Comparison of the Effect of α-Pinene and Gallic Acid on Tyrosinase Activity

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Article Info	ABSTRACT
Article Type Original Article **Article History** Received: 12 September 2024 Accepted: 16 November 2024 © 2012 Iranian Society of Medicinal Plants. All rights reserved. ***Corresponding author** mazarei@uok.ac.ir	Tyrosinase is the key enzyme in melanin synthesis. Therefore, many Tyrosinase inhibitors have been tested in cosmetics and pharmaceuticals. The aim of this study was to compare the anti-Tyrosinase potential of Gallic acid and α-Pinene. The initial analysis was conducted using molecular docking methods. Then, laboratory experiments were performed using mushroom Tyrosinase, with catechol as the substrate and Kojic acid as the standard inhibitor of the enzyme. The antioxidant activity of Gallic acid and α-Pinene was evaluated using DPPH radicals. Docking scores showed that Gallic acid has a strong binding affinity towards Tyrosinase (ΔG = -6.33 Kcal/mol) forming an H-bond with Met 280 and a pi-pi stacking with His 263. α-Pinene could only bind to the active pocket via hydrophobic interactions, resulting in a lower binding affinity (ΔG = –3.89 Kcal/mol). Gallic acid showed the highest inhibitory effect (IC_{50} = 0.130 mg/mL), whereas α-Pinene showed a lower inhibitory capacity (IC_{50} = 0.392 mg/mL). The types of inhibition were competitive inhibition for Kojic acid and uncompetitive inhibition for Gallic acid. In the DPPH radical scavenging test, EC_{50} value for Gallic acid and α-Pinene was 0.269 mg/mL and 251.2 mg/mL, respectively. Both *in silico* and laboratory results were nearly identical. While α- Pinene is not as powerful an inhibitor of Tyrosinase as Gallic acid is, its effect will be increased perhaps by increasing its concentration. The anti-oxidant potential of Gallic acid is significantly higher than that of α-Pinene, so from this point of view also Gallic acid is more harmless and applicable with a higher degree of safeness.

Keywords: Tyrosinase, α-Pinene, Gallic acid, Melanin |

INTRODUCTION

Tyrosinase (EC 1.14.18.1) belongs to the type 3 copper-containing protein family [1]. The two copper ions in the conserved active site, Cu-A and Cu-B, are coordinated by six histidine residues [2]. Tyrosinase is also an important factor in cuticle formation in arthropods and browning in plants [3]. It is also involved in wound curing, ultraviolet protection, and detoxification of phenols [4]. Tyrosinase like an oxidase is a fundamental enzyme in melanogenesis in many organisms, which is essential for pigmentation. The catalysis of L-tyrosine conversion to L-dopa is the rate-limiting step of the enzymatic pathway of melanin formation [5]. In 1895, Bourquelot and Bertrand were the first to isolate Tyrosinase from mushrooms. After that, Tyrosinase has been isolated and purified from several bacteria, fungi, plant, and animal sources. The structure of Tyrosinase contains three domains: N-terminal, central, and C-terminal domains [6].

There are many types of Tyrosinase inhibitors, and most of them have been tested with commercial mushroom Tyrosinase for use, contradicting mammalian Tyrosinase. However, recent research has reported significant differences in inhibitor effectiveness between mushroom Tyrosinase and human Tyrosinase [7]. The inhibitory effect of several Tyrosinase inhibitors suggested that ascorbic acid was the best inhibitor of human Tyrosinase and mushroom Tyrosinase as well as when determined by the lowest IC_{50} values [8]. Hydroquinone, Kojic acid, and arbutin are the best-known Tyrosinase inhibitors but they have serious side effects such as permanent depigmentation, erythema, and contact

dermatitis [9]. Also, Tyrosinase inhibitory activity of 91 native plants from central Argentina was carried out by Chiari et al [10]. Although numerous synthetic Tyrosinase inhibitors have been reported, only a few of them such as arbutin and Kojic acid, are commercially used, mainly due to disadvantages like high cytotoxicity, insufficient penetrating power, low activity, and low stability [11]. Hydroquinone is also potentially mutagenic in mammalian cells and is associated with several side effects, including contact dermatitis [12]. Arbutin is chemically unstable in its natural form and potentially toxic to the bone marrow [13]. Kojic acid is restricted to use in cosmetics [14]. Ellagic acid functions as an alternative substrate for Tyrosinase rather than serving as an effective inhibitor. Its use is limited due to concerns regarding its carcinogenic properties and instability during storage. Additionally, ellagic acid is water-insoluble and possesses low bioavailability [15]. Similarly, ascorbic acid is heat-sensitive and prone to decomposition [16]. These factors contribute to the limited applicability of previously utilized Tyrosinase inhibitors, often confining their use to low concentrations.

Tyrosinase inhibitors attract much attention in the cosmetic industry as global market demand has increased for skin-whitening agents for those who want to obtain lighter skin color [17]. Human Tyrosinase inhibitors are particularly useful in pharmaceutics and cosmetics. The discovery of whitening agents began in the 1960s with the discovery of the mono benzyl ether of hydroquinone. Generally, the typical whitening agent has been L-ascorbic acid (vitamin C) due to its melanin synthesis inhibition. L-ascorbic acid

inhibits melanin synthesis via the reduction of dopaquinon to L-dopa. However, L-ascorbic acid is unstable in formulations for cosmetics. To solve this problem, several L-ascorbic acid products have been developed, including magnesium ascorbyl phosphate, ascorbyl palmitate, and ascorbyl stearate, but these agents are precipitated and oxidized during solubilization. The currently known skin-whitening and anti-melanin molecules include arbutin, deoxy-arbutin, hydroquinone, deoxy-arbutin derivatives, resorcinol, vanillin, niacin amide, Kojic acid, arbutin-mimic isotachioside, hydroquinone derivatives (α and β-arbutin), azelaic acid, L-ascorbic acid, Ellagic acid, and Tranexamic acid [18]. This research aims to determine the Tyrosinase inhibitory ability of α-Pinene and Gallic acid, which are members of two different Tyrosinase inhibitor groups: α- Pinene (Terpenoid group) Gallic acid (phenol group and flavonoid group), and finally make some comparisons between them. Choosing these two compounds is based on their natural origin, so we hope to add a new Tyrosinase inhibitor to the list of Tyrosinase inhibitors.

MATERIAL AND METHODS

Docking Analysis and Visualization of Binding Conformations

To compare the effects of α-Pinene and Gallic acid on Tyrosinase activity, crystal structure of PPO3, a Tyrosinase from *Agaricus bisporus* was downloaded from the Protein Data Bank (PDB ID: 2Y9X). Both α-Pinene and Gallic acid were prepared using Auto Dock 4.2.6, where the native ligand was separated from the crystal structure, all water molecules were removed and polar hydrogens were added. The receptor crystal structure was subjected to energy minimization using the Auto Dock Tools (ADT, v1.5.6) prepare_receptor4.py command with Kollman-united charges was used for calculating the partial atomic charge. For the ligand's preparation, the ADT prepare_ligand4.py command was manipulated. Auto Dock 4.2.6, utilizing the Chimera interface, was used for docking. The energy minimization was set to 2.500.00, and the population size to 150, utilizing the Lamarckian genetic algorithm. Ten binding modes were produced for each ligand, with a maximum of 3 kcal/mol energy difference between each mode. The best conformations, showing the lowest binding free energy, were selected. BIOVIA Discovery Studio Visualizer 2021 was used to generate 2D interaction figures.

Mushroom Tyrosinase Inhibition Assay

In this study, Khatib's method with brief changes was used to measure inhibition of Tyrosinase enzyme activity [19]. Measurements were performed in 96-well microplates in a final volume of 200 microliters using a microplate reader (Tecan Sunrise model) at 492 nm. Potassium Phosphate buffer solution 50 mM and pH=6.5 was the main buffer in all experiments, and reactions were performed at room temperature. Each of the measurements was done in three repetitions, and the blank well contained all the materials of the test well except for the enzyme (buffer was added instead). During the analysis of the results, the absorbance values of the empty plate were deducted from those of the test plate. Additionally, the absorbance of the blank in the test wells was subtracted from the corresponding test wells to ensure accurate measurements. As a result, the final absorption was only the result of enzyme activity. For the negative control, the final absorbance was calculated in the same way. The absorbance obtained for the test sample and the negative control were plotted against the time, and the linear area of the graphs was used to calculate the slope of the tangent line. The percentage of inhibition

was calculated for three repetitions of each test. The following equation was used to determine the amount of percent inhibition.

Inhibitory % = {(Control OD − Sample OD)/Control OD)} × 100

Kinetic Analysis of the Inhibition of Tyrosinase

In order to determine the type of inhibition exerted by the Gallic acid and α-Pinene with the highest percentage of inhibition, the Lineweaver-Burke double reciprocal plot was drawn based on the enzyme reaction in the presence of different inhibitor concentrations. Real V_{max} and K_m for control and apparent V_{max} and K_m for each inhibitor concentration were determined.

Free Radical Scavenging Assay

The scavenging activity of Gallic acid and α-Pinene on DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical was determined using the method used by Fu *et al*. [20]. 100 microliters of methanol or different concentrations (0.01, 0.02, 0.1, 0.2, 0.5, and 1 mg/mL) of Gallic acid and α-Pinene solutions were mixed with 100 μL of freshly prepared 0.1 mM DPPH in methanol. DPPH absorbance was then measured at 517 nm, using an ELISA reader (TECAN, Austria). Each test was carried out in triplicate. Percent activity was calculated using the following equation:

$$\text{Activity}\,(\%) = 1 - \left(\frac{A_{\text{Sample}}}{A_{\text{Blank}}} \right) \times 100.$$
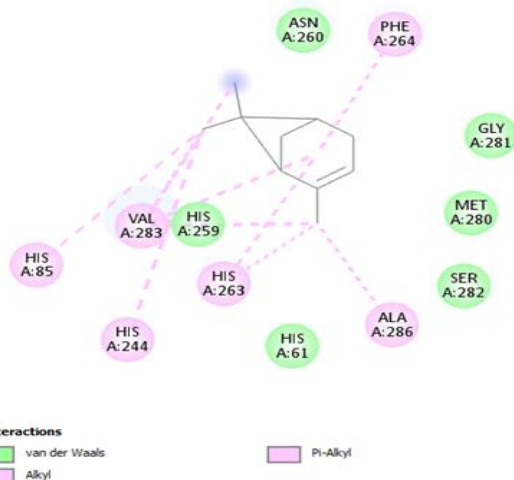
RESULTS

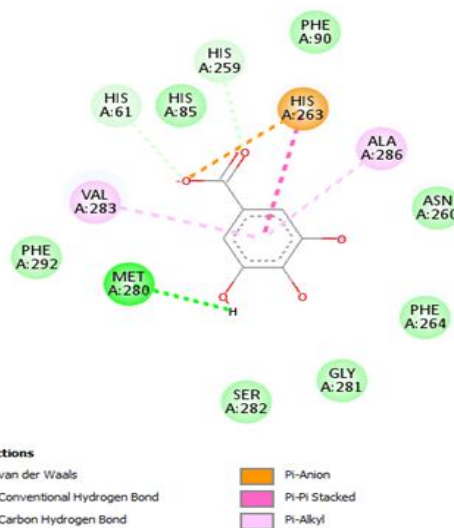**Fig. 1** 2D interactions of α-Pinene on PDB ID. 2Y9X

Fig. 2 2D interactions of Gallic acid on PDB ID. 2Y9X

Docking scores showed that Gallic acid has a strong binding affinity towards Tyrosinase enzyme ΔG = -6.33 Kcal/mol (Fig. 1) forming an H-bond with Met 280 and a pi-pi stacking with His 263. On the other hand, α-Pinene was only able to bind to the active pocket via hydrophobic interactions which is why it scored very low binding affinity (ΔG = -3.89 Kcal/mol) (Fig. 2).

The conformational changes of the protein-ligand complex were examined using two methods; root-mean-square deviation (RMSD) and solvent-accessible surface area analysis through the 50 ns MD simulation to assess the stability of the simulated system. These parameters were calculated after re-centering and re-wrapping the complex within the unit cell using the trjconv function of GROMACS.
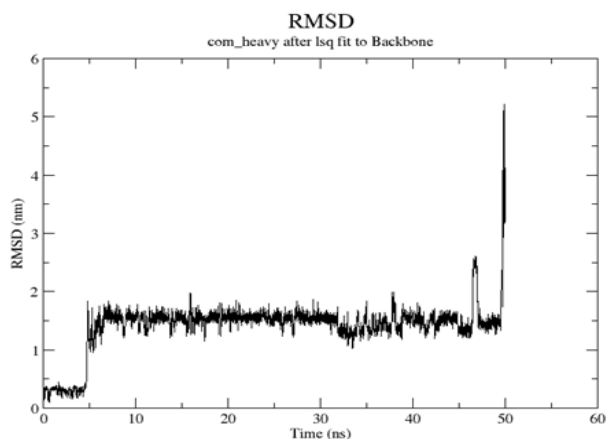
Fig. 3 Structural dynamics calculated during the 50 ns of MD trajectories; α-Pinene Root Mean Square fluctuation (RMSF) calculated during the 50 ns of MD trajectories
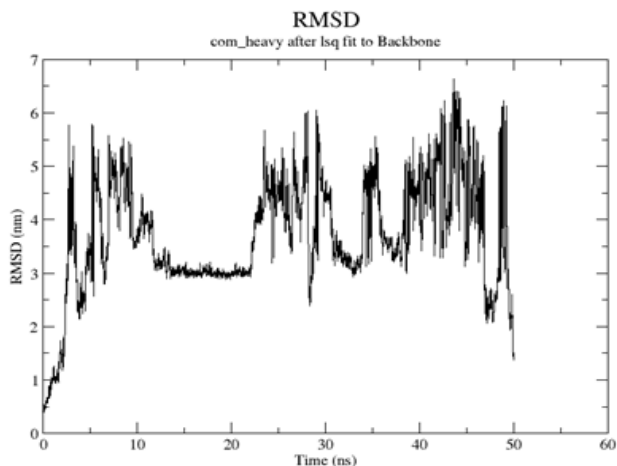
Fig. 4 Structural dynamics of Gallic acid calculated during the 50 ns of MD trajectories; Root Mean Square fluctuation (RMSF) of calculated during the 50 ns of MD trajectories

The RMSD plot (Fig. 3) indicates the stability of α-Pinene when bound to the Tyrosinase pocket and gives insight into the conformation changes throughout the 50 ns MD simulation. The RMSD of α-Pinene aligned to the protein backbone shows very stable fluctuation after an initial 5 ns of the simulation. This result indicates its binding stability throughout the whole simulation. This stability was further confirmed by the SASA plot which showed to be very stable, with minimal fluctuation ranging between 210 and 220 nm2 during the whole simulation (Fig. 4). Contrary to the docking results, Gallic acid interaction with the enzyme active pocket was observed to be very unstable with very

high fluctuation during most of the trajectory (Fig. 5). And similarly higher fluctuations were observed than those of α-Pinene (Fig. 6).
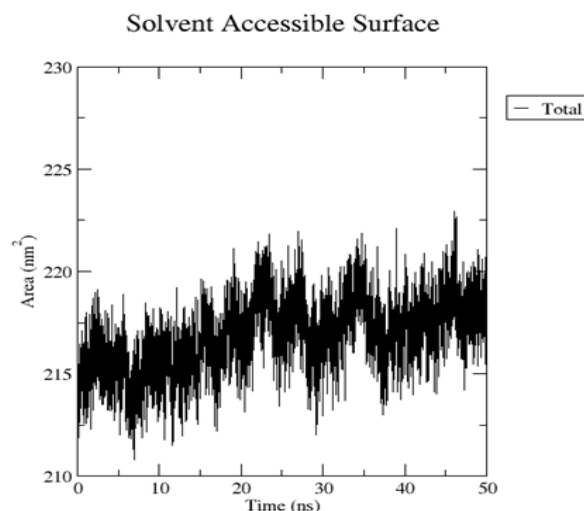
The root mean square fluctuation (RMSF) of the backbone residues was also calculated to evaluate the rigidity/flexibility of residues throughout the 50 ns MD simulation. As shown in (Fig 7) and (Fig. 8), residues involved in the interaction with the ligand show very low fluctuation (< 0.2 nm).

To assess the stability of this binding number of H-bonds formed during the 50 ns simulation was calculated (Fig. 9 and Fig.10). It is clear that no H-bonds are formed with α-Pinene, which confirms docking results that only hydrophobic interaction took place. However, Gallic acid was able to form 1-3 H-bonds during the 50 ns simulation with one permanent H-bond which is mainly with Met 280.

Kojic acid is one of the most common inhibitors of Tyrosinase, so it has been used to compare the performance results of different inhibitors. In this experiment, the inhibitory activity of Kojic acid was measured at four different concentrations and the percentage of inhibition for each concentration was calculated (Fig. 11). In the same way the percentage of Tyrosinase inhibitory activity of Gallic acid was assayed at four different concentrations (Fig. 12). The percentage of Tyrosinase inhibitory activity of α-Pinene at six concentrations is shown in Fig. 13.
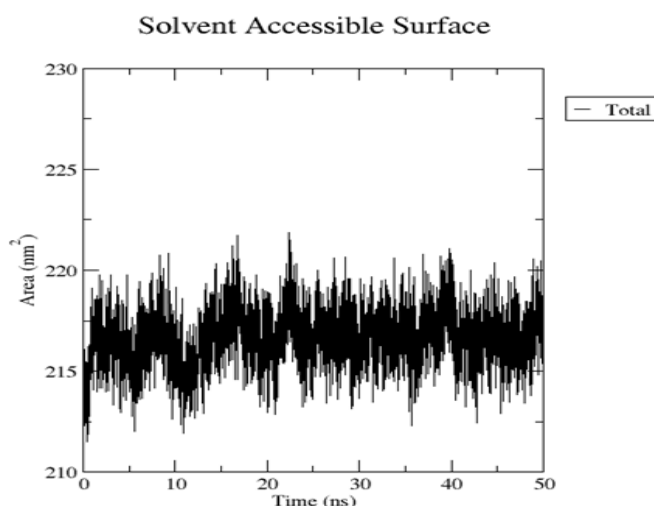
Fig. 5 SASA values of α-Pinene calculated during the 50 ns of MD trajectories

Fig. 6 SASA values of Gallic acid calculated during the 50 ns of MD trajectories

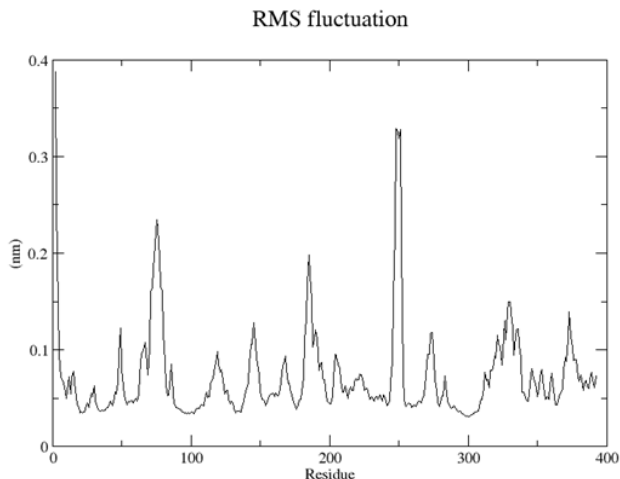**Fig. 7** Structural dynamics calculated during the 50 ns of MD trajectories; Root Mean Square fluctuation (RMSF) of α—Pinene
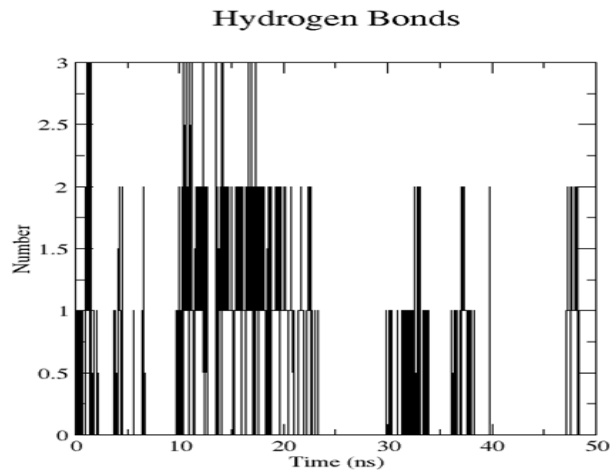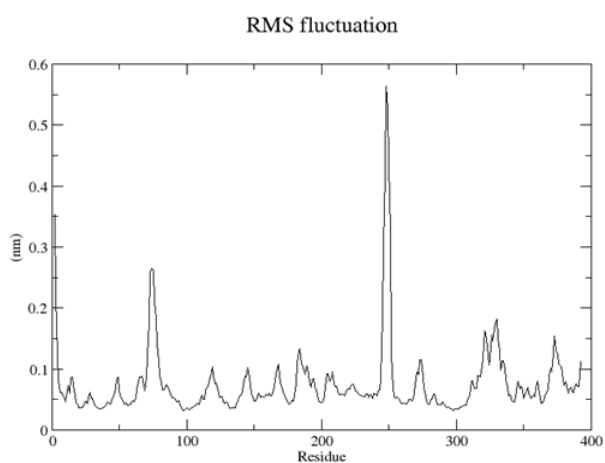

Fig. 8 Structural dynamics calculated during the 50 ns of MD trajectories; Root Mean Square fluctuation (RMSF) of Gallic acid.

Fig. 9 Number of H-bonds formed with α-Pinene.

Fig. 10 Number of H-bonds formed with Gallic acid.

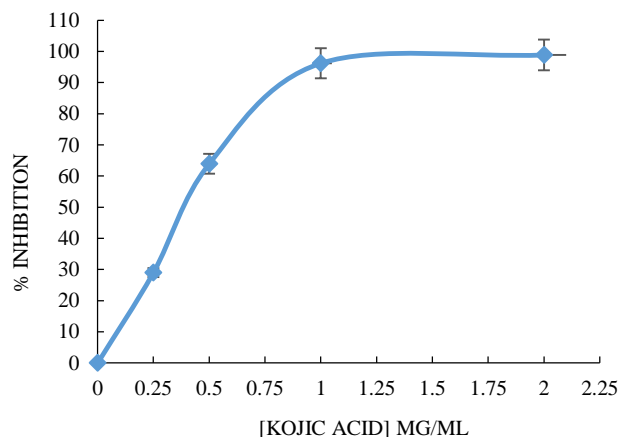**Fig. 11** Chart of changes in the percentage of inhibition of Tyrosinase enzyme vs. different concentrations of Kojic acid, IC_{50}=0.360 mg/mL.
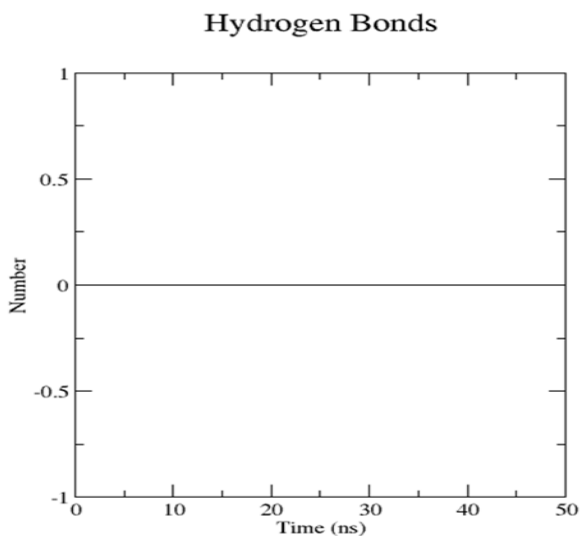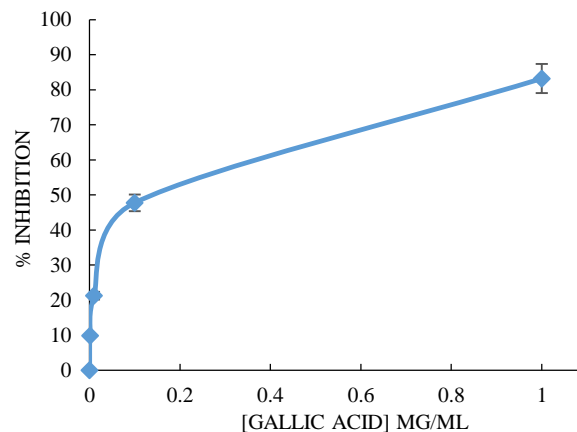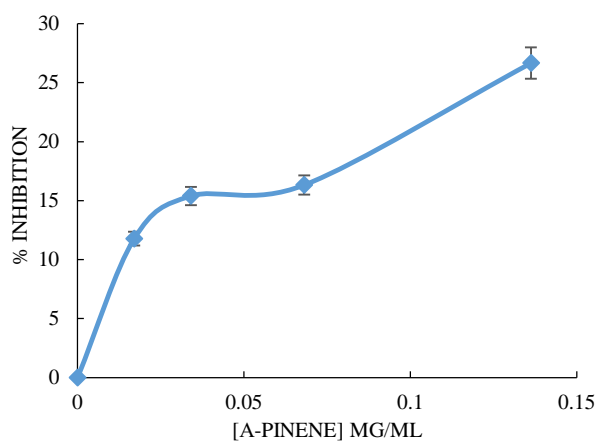

Fig. 12 Chart of changes in the percentage of inhibition of Tyrosinase enzyme vs. different concentrations of Gallic acid, IC_{50}= 0.130 mg/mL.

The Tyrosinase bioassay with pure Gallic acid showed a concentration-dependent inhibitory effect on the oxidation of pyro-catechol by mushroom Tyrosinase. The IC_{50} was determined as 0.130 mg/mL (0.764 mM) which is about 3-fold more potent than that of Kojic acid, a well-known Tyrosinase inhibitor (Table 1). α- Pinene in contrast showed weaker anti-Tyrosinase activity (Fig 3.13) and its percentage inhibition did not reach a notable point for IC_{50} calculations, or enzyme inhibition kinetics analysis.

Lineweaver-Burk method of enzyme inhibition analysis was used to determine the kind of inhibition exerted by each of under study materials. Substrate concentrations were prepared based on Lineweaver-Burk correction coefficients and practically substrate solutions were prepared in four different concentrations (3, 6, 12, and 24 mM). Kojic acid demonstrated a competitive pattern of Tyrosinase inhibition (Fig. 14). On the other hand, the other hand, Gallic acid inhibited Tyrosinase as an uncompetitive inhibitor (Fig. 15), so it binds Tyrosinase just after the formation of enzyme – substrate (ES) complex and didn't attach free enzyme. It can be concluded that Gallic acid's binding site on Tyrosinase is far from the enzyme's active site.

Fig. 13 Chart of changes in the percentage of inhibition of Tyrosinase enzyme vs. different concentrations of α-Pinene, IC_{50} = 0.392 mg/mL.
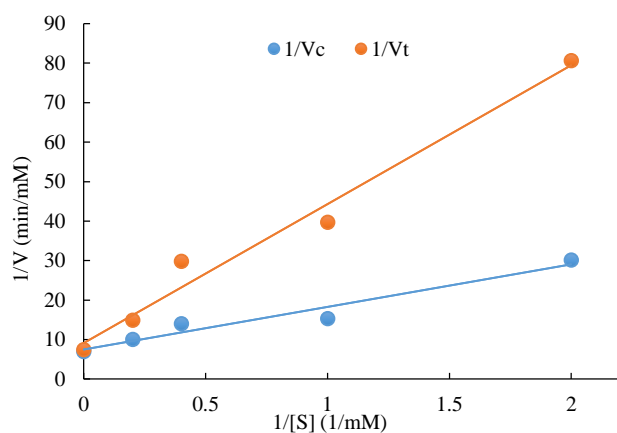
Fig. 14 Lineweaver-Burk plot of Tyrosinase inhibition, Pyro-catechol as substrate at 4 concentrations with 1 mg/mL concentration of Kojic acid as inhibitor
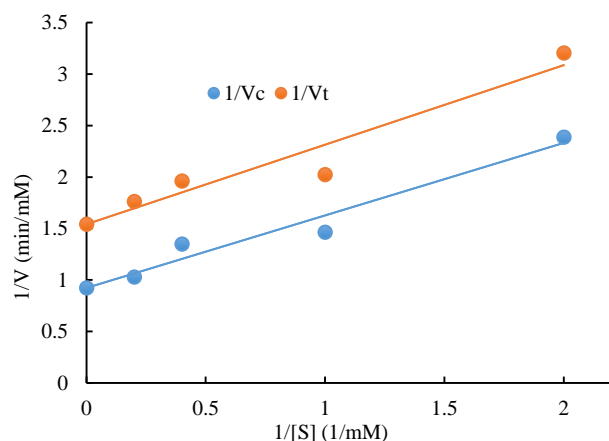
Fig. 15 Lineweaver-Burk plot of Tyrosinase inhibition, Pyro-catechol as substrate at four concentrations with 0.1 mg/mL concentration of Gallic acid as inhibitor.

To evaluate the antioxidant activity of Gallic acid and α-Pinene, free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging activity was assayed. DPPH methanolic solution has a violet color which upon reduction by an electron-donor agent (antioxidant

compounds such as ascorbic acid), turns to yellow non-radical diphenyl-picryl-hydrazine. DPPH radical scavenging activity and EC_{50} value were determined for Ascorbic acid as the positive control (Fig. 16), Gallic acid (Fig. 17), and α-Pinene (Fig. 18) as test materials. The radical scavenging activity, which can be measured as a decolorizing activity following the trapping of the unpaired electron of DPPH, was examined. In fact, Gallic acid was almost intermediate potent radical scavenging, but α-Pinene appeared as the least potent radical scavenging substance in this test (Table. 2).

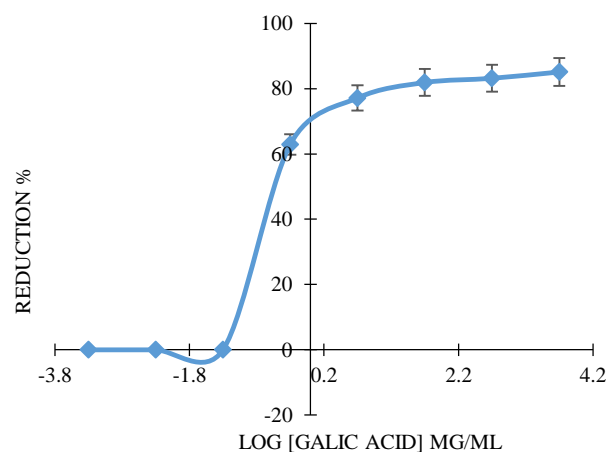**Table 1** Tyrosinase inhibitory activity of studied compounds

Inhibitor	IC_{50} (mg/mL)	Ratio to Kojic acid
Kojic acid	0.360	1
Gallic acid	0.130	0.36
α-Pinene	ND *	ND*

*See the text
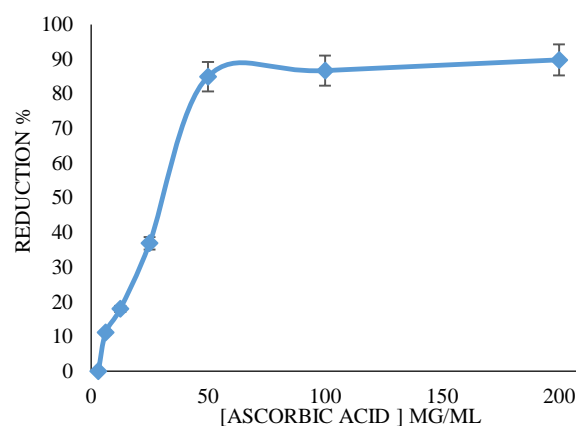
Fig. 16 Plot of DPPH reduction test for Ascorbic acid EC_{50} = 0.03 mg/mL

Fig. 17 Plot of DPPH reduction test for Gallic acid EC_{50} = 0.269 mg/mL

Table 2 Anti-oxidative activity of studied compounds

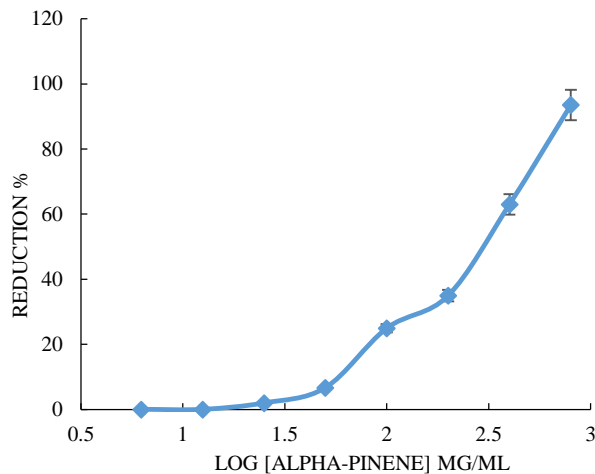| Compound	EC_{50} (mg/mL)	Ratio to Ascorbic acid
Ascorbic acid	0.03	1
Gallic acid	0.269	9
α-Pinene	251.2	707

Fig. 18 Plot of DPPH reduction test for α-Pinene $EC_{50} = 251.2$ mg/mL

DISCUSSION

The results demonstrated that both *in silico* and laboratory findings were nearly the same, according to molecular docking results, Gallic acid has a very good binding affinity towards Tyrosinase enzyme ($\Delta G = -6.33$ Kcal/mol), and also Gallic acid has the ability to form three hydrogen bond with Tyrosinase enzyme, an H-bond with Met 280 and a pi-pi stacking with His 263. While α-Pinene has a very low binding affinity ($\Delta G = -3.89$ Kcal/mol) and does not form any hydrogen bond with Tyrosinase. α-Pinene just has the ability to make a weak hydrophobic bond with the enzyme. Interestingly, Gallic acid was found to show a significantly stronger inhibitory effect than Kojic acid. This result is similar to findings from research conducted in 2022, which showed that a series of Gallic acid-benzylidene-hydrazine hybrids and among them, compounds 5d and 5f displayed an excellent ability in inhibiting Tyrosinase with IC_{50} values of 15.3 ± 0.8 μM and 3.3 ± 0.2 μM, respectively. The inhibition ability of these compounds was significantly ($p < 0.05$) stronger than that of the standard drug Kojic acid ($IC_{50} = 44.4 \pm 1.7$ μM) [21]. The Tyrosinase inhibition part of this study showed that α-Pinene is not as powerful an inhibitor as Gallic acid. As IC_{50} of Gallic acid and α-Pinene was 0.130 mg/mL and 0.392 mg/mL respectively. These results are in correlation with the results of Kim [22] and Kumar [23] with some little differences. Kürkçüoğlu et al (2002) reported that the root oil of *Ferulago longistylis*, highly rich in α-Pinene, exerted very low antioxidant activity, and no activity was recorded for Tyrosinase inhibition [24], which confirms the results of this work.

CONCLUSION

The results demonstrated that both *in silico* and laboratory findings were nearly the same, as well as our prediction for the presence of Tyrosinase inhibitory property in (α-Pinene, Gallic acid), was correct; both of them may be regarded as Tyrosinase an inhibitor but by different forms and conditions. While α- Pinene is not as powerful inhibitor as Gallic acid, perhaps by increasing its concentration its effect will be increased. Anti-oxidant potential of Gallic acid is so much higher than α-Pinene, so from this point of view Gallic acid is also more harmless and applicable with higher safety. Both of them could be prepared from natural sources since both cosmetic and agricultural fields require safety considerations.

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgment

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