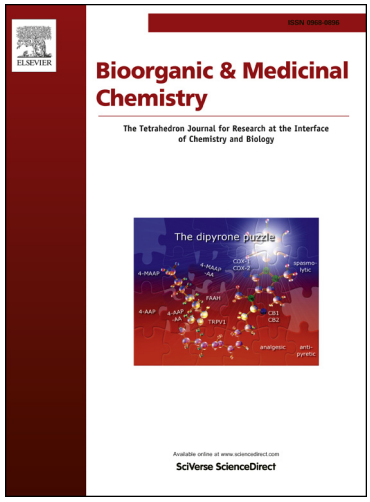

Synthesis of novel compounds containing morpholine and 5(4H)-oxazolone rings as potent tyrosinase inhibitors

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Synthesis of novel compounds containing morpholine and 5(4H)-oxazolone rings as potent tyrosinase inhibitors

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Abstract

In this study, six new compounds containing morpholine and 5(4H)-oxazolone rings were synthesized. Structures of the new compounds using IR, ^{1}HNMR, mass spectroscopy and elemental analysis were characterized. All new compounds (**4a-4f**) have a strong inhibitory effect against mushroom tyrosinase. And the inhibitory effects of these compounds were compared with Kojic acid as standard.

Keywords: Tyrosinase inhibitor, 5(4H)-Oxazolone, Morpholine, Synthesis.

1. Introduction

Tyrosinase (EC1.14.18.1), also known as polyphenoloxidase (PPO), is a copper containing enzyme involved in melanin synthesis and widely distributed in nature. Two reactions involved in melanin biosynthesis pathways catalyzed by tyrosinase are hydroxylation of monophenols to *o*-phenols and the oxidation of the *o*-phenols to *o*-quinones.[1] Tyrosinase catalyzed the formation of quinones and mediated the development of brown colour pigment through spontaneous polymerization of highly reactive quinones.[2]

In mammals, L-tyrosine serves as a typical monophenol substrate. Numerous phenolics having monophenol and *o*-diphenol structures found in plants and insects

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are similarly oxidized by tyrosinase.[3] This enzymatic oxidation is the ratelimiting step in several phenomena of color development and cuticle sclerotization; the remainder of the reaction sequence proceeds spontaneously at physiological conditions.[4]

Tyrosinase inhibitors are recommended for the treatment of melanin hyperpigmentation and used in cosmetics for whitening and depigmentation after sunburn.[5]Tyrosinasehas an important role in the regulation of melanin biosynthesis. Therefore, many tyrosinase inhibitors that prevent melanogenesis have been widely considered with the aim of developing for the treatment of hyperpigmentation.[6]

5(4H)-Oxazolones are profitable intermediates for the synthesis of several biological compounds such as amino acids and peptides.[7-8] Also some of the these compounds represent an extensive range of pharmacological activities including Anti-inflammatory,[9] anticancer,[10] antagonistic and antiangiogenic,[11-12] tyrosinase inhibitor[13] and etc.

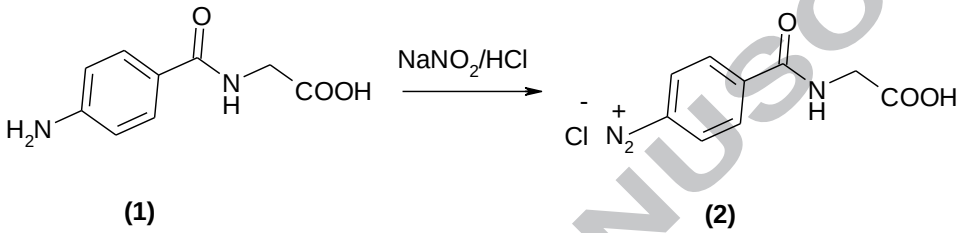
In continuation of our research on 5(4H)-oxazolones as potential compounds in our drug discovery program, [13-14] we synthesized some of new 5(4H)-oxazolone derivatives and screened them for their tyrosinase inhibitory properties. We also briefly studied the structure–activity relationships of these compounds to find out possible lead compound for the treatment of tyrosinase-related diseases.

2. Results and disscusion

2.1. Chemistry

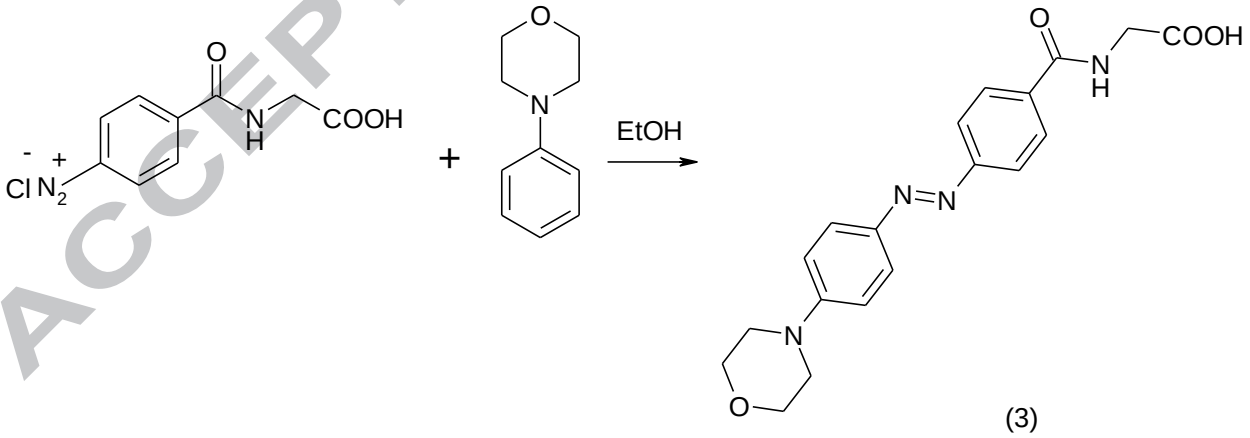
Diazonium salts could react readily with nucleophiles as an aromatic compounds containing amino or hydroxyl group, which have been extensively researched and

widely used for the preparation of molecules with significance for both academic and industrial applications. 4-Amino hippuric acid is dissolved in a 2.5% sodium carbonate solution by heating and stirring. In the solution of 4-Amino hippuric acid, sodium nitrite is dissolved and 4-Amino hippuric acid was diazotized by slow addition of conc. HCl at 0°C.A yellow precipitate of the diazonium salt **(2)** was formed (Scheme 1).[15]



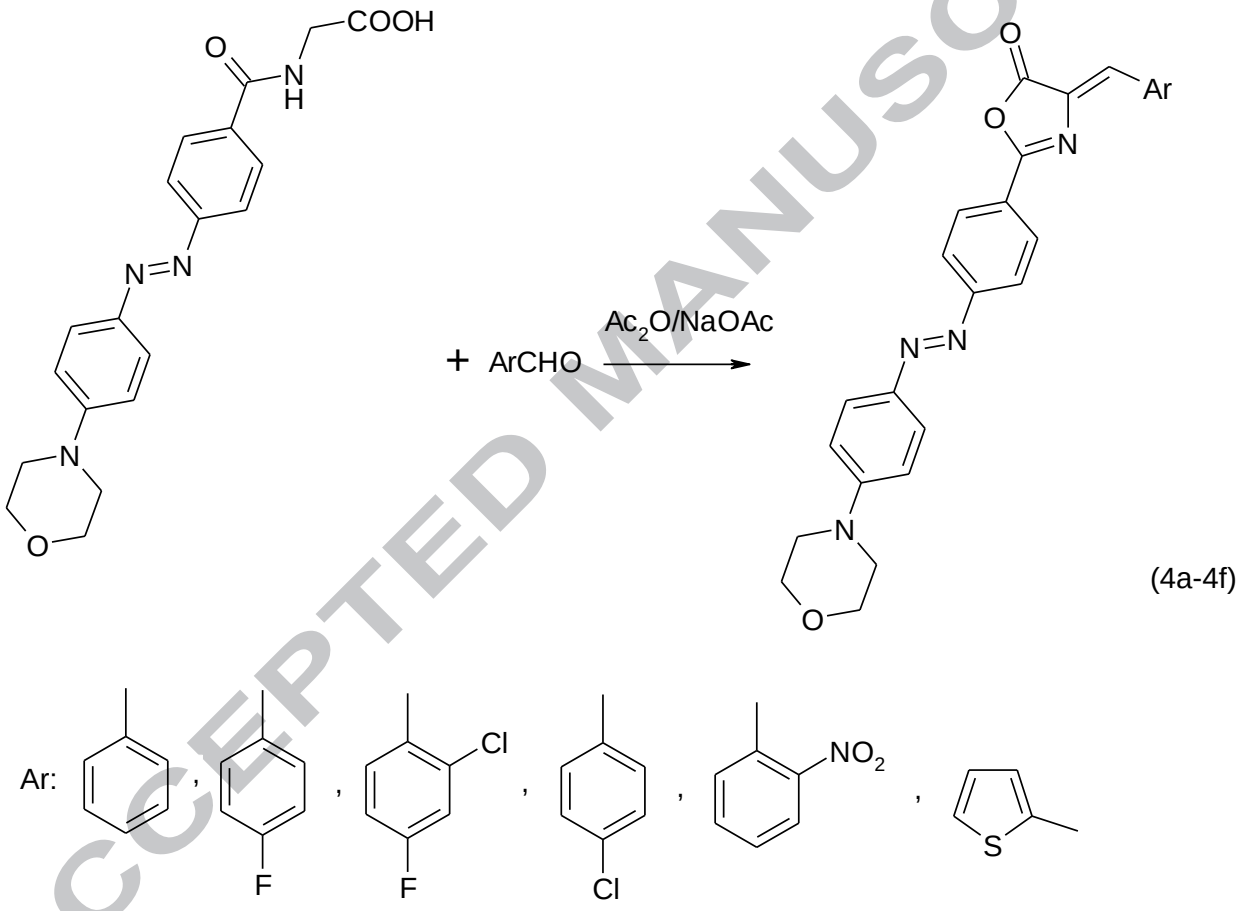
Scheme 1. Diazotisation of 4-aminohippuric acid.

N- phenylmorpholine (Coupling component) is added to diazonium salt of 4-aminohippuric acid. Azo dye **(3)** is produced in good yields. Diazonium salt is coupled to the *para*-position of the morpholino group (Scheme 2).



Scheme 2. Coupling of diazonium salt with aromatic compounds.

Then 4-arylidene-5(4H)-oxazoloneazo dyes **(4a–4f)** are synthesized by classical Erlenmeyer reaction, involving condensation of compound **(3)** with 4-fluoro benzaldehyde, benzaldehyde, 2-chloro-4-fluoro benzaldehyde, 4-chloro benzaldehyde, 2-nitro benzaldehyde and thiophen 2- carbaldehyde in presence of acetic anhydride and sodium acetate under refluxing condition at 100 °C for 4 h (Scheme 3).

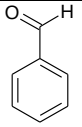
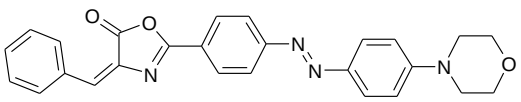
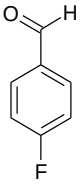
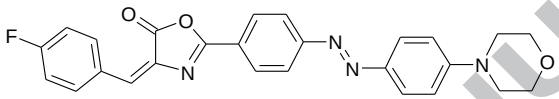
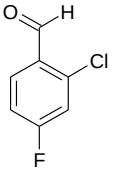
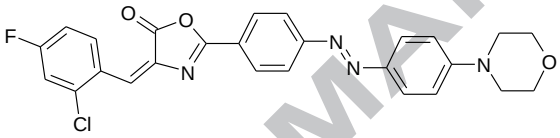
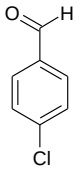
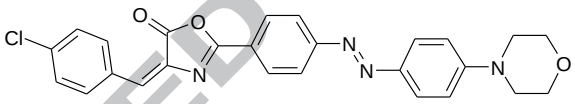
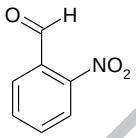
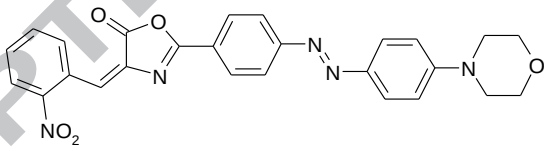
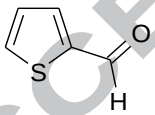
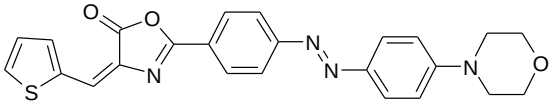


Scheme 3. Synthesis of new 5(4H)-oxazolone compounds **(4a-4f).**

The synthesized compounds obtained varied in color from red to brown, a suitable method of measuring the color of the compound was to study the absorption spectra of their solutions. The visible absorption maximums for the synthesized

compounds were measured in DMSO at the concentration of 10^{-6} M and are listed in Table 1. The absorption maximum of the synthesized dyes changed from 340 to 653 nm. Structures of synthesized compounds were determined by ^{1}H NMR, mass spectroscopic and elemental analysis.

Table 1. Structure, yields and λ_{max} of new azo dyes **(4a-4f)**

Entry	ArCHO	Product		λ_{max} (DMSO)	Yield(%)[a]
1			**4a**	653	60
2			**4b**	644	64
3			**4c**	624	73
4			**4d**	641	70
5			**4e**	340	65
6			**4f**	351	55

[a] Isolated yields

2.2. Inhibitory activity of tyrosinase

IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determined by interpolation of the dose–response curves (Figure 1).

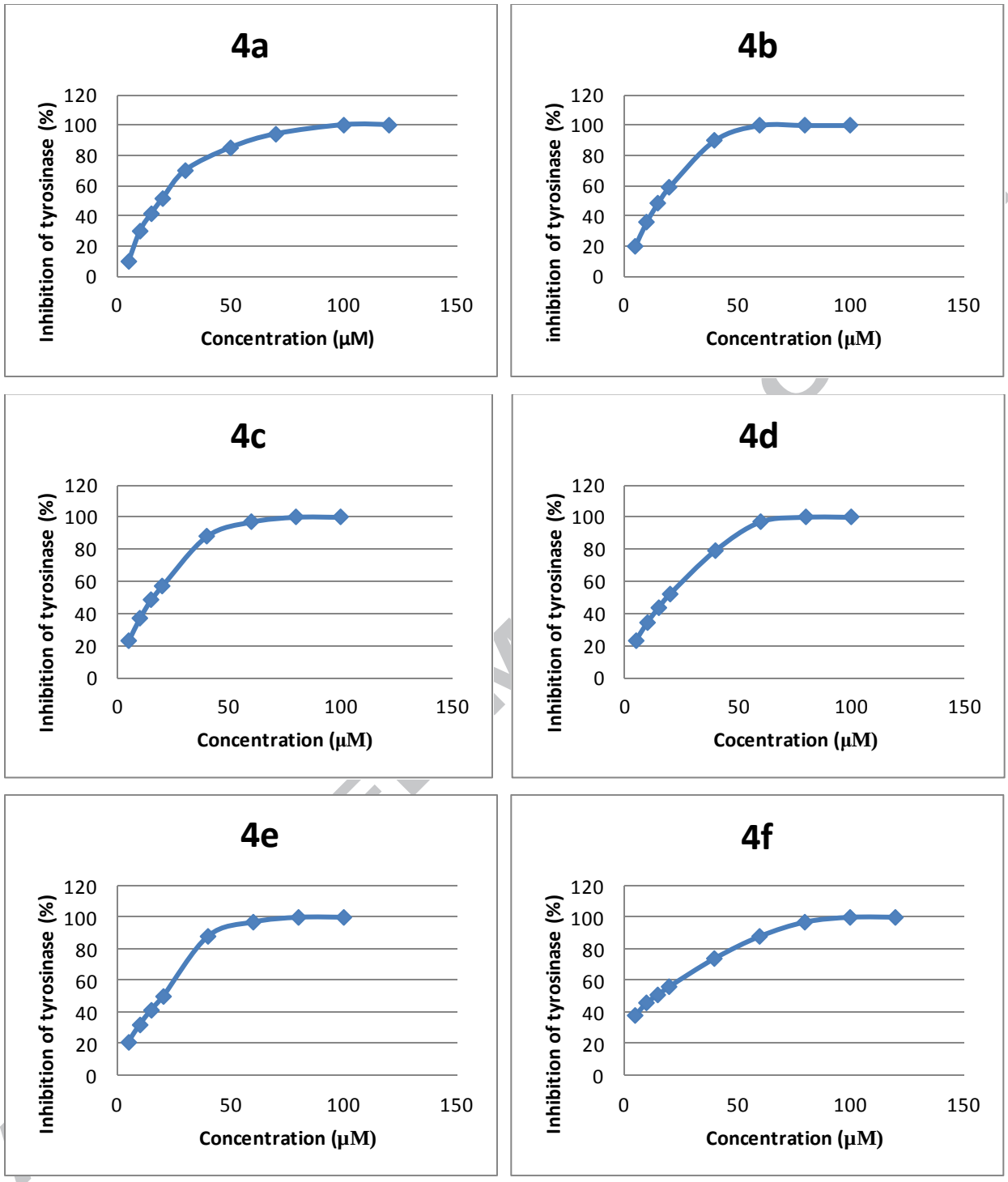


Figure 1. Dose-Dependent curves of compounds (**4a-4f)** on tyrosinase activity.

The compounds **(4a–4f)** exhibited excellent in vitro tyrosinase inhibitory properties having IC_{50} values in the range of 20.33 ± 0.47 to 13.65 ± 0.22 µM, since standard inhibitor, Kojic acid, have IC_{50} value 16.01 ± 0.56 µM, respectively (Table 2).

Table 2. Tyrosinase inhibitory activities of the compounds **(4a-4f)**, as compared to the standard inhibitor

Entry	Compound	IC_{50} ± SEM[a] (µM)
1	**4a**	17.22 ± 0.41
2	**4b**	15.78 ± 0.24
3	**4c**	16.02 ± 032
4	**4d**	18.91 ± 0.15
5	**4e**	20.33 ± 0.47
6	**4f**	13.65 ± 0.22
7	Kojic acid[b]	16.01± 0.56

[a] SEM is the standard error of the mean.
[b] Standard inhibitor of the enzyme tyrosinase.

Compound **(4f)** having IC_{50} value 13.65 ± 0.22 µM, was found to be very active members of the series, even better than the standard inhibitor. 2-{4-[2-(4-morpholinophenyl)-1-diazenyl]phenyl}-4-[1-(2-thianyl) methylidene)]-1,3-oxazol-5(4H)-one**(4f)** was found to be the most active one having IC_{50} = 13.65 ± 0.22 µM among all tested compounds.

Comparing the activities with the structures of compounds, it clear that the tyrosinase activity is mainly relevant on the substituents present at C-4 position of oxazolone ring. When tyrosinase inhibitory activity of the most active compound **(4f)** was compared with other compounds, it was observed that it has a thiophen ring on the aliphatic double bond at C-4.

This shows that expansion of conjugation through an aliphatic double bond could be the prerequisite for activity rather than expansion through an aromatic ring.

The minimum activity of compound **(4e)** (IC_{50} 20.33 ± 0.47 µM) may be due to changing the substituent in phenyl rings present at C-4.

Effect of the compound **(4f)** on tyrosinase tertiary structure was considered by measurements of intrinsic fluorescence. We found that **(4f)** had a quenching effect on the intrinsic fluorescence, which gradually occurred with increasing concentration. Consequently, compound **(4f)** effects on tyrosinase activity by destruction of tertiary structure it.

2.3. Melanin production inhibition and cytotoxicity

The inhibitory of the compounds **(4a-4f)** were also tested on melanin production and their cytotoxicity on B16F10 mouse melanoma cells at concentrations of 20 µg/ml. The results of melanin production inhibition and cytotoxicity by the compounds**(4a-4f)** are showed in Table 3.Compounds 4a–4f prevented melanin production by 40.15%, 38.83%, 32.40%, 34.37%, 30.68% and 31.17%, respectively, at concentrations of 20µg/ml. On the other hand, compounds **(4a–4f)** have shown mediate inhibition of melanin production. Cytotoxicity of new compounds **(4a–4f)** was appraised and was explained that all compounds were the more toxic than kojic acid (Table 3).

Table 3. Melanin production and cytotoxicity

Entry	Compound	Melanin production Inhibition (%)	Cytotoxicity Cell viability (%)
1	**4a**	40.15 ± 4.72	62.87 ± 4.11
2	**4b**	38.83 ± 1.50	57.24 ± 1.42
3	**4c**	32.40 ± 1.13	60.66 ± 3.33
4	**4d**	34.37 ± 2.69	61.87 ± 1.96
5	**4e**	30.68 ± 0.56	58.11 ± 6.32
6	**4f**	31.17 ± 1.25	56.71 ± 2.91
7	Kojic acid[a]	16.90 ± 3.71[b]	80 ± 3.26[b]

[a] Standard inhibitors of the enzyme tyrosinase.
[b] Tested at 200 µg/ml.

3. Experimental

3.1. General

All the chemicals were obtained from Merck, Fluka, Sigma and Aldrich Companies and used without further purification. Melting points were measured using Thermo Fisher Scientific. IR spectra were recorded Bruker tensor 27, FT- IR Spectrophotometer. All ^{1}H NMR spectra were recorded on a Bruker 500 MH_Z Spectrophotometer. Chemical shifts are reported in parts per million (ppm) using tetramethylsilane (TMS) as an internal standard. Ultraviolet-visible (UV-vis) absorption spectra were recorded on a Perkin-Elmer spectrophotometer at the wavelength of maximum absorption (λ_{max}) in a range of DMSO at same concentrations (1×10^{-6}M). The mass Spectra were run on a Shimadzu Qp 5050 Ex Spectrometer. The microanalyses for C, H and N were performed on perkin-elmer elemental analyzer.

The fluorescence emission spectra were determined with a RF-5301PC Shimadzu spectrofluorometer using a cuvette with a 1 cm path length. An excitation wave length of 280 nm was used for the tryptophan fluorescence measurements, and the emission wavelength ranged between 300 and 420nm.

3.2. Preparetion of diazonium salt of 4-aminohippuric acid (2)

In a 125- mL Erlenmeyer flask, 4-aminohippuric acid (0.01 mol) was added to 2% sodium carbonate solution (30 mL) until it was dissolved by boiling. The solution was then cooled and sodium nitrite (0.01mol) was added, with stirring, until it was dissolved. The solution was cooled by placing it in an ice bath, and then concentrated hydrochloric acid (2 mL) and water (3 mL) were added. By acidifying the solution, a powdery yellow precipitate of the diazonium salt was separated. [15]

3.3. Preparetion of Sodium2-({4-[2-(4-morpholinophenyl) -1-diazenyl]benzoyl}amino)acetate (3)

N-phenylmorpholin (0.01mol) in 96% ethanol (20 ml) was dissolved by heating. The solution N-phenylmorpholine was added to suspension of diazotized hippuric acid, with stirring, and acid-stable form of the dye was separated.

Red powder; decomposed >256 °C. Yield: 88%. IR (KBr) υ: 3361, 1712, 1674cm^{-1}. ^{1}H NMR (500 MHz, DMSO-d6): 3.22 (t, 4H), 3.64 (d, 2H), 3.80 (t, 4H), 6.84 (d, 2H, J 8.9 Hz, ArH), 6.93-8.82 (m, 9H, ArH, NH) ppm. Anal.Calcd.for $C_{19}H_{19}N_4O_4Na$: C, 58.46; H, 4.87; N, 14.36. Found: C, 58.65; H, 5.05; N, 14.48.

3.4. General procedure for synthesis of compounds (4a-4f):

A mixture of anhydrous sodiumacetate (0.001 mol), 4-fluoro benzaldehyde , benzaldehyde, 2-chloro-4-fluoro benzaldehyde, 4-chloro benzaldehyde, 2-nitro benzaldehyde and thiophen 2- carbaldehyde (0.001 mol), sodium salt of azo dye **(3)** (0.001 mol) and acetic anhydride (10 mL) was heated with stirring until the mixture is transformed from an orange semi-solid mass to a deep red liquid (4 h). After cooling, the precipitated product was filtered and recrystallized in ethanol.

3.4.1. 2-{4-[2-(4-morpholinophenyl) -1-diazenyl]phenyl}-4-[1-phenylmethylidene]-1,3-oxazol-5(4H)-one (4a):

Red powder; mp: 180-182 °C. IR (KBr) υ: 1787, 1650 cm^{-1}. ^{1}H NMR (DMSO-d6, 500 MHz) δ: 3.27 (t, 4H), 3.78 (t, 4H), 6.88–8.22 (m, 14H). MS (EI) m/z (%): 438 (M^+, 66), 294(43), 248(2),190(8), 162(100), 104(81), 77(29).Anal.Calcd.for $C_{26}H_{22}N_4O_3$: C, 71.23; H, 5.02; N, 12.78. Found: C, 71.12; H, 4.89; N, 12.97.

3.4.2 4-[1-(4-flourophenyl)methylidene]-2-{4-[2-(4-morpholinophenyl)-1-diazenyl] phenyl}-1,3-oxazol-5(4H)-one (4b):

Light brown powder; mp: 200-201 °C. IR (KBr) υ: 1790, 1651 cm^{-1}. ^1H NMR (DMSO-d6, 500 MHz) δ: 3.20 (t, 4H), 3.91 (t, 4H), 7.16–8.31 (m, 13H). MS (EI) m/z (%): 456(M^+, 27), 294(34), 236(30), 162(100), 104(89), 120(27), 77(40). Anal. Calcd for $C_{26}H_{21}N_4O_3F$: C, 68.42; H, 4.61; N, 12.28. Found: C, 68.57; H, 4.44; N, 12.08.

3.4.3. 4-[1-(2-chloro-4-flourophenyl)methylidene]-2-{4-[2-(4-morpholinophenyl)-1-diazenyl] phenyl}1,3-oxazol-5(4H)-one (4c):

Brown powder; mp: 230-232 °C. IR (KBr) υ: 1796, 1600 cm^{-1}. ^1H NMR (DMSO-d6, 500 MHz) δ: 3.36 (t, 4H), 3.88 (t, 4H), 7.02–8.18 (m, 12H). MS (EI) m/z (%):492(M+2, 2), 490(M^+, 6), 311(82), 162(100), 104(74), 120(53), 77(42). Anal. Calcd. for $C_{26}H_{20}N_4O_3ClF$: C, 63.61; H, 4.08; N, 11.42. Found: C, 63.43; H, 3.93; N, 11.39.

3.4.4. 4-[1-(4-chlorophenyl)methylidene]-2-{4-[2-(4-morpholinophenyl)-1-diazenyl]phenyl}-1,3-oxazol-5(4H)-one (4d):

Dark brown powder; mp: 220-222 °C. IR (KBr) υ: 1792, 1647 cm^{-1}. ^1H NMR (DMSO-d6, 500 MHz) δ: 3.49 (t, 4H), 3.94 (t, 4H), 7.06–8.27 (m, 13H). MS (EI) m/z (%): 474(M+2, 12), 472(M^+, 36), 249(27), 162(100), 120(92), 104(52), 77(23). Anal. Calcd for $C_{26}H_{21}N_4O_3Cl$: C, 66.03; H, 4.44; N, 11.85. Found: C, 65.90; H, 4.53; N, 12.09.

3.4.5. 2-{4-[2-(4-morpholinophenyl)-1-diazenyl]phenyl}-4-[1-(2-nitrophenyl)methylidene]-1,3-oxazol-5(4H)-one (4e):

Light red powder; mp: 256-257 °C. IR (KBr) υ: 1794, 1642, 1599, 1339 cm^{-1}. ^1H NMR (DMSO-d6, 500 MHz) δ: 3.40 (t, 4H), 3.96 (t, 4H), 7.00–8.72 (m, 13H). MS (EI) m/z (%): 483(M^+, 7), 162(94), 120(47), 104(100), 92(48), 76(58), 65(41). Anal. Calcd for $C_{26}H_{21}N_5O_5$: C, 64.60; H, 4.35; N, 14.49. Found: C, 64.75; H, 4.21; N, 14.28.

3.4.6. 2-{4-[2-(4-morpholinophenyl)-1-diazenyl]phenyl}-4-[1-(2-thianyl)methylidene)]-1,3-oxazol-5(4H)-one (4f):

Purple powder; mp: 230-231 °C. IR (KBr) υ: 1786, 1643 cm^{-1}. ^1H NMR (DMSO-d6, 500 MHz) δ: 3.35 (t, 4H), 3.88 (t, 4H), 7.02–8.18 (m, 12H). MS (EI) m/z (%): 444(M^+, 13), 294(19), 178(11), 162(100), 120(90), 104(90), 77(37). Anal. Calcd for $C_{24}H_{20}N_4O_3S$: C, 64.86; H, 4.50; N, 12.61. Found: C, 65.02; H, 4.67; N, 12.89.

3.5. Biology

3.5.1. Tyrosinase inhibition assay

Tyrosinase inhibition was assayed according to the method of Ref.[16] Briefly, all the synthesized compounds were sieved for the diphenolase inhibitory activity of tyrosinase using L-DOPA as substrate. All the compounds were dissolved in DMSO. The final concentration ofDMSOin the test solution was 2.0%. Phosphate buffer, pH 6.8, was used to dilute the DMSO stock solution of test compounds. Thirty units of mushroom tyrosinase (0.5 mg/ml) were first pre-incubated with the compounds, in 50 mM phosphate buffer (pH 6.8), for 10 min at 25 °C. Then the LDOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm of formation of the L-DOPA chrome for 10 min. The measurement was performed in triplicate for each concentration and averaged before further calculation. The percent of inhibition of tyrosinase reaction was calculated as following:

Inhibition (%) = [B – S/B] × 100

Here, the B and S are the absorbances for the blank and samples. All the experiments were carried out at least in triplicate and the results represent means ± SEM (standard error of the mean). Kojic acid was used as reference standard inhibitors for comparison.

3.5.2. Inhibition of melanin production

Melanin production inhibition was confirmed by method of Wang et al.[17] A total of 8×10^4 cells were added to 60 mm plates, and were incubated at 37 °C in a CO_2 incubator then 10 µl test samples in DMSO were added to plates and were incubated for 72 h at 37 °C in a CO_2 incubator. After washing with PBS, cells were destroyed with 1 ml of 1 N NaOH, and 200 µl portions of raw cell extracts were moved to 96-well plates. Melanin production inhibition was determined by recording absorbance at 475 nm. The effects of test samples on melanin contents are explained as percent inhibitions of the value obtained in B16F10 mouse melanoma cells which were cultured with DMSO alone.

3.5.3. Cytotoxicity assay

Cytotoxicity assays were implemented using a micro-culture MTT method described by Han et al.[18] A B16F10 mouse melanoma cell suspensionwas poured into a 96-well plate (10^3 cells/well) and cells were allowed to completely stick to each other overnight. Test samples were then added to the plate and were incubated at 37 °C for 72 h in a CO_2 incubator. 20 µl of MTT solution (2 mg/ml) was then added per well and incubated for 4 h. Supernatant was then removed and formazan was soluiblized by adding 150 µl DMSO to each well with mild shaking. Absorbance at 490 nm was recorded using an ELISA plate reader.

Acknowledgements

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