Alfarama Journal of Basic & Applied Sciences

Faculty of Science Port Said University

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July 2024, Volume 5, Issue III

DOI: 10.21608/AJBAS.2023.248584.1197

ISSN 2682-275X

Submitted: 14/11/2023
Accepted: 14/12/2023

Pages: 309 - 319

Potential inhibition of selected plant extracts on tyrosinase activity from *Aspergillus nidulans* and their antioxidant activities

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ABSTRACT

Tyrosinase (EC 1.14.18.1) was obtained from *Aspergillus nidulans* (Eidam) G. Winter (AUMC No. 7147). It is an important enzyme in biosynthesis of melanin and hydroxylation L-tyrosine to dihydroxy-phenylalanine and oxidation to dopaquinone. Tyrosinase was purified from *Aspergillus nidulans* by ammonium sulphate precipitation, DEAE-cellulose and Sephadex G-200 with specific activity of 230.76 units mg/ protein and the molecular weight was 40 kDa. Ethanolic extracts from *Morus alba* fruits and leaves of *Chenopodium album*, *Rumex dentatus*, *Eruca sativa* and *Urtica urens* showed an inhibitory effect against tyrosinase activity. The extract from *Rumex dentatus* leaves expressed the lowest IC_{50} value (The half-maximal inhibitory concentration) (33.8%), while *Urtica urens* leaves expressed the highest IC_{50} value for tyrosinase inhibition (82.6 µg/ mL). The contents of total phenols and flavonoids are investigated in the various plant extracts. *Morus alba* fruit extract expressed the highest content of phenols (328 mg/ g) and flavonoids (241 mg/ g) compared to the other plant extracts. All plant extracts exhibited appreciable scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) method. *Eruca sativa* leaves exhibited the highest scavenging activity of 150.9 µg/ mL with DPPH and 142.4 µg/ mL with $ABTS^+$ .This present article reveals that the potential of leaf extract from *Eruca sativa* is a hopeful whitening agent of natural source versus skin hyperpigmentation.

Key Words:

Antioxidant, *Aspergillus nidulans*, inhibition, plant extracts, tyrosinase.

1. INTRODUCTION

Tyrosinase is mono-oxygenase contains copper which catalyzes the *o*-hydroxylation of monophenols to catechols (monophenolase) and the oxidation of monophenols to the corresponding *o*-quinones (diphenolase) [1].

Melanin is a dark pigment found in vertebrates, fungi, plants, bacteria and insects. There are two types of melanin: eumelanin and pheomelanin in mammals which, are responsible for yellow to pink and brown to black color [2]. The essential feature is the development of colors in the skin, hair, feathers and pupils of melanin in animals [3]. Melanin is released by the help of melanocytes located inside the basal epidermal layer [4].

Tyrosinase acts on tyrosine and L-DOPA as substrates which are different from other substrates such as phenols and diphenols that are transformed by the same enzyme to diphenols and *o*-quinones [5]. The *o*-quinone can be changed into melanin through several enzymatic and non- enzymatic reactions [6, 7].

Tyrosinase is a significant enzyme that catalyzes the rate-limiting steps of the entire pathway of melanin. The transformation occurs in the preserved active site that is included six histidine residues with two copper ions [8]. Tyrosinase is accountable for changing of beverages, vegetables and fruits into brown color that reduces its shelf-life because of formation of dark pigments and changing of the taste [9].

Aberg *et al*. [10] used tyrosinase for joining of phenolic part or protein with chitosan. Chen *et al.* [11] applied tyrosinase in conjugation of the protein gelatin to chitosan. Anghileri *et al.* [12] utilized tyrosinase to yield sericin conjugates that are found in wastewater of some industries.

Furthermore, it is important to look for the selective and the effective tyrosinase inhibitors because of resistance of the enzyme to the inhibitors from synthetic or natural sources such as hydroquinone and kojic acid [13].

The goal of the current study was to investigate some natural inhibitors of tyrosinase through investigating the influence of some plant extracts on the activity of tyrosinase. Also, the total phenol as well as the total flavonoid was measured of the five tested plants.

2. METHODS AND MATERIALS

2.1. Plant materials:

The five plant listed in Table 1 were collected from Mansoura area, washed carefully and used for extraction. These plants belong to five families.

Table 1: The tested plants and their families.

Plant	Family	Plant part
Chenopodium album	Chenopodiaceae	Leaves
Eruca sativa	Brassicaceae	Leaves
Morus alba	Moraceae	Fruits
Rumex dentatus	Polygonaceae	Leaves
Urtica urens	Urticaceae	Leaves

2.2. Methods

Experimental microorganism

Aspergillus nidulans (Eidam) G. Winter (AUMC No. 7147) was purchased from Mubasher Mycological Center (AUMMC), Assiut University, Egypt – 71516.

Fungal growth and tyrosinase production

The tyrosinase was achieved by using modified tyrosine- glucose liquid medium as described by [14]. Triplicate sets were prepared from 250-ml Erlenmeyer flasks that contain 50 mL of sterile medium (g/L): glucose 10; tyrosine 5.0; $CaCl_2.2H_2O$ 0.1; $MgCl_2.6H_2O$ 0.5, KH_2PO_4 1.0; $NaNO_3$ 1.0; $ZnCl_2$ 0.02 and $FeCl_3.6H_2O$ 0.02 were applied. The pH of the growth medium was 7.0. The medium was inoculated with 0.1 ml of spore suspension then incubated for 7 days at 30°C.

Preparation of fungal tyrosinase from *A. nidulans*

A. nidulans was grown for 7 days as described above and growth medium was filtered to separate the fungal mycelium. The filtrate was taken as the crude extract of tyrosinase and the enzyme activity was determined in the filtrate.

Purification of tyrosinase

The purification of tyrosinase from *Aspergillus nidulans* was carried out through three steps using ammonium sulphate precipitation (80%), ion exchange chromatography and gel filtration according to [15]. Ammonium sulphate was added to the crude enzyme extract up to 80 % (w/v) saturation under constant stirring at 4°C for 1h then left to settle over night at 4°C. Later, the filtrate was centrifuged at 3900 rpm for 20 min at 4°C, the precipitate was collected then desalted by dialysis against 0.5 M phosphate buffer (pH 7.0) for 24 h and the buffer was changed twice for desalting. DEAE-Cellulose column (25 × 2 cm) was equilibrated with the 0.5 M phosphate buffer, pH 7.0 [15]. The column was washed with the same buffer (pH 7.0) for collecting the unbound protein. The bound protein was 0.5 ml of NaCl in the same buffer. The enzyme fractions were eluted at flow rate of 0.5 min^{-1}. Both bound and unbound proteins were examined for tyrosinase activity and the enzyme-active fractions were pooled for the next step. A Sephadex G-200 resin was soaked in 0.5 M phosphate buffer (pH 7.0) and allowed to swell. The swollen bead is poured down into column chromatography (50 × 2 cm) and left to settle without pressure. The enzyme active sample from the DEAE-cellulose column was applied to the column of Sephadex G-200 equilibrated and eluted with 0.5 M phosphate buffer (pH 7.0) at 4°C with flow rate of 0.5 min^{-1}. Eliot (1 ml-fraction) was collected separately for the measurement of enzyme activity and protein content. Enzyme active fractions were pooled and the enzyme purity was verified by SDS-PAGE according to [16]. Purity of tyrosinase was checked by SDS – PAGE according to protocol proposed by [16]. The molecular weight of tyrosinase from *A. nidulans* was determined using standard markers with wide range of molecular weights from (18-66 kDa).

Assay of tyrosinase activity

The reaction mixture for assaying of tyrosinase included in 3 ml of 150 mM of phosphate buffer, pH 7.0, 0.5 mg of the purified enzyme protein and 0.3 mM of 3, 4-dihydroxyphenylalanine (L-DOPA) as substrate. After incubation for 15 min at 37°C, the dopachrome production was assayed by reading the absorbance at 475 nm. One unit of tyrosinase activity is the amount of the enzyme that catalyzes the production of one µmole of dopachrome in one min. The dopachrome resulted in the reaction was measured. The specific activity of tyrosinase was expressed according to [17].

Plant extract preparation

The used plants were *Morus alba* (fruits), *Chenopodium album* (leaves), *Rumex dentatus* (leaves), *Eruca sativa* (leaves), and *Urtica urens* (leaves). Each plant material was cleaned with water and then frozen at -80°C. The plant material was dried by air and powdered. All plants samples were extracted with 95 % ethanol and the mixture was stirred for 10 h at room temperature then the resulting homogenate was filtered using muslin. Rotary evaporator was used for concentration of plant extract and then kept at 2ºC [18].

Inhibition of tyrosinase by plant extracts

Tyrosinase (100 units/ mL) was mixed with 150 mM of phosphate buffer (pH 7.0) and 0.3 mM of 3, 4-dihydroxyphenylalanine (L-DOPA) as substrate. Each plant extract was added at 80 mg m/ L and the mixture was incubated for 2h at 37°C. The absorbance was recorded at 475 nm after 2h of incubation followed by measuring the enzyme activity as mentioned above [19].

The inhibition percent (%) = (treated enzyme/ untreated enzyme) ×100**.**

Determination of total phenolic content

The content of total phenol was measured according to Folin–Ciocalteu [20]. Overall, 50 µL of extract solution was added to 1.16 ml distilled water and 100 µL of the reagent, followed by 300 µL Na_2CO_3 solution (200 g/L). The mixture was incubated at 40°C in a water bath for 30 min and the absorbance was recorded at 760 nm. Gallic acid was applied for preparing of standard curve. The total phenolic content was calculated from standard curve of gallic acid.

Determination of the total flavonoid content

The total flavonoid content was determined by $AlCl_3$ method [21]. Sample of plant extract was mixed with 1ml of potassium acetate, 3 mL distilled water and 1 mL of 10 % $AlCl_3$. The mixture was incubated at room temperature for 30 min and then the absorbance was recorded at 415 nm. The total flavonoid content was determined from the standard curve of catechin.

Assay of antioxidant activity

The scavenging activity of each plant extract was measured by 2, 2′-azinobis-(3- ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) and 2, 2- diphenyl -1- picrylhydrazyl (DPPH) [22, 23] and Trolox was used as standard. The % scavenging activity was calculated according to the following calculation:

Scavenging activity (%) $[(A_0 – A_1) / A_0] \times 100$

Where A_0 and A_1 are the absorbance values in the absence and presence of the test sample, respectively.

Statistical analysis

All the results in this investigation are the mean of three measurements ± SD.

3. RESULTS

Purification of tyrosinase from *A. nidulans*

The isolated enzyme was purified using ammonium sulphate precipitation (80%), DEAE-cellulose and Sephadex G-200. The results in Table 2 reveal that the purification procedure was successful and the final specific activity from Sephadex G-200 column was 230.76 units mg/ protein. The molecular weight of tyrosinase from *A. nidulans* was 40 kDa Fig. 1.

Table 2. Purification of tyrosinase from *A. nidulans*.

Purification Step	Protein (mg)	Total Units (U)	Specific Activity (U mg/ protein)	Purification Fold	Yield (%)
Crude extract	600	1043	1.73	1	100
Ammonium sulphate (80%)	105	846	8.05	4.65	81.11
DEAE – Cellulose	11	266	24.18	13.97	25.50
Sephadex G-200	0.13	30	230.76	133.38	2.87

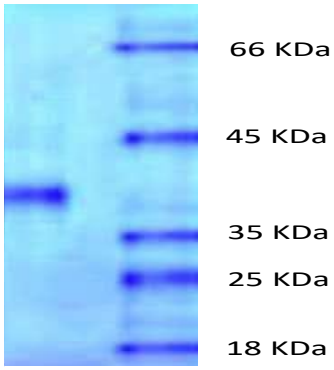

Fig. 1: SDS-PAGE of purified tyrosinase from *Aspergillus nidulans*.

Tyrosinase inhibition

Table 3 indicates the tyrosinase was inhibited by various plant extracts. The ethanolic plant extracts expressed appreciable effect in the inhibition process. It is apparent that ethanolic extract of *Rumex dentatus* leaf was the most potent inhibitor (80%) followed by *Morus alba* fruit extract (59%), *Chenopodium album* leaf extract (40.8%), *Eruca sativa* leaf extract (37.8%) and later *Urtica urens* leaf extract (21.2%) .

Table 3. Inhibition of tyrosinase by various plant extracts.

Plant extracts	Part	% inhibition
Chenopodium album	Leaves	40.8 ± 0.8
Eruca sativa	Leaves	37.8 ± 0.7
Morus alba	Fruits	59.8 ± 0.9
Rumex dentatus	Leaves	80.0 ± 1.3
Urtica urens	Leaves	21.2 ± 0.5

IC_{50} for inhibition of tyrosinase

The IC_{50} of tyrosinase inhibition by the different plant extracts were calculated as shown in Table 4. The results designate that *Rumex dentatus* leaf extract was the most effective inhibitor since it expressed the smallest value of IC_{50} (33.8 µg/ mL) for enzyme inhibition.

Table 4. The IC_{50} for tyrosinase inhibition by various plant extracts.

Plant extracts	Part	IC_{50} (µg/ mL)
Chenopodium album	Leaves	57.6 ± 1.0
Eruca sativa	Leaves	76.1 ± 1.2
Morus alba	Fruits	44.3 ± 0.8
Rumex dentatus	Leaves	33.8 ± 0.6
Urtica urens	Leaves	82.6 ± 1.5

The total phenolic content

The content of the total phenolic of the tested plant extracts is recorded in Table 5. *Morus alba* fruit extract showed the highest total phenol content (328 mg/ g) followed by *Eruca sativa* leaf extract (70.0 mg/ g), *Rumex dentatus* leaf extract (40.0 mg/ g), *Chenopodium album* leaf extract (36.4 mg/ g) and *Urtica urens* leaf extract (25.0 mg/ g).

Table 5. Total phenolic content in the tested plant extracts.

Plant extracts	Part	Total phenols (mg/ g)
Chenopodium album	Leaves	36.4 ± 0.4
Eruca sativa	Leaves	70.0 ± 1.3
Morus alba	Fruits	328 ± 2.8
Rumex dentatus	Leaves	40.0 ± 0.8
Urtica urens	Leaves	25.0 ± 0.5

The total flavonoid content

The content of the total flavonoid for the tested plants was shown in Table 6. *Morus alba* fruit extract expressed the highest content (241 mg/ g) followed by *Eruca sativa* leaf extract (30.0 mg/ g), *Chenopodium album* leaf extract (19.0 mg/ g), *Rumex dentatus* leaf extract (13.3 mg/ g) and *Urtica urens* leaf extract (12.4 mg/ g).

Table 6. Total flavonoids content in the tested plant extracts.

Plant extracts	Part	Total flavonoids (mg/ g)
Chenopodium album	Leaves	19.0 ± 0.5
Eruca sativa	Leaves	30.0 ± 0.6
Morus alba	Fruits	241 ± 2.8
Rumex dentatus	Leaves	13.3 ± 0.4
Urtica urens	Leaves	12.4 ± 0.5

4. DISCUSSION

Tyrosinase is a copper-containing enzyme fruitfully used as an inhibitor for the treatment of melanogenesis and it's a key enzyme target to plan new chemical ligands as anti-melanogenesis [2].

Tyrosinase was purified from *Aspergillus nidulans*. The final specific activity from Sephadex G-200 column was 230.76 units mg^{-1} protein. Low specific activities of 63.7, 52.2, 46.4, 28.1 and 23.2 units mg^{-1} protein was reported for tyrosinase from *Fusarium solani* [24], mushroom [25], *Pleurotus ostreatus* [26], *Aspergillus terreus* [27] and *Penicillium capticola* [28], respectively. The purity of tyrosinase was confirmed by SDS-PAGE which exhibited a single band at 40 kDa. Lower molecular weight of 35 kDa [27] was reported for the enzyme from *Aspergillus terreus* and *Penicillium capticola* [28]. Higher molecular weights (62, 75 and 95 kDa) were reported for the enzyme from *Helianthus tuberosus* [28], *Pleurotus ostreatus* [26] and mushroom [25].

The importance of finding natural inhibitors of tyrosinase with antioxidant activity is of enormous awareness. Because tyrosinase has a crucial role in melanin biosynthesis, the research for natural inhibitors of this enzyme has become progressively significant for cosmetic pharmaceuticals that might be applied for whitening of skin and in skin disorders. There is still a serious need to obtain compounds that are both safe and effective without any obvious side effects that could be commonly practical in cosmetology, industry, medicine and food [2].

Extracts from each tested plant inhibited tyrosinase activity; however the rate of inhibition varied from extract to another. *Rumex dentatus* leaf extract was the most potent inhibitor followed by *Morus alba* fruit extract. In support, tyrosinase from mushroom was inhibited by *Morus nigra* leaf extract [29]. Also, *Morus alba* leaf extract inhibited tyrosinase from *A. nidulans* [30] and *Chenopodium album* leaf extract inhibited tyrosinase from human epidermal melanocytes (HEM) cell culture [31]. *Morus* species are famous as plants rich in polyphenols and its extracts have been used as a non-toxic natural therapeutic agent, which also have high capacity in applications as skin-whitening agents due to many potent tyrosinase inhibitors being isolated from different parts of this plant [32].

Eruca sativa leaf extract was reported to retard the protein expression of tyrosinase and the authors suggested that *Eruca sativa* leaf extract inhibited melanin biosynthesis through inhibiting tyrosinase expression and tyrosinase activity. Thus, the results in the present work indicate that *Eruca sativa* leaf extract can be an applicable source for cosmetics in skin whitening [33].

It is reported that polyphenols and flavonoids are inhibitors of reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical and H_2O_2 and thus could be used as anti-melanogenic activity [34]. Phenolic compounds having at least one fragrant ring and one (or more) hydroxyl group may be considered primarily based on the quantity of their carbon atoms and connection among them [35]. There is a big variety of phenolic compounds from small to large complicated polyphenols varying by their molecular weight and mostly all of these products are promising inhibitors of melanogenesis [36]. The flavonoid derivatives that are mainly present in natural plants are used as the best tyrosinase inhibitors

[37, 38]. It has been reported that there is a correlation between the inhibitory efficiency of flavonoids on tyrosinase and synthesis of melanin in melanocytes [39].

The inhibition of tyrosinase is important for accumulation of melanin in skin. Thus, tyrosinase inhibitors are essential in synthesis of cosmetics for skin disorders. The tested plant extracts may be applied for preparations of products with bioactive phytochemicals or for production of new drugs with less toxic side effects compared with the artificial compounds.

All the tested plant extracts expressed antioxidant scavenging activity using DPPH and $ABTS^{+}$ and this is possibly due to their contents of total phenol and total flavonoids which are famous non-enzymatic antioxidant [40, 41].

5. CONCLUSION

In conclusion, tyrosinase was purified successfully from *Aspergillus nidulans*. The results illustrated that ethanolic extracts from *Morus alba* fruits, *Chenopodium album* leaves, *Rumex dentatus* leaves, *Eruca sativa* leaves and *Urtica urens* leaves have considerable tyrosinase inhibitory activity. This effect is more or less similar to the standard inhibitors of tyrosinase. Moreover, the ethanolic extract of the tested plants exhibited appreciable scavenging activity due to presence of phenols as well as flavonoids as pointed by the results. These results suggest that the tested plant extracts could be applied as supplier for bioactive phytochemicals to control hyperpigmentation and for whitening of skin.

Recommendations:

The results recommend to use the plant extract as inhibitors for fungal tyrosinase to avoid pigment formation.

Conflict of interest:

The authors declare that there is no conflict of interest.

Fund: No fund

Data availability: Available under request

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