ORIGINAL ARTICLE

Tyrosinase kinetic in the presence of three inhibitory medicinal plants

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The present study is aimed to study the tyrosinase inhibitory effect and kinetic properties in the presence of aqueous and methanolic extracts from *Quercus infectoria, Terminalia chebula* and *Linum usita-tissimum*. Different concentration of the extracts was examined against L-tyrosine oxidation in comparison to kojic acid and their IC_{50} were calculated by probit analysis. The tyrosinase inhibition kinetics, analyzed by Lineweaver–Burk plots, and the parameters of K_m and V_{max} for each extract were calculated using the Linweaver--Burk equation. The results show that the methanolic extracts of *Q. infectoria* (MEQI) and *T. chebula* (METC) potentially inhibit mushroom tyrosinase with IC_{50} values of 3.34 and 3.87 µg/ml respectively in comparison to kojic acid (IC_{50} value = 1.56 µg/ml). The MEQI and METC exhibited the most V_{max} (81.96 and 78.74 µg/ml/min respectively). This activity is comparable with one of kojic acid (V_{max}= 103 µg/ml/min). The MEQI and METC also have shown the lowest value of K_i (0.20 and 0.44 respectively) in comparison to kojic acid (K_i= 0.18). The MEQI and METC would be developed further as a natural source of tyrosinase inhibitors.

Key words: *Linum usita-tissimum*, *Quercus infectoria*, tyrosinase kinetic, *Terminalia chebula*

INTRODUCTION

The formation of melanin is due to the tyrosinase activity which catalyzes the hydroxylation of l-tyrosine to 3,4-dihydroxyphenyl)-l-alanine (DOPA) and induces pigments of melanin.[1] Tyrosinase inhibitors are becoming increasingly important due to their antipigmenting effects.[2] We previously reported the tyrosinase inhibitory effects of *Quercus infectoria* Olive and *Terminalia chebula* Retz.[3] Linoleic acid (C18:2) is a major component of biological cell membrane which can lighten the hyper pigmented skin induced by UV.[4,5] So the present work aims to study the tyosinase kinetic in the presence of aqueous and methanolic extracts of *Quercus infectoria*, *Terminalia chebula*, and *Linum usita-tissimum*.

MATERIALS AND METHODS

Chemicals

l-tyrosine, tyrosinase, and kojic acid were purchased from Sigma-Aldrich, USA. Methanol and all other chemicals used were of analytical grades.

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Plant Materials

The dried galls of *Q. infectoria,* fruits of *T. chebula* and seeds of *L. usita-tissimum* were purchased from the local market and euthanatized by Dr. Sharififar in Department of Pharmacognosy, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.

Preparation of Plant Extract

A total of 200 g of each plant was extracted with methanol and water separately using percolation method for 72 hours at room temperature. The resultant extracts were lyophilized using freeze dryer. The extracts were stored at –20°C with suit adsorbent until testing.

Enzymatic Assay of Tyrosinase

The inhibitory activity of the samples against hydroxylation activity of the mushroom tyrosinase was determined in accordance with the methods of Chang *et al*.[6] A total of 880 µl of 2 mM substrate (L-tyrosine) dissolved in a 50 mM phosphate buffer (pH=6.8) was mixed with 100 µl of the tested extracts (different concentrations in methanol) at 25°C for 2 minutes. Then, 20 µl of tyrosinase (1000 U/ml in a phosphate buffer) was added to initiate the reaction. The assay mixture was incubated at 25°C for 10 min. The increase in absorbance at 475 nm, due to the formation of dopachrome was

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measured with a spectrophotometer. The percentage of inhibition of tyrosinase activity was calculated as follows: % Inhibition=(A−B/A) ×100, where A is the absorbance at 475 nm with methanol instead of the extract and B is the absorbance at 475 nm with the extract. The concentration of a compound at which 50% of the enzyme activity was inhibited (the IC_{50} value) was obtained by probit analysis program.

Tyrosianse Kinetic in a Time-dependent Manner

The activity of tyrosinase has been evaluated on the basis of conversion of tyrosine to dopa chrome. The enzyme activity of tyrosinase was determined in a time-dependent manner as described by Baek with some modifications.[7] A mixture of 0.1 ml of different concentrations of each extract (25, 50, and 100 µg/ml concentrations) and 0.1 ml of tyrosinase (33 units in a phosphate buffer, pH= 6.8) was incubated at 31°C for 10 min. A total of 2.8 ml of tyrosine (0.5 mM in a phosphate buffer, pH=6.8) was warmed to 31°C and added to the mixture. Kojic acid and the solvent were used as positive and negative controls. The activity of tyrosinase was measured continuously for 30 minutes using a UV spectrophotometer (Shimadzu, Japan). For determining the kinetic parameters of enzyme inhibition in fixed substrate concentration in a time-dependent manner, the absorbance was measured at wavelength 492 nm (1-minute intervals) at 31°C in the presence of each extract. The individual parameters for each curve data were calculated.[8,9] The percentage of enzyme inhibition was determined according to the following equation: where A is the absorbance of negative control in the time of t_n , B the absorbance of the extract in the time of t_n. The test was done in triplicate and data were reported as mean±SD.

$$\text{Percent of inhibition} = \left(\frac{A_{t_n} - B_{t_n}}{A_{t_n}} \right) \times 100$$

The kinetic parameters of tyrosinase were determined in the presence of the tested extract using the lineweaver-burk equation. Affinity constant (K_i) was calculated in the following way:

$$k_i = \frac{IC_{50}}{1 + \frac{[S]}{Km}},$$

where [S] is the concentration of substrate.

Statistical Analysis

Results are expressed as mean±S.E.M. Differences between the control and treated groups were tested for significance using a one-way analysis of variance (ANOVA).

RESULTS

Inhibitory Activity Against Tyrosinase in Hydroxylation of L-tyrosine

The inhibitory effect against tyrosinase activity in hydroxylation of l-tyrosine for both aqueous and methanolic extracts of tested plants was determined at different concentrations. The IC_{50} value for each extract and the results of extraction have been given in Table 1. These results indicated that the methanolic extracts of *Q. infectoria* (MEQI) and *T. chebula* (METC) have shown the least IC_{50} values (3.34 and 3.87 µg/ml respectively) in comparison to that of kojic acid (IC_{50}= 1.56 µg/ml). These two extracts inhibited the tyrosianse activity in a concentration-dependent manner. Among the tested extracts the most percentage of inhibition was shown by MEQI (85.9%) in concentrations of 100 µg/ml [Figure 1]. The least of inhibition was exhibited by the methanolic extract of *L. usita-tissimum* (MELU) and aqueous extract of *L. usita-tissimum* (AELU). The methanolic extracts of the all of three plants exhibited more tyrosinase inhibition activity than the aqueous ones.

The Results of the Kinetic Study of Tyrosinase

The tyrosinase kinetic was studied in the presence of tested extracts for 30 minutes. Figures 1-3 show the results of a time-response inhibition for kojic acid, MEQI, and METC at the

Table 1: The results of extraction and IC_{50} values for tyrosinase inhibition of different tested extracts

Name	Methanolic/ aqueous extract (%), g/g	IC_{50} (µg/ml) of methanolic extracts	IC_{50} (µg/ml) of aqueous extracts
Q. infectoria	18/15.2	3.34	14.7
T. chebula	17.7/12.6	3.87	266.4
L. usita-tissimum	10.4/9.5	292.1	5427.0
Kojic acid	-	1.56	1.56

l-tyrosine was mixed with tested extracts (in different concentrations). The IC_{50} of extracts was determined by the probit analysis program

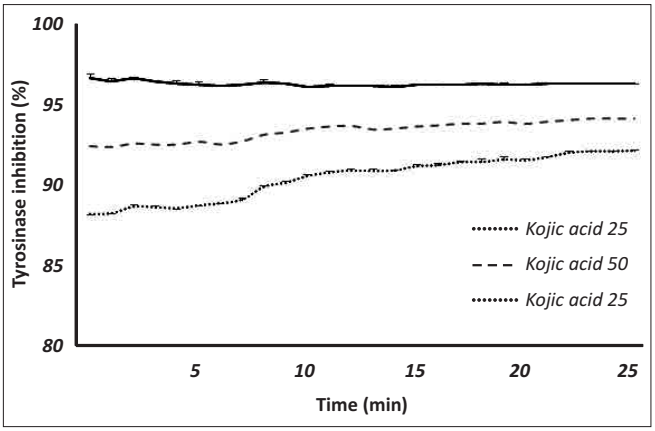

Figure 1: Time- course oxidation of l-tyrosine by tyrosinase in the presence of different concentratins of kojic acid. After adding tyrosinase, incubated at 25°C for 10 min. Increase in absorbance at 475 nm, due to dopachrome formation was measured

concentrations of 25, 50, 100 µg/ml respectively. As shown in the Figures 1 and 2, kojic acid and MEQE potentially can inhibit tyrosinase activity at three used concentrations which increased with time. The kinetic parameters were analyzed under our experimental conditions and K_m and V_{max} of each extract were calculated using the Linweaver--Burk equation by plotting the reverse of velocity versus reverse concentration. More V_{max} indicated more inhibition potency. The results have been given in Table 2. These results indicated that among the tested extracts, the MEQI and METC exhibited the most inhibition of tyrosinase (V_{max} = 81.96 and 78.74 µg/ml/min respectively). This activity is comparable with that of kojic acid (V_{max}=103 µg/ml/min). The lowest value of K_i also belongs to MEQI and METC (0.20 and 0.44 respectively) in comparison to kojic acid (K_i= 0.18) [Table 2] which indicates the low affinity of binding while the AELU showed the highest capacity of binding (K_i= 1243.29)

DISCUSSION

Kojic acid is a secondary metabolite of some species of *Aspergillus* and *Penicillium* which has been used in cosmetic preparations as a hypopigmentation agent[10] and competitively inhibits the tyrosinase. In the previous study using mushroom tyrosinase, MEQI and METC significantly inhibited the oxidation of both L-tyrosine and L-DOPA.[3] In the present work, the kinetic behaviors of the oxidation of L-tyrosine catalyzed by mushroom tyrosinase were studied at different concentrations of aqueous and methanolic extracts of three medicinal plants. The results indicated that among the tested extracts, two extracts of MEQI and METC can potentially inhibit tyrosinase activity. The IC_{50} of the extracts, as a parameter of potency, indicated that the MEQI and METC with least IC_{50} exhibited the most tyrosinase inhibition. As shown in Table 1, the IC_{50} of these two extracts are two times more than those of kojic acid. The aqueous and methanolic extracts of *L. usita-tissimum* exhibited the highest IC_{50} value. The results of the kinetic study of tyrosinase in the presence of kojic acid and different extracts have been given in Table 2. Under these conditions, K_m and V_{max} of the L-tyrosine oxidation by the tyrosinase in the presence of MEQI were found to be 5.4 µg/ml and 81.96 µg/ml/min respectively. The V_{max} of MEQI is comparable with the one of kojic acid (V_{max} = 103 µg/ml/min). The highest value of V_{max} obtained in the recent work is due to MEQI followed by METC (V_{max} = 78/74 µg/ml/min), whereas AELU exhibited the least V_{max} value (22.62 µg/ml/min). The K_i of each extract was determined from the kinetic study of tyrosinase. The MEQI has shown the lowest value of K_i among the tested

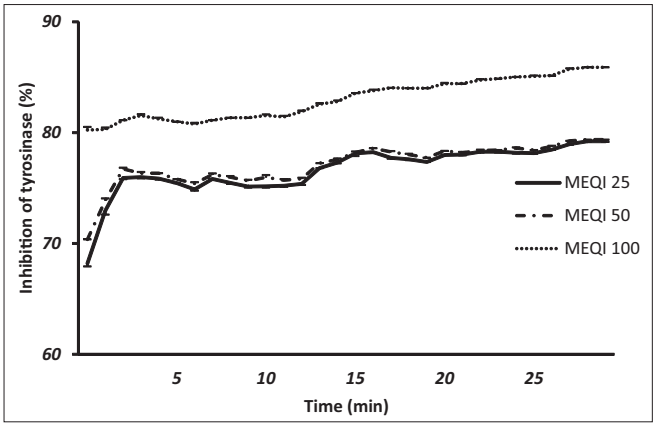

Figure 2: Time- course oxidation of l-tyrosine by tyrosinase in the presence of different concentratins of MEQI. After adding tyrosinase, incubated at 25°C for 10 min. Increase in absorbance at 475 nm, due to dopachrome formation was measured

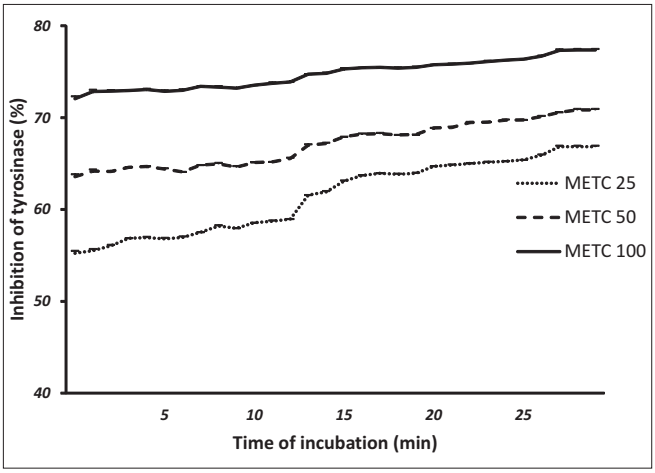

Figure 3: Course of oxidation of L-tyrosine by tyrosinase in the presence of different concentratins of methanol extract of *Terminalia chebula* (METC)

Table 2: The results of kinetic parameters for tyrosinase inhibition in the presence of different tested extracts

Sample	V_{max} (µg/ml/min)	K_m (µg/ml)	Regression equation	K_i
MEQI	97/81	41/5	y=0.006x+0.0122; R2=0.754	201/0
METC	74/78	83/10	y=0.138x+0.13; R2=0.986	440/0
MELU	59/37	86/10	y=0.289x+0.027; R2=0.683	285/33
AEQI	05/54	60/8	y=0.152x+0.019; R2=0.976	357/1
AETC	16/40	54/8	y=0.213x+0.025; R2=0.977	464/24
AELU	62/22	04/25	y=1.107x+0.044; R2=0.998	294/1241
Kojic acid	103/00	29/11	y=0.110x+0.01; R2=0.945	179/0

MEQI: Methanolic extract of *Q. infectoria*; METC: Methanolic extract of *T. chebula*; MELU: Methanolic extract of *L. usita-tissimum*; AEQI: Aqueouse extract of *Q. infectoria*; AETC: Aqueouse extract of *T. chebula*; AELU: Aquouse extract of *L. usita-tissimum*, Different concentrations of extract and tyrosinase were incubated at 31°C for 10 minutes and added to tyrosine. The activity of tyrosinase was measured continuously for 30 minutes. The absorbance was measured at wavelength 492 nm (1-minute intervals). The test was done in triplicate and data were reported as mean±SD. The kinetic parameters of tyrosinase were determined in the presence of tested extract using the Lineweaver–Burk equation.

extracts which indicates the most its effective capacity for binding to the tyrosinase whereas MELU and AELU showed the least binding capacity followed by METC and AEQI in decreasing order.

The plot of the percentage of inhibition versus the time of incubation for each extract shows that when the concentration increases, the more inhibition induces. The most inhibition was considered within the time of 15-25 minutes after incubation for all the extracts whereas in the last 5 minutes of incubation, the least of inhibition occurs. As IC_{50} and K_i of these two plants indicate, these plants show less effective tyrosinase inhibition than kojic acid, which might be due to the presence of a complex of phytochemicals with different biological functions. Flavonoids and phenolic acids were found to be the main constituents of galls of *Q. infectoria* and fruits of *T. chebula*. Major phenolic acids are *gallic acid* and *ellagic acid*.[11,12] Ellagic acid shows high affinity to two copper ions of tyrosinase.[13] Furthermore the flavonoid derivatives chelate the copper ions of tyrosinase.[14] Tyrosinase activity depends on the function of copper ions, so the agents with affinity to binding to these ions such as kojic acid or gallic acid can inhibit the activity of tyrosinase.[15] In the present work, two extracts of MEQI and METC show similar activity to kojic acid but with lesser potency. The high yield of extraction for MEQI and METC (34 and 29% respectively) indicates that each of these plants would be a valuable source of tyrosinase inhibitors. Our previous study showed the high antioxidant effects of MEQI and METC[3] which somehow may be related to their potential tyrosinase inhibition effects. The results of this work also suggest that these extracts are worthy for further studies and would be developed further as a natural source of tyrosinase inhibitors.

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