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In Silico and in Vitro Comparison of Anti-tyrosinase Potential of Crocin and Quercetin

Ruing Title: Anti-Tyrosinase Potential of Crocin and Quercetin

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ABSTRACT

Tyrosinase inhibitors are often used in two primary ways: in the cosmetics industry to lighten skin and treat certain dermatological conditions, and in agriculture to stop fruit and vegetables from browning caused by enzymes. This study aimed to compare the anti-tyrosinase and antioxidant potential of Crocin and Quercetin. The work was started by molecular docking (in silico). The experiment was then carried out in a lab setting using a commercial mushroom tyrosinase, pyrocatechol as the substrate, and Kojic acid as a typical enzyme inhibitor. Crocin and Quercetin antioxidant activity was assessed using DPPH radicals. Docking scores presented that Crocin has a great binding affinity towards tyrosinase, ΔG = -6.1 Kcal/mol. Quercetin had a binding energy of ΔG = -8.0 (kcal/mol). Quercetin exhibited a higher tyrosinase inhibitory effect with an IC_{50} of 0.158 mM, whereas Crocin showed a lower capacity of inhibition with an IC_{50} of 2.49 mM. The types of inhibition were competitive inhibition for Quercetin and mixed inhibition for Crocin. For DPPH radical scavenging activity, the EC_{50} value for Ascorbic acid was 0.179 mM; this value for Crocin and Quercetin was 1.74 mM and 0.031 mM, respectively. Quercetin is a powerful and mechanistically simple inhibitor of mushroom tyrosinase, so it is a valuable candidate for several applications targeted at regulating enzymatic browning and melanogenesis. Despite having antioxidant and inhibitory qualities, Crocin may not be as efficient as it may be as a main tyrosinase inhibitor due to its mixed mechanism of inhibition and its lower inhibitory potential.
Keywords: Tyrosinase, Crocin, Quercetin, Melanin

INTRODUCTION

Tyrosinase (EC:1.14.18.1) is a copper-containing oxidase that catalysis the first two stages in mammalian melanogenesis. It causes enzymatic browning responses in damaged fruits during post-harvest handling and processing. Hyperpigmentation in human skin and enzymatic browning in fruits are both undesirable. These events have prompted researchers to seek out novel, prevailing tyrosinase inhibitors for use in foods and cosmetics [1, 2]. Tyrosinase inhibitors are substances that inhibit tyrosinase. While several natural and synthetic tyrosinase inhibitors have been discovered, only a handful have gained widespread use due to concerns regarding effectiveness, toxicity, and adverse effects [3, 4]. The most well-known tyrosinase inhibitors are hydroquinone, kojic acid, and arbutin; nevertheless, they have major side effects such as irreversible depigmentation, erythema, and contact dermatitis [5]. The discovery of novel, powerful tyrosinase inhibitors for use in food, cosmetics, and medicine inspired scientists and researchers to concentrate on their discovery, isolation, synthesis, and characterization [1, 3].

Crocin, as a bioactive substance, is found naturally in various therapeutic plants, most notably saffron (*Crocus sativus* L.). Crocin is the glycosyl ester of crocetin [6]. Many of the beneficial properties of saffron are attributed to its Crocin, especially alpha Crocin, which has been shown in previous investigations to possess antioxidant, anti-inflammatory, and radical-quenching qualities [7, 8].

For generations, humans have included Quercetin in their diets. Several studies have been conducted on its numerous health advantages, which cover anticancer, antiviral, antibacterial, anti-inflammatory, and antioxidant potentials [9]. It has its place in a group of plant dyes called flavonoids that give color to many fruits, flowers, and vegetables. These secondary metabolites serve as a buffer against biological and environmental stress. The highest known concentration of Quercetin is found in onions [10, 11]. Quercetin is a viable option for the prevention and treatment of cardiovascular illnesses since it lowers blood pressure, lowers cholesterol, and improves endothelial function, among other cardiovascular benefits [12].

As previously alleged, several researchers are now searching for better tyrosinase inhibitors based on two primary criteria: safety and efficacy. Despite these efforts, we are unable to name a single tyrosinase inhibitor as the best at this time. We might be able to identify a good one in the future with the aid of molecular docking and other in-silico techniques. This research aimed to determine the tyrosinase inhibitory ability of Crocin and Quercetin and compare them to determine a significant difference between their inhibition potential, which are members of two different tyrosinase inhibitor groups: Crocin (carotenoid group) and Quercetin (flavonoid group), and make a comparison between them. Choosing these two compounds is based on the condition that both of them can be obtained from natural resources.

MATERIAL AND METHODS

Protein and ligand Preparation for Docking

To compare the effect of Crocin and Quercetin on the tyrosinase enzyme, the crystal structure of PPO3, a *mushroom tyrosinase (abPPO4)*,

was downloaded from the Protein Data Bank (PDB ID: 4OUA). Both Crocin and Quercetin 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, where Kollman-united charge was used for calculating the partial atomic charge. For the ligand's preparation, the ADT prepare_ligand4.py command was manipulated. AutoDock 4.2.6, utilizing the ChEMBL interface, was used for docking. The energy minimization was set to 2.500 and the population size to 150, utilizing the Lamarckian genetic algorithm. With the grid box of (center_x = -5.826000, center_y = 26.962737, center_z = 63.207025 and size_x = 50, size_y = 50, size_z = 50). A total of ten binding modes were produced for each ligand, with a maximum energy variance of 3 kcal/mol between the least favorable and optimal conformations. The optimal conformations were shown based on their lower binding free energy. To visualize the interactions, 2D interaction figures were created using BIOVA Discovery Studio 2021.

Tyrosinase Inhibition Assay

The protocol employed Khatib's method with slight modifications to assess the inhibitory effect on tyrosinase activity [13]. All measurements were performed in 96-well microplates in a final volume of 200 microliters using a microplate reader (Tecan Sunrise model). Each measurement was performed in three repetitions, and the blank well contained all the test materials except for the enzyme. The absorption of interfering factors was measured, after which the absorption of the test well was subtracted from the measurements. Next to the finish of the measurements, the absorbance of the empty plate was subtracted from the absorbance of the matching wells, as well as the absorbance of the blanks from the test wells. 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 was plotted against 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 extract. The equation outlined below was employed to determine the percent inhibition.

IC_{50} values Calculation

Semi-highest inhibitory concentration (IC_{50}) is a measure of the effect of a substance in inhibiting a specific biological or biochemical process. IC_{50} is a numerical grade that displays how much of a particular inhibitory material (e.g., drug) is necessary to inhibit, *in vitro*, a certain biological process or biological component by 50% [14]. IC_{50} was determined by plotting the variations in percentage of inhibition against the inhibitor concentration.

Kinetic Analysis of Tyrosinase Inhibition

To determine the inhibition type, exerted by Crocin and Quercetin by the uppermost percentage of inhibition, the Lineweaver-Burke plot was drawn based on the enzyme reaction in the presence of different inhibitor concentrations. V_{max} and K_m for the control and test were determined.

DPPH Scavenging Assay

The scavenging action of Crocin and Quercetin on DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical was quantified using a method introduced by Fu [15]. A total of 100 microliters of five different concentrations of methanol, along with solutions of Crocin and Quercetin, were mixed with 100 μL of freshly prepared 0.1 mM DPPH in methanol. Absorbance of the DPPH solution was then measured at 492 nm. All tests were carried out in triplicate.

RESULTS

Molecular Docking

Two criteria are important in determining the best docked state: one is the largest (most negative) binding free energy (ΔG binding), which was estimated, and the second is the most suitable interactions by the main amino acids of the enzyme active site. Crocin with a binding energy of ΔG = -6.1 (kcal/mol) (Fig.1) created interactions with the amino acid residues in the active site of *mushroom tyrosinase (abPPO4)*. This compound created Alkyl and Pi-Alkyl bonds with LEU B:365, TYR B:369, VAL B:358, and van der Waals interactions with each of ASP B:368, ASP B:372, and LYS B:357. Quercetin with binding energy ΔG = -8.0 (kcal/mol) created interactions with the amino acid residues in the active site of *mushroom tyrosinase (abPPO4)* (Fig.2). This compound created a conventional hydrogen bond with THR B:345, and van der Waals interaction with ASP B:344, ALA B:346, THR B:343, SER B:291, GLN B:294, VAL B:366, and created π-π-interaction with PHE B:355, Pi-alkyl interaction with ALA B:295.

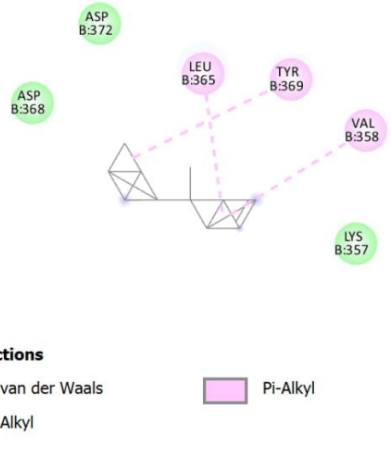

Fig. 1 2D interactions of Crocin on PDB ID. 4OUA

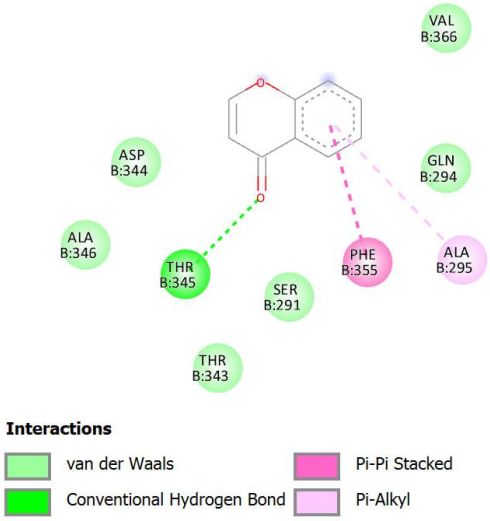

Fig. 2 2D interactions of Quercetin on PDB ID. 4OUA

Tyrosinase Inhibition Assays

As Kojic acid is one of the most common inhibitors of Tyrosinase, it is consequently used as a positive control to compare the performance results of different inhibitors. Inhibitory activity of Kojic acid was measured at 4 dissimilar concentrations and the percentage of inhibition for each concentration was calculated showing in (Fig. 3). The percentage of inhibitory activity of Tyrosinase, related to Crocin in four concentrations, is shown in (Fig. 4). The ratio of Tyrosinase inhibitory activity of Quercetin, in 4 concentrations, is shown in (Fig. 5) and IC_{50} for all the tree compounds with the ratio to positive control are shown in Table 1.

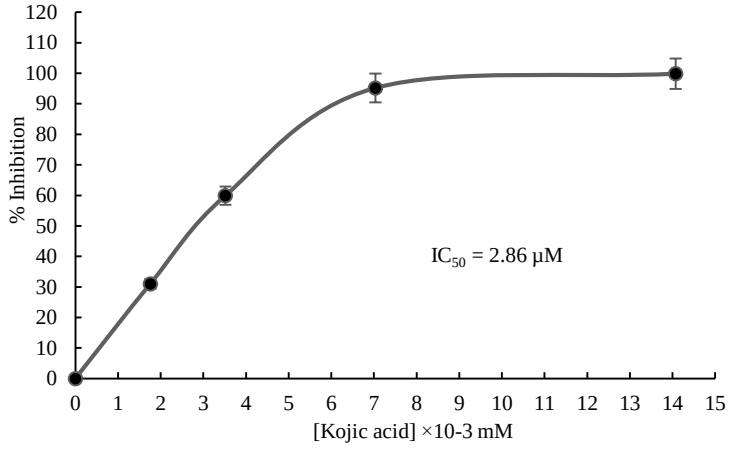

Fig. 3 Diagram of changes in the percentage of inhibition of the Tyrosinase vs. different concentrations of Kojic acid (Positive control).

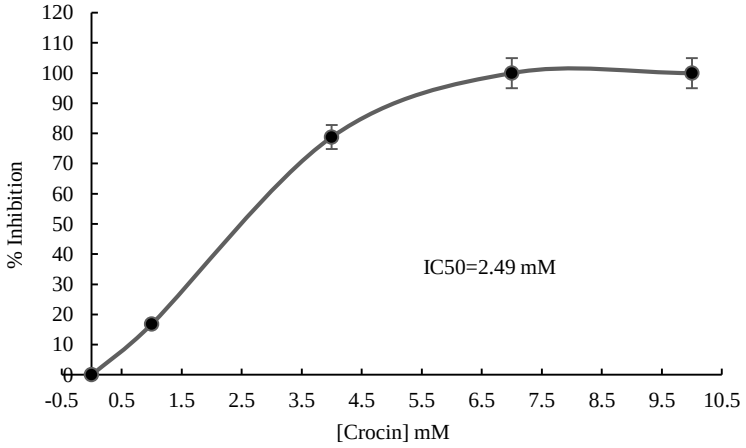

Fig. 4 Diagram of changes in the percentage of inhibition of the Tyrosinase vs. different concentrations of Crocin.

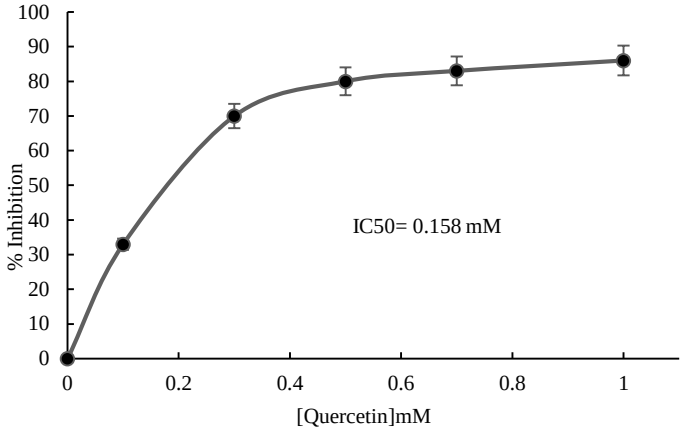

Fig. 5 Diagram of changes in the percentage of inhibition of the Tyrosinase vs. different concentrations of Quercetin.

Table 1 Tyrosinase inhibition of the considered compounds, IC_{50} for all tree compounds with the ratio to the positive control.

Inhibitor	IC_{50} µM	Ratio to Kojic acid
Kojic acid	2.86	1
Crocin	2490	870
Quercetin	158	55

Kinetic Analysis of Tyrosinase Inhibition

The double reciprocal approach, Lineweaver-Burk, was employed to determine the type of inhibition exercised by the under-study materials. Primarily based on the enzymatic response in the presence of inhibitors in 4 distinct concentrations of pyro-catechol substrate, have been plotted. Substrate concentrations were prepared based on Lineweaver-Burk correction coefficients and practical substrate solutions in four concentrations, which are 3, 6, 12, and 24 mM Kojic acid, showing the competitive pattern of tyrosinase inhibition (Fig. 6). Again, Crocin inhibited tyrosinase as a mixed competitive-uncompetitive inhibitor (Fig. 7). While Quercetin's pattern of inhibition on tyrosinase was competitive too (Fig. 3.8) (Table 2).

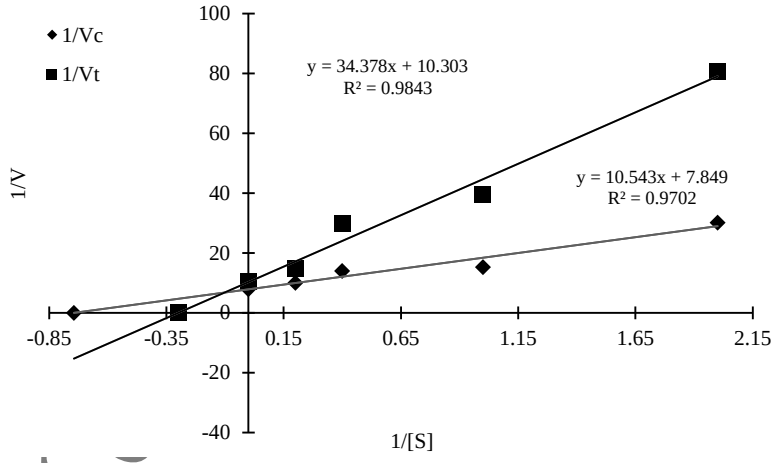

Fig. 6 Double reciprocal plot of Tyrosinase inhibition, Pyro-catechol as substrate, and 1 mM concentration of Kojic acid.

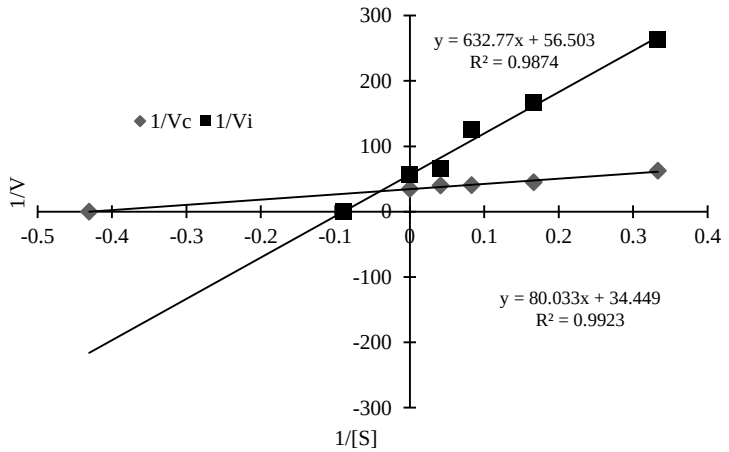

Fig. 7 Double reciprocal plot of Tyrosinase inhibition, Pyro-catechol as substrate with 0.1 mM concentration of Crocin.

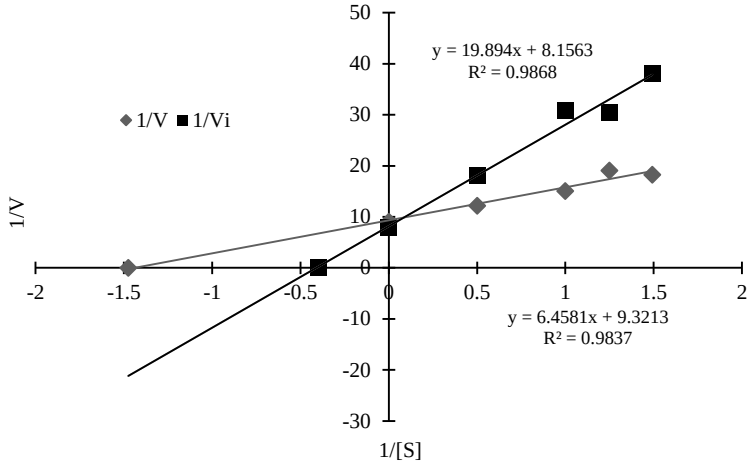

Fig. 8 Double reciprocal plot of Tyrosinase inhibition, Pyro-catechol as substrate with 24 mM concentration of Quercetin.

Table 2 Results of kinetic parameters of different compounds in the Lineweaver–Burk diagram

Inhibitor	Vmax (mM/min)	Km (mM)	Inhibition Type
Control	0.95	2.53	-
Kojic acid	0.12	2.95	Competitive
Crocin	0.02	1.17	Mixed
Quercetin	0.11	2.5	Competitive

Antioxidant Activity

To evaluate the antioxidant action of Crocin and Quercetin, their free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity was assayed. The first approach is to trap the DPPH, a violet stable radical, which on reduction by an electron-donor agent (antioxidant compounds such as vitamin C), goes yellow and non-radical diphenyl picryl hydrazine. DPPH radical scavenging activity and EC_{50} values were determined for Vitamin C as a positive control (Fig. 9), Crocin (Fig. 10), and Quercetin (Fig. 11) (Table 3).

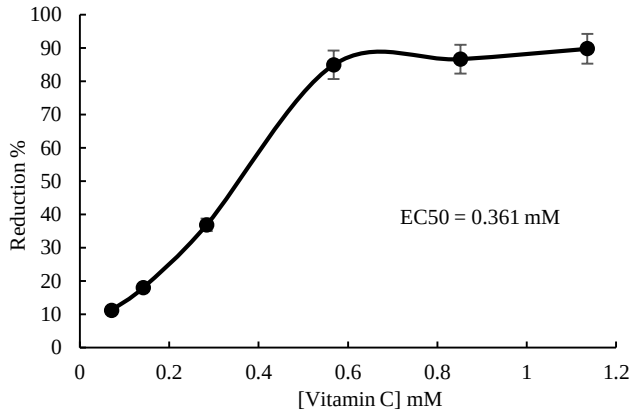

Fig. 9 Plot of DPPH reduction test for Vitamin C.

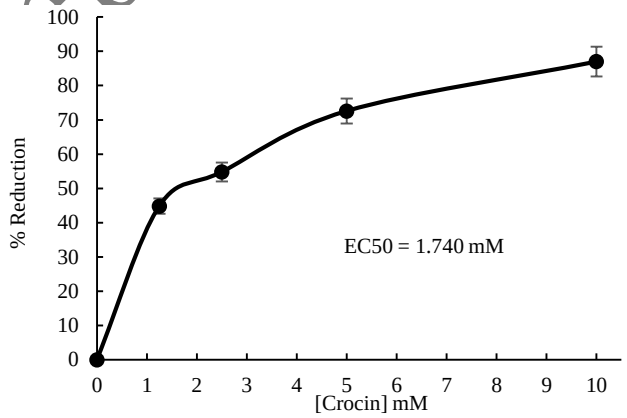

Fig. 10 Plot of DPPH reduction test for Crocin.

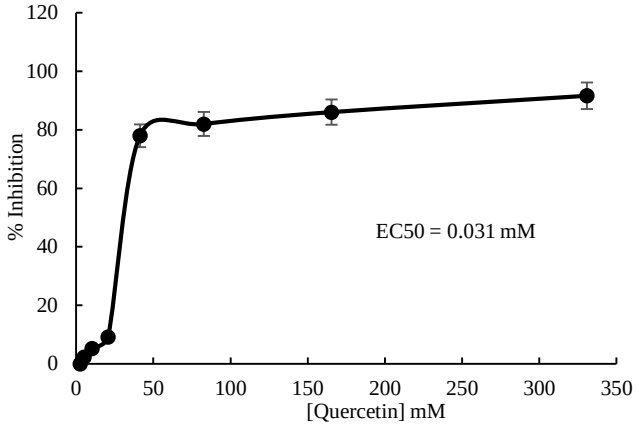

Fig. 11 Plot of DPPH reduction test for Quercetin.

Table 3 DPPH radical scavenging activity and EC_{50} values of studied compounds

Inhibitor	EC_{50} mM	Proportion to Ascorbic acid
Ascorbic acid	0.361	1
Crocin	1.740	4.82
Quercetin	0.031	0.086

DISCUSSION

Docking studies can determine the important amino acid residues involved in the interaction and estimate the binding affinity by mimicking the non-covalent interactions between the inhibitor and the enzyme [16]. Quercetin has better inhibitory activity over Crocin, as indicated by molecular docking, suggesting a more structurally complementary and energetically favorable interaction with the tyrosinase active site. The spontaneity and stability of the ligand-protein complex are reflected in the Gibbs free energy of binding (ΔG), a thermodynamic parameter computed during docking simulations. Quercetin would have a stronger and more persistent interaction with tyrosinase because its ΔG value, binding energy, ΔG = -8.0 (kcal/mol), was more negative than Crocin's binding energy, ΔG = -6.1 (kcal/mol). A better overall fit inside the active site cavity, reducing steric conflicts and optimizing advantageous electrostatic and hydrophobic interactions, may be the cause of this increased affinity. Quercetin may be able to form a more extensive network of contacts through the amino acid residues lining the enzyme's active site. Strategic Involvement of Important Active Site Residues Tyrosinase's catalytic activity depends on two copper ions (CuA and CuB) that are present in its active site [17]. In order to prevent substrate binding or interrupt the catalytic cycle, inhibitors usually work by either directly interacting with these copper ions or by attaching to residues nearby. Quercetin probably interacts more frequently or more strongly with important amino acid residues that are known to be essential for tyrosinase activity. These interactions involve van der Waals forces that support the complex's overall stability and hydrogen bonds.

Optimal Orientation and Spatial Positioning within the Active Site: An inhibitor's effectiveness depends on both its orientation inside the active site and the strength of its binding [18]. An inhibitor that is oriented correctly can either cause conformational changes in the enzyme that hinder its activity or successfully prevent the substrate from reaching the catalytic center. Quercetin may have been able to directly obstruct the substrate-binding channel or obstruct the exact placement of the substrate needed for catalysis by adopting a more advantageous spatial arrangement inside the tyrosinase active site, as shown by molecular docking. Induction of Steric Hindrance and Conformational Changes: An inhibitor's effectiveness may be greatly influenced by its size and shape. Because of its glycosylated carotenoid structure, Crocin is a bigger molecule than Quercetin and may provide more steric hindrance in the active site or nearby [19, 20]. The conformational flexibility necessary for the catalytic cycle of the enzyme may be disrupted, or substrate binding may be physically prevented due to this steric interference. Additionally, Crocin binding may result in mild allosteric effects, which alter the enzyme's structure and hence lower its activity. While Quercetin can also cause some steric hindrance, Crocin's longer and more intricate structure probably permits a more noticeable steric effect [21].

Although molecular docking offers useful theoretical insights into possible interactions between an inhibitor and its target enzyme, actual research is necessary to thoroughly test these predictions. The computational results are greatly supported by the reported concordance between molecular docking results and in vitro testing, which shows a better inhibitory action of Quercetin. Quantitative information on the inhibitor's efficacy is provided by in vitro enzymatic tests, which evaluate the enzyme's activity directly in the presence of different inhibitor doses. Several important factors may explain Quercetin's apparent advantage in these tests, as well as reduced IC_{50} Value and Inhibition Mechanism. Quercetin has a far lower IC_{50} (0.158 mM) value than Crocin (2.29 mM). Quercetin exhibits a remarkably lower IC_{50} value, approximately 15.76 times lower than that of Crocin. This signifies that Quercetin is significantly more potent in inhibiting mushroom tyrosinase, needing a lower dosage to produce the same degree of enzyme inhibition. This variation in IC_{50} values is a clear indication of Quercetin's improved enzymatic mode of action and stronger contact [15, 22, 23, 24]. The mechanism by which an inhibitor works can be clarified by in vitro kinetic experiments. Common mechanisms include uncompetitive-inhibition, which happens when the inhibitor only binds to the complex of the enzyme with its substrate, non-competitive inhibition, which happens when the inhibitor binds to a place different from the active site and affects the activity of the enzyme, and competitive inhibition, which happens when the inhibitor and the substrate compete for binding to the active site [25]. In order to understand why Quercetin is more potent than Crocin, it may be helpful to identify the mechanism of inhibition for both compounds [26]. For example, Quercetin, as a competitive inhibitor, directly competes with the substrate (L-tyrosine or L-DOPA) for binding by binding reversibly to the enzyme's active site. While the maximum velocity Vmax stays constant, this kind of inhibition raises the enzyme's apparent Michaelis-Menten constant (Km) for the substrate,

suggesting a decreased affinity. The concentration of the substrate has a significant impact on how effective a competitive inhibitor is at high substrate concentrations; the inhibitor's activity can be counteracted. Given that Quercetin competitively inhibits substrate binding [22]. Its structural characteristics most likely resemble the substrate or interact with important residues in the active site. This competitive binding may be greatly aided by Quercetin's phenolic hydroxyl groups, especially the catechol moiety, which may interact with the copper ions or amino acid residues implicated in substrate identification [27]. When an inhibitor has varying affinities for binding to both the free enzyme and the enzyme-substrate complex, this is known as mixed inhibition. Both substrate binding and catalysis may be impacted if the inhibitor attaches to the enzyme at a location other than its active site. If the inhibitor binds preferentially to the free enzyme, mixed inhibition results in a drop in the apparent Vmax and an increase in the apparent Km. The free enzyme-attaching capacity of the inhibitor gives it a competitive constituent, but its binding to the enzyme-substrate complex gives it an uncompetitive component.

Crocin's dual method of inhibition points to a more intricate relationship with tyrosinase. Both substrate binding and the catalytic turnover of the enzyme-substrate complex may be impacted by interactions between the gentiobiose moieties or the Crocetin core and areas inside and outside the active site. Though not as strongly as Quercetin's direct active site competition, this dual mechanism may help explain its inhibitory action. In the assay system, stability and solubility: The physicochemical characteristics of the inhibitors, such as their stability and solubility in the in vitro test conditions, might indirectly affect the measured inhibitory efficacy, even if the direct interaction with the enzyme is the main emphasis. Crocin's superiority over Quercetin, which may be less stable or soluble, may be due to its greater solubility and stability under test conditions, which would allow it to interact with the tyrosinase enzyme more easily [28, 29]. Structure-Based Differences: Connecting Inhibitory Efficacy with Molecular Architecture. By analyzing their different chemical structures, Crocin and Quercetin's opposing inhibitory activities may be explained:

Compared to the more flexible and extended Crocin molecule, Quercetin's planar structure may also limit its capacity to assume highly precise orientations inside the active site or to create severe steric hindrance. One important characteristic that sets Crocin apart is the presence of its large, hydrophilic sugar moieties. These sugar units may contribute to particular interactions with the enzyme, in addition to enhancing water solubility. They may permit a specific orientation of the crocetin core within the active site that maximizes its inhibitory potential, or they may interact with residues outside the primary active site, resulting in allosteric control of enzyme activity [30]. Both Crocin and Quercetin are well-known for their antioxidant qualities in addition to their tyrosinase inhibitory capacity, which may be important for food preservation and possible synergistic effects. The antioxidant activities that are offered include:

Crocin, while exhibiting lower tyrosinase inhibitory potency and antioxidant activity compared to Quercetin, still possesses these properties. Its mixed mode of inhibition might offer a different mechanism of action that could be advantageous in specific contexts or when used in combination with other inhibitors. The water solubility of Crocin, due to its glycosidic nature, might also be a factor to consider for certain applications where solubility is crucial. Capitalizing on Quercetin Potential. The strong evidence that Quercetin has better tyrosinase inhibitory action than Crocin [22, 31] has important ramifications for a number of fields: Pharmaceutical and Cosmetic Innovations: A possible natural possibility for the creation of safer and more effective depigmenting chemicals is Crocin. It may be able to treat hyperpigmentation issues more successfully due to its higher potency when compared to a well-researched natural inhibitor like Quercetin. It is necessary to conduct more studies on its dermatological uses, including formulation techniques and in vivo effectiveness tests. In comparison to synthetic inhibitors, Crocin's natural source may also be beneficial in terms of consumer acceptability and the possibility of fewer adverse effects.

CONCLUSION

Quercetin is a more powerful and mechanistically simpler inhibitor of mushroom tyrosinase than Crocin, according to the comparative analysis based on autodocking findings, IC_{50} values, mechanisms of inhibition, and antioxidant properties. It is a viable candidate for several applications aimed at regulating enzymatic browning and melanogenesis due to its superior antioxidant activity, competitive mechanism of inhibition, significantly lower IC_{50}, and stronger anticipated binding affinity. Despite having antioxidant and inhibitory qualities, Crocin may not be as efficient as it could be as a main tyrosinase inhibitor due to its mixed mechanism of inhibition and lower potency. Its special qualities, including its water solubility, warrant further research for specific uses or in combination treatments. To fully utilize the advantages of these natural chemicals, more study into their modes of action and possible combinations is essential.

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