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Glabrene and Isoliquiritigenin as Tyrosinase Inhibitors from Licorice Roots

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Tyrosinase is known to be a key enzyme in melanin biosynthesis, involved in determining the color of mammalian skin and hair. Various dermatological disorders, such as melasama, age spots, and sites of actinic damage, arise from the accumulation of an excessive level of epidermal pigmentation. The inadequacy of current therapies to treat these conditions as well as high cytotoxicity and mutagenicity, poor skin penetration, and low stability of formulations led us to seek new whitening agents to meet the medical requirements for depigmenting agents. The inhibitory effect of licorice extract on tyrosinase activity was higher than that expected from the level of glabridin in the extract. This led us to test for other components that may contribute to this strong inhibitory activity. Results indicated that glabrene and isoliquiritigenin (2′,4′,4-trihydroxychalcone) in the licorice extract can inhibit both mono- and diphenolase tyrosinase activities. The IC_{50} values for glabrene and isoliquiritigenin were 3.5 and 8.1 μM, respectively, when tyrosine was used as substrate. The effects of glabrene and isoliquiritigenin on tyrosinase activity were dose-dependent and correlated to their ability to inhibit melanin formation in melanocytes. This is the first study indicating that glabrene and isoliquiritigenin exert varying degrees of inhibition on tyrosinase-dependent melanin biosynthesis, suggesting that isoflavenes and chalcones may serve as candidates for skin-lightening agents.

KEYWORDS: Tyrosinase; whitening agents; isoflavans; glabrene; chalcones

INTRODUCTION

Melanin is the pigment responsible for the color of human skin and hair. It is secreted by melanocyte cells distributed in the basal layer of the dermis (*1*). Its role is to protect the skin from ultraviolet (UV) damage by absorbing the ultraviolet sunlight and removing reactive oxygen species (ROS). Various dermatological disorders result in the accumulation of an excessive level of epidermal pigmentation. These hyperpigmented lentigenes include melasama, age spots, and sites of actinic damage (*2*). The type and amount of melanin synthesized by the melanocyte and its distribution in the surrounding keratinocytes determine the actual color of the skin. Melanin is formed through a series of oxidative reactions involving the amino acid tyrosine in the presence of the enzyme tyrosinase. Tyrosinase (monophenol monooxygenase, EC 1.14.18.1), also known as polyphenol oxidase (PPO) (*3*), is a copper-containing monooxygenase that is widely distributed in nature. The enzyme catalyzes two distinct reactions involving molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity). Quinones are highly reactive compounds and can polymerize spontaneously to form high-molecular-weight compounds or brown pigments (melanins) or react with amino acids and proteins that enhance the brown color produced (*4*). Melanin biosynthesis can be inhibited by avoiding UV exposure, by the inhibition of tyrosinase, by the inhibition of melanocyte metabolism and proliferation (*5*, *6*), or by the removal of melanin by corneal ablation.

Standard topical treatments for hyperpigmentation disorders, such as melasma and postinflammatory hyperpigmentation, include bleaching hydroquinones, retinoids, and tyrosinase inhibitors. Among these skin-lightening agents, 1,4-dihydroquinone is one of the most widely prescribed (*2*, *7*, *8*). It causes reversible inhibition of cellular metabolism by affecting both DNA and RNA synthesis. It is also a poor substrate for tyrosinase, thereby competing for tyrosine oxidation in active melanocytes. Hence, 1,4-dihydroquinone can be considered to be a potent melanocyte cytotoxic agent and has also been reported to induce mutations. As a result of these and other side effects, such as chronosis in African nations (*2*), there has been increasing impetus to find alternative herbal and pharmaceutical depigmenting agents.

The licorice root (*Glycyrrhiza glabra L.)* has long been employed in Western countries as a flavoring and sweetening

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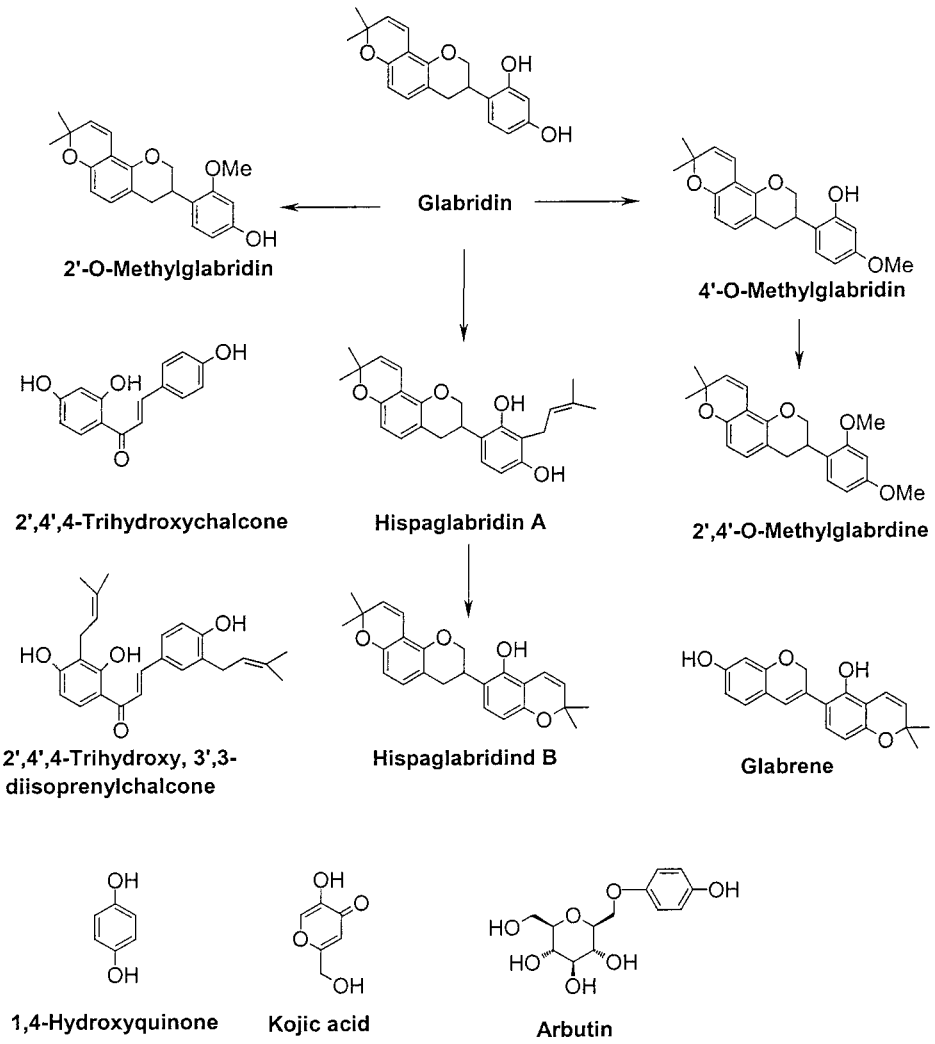

Figure 1. Molecular structure of compounds tested.

agent, as well as a demulcent and expectorant. Minor components of licorice demonstrated significant antimicrobial activity in vitro (*9*, *10*) and antioxidant activity (*11*, *12*). We reported recently that the major isoflavan, glabridin, exhibited varying degrees of estrogen receptor (ER) agonism in different estrogen-responsive tissues (*13*). Glabridin has also been reported to inhibit the tyrosinase activity of melanocytes. Yokota et al. (*14*) further showed that UV-B-induced pigmentation and erythema were inhibited by topical application of 0.5% glabridin (*14*). The main drawbacks of glabridin are its poor skin-penetrating ability and its instability in formulations.

Glabrene, an isoflavene isolated from licorice root in our laboratory (*12*), has two hydroxyl groups at the 2′ and 7 positions, a 2,2-dimethyl-γ-pyran ring fused to the B ring, and a double bond between carbon atoms 3 and 4 in the C ring, which confers maximal conjugation of the double bonds on the molecule (**Figure 1**). This conjugation increases the stability of the phenoxyl radical which is formed after the donation of an electron, resulting in increased antioxidative activity (*12*, *15*). Recently, Tamir et al. reported that glabrene is an isoflavene with phytoestrogen characteristics (*16*).

Hispaglabridin A and hispaglabridin B (**Figure 1**), two additional derivatives isolated from licorice root, were also tested for their ability to inhibit tyrosinase activity. The structures of these two isoflavans are similar to that of glabridin, except that hispaglabridin A has an isoprenyl side chain attached to the 3′ position, and hispaglabridin B has one hydroxyl group, both features increasing their lipophilicity in comparison to that of glabridin. Isoprenylchalcone and isoliquiritigenin (2′,4′,4-trihydroxychalcone) are two chalcones isolated from licorice extract (*12*). Isoliquiritigenin, the more hydrophilic of the two, was found to be more active as an antioxidant and as a phytoestrogen (*16*).

In this study, we investigated natural compounds found in licorice root—glabrene, an isoflavene, and isoliquiritigenin, a chalcone—for their tyrosinase inhibitory activity. The relationship between the structure of the new and the previously known tyrosinase inhibitors and their activity contributes to the design of an optimal inhibitor.

MATERIAL AND METHODS

Chemicals and Reagents. Tyrosinase (EC 1.14.18.1, Sigma Product T7755, with an activity of 6680 units/mg) was purchased from Sigma. Fetal calf serum (FCS), trypsin EDTA, L-glutamine, Hepes buffer, penicillin/streptomycin, sodium pyruvate solutions, and XTT Reagent Cell Proliferation Kit were all purchased from Biological Industries (Beth Haemek, Israel). Glabrene, hispaglabridin A, hispaglabridin B, and chalcones were isolated from the acetone extract of the roots of *Glycyrrhiza glabra* (from the northeast of Turkey) (*15*).

Tyrosinase Assay. Potassium phosphate buffer (0.07 mL, 50 mM) at pH 6.5, 0.03 mL of tyrosinase (333 units/mL), and 2 μL of the tested compounds (glabridin, 0.03−3.5 μM; glabrene, 0.07−50 μM; and isoliquiritigenin, 2−500 μM) dissolved in absolute ethanol were inserted into 96-well plates. After 5 min of incubation at room temperature,

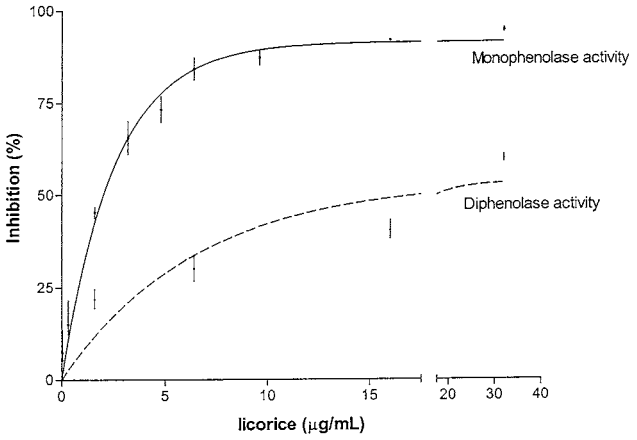

Figure 2. Effect of licorice extract on the monophenolase (■) and diphenolase (▲) activity of mushroom tyrosinase. Inhibition of L-tyrosine and L-DOPA oxidation to dopachrome by licorice extract was measured at 492 nm. Ethanol was used as a control. Data presented as percent inhibition ± SE.

0.1 mL of L-tyrosine (2 mM) or 12 mM L-DOPA was added. Optical density at 492 nm was measured (Elisa SLT Labinstruments Co. A-5082).

Cell Culture. Human melanocyte (G361) was purchased from the American Type Culture Collection. The cells were grown in DMEM (Dulbecco's Modified Eagle's Medium), supplemented with 2 μg/mL insulin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 4 mM glutamine, 10% FCS, and antibiotics (penicillin/streptomycin). All tested compounds were prepared in DMSO. After 3 days in culture, the cells were collected by trypsinization, counted by means of tryptan blue, and analyzed for melanin content.

Determination of Melanin Content in Melanocyte. Melanin content was determined according to Curto et al. (*18*). After being washed with PBS, the cells were lysed with 1 mL of 1 N NaOH, and 200 μL portions of crude cell extract were transferred to 96-well plates. Melanin content was determined at 405 nm.

RESULTS

Inhibition of Tyrosinase Activity by Licorice Extract. The use of L-tyrosine and L-DOPA as substrates enabled us to distinguish between the ability of the extract to inhibit the *o*-hydroxylation of tyrosine and its further oxidation to *o*-diquinone. The inhibitory activity was monitored by spectrophotometric measurement of dopachrome formation at 492 nm. The dry licorice extract was dissolved in ethanol and added to the reaction solution before the tyrosinase was added; ethanol was used as control. The effects of increasing concentrations of licorice extract on the monophenolase and diphenolase activated forms of tyrosinase are shown in **Figure 2**. The extract inhibited the oxidation of tyrosine by the enzyme to an IC_{50} value of 0.9 μg/mL. The maximum inhibitory activity was higher than that expected from its glabridin content (approximately 10% w/w in the extract). The extract also inhibited the oxidation of L-DOPA by the enzyme to an IC_{50} value of 53 μg/mL. But the inhibitory effect on the first step of oxidation was greater than that on the oxidation of L-DOPA.

Inhibition of Tyrosinase Activity by Glabridin, Isoliquiritigenin, and Glabrene. Glabridin, isoliquiritigenin, and glabrene were the most potent inhibitors of monophenolase activity, with IC_{50} values of 0.09, 3.5, and 8.1 μM, respectively. The IC_{50} values for diphenolase activity were 15−1000 times higher (the IC_{50} values of glabridin, isoliquiritigenin, and glabrene were 3.94, 47, and 7600 μM, respectively). All effects were dose-dependent (data not shown). The IC_{50} values for glabrene and isoliquiritigenin were found to be higher than that of glabridin but much lower than those of other known tyrosinase inhibitors, such as kojic acid, for which 0.14 mM caused 64% inhibition of monophenolase activity and 72% inhibition of the diphenolase activity (*17*), 1,4-hydroquinone with an IC_{50} value of 654 mM (*18*), and resveratrol with an IC_{50} value of 155 μM on the diphenolase activity (*19*). Hispaglabridin A and hispaglabridin B did not inhibit tyrosinase activity. Although isoliquiritigenin was active, isoprenylchalcone did not inhibit tyrosinase activity in these assays, which suggests that the addition of two isoprenyl groups on rings A and B resulted in the elimination of inhibitory activity, possibly due to increased lipophilicity or steric hindrance. Two derivatives of glabridin, 2′- and 4′-OMe glabridin, did not inhibit tyrosinase activity, which suggests the importance of both hydroxyl groups to the activity.

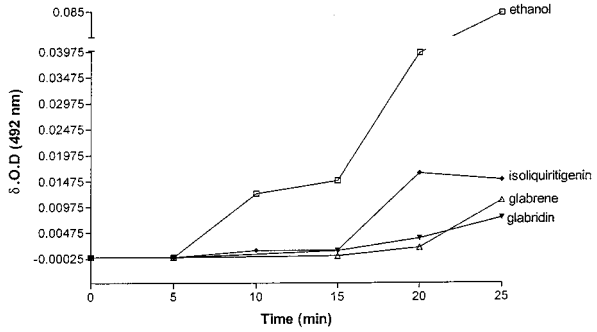

Figure 3. Effect of licorice constituents on the monophenolase activity of tyrosinase. Enzyme activity was tested in the presence of L-tyrosine, as substrate, and the inhibitors: glabridin, 0.7 μM (▼); glabrene, 7 μM (△); and isoliquiritigenin, 26 μM (●). Ethanol was used as a control (□).

Kinetic Parameters of the Effects of Glabridin, Glabrene, and Isoliquiritigenin on the Mono- and Diphenolase Activities of Tyrosinase. Glabridin has been identified as a potent tyrosinase inhibitor in mushrooms by Yokota et al (*14*). These authors determined that glabridin inhibited the tyrosinase activity in B16 melanoma cells at concentrations of 0.1−1.0 μg/mL, using L-tyrosine as substrate. No kinetic study of this inhibitory effect has been carried out prior to the current study. To study tyrosinase inhibition by the isolated licorice constituents, as compared to glabridin, we tested its effect on the mono- and diphenolase activities of the enzyme by determining the kinetic parameters (**Figures 3** and **4**).

When the enzymatic reaction was started by the action of tyrosinase on L-tyrosine, a marked lag period, characteristic of monophenolase activity (*20*), was observed, simultaneously with the appearance of the first stable product, dopachrome (**Figure 3**). All the compounds tested behaved as inhibitors of the monophenolase activity of tyrosinase. The lag period depended on both enzyme and substrate concentrations in the reaction medium and can be shortened, or even abolished, by the presence of catalytic amounts of transition metal ions or *o*-diphenols (*21*). However, the lag phase is known to be extended by some monophenolase inhibitors, such as tropolone (*22*) or cumic acid (*23*). Glabrene extended the lag phase by 15 min compared to the control, especially when its concentration was higher than 7 μM. Isoliquiritigenin did not prolong the lag phase. When the diphenolase activity of mushroom tyrosinase was assayed, using L-DOPA as substrate, the reaction reached a steady-state rate after a few seconds, and in the presence of glabridin, glabrene, and isoliquiritigenin, the diphenolase activity decreased (data not shown).

To obtain further information about the type of inhibition exerted by glabridin, glabrene, and isoliquiritigenin on mush-

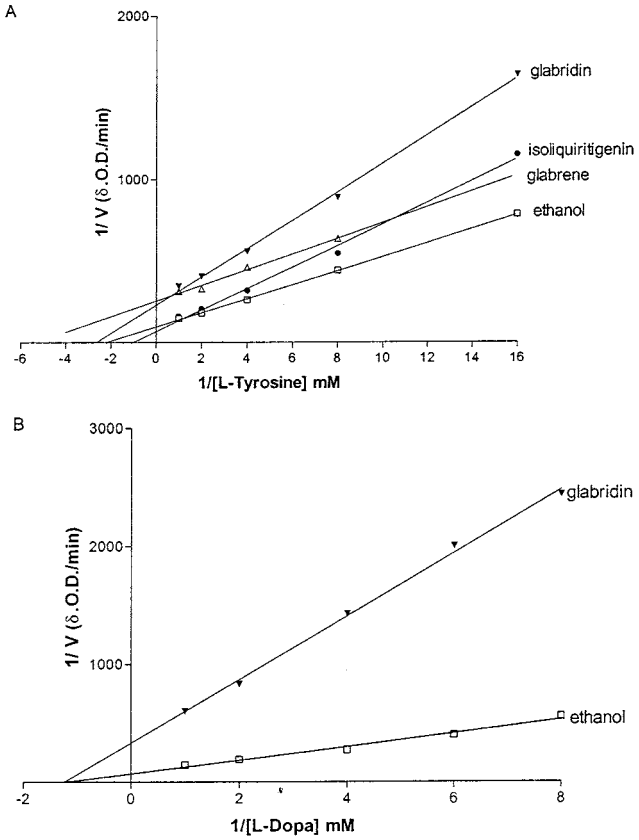

Figure 4. Lineweaver–Burk plots of mushroom tyrosinase. (A) With L-tyrosine as a substrate, in the presence of glabridin, 0.1 μM (▼); glabrene, 5 μM (△); isoliquiritigenin, 8 μM (●); and ethanol as control (□). (B) With L-DOPA as a substrate, in the presence of glabridin, 4 mM (▼), and ethanol as a control (□).

room tyrosinase, monophenolase activities were measured as a function of increasing concentration of L-tyrosine. The Lineweaver−Burk plot obtained (**Figure 4A**) shows that glabridin is also a noncompetitive inhibitor of the monophenolase activity of tyrosinase, while glabrene and isoliquiritigenin exhibited mixed inhibition. The K_i values obtained for this compounds were 0.38, 0.18, and 0.99 mM, respectively.

To ascertain whether these inhibitors behave in the same manner with regard to the diphenolase activity of mushroom tyrosinase, the rate of dopachrome accumulation was measured as a function of L-DOPA concentration. The Lineweaver−Burk plot (**Figure 4B**) shows noncompetitive inhibition for glabridin, with $K_i = 0.81$ mM.

The Effect of Licorice Components on Melanin Biosynthesis in Human Melanocytes. A general strategy was described by Dooley for the discovery and development of novel topical skin-lightening products (*7*). Desirable skin-lightening agents should inhibit the synthesis of melanin in melanosomes by acting specifically to reduce the synthesis or activity of tyrosinase, exhibit low cytotoxicity, and be nonmutagenic. **Table 1** presents the assessment of the licorice constituents in melanocyte cultures. The IC_{50} values of inhibition of cultured G361 human melanocyte pigmentation due to melanin synthesis were compared to the cytotoxicity effects of the compounds. Glabridin was a very effective agent, with an IC_{50} of about 2.4 μg/mL. Isoliquiritigenin and glabrene, which also inhibited tyrosinase activity, inhibited melanin formation with IC_{50} values of 4.73 and 6.68 μg/mL, respectively. All licorice constituents tested were less toxic than 1,4-hydroquinone (*18*).

Table 1. Effects of Glabridin, Glabrene, and Isoliquiritigenin on Melanin Synthesis in G361 Human Melanocytes

tested compound (concn)	% melanin	% survival
glabridin (10 μM)	45.15 ± 16.1[a]	92.2 ± 19.5
glabrene (10 μM)	73.6 ± 5.7[a]	71.9 ± 4.04
isoliquiritigenin (10 μM)	87.9 ± 6.8	110.2 ± 7.9
isoliquiritigenin (20 μM)	63.3 ± 4.8[a]	92.06 ± 2.6

[a] $P < 0.005$.

According to Curto et al. (*18*), a potentially efficacious skin depigmentation agent is one that inhibits tyrosinase with an $IC_{50} < 25$ μg/mL, inhibits melanocyte cell pigmentation with an $IC_{50} < 100$ μg/mL, and is noncytotoxic to cells with an $IC_{50} > 100$ μg/mL. Of the compounds tested in the present study, glabridin, glabrene, and isoliquiritigenin satisfied these requirements.

Structure−Activity Relationship Studies. The effect of modifications to the structure of glabridin on the degree of inhibition of tyrosinase was studied, using natural and semisynthetic glabridin derivatives. The structure of 4′-*O*-methylglabridin isolated from licorice root (*12*) resembles that of glabridin, with one hydroxyl at position 4′ blocked with a methyl group, leaving the second hydroxyl group at position 2′ free. Both 2′-*O*-methylglabridin and 2′,4′-*O*-methylglabridin are semisynthetic products, synthesized (*15*) from glabridin, the first with the hydroxyl at position 2′ blocked and that at position 4′ free, and the other with both hydroxyl groups blocked. Using these derivatives, the influence of the hydroxyl groups of glabridin on tyrosinase was examined. 2′-*O*-Methylglabridin and 4′-*O*-methylglabridin were found to be noninhibitory, indicating that both hydroxyl groups contribute to inhibition capacity, and when both were blocked, the inhibitory activity significantly diminished. Both chalcones were tested, and only isoliquiritigenin was found to inhibit tyrosinase. The main difference between the two chalcones is the absence of the two prenyl groups present in isoliquiritigenin.

Hispaglabridin A and hispaglabridin B, two additional derivatives isolated from licorice root, were found to have no tyrosinase inhibitory activity. The general structures of these two isoflavans are similar to that of glabridin, except that hispaglabridin A has an isoprenyl side chain attached to the 3′ position (**Figure 1**) and hispaglabridin B has only one hydroxyl group at position 2′, both of which increase the lipophilic nature of the isoflavans in comparison to that of glabridin but significantly diminish the compound's activity as a tyrosinase inhibitor.

DISCUSSION

Licorice extract exhibited a greater inhibitory effect on tyrosinase activity than was expected on the basis of its content of glabridin, a known tyrosinase inhibitor (*14*). This finding led us to examine other constituents present in the extract, including glabrene and isoliquiritigenin. By using different assay methods, we have demonstrated that glabrene and isoliquiritigenin can inhibit the mono- and diphenolase activities of mushroom tyrosinase. Furthermore, glabrene and isoliquiritigenin inhibited the biosynthesis of melanin in melanocytes. In addition, preincubation of the enzyme with these inhibitors in the absence of the substrate did not diminish enzyme activity significantly, suggesting that these compounds are inhibitors rather than inactivators of the enzyme (*22*). Kinetic analysis of both new inhibitors, glabrene and isoliquiritigenin, suggests that

they are mixed inhibitors, whereas glabridin acts as a noncompetitive inhibitor.

A review of the literature reveals that numerous depigmenting or skin-lightening agents are in use or under investigation. Some of these, such as kojic acid (*24*, *25*) and azelaic acid (*26*), are well known to most dermatologists. Others have been discovered and reported in the literature more recently (*18*, *27*−*31*). Azelaic acid is a naturally occurring, saturated dicarboxylic acid, originally isolated from *Pityrosporum ovale*, which acts as a rather weak competitive inhibitor of tyrosinase in vitro. Azelaic acid also has a cytotoxic effect on melanocytes (*26*). Kojic acid (5-hydroxy-2-methylpyran-4-one) is a fungal metabolic product, which inhibits the catecholase and monophenolase activities of tyrosinase. Melanocytes treated with kojic acid become nondendritic, with a decreased melanin content. Additionally, it scavenges radical oxygen species. It is used in concentrations ranging from 1 to 4% (*32*). Although effective as a skin-lightening gel, it has been reported to have a high sensitizing potential and hence to cause irritant contact dermatitis (*24*, *25*). Like hydroquinone, 4-hydroxyanisole is cytotoxic to melanocytes, and when used alone it did not produce significant hypopigmentation. Arbutin (hydroquinone-β-D-glucopyranoside) is a glycosylated hydroquinone found at high concentrations in certain plants. It has also been shown to inhibit melanin synthesis by inhibiting tyrosinase activity. Inhibition of melanin synthesis (about 39%) occurs at a concentration of 5×10^{-5} M. Although the effective topical concentration in treating disorders of hyperpigmentation has not been formally evaluated and published, several manufacturers are marketing arbutin as an effective depigmenting agent, at a concentration of 1% (*28*). Several other natural compounds (*19*, *33*), such as quercetin, myricetin, and a glycoside of myricetin, have been reported to have varying degrees of inhibitory activity toward tyrosinase (*27*). Structure-related activity studies of flavonoids, stilbenes, and related 4-substituted resorcinols, obtained from *Artocarpus incisus* and other plants, suggest that compounds with the 4-substituted resorcinol skeleton have a potent tyrosinase inhibitory ability (*29*). However, the effective topical concentration of these compounds in disorders of hyperpigmentation is not yet known.

Glabridin, an ingredient of licorice extract, was first reported to inhibit the tyrosinase activity of melanocytes by Yokota et al. (*14*), who further showed that UV-B-induced pigmentation and erythema were inhibited by topical application of 0.5% glabridin. The main commercial problems of glabridin are its poor skin-penetrating ability and its instability in formulations. The lack of an efficient whitening agent which does not suffer from low activity (kojic acid), high cytotoxicity and mutagenicity (hydroquinone), poor skin penetration (arbutin), or low stability in formulation (glabridin) encourages a continuation of research for new skin-lightening agents to meet medical needs.

Among the licorice constituents we have studied, glabridin exhibited stronger activity than glabrene or isoliquiritigenin. All the licorice inhibitors tested were more effective on the monophenolase than on the diphenolase activity, which underlines their role in the first step of oxidation, the rate-limiting reaction. The inhibition kinetics indicate that glabridin exhibited a noncompetitive inhibition for L-DOPA oxidation ($K_i = 0.81$ mM, $IC_{50} = 3.9$ μM) and L-tyrosine oxidation ($K_i = 0.38$ mM, $IC_{50} = 0.09$ μM) by mushroom tyrosinase (**Figure 4**), and the IC_{50} values were lower than those of other known natural inhibitors. A noncompetitive inhibitory mechanism was also reported for flavonone derivatives, isolated from *Sophora flavescens* (*34*) ($K_i = 12$ μM, $IC_{50} = 4.6$ μg/mL), for 3,4-dihydroxycinnamic acid and for 4-hydroxy-3-methoxycinnamic acid, isolated from *Pulsatilla cernua* roots (*35*) ($IC_{50} =$ 0.97 and 0.33 mM, respectively), for oxyresveratrol ($IC_{50} =$ 52.7 μM, $K_i = 0.32-0.42$ μM) (*36*) and others (*35*−*37*). These data suggest that one of the structural requirements for noncompetitive inhibitors is the presence of a resorcinol moiety. Glabrene ($K_i = 0.18$ mM, $IC_{50} = 3.54$ μM) and isoliquiritigenin ($K_i = 0.99$ mM, $IC_{50} = 8.1$ μM), on the other hand, exhibited competitive inhibition with IC_{50} values in the range of other known natural inhibitors, such as 4-substituted benzaldehydes, which were reported to behave as classical competitive inhibitors ($K_i = 0.04-1.5$ mM, $IC_{50} = 0.05$ mM) by Jimenez et al. (*38*), benzoic acid (*35*) ($IC_{50} = 0.71$ mM), 4-hydroxybenzyl benzoate ($K_i = 40$ μM, $IC_{50} = 0.1$ mM), and kojic acid, which are reported to bind to the dicopper(II) complex in the first step and to the dicopper−dioxygen adduct in the second step, preventing the binding of the substrate (*39*). Kaempferol and quercetin, members of the flavonol group, were also found to be competitive inhibitors ($IC_{50} = 0.23$ and 0.07 mM, respectively). Kubo and Kinst-Hori (*31*) suggest that their inhibitory activity comes from their ability to chelate copper in the enzyme. This copper chelation mechanism can be applicable to all of the flavonols, as long as their 3-hydroxyl group is free. Catechin, 3-*O*-gallate, was also reported (*40*) to inhibit tyrosinase competitively ($K_i = 0.29$ mM, $IC_{50} = 17.34$ μM) when L-tyrosine was used as substrate. Structural analysis of these known competitive inhibitors and the newly found inhibitors from licorice root reveals that the presence of a resorcinol subunit is not essential for competitive inhibitors but plays a major role in noncompetitive inhibition of tyrosinase. In addition, most of the competitive inhibitors have the ability to chelate copper in the enzyme, implying a possible inhibitory mechanism. No activity was observed for hispaglabridin A or B, or for the glabridin derivatives, 2′-*O*-methylglabridin and 4′-*O*-methylglabridin, which were used to test the influence of the hydroxyl groups of glabridin on tyrosinase activities. It should be noted that further studies are required to understand the effect of different functional group substitutions and the mode of inhibition of tyrosinase activity.

The molecular structures of the compounds found to inhibit tyrosinase are shown in **Figure 1**. Among these compounds, glabridin was the most active of the licorice constituents with an isoflavan structure that were studied and characterized by the attachment of ring B of the molecule to position 3. Like glabrene, both such compounds exhibited phytoestrogenic activity, antioxidative properties, and tyrosinase inhibition. The structure of glabrene includes a double bond between carbon atoms 3 and 4, resembling that of *trans*-diphenylstilbenes, a group of compounds which exert a high inhibitory activity of tyrosinase (*29*). However, while glabridin lacks this double bond in ring C, it nonetheless demonstrated tyrosinase inhibitory activity, which may suggest that conjugated double bonds between rings A and B are not essential among the flavonoid class of compounds.

De Vincenzo et al. (*41*) tested the relationship of different chalcone structures to their antiproliferative activity and to their ability to bind to the human estrogen receptor in ovarian cancer cells. These authors concluded that the presence of an α,β double bond and a hydroxyl in position 2′ or 3′, and the absence of a prenyl group, were important to both proliferation and binding activities. In the present study, two chalcones, isoliquiritigenin and isoprenylchalcone, were tested, both including an α,β double bond and a hydroxyl at position 2′, with two

additional hydroxyls at positions 4′ and 4. Of the two chalcones tested as tyrosinase inhibitors, only isoliquiritigenin, which does not contain a prenylchalcone group, inhibited tyrosinase, whereas isoprenylchalcone, which contains two prenyl groups, was totally inactive. The different biological tests employed in this study also demonstrate that the presence of an isoprenyl group in the chalcone molecule resulted in its inactivity, possibly due to steric hindrance.

Safety is a primary consideration for tyrosinase inhibitors, especially for those used in food and cosmetic products, as these may be regularly utilized in unregulated quantities. Glabridin is a natural compound found in licorice roots in amounts of 0.4% (w/w), which is higher than the concentration needed to protect against enzymatic browning (*12*). The present study demonstrated that licorice extract contains constituents with tyrosinase inhibitory activity other than glabridin.

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