Design, synthesis, and biological evaluation of symmetrical azine derivatives as novel tyrosinase inhibitors

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Abstract
A series of symmetrical azine derivatives containing different substituted benzyl moieties were designed, synthesized, and evaluated for their inhibitory activity against tyrosinase. The results showed that compounds 3e, 3f, 3h, 3i, 3j, and 3k possess effective tyrosinase inhibition with IC50 values ranging from 7.30 μM to 62.60 μM. Particularly, compounds 3f displayed around three-fold improvement in the potency (IC50 = 7.30 ± 1.15 μM) compared to that of kojic acid (IC50 = 20.24 ± 2.28 μM) as the positive control. Kinetic study of compound 3f confirmed uncompetitive inhibitory activity towards tyrosinase indicating that it can bind to enzyme–substrate complex. Next, molecular docking analysis was performed to study the interactions and binding mode of the most potent compound 3f in the tyrosinase active site. Besides, the cytotoxicity of 3f, as well as its potency to reduce the melanin content were also measured on invasive melanoma B16F10 cell line. Also, 3f exhibited above 82% cell viability in the A375 cell line at 10 μM. Consequently, compounds 3f could be introduced as a potent tyrosinase inhibitor that might be a promising candidate in the cosmetics, medicine, and food industry.

Keywords: Melanin, Azine derivatives, Tyrosinase inhibitors, Molecular docking

Introduction
Melanin is known as a major pigment found in the eyes, hair, and skin of animals and humans which has protective roles against the harmful effects of ultraviolet (UV) irradiation, oxidative stress, DNA damage, and malignant transformation [1, 2]. Despite the key features of melanin, excessive production, and hyperpigmentation of melanin cause dermatological disorders such as melasma, ephelides, chloasma, freckles, melanoderma, and senile lentigines [3]. The excess melanin synthesis can also induce inflammation such as eczema, irritant and allergic eczema contact dermatitis, which may be attributed to critical and emotionally distressing difficulty [4]. Moreover, there is some evidence about the correlation between neuromelanin and the pathogenesis of Parkinson's disease [5]. Also in the agricultural industry, overproduction of melanin in fruits and vegetables causes food browning and decline in product quality [6].

Tyrosinase (Polyphenol oxidase, a copper-containing enzyme, PPO, E.C.1.14.18.1) is a critical rate-limiting enzyme in the melanogenesis pathway. Tyrosinase plays a central role in the biosynthesis pathway of melanin pigment by catalyzing the hydroxylation and oxidation of monophenols to o-diphenols (monophenolase activity) and the oxidation of o-diphenols to o-quinones (diphenolase activity) [7]. To get rid of the mentioned undesirable problems, searching for novel and effective tyrosinase inhibitors is...
hormones is highly demanded. Although a large number of tyrosinase inhibitors have been discovered [8] such as chalcones [9], stilbenes [10–13], flavonol [14], flavone [15, 16], flavanone [17], flavanol [18], kojic acid [19–23], tropolone [8, 24–27], phthalimide [28], triazole [29] thiosemicarbazide [30] and quinazoline [31] derivatives (Fig. 1), only a few of them have been applied to the market due to their harmful and undesirable side effects, such as lack of safety, low efficacy and allergenic reactions [32, 33]. Consequently, it is necessary to search for new and potent tyrosinase inhibitors with fewer adverse effects.

Azines, N–N linked diamines are useful and stable compounds with interesting chemical attributes. Recently, azine-containing compounds gain lots of attention as important scaffold for drug designing owing to their modulating behavior towards bio-receptors [34, 35]. Azines also demonstrated antibacterial, antimalarial, antiviral, antitumor, and anti-inflammatory properties [36, 37].

As a result, in this study, novel series of azine derivatives were rationally designed, synthesized, and evaluated against the tyrosinase enzyme. Molecular docking analysis of the most potent derivative was also obtained to achieve a distinct insight into the binding mode and interactions of the compounds in the tyrosinase enzyme active site. Moreover, the kinetic, cytotoxic, as well as melanin content assays, were also performed.

**Results and discussion**

**Design approach**

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) (A) is a natural antioxidant with numerous beneficial effects on human health such as neuroprotective, cardioprotective, anti-inflammatory, and anti-cancer effects which prompted the use of resveratrol as a therapeutic agent [38, 39]. Resveratrol is also known as a powerful tyrosinase inhibitor with an IC₅₀ value of 26.63 μM with no marked toxicity (main concern at doses of ≥ 0.5 g/day for long-term use) [40].

Oozeki et al. reported resveratrol base structure as strong tyrosinase inhibitors with an IC₅₀ value of 0.37 μM for the most potent compound (compound B, Fig. 2). Their results showed that the symmetry bibenzyl skeleton could easily bind to the active site of the tyrosinase and improved the inhibitory potency [41]. Furthermore, incorporation of an azo group into the linker while keeping biaryl structure gave azo-resveratrol derivatives. Particularly, compound C showed IC₅₀ = 36.28 ± 0.72 μM in

![Chemical structures of some tyrosinase inhibitors from natural or synthetic sources](image-url)
a dose-dependent manner, comparable to that of resveratrol [42].

Recently, it has been reported that some hydrazine-containing compounds, such as compounds D [43], E [44], and F [45], were effective tyrosinase inhibitors with an IC50 value of 9.9, 1.58, and 0.05 μM, respectively [46, 47]. Considering these structural features of all depicted compounds, most of them contain the same pharmacophore so that a linker with optimum length (two to four atoms) is connected to two aromatic rings (Ring X-Linker-Ring Y).

As a result, molecular hybridization and fragment-based drug design strategy were performed to develop a series of 1,2-bisaryl hydrazine derivatives as tyrosinase inhibitors. Various substituents were performed on benzylidene moieties to define beneficial structure–activity relationships (SARs).

**Chemistry**

A series of bis aryl hydrazine hybrids (3a–k) was synthesized according to the general synthetic route depicted in Scheme 1. Commercially available aryl aldehydes (1) were reacted with hydrazine hydrate (2) in refluxing ethanol for 24 h to give the corresponding compounds (3a–k). After cooling, the precipitate was filtered, washed with cold water, and recrystallized in methanol. The structures were fully characterized and confirmed by IR, 1H-NMR, 13C-NMR, MS, and elemental analysis.

**Tyrosinase inhibitory activity**

The inhibitory effects of all synthesized derivatives (3a–k) on tyrosinase are presented in terms of IC50 in Table 1. Kojic acid was used as a positive control for comparative purposes. In general, six compounds 3e, 3f, 3h, 3i, 3j, and 3k showed considerable inhibitory effects on tyrosinase with IC50 ranging from 7.3 to 62.6 μM.

The unsubstituted benzyl derivative (3a) had no significant inhibitory activity at the tested concentrations (IC50 > 100 μM).

To investigate the effect of the substituted moiety, different groups were introduced on the benzyl pendant. In the case of mono-substitution, it was found that the presence of one methoxy at different positions of benzyl ring did not show any improvement in the inhibitory activity (3b, R = ortho-OCH3 with IC50 > 100 μM and 3c, R = para-OCH3 with IC50 > 100 μM) compared to unsubstituted one. The isosteric replacement of methoxy at para position (3c), with a hydroxyl group in compound 3j, increased the inhibitory effect against tyrosinase (IC50 = 62.6 ± 0.71 μM).

Besides, compared to mono substitutions, multi-substitutions on benzyl ring significantly enhanced...
inhibitory potency as can be seen in compounds 3e, 3f, 3h, 3i, and 3k. The exception in this series came back to the compounds 3d (R = 3-OEt-4-OH, IC50 > 100 μM) and 3g (R = 3,4,5-(OMe)3, IC50 > 100 μM) which depicted weak anti-tyrosinase activity. Particularly, the most potent ligand identified in this study was compound 3f bearing 2,4-dihydroxy moiety with IC50 = 7.30 ± 1.15 μM, followed by 3k (R = 3-OH-4-OH) with an IC50 value of 12.90 ± 0.18 μM. On the other hand, the substitution of a para hydroxyl group in compound 3k (R = 3-OH-4-OH, IC50 = 12.90 ± 0.18 μM) with an acetoxy moiety resulted in 3i which showed a significant decrease in the inhibitory effect (IC50 = 28.1 μM). From the screening data, 3e containing 3,4,5-trimethoxy substituted groups at R known as the third potent compound in this series (IC50 = 20.10 ± 0.01 μM) with approximately the same value of potency compared to that of the standard kojic acid with an IC50 value of 20.24 ± 2.28 μM.

**Kinetic studies**

Kinetic studies were performed to examine the mechanism and type of inhibition by compound 3f as the most potent derivative against tyrosinase. The results are presented in Fig. 3. Lineweaver–Burk plots (plot of 1/V versus 1/[S]) for the inhibition of tyrosinase were obtained with several concentrations of 3f as the inhibitor and L-DOPA as the substrate. The analysis showed that Vmax and Km values decreased in the presence of increasing concentrations of 3f which showed a significant decrease in the inhibitory effect (IC50 = 28.1 μM). From the screening data, 3e containing 3,4,5-trimethoxy substituted groups at R known as the third potent compound in this series (IC50 = 20.10 ± 0.01 μM) with approximately the same value of potency compared to that of the standard kojic acid with an IC50 value of 20.24 ± 2.28 μM.

**Molecular docking study**

The molecular binding analysis was then performed to gain an understanding of the interactions and binding mode of 3f in the tyrosinase active site. The results are presented in Table 2. Tyrosinase has H2L2 tetramer structure with two H subunits and two L subunits. The active site placed in the H subunit comprises binuclear copper ions so that the first copper ion demonstrated interactions with three histidine residues named His 61, His 85, 

![Scheme 1](image-url)
and His95. The second copper ion coordinated by His 259, His 263, and His 296. Detailed and comprehensive studies confirmed that interactions with these critical residues can completely inactivate the monophenolase and diphenolase activity.

As shown in Fig. 4, compound 3f fitted well in the tyrosinase binding pocket by hydrogen bonding, electrostatic and hydrophobic interactions. The nitrogen atoms and the oxygen atoms of 3f formed five hydrogen bonds interacting with the Val 283, Gly 281, His 296, Met 280, and His 259 residues at distances of 2.80 Å, 3.74 Å, 2.78 Å, 2.80 Å, and 3.07 Å, respectively. Moreover, compound 3f was involved in electrostatic interactions with His263 and Arg268 as well as hydrophobic interactions with Phe264, Val283, His263, and Ala286. Docking results indicated that the substitution on the phenyl ring played an important role in forming drug-receptor interactions and binding orientation of the compound in the active site of tyrosinase by enhancing phenyl ring electron density.

**Cell viability on B16F10**

MTT assay was performed to determine the toxicity of 3f as the most potent tyrosinase inhibitor against invasive melanoma B16F10 cell line. According to the toxicity assay, 3f showed 60.15 ± 9.82% (±SD) cell viability at 10 µM.

**Measuring cell viability of A375 in response to 3f**

The A375 cell line is derived from a human skin melanoma carrying two mutant genes, B-RAF and CDKN2
which are associated with melanoma of sun-damaged skin [48]. To determine the cytotoxicity of 3f in A375 melanoma cell lines, MTT studies were conducted. Results exhibited that the cell viability was significantly higher in A375 with 82.59 ± 5.85 (% viability) compared with the B16F10 cell line at 10 µM.

Melanin content assay
It is well documented that effective tyrosinase inhibitors may reduce the phenolase activities of the enzyme which in return downregulates the melanogenesis process [49, 50]. In this regard, the potency of 3f to reduce the melanin content was evaluated on the B16F10 cell line. Kojic acid was used as the positive control. Data were expressed as mean ± SD of at least three independent experiments. According to Fig. 5, 3f significantly reduced the melanin content in skin melanoma cells to 79.58% at 10 µM in comparison with the control (P-value < 0.05).

Conclusion
Following our expertise in the rational design of tyrosinase inhibitors; herein, we designed, synthesized, and evaluated different azine substituted derivatives against tyrosinase. The most active compound 3f bearing 2,4-dihydroxy on the benzyl ring (IC50 = 7.30 ± 1.15 µM) showed 3 times better potency compared to that of kojic acid as the positive control. Moreover, it is worth mentioning that 3f showed an uncompetitive inhibition mode of action in the enzymatic assay. Molecular docking analysis demonstrated that the high potential of 3f would be due to the formation of strong interactions with the critical residues of the tyrosinase active site. In addition, cell assessments of 3f at 10 µM against B16F10 and A375 cell lines exhibited around 60% and 82% viability, respectively. Importantly, compound 3f showed approximately similar potency to reduce the melanin content on B16F10 cell lines at 10 µM compared to kojic acid as a positive control. In general, it can be concluded that the synthesized compounds can serve as structural outlines and promising lead to design and expand potential tyrosinase inhibitors.

Material and methods
General
All needed chemicals were purchased from Merck and Acros chemical companies. All reagents and solvents were dried before use according to standard methods. Mushroom tyrosinase (EC1.14.18.1), kojic acid, dimethyl sulfoxide (DMSO), and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). IR spectra were obtained on an FT-IR Perkin-Elmer Precisely system spectrophotometer (potassium bromide disks). Melting points were determined on a Kofler hot stage apparatus and are uncorrected. 1H NMR and 13C NMR spectra were recorded in DMSO using a Bruker Avance DPX instrument (1H NMR 500 MHz, 13C NMR 125 MHz). Chemical shifts (δ) were reported in parts per million (ppm) downfield from TMS as an internal standard. All of the coupling constants (J) are in hertz. The mass spectra were run on an Agilent 6410 apparatus. Merck silica gel 60 F254 plates were used for analytical thin-layer chromatography (TLC). Preparative thin-layer chromatography was done with prepared glass-backed plates (20*20 cm2) using silica gel (Merck-Kieselgel 60 HF254, Art. 7739).

Synthesis
The typical procedure for the synthesis of bis aryl hydrazide derivatives
A mixture of aryl aldehyde (1 mmol), and hydrazine hydrate (0.5 mmol) were refluxed in ethanol for 24 h. The reaction proceeding was monitored by TLC (n-hexane/ethyl acetate, 20:1 v/v). After completion of the reaction, the mixture was cooled to room temperature, the precipitated solid was isolated by filtration, all products were recrystallized from hot ethanol. Physical and spectral data of bis aryl hydrazide derivatives are given below [51–53].

1,2-di(Benzylidene)hydrazine (3a)
Yellow solid; Isolated yield: 65%; m.p: 88–93 °C; LR (KB, cm−1): 3067, 3050, 3025, 3000, 2949, 1624, 1574, 1491, 1446. 1H NMR (300 MHz, DMSO-d6) δH (ppm): 8.58 (s, 2H), 7.98–7.81 (m, 4H), 7.55–7.32 (m, 6H). 13C NMR (75 MHz, DMSO-d6) δc (ppm): 156.30, 134.20, 130.89, 128.76, 128.31. Anal. Calcd. For C14H12N2: C: 80.74; H: 5.81; N: 13.45; Found. C: 80.99; H: 5.83; N: 13.48.

1,2-bis(2-Methoxybenzylidene)hydrazine (3b)
Commercially available with CAS number of 17745-81-2. Yellow solid; isolated yield: 64%; m.p: 142–146 °C; I.R
(KBr) \( \nu_{\text{max}} \) (cm\(^{-1}\)):
- 3075, 3005, 2965, 2946, 2842, 1616, 1576, 1485, 1468, 1438, 1321, 1250.
- \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 8.94 (s, 2H), 7.98 (d, \( J = 7.3 \) Hz, 2H), 7.51 (t, \( J = 7.5 \) Hz, 2H), 7.15 (d, \( J = 7.5 \) Hz, 2H), 7.05 (t, \( J = 7.5 \) Hz, 2H), 3.89 (s, 6H).
- \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 56.27, 112.49, 121.19, 122.08, 127.00, 133.50, 157.01, 159.22.
- MS (m/z, %):
  - 77.3(41), 91.3(90), 119.3(65), 131.3(26), 150.3(35), 161.3(13), 237.3(92), 268.3(100).
- Anal. Calcd. For C\(_{16}\)H\(_{14}\)N\(_2\)O\(_2\): C: 71.62; H: 6.01; N: 10.44; Found: C: 72.92; H: 6.00; N: 10.45.

1,2-bis(4-Methoxybenzylidene)hydrazine (3c)
Commercially available with CAS number of 2299-73-2.
- Yellow solid; isolated yield: 58%; m.p.: 164–166 °C; I.R max (cm\(^{-1}\)):
  - 3518, 3468, 3214, 3097, 2998, 2936, 2891, 1616, 1589, 1534, 1453, 1251, 1121.
- \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 6.35 (d, \( J = 2.4 \) Hz, 2H), 6.41 (dd, \( J = 8.4, 2.4 \) Hz, 2H), 7.41 (d, \( J = 8.4 \) Hz, 2H), 8.77 (s, 2H), 10.27 (s, 2H), 11.39 (s, 2H).
- \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 102.95, 108.69, 110.73, 133.46, 161.16, 162.27, 162.54. MS (m/z, %): 137.3(79), 255.4(36), 272.2(M\(^+\), 100). Anal. Calcd. For C\(_{18}\)H\(_{18}\)N\(_2\)O\(_4\): C: 61.76; H: 4.44; N: 10.29; Found: C: 60.88; H: 4.45; N: 10.28.

5,5′-(Hydrazone-1,2-diylidene)bis(2-methoxyphenyl)ene diacetate (3i)
Commercially available with CAS number of 17745-86-7.
- Yellow solid; isolated yield: 94%; m.p.: 197–199 °C; I.R max (cm\(^{-1}\)):
  - 3518, 3468, 3214, 3097, 2998, 2936, 2891, 1616, 1589, 1534, 1453, 1251, 1121.
- \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 6.35 (d, \( J = 2.4 \) Hz, 2H), 6.41 (dd, \( J = 8.4, 2.4 \) Hz, 2H), 7.41 (d, \( J = 8.4 \) Hz, 2H), 8.77 (s, 2H), 10.27 (s, 2H), 11.39 (s, 2H).
- \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)) \( \delta \) (ppm): 102.95, 108.69, 110.73, 133.46, 161.16, 162.27, 162.54. MS (m/z, %): 137.3(79), 255.4(36), 272.2(M\(^+\), 100). Anal. Calcd. For C\(_{18}\)H\(_{18}\)N\(_2\)O\(_4\): C: 61.76; H: 4.44; N: 10.29; Found: C: 60.88; H: 4.45; N: 10.28.
The inhibitory activity of the tested compounds was expressed as the concentration that inhibited 50% of the enzyme activity (IC_{50}).

**Determination of the inhibition type**

A series of experiments were performed to determine the inhibition kinetics of 3f. The inhibitor concentrations were: 0, 8, 16 μM. Substrate (L-DOPA) concentrations were (0.1, 0.25, 0.5, 0.75 and 1.5 mM) in kinetic studies. Pre-incubation and measurement time was the same as discussed in the mushroom tyrosinase inhibition assay protocol. Maximum initial velocity was determined from the initial linear portion of absorbance up to 10 min after addition of L-DOPA with 1 min interval. The Michaelis constant (K_{m}) and the maximal velocity (V_{max}) of the tyrosinase activity were determined by the Lineweaver–Burk plot at various concentrations of L-DOPA as a substrate. The inhibition type of the enzyme was assayed by Lineweaver–Burk plots of the inverse of velocities (1/V) versus the inverse of substrate concentrations 1/[S] mM^{-1}.

**Molecular docking study**

Docking was done by AutoDock 4.2 (http://autodock.scripps.edu) and AutoDock Tools 1.5.4 (ADT) (http://mgltools.scripps.edu). X-ray crystal structure of agaricus bisporus tyrosinase containing tropolone in the active site (PDB ID: 2Y9X), was regaigned from protein data bank (http://www.rcsb.org). Before the docking method, the water molecules and the inherent ligand were eliminated from the protein. Hydrogens were attached and non-polar hydrogens were merged. Also, Gasteiger charges were calculated for protein 2Y9X. 3D structures of ligands were drawn and minimized under Molecular Mechanics MM^{+} and then Semi-empirical AM1 methods using HyperChem software (http://www.hyper.com/). The pdbqt formats of the ligands were prepared by adding Gasteiger charges and setting the degree of torsions. The active site which contains Cu^{2+} metal ions were chosen for docking and the grids’ center was placed on the tropolone’s binding site. The box dimensions were set to 40 × 40 × 40 with 0.375 Å grid spacing. To determine the docking parameter file, a rigid macromolecule was elected. The Lamarckian genetic search algorithm was used and the number of GA runs was determined at 100. The other parameters were left at program default values. The validity of the docking procedure was tested using the co-crystallized inhibitor as ligand and the above-mentioned protocol. Finally, conforma-
MTT assay for cell viability
The cytotoxic activity of compound was evaluated in B16F10 cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in a 96-well plate (5 × 10^3 cells/well) and incubated at 37 °C with samples at different concentrations for 48 h. Following the treatment, cells were incubated with MTT (0.5 mg mL^-1) at 37 °C for 3 h. The MTT-containing medium was then removed, and 100 μL of DMSO was added to each well, mixed thoroughly with a 10 min shake to dissolve formazan crystals. The absorbance of each well was measured at 540 nm.

Determination of melanin content
B16F10 cells were seeded in six-well plates (1.0 × 10^5 cells/well). After 24 h, the medium was substituted by a fresh one and treated with 3f, and incubated for 48 h. Then, cells were treated with 100 nM α-MSH. Kojic acid was used as positive control and for comparing the inhibitory strength of the compound. After incubation cells were washed twice with PBS and harvested using 0.25 M trypsin, then dissolved in 300 μL of 1 N NaOH/10% DMSO buffer and boiled for 2 h at 80 °C to solubilize the melanin. The absorbance of the supernatant was measured at 470 nm in a microplate reader. The obtained results were normalized using total protein content.

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Authors’ contributions
SK synthesized compounds as well as prepare the manuscript. FK synthesized compounds. MA performed the biological assay. MG performed the biological assay. SH supervised the biological tests. AS contributed to the design and characterization of compounds. YB performed the biological assay. MKabiri performed the biological assay. AI performed docking study and contributed to the preparation of the manuscript. MKhoshnevisazadeh supervised all phases of the study. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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