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# Evaluation of Tyrosinase Inhibitory and Antioxidant Activity of Bis(carboxymethoxy) Curcumin Derivatives

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## Abstract

Hyperpigmentation disorders are associated with various psychological health concerns and increased risk of skin cancer. Among potential therapeutic approaches, tyrosinase inhibitors represent promising candidates for treating these concerns. This study investigated the tyrosinase inhibitory activity and mechanisms of bis(carboxymethoxy) bismethoxycurcumin (4x), bis(carboxymethoxy) demethoxycurcumin (5v), and bis(carboxymethoxy) bisdemethoxycurcumin (6d). The results showed that 4x exhibited superior tyrosinase inhibitory activity across the tested concentrations (75 - 600 µM), decreasing enzyme activity to 78.52% - 36.72%, relative to 100% of the control, with an $IC_{50}$ value of 272.85 µM. This compound also demonstrated the highest antioxidant activity, with values of 1.61 µM Trolox equivalent per µM 4x against the ABTS radical. The modification with a carboxymethoxy ($-OCH_2COOH$) group enhanced the tyrosinase inhibitory and ABTS radical scavenging activities, while the absence of a methoxy group reduced these inhibitory activities. Molecular docking analysis indicated that these compounds interacted directly with the tyrosinase active site. Stronger interactions with copper atoms at the active site led to increased tyrosinase inhibitory activity, while the methoxy group proved significant for the tyrosinase inhibitory activity of 4x. Based on its superior inhibitory activity, 4x was selected for further kinetic and melanin inhibition studies, which revealed it to be a competitive inhibitor of tyrosinase with a $K_i$ of 42.38 µM. Moreover, this compound, at concentrations of 75 - 150 µM, reduced melanin content in B16F10 cells to 88.02% - 70.57% compared to 100% of the control, without inducing cytotoxicity. These results suggest that 4x represents a promising candidate for development as an anti-hyperpigmentation agent in both cosmetic and medical applications.

**Keywords:** Bis(carboxymethoxy) curcumin derivatives, Tyrosinase inhibitor, Antioxidant, Molecular docking, Kinetic study, Melanin

## Introduction

Melanin plays a major role in protecting the skin from ultraviolet (UV) radiation [1]. However, excessive melanin production and deposition can lead to hyperpigmentation, including melasma, chloasma, freckles, and age spots [2]. Beyond aesthetic concerns, hyperpigmentation impacts psychological well-being, lowering self-esteem and contributing to depression and anxiety [3]. In severe cases, it can increase the risk of skin cancer [4].

Several factors, particularly prolonged UV exposure and inflammation, can trigger hyperpigmentation through different signaling pathways in melanogenesis [5]. The microphthalmia-associated transcription factor (MITF) serves as the primary target for the activation of melanogenesis, resulting in an increase in melanogenic enzymes and, consequently, a rise in melanin synthesis and accumulation [6]. Among melanogenic enzymes, tyrosinase acts as the rate-limiting enzyme, catalyzing the transformation of L-tyrosine into L-dopa, which is subsequently converted into L-dopaquinone. L-dopaquinone is then converted through several steps and finally yields eumelanin and pheomelanin. Based on the rate-limiting step, the activation or inactivation of tyrosinase can effectively influence the rate of melanin synthesis [7]. Furthermore, various factors associated with hyperpigmentation often produce reactive oxygen species that can activate melanogenesis-related proteins, ultimately amplifying melanin production and hyperpigmentation disorders [8]. Therefore, compounds exhibiting both tyrosinase inhibition and antioxidant properties represent primary targets for regulating melanin synthesis.

Despite the widespread use of several tyrosinase inhibitors in cosmetic and medical treatments for hyperpigmentation, many have demonstrated significant limitations. Kojic acid shows instability in formulations and potential carcinogenicity concerns. Similarly, ascorbic acid is prone to rapid degradation, while ellagic acid exhibits poor bioavailability, limiting their therapeutic efficacy [9]. Moreover, allergic contact dermatitis has been reported after topical application of arbutin [10]. Collectively, these drawbacks emphasize the need for safer and more stable alternatives with improved anti-hyperpigmentation profiles.

Natural and semi-synthetic compounds have gained interest as tyrosinase inhibitors due to their lower cytotoxicity [11]. Curcumin, bisdemethoxycurcumin, and demethoxycurcumin are widely recognized natural compounds that are derived from *Curcuma longa*. These compounds and their derivatives demonstrate numerous biological activities, including antioxidant, anti-inflammatory, anticancer, and anti-tyrosinase [12-14]. However, significant variability in tyrosinase inhibitory potency has been reported across these compounds [13]. Specifically, carboxyl-containing compounds, including benzoic acid, cumic acid, and cinnamic acid derivatives demonstrate effective tyrosinase inhibition [15]. Meanwhile, phenolic compounds containing a carboxyl group show strong antioxidant activity [16]. The carboxyl-containing compounds, including bis(carboxymethoxy) bismethoxycurcumin (4x), bis(carboxymethoxy) demethoxycurcumin (5v), and bis(carboxymethoxy) bisdemethoxycurcumin (6d), have been previously synthesized, characterized, and extensively investigated for their anticancer properties, specifically through the mechanism of histone deacetylase inhibition [17]. However, the tyrosinase inhibitory activity of these carboxyl-containing compounds has not been previously studied. Therefore, current research aimed to investigate the tyrosinase inhibitory and antioxidant activities of 4x, 5v, and 6d. Tyrosinase inhibition was evaluated using the dopachrome method and the inhibition mechanism was investigated through molecular docking and kinetic studies. The antioxidant activity was assessed by DPPH and ABTS radical scavenging assays. Additionally, cytotoxicity and melanin inhibition were examined in B16F10 cells. This research provides evidence that 4x, 5v, and 6d are promising tyrosinase inhibitors with antioxidant properties for the potential treatment of hyperpigmentation disorders. Furthermore, the significance of the methoxy group in relation to the tyrosinase inhibitory activity and antioxidant properties of bis(carboxymethoxy) curcumin derivatives has been highlighted for the first time.

## Materials and methods

### Chemical and Reagent

2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), DPPH (2, 2-diphenyl-1-picrylhydrazyl), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), mushroom tyrosinase, 3,4-dihydroxy-L-phenylalanine (L-Dopa), dimethyl sulfoxide (DMSO), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (USA). Kojic acid was obtained from TCI (Tokyo, Japan). Fetal bovine serum (FBS) was supplied by Hyclone (USA). Dulbecco's Modified Eagle Medium (DMEM), Antibiotic-Antimycotic, and trypsin-EDTA were purchased from Gibco (USA).

## Methods

### *Synthesis of bis(carboxymethoxy) curcumin derivatives*

The synthesis and characterization of 4x, 5v, and 6d were presented in the work of Somsakeesit *et al.* [17].

### *Tyrosinase activity assay*

Tyrosinase activity was determined using a previously described method [18] with some modifications. Briefly, 50 µL of compounds (0 - 175 µM) were mixed with 40 µL of L-DOPA (4.50 mM in 125 mM phosphate buffer, pH 6.8) and 10 µL of tyrosinase (100 U/mL). The absorbance was recorded continuously every 30 s using a microplate reader at 475 nm at 25 °C. The linear slope of the plot between absorbance at 475 nm and time (min) was determined as the initial rate (ΔA 475/min). DMSO served as the negative control, while kojic acid served as the positive control. The remaining tyrosinase activity was calculated using the following Eq. (1):

$$\text{Remaining tyrosinase activity (\%)} = [(\text{Rate}\Delta\text{A475 of test}) / \text{Rate}\Delta\text{A475 of control})]\times 100, \quad (1)$$

where RateΔA475 of test and RateΔA475 of control represent the initial rate (slope) of the reaction with the inhibitor and the reaction without the inhibitor, respectively.

Half Maximal Inhibitory Concentration ($IC_{50}$) values were determined from the linear equation of the plot between log [concentration] and percentage of remaining tyrosinase activity.

### *Kinetic study*

Kinetic study was performed according to Joompang *et al.* [18] with slight modifications. Briefly, tyrosinase activity was determined using 10 U/mL of tyrosinase and L-DOPA concentrations ranging from 0 to 0.80 mM, both with and without test compounds (0 - 175 µM). A Lineweaver-Burk plot was created. The changes in the Michaelis-Menten constant ($K_m$) and maximum velocity ($v_{max}$) from the Lineweaver-Burk plot were then used to indicate the mode of inhibition. The inhibition constant ($K_i$) was estimated from the x-intercept from the plot of slope versus compound concentration.

## Molecular docking

Molecular docking was conducted using GOLD Suite 5.5 (Cambridge Crystallographic Data Center, Cambridge, UK) [19] to examine the interactions between the test compounds and tyrosinase. The tyrosinase crystal structure (PDB: 2y9x) was downloaded from the RCSB Protein Data Bank (https://www.rcsb.org/). Discovery Studio 2017 R2 Client (Dassault Systèmes BIOVIA, BIOVIA Workbook, Release 2017; BIOVIA Pipeline Pilot, Release 2017; San Diego: Dassault Systèmes) was utilized to remove water molecules and ligands from the crystal structure. The molecular structures of 4x, 5v, and 6d were constructed utilizing the ChemDraw program (ChemDraw Professional 17.1) and the energy was minimized using HyperChem (Release 8.0 for Windows, Molecular Modeling System: HyperCube, 2007). In the molecular docking setting, hydrogen bonds were added, tropolone was removed and the tropolone binding site with a 10 Å radius was set as the binding site of the protein receptor. The compounds were docked to the binding site using the GOLD scoring function with 10 genetic algorithm (GA) runs and flexible docking. The other parameters were set to default. The docking protocol was validated by conducting self-docking of tropolone into its binding site. The threshold for the self-docking protocol was accepted at a root-mean-square deviation (RMSD) of less than 2 Å. The complex with the highest GOLD score was selected and interactions between test compounds and tyrosinase were analyzed using Discovery Studio 2017 R2 Client.

### *DPPH radical scavenging activity assay*

The DPPH radical scavenging activity assay was evaluated according to Abdullah *et al.* [20], with slight modifications. Briefly, 15 µL (100 µM) of the sample was mixed with 150 µL of 0.2 mM DPPH radical in ethanol. The mixture was incubated at room temperature for 30 min in the dark, followed by absorbance measurement at 517 nm using a microplate reader. Results were quantified using a Trolox standard curve and expressed as µmol Trolox equivalent per µmol tested compound.

***ABTS radical scavenging activity assay***

The ABTS radical scavenging activity assay was performed as described by Abdullah *et al.* [20], with minor adjustments. ABTS radical was prepared by reacting 7 mM of ABTS with 2.45 mM potassium persulfate (1:1) and allowing the mixture to stand at room temperature in the dark for 12 - 16 h. The resulting ABTS radical solution was diluted with acetate buffer to an absorbance of 0.7. For analysis, 10 µL (100 µM) of the sample was mixed with 200 µL of 0.2 mM of ABTS radical and incubated at room temperature for 7 min. Absorbance was measured at 734 nm using a microplate reader. The Trolox standard curve was constructed and ABTS radical scavenging activity was expressed as µmol Trolox equivalent per µmol tested compound.

***Cell culture***

B16F10 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin solution and 5% L-glutamine. The cells were cultured in an incubator set at 37 °C, with a carbon dioxide concentration of 5% and a relative humidity of 90%. For further experiments, MTT assay was performed as described by Phosri *et al.* [21] and melanin content was carried out according to Joompang *et al.* [22] with slight modifications. Cells were seeded in 96-well plates and 12-well plates for MTT assay and melanin content determination, respectively and cultured until reaching 80% confluency. The cells were then treated with test compounds at concentrations ranging from 0 to 175 µM and incubated for 24 h before subsequent assays.

***MTT assay***

After a 24 h treatment period, the medium was removed and 150 µL of MTT solution (0.5 mg/mL) was added to each well, followed by incubation for 4 h. The resulting formazan crystals were dissolved in 150 µL of DMSO and absorbance was measured at 570 nm using a microplate reader. The percentage of cell viability was calculated using the following Eq. (2):

$$\text{Cell viability (\%)} = [(\text{A 570 of test}) / \text{A 570 of control})] \times 100, \tag{2}$$

where A 570 of test and A 570 of control represent the absorbance values with and without test compounds, respectively.

***Melanin content***

After a 24 h treatment period, the medium was removed, cells were washed with phosphate buffer saline (PBS) and 150 µL of DMSO/NaOH (1 M) mixture (1:9) was added to each well, followed by incubation at 80 °C for 1 h. Then, 100 µL of the melanin solution was transferred to a 96-well plate and absorbance was measured at 450 nm using a microplate reader. Melanin content was calculated using the following Eq. (3):

$$\text{Melanin content (\%)} = [(\text{A 450 of test}) / \text{A 450 of control})] \times 100, \tag{4}$$

where A 570 of test and A 570 of control represent the absorbance values with and without test compounds, respectively.

***Statistical analysis***

The data were represented as mean ± SD (n = 3). The one-way ANOVA analysis was performed using IBM SPSS Statistics 20. Duncan's test was used to indicate the significant differences (*p-value* < 0.05).

## Results and discussion

### Tyrosinase inhibitory activity

The inhibition of tyrosinase represents an effective therapeutic approach for the treatment or prevention of skin hyperpigmentation. Tyrosinase inhibitors derived from natural, synthetic and semi-synthetic compounds have been the subject of extensive research [23]. Curcumin and its derivatives constitute a group of compounds that have demonstrated various biological activities, including significant tyrosinase inhibitory effects [13,24].

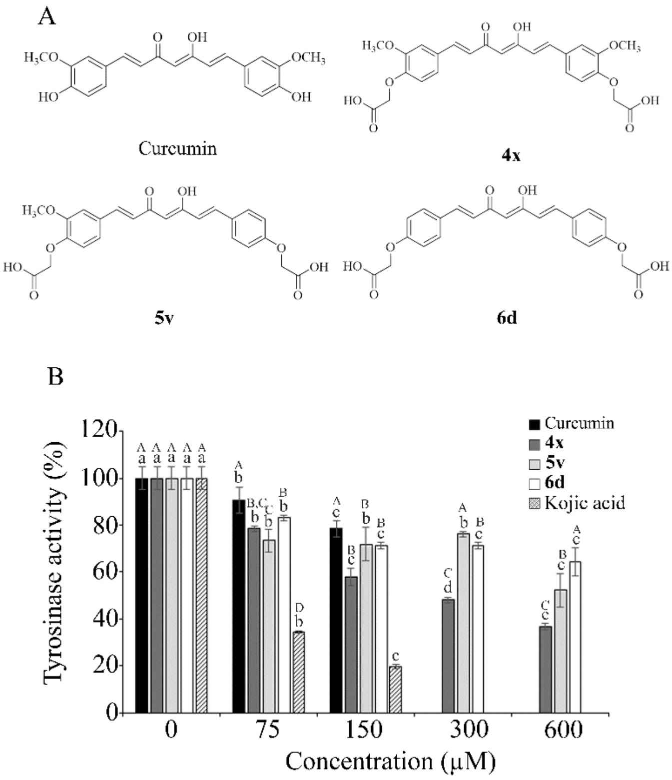

**Figure 1** Structure of bis(carboxymethoxy) curcumin derivatives and tyrosinase activity. (A) Structure of 4x (2,2'-((((*1E,3Z,6E*)-3-hydroxy-5-oxohepta-1,3,6-triene-1,7-diyl)bis(2-methoxy-4,1-phenylene))bis(oxy)) diacetic acid), 5v (2-(4-((*1E,3Z,6E*)-7-(4-(carboxymethoxy)-3-methoxyphenyl)-3-hydroxy-5-oxohepta-1,3,6-trien-1-yl)phenoxy)acetic acid), and 6d (2,2'-((((*1E,3Z,6E*)-3-hydroxy-5-oxohepta-1,3,6-triene-1,7-diyl)bis(4,1-phenylene))bis(oxy))diacetic acid). (B) Remaining tyrosinase activity. The tested compounds, curcumin and kojic acid were evaluated at concentrations of 75 - 600 µM and 75 - 150 µM, respectively. The data are presented as mean ± SD (n=3). Different lowercase letters indicate significant differences by Duncan's test (*p-value* < 0.05) between concentrations, within each compound. Different uppercase letters indicate significant differences by Duncan's test (*p-value* < 0.05) between compounds, within each concentration.

Previously, Somsakeesit *et al.* [17] synthesized and reported histone deacetylase inhibitory activity of curcumin derivatives for anticancer development. Meanwhile, our study focused on carboxyl-containing derivatives, specifically 4x, 5v, and 6d (**Figure 1(A)**). Although some carboxyl-containing compounds exhibit strong tyrosinase inhibition, the tyrosinase inhibitory activity of these particular bis(carboxymethoxy) curcumin derivatives has not been investigated until now.

Despite structural and functional differences between mushroom and human tyrosinase, leading to the same inhibitor showing different levels of inhibition and mechanisms in mushroom versus human tyrosinase [25], mushroom tyrosinase has been widely used as a model for studying tyrosinase inhibitors due to the conserved amino acids at the active site with human tyrosinase, its low cost and its widespread commercial availability [26,27]. Standard tyrosinase inhibition assays are commonly conducted at 25 °C and pH 6.8, as these conditions provide an optimal balance between enzyme activity and long-term stability, while enabling comparison of tyrosinase inhibitory activity across different studies. Therefore, in this study, the inhibitory activity of the test compounds against mushroom tyrosinase was investigated under these standard conditions.

The results of our study showed that 4x, 5v, and 6d at concentrations of 75 - 600 µM decreased tyrosinase activity to 78.52 - 36.72, 73.44 -52.34 and 83.20 - 64.45%, respectively, compared to 100% of the control (**Figure 1(B)**). Notably, the tyrosinase inhibitory activity of 4x was greater than that of curcumin at the same concentration, suggesting that the modification with the carboxymethoxy group increased tyrosinase inhibitory activity. Among the tested compounds, 4x demonstrated the highest inhibitory activity, followed by 5v and 6d. This result indicates that the removal of the methoxy group contributes to a decreased tyrosinase inhibitory activity in the bis(carboxymethoxy) curcumin derivatives.

The $IC_{50}$ value of 4x was further assessed based on its highest inhibitory activity against tyrosinase, demonstrating an $IC_{50}$ value of 272.85 μM. For comparison, under identical assay conditions, kojic acid at concentrations of 75 - 150 μM decreased tyrosinase activity to 34.59% - 19.73%, with an $IC_{50}$ value of 36.50 μM [28]. Previous studies have reported $IC_{50}$ values of curcumin derivatives against tyrosinase ranging from 8.0 to 330.9 μM [13], while other carboxyl-containing compounds demonstrated $IC_{50}$ values between 3.38 and 5.42 mM [29]. The $IC_{50}$ value of 4x remains consistent with the range observed in previously reported tyrosinase inhibitors, thereby establishing it as a promising candidate for application in hyperpigmentation treatment.

## Antioxidant activity

Reactive oxygen species play a crucial role in hyperpigmentation by activating proteins linked to melanogenesis [8]. Consequently, antioxidants show significant promise in treating hyperpigmentation. In this research, we evaluated the antioxidant activity of 4x, 5v, and 6d using ABTS and DPPH radical scavenging assays, which are widely accepted models for antioxidant assessment.

As presented in **Table 1**, curcumin exhibited a DPPH radical scavenging activity of 0.77 μM Trolox per μM curcumin, whereas this activity was not detected in bis(carboxymethoxy) curcumin derivatives. This difference can be explained through the evaluation of radical scavenging mechanisms. DPPH radical scavenging mechanism is based on the transfer of hydrogen atoms, with the phenolic hydroxyl group (phenol-OH) serving as a hydrogen atom donor to neutralize the radical [30,31]. Thus, the absence of the phenol-OH groups in bis(carboxymethoxy) curcumin derivatives following structural modification significantly diminishes their DPPH radical scavenging activity. In contrast, 4x demonstrated enhanced ABTS radical scavenging activity compared to curcumin. Unlike DPPH scavenging, the ABTS radical scavenging mechanism predominantly relies on electron transfer rather than hydrogen donation [32].

The carboxymethoxy ($–OCH_2COOH$) is generally a weak electron-donating group with a mild electron-withdrawing effect. Therefore, modification with the carboxymethoxy ($–OCH_2COOH$) group reduces the overall electron-donating effect, which would typically be expected to decrease ABTS radical scavenging activity. However, the observed increase in ABTS radical scavenging activity can be attributed to the enhanced hydrophilicity conferred by the carboxymethoxy modification. The introduction of this polar group significantly improves the water solubility of the curcumin derivatives, facilitating better interaction with ABTS radicals in the aqueous assay environment, ultimately leading to improved antioxidant efficiency.

Among the tested compounds, 4x showed the highest antioxidant activity, followed by 6d and 5v. These findings highlight the significance of the methoxy group for the antioxidant properties of 4x. The superior performance of methoxy-containing compounds can be explained by the *ortho*-methoxy group, which plays a key role as an electron-donating group [33].

Overall, this research demonstrates the significant antioxidant potential of bis(carboxymethoxy) curcumin derivatives, which could effectively reduce reactive oxygen species involved in hyperpigmentation pathways. Furthermore, our findings emphasize the critical role of methoxy groups in enhancing the antioxidant activity of bis(carboxymethoxy) curcumin derivatives through an electron transfer mechanism, providing valuable insights for the rational design and development of effective antioxidant compounds for therapeutic applications.

**Table 1** Antioxidant activity of curcumin and bis(carboxymethoxy) curcumin derivatives.

| **Compounds** | **µM Trolox/µM compound** | |
|---|---|---|
| | **ABTS radical** | **DPPH radical** |
| Curcumin | 1.17 ± 0.05$^{b}$ | 0.77 ± 0.13 |
| 4x | 1.61 ± 0.24$^{a}$ | n.d. |
| 5v | 0.48 ± 0.12$^{c}$ | n.d. |
| 6d | 0.58 ± 0.17$^{c}$ | n.d. |

The data are presented as mean ± SD (n=3). Different letters indicate significant differences by Duncan's test (*p-value* < 0.05). The abbreviation "n.d." stands for "not detected" at the tested final concentration of 10 µM.

### Molecular docking

To further understand the tyrosinase inhibitory mechanism, the interactions between 4x, 5v, and 6d with tyrosinase were elucidated using molecular docking. The results revealed that these compounds bound to tyrosinase at a site similar to that of curcumin (**Figure 2**). The GOLD scores for curcumin, 4x, 5v, and 6d were 77.81, 80.38, 86.42, and 77.09, respectively. The primary interactions of these compounds with the tyrosinase active site occurred through van der Waals forces, hydrogen bonds, and hydrophobic interactions (**Figures 3(A) - 3(D)**).

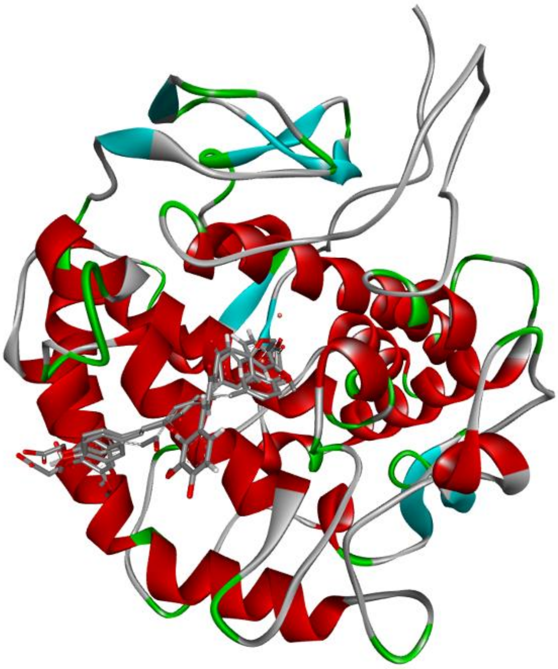

**Figure 2** The binding site of bis(carboxymethoxy) curcumin derivatives on the active site of tyrosinase. The figure was created and modified from Discovery Studio 2017 R2 Client.

The active site of tyrosinase contains six histidine residues, including His61, His85, His94, His259, His263, and His296, which interact with catalytic copper A (II) (Cu400) and B (II) (Cu401). Additionally, Phe90 and Phe292 provide stability to the copper-binding site. These amino acid residues and copper atoms constitute the key components of the tyrosinase catalytic pocket. The carboxylic group of 4x interacted with His61, His259, His263, and His296 through hydrogen bonds, while the carboxylic group of 5v and 6d formed hydrogen bonds with His61, His85, His94, His259, and His296. All three compounds interacted with Phe90 and Phe292 through van der Waals interactions. Moreover, the benzyl rings of the 4x established a hydrophobic interaction with Val283 and His259 in the catalytic pocket. 5v formed hydrophobic interactions with Val283 and His263, whereas 6d interacted solely with Val283 via a hydrophobic bond. These interactions with key amino acids in the tyrosinase catalytic site suggest that these compounds prevent substrate binding, thereby decreasing tyrosinase activity.

Moreover, Asn81, Asn260, His263, and Met280 are the key target amino acids for mushroom tyrosinase inhibitors. Well-known compounds like kojic acid, tropolone, and arbutin interact with some of these amino acids through hydrogen bonds and hydrophobic interactions [25]. Curcumin is positioned near Asn260, His263, and Met280, with its methoxy group's $CH_3$ forming a carbon hydrogen bond with Met280 and the phenol's OH creating a hydrogen bond with His263. Similarly, 4x is also located near Asn260, His263, and Met280, forming more interactions than curcumin, which include a carbon hydrogen bond ($CH_3$ of methoxy with Met280), hydrophobic interactions ($CH_3$ of methoxy with His263), a hydrogen bond (oxygen of carboxylic group with His263) and pi-lone pair interactions (phenyl ring with Asn260). The greater number of these interactions may result in higher tyrosinase inhibitory activity compared to curcumin.

For 5v and 6d, only 5v formed a hydrophobic interaction between its phenyl ring and His263, while no other hydrogen bonds or hydrophobic interactions were observed for either compound. Despite these limited molecular interactions, both compounds showed higher inhibitory activity than curcumin. The oxygen atom of the carboxylic group was positioned near the two catalytic copper atoms at a location similar to the oxygen atom of the phenol-OH in curcumin, suggesting an interaction between the oxygen atom and the catalytic copper atoms. This observation is consistent with the previous reports showing that the oxygen from phenol-OH groups in curcumin derivative aligns closely with catalytic copper atoms, demonstrating strong interactions [34]. In general, carboxyl group (-COOH) of carboxymethoxy group is more acidic than phenol-OH [35], which can be more easily deprotonated to carboxylate group and provide a stronger interaction with the copper atoms. Therefore, although 5v and 6d had fewer interactions with key amino acids (Asn81, Asn260, His263, and Met280), the carboxylic group of the carboxymethoxy group in these bis(carboxymethoxy) curcumin derivatives likely establishes a stronger coordination interaction with copper atoms compared to the oxygen atom in phenol-OH of curcumin, resulting in a greater inhibition.

The molecular docking also revealed that removing the methoxy group led to a loss of interaction between 4x and key amino acids, as mentioned above. Moreover, the hydrophobic interaction between 4x and Val286 was also eliminated, resulting in reduced tyrosinase inhibitory activity. This finding aligns with the recent research demonstrating that removal of both methoxy groups reduces curcumin's tyrosinase inhibitory activity [36]. Our results indicate that the methoxy group is essential for the tyrosinase inhibition activity of 4x. Based on these findings and its superior inhibitory activity, 4x was selected for further evaluation of its kinetic properties and melanin inhibition capacity.

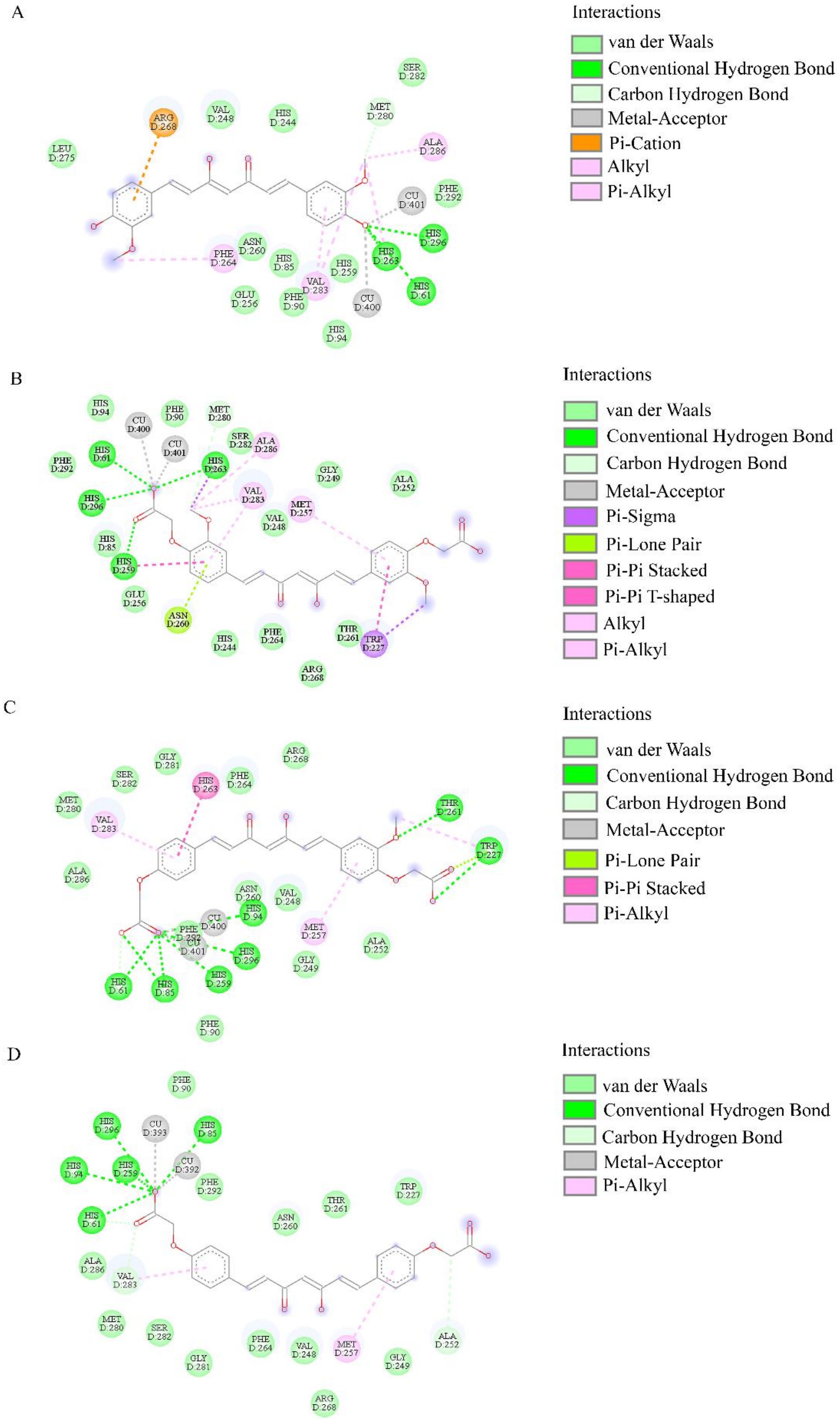

**Figure 3** Bis(carboxymethoxy) curcumin derivatives-tyrosinase complex. (A) Curcumin, (B) 4x, (C) 5v, and (D) 6d-tyrosinase complex. The interactions between the complexes are indicated by the dashed lines. The figure was created and modified from Discovery Studio 2017 R2 Client.

**Kinetic study**

To determine the type of inhibition, the enzyme kinetics were analyzed using the Lineweaver-Burk plot. The results showed that increasing concentration of 4x resulted in elevated $K_m$ values, while $v_{max}$ remained constant (**Figure 4(A)** and **Table 2**), indicating the presence of a competitive inhibitor. This implies that 4x directly competes with L-Dopa for binding to the tyrosinase active site, thereby reducing enzyme activity. The x-intercept of slope replot analysis revealed that 4x exhibits a $K_i$ value of 42.38 µM (**Figure 4(B)**). According to the previous report, a tyrosinase inhibitor with a $K_i$ in the micromolar range was classified as a weak inhibitor [37]. Therefore, 4x falls into the weak inhibitory category. However, weak inhibitors may be advantageous for developing anti-hyperpigmentation agents intended for long-term use, as they are less likely to cause adverse effects while maintaining therapeutic efficacy.

**Table 2** Kinetic parameter of tyrosinase in the absence and presence of bis(carboxymethoxy) bismethoxycurcumin.

| Compound | Concentration (µM) | $K_m$ (mM) | $v_{max}$ ($\triangle A_{475}$/min) | Type of inhibition |
|---|---|---|---|---|
| 4x | 0 | $0.176 \pm 0.026^a$ | $0.096 \pm 0.011^a$ | Competitive inhibitor |
| | 75 | $0.324 \pm 0.082^a$ | $0.097 \pm 0.012^a$ | |
| | 150 | $0.677 \pm 0.193^b$ | $0.110 \pm 0.026^a$ | |

The data are presented as mean ± SD (n = 3). Different letters indicate significant differences by Duncan's test (*p-value* < 0.05).

These kinetic findings corroborate our molecular docking results, which demonstrated that 4x binds to the tyrosinase active site and effectively blocks substrate binding. Our results are consistent with previous studies reporting competitive inhibition by other carboxylic-containing compounds, including 3-phenyllactic acid, azelaic acid, ferulic acid, and chlorogenic acid [29,38,39]. Rocchitta *et al.* [34] found that curcumin derivative compounds 1 and 6 acted as competitive inhibitors [34]. In contrast, other curcuminoid derivatives, including 5, 12, and 17, demonstrated different inhibition mechanisms and acted as mixed, non-competitive and mixed inhibitors, respectively [13]. Furthermore, chemically modified curcumin derivatives (CMC), CMC2.24 and CMC2.23, demonstrated mixed inhibition mode with a predominant competitive inhibition [40].

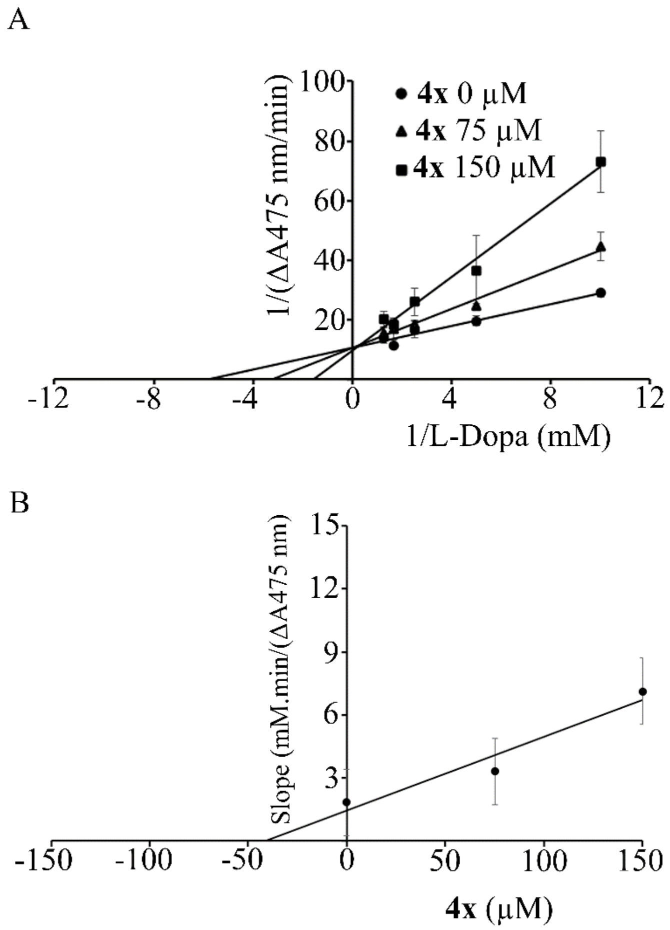

**Figure 4** Kinetic analysis of tyrosinase inhibition by 4x. (A) Lineweaver-Burk plot and (B) the slope replot between 4x concentration (0 to 150 µM) and slope. Tyrosinase activity was measured using L-Dopa concentrations ranging from 0.1 to 0.8 mM in the presence and absence of bis(carboxymethoxy) bismethoxycurcumin (75 to 150 µM). The data are represented as mean ± SD (n = 3).

This study examined the tyrosinase inhibitory activity of curcumin derivatives using mushroom tyrosinase as a model system. Although mushroom tyrosinase is widely used for screening tyrosinase inhibitors due to its availability and cost-effectiveness, its three-dimensional structure and kinetic properties differ from those of human tyrosinase. Consequently, the same inhibitor can exhibit different inhibitory potencies, interactions, and kinetic properties between mushroom and human tyrosinase [25,41]. Therefore, further investigation of these curcumin derivatives against human tyrosinase is essential to validate their inhibitory mechanisms and evaluate their potential as anti-hyperpigmentation agents for therapeutic applications.

### Cytotoxicity and melanin content

The effect of 4x on melanin content was investigated following the assessment of its cytotoxicity profile. The result showed that when B16F10 cells were treated with 4x at concentrations 75 - 150 µM, no significant cytotoxicity was observed, as evidenced by comparable cell viability percentages between treated and untreated cells (**Figure 5(A)**). While untreated cells exhibited a melanin content of 100.00%, cells treated with 4x (75 - 150 µM) showed a decrease in melanin content to 88.02% - 70.57% (**Figure 5(B)**). Previous studies found that curcumin derivative compounds 1, 6, and 7 at 100 µM reduced melanin content in B16F10 cells to 5.8%, 3.9%, and 78.3%, respectively [34]. Moreover, curcumin and its derivatives CMC2.14, CMC2.23, and CMC2.24 at 10 µM suppressed melanin with percentage inhibitions of 20.92%, 28.42%, 28.63%, and 32.54%, respectively [40]. These results demonstrate that 4x effectively reduces melanin production in B16F10 cells without inducing cytotoxicity, positioning itself as a promising candidate for development as an anti-hyperpigmentation agent. However, during development, the low stability of the compound can limit its use. For instance, kojic acid, a popular commercial whitening agent, exhibits low stability, which constrains its application [9]. Conversely, several curcumin derivatives have been reported to have improved stability, which can increase their usability [24,40]. Therefore, investigating the stability of 4x is important for future research to discover more potential applications.

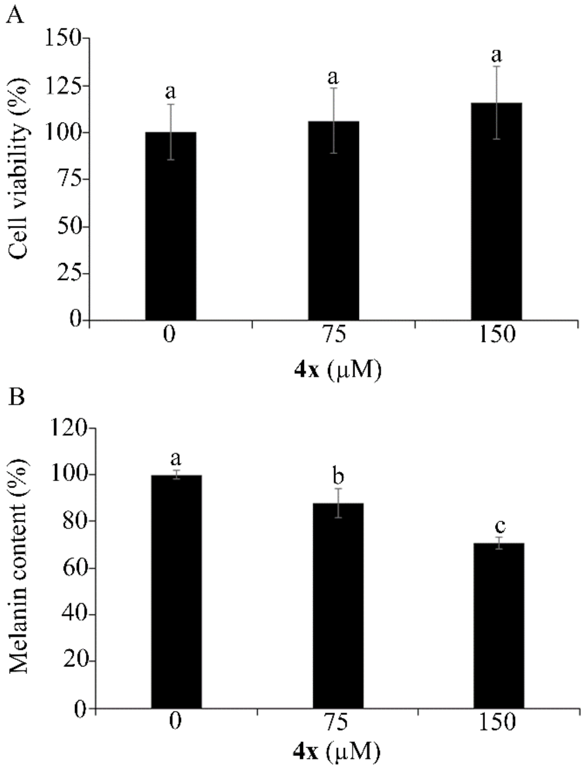

**Figure 5** Cytotoxicity and melanin content of 4x. (A) Percentage of cell viability of B16F10 cells and (B) percentage of melanin content. 4x was tested at concentrations ranging from 75 to 150 μM. The data are presented as mean ± SD (n=3). Different letters indicate significant differences by Duncan's test (*p-value* < 0.05).

## Conclusions

This research demonstrates the potential of 4x, 5v, and 6d as effective tyrosinase inhibitors. The modification with carboxymethoxy groups significantly enhances the tyrosinase inhibitory activity of curcumin, with 4x exhibiting superior tyrosinase inhibitory and ABTS radical scavenging activities. Moreover, the results highlighted the important role of the methoxy group in conferring antioxidant properties through electron transfer of bis(carboxymethoxy) curcumin derivatives. Molecular docking analyses revealed that these compounds interact with the active site of tyrosinase mainly through van der Waals forces, hydrogen bonds, and hydrophobic interactions. Compounds exhibiting stronger interactions with copper demonstrated greater inhibitory activity. In addition, the methoxy group proved to be crucial for the tyrosinase inhibitory activity of 4x. Moreover, 4x was found to be a competitive inhibitor that effectively reduced melanin in B16F10 cells without inducing cytotoxicity. Therefore, 4x presents itself as a promising candidate for further development as an anti-hyperpigmentation agent in both cosmetics and medical applications.

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## Declaration of Generative AI in Scientific Writing

No content generation or data interpretation was performed by AI. The authors take full responsibility for the content and conclusions of this work.

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