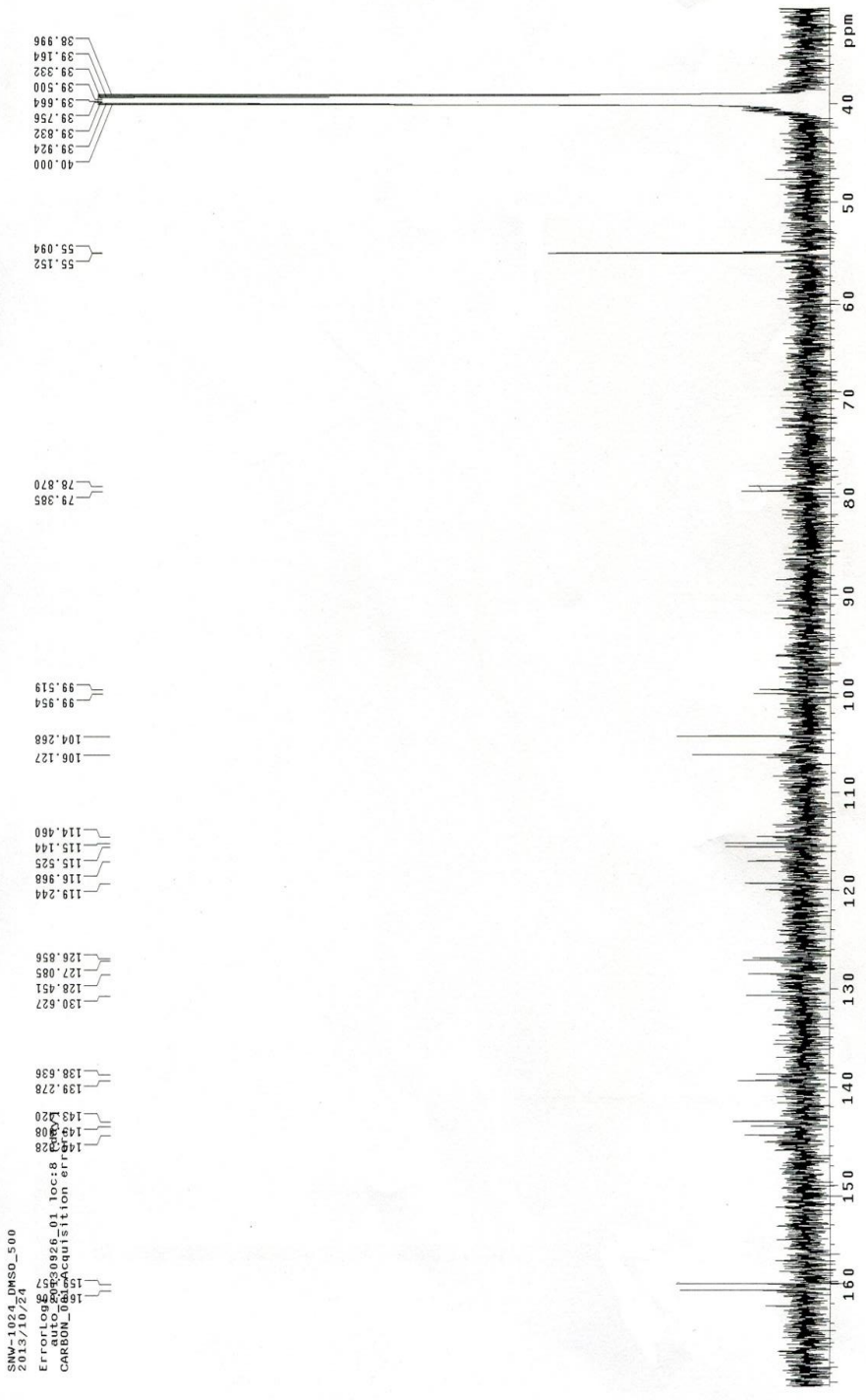


Figure 13. ^{13}**C-NMR spectrum of 3´-hydroxypterostilbene *trans* dehydrodimer**



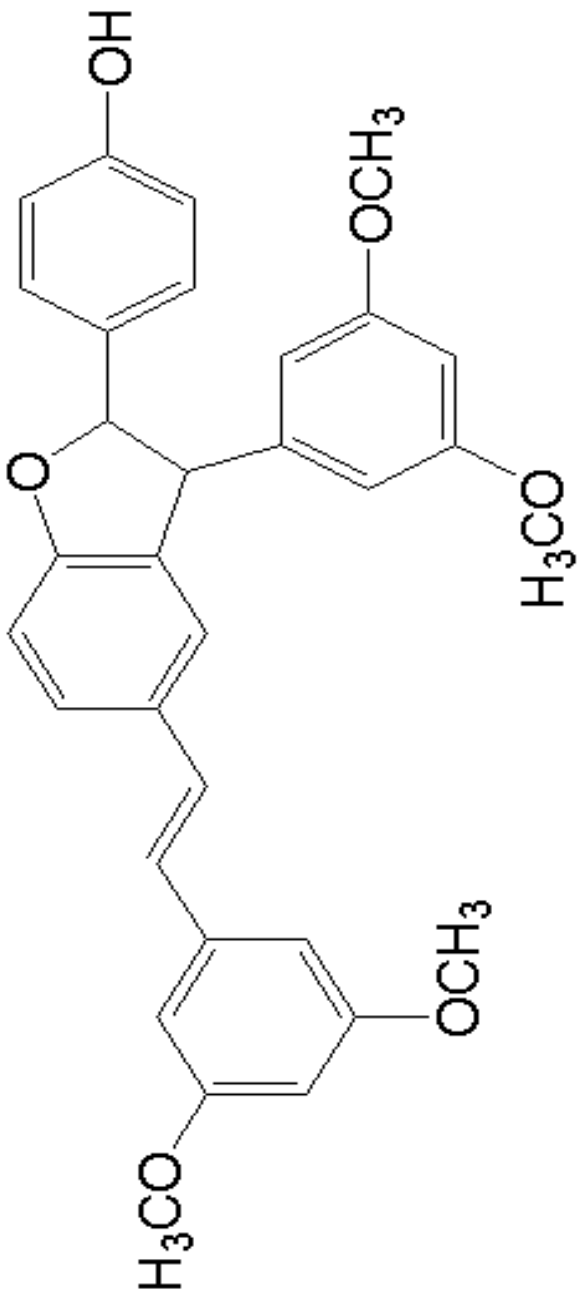


Figure 14. Structure of pterostilbene *trans* dehydrodimer

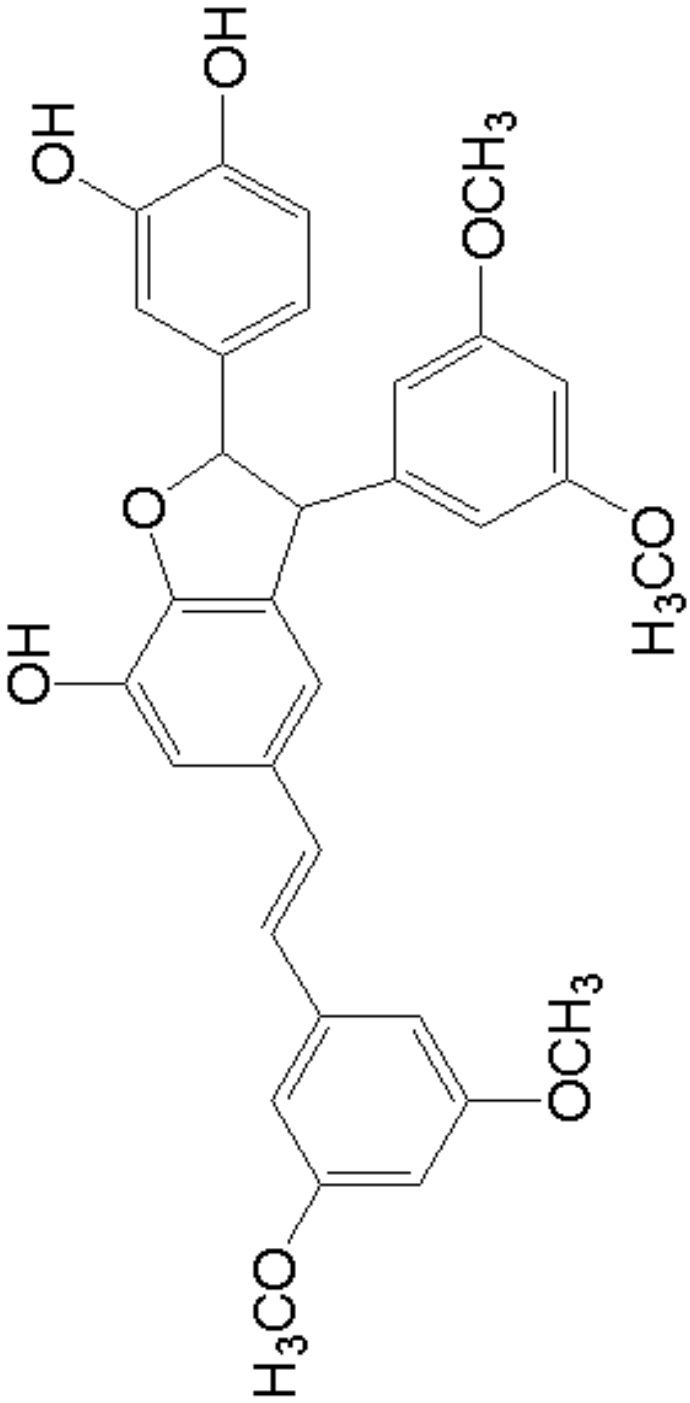


Figure 15. Structure of 3´-hydroxypterostilbene *trans* dehydrodimer

Figure 16. Nitrite production of RAW264.7 cells treated with pterostilbene, 3´-hydroxypterostilbene, and pterostilbene *trans* dehydrodimer

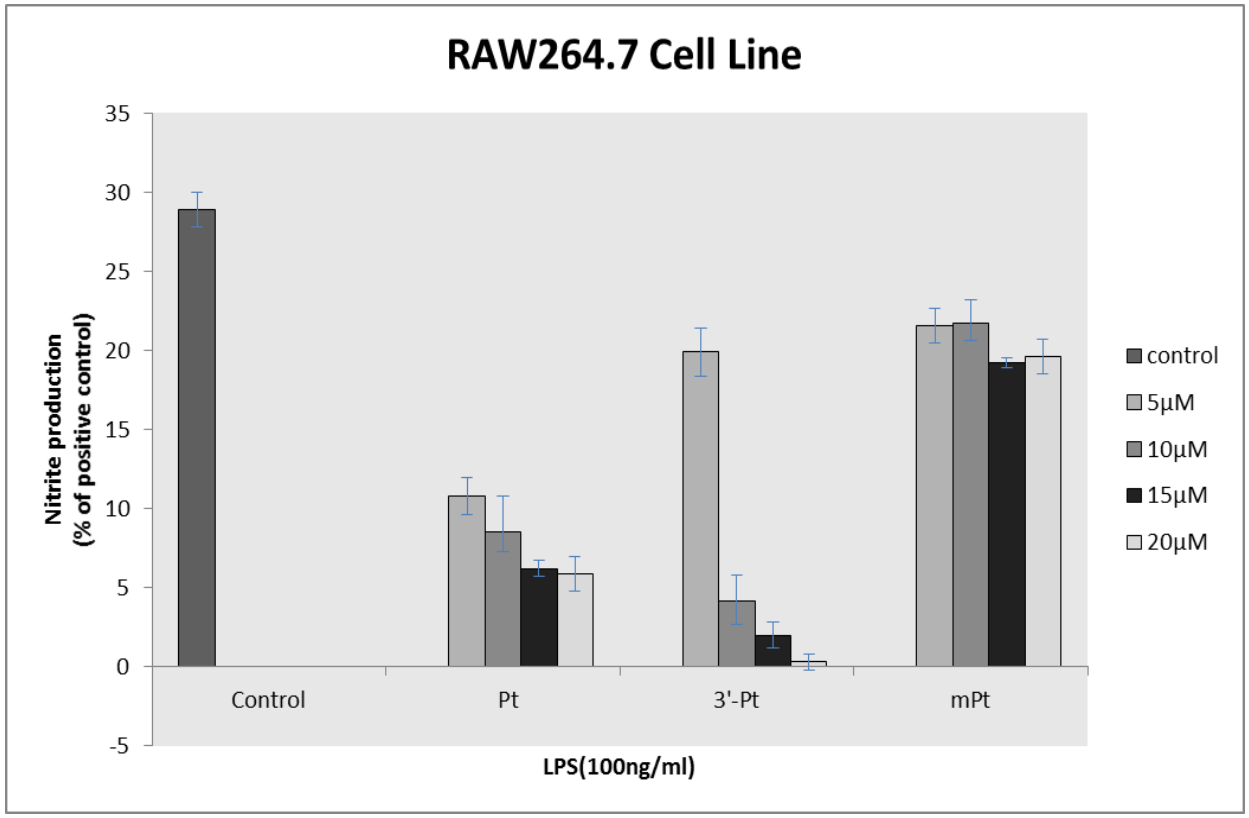


Figure 17. Nitrite production of RAW264.7 cells treated with 3´-hydroxypterostilbene *trans* dehydrodimer

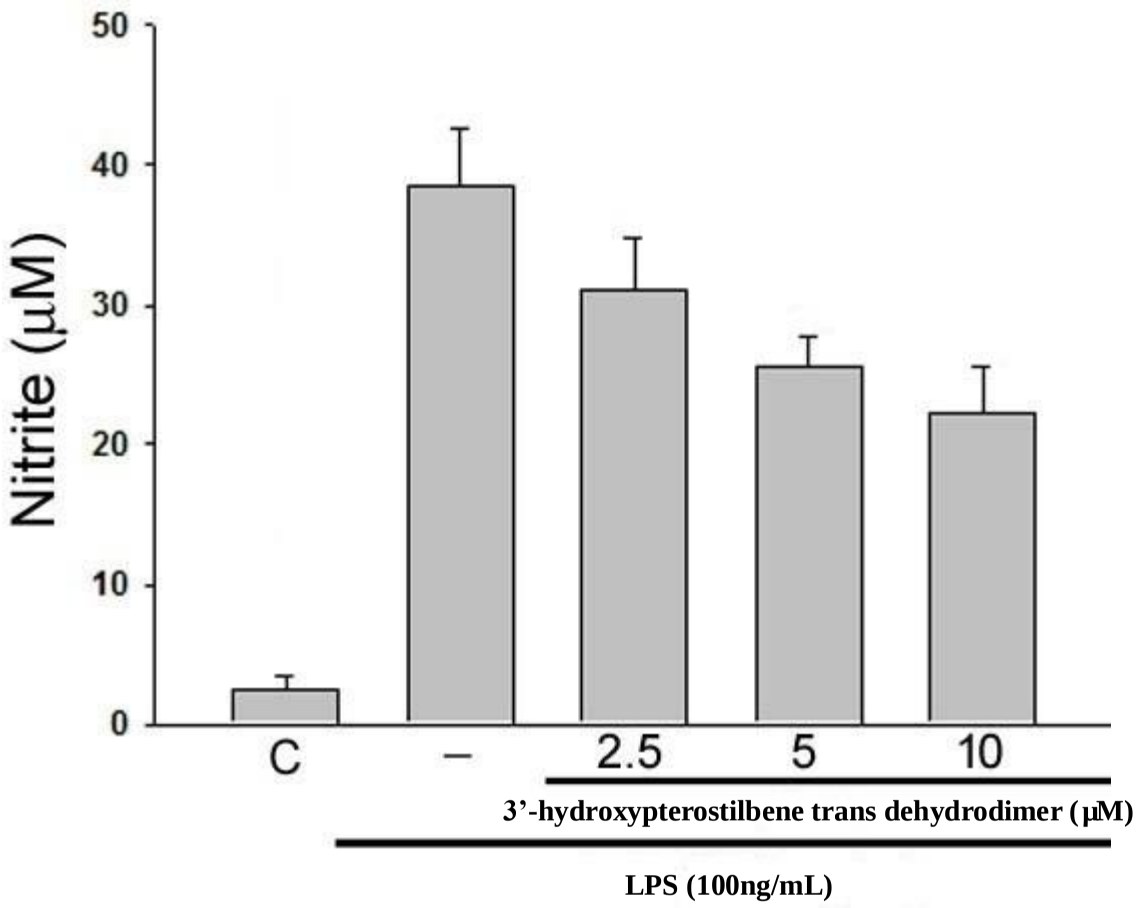


Figure 18. Cell viability of HL-60 cells treated with pterostilbene, 3´-hydroxypterostilbene, and pterostilbene *trans* dehydrodimer

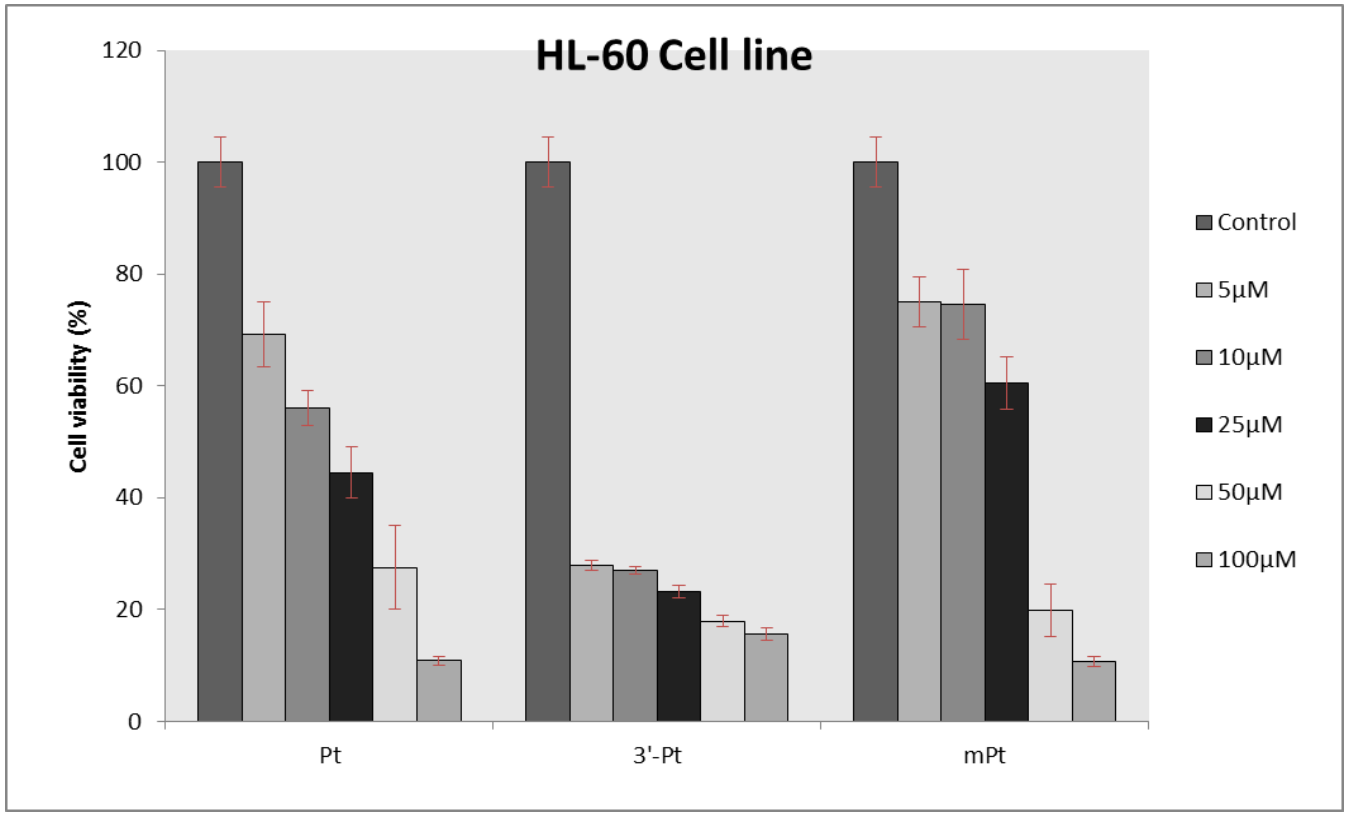


Figure 19. Cell viability of MDA-MB231 cells treated with pterostilbene, 3´-hydroxypterostilbene, and pterostilbene *trans* dehydrodimer

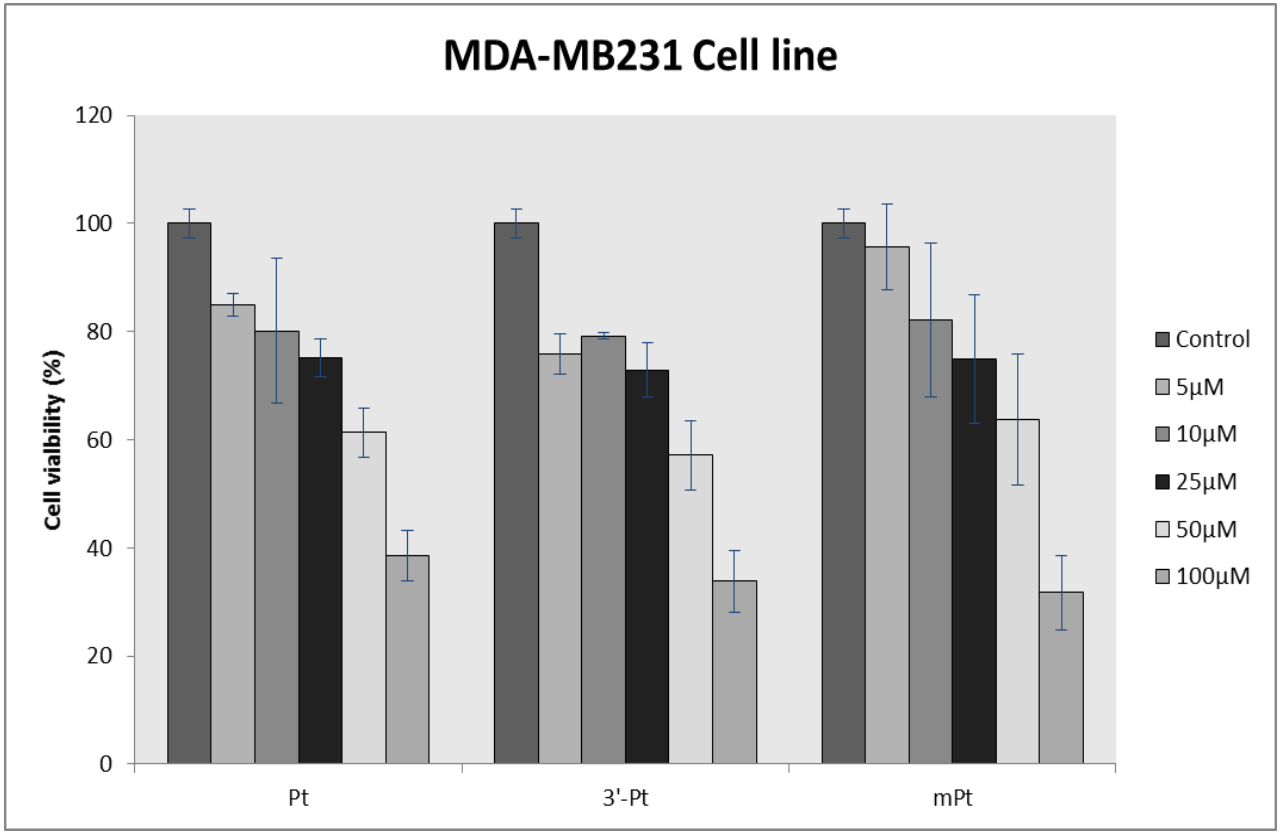
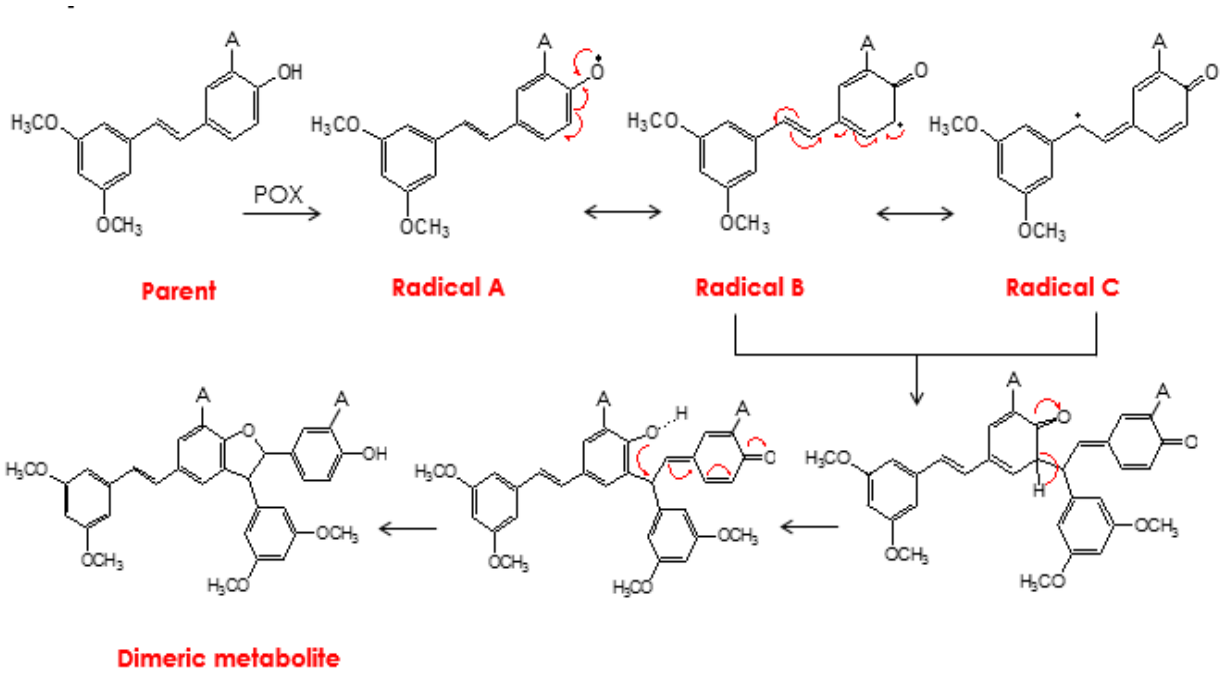


Figure 20. Proposed pathway of pterostilbene and 3´-hydroxypterostilbene enzymatic oxidation



Pterostilbene: A=H
3´-hydroxypterostilbene: A=OH

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