CHAPTER II

HISTORICAL

In the past three decades, tyrosinase became the more interesting target for researchers in medical, cosmetic, agricultural , and food fields due to their important key enzymes in various process in animals, insects, plants and microorganisms. In order to explore safer and more potent compounds for the tyrosinase inhibitors currently in use, the details of melanin synthesis and how to control it, studies on tyrosinase , its substrate and inhibitors are need.

1. Tyrosinase

Tyrosinase (EC 1.14.18.1) is a copper-containing mixed-function oxidase widely distributed in nature and is mainly involved in formation of pigments such as melanin and other polyphenolic compounds (van Gelder *et al.,* 1997). Tyrosinase is also known as polyphenol oxidase. It is well-known that tyrosinase catalyzes two different reactions: the hydroxylation of monophenol to *o*-diphenol (monophenolase or cresolase activity) and oxidation of *o*-diphenol to *o*-quinones (diaphenolase or catecholase activity). Quinones are highly reactive compounds which can spontaneously polymerize to form a high molecular weight compound, melanins (Briton, 1983; van Gelder *et al.*, 1997; Sanchez-Ferrer *et al.*, 1995; Seo *et al.*, 2003).

Tyrosinase has been adapted to serve diverse physiological role in different organisms. In fungi and vertebrates, tyrosinase catalyzed the initial step in formation of the pigment melanin from tyrosine (Kim and Uyama, 2005). The melanin biosynthesis pathway was proposed by Raper and Mason as shown in Figure 2 (Briton, 1983). Tyrosine is oxidized in a two-step reaction to give DOPAquinone. This compound is then transformed into an intermediate name DOPAchrome, a red pigment which has been used as a target for the measurement of tyrosinase inhibitor

in several *in vitro* assays (Iida *et al.*, 1995; Likhitwitayawuid and Sritularak, 2001; Nerya *et al.*, 2003; Shin *et al.*, 1998). DOPAchrome is converted through several biochemical reactions to indole 5,6-quinone which is subsequently polymerized to form melanin. In higher plants, the enzyme protects plants against insects and microorganisms by catalyzing the formation of an impervious scab of melanin against further attack. In insects, tyrosinase is involved in sclerotization of the exoskeleton and in protection against other organisms by encapsulation in melanin. In most fruits and vegetables, it is responsible for enzymatic browning when tissues are injured (van Gelder *et al.*, 1997).
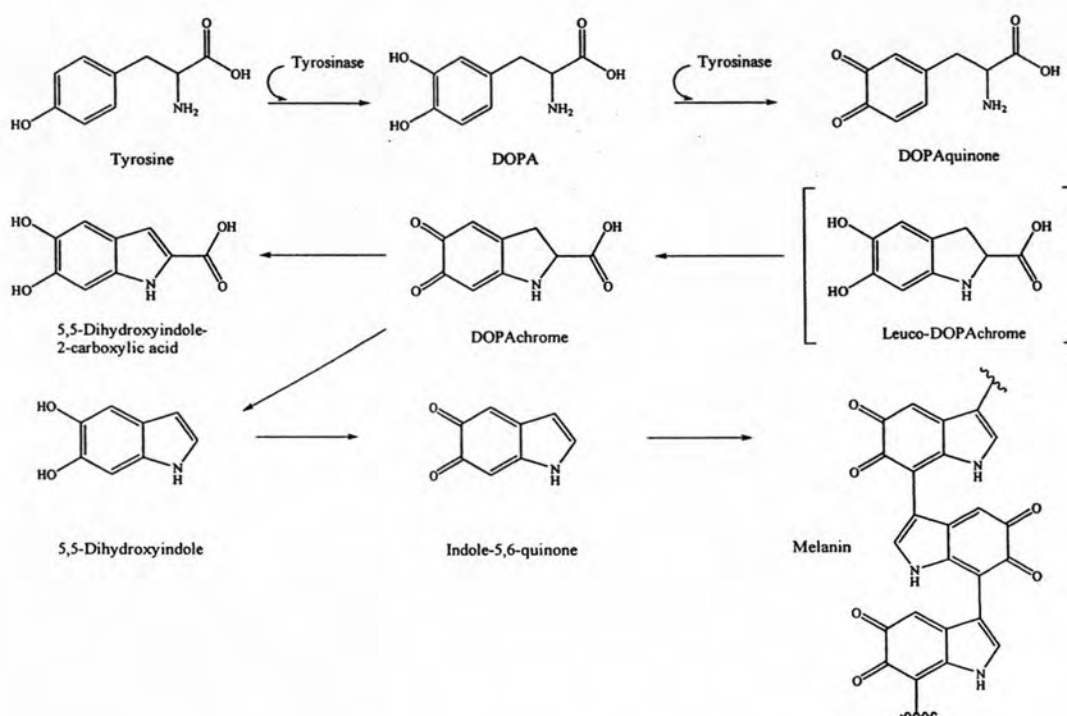

Figure 3. The Raper-Mason scheme of melanogenesis (Briton, 1983).

1.1 Structure of Active Center

Mushroom tyrosinase is popular among researchers as it is commercially available and inexpensive, and also there are easy tools to investigate the feature of this enzyme (Seo *et al.*, 2003). *Agaricus bisporus* tyrosinase has been reported to be a heterotetramer comprising two heavy and light chains with a molecular mass of 120

kDa. It is a soluble cytosolic enzyme, while human tyrosinase is a membrane-bound glycoprotein located inside the melanosome (Kim and Uyama, 2005; van Gelder *et al.*, 1997). The most prominent features observed in all tyrosinase sequences are the two Copper (Cu) binding sites, called Cu A and Cu B (Figure 4). Several conserved sequences are found to be present in tyrosinases from different sources as shown in Figure 5. In fact, when all tyrosinase sequences are compared, the only conserved domain seems to be the central copper-binding domain, which also shares sequence homology with hemocyanins, copper-containing oxygen carriers from the hemolymph of many molluscs and arthropods. Six conserved histidine residues bind a pair of copper ions in the active site of the enzyme tyrosinase, which interact with both molecular oxygen and its phenolic substrate. The location of cysteine (Cys) also plays an important role in the formation of disulfide linkages, which stabilize the protein structure. The number of Cys residues varies from one organism to another, as along the N-terminal and central part of the protein, Human and mouse tyrosinases have 17 Cys residues and plants have 11, whereas the C-terminal domain contains 1 Cys residue. Inhibitors and activators modulate the enzyme activity by binding to this site (Seo *et al.*, 2003).
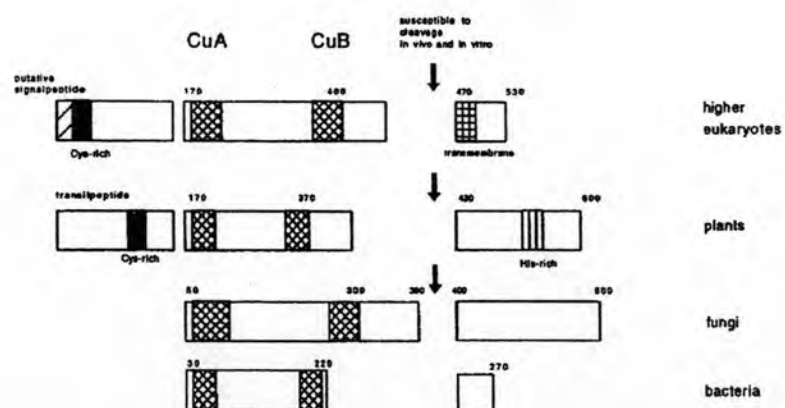

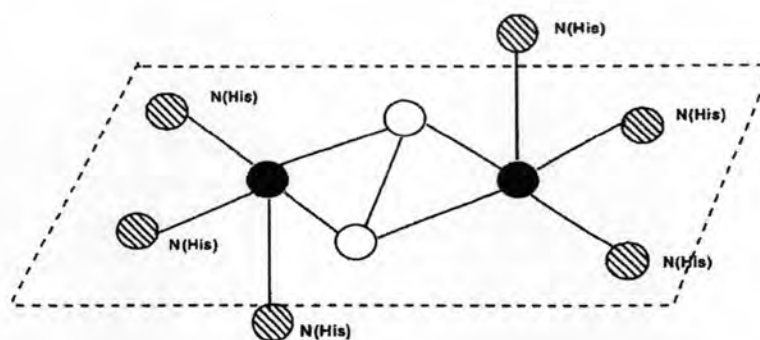Figure 4. Domain structure of tyrosinase from different groups of species (van Gelder *et al.*, 1997)

Figure 5. Schematic representation of the binuclear copper center (van Gelder *et al.*, 1997). The oxygen is bound as peroxide and each Cu ion is bound to three histidine nitrogen atoms. Black symbols--Cu-ions; white symbols--oxygen; symbols dashed vertically -- His-N.

1.2 Mechanism of Tyrosinase Action

There are three types of tyrosinase: met-, oxy- and deoxytyrosinase, with different binuclear copper structure of active site. The mechanism for the monophenolase and diaphenolase of tyrosinase has widely been studied based on these three forms of the enzyme (Sanchez-Ferrer *et al.*, 1995; Kim and Uyama, 2005; Seo *et al.*, 2003). In the monophenolase cycle, the monophenol can react only with the oxy form and binds to the axial position of one of the coppers of this form. Rearrangemant through a triagonal bipyramidal intermediate leads to o-hydroxylation of monophenol by the bound peroxide as shown in Figure 6. This generates the coordinated *o*-diphenol and resulting in a deoxy form ready for further dioxygen binding. In diaphenolase cycle, both the oxy and met forms can react with *o*-diphenol and oxidise it to the *o*-quinone.
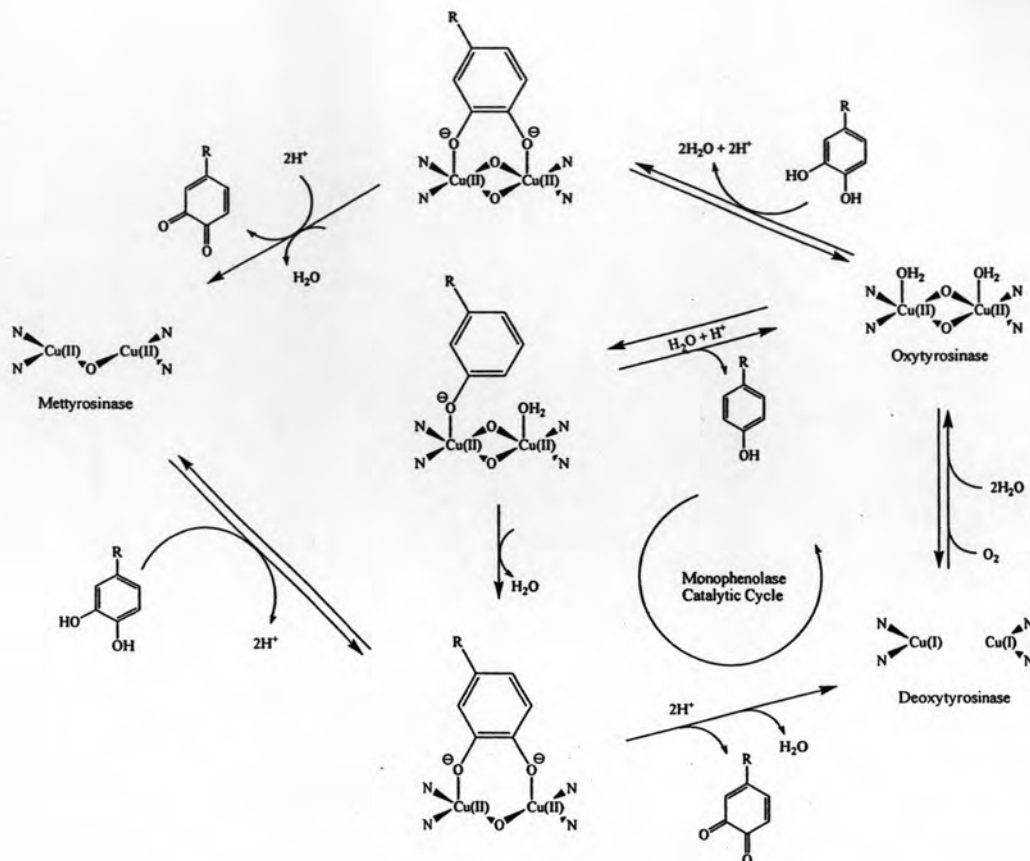
Figure 6. Catalytic cycles of the hydroxylation of monophenol and oxidation of *o*-diphenol by tyrosinase (Kim and Uyama, 2005).

2. Tyrosinase inhibitors

Since tyrosinase plays critical role in melanin synthesis as well as browning of plant-derived food and beverages, These reasons encourage the researchers to seek the potential tyrosinase inhibitors. A number of different types of compounds from both natural and synthetic sources have been investigated.

2.1 Tyrosinase Inhibitors from Natural Sources

As plants are the rich source of several bioactive compounds, which are mostly free from harmful side effects, a number of compounds from them have been studied for mushroom tyrosinase inhibitory activity. Tyrosinase inhibitors from natural sources can be divided into 3 main subgroups: plant polyphenols, aldehydes and other derivatives from plants, and fungal metabolites.

2.1.1 Plant Polyphenols

Polyphenols are usually referred to a group of compounds containing multiple phenolic functionalities. They are widely distributed in the most higher plant in which they have numerous biological activities. Among these, flavonoids has been reported as potent tyrosinase inhibitors and their structure activity relationship have also been studied. It was found that flavonoids possessing an α–keto group show potent tyrosinase inhibitory activity. This may be explained in terms of similarity between the dihydroxyphenyl group of L-Dopa and the α–keto group of flavonoids (Kubo et al., 1994).
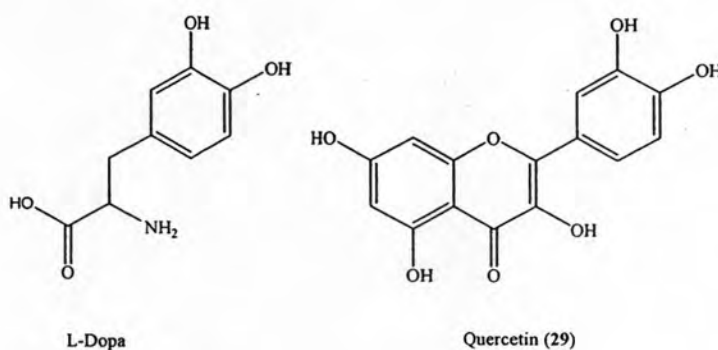
L-Dopa   Quercetin (29)

Figure 7. The structure similarity between L-Dopa and quercetin (29) (Kubo et al., 1994).

Quercetin (29) and kaempferol (30), flavonols containing 3-hydroxy-4-keto moiety, exhibited their tyrosinase inhibitory activities by chelating the copper in active site, leading to inactivation of the enzyme activity. Kubo et al. (1994) reported that both kaempferol (30) and quercetin (29) chelate copper in the met form to tyrosinase differ from that of kojic acid (2) which chelate via the oxy form (Kim and Uyama, 2005). The chelation mechanism seems to be specific to flavonols as long as the 3-hydroxyl group is free. But this is not essential requirement for tyrosinase inhibition of other types of flavonoids such as luteolin (31) and luteolin 7-O-glucoside (32) (Chen and Kubo, 2002; Seo et al., 2003).

Structure activity relationship of flavonoids (**33-36**), stilbenes (**37-39**) and related 4-substituted resorcinols isolated from *Artocarpus incisus* (Shimizu *et al.*, 1998) has been studied by Shimizu, Kondo and Sakai (2000). Compounds with the 4-substituted resorcinol skeleton showed potent tyrosinase inhibitory activity (Figure 8). In the case of stilbenes, such as oxyresveratrol (**40**), the 4-substituted resorcinol skeleton must be the most important feature for revealing potent tyrosinase inhibition. For flavonoids, not only this skeleton but also addition structural factors are nesscessary to reveal tyrosinase inhibitory activity. Introduction of substituted group to C3 position of flavanone and flavone type dramatically decreased their activities. The summarized structure activity ralationships of compounds having 4-substituted resorcinol skeleton are demonstrated in Figure 9.

(+)-Dihydromorin (**33**), R = OH IC_{50} =25 µM
(+)-Norartocarpanone (**34**), R = H IC_{50} =1.76 µM

Artocarpesin (**35**), R = Pr IC_{50} =13.5 µM
Isoartocarpesin (**36**), R = CHCHCH(CH$_3$)$_2$ IC_{50} = 21.1 µM

4-Prenyloxyresveratrol (**37**), R = Pr IC_{50} = 0.66 µM
Chlorophorin (**38**), R = Ger IC_{50} = 0.26 µM

Artocarbene (**39**), IC_{50} = 2.45 µM
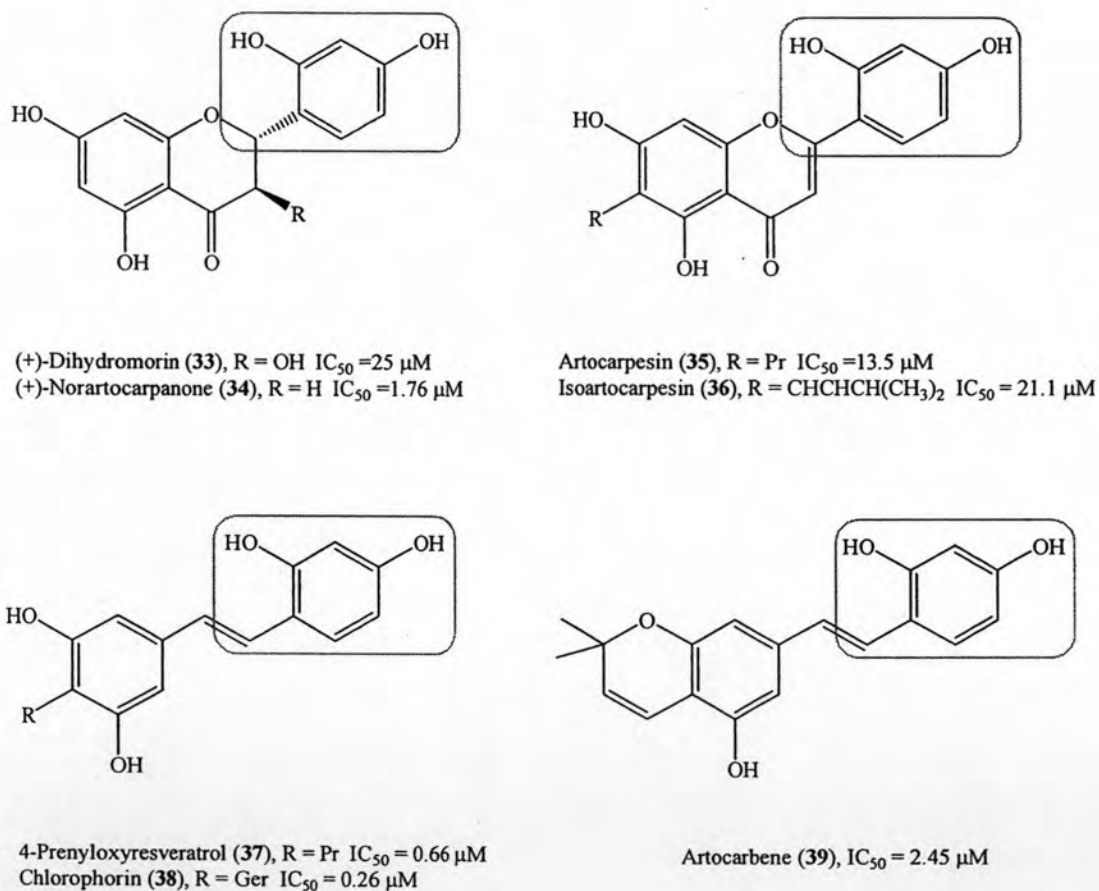
Figure 8.   The chemical structure and IC_{50} of active components from *A. incisus*. The boxed part: 4-substituted resorcinol skeleton (Shimizu *et al.*, 2000).

Hydrophobic and less bulky substituents are preferred. Replacement of substituent with hydrogen atom dramatically decrease activity

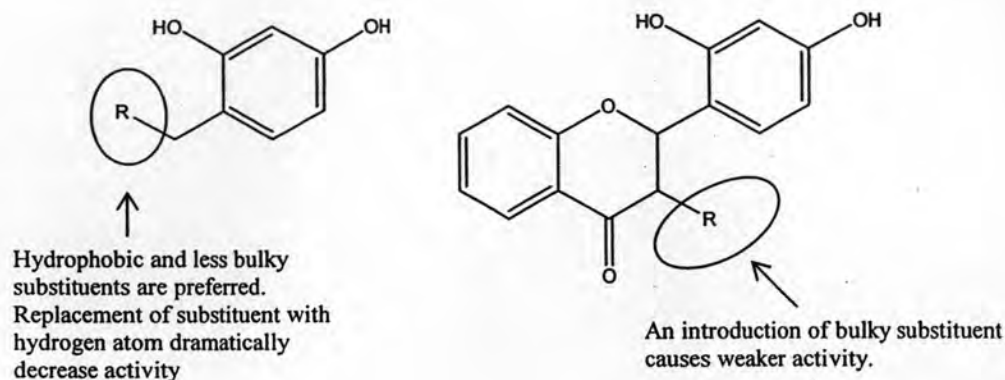An introduction of bulky substituent causes weaker activity.

Figure 9. Summarized structure activity ralationships of compounds having 4-substituted resorcinol skeleton (Shimizu *et al.*, 2000).

Among licorice flavonoids, glabridin (**4**) , glabrene (**7**) and isoliquiritin (**10**) exhibited high tyrosinase inhibitory activity. Glabridin was the most potent inhibitor of licorice constituents with 4-substituted resorcinol structure (Nerya *et al.*, 2005). Like glabridin, glabrene exhibited tyrosinase inhibitory activity. The structure of glabrene includes a double bond between carbon atom 3 and 4 of ring C, while glabridin lacks of the double bond, which may suggest that conjugated double bonds between ring A and B are not essential among the flavonoids. Hispaglabridin A (**8**) and B (**9**) did not inhibit tyrosinase activity. Structure of the two isoflavans are similar to that of glabridin, except hiapaglabridin A has an isoprenyl side chain attached to the 3′ position and hispaglabridin B has only one hydroxyl group, both features increasing their lipophilicity in comparison to glabridin. The two derivatives of glabrin, 2′-methylglabridin (**41**) and 4′-methylglabridin (**5**), also did not inhibit tyrosinase activity, which suggests the importance of both hydroxyl groups to the activity.
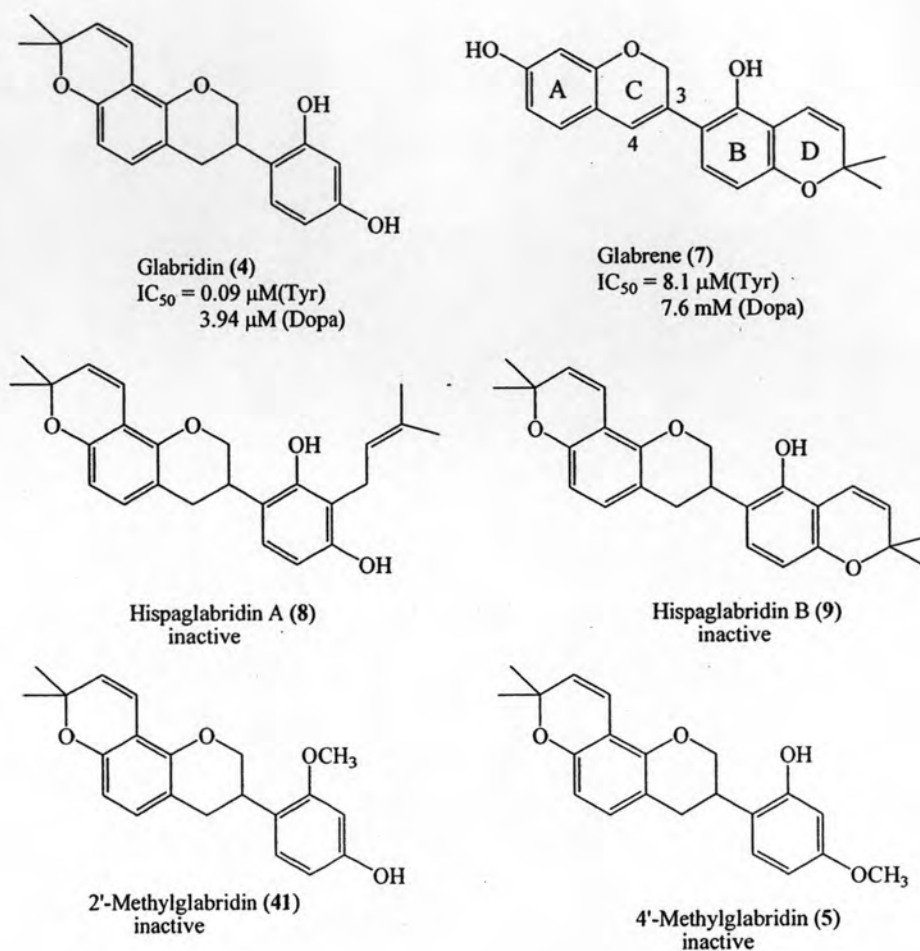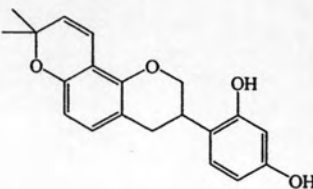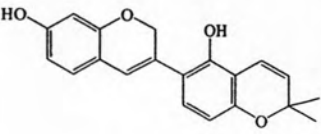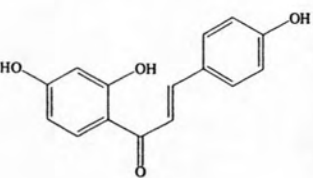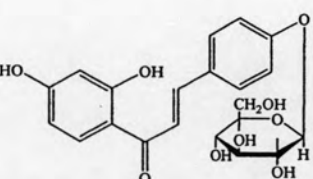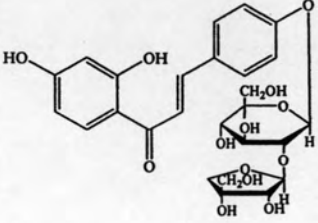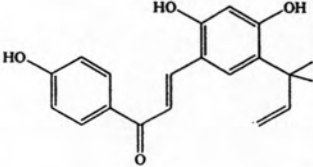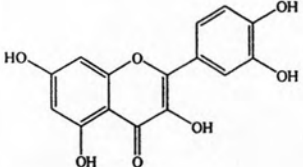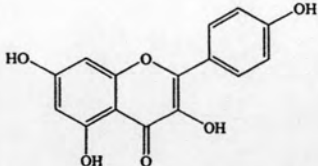
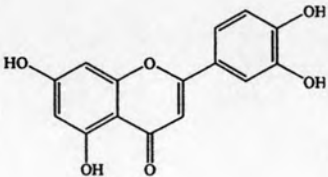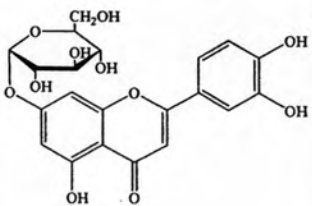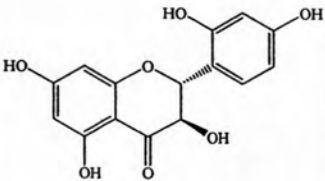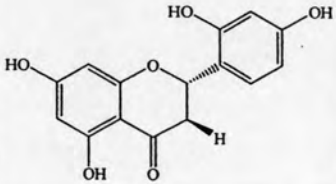Figure 10. Licorice isoflavonoids and their tyrosinase inhibitory activities.

Another important compound of this group is gallic acid. Gallic acid esters (42-44) are identified as strong tyrosinase inhibitors. The flavon-3-ol skeleton with a galloyl moiety at the 3-position is an important structural requirement for tyrosinase inhibition (No *et al.*, 1999).
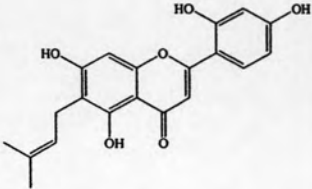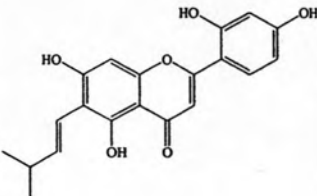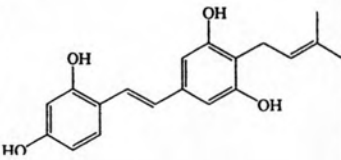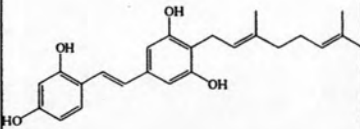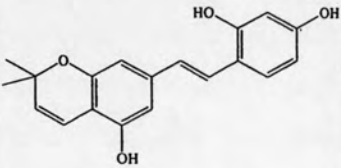
As summarized in Table 2, a number of plant polyphenols have been reported to inhibit mushroom tyrosinase. The potency of each compound was demonstrated as its IC_{50} value. Since different methods of evaluation have been used in different investigations, it would be difficult to make a direct comparison of the inhibitory activity of these compounds based on the reported IC_{50} values. However, these data can be used as a guideline for searching a potent tyrosinase inhibitor.
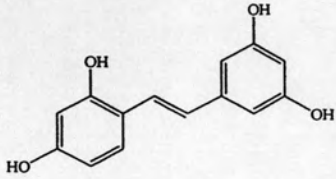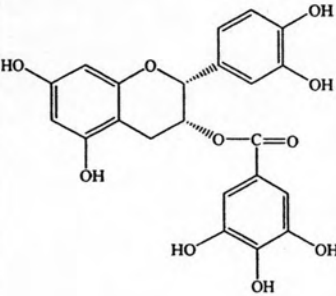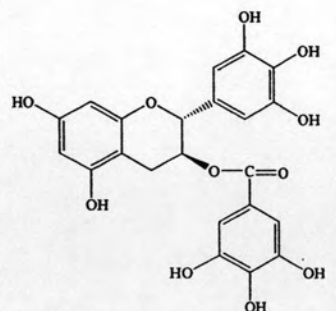
Table 2. Tyrosinase inhibitors from plant polyphenols.
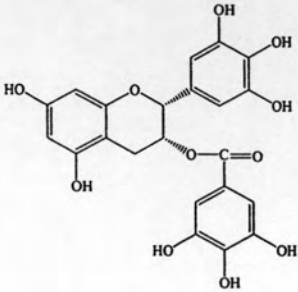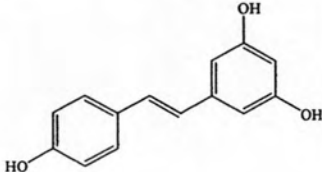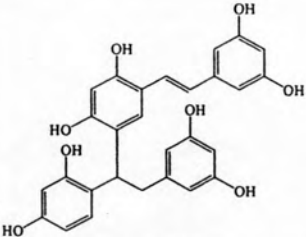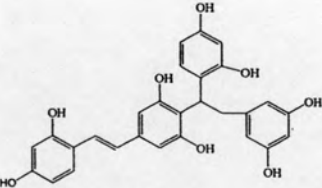
Compounds	Source	Type of inhibitor	IC_{50} (mM)	Reference
Glabridin (4)	*Glycyrrhiza glabra*	noncompetitive	0.00009^{a} 0.0039^{b}	Nerya *et al.*, 2003.
Glabrene (7)	*G. glabra*	mixed-type	0.0081^{a} 7.6^{b}	Nerya *et al.*, 2003.
Isoliquiritigenin (10)	*G. glabra*	mixed-type	0.0035^{a} 0.047^{b}	Nerya *et al.*, 2003.
Isoliquiritin (11)	*G. uralensis*	competitive	0.038	Fu *et al.*, 2005.
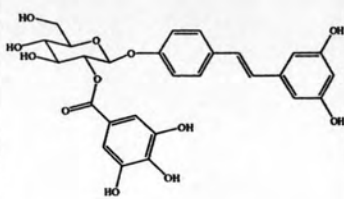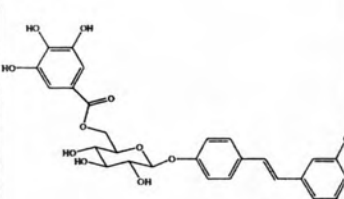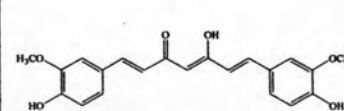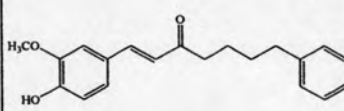
Compounds	Source	Type of inhibitor	IC_{50} (mM)	Reference
Licuraside (**12**)	*G. uralensis*	competitive	0.072	Fu *et al.*, 2005.
Licochalcone (**15**)	*G. inflate*	competitive	0.0258	Fu *et al.*, 2005.
Quercetin (**29**)	*Heterotheca inuloides*	competitive	0.070	Kubo, Kinst-Hori, Chauhuri *et al.*, 2000.
Kaempferol (**30**)	*Crocus sativus*	competitive	0.230	Kubo and Kinst-Hori, 1999a; Kubo Kinst-Hori, Chauhuri *et al.*, 2000.
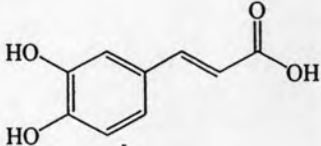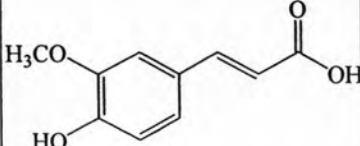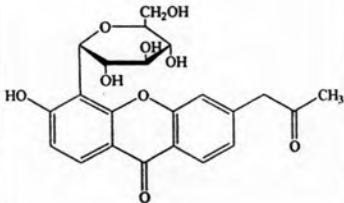
Compounds	Source	Type of inhibitor	IC$_{50}$ (mM)	Reference
Luteolin (**31**)	*Glycine max*	noncompetitive	0.19	Kubo Kinst-Hori, Chauhuri *et al.*, 2000.
Luteolin-7-O-glucoside (**32**)	*G. max* *Marrubium velutinum*	noncompetitive	0.5	Kubo Kinst-Hori, Chauhuri *et al.*, 2000.
Dihydromorin (**33**)	*Artocarpus incisus*	-	0.025	Shimizu *et al.*, 1998.
Norartocarpanone (**34**)	*A. incisus*	competitive	0.00176	Shimizu *et al.*, 1998; Shimizu *et al.*, 2000.

Compounds	Source	Type of inhibitor	IC_{50} (mM)	Reference
Artocarpesin (35)	*A. incisus*	-	0.0135	Shimizu *et al.*, 1998.
Isoartocarpesin (36)	*A. incisus*	-	0.0211	Shimizu *et al.*, 1998.
4-Prenyloxyresveratrol (37)	*A. incisus*	competitive	0.00066	Shimizu *et al.*, 1998; Shimizu *et al.*, 2000.
Chlorophorin (38)	*A. incisus*	competitive	0.00026	Shimizu *et al.*, 1998; Shimizu *et al.*, 2000.
Artocarbene (39)	*A. incisus*	competitive	0.00245	Shimizu *et al.*, 1998; Shimizu *et al.*, 2000.

Compounds	Source	Type of inhibitor	IC$_{50}$ (mM)	Reference
Oxyresveratrol (**40**)	*Morus alba*	noncompetitive	0.000001	Shin *et al.*, 1998.
(-)-Epicatechin-3-O-gallate (**42**)	*Thea sinensis*	competitive	0.03458	No *et al.*, 1999.
(+)-Gallocatechin-3-O-gallate (**43**)	*T. sinensis*	competitive	0.01734	No *et al.*, 1999.

Compounds	Source	Type of inhibitor	IC_{50} (mM)	Reference
(-)-Epigallocatechin-3-O-gallate (44)	*T. sinensis*	competitive	0.034	No *et al.*, 1999.
Resveratrol (45)	*Morus alba*	-	0.155	Shin *et al.*, 1998.
Artogomezianol (46)	*Artocarpus gomezianus*	-	0.068	Likhitwita yawuid and Sritularak, 2001.
Andalasin (47)	*A. gomezianus*	-	0.039	Likhitwita yawuid and Sritularak, 2001.

Compounds	Source	Type of inhibitor	IC$_{50}$ (mM)	Reference
3,4′,5-Trihydroxy stilbene-4′-O-β-D-(2″-O-galloyl)glucopyrano side (48)	*Rheum officinale*	competitive	0.0067[a] 0.246[b]	Iida *et al.*, 1995.
3,4′,5-Trihydroxy stilbene-4′-O-β-D-(6″-O-galloyl)glucopyrano side (49)	*R. officinale*	competitive	0.0147[a] 0.0823[b]	Iida *et al.*, 1995.
Curcumin (50)	*Curcuma longa*	-	0.047	Shirota *et al.*, 1994.
Yakuchinone B (51)	*Alpinia oxyphylla*	competitive	0.057	Shirota *et al.*, 1994.

Compounds	Source	Type of inhibitor	IC$_{50}$ (mM)	Reference
3,4-Dihydroxycinnamic acid (52)	*Pulsatilla cernua*	noncompetitive	0.97	Lee, 2002.
4-Hydroxy-3-methoxy-cinnamic acid (53)	*P. cernua*	noncompetitive	0.33	Lee, 2002.
Aloesin (54)	*Aloe vera*	noncompetitive	0.10	Yagi, Kanbara and Morinobu, 1987

[a] Monophenolase activity (Tyrosine was used as a substrate).

[b] Diaphenolase activity (L-Dopa was used as a substrate).

2.1.2 Aldehyde and Other Derivatives from Plants

A large number of aldehydes and other compounds were also isolated and identified as tyrosinase inhibitors such as anisaldehyde (55) (Kubo and Kinst-Hori, 1998a), cuminaldehyde (56), cumic acid (57) (Kubo and Kinst-Hori, 1998b), cinnamaldehyde (58) (Lee, 2002), 2-hydroxy-4-methoxybenzaldehyde (59) (Kubo

and Kinst-Hori, 1999b), and 4-isopropyl-salicylaldehyde (2-hydroxy-4-isopropyl benzaldehyde or chamaecin, **60**) (Nihei *et al.,* 2004; Song, Lin and Chen, 2005). As the aldehyde group is known to react with biologically important nucleophillic groups such as sulhydryl, amino and hydroxyl groups. Their tyrosinase inhibitory mechanisms have been proposed due to the formation of Schiff base with primary amino group of the enzyme. Interestingly, the introduction of an electron-donating group (isopropyl and methoxyl) to the *para* position of benzaldehyde as in cuminaldehyde and 2-hydroxy-4-methoxybenzaldehyde, increased inhibitory activity, probably stabilizing the Schiff base at the active site of enzyme. Furthermore, the *ortho*-hydroxybenzaldehyde moiety in 2-hydroxy-4-methoxybenzaldehyde forms a quasi six-membered ring through intramolecular hydrogen bonding and produces a more stable chelate structure as shown in Figure 11.
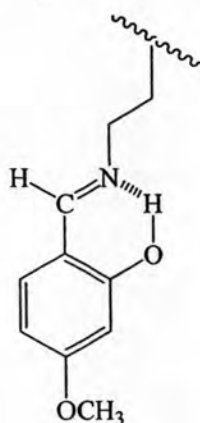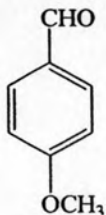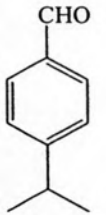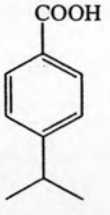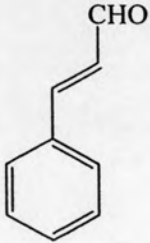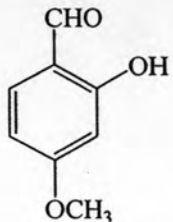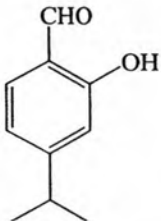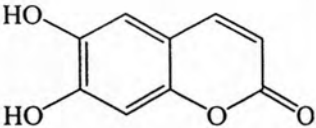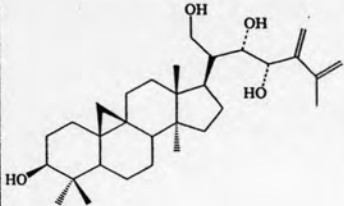
Figure 11. Structure of Shiff base adduct of 2-hydroxy-4-methoxybenzal-dehyde (Kubo and Kinst-Hori, 1999b).
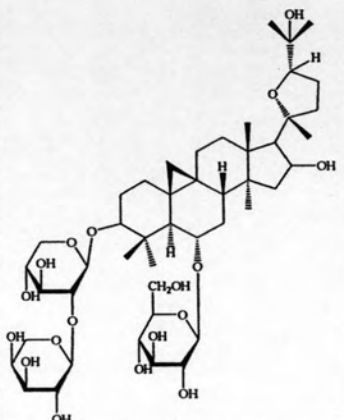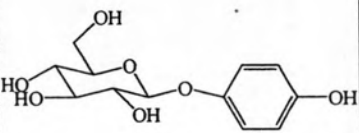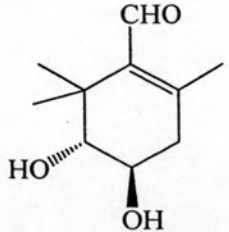
Masamoto *et al.* (2003) found that esculetin (**61**), a coumarin derivative isolated from *Euphobia lathyris*, inhibited tyrosinase activity with an IC_{50} of 43 µM. The hydroxyl group at the carbon atom 6 and 7 positions of coumarin skeleton were suggested to play critical role in exhibiting the tyrosinase inhibitory activity. Recently, cycloartane type tritepenoids such as 3β,21,22,23-tetrahydroxy-cycloart-24(31),25(26)-diene (**62**) and askendoside B (**63**) have been reported as tyrosinase inhibitors (Khan, Khan and Ather, 2006; Khan *et al.*, 2006).

A number of compounds as well as aldehydes, which possess tyrosinase inhibitory activity, are summarized in Table 3.

Table 3. Tyrosinase inhibitors from aldehydes and other derivatives.

Compounds	Source	Type of inhibitor	IC_{50} (mM)	Reference
Anisaldehyde (55)	*Pimpinella anisum*	noncompetitive	0.32	Kubo and Kinst-Hori, 1998a.
Cuminaldehyde (56)	*Cuminum cyminum*	noncompetitive	0.05	Kubo and Kinst-Hori, 1998b.
Cumic acid (57)	*C. cyminum*	noncompetitive	0.26	Kubo and Kinst-Hori, 1998b.
Cinnamaldehyde (58)	*Cinnamomun cassia*	noncompetitive	0.97	Lee, 2002.

Compounds	Source	Type of inhibitor	IC$_{50}$ (mM)	Reference
2-Hydroxy-4-methoxy-benzaldehyde (59) CHO OH OCH$_3$	*Mondia whitei, Rhus vulgaris, Sclerocarya caffra*	mixed-type	0.30	Kubo and Kinst-Hori, 1999b.
4-Isopropylsalicyl-aldehyde (60) CHO OH	*Eucalyptus cneralifolia, Chamaecyparis taiwanensis*	mixed-type	0.0023[b] 0.106[a] 0.0014[b]	Nihei *et al.*, 2004; Song *et al.*, 2005.
Esculetin (61) HO HO O	*Euphobia lathyris*	competitive	0.043	Masamoto *et al.*, 2003
3β,21,22,23-Tetrahydroxy-cycloart-24(31),25(26)-diene (62)	*Amberboa ramose*	-	0.0013	Khan, Khan and Ather, 2006.

Compounds	Source	Type of inhibitor	IC_{50} (mM)	Reference
Askendoside B (63)	*Astragalus taschkendicus*	-	0.0139	Khan *et al.*, 2006.
β-Arbutin (64)	*Uva ursi*	competitive	8.40	Funayama *et al.*, 1995.
Crocusatin K (65)	*Crocus sativus*	-	0.26	Li, Lee and Wu, 2004

[a] Monophenolase activity (Tyrosine was used as a substrate).

[b] Diaphenolase activity (L-Dopa was used as a substrate).

2.1.3 Fungal Metabolites

Besides the higher plants, some compounds from fungal metabolites have been reported for their tyrosinase inhibitory activities. Kojic acid (2) is produced by many species of *Aspergillus* and *Penicillium* (Chen, Wei and Marshall, 1991). It is a good chelator of transition metal ions and a good scavenger of free radicals. Agaritine (66) showed depigmentaing effect to prevent melanin formation. The inhibitions were uncompetitive for diaphenolase activity and partially competitive for monophenolase activity. Since agaritine is very abundant in *Agaricus bisporus* mushroom, suggested that agaritine could be an endogenous regulator of mushroom tyrosinase activity (Espin, Jolivert and Wichers, 1998). Metallothioneins (67), the low-molecular weight cystein-rich heavy-metal-binding proteins, have strong avidity to chelate copper at active site of the enzyme tyrosinase (Goetghebur and Kermasha, 1996). Indole-3-carbaldehyde (68) showed inhibitory activity on mushroom tyrosinase and on melanin formation in B16 melanoma cell (Shimizu *et al.*, 2003).

Table 4. Tyrosinase inhibitors from fungal metabolites.

Compounds	Source	Type of inhibitor	IC_{50} (mM)	Reference
Kojic acid (2)	*Aspergillus* spp., *Penicillium* spp.	Mixed-ytpe[b]	0.014	Chen *et al.*, 1991.
Agaritine (66)	*Agaricus bisporus*	Competitive[a] Uncompetitive[b]	-	Espin *et al.*, 1998.

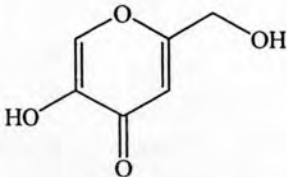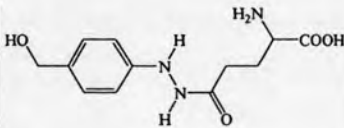| Compounds	Source	Type of inhibitor	IC$_{50}$ (mM)	Reference
Metallothioneins (67)	*Aspergillus niger*	mixed-type	0.22	Goetghebur and Kermasha, 1996.
Indole-3-carbaldehyde (68)	Fungus YL185	-	1.3	Shimizu *et al.*, 2003.

[a] Monophenolase activity (Tyrosine was used as a substrate).

[b] Diaphenolase activity (L-Dopa was used as a substrate).

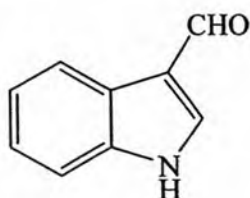## 2.2 Tyrosinase Inhibitors from Synthetic Sources

A number of drugs and chemicals such as captopril (69), methimazole (70), cupferron (71) and tropolone (72), have been reported as tyrosinase inhibitors. The antihypertensive drug, captopril (69), showed noncompetitive inhibition on monophenolase and competitive inhibition on diaphenolase activities of mushroom tyrosinase. Captopril (69) is also known as a copper chelator. Therefore, captopril exerted its inhibitory effect by chelating copper ion at the active site of the enzyme. In addition, disulfide interchange reactions between captopril and cystein rich domains at the active center of tyrosinase may be involved the inhibition process (Espin and Wichers, 2001).

Methimazole (70), an antithyroid drug, also inhibited both monophenolase and diaphenolase activities of tyrosinase as a mixed-type inhibitor. Its inhibition process

were conjugating with o-quinones and chelating copper ion at the active site of tyrosinase enzyme (Andrawis and Kanh, 1986).

Cupferron (71) has been recognized as a competitive inhibitor for mushroom tyrosinase with the IC_{50} value of 0.52 µM for monophenolase and 0.84 µM for diaphenolase activities (Xie *et al.*, 2003). Another effective copper chelator, tropolone (72) exhibited slow-binding inhibition on tyrosinase and could only bind the oxy form of the enzyme (Espin and Wichers, 1999).

Shiino *et al.* (2001, 2003) synthesized a series of N-substituted N-nitroso-hydroxylamines and studied for their tyrosinase inhibitions. It was found that the potency of these compounds was affected by the N-substituent groups. The length of alkyl chain did not affect the activities of the N-n-alkyl derivatives, while inhibitory activities of the compounds containg phenyl substituent at the α–carbon of the N-nitroso-N-hydroxylamine dramatically decreased with an increase in the length of the alkyl chain. These may be suggested that the phenyl or alkyl substituent at α–carbon of the N-nitroso-N-hydroxylamino group may cause steric hindrance for the approach of the inhibitors to the active site of the enzyme. Moreover, the substituents on the benzene ring of N-hydroxybenzyl derivatives slightly decreased the inhibitory activity, regardless of nature of substituent or its position, confirming that the inhibitory activity was caused by the chelation of the copper ion in the active site by the N-nitrosohydroxylamino group. Thus, both N-nitroso and N-hydroxyl groups are essential for inhibitory activity. Among these amines, N-cyclopentyl-N-nitrosohydroxylamine (73) was the most potent tyrosinase inhibitor with the IC_{50} value of 0.6 µM.

The 4-substituted resorcinols, which are structurally related to phenolic substrates have been identified as tyrosinase inhibitors (Chen *et al.*, 2004). Hexylresorcinol (74) and dodecylresorcinol (75) showed potent inhibition on mushroom tyrosinase with IC_{50} value of 1.24 and 1.15 µM on monophenolase, and 0.85 and 0.80 µM on diaphenolase activity of the enzyme, respectively.

Recently, a large number of natural-derived compounds have been modified in order to search for the potential tyrosinase inhibitors. A series of amino derivatives of kojic acid have been synthesized and their tyrosinase inhibitory activities have been

investigated (Kobayashi *et al.*, 1995; 1996). *N*-carbobenzoxy-L-phenylalanine derivative (76) showed potent tyrosinase inhibitory activity (IC_{50} = 0.28 µM). Furthermore, the *N*-kojic-L-phenylalanyl kojiate (77) exhibited the most potent inhibition with the IC_{50} value of 0.06 µM.

The tyrosinase inhibitory activity of gallic acid (78) and its alkyl esters were examined by Kubo *et al* (Kubo, Kinst-Hori, Kubo *et al.*, 2000; Kubo *et al.*, 2003). Gallic acid and its short alkyl chain (<C10) esters were oxidized as substrate yielded yellowish products. On the other hand, the long alkyl (>C10) gallates inhibited the enzyme activity without being oxidized, indicating the carbon chain length is related to their tyrosinase inhibitory activities. Dodecyl gallate (79) was a mixed-type inhibitor (IC_{50} = 1.55 mM). The mechanism of activity was proposed that the bulky hydrophobic portions were apparently better embraced by the hydrophobic pocket surrounding the binuclear copper active site.

In order to improve the use of stilbenes as a potent tyrosinase inhibitors, various designs and syntheses of effective natural product-based inhibitors were performed. 2,4,3',5'-Tetrahydroxybibenzyl (80) has been modified from oxyresveratrol (40) and exhibited more potent tyrosinase inhibitory activity than its parent compound, without cytotoxicity (Likhiwitayawuid *et al.*, 2006).

The inhibitory effects of *cis*- and *trans*-isomers (81-82) of 3,5-dihydroxy-stilbene on mushroom tyrosinase activity have been studied (Song *et al.*, 2006). The inhibitory capacity of *cis*-isomer (IC_{50} = 0.405 mM) was stronger than that of corresponding *trans*-isomer ((IC_{50} = 0.705 mM). This result indicated that if one of the two benzene rings in stilbenes has no substituted groups, one end of the compound is hydrophobic and another end is hydrophilic. The *cis*-conformation will aid the hydrophilic end of the inhibitor to interact with the enzyme active site, and hydrophobic end interact with the hydrophobic vicinity of the tyrosinase active site. Thus, the hydrophobic vicinity of the tyrosinase active center was very important for effectors interacting with the enzyme.

A series of hydroxyl substituted phenyl-naphthalenes were synthezied as potent tyrosinase inhibitors (Song *et al.*, 2007). The isostere of oxyresveratrol (40), HS-1713 (83) and the isostere of resveratrol (45), HS-1784 (84), showed IC_{50} value of

0.49 and 16.52 μM, respectively. HS-1784 demonstrated the stronger inhibitory activity than its parent compound which showed IC$_{50}$ value of 55.61 μM. Among these, HS-1793 (**85**) was the most potent tyrosinase inhibitor (IC$_{50}$ = 0.034 μM). As comparison to HS-1784, HS-1793 which possessed 2,4-dihydroxy substituted, exhibited better inhibitory activity on mushroom tyrosinase.
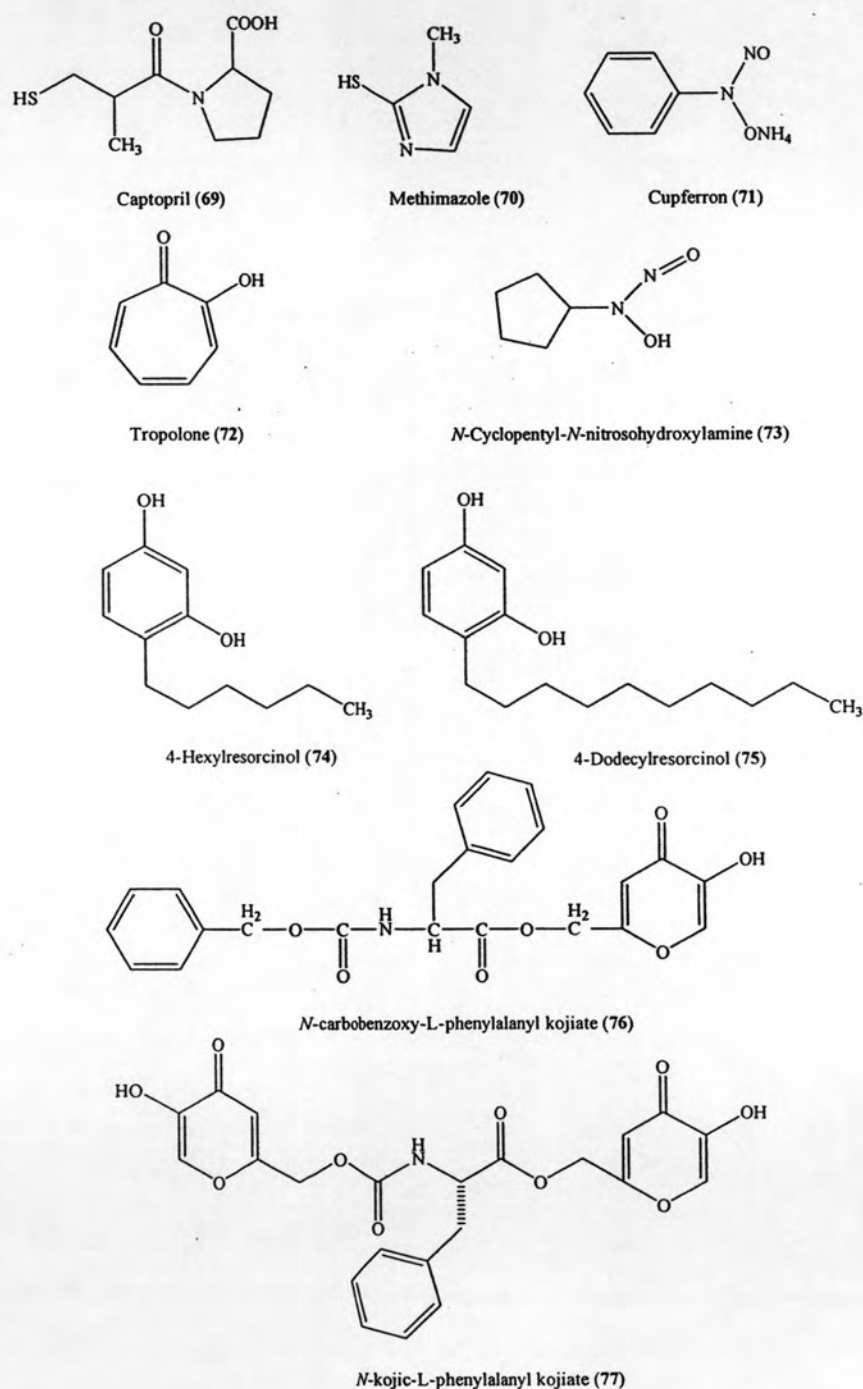
Captopril (69)　　　　　Methimazole (70)　　　　　Cupferron (71)

Tropolone (72)　　　　　　N-Cyclopentyl-N-nitrosohydroxylamine (73)

4-Hexylresorcinol (74)　　　　　4-Dodecylresorcinol (75)

N-carbobenzoxy-L-phenylalanyl kojiate (76)

N-kojic-L-phenylalanyl kojiate (77)

Figure 12. Tyrosinase inhibitors from synthetic sources.

Gallic acid (78)

Dodecyl gallate (79)

2,4,3',5'-tetrahydroxybibenzyl (80)

cis-3,5-dihydroxystilbene (81)

trans-3,5-dihydroxystilbene (82)

HS-1713 (83)

HS-1784 (84)

HS-1793 (85)
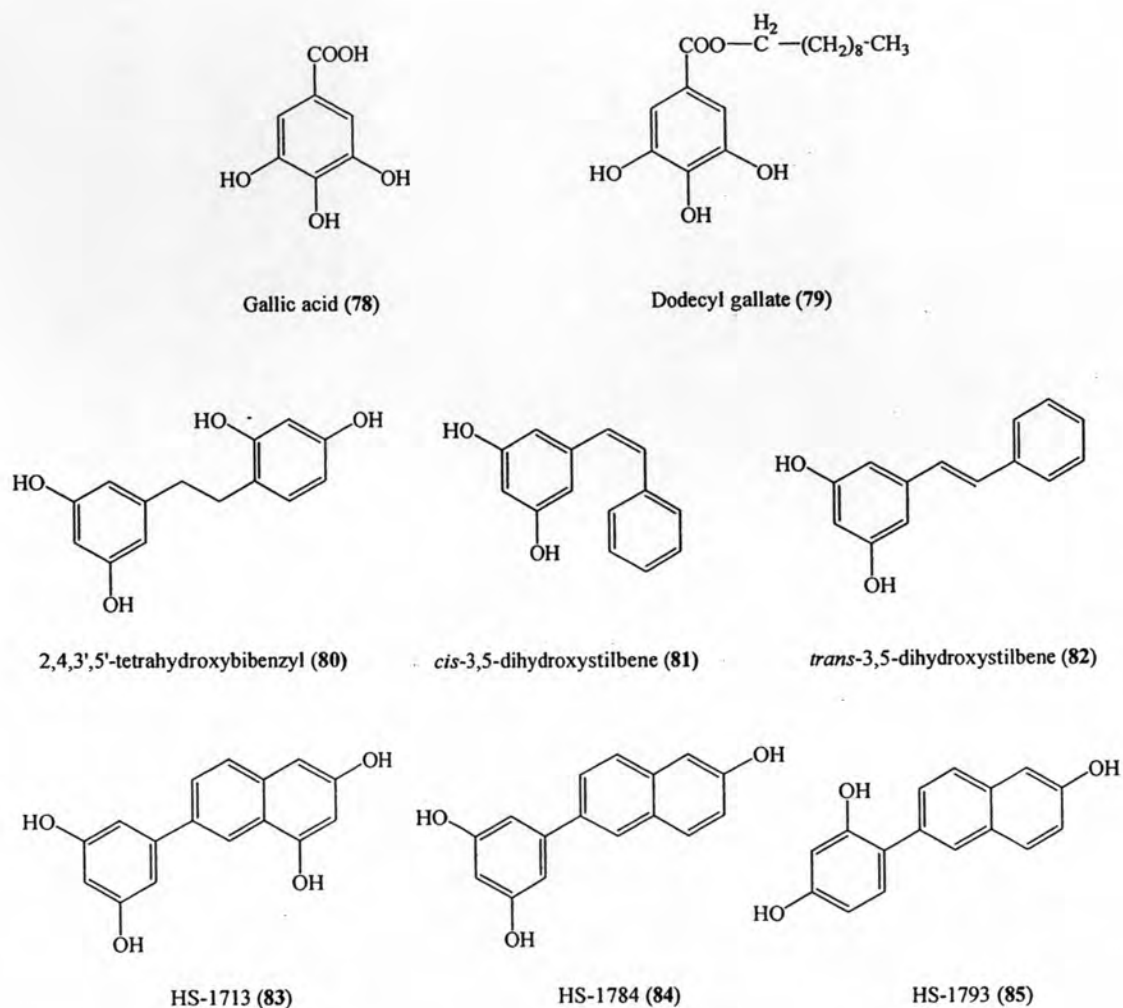
Figure 12. Tyrosinase inhibitors from synthetic sources (continued).

The study of effects of the hydroxyl group at different position of tetra-hydroxychalcone was performed by Khatib et al. (2005). The results indicated that a 2,4-substituted resorcinol subunit on ring B contributed the most to inhibitory potency. Changing this substitute to 3,5-position or placing it on ring A significantly diminished the inhibitory activity of compounds. The catechol subunit on ring A chelated the copper ions at the active site of the enzyme as a competitive inhibitor, while the catechol subunit on ring B was oxidized to o-quinone. Besides, the requirement was the resorcinol construction on ring B must be at position 2 and 4 rather than at position 3 and 5. The two chalcone derivatives, 2,4,3',4'-terahydroxychalcone (86) and 2,4,2',4'-terahydroxychalcone (87), were reported as

potent tyrosinase inhibitors with IC_{50} of 0.2 and 0.02 μM. Further study of structure related activity of chalcones have been investigated by Jun *et al.* (2007). Their results confirmed that the 2,4-hydroxylated resorcinol structure on ring B of chalcones were effective for tytrosinase inhibition. Moreover, 2',4',6'-trihydroxy structure enhanced potential tyrosinase inhibitory activity. The 2,4,2',4',6'-Pentahydroxychalcone (**88**) showed the highest tyrosinase activity with IC_{50} value of 1 μM.
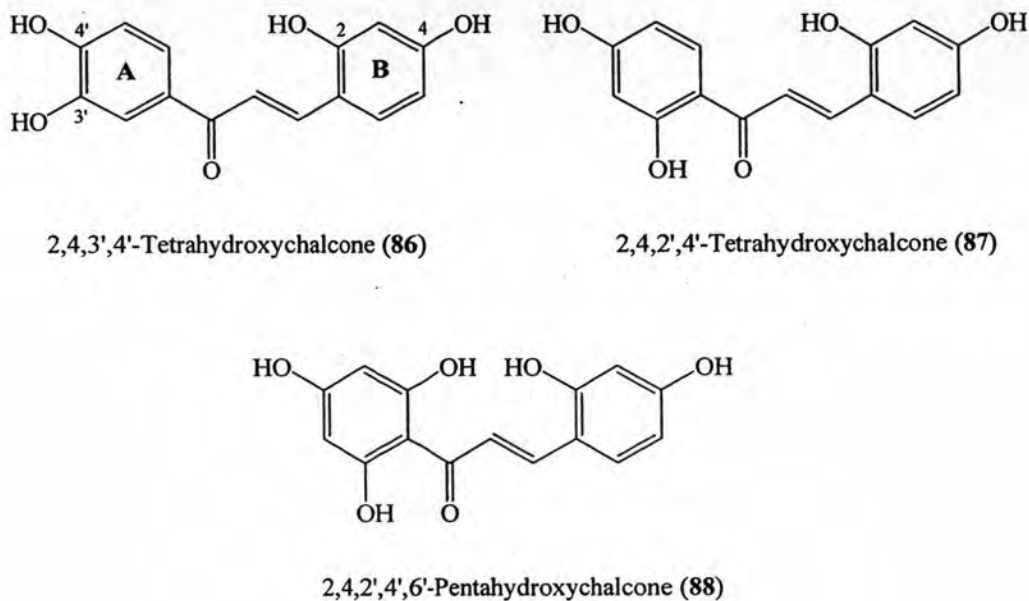

2,4,3',4'-Tetrahydroxychalcone (**86**)   2,4,2',4'-Tetrahydroxychalcone (**87**)

2,4,2',4',6'-Pentahydroxychalcone (**88**)

Figure 13. The inhibitory effects of hydroxylated chalcones and their tyrosinase inhibitory activities on mushroom tyrosinase.

3. Application of tyrosinase inhibitors

3.1 Medicinal and Cosmetic Industries.

Since, tyrosinase plays a crucial role in melanin synthesis, the utility of tyrosinase inhibitors is becoming increasingly important in medicinal and cosmetic industries due to their preventive effect on hyperpigmentation disorders. Tyrosinase inhibitors can reduce the melanin formation, thus it is used as skin-whitening agents in many cosmetic products. Tyrosinase and its inhibitors may also be targets for

developing medicine to treat hypopigmentation related problem such as albinism and piebaldism (Kim and Uyama, 2005). A number of tyrosinase inhibitors, both from natural and synthetic sources, have been reported, but only a few of them are used as skin-whitening agent due to various safety concerns.

Kojic acid is a well-known skin-whitening agent currently in use, but also exhibits side effects. Arbutin and aloesin are tyrosinase inhibitors and are used as skin-whitening agents. The co-treatment of aloesin and arbutin inhibited tyrosinase activity in a synergistic manner by acting through different mechanism: aloesin inhibited noncompetitively, while arbutin inhibited competitively. This indicated the beneficial of using aloesin and arbutin as a mixture for depigmentating effect, since the co-treatment reduce the effective dose of these agents for the same inhibitory effect as well as reduce adverse side effects (Kim and Uyama, 2005). Presently, various plant extracts such as licorice extract and mulberry extract, are used as skin-whitening agents in many cosmetic products.

3.2 Agricultural and Food Fields.

Tyrosinase is responsible for the enzyme browning in fruits and vegetables. The current conventional techniques to avoid browning include autoclave and blanching methods to inactivate tyrosinase activity, but these processes can cause important weight and nutrition loss in plant-derived products. Another alterative method, microwave energy will generate gradient temperature within the samples during microwave heating. Hence, an inhibitor of tyrosinase should be useful in Food industry. The number of chemicals that can actually be used in food products is limited due to off-flavors, off-odors, toxicity and economic feasibility (Kim and Uyama, 2005). Sulfiting agent are widely used to prevent enzymatic browning in plant and seafood products, but the use of sulfites is becoming more restricted due to potential health hazards. While ascorbic and citric acid are less effective than sulfites. Presently the use of 4-hexylresorcinol is considered to be safe in the food industry. It is quite effective in prevention of shrimp melanosis and for browning control in fresh and dried fruits. However, as safe is of prime concern for an inhibitor to be used in food industry, there is a constant search for better inhibitors from natural sources as

they are largely free of any harmful side effects (Kim and Uyama, 2005; Seo *et al.*, 2003). Furthermore, tyrosinase is one of the most important key enzymes in insect molting process, tyrosinase inhibitors have become an active alterative approach to control insect pests.