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*Full Length Research Paper*

# Determination of total polyphenol content and antityrosinase capacity of mulberrymedicine (*Morusnigra* L.) extract

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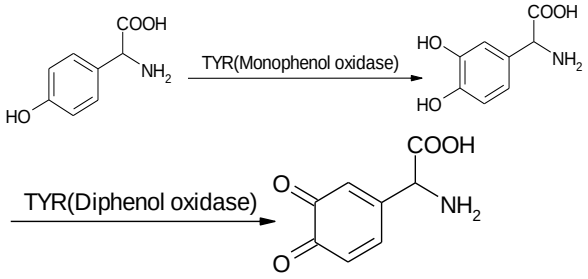
**Total polyphenol content and antityrosinase capacity of mulberry medicine extract were evaluated for its development and utilization. Total polyphenols were extracted from mulberry medicine with 70% alcohol solution. The content of total polyphenols was determined by ultraviolet spectrophotometer and the capacity of antityrosinase was evaluated by using the enzyme-linked immunosorbent *assay*(ELISA) method. Results show that the content of polyphenols was up to 8.668 mg/g and tyrosinase half-inhibitory concentration ($IC_{50}$) was 12.12 mg/ml. Kinetic study indicated that the mechanism of tyrosinase inhibition was mixed type inhibition.**

**Key words:***Morusnigra* L., extraction of medicine mulberry, polyphenols, tyrosinase.

## INTRODUCTION

Tyrosinase (TYR) is widely distributed in human beings, animals, plants and microorganisms. It is the crucial enzyme which restricts the output of melanin (Fenoll et al., 2004). Not only does it have the activity of monophenol oxidase, but it also has the activity of diphenol oxidase(Gong et al., 2005; Shi et al., 2005). It catalyzes the following reactions:




Disorders of melanin pigmentation and melanoma can be attributed to the malfunction of tyrosinase. Tyrosinase existing in plants is the main cause of their enzymatic browning, while tyrosinase existing in insects plays an important role in their tanning process (Fenoll et al., 2004; Kubo et al., 2003). By inhibiting the activity of tyrosinase, tyrosinase inhibitors can restraint the production of melanin; therefore, they have an extensive prospect in cosmetic,medicine and agriculture. In recent years, the tyrosinase inhibitors have been investigated extensively (Chang et al., 2011; Prasad et al., 2010; Behera et al., 2007; Rangkadilok et al., 2007).

Polyphenols, possessing several phenolic hydroxyls, are excellent hydrogen or electron donors and have an amount of antioxidant activity. They can effectively remove superoxides and hydroxyl radicals (Bouaziz et al., 2009). Previous researches have already shown that polyphenols are effective tyrosinase inhibitors (Bouaziz et al., 2009; Jewell and Ebeler, 2001). Thus, polyphenols can be regarded as a kind of tyrosinase inhibitors.In addition to possessing good antioxidation and antityrosinase activity, polyphenols have some other functions. For example, the green and black tea polyphenols have the activity of anti-cervical cancer which is the second most common malignant neoplasm in women, in terms of both incidence and mortality rates worldwide (Singh et al., 2010). Red wine polyphenols cause growth inhibition and apoptosis in acute lymphoblastic leukaemia cells by inducing a redox-sensitive up-regulation of p73 and down-regulation of UHRF1 (Sharif et al., 2010).

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**Figure 1.**Medicine mulberry.

Mulberry medicine (Figure 1) is a half-domesticated plant. In plant taxonomy, it is black mulberry species (*Morusnigra* Linn). It was originated in Iran, and cultivated in Xinjiang Province in the 16th century. Mulberry medicine is the only black mulberry species which has 22 haploid sets of chromosomes in a body cell. It is also a very precious officinal resource in nature. Mulberry medicine is a traditional Uyghur medicine in china and has the efficacy of anti-oxidation (Lqbal et al., 2010), delaying aging (Jiang, 2010), protecting liver and decreasing blood sugar level (Hemmati et al., 2010). The juice of mulberry medicine has a lot of active contents, including polyphenols, alkaloid, vitamin C, amino acids, polysaccharose etc(Ercisli et al., 2008, 2007, 2010; Koyuncu et al., 2004). These contents have the property of removing free radicals and are potent antioxidants. The objectives of this study were to determine the content of total polyphenols in mulberry medicine extraction, and investigate its inhibition effect on tyrosinase.

## MATERIALS AND METHODS

Mature mulberry medicine (from The Germ Plasm Resource Garden of Institute of Sericulture of Xinjiang Province) was dried naturally and ground into powder.

Ethanol, phosphoric acid, hydrogen peroxide, lithium sulfate, concentrated hydrochloric acid, natriumcarbonicumcalcinatum, sodium methoxide, gallic acid, sodium wolframate, sodium nitrite, aluminum nitrate, sodium hydroxide, rutin, monopotassium phosphate, potassium hydroxide and dopa (L-dopa) were of analytical pure grade and purchased from Taixin Reagent Company (Chongqing, China). Arbutin and mushrooms tyrosinase were of HPLC grade and obtained from Dingguo Reagent Company (Chongqing, China).

Electronic balance was obtained from FA2004A, Shanghai Jingtian Electronics Co., Ltd; ultraviolet spectrophotometer, Xinshiji T6, Beijing Puxi General Co., Ltd; grinder, FFC-45D, LinyiDahua Machinery Works; ELIASA, BIO-RAD iMarkmicroplate Reader, Japan.

### Extraction of total polyphenols

Twenty five grams of mulberry medicine powder was immersed in 40 ml 70% ethanol solution for 4 h, and then filtered. The process was repeated twice and all of them were done under room temperature. The filtrate was added with 70% ethanol solution to 100 ml.

### Determination of total polyphenols

This was carried out according to the method of Bae and Suh (2007), with some modifications. In brief, 20 g of sodium wolframate and 5 g of sodium methoxide were placed in round bottomed flask and dissolved with 140 ml of distilled water.Then, 10 ml of 85% phosphoric acid and 20mL of concentrated hydrochloric acid were added and refluxed for 2 h at 100°C. We added 3 g lithium sulfate and 15 ml 30% hydrogen peroxide to the solution, and then boiled the solution until it became bright yellow(about 15 min). After that the solution was cooled and fixed to a constant volume of 250 ml, stored in a brown bottle and kept in dark place.

### Establishment of standard curve

0.50 g of gallic acid standard sample was accurately weighed,

**Determination of total polyphenols in sample**

0.01 ml of solution mentioned above was put in 10 ml centrifuge tube, added with 1 ml forint reagent, 3 ml 200 g/L sodium carbonate solution and 0.99 ml distilled water. After shaking up, it was placed under room temperature for 2 h. 70% ethanol was used as a blank. Absorbance was measured at 765 nm.

**Determination of antityrosinase activity**

The antityrosinase activity was investigated by the method of Shin(Shin et al., 1998), with some modifications. In brief, 50 µl of L-DOPA solution (3 mg/ml, dissolved in 0.1 mol/L phosphate buffer, pH 6.8) and 50µl of medicine mulberry medicine extract (dissolved in 70% ethanol) were placed in a 96-well microtiter plate, and then added to 50 µl of tyrosinase solution (100 u/ml, dissolved in 0.1mol/L phosphate buffer, pH 6.8).The volume was adjusted to 250 µl by adding 100µl PBS buffer solution (0.1 mol/L, pH 6.8). The solution was incubated at 37°C for 15 min and its absorbance was measured at 490 nm. PBS buffer solution was used as a blank. Percent of tyrosinase inhibitory activity was calculated using the following formula:

$$I=[(A - B) - (C - D)]/(A - B) \times 100\%$$

Where, A is the absorbance of the mixed solution without sample, 50 µl 0.3% L-DOPA + 50 µl 70% alcohol + 100 µl PBS (pH 6.8) + 50µl enzyme solution; B is the absorbance of the mixed solution without enzyme, 50 µl 0.3% L-DOPA + 50 µl 70% alcohol + 150 µl PBS (pH 6.8); C is the absorbance of the mixed solution with the sample, 50 µl 0.3% L-DOPA + 50 µl sample + 50 µl PBS (pH 6.8) + 50 µl enzyme solution; D is the absorbance of the mixed solution without enzyme, 50µl 0.3% L-DOPA + 50 µl sample + 150 µl PBS (pH6.8). Half effective concentration ($IC_{50}$) was obtained by using the logarithm concentration-enzyme inhibition ratio regression equation.

**Mechanism of antityrosinase activity**

The mechanism of antityrosinase activity was analysed according to the method of Zhang and Chen (Zhang et al., 2006; Chen et al., 2002), with some modifications. In brief, L-DOPA (50 µl, 3 mg/ml, dissolved in 0.1 mol/L phosphate buffer, pH 6.8) was mixed with differentvolumes (10, 30, 50 and 70 µl) of tyrosinase solution (100 u/ml, dissolved in 0.1 mol/L phosphate buffer, pH 6.8) in a 96-well microtiter plate, and then the differentvolumes (10, 30 and 50 µl) of extract were added. The volume was adjusted to 250 µl by adding PBS buffer solution. After that the solution was incubated at 37°C for 15 min and its absorbance was measured at 490nm by ELIASA. PBS buffer solution was used as a blank.

**Determination of inhibitory pattern**

The inhibitory pattern of anti-tyrosinase was determined according to the method of Zhang and Chen (Zhang et al., 2006; Chen et al., 2002), with some modifications. In brief, tyrosinase solution (50µL, 100u/mL dissolved in 0.1mol/L phosphate buffer, pH 6.8) was mixed with differentvolumes (10 µl, 30, 50 and 70 µl) of L-DOPA (50 µl, 3 mg/µl, dissolved in 0.1mol/L phosphate buffer, pH 6.8) in a 96-well microtiter plate, and then the differentvolumes (10, 30 and 50 µl) of extract were added. The volume was adjusted to 250 µl by adding PBS buffer solution. After that the solution was incubated at 37°Cfor 15 min and its absorbance was measured at 490nm by ELIASA. PBS buffer solution was used as a blank. Samples inhibition type was determined by graphs constructed, using Line weaver-Burk double reciprocal.

## RESULTS

### Determination of total polyphenol content

The concentration of gallic acid standard solution had a linear relation with its absorbance within concentration range of 0.005 to 0.1 mg/ml. The corresponding equation of linear regression was y = 0.0486x - 0.0015 $R^2$ = 0.9994. Total polyphenol content was calculated according to standard curve and the result is shown in Table 1. As illustrated in Table 1, the content of total polyphenolsin mulberry medicine wasup to 8.668mg/g. Multipledetermination coefficient of variation was 0.022, which indicated the result was reliable.

### Determination of anti-tyrosinase activity

Using effector concentration as abscissa, inhibition ratio as ordinate, we constructed fitted curve by Origin8 analysis software, and showed the result in Figure 2. As illustrated in Figure 2, relative inhibition ratio increased dramatically with the increase of extract concentration in low concentration range (0 to 0.02 mg/ml). In high concentration range (>0.02 mg/ml), the inhibition ratio increased much slower. Half inhibition concentration of extract on tyrosine ($IC_{50}$) was approximately 12.12 mg/ml.

### Mechanism of anti-tyrosinase activity

Different concentrations of mulberry medicine extract were added to enzyme activity determination system. A group of straight lines coming across the coordinate origin were obtained in the enzyme activity of the enzyme concentration plot (Figure 3). Withthe increase of extract concentration, the slope of the straight line became lower and lower, which meant the inhibition of mulberry medicine extract on tyrosinase was reversible inhibition. Its inhibition on tyrosinase was not achieved by reducing the enzyme's catalysis ability but by reducing the amount of enzyme.

### Determination of inhibitory pattern

The concentration of tyrosinase was fixed in the activity measurement system, and then concentration gradient of L-DOPA was set. We investigated the impact of different concentrations of mulberry medicine extract on tyrosinase activity, and constructed a group of hyperbolic curves by using the initial enzyme reaction rate on substrate concentration plot (Figure 4). As shown in Figure 4, the enzymatic reaction follows Michaelis' (Michaelis-Menten) kinetic equation. To study the

**Table 1.**The content of polyphenols in mulberry fruit.

| Parameter | Parallel experiment | | | | | Average value | Standard deviation | Coefficient of differentiation |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | | |
| Content (mg/g) | 8.664 | 8.949 | 8.723 | 8.424 | 8.580 | 8.668 | 0.193 | 2.2% |

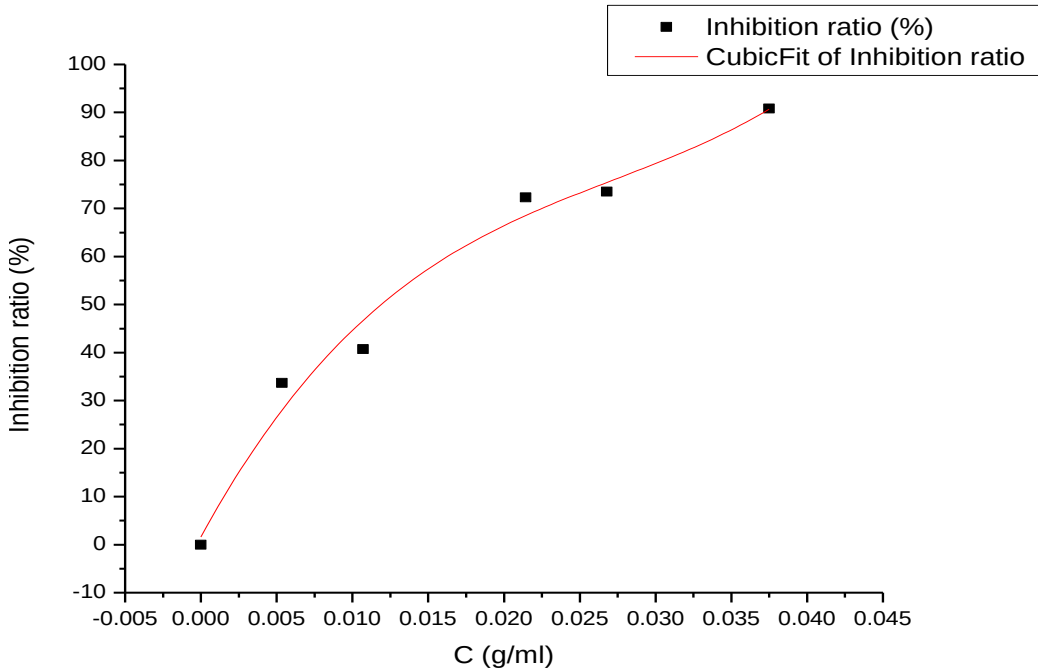


**Figure 2.**Effect of water extract from medicine mulberry on the activity of tyrosinase.

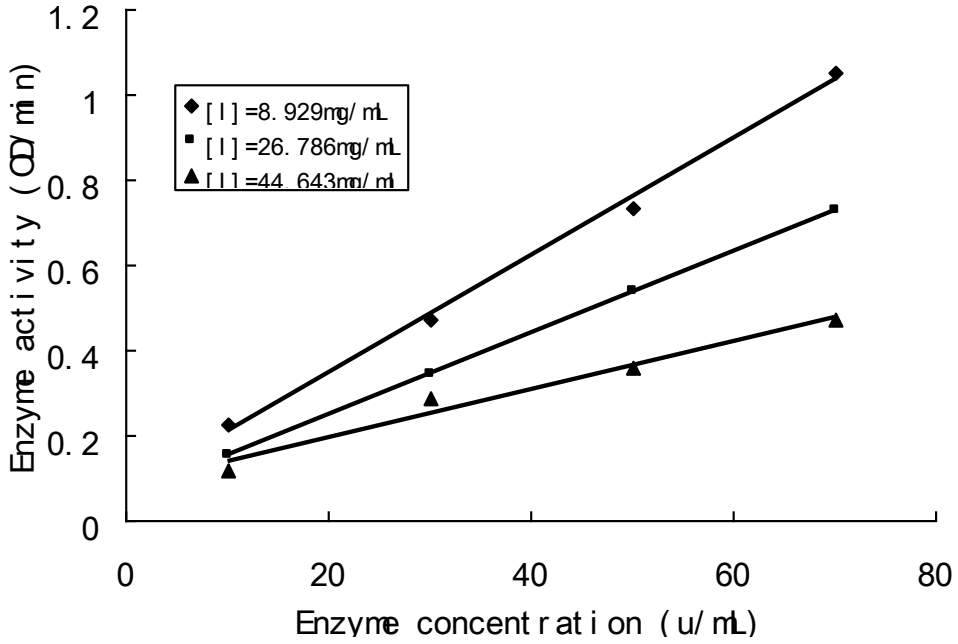


**Figure 3.**Inhibitory mechanism of ethanolic extract from medicine mulberry on tyrosinase. [I] Denotes the concentration of inhibitor (the ethanolic extract from medicine mulberry).

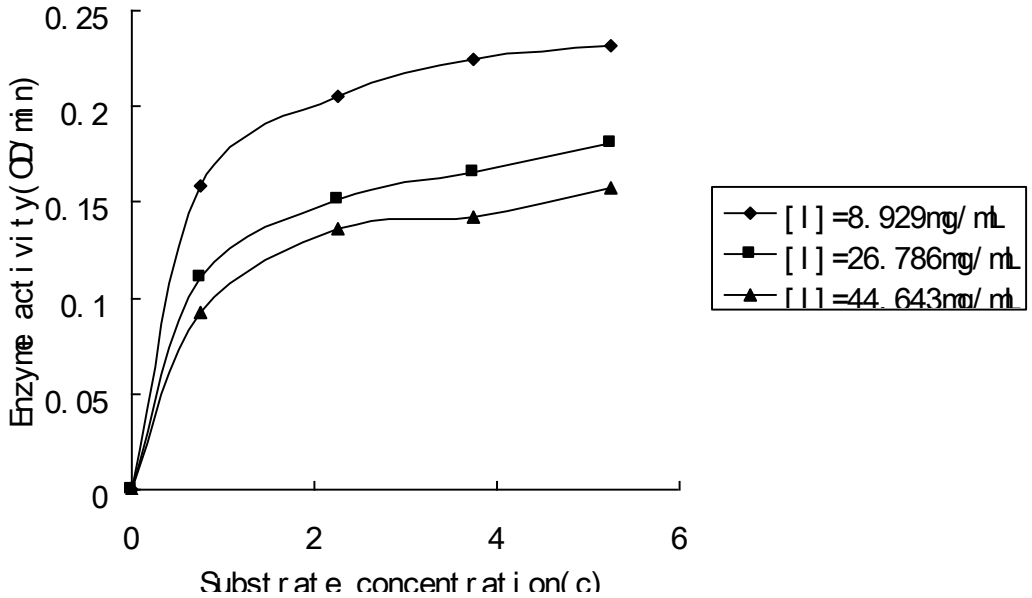


**Figure 4.**The relationship between enzyme activity and substrate concentration. [I] Denotes the concentration of inhibitor (the ethanolic extract from *Morusnigra* L).

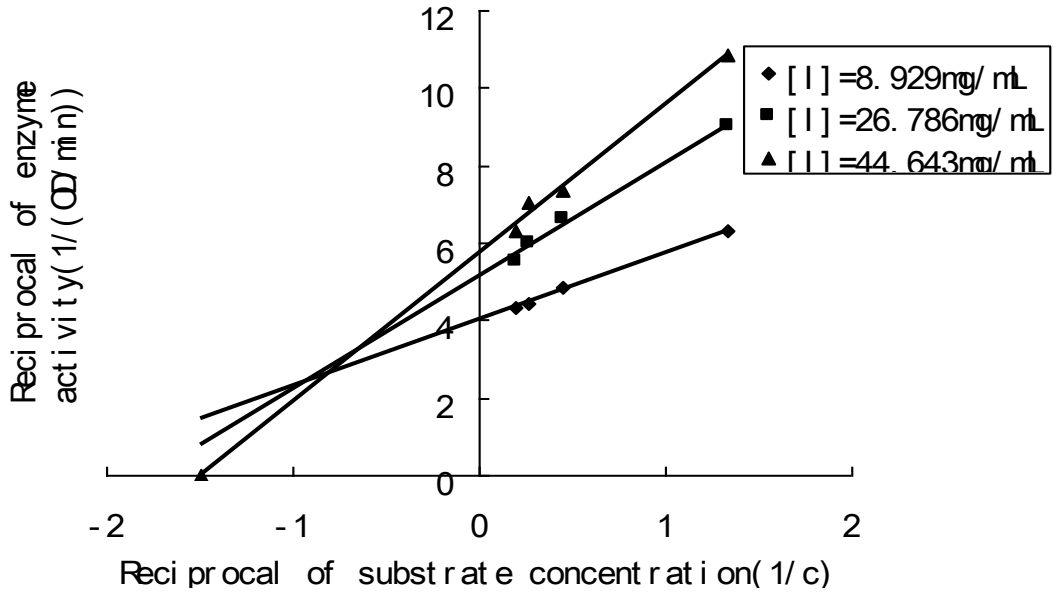


**Figure 5.** Lineweaver-Burk plots for inhibition of the ethanol extract of medicine mulberry on tyrosinase.[I]denotes the concentration of inhibitor (the ethanolic extract from medicine mulberry).

inhibition type, Lineweaver-Burk double-reciprocal plots was used. The results are shown in Figure 5. The double-reciprocal plots yield a family line with different slopes and different intercepts, and they intersect one another in the second quadrant. This behavior indicatesthat mulberry medicine extract can bind, not only with free enzyme, but also with the enzyme-substrate complex, which shows its inhibition mechanism was mixed type.

## DISCUSSION

In this study, we found that the total polyphenols content in the dried sample of mulberry medicine was 8.668 mg/g. It is a little lower than black mulberry fruits grown in East Anatolia Region of Turkey, in which the total phenolic content was 1422 mg gallic acid equivalents/100g fresh matter (Ercisli et al., 2007).

Polyphenols are potent antioxidants and can clear away free radicals effectively (Isabelle et al., 2008; Segev et al., 2010). The content of polyphenols in the ethanol-water extract of mulberry medicine is up to 8.668mg/g. Therefore, it explains why mulberry medicine has been used as a traditional medicine for so long.

The ethanol extract of mulberry medicine was an efficient inhibitor of tyrosinase, its $IC_{50}$ was 12.12 mg/ml, and its inhibition type was mixed type inhibition. Walker and Wilson (1975) suggested the existence of two distinct sites on the enzyme: one site for the binding of the substrate and another site, adjacent, for binding the inhibitor. Some inhibitors can bind not only with the free enzyme but also with the enzyme-substrate complex. So mixed type inhibition of the ethanol extract of mulberry medicine may be due to various substances it contains. The antityrosinase activityof mulberry medicine indicates that it can be used in cosmetics, treatment of skin disease and retain freshness of fruits and vegetables etc. It will also provide us with insight into finding new, efficient and safe tyrosinase inhibitors. This study is the first report on total polyphenols content and antityrosinase activity of mulberry medicine. Further investigation is required to purify phenolic compounds from mulberry medicine which have antityrosinase activity.

## ACKNOWLEDGEMENTS

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