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Research Paper

Humic and Fulvic Acids Induced Thermodynamic and Structural Instability of Tyrosinase With Antiproliferative Effect on A375 Melanoma Cancer Cell Line

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ABSTRACT

Background: The tyrosinase enzyme catalysis monophenols to melanin pigments through the melanogenesis process. For this reason, various inhibitors have been studied for enzyme regulations in melanogenesis abnormalities in both the food and cosmetics industries. In this study, the effect of humic acid (Hu) and fulvic acid (Fu) on the structure, activity, and stability of mushroom tyrosinase (MT) was investigated.

Methods: These two organic acids are the main components of soil humus. Assessment of the thermodynamic and structural stability of enzymes was obtained through thechemical and thermal denaturations and (8-anilino-naphthalene sulfonic acid) ANS fluorescence analysis. The Hu and Fu impact on A375 melanoma cancer cell viability was achieved by MTT assay.

Findings: The results of enzyme half denaturation concentration (Cm), melting points (T_m), ΔG^0 values and external fluorescence emissions in the presence of Hu and FA proved the reduction of the thermodynamic and structural stability of MT by these compounds. The anti-proliferation effects of the compounds were confirmed by the inhibitory concentrations of 50% (IC_{50}) of 31.5 and 42.7 µM and 12.5 and µM at time points of 24 and 48 hours treatments of the A375 melanoma cell line by Hu and Fu, respectively.

Conclusion: Humic and fulvic acids can be expected to contribute to advancing skin disorder science play a crucial role in tyrosinase related disorders and anti-cancer effects, and good candidates for medical applications.

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1. Introduction

The Tyrosinase enzyme has been studied as a suitable model for various scientific topics. The first studies on this enzyme began in 1895 when the damaged edible mushroom was exposed to the air [1]. It was later named tyrosinase because of color changes [2]. The tyrosinase enzyme is a structure of copper-containing polyphenol oxidase that is involved in the process of melanogenesis. This enzyme is widely found in plants and animals [3]. This enzyme is responsible for catalyzing the two continuous reactions of monophenolase and diphenolase. The cresolase activity occurred through the orthohydroxylation of monophenols, and in the catecholase reaction, ortodiphenols were oxidized to otoquinon [4, 5]. Today, the known structure of tyrosinase is Agaricus bisporus, which is a hetero-tetramer of two heavy chains and two light chains with a total molecular weight of 120 kDa [6].

Tyrosinase plays a crucial role in the formation of melanin and processes such as internal pigments and their browning in nature. Browning is the cause of changes in the properties of food products [7]. Normalin formation in the brain is another critical role of tyrosinase that can be used to control dopamine and be associated with neuronal production in the brain. It may also be associated with Parkinson disease [8]. Tyrosinase is also used in immunoassay as an antigen to diagnose melanoma [9]. The role of this enzyme is known in wound healing, defense response, and important physiological processes. Melanin produced by tyrosinase changes the color of the hair and the surface of the body. Thus, it protects the skin and eyes, resists ultraviolet rays, and prevents overheating internal tissues [10]. Mammalian tyrosinase is commonly found in melanocytes, which are pigment-producing cells found in the hair follicles, skin, and eyes [11, 12]. Tyrosinase function is affected and causes disorders such as epilepsy and albinism when it is diminished or deleted. Tyrosinase deficiency is connected with recessive autosomal disorders in both animals and humans [13].

Among many applications of tyrosinase inhibitors, the cosmetic industry can be mentioned, which are used due to their whitening effects on the skin. Kojic acid, hinocytol, natural hydroquinones, and cresolase are effective bleaching inhibitors, but they also have side effects. Aloesin and arbutin are now widely used in the cosmetics industry. These compounds have the inhibitory power of human tyrosinase [14].

As depicted in Figure 1, humic acid (Hu) and fulvic acid (Fu) are among two important molecules with crucial biological functions. Hu has a variety of roles in the biological world; for example, by increasing cell wall permeability and accelerating the production of proteins and nucleic acids within the cell and other processes. It also helps the growth and proliferation of any other living organism. Hus is a group of large molecules and heavy polymers formed from the decomposition of organic matter, especially dead plants and animals. This molecule is also an influential factor in soil fertility. Since research on this molecule has determined that its structure contains active, weakly acidic, carboxyl benzoic, and phenolic agents, which give Hu a superior chelating quality, in addition to high-capacity cation exchanges, heavy ions should also be considered. These macromolecules are soluble in alkali and insoluble in water, acids, and alcohols [15]. Significant effects of Fu products include the prevention of gastric ulcers, seizures, and allergic reactions. They also have an active role in combating tumor cells. Fu is a yellow-brown substance found in natural materials such as peat, soil, coal, and water or lake streams. Fu is formed when plants and animals break down. Today, it is added to anti-dandruff shampoos and skin creams to treat acne, eczema, and psoriasis. It also has anti-inflammatory and anti-bacterial effects, although these are among the effects of local Fu treatment. This substance is easily absorbed into the bloodstream. Fu can remove all toxins from the body, so it is now considered the first line of defense in treating skin diseases. Traditional medicine claims Fu can rehabilitate muscles, bones, and nerves and treat signs of aging such as arthritis, diabetes, allergic manifestations, and insanity [16]. Finding new candidates for anti-browning to avoid low-cost agricultural products and new cosmetic and whitening agents for controlling melanogenesis and the medical drugs for inhibition of skin unwanted browning and hyperpigmentation led us to design this study on Hu and Fu assessments on tyrosinase thermodynamic and structural stability and their impact on a cellular melanoma cancer A375 cell line.

2. Materials and Methods

Mushroom Tyrosinase (specific activity 3,600 units/mg, EC 1.14.18.1), L-DOPA, Hu, and Fu were purchased from Sigma-Aldrich. Hu and Fu powders are soluble in phosphate buffer (PBS; Na_2HPO_4 and NaH_2PO_4; 10 mm; pH 6.8). In this study, isopropanol (2-propanol) was used to prepare enzyme and substrate solutions. All substances, such as enzymes, substrates, and inhibitors, were used in freshly prepared solutions.

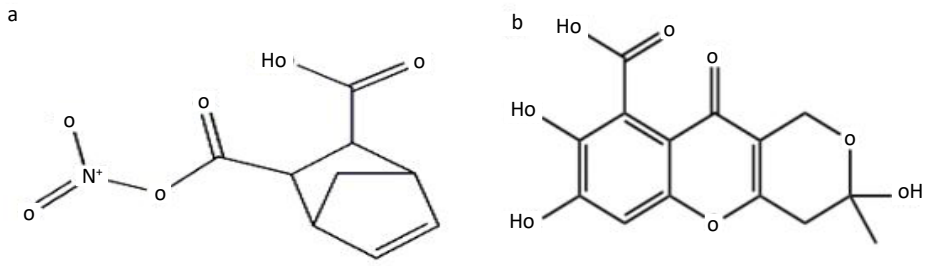

Figure 1. Molecular structure of humic

a) Fulvic; b) Acids.

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Enzyme thermal denaturation with differential scanning calorimetry

Thermal denaturation of the tyrosinase enzyme was performed in the presence of Hus and Fus using differential scanning calorimetry (DSC). This method can be used to obtain thermodynamic parameters of ligand-protein interactions. DSC was performed using Nano DSC 3 (TA Instrument USA), and data analysis was performed using TM-Nano analysis software in the presence of MT (40 units), HA, and FA concentrations of 12 µM. Finally, after incubation in the cuvette for 3 min, thermal denaturation spectra were obtained at a rate of 1˚C per minute from 273-373K, as depicted in Figure 2. The changes in protein molar heat capacity (Cp) were illustrated at temperatures ranging from 273 to 373 K. In thermal denaturation, the melting point of a protein (Tm) is the temperature at which half of the proteins are denatured.

Enzyme chemical denaturation with urea

A Double beam UV 2100 model spectrophotometer was used to assess enzyme chemical denaturation by urea at an optical density of 280 nm. Gradual titration of the enzyme with urea as a chemical denaturant was used to evaluate enzyme chemical stability with and without the presence of Hus and Fus. The stability of the enzyme under chemical stress conditions was evaluated, and the free denaturation energy of Gibbs in the absence of denaturant (ΔG_{H2O}), as an indicator of the chemical stability of the enzyme, was obtained based on a two-state model of denaturation (Equation 1).

1. *Native (N)<=>Denatured (D)*

Assuming a two-state mechanism for enzymatic denaturation, the process can be explained by monitoring changes in enzyme absorbance at titration with urea and calculating the denaturation fraction (F_d), and determin-

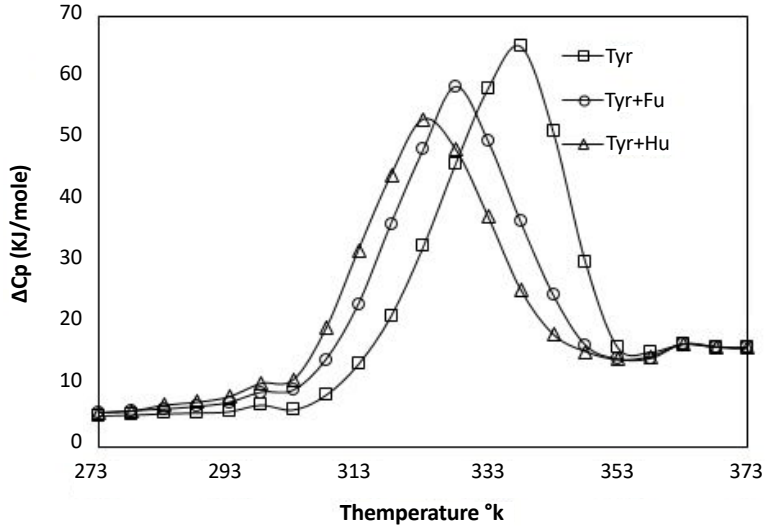

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Figure 2. Thermal denaturation of tyrosinase with and without the presence of humic and fulvic acids and the ΔCp spectra obtained using differential scanning calorimetry technique

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ing constant equilibrium (K), as shown below (Equation 2 and 3).

2. $F_d = \frac{(Y_N - Y_{obs})}{(Y_N - Y_D)}$

3. $\frac{(Y_N - Y_{obs})}{(Y_{obs} - Y_D)} = \qquad K = \frac{F_d}{(1 - F_d)}$

Y_{obs} is the observed absorption variable parameter, and Y_N and Y_D are the absorption values for the enzyme in both normal and denatured states, respectively. The following equation for the Gibbs free energy standard (ΔG^0) for enzyme denaturation is given (Equation 4):

4. $\Delta G^0 = -RT\ln K$

In the above equation, R is the universal constant of gases, and T is the absolute temperature. ΔG^0 changes linearly with temperature (T) in a finite region.

From the following equation, the valuesof ΔG_{H2O} and $D_{1/2}$ are calculated in the denaturation conditions for the enzyme alone and the enzyme in the presence of Fu and Hu (Equation 5).

5. $\Delta G^0 = \Delta G_{H2O} - m\,[D]$

Thermodynamic parameters such as ΔG (Gibbs free energy), ΔH (enthalpy), and ΔS (entropy) can be calculated by the main thermodynamic Gibbs free energy equation as below (Equation 6):

6. $\Delta G^0 = \Delta H - T\Delta S$

ANS external fluorescence as a structural analysis of the enzyme

A solution of 40 mM ANS in distilled water and deionized water was prepared and stimulated at 350 nm. After stimulation of the enzyme incubated with Hu and Fu and 50 µM ANS at 350 nm, the fluorescence emission spectra of solutions were achieved at 400-550 nm using a spectrofluorometer (Cary Eclipse model bio-100). These spectra were recorded in the presence of ANS alone (50 µM), ANS, and enzyme (concentration 0.17 mg/mL). ANS plus enzyme was incubated with Hus and Fus at two concentrations of 2 and 8 µM in 10 mM PBS buffer at pH=6.8 and 25°C.

MTT assay and cell viability of A375 melanoma cancer cell line

The MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as a vital compound that is catalyzed by mitochondrial succinate dehydrogenase has been widely used in cell viability assessment. The MTT assay is a colorimetric method by which the cell cultures were incubated for 2 hours in a culture medium or a Krebs–Hensleit–HEPES buffer (5 mM KCl, 115 mM NaCl, 2 mM CaCl2, 1.2 mM MgSO4, 1 mM KH2PO4, and 25 mM HEPES at pH 7.4) containing 0.5 mg/mL MTT. The incubation buffer was removed after 2 hours, and the blue MTT-formazan product was extracted with acidic isopropyl alcohol (0.04 N HCl). The absorbance of the formazan solution was measured by spectrophotometric technique at 570 nm wavelength after 30 minutes of extraction at room temperature.

3. Results

Thermodynamic parameters from thermal and chemical denaturation analysis

The enzyme thermal denaturation using the DSC technique showed changes in ΔCp values as the protein heat capacity in a stationary pressure in the presence and absence of Hus and Fus (Figure 2). From the thermodynamic equations mentioned in methods, the analysis of ΔCp spectra in Figure 2 resulted in the thermodynamic parameters including Tm (melting point of enzyme), ΔG (Gibbs free energy), ΔH (enthalpy), and ΔS (entropy), with their magnitudes reported in Table 1. The chemical denaturation of the enzyme with urea was determined based on the changes in its absorption spectrum at 280 nm with and without the presence of Hus and Fus (Figure 3a). These sigmoidal plots of the two-state chemical denaturation process were further analyzed for achieving the ΔG_{H2O} and $D_{1/2}$ or Cm values in Figure 3b and the values were reported in Table 1. Gibbs free energy of enzyme denaturation at standard conditions (ΔG^0) is used as a criterion for the structural stability of tyrosinase.

Enzy m e structural analysis with ANS external fluorescence

The effect of Fus and Hus on the structure of tyrosinase was investigated by measuring fluctuations in extrinsic protein fluorescence (Figure 4a-b). The figures show the enzyme tertiary structure changing with the presence of acids resulting, in a gradual increase, in ANS emission inten sities. This external fluorophore has an emission spectrum between 400 and 550 nm after exciting with

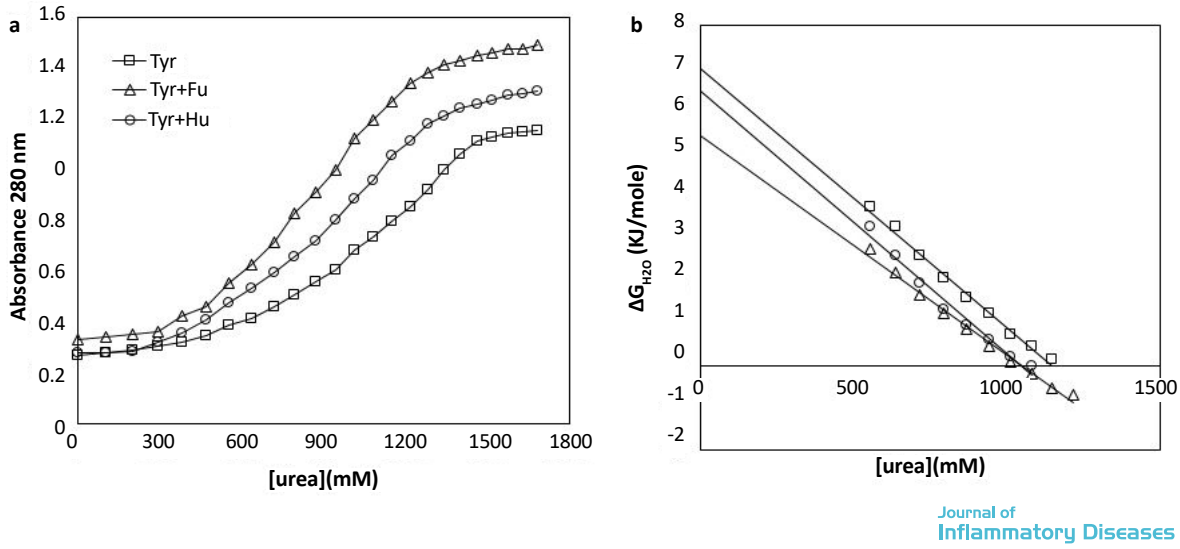

Figure 3. Chemical denaturation of tyrosinase with and without the presence of humic and fulvic acids in the enzyme absorption at 280 nm and titration with different concentrations of urea

a) The sigmoidal spectra of the enzyme; b) The secondary Plots of Gibbs free energy of denaturation.

350 nm. ANS alone and with the enzyme show emissions, and its interaction with the enzyme hydrophobic patches increases its emission intensities.

MTT assay and antiproliferative of humic acids and fulvic acids on the A375 melanoma cell line

The survival rate of the A375 cell line was investigated under the treatment of 10-60 µM Hus and Fus concentrations in different periods of 24 and 48 hours. As illustrated in Figures 5a and 5b, the cell viability of the A375 cancer cell line was affected by the treatment with different concentrations of these compounds in a time and concentration-dependent manner. Finally, the IC_{50} values were obtained at 31.5 and 42.7 µM for Hu and 12.5 and µM for Fu at time points of 24 and 48 hours, respectively.

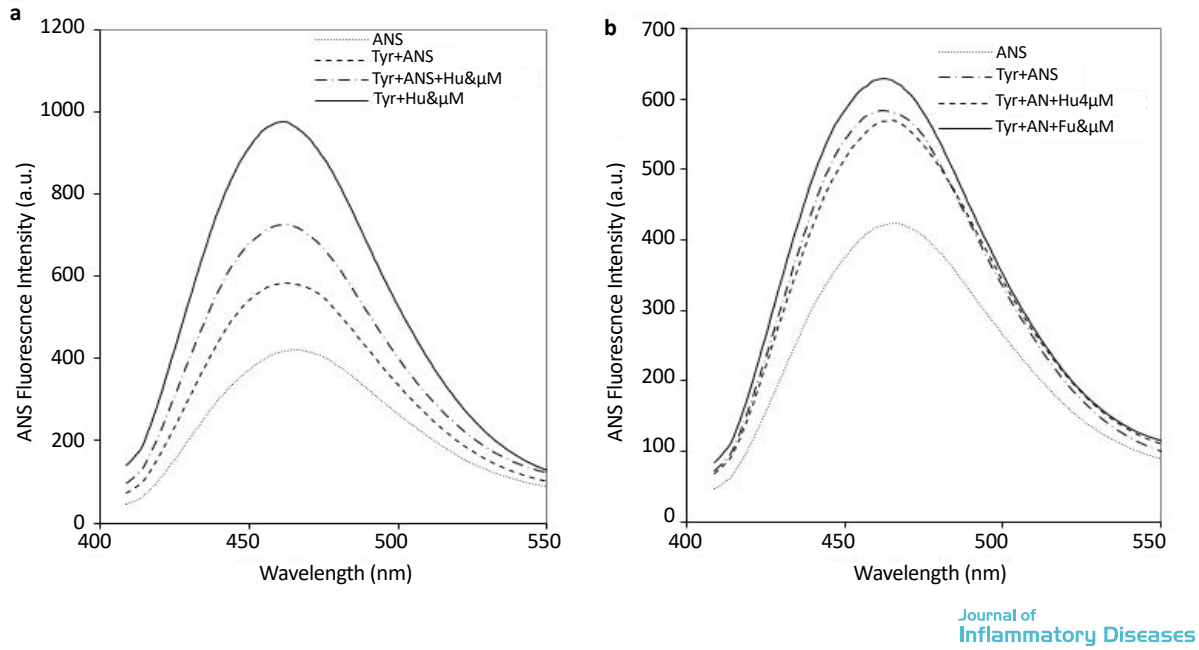

Figure 4. External emission of ANS fluorescence after its incubation with enzymes and the presence of humic

a) Fulvic; b) Acids.

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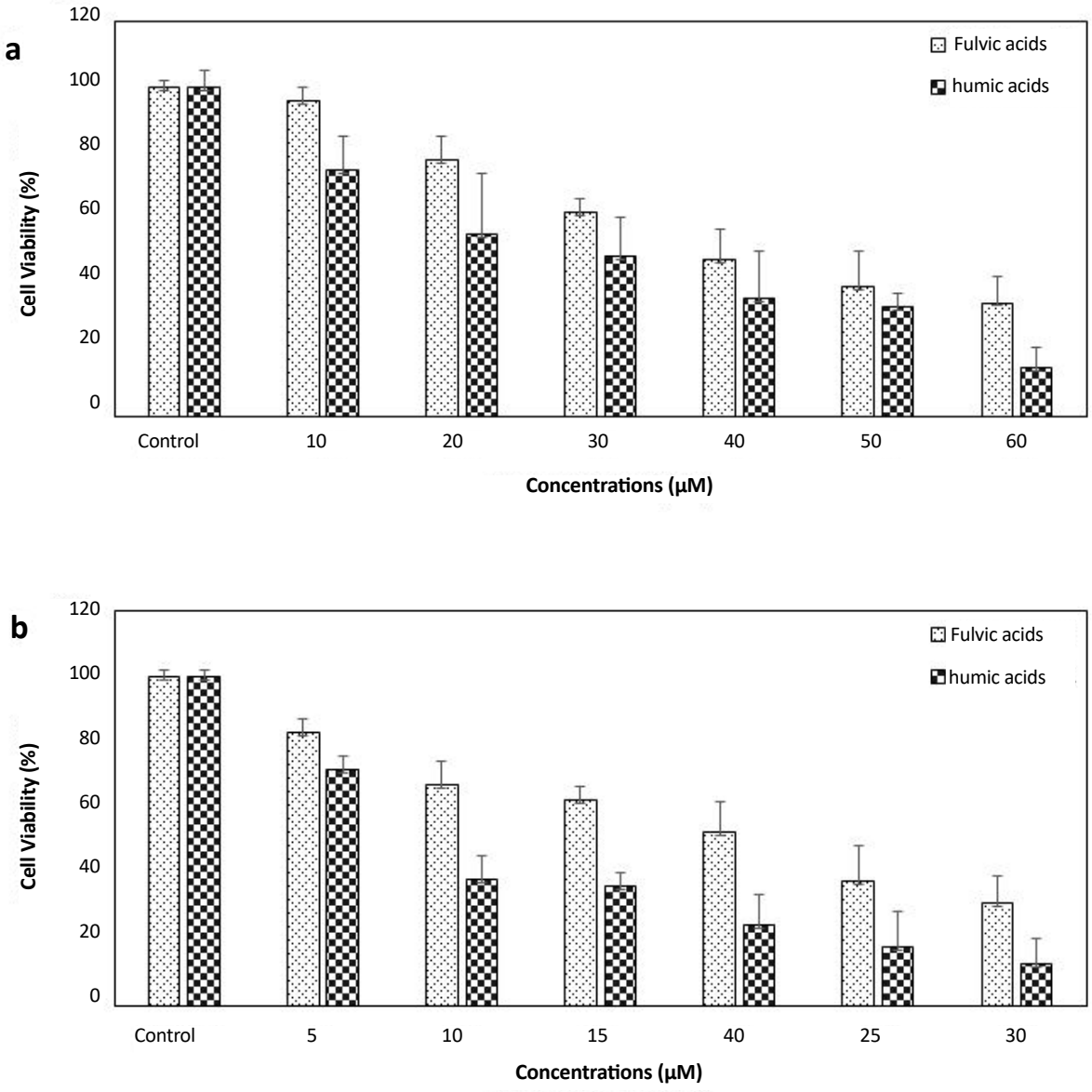

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Figure 5. The change of A375 melanoma cancer cell line viability by MTT assay in the presence of different concentrations of humic

a) Fulvic acids at 24 hours; b) 48 hours acids.

4. Discussion

The tyrosinase thermodynamic and its structural stability were affected by Hus and Fus as two important natural substances. The reduction in both structural and thermodynamic stabilities was obtained by assessment of different parameters such as Tm, Cm, ΔG^0, ΔH, and ΔS. In line with the above effects of Hus and Fus on tyrosinase as the major enzyme of melanogenesis, from a cellular point of view, their anticancer and antiproliferative effects were proven on the A375 melanoma cell line by MTT assay. Mushroom tyrosinase is a good model system for investigations of the experimental analysis of enzyme inhibitors interactions and generalizing the results for human melanogenesis and related skin cancer disorders [17, 18]. Besides, the assessment of tyrosinase activity in our previous study showed inhibitory effects of Fus and Hus with Ki of 2.02 and 5.2 µM, respectively [19]. The Cp spectra in Figure 2 which were obtained by the DSC technique were used for calculating the Tm as the major thermal stability parameter of the enzyme in Table 1. The Tm values of 338K for the sole enzyme and 323 and 328 for the enzyme incubated with Hus and Fus proved the induction of tyrosinase instability

Table 1. Thermodynamic parameters of tyrosinase from chemical and thermal denaturation in the absence and presence of humic and fulvic acids

Complex	Chemical Denaturation		Thermal Denaturation			
	ΔG_{H2O}(kJmol^{-1})	Cm (M)	Tm (K)	ΔG (kJmol^{-1})	ΔH (kJmol^{-1})	ΔS (Jmol^{-1}K^{-1})
Enzyme	7.02	1.15	338	-	-	-
Enzyme+Hu	5.44	1.05	323	0.98	32.5	0.1
Enzyme+Fu	6.5	1.05	328	0.2	194.5	0.59

Hu: Humic acid; Fu: Fulvic acid.

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with these natural compounds. The same results were obtained after the chemical denaturation of the enzyme with the urea and $[\Delta G]_{H2O}$ and Cm values from Figures 3a and 3b, which are depicted in Table 1, emphasizing the induction of chemical instability of Hus and Fus. These figures show the sigmoid curve of denaturation of the compounds, in which the enzyme is unfolded through a two-state manner of denaturation. According to a theory developed by Pace et al., Gibbs free energy for denaturation (ΔG^0) was calculated as a measure of the structural stability of a spherical protein [20-22]. Besides, the change in thermodynamic and chemical stability of tyrosinase, its structural analysis with ANS as the tag of hydrophobic patches is illustrated in Figures 4a and 4b. External fluorescence spectroscopy of the enzyme showed a gradual increase in ANS emission with increasing concentrations of Hus and Fus, which indicates a partial unfolding and fluctuation of the tertiary structure of the enzyme. ANS is a substance that tags a hydrophobic patch in the hydrophobic domain of proteins structure; in denaturation studies, as the structure of the protein becomes more open and the denaturation increases, the emission of ANS also increases, and the higher the amount of this intensity, the greater the denaturation. In this study, this increase indicates the instability of the enzyme structure under the influence of Hus and Fus. As reported in Table 1, a decrease in Tm and ΔG^0 values was observed in the combination of the enzyme with the mentioned acids, which indicates a decrease in its resistance to thermal and chemical denaturation, decreasing the thermodynamic stability of the enzyme. These results are in agreement with the other biological compounds on mushroom tyrosinase thermodynamic and structural stability. In this regard, kinetics and thermodynamic parameters of the native tyrosinase enzyme and modified enzyme were reduced after it incubated pyruvic, acrylic, 2-oxo-butanoic, 2-oxo octanoic, and propanoic acids [23].

Furthermore, mono-unsaturated fatty acids (UFAs) produced a decrease in the secondary structure of recombinant S100A8/A9, whereas arachidonic acid generated less instability in the protein structure than the other UFAs studied. These findings could be related to the number of double bonds in UFAs [24]. The tyrosinase activity with gallic acid showed a non-competitive manner, but the chrysin, naringin, and quercetin induced a competitive type of inhibition [25]. From the results of this study using Hus and Fus and the above-mentioned references, the use of biological compounds is a strategy for the control of tyrosinase activity in both melanogenesis and its gene expression in all skin-related disorders. So, decreasing tyrosinase activity has been targeted to prevent and improve skin hyperpigmentation, such as melanoma and age-related blemishes. In another research, the recombinant S100A8/A9 (calprotectin) and its modified form reduced the activity of tyrosinase by modifying the structure, which can control malignant melanoma, the most dangerous type of skin cancer [26]. Moreover, the inhibitory effect of Cu^{+2} and Ni^{+2} cations on tyrosinase, and the results of this study showed that both cations make tyrosinase more fragile and less active [27].

The previous works showed that tyrosinase and the tyrosinase-related proteins 1 and 2 as the main proteins involved in melanogenesis were overexpressed in the A375 melanoma cell line [28, 29]. Tyrosinase, as a glycoprotein enzyme, is one of the most important markers of malignant melanoma [30]. The action of tyrosinase downstream is regulated by a microphthalmia-associated transcription factor, which is an oncogene in the melanoma malignancy process [31]. As presented in Figures 5a and 5b, the treatment of the A375 melanoma cancer cell line with different concentrations of Hus and Fus at time points of 24 and 48 hours confirmed the antiproliferation effects of the compounds with IC_{50} of 31.5 and 42.7 µM and 12.5 and µM, respectively. There are various reports on the role of tyrosinase in the control of

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cancer, e.g. the role of tumor suppressor for edible fungal tyrosinase (mushroom tyrosinase) which was used in this study, and others have reported mutagenic properties for this protein. Gamma-L-glutaminyl-4-hydroxybenzene (GHB) is a phenolic substance that is converted by tyrosinase to a quinone and a by-product of its oxidation that blocks both mitochondrial energy production and nucleic acids and proteins synthesis [32]. Treatment of two cell cultures containing melanoma and leukemia cells with purified quinone eliminated tumor growth. In the presence of GHB, suppression of tumor growth is seen in melanoma cells, while in leukemia cells, tumor growth continues due to a lack of tyrosinase. The toxic effect of GHB is dependent on the presence of tyrosinase [33]. Also, there are reports of a negative effect of tyrosinase on cancer. For example, a study has shown that tyrosinase increases the mutagenicity of edible fungi. This may be due to the production of phenolic and quinone compounds. This mutagenicity can be eliminated by catalase, superoxide dismutase, glutathione, and dimethyl sulfoxide, which confirms the role of phenolic and quinone compounds in the production of active oxygen [34]. Increased mutagenicity has also been reported in samples of cooked edible mushrooms [35]. Aromatic hydrazine plays an important role in fungal carcinogenesis, so studies have been performed on the relationship between hydrazine and edible fungal tyrosinase [36, 37]. Other studies on the mutagenicity of agaritine metabolites in the presence of the fungal tyrosinase show that among these metabolites, the tyrosinase mutagenicity of N-acetyl-4- (hydroxymethyl) phenylhydrazine raises [38]. So, with the above different roles of tyrosinase enzyme and its related metabolites in different levels of life span, focusing on its new biological inhibitors is a good strategy to control its unwanted roles. It should be noted that in previous research, a similar study was conducted in 14 hours, and Hus and Fus showed antiproliferative and anticancer effects [39]. In line with this study, other studies have shown various effects of Hu in health promotion such as ultraviolet protective properties, anti-inflammatory, anti-neoplastic, anti-oxidant, and pro-apoptotic effects in some other cancer cell lines [40].

According to a new study, Fu efficiently suppresses the cell survival of human cancer cells lines such as Hep3B, HL60, LNCaP, and MCA-102 fibrosarcoma cells [41, 42]. According to the latest studies, Fu and Hu are effective in the regulation of apoptosis procedures in some cell lines such as cervical cancer cells [43], Hep G2 [44], and HL-60 [45].

5. Conclusion

The overall results from this study show the effects of Hu and Fu on lowering the chemical and physical stability and partially unfolding tyrosinase structure in an in vitro study and their treatments on A375 melanoma cancer cell line inducing anti-proliferative effects and cell toxicity. Tyrosinase presented is a good target for cancer therapy especially when the Hus and Fus confer their activity in both in vitro and in vivo experiments. Thus, Hus and Fus can be presented as good candidates for medical, food, and agricultural applications because of their inhibitory and anticancer effects.

Ethical Considerations

Compliance with ethical guidelines

This article does not include any experiments with animals performed or human participants by any of the authors.

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Authors' contributions

Conceptualization and supervision: Hossein Piri; Methodology: Azadeh Hekmat; Investigation, writing–original draft, and writing–review & editing: All authors; Data collection: Negar Taherkhani; Data analysis: Hossein Piri, Azadeh Hekmat and Kamahldin Haghbeen.

Conflict of interest

The authors declared no conflicts of interest.

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