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**ORIGINAL ARTICLE**

# Kojic Acid Effect on the Inhibitory Potency of Tyrosinase

**Forogh Azami [1], Elham Tazikeh-Lemeski[*2], Mehr-Ali Mahmood-Janlou[1]**

[1]Department of Biology, Gorgan Branch, Islamic Azad University, Gorgan, Iran

[2] Department of Chemistry, Gorgan Branch, Islamic Azad University, Gorgan, Iran

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**KEYWORDS**

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**ABSTRACT:** In recent years, enzymatic activity of tyrosinase has been the focus of investigation due to its potential applications in medicine, agriculture and cosmetics. Tyrosinase, entitled polyphenol oxidase, is a key enzyme that catalyzes synthesis of melanin in plants, microorganisms and mammalian cells. Presence of some antioxidants can delay or inhibit the activity of this enzyme as well. In this survey, molecular docking calculation method using *Autodock 4.0* software for prediction of binding energy of the protein with some antioxidant ligands was executed. The pose with the lowest energy of binding or inhibition constant was extracted at 298.15 K for kojic acid. Number of conformations in the cluster of rank was 13. The first and second boxes free energy and the inhibition constant were as follows: -5.60 kcalmol$^{-1}$, 78.99 µM and -3.32 kcalmol$^{-1}$, 3.66 µM, respectively. Since the first box presented a lower value of free energy, it was considered as the best mode of structure of kojic acid and the protein docking for further analysis. Thus, our present study could contribute to development and discernment of tyrosinase inhibitors in order to prevent hyper pigmentation.

## INTRODUCTION

Oxidation is a reaction that transfers electrons from a substance to oxidizing agent. Oxidizing agents and radicals are known as the mediating factors of these disorders, but in healthy individuals, they are usually surpassed by antioxidant enzymes. These reactions produce free radicals starting a series of reactions that can damage cells. Although oxidation reactions can lead to cellular damage, but it is vital to life, therefore plants and animals have complex systems consisting of different types of antioxidants that neutralize the harmful effects free radicals [1]. Free radicals can cause diseases and injuries to the lungs, heart, cardiovascular system, kidneys, liver, eyes, skin, muscle, brain and contribute to cellular aging. Antioxidants neutralize free radicals and put an end to these chain reactions. Kojic acid is one of the compounds available in skin lightening products. Kojic acid skin lightening effect is due to tyrosinase inhibition that prevents the production of melanin. In

* Corresponding author: elham_tazike@yahoo.com (E. Tazike).

fact, hydroquinone seems to play an important role in inhibition of tyrosinase activity.

Kojic acid ($C_6H_6O_4$) is a *yellowish brown* naturally occurring hydrophilic fungal metabolite obtained from species of mushrooms and other plant materials. It is believed to inactivate tyrosinase and induce skin lightening and is a substance with the *potential* to *cause allergic contact dermatitis* in some people [2]. Tyrosinase is a key enzyme involved in melanogenesis, which is essential for melanin production in skin and hair in a wide range of organisms. Tyrosinase is a metallo-enzyme contains copper atom and oxygen. Tyrosinase is widely available in nature and this indicates the importance of its application in biological systems [3]. Regulating the activity of tyrosinase due to its potential applications in medicine, agriculture and cosmetics has been *the subject of research* in recent years [4]. Molecules are essential for the catalytic activity of tyrosinase. This enzyme causes skin, hair and eye colors in epidermal cells of animals. In plants, they play a role in defense mechanisms against pathogens and insects; furthermore, it is involved in molting processes and forming exoskeleton of the insects [5].

Melanin synthesis consists of a bipartite process involving structural proteins exportation from the endoplasmic reticulum and their fusion with melanosome-specific regulatory glycoproteins, which are released in coated vesicles from the Golgi-apparatus. Subsequently, melanin synthesis proceeds to the sorting and trafficking of these proteins to the melanosome . Specifically, tyrosinase enzyme catalyzes the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to DOPA quinone. When there is no tyrosinase activity in melanocytes, the pigment is not formed, and it leads to localized albinism such as white spots on the skin of some animals. Melanin produced in melanocytes is transferred to the keratinocytes upper layer cells called melanophore by the buds of cytoplasm. The melanosomal transfer from melanocytes to keratinocytes, followed by melanosome processing in the recipient keratinocytes plays a critical role in skin pigmentation [6].

Natural whitening products may have a role in decreasing skin pigmentation by their interfering with the pigmentary processes [7]. The inhibitory activity of 30 different plants using mushroom tyrosinase inhibition was studied. The results demonstrated that two plants extracts; *Vitis vinifera* in water and *Citrus sinensis* in ethyl acetate, showed a noticeable level of mushroom tyrosinase inhibition compared to positive controls of kojic acid in the same solvent systems [8]. Two plant extracts in vitro; *C. sinensis* in ethyl acetate and *V. vinifera* in water, revealed a considerable level of mushroom tyrosinase inhibition compared to positive controls of kojic acid in the same solvent systems The antioxidant activity of methanolic extract of crocus sativus flowers (saffron) was evaluated. It was about 11 times lower than that of ascorbic acid. The extract showed a significant inhibitory effect on tyrosinase activity [9]. In order to study the inhibition of tyrosinase by kojic acid using the influence of divalent cation of a metal ion, experimental kinetics and computational analysis were used [10]. Another study refers to the affinity and binding energy of kojic acid and tyrosine docked to Streptomyces castaneoglobisporus tyrosinase and embelin to agaricus bisporus by molecular docking [4]. We used mushroom tyrosinase as an appropriate model to define the anti-tyrosinase activity of different plant extracts. Anti-tyrosinase activity does not depend on dose of the plant extract, besides we can use the plant extract as skin is whitening and anti-ageing agents.

The antioxidant and anti-tyrosinase activity the piper officinarum was studied by different chromatography methods [11]. This plant has noteworthy antioxdidants activity and act as natural antioxidants [12]. In another case, the methanolic extraction of *Pleurotus ostreatus* has more antioxidant activity and inhibition tyrosinase effects compared with acetone and this mushroom can be a natural antioxidant [13]. The interactions occurring in

tyrosinase have been illustrated by means of molecular docking models in complex with some ligands, as isophthalic acid [14], hesperetin [15] and oxymatrine [16]. Tyrosinase is considered as an effective enzyme in different levels of life. It is important in terms of performance, structure and stability of the enzyme. Since human tyrosinase is not available and plants and mushrooms tyrosinase shows very high similarities compared to human tyrosinase, mushroom can be a prosperous source for conducting in this research.

In the present study, we evaluated its docking behavior of kojic acid with the mushroom tyrosinase by molecular docking.

## MATERIALS AND METHODS

Molecular docking was carried out using *AutoDock 4.0* software using a Lamarckian genetic algorithm for prediction of all binding structures of the protein by kojic acid ligands at 298.15K. Chemical structures of kojic acid [CID no: 3840] showed in Figure 1, was extracted from Pubchem compound database. The three-dimensional structures of the copper-bound Mushroom tyrosinase were obtained from the RCSB Protein data bank (http://www.rcsb.org) [17] and entry was PDB ID: 2y9w [18].

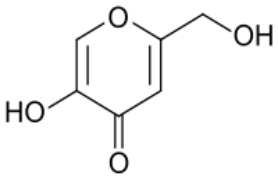


**Figure 1**. Kojic Acid Structure (Molar mass: 142.11 $gmol^{-1}$)

## RESULTS

### *Docking setup*

Autodock combines energy evaluation through pre-calculated grids of potential affinity employing various search algorithms to find the suitable binding position of a ligand on a given protein. At first, two grid boxes were created. Three-dimensional structure of kojic acid was constructed and optimized using Polak-Ribiere conjugate gradient algorithm and AMBER95 force field implemented in Hyper Chem (Hyper Cube Inc., Gainesville, FL). The protein has two forms, monomer and dimer. Its active form (monomer) was studied and saved in pdb file. The ligand was stored by Chemspider server (http://www.chemspider.com). The mol files of ligands were transformed into a pdb file format using webqc server (http://www.webqc.org). Using a plain text editor all the water molecules were removed, then missing hydrogens and Kollman united atom charges and polar hydrogens were added to the protein using Autodock tools [19]. Finally, non-polar hydrogens were merged to their corresponding carbons, and desolvation parameters were assigned to each atom and after merging non-polar hydrogens, rotatable bonds were assigned. Rotatable bonds in the ligands were kept free to allow for flexible docking. Then in the Auto Dock Tools package, the partial atomic charges were calculated [20]. Grid size was set to 126 x 126 x 126 grid points (x, y and z), with spacing between grid points kept at 0.375 Å and the coordinate of central grid point of maps was set to 10.257 x 20.904 x 89.291 points (x, y and z). The Lamarckian genetic algorithm was chosen to search for the best conformers. Standard docking protocol was applied. One hundred independent docking runs were carried out for each box. The ligand and the protein binding sites were labeled Using chimera 1.7s software.

## DISCUSSION

The active site of tyrosinase can be divided into three regions; 1) a substrate-binding pocket (six histidine residues along with the dinuclear copper ions) surrounded by charged residues such as Glu256 and Glu322 residues; 2) a hydrophobic region, consisting of Val248, Phe264, Val283, and Pro284 residues; and 3) a solvent-exposed region, composed of Glu189 and Arg268 residues.

In this study, we have compared docking energy of some inhibitors such as; kojic acid, tyrosol, arbutine, hydro quinone, niacin amid and azelaic acid with tyrosinase by Auto dock tool. To conclude that which ligand unfolds or which of them has more effect on the enzyme stability we have calculated their free energies. According to docking results in Table 1, kojic acid has the most interactions with tyrosinase. In conclusion, kojic acid binds better to the enzyme. The obtained results can support the future design of newer compounds with better tyrosinase aggregation inhibitor activity.

**Table 1.** The number of residues in the active site of tyrosinase- ligand complex and the binding energy values of these complexes.

| Antioxidant Name | The numbers of residues in the active site of tyrosinase | Binding Energy ($kJmol^{-1}$) |
|---|---|---|
| **Azelaic Acid** | 6 | -3.89 |
| **Niacin Amid** | 6 | -5.24 |
| **Arbutin** | 7 | -5.36 |
| **Kojic Acid** | **6** | -5.60 |
| **Hydroquinone** | 5 | -5.30 |
| **Tyrosol** | 11 | -5.38 |

According to ligand binding energy in mushroom tyrosinase enzymes, kojic acid has the most negative and azelaic acid showed the least free binding energy. Therefore, for further studies we will focus our results on kojic acid antioxidant.

In order to find the most possible binding sites of kojic acid–tyrosinase complex, a preliminary docking using a grid map as big as whole tyrosinase molecule was carried out using maximum number of independent runs supported by AutoDock. Since the target protein was too large, two grid boxes were generated and clustered into 13 different conformations (with an RMSD cut-off of 0.5 Å). The pose with the lowest energy of binding or inhibition constant were extracted and saved in pdb format for further analysis as it is listed in Table 2. The first box had the free energy of binding; from -4.68 to -5.60 kcal/mol and the second box had the free energy of binding; from -2.31 to -3.32 kcal/mol shown in Figure 2. Since the first box presented a lower value (-5.60 kcal/mol) it is considered as the best mode of pdb.

**Table 2**. The inhibition constant and free energy of binding and other energy values of kojic acid–tyrosinase complex

| *Energy* | First Box | Second Box |
|---|---|---|
| $K_{i\ (micro\ molar)}$ | 78.99 | 3.66 |
| Binding Energy ($kcalmol^{-1}$) | -5.60 | -3.32 |
| Intermolecular Energy($kcalmol^{-1}$) | -5.89 | -3.62 |
| Internal Energy($kcalmol^{-1}$) | -0.07 | -0.08 |
| Torsional Energy($kcalmol^{-1}$) | 0.3 | 0.3 |
| Internal Energy ($kcalmol^{-1}$) | -5.04 | -2.99 |
| Entropy ($kcalmol^{-1}K^{-1}$) | 9.15 | 9.15 |

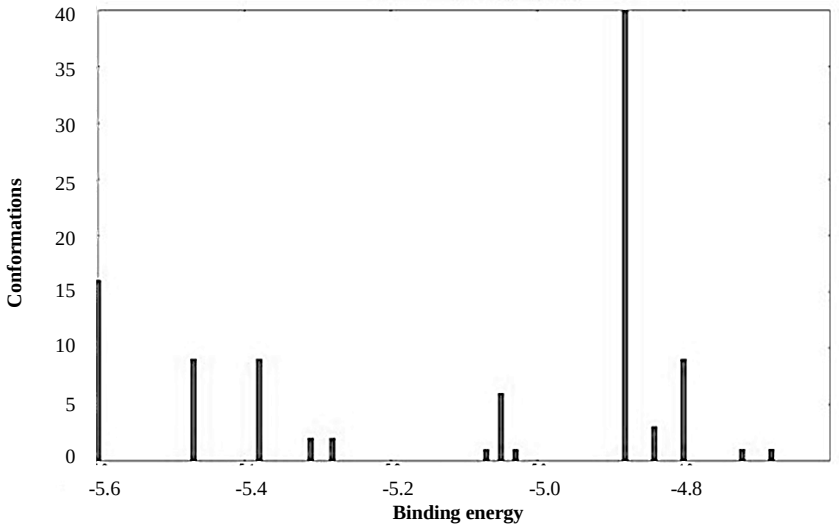


**Figure 2**. The binding energy range of kojic acid-tyrosinase complex

The results indicate that the ligand and the protein bound with amino acids in 6 points of binding sites. All of mentioned amino acids in this region are histidine (His) were listed in Table 3 that it is positive charged and a hydrophilic amino acid. In addition, Table 4 shows the details of the lowest binding energy of this complex in every cluster.

**Table 3**: Amino acide's type and numbers in the binding site.

| Amino Acid | Amino Acid No. |
|---|---|
| ***His*** | 61 |
| ***His*** | 85 |
| ***His*** | 94 |
| ***His*** | 259 |
| ***His*** | 263 |
| ***His*** | 296 |

Minimum coordinates in grid = (-13.368, -2.721, 65.666)

Maximum coordinates in grid = (33.882, 44.529, 112.916)

Coordinates of Central Grid Point of Maps = (10.257, 20.904, 89.291)

Initial quaternion, (x,y,z,w) = ( -0.477, 0.735, 0.476, 0.077 )

Initial translation = (5.541, -0.226, 70.077)

Small molecule center of rotation = (-9.158, -19.725, +6.981)

Number of distinct conformational clusters found = 13, out of 100 runs

**Table 4**. Binding free energy for tyrosinase-kojic acid calculated by Autodock.

| Cluster Rank | Lowest Binding Energy | Run | Mean Binding Energy | Num In Claus |
|---|---|---|---|---|
| **1** | -5.60 | 100 | -5.52 | 16 |
| **2** | -5.47 | 10 | -5.39 | 9 |
| **3** | -5.38 | 6 | -5.25 | 9 |
| **4** | -5.31 | 77 | -5.23 | 2 |
| **5** | -5.28 | 68 | -5.27 | 2 |
| **6** | -5.07 | 67 | -5.07 | 1 |
| **7** | -5.05 | 95 | -4.99 | 6 |
| **8** | -5.03 | 60 | -5.03 | 1 |
| **9** | -4.88 | 39 | -4.81 | 40 |
| **10** | -4.84 | 45 | -4.77 | 3 |
| **11** | -4.80 | 81 | -4.78 | 9 |
| **12** | -4.72 | 41 | -4.72 | 1 |
| **13** | -4.68 | 72 | -4.68 | 1 |

Statistical mechanical analyses are listed here;

Partition function, Q = 100.85 at T = 298.15 K

Free energy, A ~ -2733.52 kcal/mol at T = 298.15 K

Internal energy, U = -5.04 kcal/mol at T = 298.15 K

Entropy, S = 9.15 kcal/molK at T = 298.15 K

In this case, we show the class of an analysis, as follows:

Run = 100, Cluster Rank = 1, Number of conformations in this cluster = 16

RMSD from reference structure = 99.470 A

**Estimated Free Energy of Binding = -5.60 kcal/mol** [= (1) + (2) + (3) - (4)]

**Estimated Inhibition Constant, Ki = 78.99 uM (micromolar) at** [T = 298.15 K]

Final Intermolecular Energy = -5.89 kcal/mol

vdW + Hbound + desolv Energy = -5.81 kcal/mol

Electrostatic Energy = -0.09 kcal/mol

Final Total Internal Energy = -0.07 kcal/mol

Torsional Free Energy = +0.30 kcal/mol

Unbound System's Energy [= (2)] = -0.07 kcal/mol

Tyrosinase is a metallo-enzyme containing copper ion (+2) and performed the main role in the presence of these ions. However, the results from tyrosinase structure study indicated that copper ions are essential for kojic acid and tyrosinase bounding. As a result, kojic acid is believed to inactivate tyrosinase by chelating copper ions of tyrosinase and reduce melanin formation. According to the labeled binding sites, kojic acid tendency to coordinate histidine residues in the active site of the target amino acids (a positive charge) is expected.

On the other hand, electrostatic surfaces corresponding to the most negative docking sites (first negative rank) in interaction of kojic acid with tyrosinase are shown in Figure 3. In Figure 4, the blue color represents the positive charge and red color represents the negative charge of residues. Using chimera 1.7s, the protein-ligand complex is labeled and shown in Figures

3, 4. In addition, H-bounds formation in this combination is available in Figure 5.

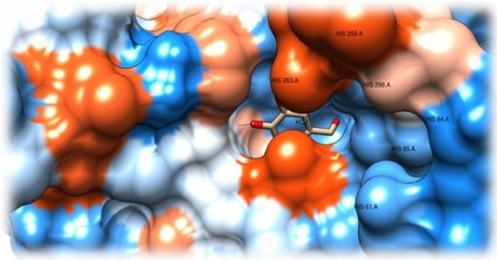

**Figure 3**. The electrostatic surface potential obtained from docking of kojic acid to tyrosinase from two aspects. Blue, red and white colors are representative of positive, negative and neutral charges, respectively.

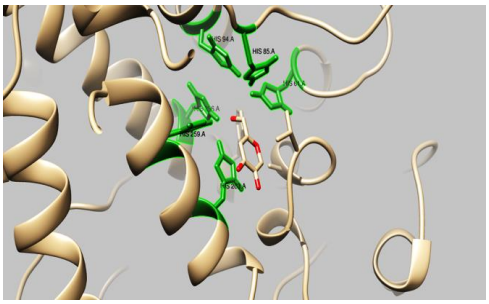

**Figure 4**. The binding sites of the most negative clusters of kojic acid near the tyrosinase

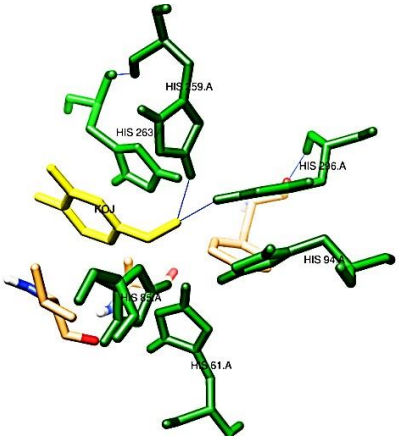


**Figure 5**. Intermolecule of H-Bound formations in tyrosinase-kojic acid complex.

## CONCLUSIONS

In this study, the results of the ligand docking showed that the binding pocket involves the amino acid; His263, His259, His296, His94, His85, His61 combine to kojic acid. According to the above-mentioned binding structure of tyrosinase, site 1 is the main binding site of this enzyme for kojic acid. On the other hand, by doing more research on other antioxidants such as hydroquinone, tyrosol, niacin amide, arbutin and azelaic acid by molecular docking method, it was seen that tyrosol same as kojic acid is connected to the region 1 of tyrosinase enzyme including mentioned six residues. Hence, these two antioxidants compete in combining to tyrosinase. The

results is gained by estimating the inhibition constant of these combinations ($k_{i\ tyrosol}$: 114 micro molar and $k_{i\ kojic\ acid}$: 78 micro molar) show that tyrosol is winner in this competition ($k_{i\ tyrosol} > k_{i\ kojic\ acid}$) and probably where both of these antioxidants are included in drugs or cosmetic combinations, tyrosol can inhibit the actual effect of kojic acid. Indeed, inhibiting and understanding tyrosinase would be a significant issue in medicine due to its clear role in melanoma, hyper pigmentation and Parkinson's disease. Hence, our present study results provide new insight in understanding the kojic acid as a potent tyrosinase inhibitor. Thus, our present molecular docking studies could contribute for the further, development and understanding of tyrosinase inhibitors for the prevention of hyper pigmentation. Studies determining the mechanism of the action and binding sites of antioxidant and enzymes can be used in fields of competitive effects, drug interferences and synergy effects of drugs in the future.

## ACKNOWLEDGEMENTS

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