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TYROSINASE INHIBITORY FLAVONOIDS FROM *HETEROTHECA INULOIDES* AND THEIR STRUCTURAL FUNCTIONS

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Abstract. By bioassay-guided fractionations, quercetin was isolated as the principal tyrosinase inhibitor from the dried flowers of *Heterotheca inuloides* CASS (Compositae). This flavonol inhibited the oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) by mushroom tyrosinase (EC 1.14.18.1) with an ID_{50} of 22 μg/ml (0.07 mM). Interestingly, quercetin 3-β-glucoside did not inhibit this oxidation up to 1 mg/ml. Kinetic study have found quercetin to be a characteristic competitive inhibitor for the L-DOPA oxidation by the mushroom tyrosinase. Based on the above findings, several related flavonoids were also examined for comparison.

As part of our continuing investigation of naturally occurring alternative insect control agents[1], we have been searching for tyrosinase inhibitors from plants[2] since tyrosinase is one of the key enzymes in the insect molting process[3,4]. Thus, tyrosinase inhibitors might ultimately provide clues to control insect pests. In addition, tyrosinase inhibitors have become increasingly important in cosmetic[5] and medicinal[6] products in prevention of hyperpigmentation[7,8]. In our preliminary screening using the mushroom tyrosinase, the methanol extract of the dried flowers of *H. inuloides*[9], a Mexican medicinal plant locally known as "arnica", was found to show significant inhibitory activity with an ID_{50} of 190 μg/ml.

The methanol extract was suspended in water and extracted with *n*-hexane, ethyl ether and ethyl acetate in this order. The subsequent bioassay[10] found the ethyl acetate fraction to be active. The bioassay-guided fractionations of the ethyl acetate portion by various chromatographic methods using mushroom tyrosinase led to the isolation of the principal inhibitor which was characterized by spectroscopic method as the common flavonol, quercetin (**1**). Quercetin has previously been isolated from many plants, including the dried flowers of *H. inuloides* themselves[11]. Based on the characterization of quercetin as the principal active compound, four additional flavonols, quercetin 3-β-glucoside (**2**) otherwise known as isoquercitrin, kaempferol (**3**), kaempferol 3-β-glucoside (**4**), and quercetin 3-β-rutinoside (**5**) otherwise known as rutin[12], were also isolated from the same source and their activity was studied.

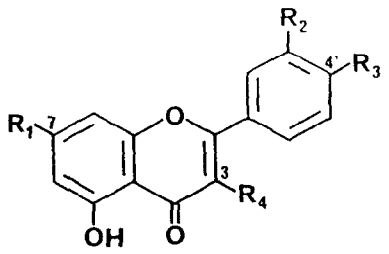
The bioassay with the purified quercetin was found to show a concentration-dependent inhibitory effect on the oxidation of L-DOPA by mushroom tyrosinase. The addition of 18 μg/ml of quercetin to the assay system containing L-DOPA caused the inhibition of tyrosinase by 40%. The inhibition was elevated to 80% when 45 μg/ml of quercetin was added. The ID_{50} of quercetin was found to be 22 μg/ml (0.07 mM). The yield of quercetin from the dried flower of *H. inuloides* was 0.25%. This result is in general agreement with those reported earlier[11] and the initial activity of the methanol extract can be explained with this flavonol. Interestingly, isoquercitrin, quercetin 3-β-glucoside (**2**), did not exhibit any activity up to 1 mg/ml. This means that the free hydroxyl group at the C-3 position seems to play an important role in eliciting activity. This hypothesis is also supported by similar observations between kaempferol (**3**) and its glycoside, kaempferol 3-β-glucoside (**4**). That is, kaempferol inhibited the mushroom tyrosinase oxidation of L-DOPA with an ID_{50} of 67 μg/ml (0.23 mM), whereas kaempferol 3-β-glucoside did not show activity up to 1 mg/ml. As expected, rutin (**5**)[12] did not show activity up to 200 μg/ml either. Higher concentration of these glycosides could not be examined because of their limited availability. Nevertheless, as far as flavonols are concerned, it seems that aglycones exhibited the tyrosinase inhibitory activity but not their 3-glycoside analogues.

In addition, we have recently characterized two tyrosinase inhibitory flavonols, buddlenoids A (**6**) and B (**7**), isolated from *Buddleia coriacea* (Loganiaceae), with ID_{50}s of 0.39 mM and 0.44 mM, respectively[2]. Both flavonols possess the free hydroxyl group at the 3-position, hence their activity corroborates the above observation. Although this hydroxyl group somehow relates to the activity, it may not be essential because several flavones, such as luteolin (**8**) and luteolin 7-β-glucoside (**9**), which lack this 3-hydroxyl group still showed activity.

The additional hydroxyl group at the 3`-position also somewhat affected the activity since quercetin exhibited slightly more potent activity than the more lipophilic kaempferol.

In addition, the inhibitory kinetics of quercetin were analyzed by a Lineweaver-Burk plot as shown in Figure 1. The slopes, obtained from the uninhibited enzyme and from the two different concentrations of quercetin, intercepted at the origin. This result indicates that quercetin exhibited a characteristic competitive inhibition for the oxidation of L-DOPA by mushroom tyrosinase. This was also supported by the results of the pre-incubation experiment[13]. Pre-incubation of the enzyme in the presence of 0.07 mM of quercetin and in the absence of the substrate resulted in quercetin being a direct inhibitor of this enzyme since it significantly decreased the activity of the enzyme. Thus, the inhibition by quercetin was increased from 45% to 77%.

In general, most competitive inhibitors closely resemble, at least in part if not all, the structure of the substrate. Based on this concern, it is fair to conclude that part of the structure of quercetin, as illustrated with the bold line in Figure 2, overlaps with L-DOPA. This part of the quercetin molecule fits loosely into the active site of the mushroom tyrosinase and prevents the entry of L-DOPA. In addition, as



1 R_1=R_2=R_3=R_4=OH
2 R_1=R_2=R_3=OH, R_4=OGlc
3 R_1=R_3=R_4=OH, R_2=H
4 R_1=R_3=OH, R_2=H, R_4=OGlc
5 R_1=R_2=R_3=OH, R_4=OGlc^6-Rha
6 R_1=OGlc^6-*p*-coumaroyl, R_2=H, R_3=R_4=OH
7 R_1=OGlc^6-*p*-coumaroyl, R_2=OCH_3, R_3=R_4=OH
8 R_1=R_2=R_3=OH, R_4=H
9 R_1=OGlc, R_2=R_3=OH, R_4=H
10 R_1=OH, R_2=R_3=R_4=H

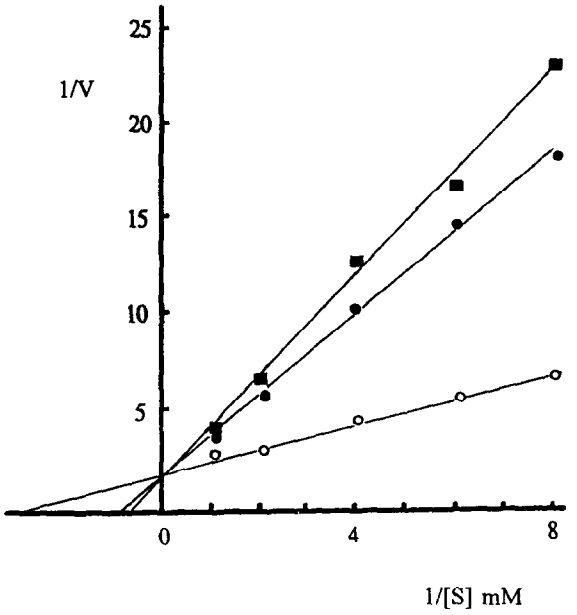


Figure 1. Lineweaver-Burk plots of mushroom tyrosinase and L-DOPA without (-○-, control) and with [-●-, 12 μg/ml (0.04 mM); -■-, 15 μg/ml (0.05 mM)] of quercetin. 1/V: 1/Δ475 nm/min.

discussed above, the 3-hydroxyl group in flavonols seems not to be essential in eliciting the activity. However, a bulky sugar moiety attached to the 3-hydroxyl group in the flavonols (**2**,**4**,**5**) may hinder their approach to the active site in the enzyme. In contrast, the three flavone glycosides (**6**,**7**,**9**) of which the sugar moiety is located on the place which does not block the active site in the enzyme showed the activity. Since chrysin (**10**) did not show activity up to 1 mg/ml, at least one hydroxyl group on the C-ring, most likely at the 4'-position, seems to be essential. This is also supported by the fact that tyrosinase is known as monophenol monooxygenase[14].

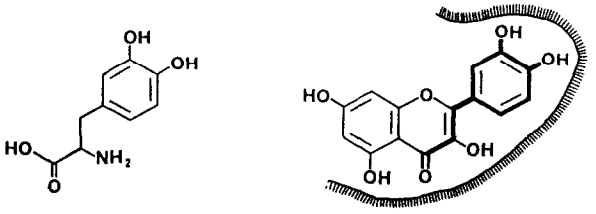


Figure 2. L-DOPA and quercetin, and their structural similarity

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9. The dried fluffy flowers of *H. inuloides* were purchased at market places in Guadalajara, Mexico. The plant was identified by Dr. D. N. Pelaez, School of Biology, Universidad Autonoma de Guadalajara where a voucher specimen was deposited.
10. The mushroom tyrosinase (EC 1.14.18.1) used for the bioassay was purchased from Sigma Chemical Co. (St. Louis, MO). Although mushroom tyrosinase differs somewhat from animal tyrosinase, the mushroom tyrosinase was used for this study due to its easy availability. All the samples were first dissolved in DMSO and used for the actual experiment at 30 times dilution. The assay was performed as previously described[7,15]. Thus, 1 ml of 2.5 mM L-DOPA solution was mixed with 1.8 ml of 0.1 M phosphate buffer (pH 6.8), and incubated at 25°C for 10 min. Then, 0.1 ml of sample solution and 0.1 ml of the aqueous solution of the mushroom tyrosinase (138 units) was added to the mixture to immediately measure the initial rate of linear increase in optical density at 475 nm, based on the formation of dopachrome. The extent of inhibition by the addition of samples is expressed as the percentage necessary for 50% inhibition (ID_{50}).
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13. The pre-incubation mixture consists of 1.8 ml of 0.1 M phosphate buffer (pH 6.8), 0.6 ml of water, 0.1 ml of the sample solution containing 0.07 mM of quercetin which inhibits about 50% of the enzyme reaction (=ID_{50}), and 0.1 ml of the aqueous solution of the mushroom tyrosinase (138 units). The mixture was pre-incubated at 25°C for 5 min. Then, 0.4 ml of 6.3 mM L-DOPA was added and the reaction was monitored at 475 nm for 2 min.
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