ANTI-TYROSINASE AND ANTIOXIDANT ACTIVITY OF *LAVANDULA SP*. EXTRACTS

Sariri R.*, Seifzadeh S. and Sajedi R.H.

Department of Biology, Faculty of Science, University of Guilan, Rasht, Iran

Summary

Background: Tyrosinase catalyzes melanin biosynthesis in human skin and the epidermal hyperpigmentation results in various dermatological disorders, such as melasma, freckles and age spots. Therefore, safe and effective tyrosinase inhibitors have become important for their potential applications in preventing pigmentation disorders and other melanin-related health problems in human skin.
Aims: Since most plants including lavender are rich sources of bioactive chemicals, the aim of this research was screening lavender extracts for total antioxidant and anti-tyrosinase activity.
Methods: Water extracts of dried lavender leaves were used for both experiments. Four lavender species from North of Iran: *Lavandula angustifolia*, *Lavandula stoechas*, *Lavandula dentata* and *Lavandula latifolia* were tested. The biological activity of mushroom tyrosinase on dopamine hydrochloride was measured in the absence and presence of lavender extracts.
Results: The order of potency as tyrosinase inhibitors were found to be *Lavandula angustifolia*< *Lavandula stoechas*< *Lavandula dentata*< *Lavandula latifolia*. Kinetics studies showed that inhibition was of mixed type in all cases. Total antioxidant activity of *Lavandula angustifolia*, *Lavandula stoechas*, *Lavandula dentata* and *Lavandula latifolia* extracts was 9.2, 12.5, 38.7 and 65.1 μg/ml depending on the respectively.

Key words: Lavender, Lavandula antioxidant, tyrosinase, inhibitors.

Introduction

Tyrosinase (oxygen oxidoreductase, polyphenol oxidases PPO EC 1.14.18.1) is a multifunctional copper-containing enzyme mainly involved in the first two steps of the melanin biosynthesis. During enzymatic biosynthesis of melanin, l-tyrosine (monophenolase activity) is hydroxylated and the hydroxylation product, l-Dopa (diphenolase activity), is further oxidized into the corresponding *o*-quinone (Fig. 1). Tyrosinase catalyzes the reaction of melanin biosynthesis in human skin and the epidermal hyperpigmentation results in various dermatological disorders, such as melasma, freckles and age spots [1]. Enzymatic browning in cut vegetables and flowers and raw fruits is also the result of polyphenol oxidases action [2]. Moreover, PPOs are also efficient reagents used for treatment of wastewater containing polyphenols due to their ability for oxidation of phenols [3-5].

Designing safe and effective tyrosinase inhibitors for pharmacological preparations have become important aims of many research works during the last decade. They have shown potential applications in preventing pigmentation disorders and other melanin-related health problems in human [6]. Research in this area includes both synthetic [7-11] and natural [12-16] compounds acting as tyrosinase inhibitors as well as antioxidants [17, 18]. Furthermore, tyrosinase inhibitors are also important in cosmetic applications for skin whitening effects, as many people prefer lighter skin. Most plants sources are rich in bioactive chemicals, mostly free of potent side effects. Therefore, search of natural tyrosinase inhibitors from plant sources would have many industrial outcomes.

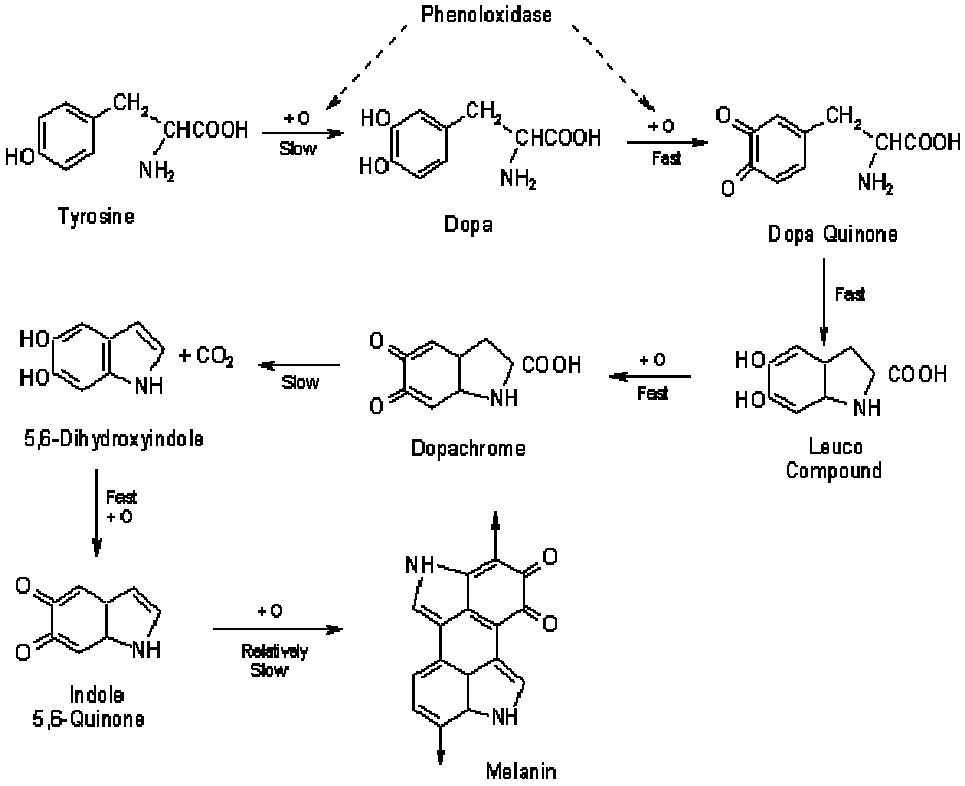

Figure 1. Formation of melanin from amino acid, tyrosine. The monophenolase and diphenolase activity of polyphenol oxidase are pointed by doted arrows.

Lavander has long been traditionally used as dried flower arrangements, placed among stored clothing to give a fresh fragrance and as a deterrent to moths. The plant has also been grown commercially for extraction of its essential oil. Lavender essential oil could be used as an antiseptic and for aromatherapy. Lavender flowers contain abundant nectar which yields a high quality honey for beekeepers. Lavender is also used to flavour sugar, and the flowers are occasionally sold in a blend with black, green, or herbal tea, adding a fresh, relaxing scent and flavour. The Lavenders, *Lavandula* are a genus of about 25-30 species of flowering plants in the mint family. The genus includes annuals, herbaceous plants, subshrubs, and small shrubs. The native range extends across the Canary Islands, North and East Africa, south Europe and the Mediterranean, Arabia, and India. Because the cultivated forms are planted in gardens world-wide, they are occasionally found growing wild, as garden escapees, well beyond their natural range.

Although many lavender species have been used either dried or as an essential oil for centuries for a variety of therapeutic and cosmetic purposes, there is still considerable debate about whether lavender essential oil could show significant clinical potential [19]. The objective of present study is screening lavender species found in our part of the world for total antioxidant and anti-tyrosinase activity.

Materials and Methods

Materials

Mushroom tyrosinase, dopamine hydrochloride and DMF were purchased from Sigma Chemical Company. Sodium mono-phosphate, sodium di-phosphate, propylene glycol, DMSO, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and MBTH were obtained from Merck representative in Iran. Nylon membrane (13 mm) was obtained from Orange Scientific, Belgium. Lavender species (leaves, stems and flowers) were collected from different parts of Gilan.

Preparation of the enzyme and substrate solution

a) Enzyme solution: Pure mushroom tyrosinase (1mg/ml) was used without further purification and diluted to 1/160 of its original concentration.
b) Substrate solution: Dopamine hydrochloride (44 mM) was freshly prepared in phosphate buffer (pH 6.8) containing 2% (v:v)DMF and 5 mM MBTH. This solution was stored in dark, as the direct light changed its color.

Preparation of lavender extracts

Fresh leaves and flower buds of four lavender species, *Lavandula angustifolia*, *Lavandula stoechas*, *Lavandula dentata* and *Lavandula latifolia* cultivated in the Faculty of Agriculture, University of Guilan. Selected fresh leaves and flower buds were harvested, washed, dried and stored in dark and cool place for later extraction. Fresh leaves were also used for preparation of extracts, but only slight difference between dry and fresh samples were observed. Therefore, only the dried samples were used all through the research. 500 g of each dried lavender powder was soaked in 2 L water (fixed temperature of 50 °C) for at least 2 hours prior to extraction. The mixture was then covered and stirred using a magnetic stirrer at the same conditions for another 1 hour, filtered and stored at 4°C. The residue was re-extracted twice with 2 L of hot water each time. The three water extracts were combined, concentrated and lyophilized using a freeze-dryer. The percentage yield of extracts was between 15% and 21% depending on cultivars. To study the antioxidant activity, samples were dissolved in DMSO (100%) at a final concentration of 5 mg/ml prior to chemical assay.

Scavenging of diphenyl-picrylhydrazyl (DPPH) radicals

2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical was used as active scavenger of free radicals in the extracts. The reduced DPPH formazan form was determined using a spectrophotometer. A modification of the assay method described by van Amsterdam et al [20] was used. In a typical experiment, 5µl of each lavender extracts, 100% DMSO (as a negative control) and 10 mM ascorbic acid (as a positive control as well as a blank for background subtraction) were allowed to react with 195 µl of 100 µM DPPH aqueous solution in a 96-well micro-plate. The plate was then incubated at 37 °C for 30 min after which the absorbance was measured at 515 nm using a UV–VIS micro-plate reader. Scavenging capacity of the each extract was compared to that of DMSO (0% radical scavenging) and ascorbic acid as positive control (100% radical scavenging). The results expressed as the concentration of the extracts or pure compounds which scavenged free radicals by 50% (SC_{50}).

Assay and inhibition of tyrosinase

The diphenolase activity of mushroom tyrosinase was measured spectrophotometrically using kojic acid as a standard tyrosinase inhibitor, a modified method described by Yi, et al [10]. The enzymatic reaction was initiated by addition of a known amount of the enzyme to a solution of substrate, dopamine hydrochloride containing dimethyl formamide (DMF and MBTH). In the presence of DMF, the resulting colored complex remained in soluble state during the course of investigations. The progress of the reaction was followed by measuring the intensity of the resulting pink color at 505 nm. A typical reaction mixture with a total volume of 1.0 ml contained 100 µl enzyme solution (a), 500 µl substrate solution (b) and 400 µl phosphate buffer (pH 6.8). To investigate the effect of lavender extracts, tyrosinase activity was measured by replacing the phosphate buffer with 400 µl of extracts (3-6 mg/ml). The 50% inhibition (IC_{50}) of tyrosinase activity was calculated as the concentrations of each sample that inhibited 50% of tyrosinase activity. The data were expressed as percentage of inhibition of tyrosinase activity.

Kinetic parameters, K_m and $V_{max,}$ were calculated by linear regression from Lineweaver–Burk plots. The inhibition constant (K_i) of an inhibitor was obtained from the secondary plot of Lineweaver–Burk plots, the slope for the vertical axis, and inhibitor concentration for the horizontal axis. The intercept on the horizontal axis, (I), was the absolute value of the K_i. Triplicate measurements were performed in all experimental procedures and the results were expressed as the mean value of the three tests.

Results

Free radical scavenging ability of lavender extracts (DPPH assay)

It is known that polyphenols are good scavengers of free radicals. $DPPH^{\cdot}$ is a stable free radical and accepts hydrogen radical to become a stable diamagnetic molecule, yellow coloured diphenylpicrylhydrazine [20]. The $DPPH^{\cdot}$ scavenging capacity of most plant extracts may be mostly related to their phenolic hydroxyl groups. In the present study, hot water extracts of lavender evaluated for total antioxidant activities, i.e. their abilities to neutralize the stable DPPH free radicals. The SC_{50} values (the concentration that scavenges 50% of the DPPH radical) for *Lavandula angustifolia*, *Lavandula stoechas*, *Lavandula dentata* and *Lavandul latifolia* extracts were between 29.2 and 95.1 µg/ml depending on the lavender species. In addition, standard pure ascorbic, gallic and ellagic acid showed very high activity towards DPPH radicals with SC_{50} 2.0, 2.4 and 2.9 µg/ml, respectively (Table I). It has been postulated that the total antioxidant activity of the each extract is the sum of the individual activities of each phenolic compound present, and that also these compounds might have synergistic effects. Dorman et al. [16] demonstrated that the $OH^{\cdot}$ scavenging activities does not depend on the total phenolic content. They found that free radical scavenging activity of rosemary and thyme extracts were similar. However, total phenolic content of rosemary was 2-fold higher than thyme.

Inhibition of tyrosinase activity

The rate of tyrosinase reaction on dopamine hydrochloride was obtained at 20ºC in the absence and presence of various lavender extracts. The hyperbolic dependence of the rate on substrate concentrations confirmed nonlinear regression to the Michaelis equation.

Hot water extracts of all lavender species showed anti-tyrosinase activity depending on their concentrations, i.e. lavender species (Figure 3). The IC_{50} values for *Lavandula angustifolia*, *Lavandula stoechas*, *Lavandula dentata* and *Lavandula latifolia* extracts were 43.6, 33.3, 28.9 and 22.1 µg/ml, respectively.
Table II shows that anti-tyrosinase activity of lavender extracts are mostly lower than a known potent tyrosinase inhibitors, kojic acid (IC_{50} = 8.9 µg/ml). A variety of synthetic compounds, such as hydroquinone, hinokitiol, methyl p-hydroxybenzoate, resorcinol and L-ascorbic acid are tyrosinase inhibitors, mostly competitive type [21, 22].

Table I. Total free radical scavenging activities (DPPH assay) of lime extracts and some pure reference standards.

Lavender xtracts or pure standards	DPPH assay, SC_{50} (µg/ml)
Lavandula angustifolia	29.2 ± 1.2
Lavandula stoechas	32.5 ± 0.8
Lavandula dentata	48.7 ± 1.4
Lavandula latifolia	95.1 ± 0.9
Ascorbic acid	2.0 ± 0.11
Gallic acid	2.4 ± 0.7
Ellagic acid	3.9 ± 0.10

Table II. IC_{50} and Kinetic parameters for mushroom tyrosinase in the presence and absence of IC_{50} of lavender extracts.

Inhibitor	IC_{50} (µM)	K_m (µM)	V_{max} (µmol/min)	K_i (µM)
None	--	12.4 ± 0.14	4.8×10^{-3}	--
Lavandula agustifolia	43.6 ± 0.14	12.8 ± 0.13	4.7×10^{-3}	4.12 ± 0.09
Lavandula stoechas	33.3 ± 0.11	13.2 ± 0.15	4.6×10^{-3}	3.42 ± 0.08
Lavandula dentata	28.9 ± 0.12	14.8 ± 0.11	4.7×10^{-3}	2.27 ± 0.07
Lavandula latifolia	22.1 ± 0.08	16.2 ± 0.08	4.8×10^{-3}	2.06 ± 0.10
Kojic acid	8.9 ± 0.15	22.8 ± 0.06	4.5×10^{-3}	0.06 ± 0.05

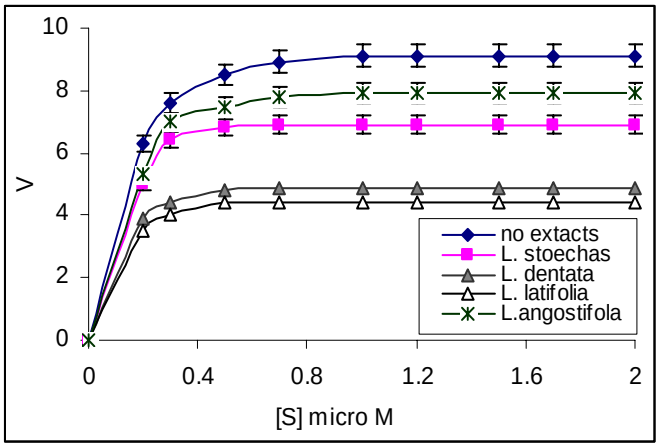

Figure 3. Dependence of rate (µM/min) on dopamine hydrochloride concentration in absence and presence aqueous extracts of various lavender species.

A competitive inhibitor binds to the active site of enzyme and increasing the substrate concentration can overcome its effect. This plot shows that the K_m value is increased in the presence of *Lavandula latifolia* extracts. The value of K_m indicates the affinity of an enzyme towards its substrates; the greater the value of K_m, the less is the affinity [23]. Lavender extracts, therefore, do not change the affinity of tyrosinase towards dopamine hydrochloride, but they reduce the rate of enzymatic reaction (V_{max}). Our kinetic studies showed that all lavender extracts inhibited tyrosinase activity also in a competitive manner.

To study the nature of inhibition, the double reciprocal Lineweaver-Burk plot obtained in the presence and absence of lavender extracts. A typical plot obtained for *Lavandula Latifolia* is presented in Fig. 4.

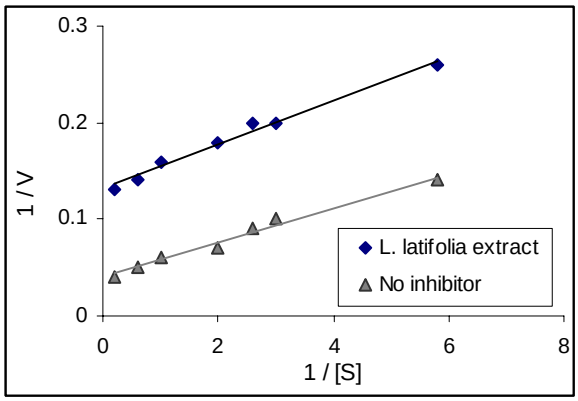

Figure 4. Double reciprocal Lineweaver-Burk plot (1/V in μM/min versus 1/[S] in μM) of inhibition of mushroom tyrosinase by *Lavandula latifolia* extracts.

Discussion

The reactive oxygen species (ROS) include superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) and accumulation of these ROS can result in oxidative stress that has been related to human diseases such as cardiovascular diseases, cancers, aging, diabetes, and atherosclerosis [17]. In addition to their use in cosmetic and food industry to prevent enzymatic browning, lavender extracts can also be used in traditional medicine as anti-tyrosinase agents. On the other hand, lavender extracts having high total antioxidant activity, are able scavenge excessive free radicals such as superoxide anion radical ($O_2^{\cdot-}$) and peroxyl radical ($ROO^{\cdot}$) in the body and protect human cells or tissues against oxidative stress.

In this study, multiple extraction and mechanical process (stirring 1 h each time, 3 times) to increase the efficiency of the extraction method. Considering that phenolic compounds are partially non-polar, it is expected that adding some percent of non-polar solvent may add the extraction yield. The reason for not using alcohols as extraction solvent was that they may exhibit anti-tyrosinase effects and traces of these compounds in the lavender extract could interfere with the results.

It is known that the total antioxidant activity of the each extract is the sum of the individual activities of each phenolic compound present. However, the SC_{50} values of four lavender species obtained from the DPPH assay was 29.2-95.1 μg/ml. These results indicate that total antioxidant activity in lavender extracts is lower than the SC_{50} of individual pure phenolic acids such as

ascorbic, gallic and ellagic acid (Table I). Therefore, individual phenolic acids are not the only contributors to the high antioxidant effects of lavender extracts. Considering the results obtained from the present study, it is expected that other polyphenolic/flavonoid plant glycosides or ellagitannins, which can be easily extracted in hot water, may also contribute to the potent antioxidant activity of these extracts. Dorman et al. [16] have also demonstrated that radical scavenging activities does not necessarily depend on the total phenolic content

It was found that, *Lavandula latifolia* extract scavenged DPPH, superoxide ($O_2^{\cdot-}$), and peroxyl ($ROO^{\cdot}$) radicals. These are the most important free radicals which can be generated as harmful by-products implicating in lipid peroxidation and some diseases [17]. The $ROO^{\cdot}$ radical is generated in normal metabolic reactions by all aerobic organisms and is also the source of the highly biologically reactive, hydroxy radical ($OH^{\cdot}$). Therefore, free radicals scavengers present in natural lavender extracts can be effective in preventing a living organism against oxidative stress. In addition, *Lavandula latifolia* extract had significant antityrosinase activity, although, its activity was lower than kojic acid. In conclusion, it is suggested that lavender extracts especially from *Lavandula latifolia* may be developed and used as natural sources of free radical scavenging and anti-tyrosinase phytochemicals to be used in pharmaceutical, food and cosmetic industries.

The results indicate that water extracts of all lavender species are competitive inhibitor for the reaction of tyrosinase on dopamine hydrochloride, its most common substrate. The double reciprocal Lineweaver-Burk plot indicated that values of K_m increased while V_{max} remained constant, typical kinetic behavior of competitive inhibitors. Numeric values of K_i, calculated from Dixon plots [24], K_m and IC_{50} for lavender extracts are presented in Table II. As the value of K_m indicates the affinity of an enzyme towards its substrates, water extracts of lavender species have decreased the affinity of tyrosinase towards dopamine hydrochloride.

Conclusions

In this study we explored some novel properties of lavender not explored so far, the antioxidant and anti-tyrosinase activity. Although, all the presented species were not potent tyrosinase inhibitors, but the nature of inhibition showed that they can be of interest in cosmetic and pharmaceutical applications. On the other hand, the free radical scavenging activity of lavender extract makes the suitable candidates to be used in many drug preparations designed for neurodegenerative diseases. Both tyrosinase inhibitor and antioxidant properties have only rarely reported in literature and more research is needed to compare the extracts of various parts of the plant, i.e. flower buds, very young shoots or wastes. It is also needed to compare different species and search for other species of lavender found in this area of the world.

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