CHAPTER II
LITERATURE REVIEWS

1. *Morus alba*: An Overview

Figure 1 *Morus alba* L.

1.1 Introduction

Morus species (mulberry) is a genus belonging to the Moraceae family. There are about 68 species and over a thousand varieties of this deciduous woody tree. The mulberries are under cultivation in many regions including Asia, Middle East, Central America and South America (Datta, 2000). Mulberry leaves have been widely used in silkworm and ruminant feeding. Moreover the leaves are recently claimed to be an anti-diabetic drink (Hansawasdi, Kawabata, 2006). Mulberry fruits are edible and also used for making food and drink products such as jam, jelly and juice. Additionally, the fruit, leaves, branches, root and root bark of mulberry has been used as herbal medicine for various therapeutic purposes, including anti-inflammation, anti-asthmatic, function tonic and anti-diabetic (Kim et al., 2002; Kumar, Chauhan, 2008; Piao et al., 2010; Hunyadi el al., 2012). Furthermore, root bark, twig and leaf of mulberry are also potential sources of anti-oxidant and whitening compounds for cosmetic industry (Kim et al., 2002; Piao et al., 2010; Chang et al., 2011).

Among 68 species, *M. alba* L. (white mulberry, Figure 1) is considered to be a major species mostly used in sericulture and exclusively used in Chinese medicine. The characteristic feature of *M. alba* was described as follows, a medium sized monoecious tree, teeth of leaves uniform, usually blunt, segments of the perianth of female flowers in four numbers, the two outer keeled, very short style, white and black fruit color, brown color bud and oval round in shape (Tikader, Kamble, 2008; Kumar, Chauhan, 2008).

1.2 Bioactive Secondary Metabolites

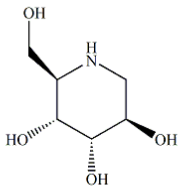
M. alba is a potential source of bioactive secondary metabolites (Table 1). For an example, the leaves of this medical plant were reported to exhibit anti-hyperglycemic activity (Nawaboot et al., 2009). It contains 1-deoxynojirimycin (Figure 2A), a potent anti-α-glucosidase iminosugars (or piperidine alkaloids), flavonoids including rutin (Figure 2C) and others anti-hyperglycemic constituents such as chlorogenic acid (Figure 2B), polysaccharides, glycopeptides and ecdysteroids (Hunyadi et al., 2012). The leaves also contain mulberroside F (Figure 2D), which shows inhibitory effect on tyrosinase activity and also exhibits superoxide scavenging activity (Lee et al., 2002).

In Turkey and Greece, mulberry fruits have certain application in some traditional foodstuffs. They are rich in antioxidants anthocyanins including cyanidin 3-rutinoside and cyanidin 3-glucoside (Hsieh et al., 2006). Cyanidin 3-*O*-β-D-glucopyranoside isolated from fruit of *M. alba* was reported to have free radical scavenging and inflammation suppressing activities and brain protecting from endothelial dysfunction (Serraino et al., 2011). In addition, flavonoids and ascorbic acid were also found in the fresh fruit (Ercisli, Orhan, 2006; Lu et al., 2009).

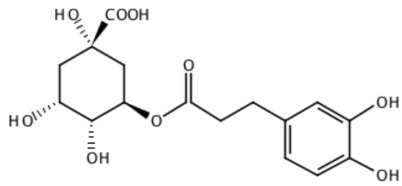
Ramulus mori (Sang zhi), the branches of *M. alba*, was used as traditional Chinese medicine to expel wind, dredge the meridians, and ease joint pain (Ou, 1992). Besides that, it was found to potentially inhibit α-glucosidase activity (Ye et al., 2002).

The *M. alba* root is one of the constituent of a Chinese drug named "Sohaku hi", used for astringent, anthelmintic and reduce blood pressure. Cortex Mori or Sang bi pi (derived from the root bark), one of the well-known traditional Chinese herbal, has been used as an anti-tussive, anti-asthmatic and antithelmintic agents (Kumar, Chauhan, 2008; Piao et al., 2010 cited from Chinese Pharmacopoeia

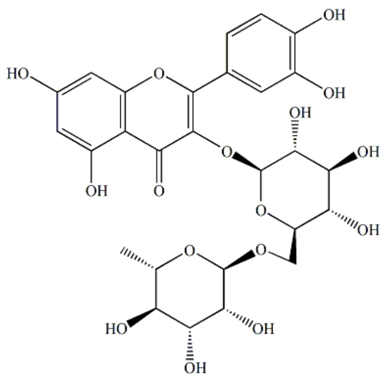
Commission, 2005). MuA, the highest stilbene glycosides accumulated in *M. alba* root, is a major bioactive compound. Moreover, the root bark also contains bioactive Diels Alder adducts, flavonoids and alkaloids (Bhattari, 2002; Kumar, Chauhan, 2008; Piao et al., 2010).



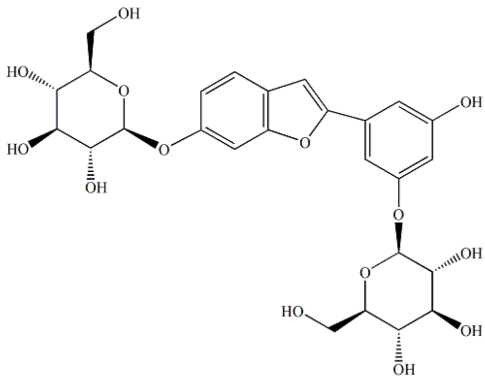
(A) 1-Deoxynojirimycin



(B) Chlorogenic acid



(C) Rutin



(D) Mulberroside F

Figure 2 Structures of bioactive compounds from *M. alba*

Table 1 Bioactive chemical constituents of *M. alba*

Compound	Part of *M. alba*	Bioactive activity	Reference
Albanins A -H	Root bark	Anti-microbial activity	Asano et al., 1994
Cathayanon A-B	Root bark	Anti-inflamation	Rui and Mao, 2001

Table 1 Bioactive chemical constituents of *M. alba* (cont.)

Compound	Part of *M. alba*	Bioactive activity	Reference
Chlorogenic acid	Leaf	Anti-hyperglycemic	Hunyadi et al., 2012
Cis-mulberroside A	Root bark	Anti-inflamation	Piao et al., 2010
Cyanidin 3-glucoside	Fruit	Anti-cancer, Antioxidant	Hsieh et al., 2006
Cyanidin 3-rutinoside	Fruit	Antioxidant	Hsieh et al., 2006
Cyanidin 3-*O*-b-D-glucopyranoside	Fruit	Inflammation suppressing, Antioxidant	Serraino et al., 2011
1-Deoxynojirimycin	Root, leaf, fruit	Alpha-glucosidase inhibitor	Hughes, Rudge, 1994; Shi-De et al., 1995
20-Hydroxyecdysone and inokosterone	Leaf	Anti-diabetic activity, enhance tissue sensitivity to insulin	Hunyadi cited fom Takemoto et al., 1967, Lafont et al., 2003
Kwanon H	Root bark	Anti-HIV	Hughes, Rudge, 1994
Kwanon J	Stem bark	Anti-oxidant, anti-inflammatory	Yu et al., 2004
Moracin A-Z	Root bark	Anti-microbial activity	Asano et al., 1994
Morusin	Root bark	Anti-HIV, inhibits tumor promotion	Shi-De et al., 1995
Morusin 4’- glycoside	Root bark	Anti-HIV	Hughes, Rudge, 1994
Mulberrofuran G	Stem bark	Anti-oxidant	Yu et al., 2004

Table 1 Bioactive chemical constituents of *M. alba* (cont.)

Compound	Part of *M. alba*	Bioactive activity	Reference
Mulberrofuran J-K	Stem bark	Anti-oxidant, anti-inflammatory	Yu et al., 2004
Mulberroside A	Root bark, twig	Anti-inflamation, anti tussive, antithelmintic	Piao et al., 2010; Zhang et al., 2008
Mulberroside F	Leaf	Anti-tyrosinase inhibitor	Lee et al., 2002
Oxyresveratrol	Root, fruit, twig	Anti-inflamation, anti-microbial activity, Anti-oxidant, tyrosinase inhibitor, antithelmintic	Lu et al., 2009; Lipipun et al, 2011, Likhitwitayawuid et al 2006; Zhang et al., 2008
Quercetin	Leaf, fruit	Anti-inflamation	Lu et al., 2009
Resveratrol	Fruit	Anti-microbial, Antioxidant	Lu et al., 2009
Rutin	Leaf, fruit	Anti-inflamation	Lu et al., 2009

2. Mulberroside A

2.1 Biosynthetic pathway

Stilbenes are a small family of plant secondary metabolites derived from the phenylpropanoid pathway. They play roles in plant resistance to fungal pathogens and their biological effects. The essential structural skeleton of stilbenes comprises two aromatic rings joined by a methylene bridge. Most naturally occurring stilbenes are *trans* configuration, but *cis* configuration stilbenes also have been encountered (Chong et al., 2009).

The phenylalanine ammonia lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL) and especially stilbene synthase (STS) are key enzymes in the pathway. The STS, a member of the chalcone synthase (CHS), is characteristic of stilbene-producing plants and catalyzes, in a single reaction, the biosynthesis of the stilbene backbone from three malonyl-CoA and one CoA-ester of a cinnamic acid derivative (Chong et al., 2009). The difference of the substrates and specific enzyme responsibility result in producing different stilbenes. Both free forms and glycosides of stilbenes are naturally occurred in stilbene-producing plants. Because of the glycosylated forms could be involved in their storage, transport from cytoplasm to apoplasm, and protection from peroxidative degradation. While, genetic engineered plants do not normally produce glycosylated stilbenes (Chong et al., 2009).

Mulberroside A (MuA, Figure 3), a stibene glycosides isolated from *M. alba* is simple stilbenes having oxygen functions and glycosides on aromatic rings (Xiao et al., 2008; Chong et al., 2009).

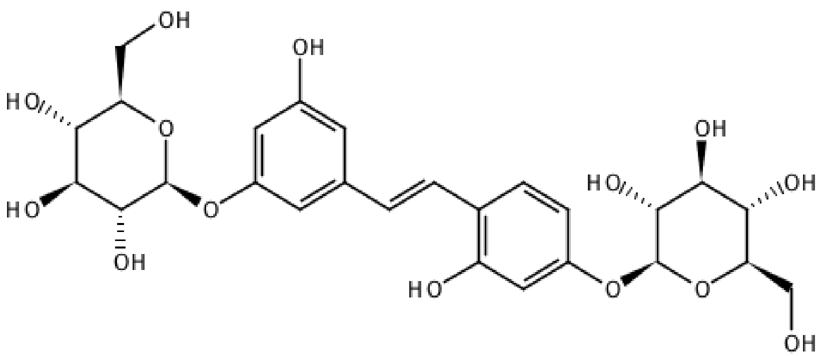


Figure 3 Structure of MuA

The biosynthetic study of MuA in *M. alba* callus culture indicated that the MuA is biosynthesized via cinnamoylpolyketide intermediate and not only L-phenylalanine but also L-tyrosine contribute to the synthesis of the cinnamoyl part of MuA (Figure 4, Shimazaki et al., 2000).

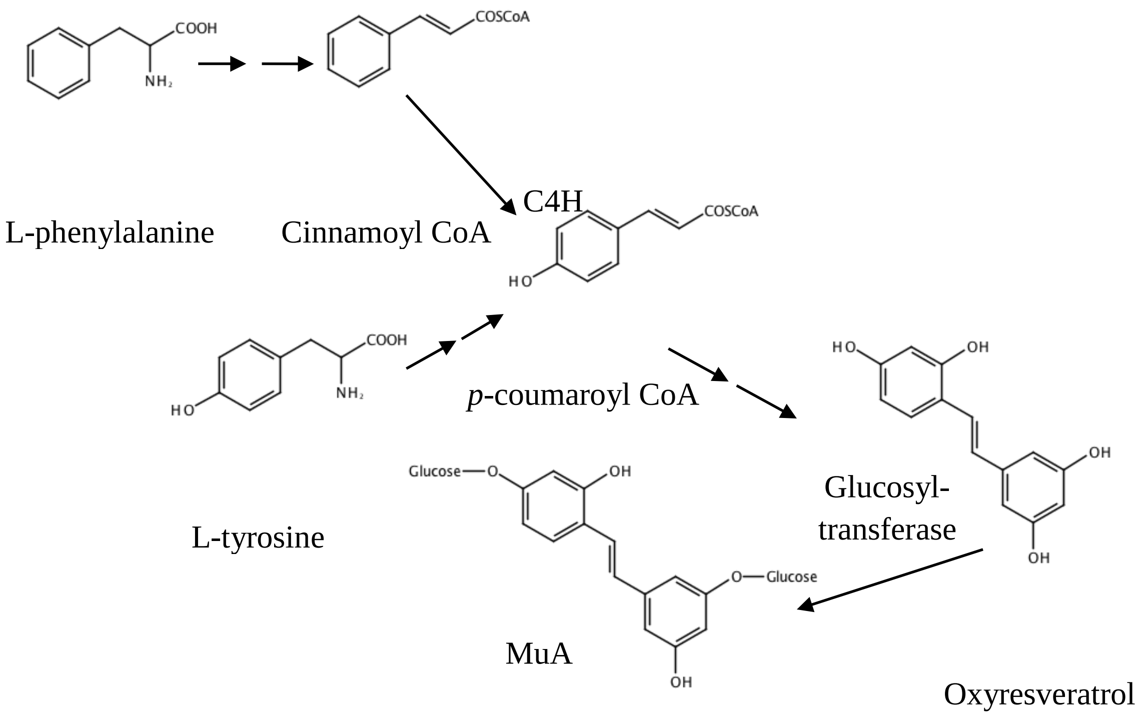


Figure 4 The biosynthesis of MuA

2.2 Bioactivities

The MuA is a major stilbene glycoside from root bark of *M. alba*. It has been identified as the major active compound. MuA can be converted by microbial intestine or directly by glycosidase to aglycone part, oxyresveratrol (Kim et al., 2010; Mei et al., 2012). Oxyresveratrol and MuA have been reported for many pharmacological effects.

2.2.1 Antiviral activities

The oxyresveratrol was reported on anti-viral effects. For an example oxyresveratrol exhibited the inhibitory activity on viral replication at the early and late phase and herpetic skin lesion developments were also significantly delayed in mice orally treated with oxyresveratrol. Application of 20% oxyresveratrol also significantly delayed the development of skin lesions and protected mice from death compared with control and non significanty compared with 5% acyclovir, a topical anti-herpes cream. The concentration of oxyresveratrol can be reduced to 10% or less, depend on pharmaceutical excipients and formulation (Chuanasa et al., 2008; Lipipun et al., 2011). In addition, oxyresveratrol shows a potent, dose-dependent inhibitory activity on African swine fever virus replication (Galindo et al., 2011).

2.2.2 Cytotoxic activities

The low cytotoxic activities on both normal and cancer cells provide benefits of oxyresveratrol as food constituents, food supplement, medicines and cosmetic products. However, the conversion into *cis*-form and transformation by *O*-methylation can increase the cytotoxic activites. A tetra-*O*-methylated analogue of *cis*-oxtresveratrol showed very strong cytotoxicity against the human cancer cells KB, BC, and NCI-H187, with potency comparable to ellipticine and doxorubicin, the anticancer agents (Likhitwitayawuid et al., 2006).

2.2.3 Whitening effect

The oxyresveratrol show stronger inhibition on tyrosinase activity than resveratrol, a well-known active stilbenes compound (Figure 5) due to the greater number of hydroxyl substitution. The inhibitory activity of oxyresveratrol is also greater than kojic acid, a well-known whitening agent used in cosmetic industry. In contrast, *cis*-configuration and methyl substitution decrease the inhibitory activity. The hydrogenation product of oxyresveratrol also exhibited stronger tyrosinase inhibitory activity than the parent compound (Likhitwitayawuid et al, 2006; Wen et al., 2008).

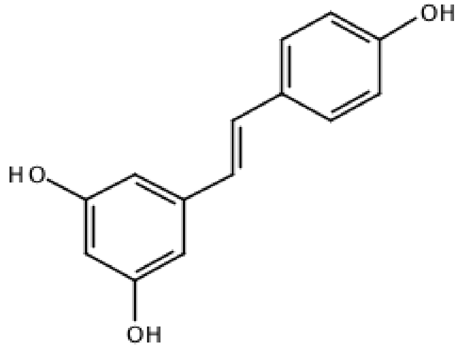


Figure 5 Structure of resveratrol

Topical application of MuA, oxyresveratrol and oxyresveratrol-3-*O*-glucoside solution show inhibitory effect on melanogenesis induced by ultraviolet B irradiation in mice. Among three solution, oxyresveratrol shows highest activity. The activities are inversely proportional to number of sugar substituted (Lim et al., 2011). Furthermore, the heartwood extract of *Artocarpus lakoocha* (a oxyresveratrol is major active compound) was given the shorter onset of significant whitening effect than 0.25% licorice extract and 3% kojic acid (Tengamnuay et al., 2006).

2.2.4 Antioxidant

The higher number of hydroxyl group is also the reason that oxyresveratrol shows greater solubility, antioxidant and oxidative stress protection effects comparing to resveratrol. Because of these reasons and its blood-brain barrier permeativity, the oxyresveratrol shows neuroprotective effects and inhibits the apoptotic cell death in transient cerebral ischemia orally (Chao et al., 2004; Horn et al., 2004). Protective effects of MuA and oxyresveratrol against ethanol-induced hepatic damage were also reported (Zhang et al., 2008).

2.2.5 Anti-inflammation

The analgesic and anti-inflammatory activities of the stilbenes were reported. The oxyresveratrol isolated from *Artocarpus heterophyllus* exhibited *in vitro* anti-inflammatory effects by inhibition of nitric oxide production (Yen et al., 2008). The *cis*-MuA isolated from *Ramulus mori* exhibited anti-inflammatory activities in several models of inflammatory pain in mice. The results support the potential anti-rheumatoid activity of *Ramulus mori* in Chinese traditional medicine (Shi et al., 2010).

3. *In Vitro* Cultures of *M. alba*

3.1 Tissue Cultures for Production of Plant Secondary Metabolites

Plant tissue culture technique was developed for preservation, micropropogation and production secondary metabolites from medical plants. This technique is an appropriate method for studying plant bioactive metabolites under different culture environmental conditions such as nutrients, light, temperature, pH, these environment factors are controllable (Dicosmo, Misawa, 1995; Yoshimatsu, 2008). This method provides high potential choice for production of high-value secondary metabolites and may be an effective tool to solve problem related to production of secondary metabolites from natural plants including environmental factors, political and labor instability (Smetanska, 2008). Some plant cell cultures can grow with short growth cycles in the laboratory and produce a higher amount of secondary metabolites than their parent plants. The specifically problems can be occurred in the metabolite production of some plants by this method including the instability of cell lines, low yields, slow growth and scale-up problems. However, there are many strategies that have been developed and adopted for the enhancement of metabolites including specific organ cultures, elicitation, mutation, hairy root induction, immobilization and optimization of culture conditions (Rao, Ravishankar, 2002).

3.2 Induction of *In Vitro* Cultures of *M. alba*

In vitro proliferated shoots of *M. alba* were multiplied rapidly by culture of shoot tips and nodal explants on Murashige and Skoog medium (MS) with 6-Benzylaminopurine (benzyladenine or BAP) and 1-naphthaleneacetic acid (NAA) as supplements (Anis et al., 2003). Root cultures of the *M. alba* can be induced from microshoots on MS medium supplemented with NAA. The results indicated that the tissue culture technique is a promising method for rapid propagation for commercial scale (Anis et al., 2003). In addition, the *in vitro* culture of *M. alba* was also used in the biosynthetic study of the MuA (Shimazaki et al., 2000).

4. Elicitation

Elicitors are molecules that stimulate any of a number of defense responses in plants. According to plant secondary metabolites are a group of compounds, which play a major role in the plant defense mechanisms, elicitation could be an effective strategy to increase the secondary metabolites accumulation. Recognition and sensitivity of defensive response are important factors for elicitation. Microbial components, several physical stress, harmful chemicals and phytohormones were reported as biotic and abiotic elicitors (Hahn, 1996; Gao et al., 2010).

4.1 Microbial elicitors

Recently, not only pathogenic microbes were reported for the exhibition of elicitation effects but common and endophytic microbiales found in plants also stimulate their host bioactive metabolites productions. For an example addition of *Arbuscular mycorrhiza* (isolated from *M. alba*) extracts were found to be equivalent to indole-3-butyric acid (IBA) in promoting rhizogenesis, root growth and proliferation of *in vitro M. alba* shoot cultures (Shama et al., 2005). Besides, sterilized microbial components, living microorganisms have also been reported for elicitation effects including *Bacillus* sp. enhancing the production of resveratrol and resistance to *Botrytis cinerea*, a phytopathogen in grapevine (Paul et al., 1998). *Fusarium mairei*, an taxol-producing endophytic fungi was reported its synergistic effects on taxol production with *Taxus chinensis* var. *mairei* in bioreactor scale cultured by co-culture method (Li et al., 2009).

For these reasons tissue culture method with elicitation treatment is an interesting method for the production of bioactive metabolites in large scale.

. **4.2 Stilbenes elicitation**

Stimulating STS and involved enzymes including PAL and C4H can enhance stilbenes production in plants. Reported stilbeniod elicitors including pathogenic fungi, UV radiation, aluminium ion, methyl jasmonate, ethylene, yeast, salicylic acid, and chitosan can enhance the accumulations and reduce production time of stilbeniod compounds in plants (Aziz et al, 2006; Roat, Ramawat, 2009; Belhadj et al., 2006; Chong, 2009).

5. Endophytic Microooganism

Endophytes are microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects (Bacon, White, 2000).

It has been reported that plant growth regulators such as auxins, cytokinins and gibberellin were produced by endophytic bacteria (Liu et al., 2010; Hurek RB, Hurek T, 2011). Endophytes were also used as elicitors. For an example, mycelium suspension of *Colletotrichum* sp. was used to enhance artemisinin production in hairy root culture of *Artemisia annua* (Wang et al., 2001).

To date, diverse bioactive metabolites (such as insecticidal, antioxidant, antibiotics, antiviral and anticancer agents) were obtained from endophytes (Strobel et al., 2004). Furthermore, some endophytes can produce similar or identical compounds which produced by host plants (Gunatilaka, 2006). One of interesting strategies to discover endophytes is the selection of ethnobotanical plants (Strobel et al, 2004). The most frequently isolated endophytes from medicinal plants are fungi

(Strobel et al., 2004). For examples, there are reports that *Taxomyces andreanae*, endophytic fungus from *Taxus brevifolia* and other endophytes can produce taxol (Stierle et al.,1993; Zhou et al., 2010) and endophytic fungus isolated from *Vinca minor* can produce vincamine (Yin, Sun, 2011). Therefore, isolation and screening for endophytic fungi producing bioactive compounds are interesting points. Several strategies were used for screening plant-associated microorganism, for examples, monoclonal antibodies specific to taxol was used as a screening tool for taxol-producing fungi (Stierl et al., 1995) and the assay of α-glucosidase inhibition was used to detect glucosidase inhibitor producing endophytic actinomyces (Pujiyunto et al., 2011).

6. Polyclonal Antibody

The New Zealand White rabbit is most frequently used animal for the production of polyclonal antibodies according to it easy to maintain, and low batch-to-batch variations. The dose of immunization traditionally start from 10 µg to 200 µg of an antigen suspended in an equal volume of adjuvant. In rabbits, volumes of 0.1-0.5 ml are usually given intradermally and distributed over several sites for generation of an immune response. Blood is generally harvested from the ear (Boenisch, 2001).

Polyclonal antibodies can be used for the development of enzyme-linked immonosorbent assay (ELISA) method. ELISA is a rapid, simple and high sensitivity method for the determination of secondary metabolites. For examples, the determination of isoflavonoids in *Pueraria candollei* (Pongkitwitoon et al., 2010), aculeatiside A in *Solanum aculeatissimum* (Putalun et al., 2002) and asiaticoside in *Centella asiatica* (Tassanawat et al., 2012).