Synthesis, Characterization, Aggregation, Antioxidant and Anti-inflammatory activities of novel metallophthalocyanines bearing 2(2-methoxyphenyl) ethanol and 3-Hydroxybenzaldehyde groups

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Abstract: Novel metallophthalocyanines (M: Co, Cu, Ni) containing 2(2-methoxyphenyl)ethanol and 3-Hydroxybenzaldehyde groups were synthesized and characterized.

The structures were confirmed by IR, UV/vis, 1H NMR, 13C NMR, mass spectroscopy and elemental analysis. The aggregation behavior of these compounds was investigated in DMSO. In addition; the compounds 3-8 were investigated for anti-oxidant activities by super oxide radical; DPPH (2,2-Diphenyl-1-picrylhydrazyl); and hydroxyl radical scavenging assays; in which most of them displayed significant antioxidant activities. Furthermore; compounds 3-8 were evaluated for anti-inflammatory activity by indirect hemolytic and lipoxygenase inhibition assays and revealed good activity.

Keywords: Metallophthalocyanine; Synthesis; Phthalonitrile; Antioxidant and anti-inflammatory activities

Introduction

Phthalocyanines (Pcs) are 18 π - electron disk-like aromatic macrocycles with 2D π-electron delocalization over the whole molecule 1. Since their discovery, Pcs have attracted attention as functional chromophores for various applications such as liquid crystals, chemical sensors, electrochromic compounds; and nonlinear optical and photovoltaic cells 2-7. The physicochemical properties of Pcs depend on the nature of the peripheral or nonperipheral functional groups, as well as the electronic properties of the central metal cations in the Pc core 8. The substitution by functional groups is advantageous because it gives exibility in solubility and also efficiently tunes the color of the material.

One important problem related to Pc derivatives is their low solubility in several organic media and water because of aggregation phenomena. The solubility of Pc compounds can be improved via nonperipheral or peripheral substitution 9,10. Placing substituents on nonperipheral positions of the Pc ring may reduce the detrimental effect of the substituents on the strong π-π interaction between Pc molecules.

On the other hand, antioxidant intake has emerged as an alternative therapeutic approach for several pathological conditions related to oxidative damage in the biological systems responsible for normal cell functions 14-15. Oxidative stress is involved in the etiology of several chronic diseases, including cardiovascular disease, diabetes, cancer, and neurodegenerative disorders.

Traditional synthetic routes to Pcs need long reaction times and very high temperatures 16. In this regard, we report herein the synthesis of metallophthalocyanines 3-8 carrying 2(2-methoxyphenyl) ethanol and 3-hydroxybenzaldehyde groups on the nonperipheral positions. The spectroscopic characterization and the electronic and aggregation behaviors of these newly synthesized complexes are also presented. Furthermore, the antioxidant and anti-inflammatory activities for the obtained metallophthalocyanines were also investigated.

Results and Discussion

The synthesis of substituted phthalonitrile derivatives is an important step in Pc synthesis. Nonperipherally substituted phthalonitrile derivatives are synthesized through reactions between 4-nitrophthalonitrile and O-, S-, or N-nucleophiles 17,18. Using this synthetic strategy, the synthesis and characterization of metallophthalocyanines 3-8 and their precursor 1-2 are reported. The synthesis of 4-[2-(2-methoxyphenyl)ethoxy]phthalonitrile (1) was achieved in 78% yield through base-catalyzed
aromatic displacement of 4-nitrophthalonitrile with 2(2-methoxyphenyl) ethanol using K$_2$CO$_3$ as the base in dry DMF. The reaction was carried out at room temperature under N$_2$ atmosphere for 72 h.

The synthesis of 4-(3-formylhenoxy) phthalonitrile (2) was achieved in 74% yield using K$_2$CO$_3$ as the base in dry DMF. The reaction was carried out at room temperature under N$_2$ atmosphere for 24 h. The synthetic route is shown in the Scheme1.

Characterization of the products 1-2 involved a combination of methods, including elemental analysis, IR, $^1$HNMR and $^{13}$CNMR, NMR 2D and Mass spectroscopy techniques.

IR spectrum of phthalonitrile compound 1 indicated a CN group by the presence of intense stretching vibration band at 2233. Moreover, the aromatic C–H peaks appeared at 3049 cm$^{-1}$. The $^1$H NMR spectrum of compound 1 exhibited the characteristic chemical shifts for the structure, as expected. The aromatic protons appeared between 6.81 and 8.04 ppm. The elemental analyses data for compound 1 gave satisfactory results.

In the IR spectrum of 2, the broad peak for the OH bands for 3-Hydroxybenzaldehyde disappeared and the characteristic vibrations of the C≡N peak appeared at 2227 cm$^{-1}$, respectively. In the $^1$H NMR spectrum of 2, the disappearance of the OH peaks of 3-Hydroxybenzaldehyde, besides the presence of additional aromatic protons indicated that nucleophilic aromatic nitro displacement was achieved. The $^{13}$C NMR spectrum of 1 and 2 indicated the presence of nitrile carbon atoms in 1 and 2 at (120.9, 121.4 ppm) for 1, (115.7, 116.2 ppm) for 2.

In the mass spectra of compounds 1-2, the presence of the characteristic molecular ion peaks at m/z = 278.30 [M]+ for 1, m/z = 247.22 [M]+ for 2, confirmed the proposed structures 1-2.

Conversion of 1 and 2 into metallophthalocyanine derivatives 3-8 were accomplished through the cyclotetramerization reactions in the presence of corresponding metal salts CuCl$_2$·2H$_2$O, CoCl$_2$·6H$_2$O and NiCl$_2$·6H$_2$O in 2-(dimethylamino) ethanol and DBU at 160°C for 24h under a nitrogen atmosphere with the yields between 39 and 66% after purification by column chromatography method utilizing chloroform/methanol(80 :20) as eluent. Scheme 3.
The new obtained complexes were characterized by elemental analysis and by their spectral data (1H NMR, IR, mass, and UV-Vis spectra). The data are consistent with the assigned structures.

The dark green products 3-8 are extremely soluble in polar and apolar solvents such THF, DMF and DMSO.

In the 1H NMR spectrum of 3, taken in DMSO at room temperature the aromatic protons appeared at between 6.87 and 7.72 ppm as a multiplet, and methoxylic protons appeared at 3.75 ppm as singlets.

Paramagnetic compounds would affect the magnetic shimming so, 1H NMR measurements were precluded owing to its paramagnetic nature due to the paramagnetic cobalt and copper atoms in the phthalocyanines 4, 5, 7 and 8 cavity.

The IR spectra of metallophthalocyanines 3-8 are very similar. The intense absorption vibrations at 2227 cm\(^{-1}\) corresponding to the CN groups for phthalonitrile compound 2 disappeared after their
conversion into the metallophthalocyanines 3-8. The characteristic vibrations corresponding to CC groups at ~1570 cm⁻¹ and aromatic CH stretching at 3041-3056 cm⁻¹ were observed for all complexes. The elemental analyses results of metallophthalocyanine complexes 6-8 showed a good agreement with calculated values.

**UV–Vis absorption spectra**

The best indication for the phthalocyanine systems is their UV-vis spectra in solutions.

The electronic spectra of phthalocyanines show two strong absorption regions, one in the UV region at about 300-350 nm (B band) and the other in the visible region at 600-700 nm (Q band). Both correlate to π-π* transitions 21,22. The electronic spectrum of compound 3 showed characteristic two absorptions in the UV-vis region in DMSO: one of them was in this region at about 650-679 ppm. UV-Vis spectral data for **Pcs 3-5** in various solvents at a concentration of 10⁻⁵ M are given in **Table 1**.

**Table 1.** UV-Vis spectral data for **Pcs 3-5** in various solvents at a concentration of 10⁻⁵ M.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Pcs</th>
<th>Q-Band, λ (nm)</th>
<th>log ε</th>
<th>B-Band, λ (nm)</th>
<th>log ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMF</td>
<td>3 : Ni</td>
<td>650 - 679</td>
<td>5.10</td>
<td>321 - 358</td>
<td>5.19</td>
</tr>
<tr>
<td></td>
<td>4 : Cu</td>
<td>650 - 684</td>
<td>4.96</td>
<td>305 - 361</td>
<td>4.08</td>
</tr>
<tr>
<td></td>
<td>5 : Co</td>
<td>651 - 687</td>
<td>4.84</td>
<td>306 - 357</td>
<td>4.95</td>
</tr>
<tr>
<td>DMSO</td>
<td>3 : Ni</td>
<td>653 - 702</td>
<td>4.83</td>
<td>318 - 371</td>
<td>4.61</td>
</tr>
<tr>
<td></td>
<td>4 : Cu</td>
<td>660 - 714</td>
<td>4.97</td>
<td>307- 357</td>
<td>5.14</td>
</tr>
<tr>
<td></td>
<td>5 : Co</td>
<td>649 - 703</td>
<td>5.08</td>
<td>309 - 377</td>
<td>5.22</td>
</tr>
<tr>
<td>THF</td>
<td>3 : Ni</td>
<td>640 - 697</td>
<td>4.99</td>
<td>310 - 379</td>
<td>5.06</td>
</tr>
<tr>
<td></td>
<td>4 : Cu</td>
<td>664 - 709</td>
<td>5.01</td>
<td>305 - 344</td>
<td>5.14</td>
</tr>
<tr>
<td></td>
<td>5 : Co</td>
<td>659 - 711</td>
<td>5.08</td>
<td>313 - 364</td>
<td>5.08</td>
</tr>
</tbody>
</table>

The Q bands of the complex 7 were observed at 650-684 nm with a shoulder at 615 nm in DMSO. On the other hand, the Q bands of metallophthalocyanines 6-8 were observed expectedly at 650-684, 651-687, and 635-690 respectively. The B band absorptions of phthalocyanines 6-8 describing the transition of deeper π levels to LUMO were observed at 305-361,306-357 and 310-350 respectively in DMSO. **Table 2**.

**Table 2.** UV-Vis spectral data for **Pcs 6-8** in various solvents at a concentration of 10⁻⁵ M.

<table>
<thead>
<tr>
<th>solvent</th>
<th>Pcs</th>
<th>Q-Band, λ (nm)</th>
<th>log ε</th>
<th>B-Band, λ (nm)</th>
<th>log ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>6 : NiPc</td>
<td>650 - 684</td>
<td>4.96</td>
<td>305 - 361</td>
<td>4.08</td>
</tr>
<tr>
<td></td>
<td>7 : CuPc</td>
<td>651 - 687</td>
<td>4.84</td>
<td>306 - 357</td>
<td>4.95</td>
</tr>
<tr>
<td></td>
<td>8 : CoPc</td>
<td>635 - 690</td>
<td>4.73</td>
<td>310 - 350</td>
<td>4.80</td>
</tr>
</tbody>
</table>

UV-Vis spectral data for **Pcs 6-8** in various solvents (DMF and THF) at a concentration of 10⁻⁵ M were listed in the following **Table 3**.

**Table 3.** UV-Vis spectral data for **Pcs 6-8** in various solvents (DMF and THF) at a concentration of 10⁻⁵ M.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Pcs</th>
<th>Q-Band, λ (nm)</th>
<th>log ε</th>
<th>B-Band, λ (nm)</th>
<th>log ε</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMF</td>
<td>6 : NiPc</td>
<td>629 - 694</td>
<td>5.15</td>
<td>370</td>
<td>4.29</td>
</tr>
<tr>
<td></td>
<td>7 : CuPc</td>
<td>649 - 703</td>
<td>5.08</td>
<td>309 - 377</td>
<td>5.22</td>
</tr>
<tr>
<td></td>
<td>8 : CoPc</td>
<td>660 - 714</td>
<td>4.97</td>
<td>307- 357</td>
<td>5.14</td>
</tr>
<tr>
<td>THF</td>
<td>6 : NiPc</td>
<td>640 - 697</td>
<td>4.99</td>
<td>310 - 379</td>
<td>5.06</td>
</tr>
<tr>
<td></td>
<td>7 : CuPc</td>
<td>664 - 709</td>
<td>5.01</td>
<td>305 - 344</td>
<td>5.14</td>
</tr>
<tr>
<td></td>
<td>8 : CoPc</td>
<td>659 - 711</td>
<td>5.08</td>
<td>313 - 364</td>
<td>5.08</td>
</tr>
</tbody>
</table>

The electronic absorption spectra of Pcs 6-8 showed characteristic intense Q bands at 676 (6), 650 (7) and 690 (8) nm in DMSO. The B bands were observed around 310-379 nm. The wavelengths of the absorption of the Q band of 6-8 follow the order
of Ni > Cu > Co, due to the nature of the central metal ion.

**Aggregation studies**

The aggregation tendency of phthalocyanines is owing to the interactions between their 18 π electron systems, which often cause weak solubility in many solvents.

In this study, the aggregation behavior of complexes 6-8 was examined at different concentrations in DMSO (Schemes 4-5 shows the series of spectra for complex 6-8)

![Absorbance vs Wavelength for complexes 6-8 in different solvents](image)

**Scheme 4.** Absorption spectra of complex 6 in different solvents (DMSO, DMF, THF) at 10⁻⁵ M.

![Absorbance vs Wavelength for complexes 6-8 in different solvents](image)

**Scheme 5.** Absorption spectra of complex 8 in different solvents (DMSO, DMF, THF) at 10⁻⁵ M.

As the concentration M was increased, the intensity of absorption of the Q band also increased. No new band due to the formation of aggregated species was observed 23-24. This means that the Pc derivatives 6-8 did not show aggregation in DMSO.

**Pharmacology**

**Antioxidant activity**

In the current study, we investigated and clarified the antioxidant properties of the synthesized MPCs, because of the relevance of these compounds in the contexts of oxidative stress, disease etiology, and for the progress of medicine 25. Thus we considered that it was worthwhile to study the potential aspects of these new copper, cobalt and nickel phthalocyanines carrying four 2(2-methoxyphenyl)ethanol and 3-Hydroxybenzaldehyde groups on the peripheral positions 3-8 for antioxidant activity according to our initial planning. The synthesized compounds 3-8 were tested for in vitro antioxidant activity by DPPH radical, hydroxyl
radical and superoxide radical scavenging assays. The IC50 values of the standards and test samples are summarized in Table 4. In all three antioxidant assays copper, cobalt and nickel phthalocyanines 5, 6 showed antioxidant activities at 14 µM concentration. But, phthalocyanines 4 and 7 showed good antioxidant activity (12 - 13 µM).

Table 4. IC50 values of copper, cobalt and nickel phthalocyanines 3-8 in anti-oxidant assays.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>DPPH radical scavenging assay</th>
<th>Hydroxy radical scavenging assay</th>
<th>Superoxide radical scavenging assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>12</td>
<td>12.3</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>13.1</td>
<td>10.8</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>14</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>12.1</td>
<td>12.2</td>
<td>-</td>
</tr>
<tr>
<td>Quercetin</td>
<td>-</td>
<td>-</td>
<td>10.9</td>
</tr>
</tbody>
</table>

Anti-inflammatory activity

As per our objective, we next examined the anti-inflammatory activities of copper, cobalt and nickel phthalocyanines bearing coumarin derivatives 3-8 by lipoxgenase inhibition and phospholipase A2 (PLA2) inhibition assays. The IC50 values of the standards and test samples in both assays are given Table 5. In both the assays, phthalocyanines 4 and 5 showed potent anti-inflammatory activity in lipoxgenase inhibition assay (5.0 – 5.1 µM) and PLA2 inhibition assay (26.5 - 34.9 µM). Notably, compound 3 is not active. It should be noted that 4 and 5 nearly have anti-inflammatory activities, as that of standards Indomethacin and Aristolochic acid.

Table 5. IC50 values of copper, cobalt and nickel phthalocyanines 3-8 for anti-inflammatory activity.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Lipoxygenase inhibition assay</th>
<th>PLA2 inhibition assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>10.2</td>
<td>1105</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>26.5</td>
</tr>
<tr>
<td>5</td>
<td>5.1</td>
<td>34.9</td>
</tr>
<tr>
<td>6</td>
<td>11.4</td>
<td>61.1</td>
</tr>
<tr>
<td>7</td>
<td>11.3</td>
<td>61.4</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>62</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>4.8</td>
<td>-</td>
</tr>
<tr>
<td>Aristolochic acid</td>
<td>4.9</td>
<td>25.0</td>
</tr>
</tbody>
</table>

Conclusion

In the presented work, the synthesis of novel non-peripherally substituted metallophthalocyanines (M = Co, Cu, and Ni) with different groups was achieved. The characterizations, aggregation behavior of these new metallophthalocyanines were investigated. The preparations of the new products are supported by elemental analyses, IR, 1H/13C NMR, MS and UV–Vis spectroscopy. The compounds 3-8 were investigated for antioxidant activities by super oxide radical; DPPH (2,2-Diphenyl-1-picyrylhydrazyl); and hydroxyl radical scavenging assays; in which most of them displayed significant antioxidant activities. Furthermore; compounds 3-8 were evaluated for anti-inflammatory activity by indirect haemolytic and lipoxgenase inhibition assays and revealed good activity.

Experimental Section

Materials and equipment

All reagents were obtained from Fluka and Aldrich. The purity of the products was tested in each step by TLC (SiO2, CHCl3/MeOH and THF/MeOH). Melting points were determined using an electrothermal apparatus and are uncorrected. 1H NMR and 13C NMR spectra were carried on a Varian Gemini 400 (400 MHz) spectrometer using TMS as internal standard (δ = 0 ppm). IR spectra were recorded on a Perkin-Elmer 398 Spectrophotometer. MS were recorded on a LC-MS-MS 8030 Shimadzu. Elemental analyses were performed on Perkin-Elmer 2400 elemental analyzer, and the values found were
within ±0.3% of the theoretical values. The UV spectra were recorded on a Perkin Elmer Lambda 11 spectrophotometer.

**Synthesis of 4-[2-(2-methoxyphenyl)ethoxy]phthalonitrile (1).**
4-Nitrophthalonitrile (0.5 g, 2.8 mmol) was dissolved in 20 mL of dry DMF and 2-(2-methoxyphenyl)ethanol (0.90 g, 4.22 mmol) was added. After stirring for 15 min, 1.2 g of finely ground anhydrous K$_2$CO$_3$ (8.65 mmol) was added in small portions for 2 h with efficient stirring. The reaction mixture was stirred under nitrogen at room temperature for 72 h. The mixture was then poured into 200 mL of the ice-water mixture and the precipitate was filtered and washed with water until the filtrate was neutral, and dried in vacuo. Finally, a white product was crystallized from ethanol. Yield: 1.44 g (78%).

**General procedure for synthesis of metallophthalocyanines (3-8)**
Compound (1) (0.24 mmol) or compound 2 (0.24 mmol) N,N-dimethylyaminoethanol (DMAE) (4 ml), 1,8-diazabicyclo[4.5.0]undec-7-ene (DBU) (3 drops) and (0.06 mmol) of corresponding metal salts (CuCl$_2$·2H$_2$O, CoCl$_2$·6H$_2$O and NiCl$_2$·6H$_2$O) were added in a shink tube. The mixture was heated at reflux temperature of 160°C for 24 h under N$_2$ atmosphere. Then the mixture was left for cooling at room temperature then treated with ethylacetate to precipitate the product which was then filtered off and suddenly washed with water. The green solid product was washed with hot ethanol and hot acetic acid and dried in vacuum. The raw product was purified by chromatography on silica gel column.

**Ni(II)Pc (3)**
Elution solvent system: chloroform: methanol (100:3) as eluent.
Yield: 206 mg (75%)
Mp =320°C
Calc. for Ca$_8$H$_8$N$_8$O$_4$Ni: C, 69.62%; H, 4.816%; N, 5.008%; Found: 69.7%; H, 5.1%, N, 5.4%

**FT-IR (KBr) n, cm$^{-1}$:** 3021 (C-H), 1630 (C=C), 1482 (C=N); 908 (Co-N)

**Co(II)Pc (4)**
Elution solvent system: chloroform: methanol (100:3) as eluent.
Yield: 121 mg (80%)
Mp =315°C
Elemental analysis C$_{72}$H$_{60}$NiO$_2$Co: Calculated C, 69.678%; H, 4.815%; N, 9.560%; Found: C, 69.8; H, 4.9, N 9.7.

**Cu(II)Pc (5)**
Elution solvent system: chloroform: methanol (100:3) as eluent.
Yield: 190 mg (85%)
Mp =320°C
Elemental analysis C$_{72}$H$_{60}$NiO$_2$Cu: Calculated C, 66.681%; H, 4.974%; N, 8.640%; Found: C, 66.7; H, 5.1, N 8.7.

**Ni(II)Pc (6)**
Elution solvent system: chloroform: methanol (100:3) as eluent.
Yield: 95%    
Mp =315°C
**FT-IR (KBr) n, cm$^{-1}$:** 3016 (C-H), 1630 (C=C), 1482 (C=N); 908 (Co-N)

**Ni(II)Pc (7)**
Elution solvent system: chloroform: methanol (100:3) as eluent.
Yield: 85%   
Mp =330°C
FT-IR (KBr) n, cm$^{-1}$:** 3018 (C-H), 1394 (C=C); 1236 (C-N); 1611 (C=C); 1485 (C=C); 905 (Ni-N)

**Elemental analysis C$_{60}$H$_{52}$NiO$_3$Ni: Calculated C, 65.526%; H, 3.067%; N, 10.655%; Found: C, 68.7; H, 3.1, N 10.7.

**Ni(II)Pc (8)**
Elution solvent system: chloroform: methanol (100:3) as eluent.
Yield: 85%  
Mp =330°C
FT-IR (KBr) n, cm$^{-1}$:** 3018 (C-H), 1394 (C=C); 1236 (C-N); 1615 (C=C); 1476 (C=C); 903 (Co-N)
Elemental analysis C_{6}H_{5}N_{2}O_{2}Co: Calculated C 68.510%; H, 3.066%; N 10.653% Found: C 68.8; H, 3.1, N10.67.

Cu(II)Pc (8).
Elution solvent system: chloroform: methanol (100:3) as eluent.
Yield: (72%)
Mp =320°C
FT-IR (KBr) v, cm\(^{-1}\): 3018 (C-H\(_{\text{arom}}\)); 1392 (C-C); 1235 (C-N); 1615 (C=C); 1476 (C≡N); 905 (Cu-N)
Elemental analysis C_{60}H_{58}N_{3}O_{3}Cu: Calculated C 68.211%; H, 3.053%; N 10.606% Found: C 68.5; H, 3.2, N10.7.

Antioxidant activity

DPPH radical scavenging assay: The free radical scavenging activity of DPPH radicals was performed as described previously [26]. In brief, reaction mixture containing 200 μL of 0.1 mM DPPH-ethanol solution, 90 μL of 50 mM Tris-HCl buffer (pH 7.4) and 10 μL of deionized water (as control) and various concentrations of compounds 3-8 (3.0 – 16.0 μM), and ascorbic acid was used as a control. The reaction mixture was incubated for 30 min at room temperature and absorbance was read at 540 nm. The percentage radical scavenging activity was calculated according to the following formula:

\[
\text{Inhibition (\%)} = \left(1 - \frac{\text{Absorbance sample}}{\text{Absorbance Control}}\right) \times 100
\]

Hydroxyl radical scavenging assay: The hydroxyl radical (\(\cdot OH\)) scavenging activity of newly synthesized compounds was determined previously [27]. We used Fe (III) -ascorbate – ethylenediaminetetraacetic acid – hydrogen peroxide system (Fenton’s reaction) to generate hydroxyl radical. In brief, reaction mixture containing 0.01 mL of FeCl\(_3\) (10 mM), 0.1 mL of EDTA (1 mM), 0.36 mL of deoxyribose (10 mM), 0.1 mL of H\(_2\)O\(_2\) (10 mM), 1 mL of the compounds 3-8 (concentrations ranging from 3.0 – 16.0 μM), 0.33 mL of phosphate buffer (50 mM, pH 7.4) and 0.1 mL ascorbic acid (1 mM) was added. The mixture was incubated at 37°C for 1 h and 1 mL of the incubated mixture was mixed with 1 mL of 10% trichloroacetic acid (TCA) and 1 mL of thiobarbituric acid (TBA) (1% in 0.025 M NaOH), the resulting mixture was incubated in water bath at 90°C for 20 min. The absorbance was measured at 532 nm. Ascorbic acid was used as a positive control. The percentage of hydroxyl radical scavenging activity was calculated using the formula (1).

Superoxide anion radical scavenging assay: The superoxide anion radical scavenging activity of newly synthesized compounds were determined previously [28]. 1 mL of Nicotinamide adenine dinucleotide (NADH) (468 μM in 100 mM phosphate buffer of pH 7.4), 1 mL of Nitro blue tetrazolium (NBT) (156 μM NBT in 100 mM phosphate buffer of pH 7.4), and different concentration of compounds 3-8 (3.0 – 16.0 μg/mL) were added to get the final volume of 3 mL. The reaction was started by the addition of 100 μL of PMS (60 μM in 100 mM phosphate buffer of pH 7.4). The mixture was incubated for 5 min at 25°C and the absorbance was measured at 560 nm. Quercetin was used as a control. The percentage radical scavenging activity was calculated using the formula (A).

Anti-inflammatory activity

Lipoxygenase inhibition assay: The lipoygenase inhibition assay was performed according to the method previously described [29]. Briefly, to a solution of 0.1 mL of 0.2 M borate buffer (pH 9.0), 0.1 mL of 1000 units lipoydase enzyme solution, test compounds 3-8 dissolved in DMSO (3 - 16 μM) was added, agitated and incubated at room temperature for 5 min. Later, 2.0 mL of 0.6 mM linoleic acid was added and the absorbance was measured at 234 nm. Indomethacin was used as a standard. The percent (\%) inhibition was calculated by the following equation (A).

Indirect haemolytic assay: Indirect haemolytic assay was performed according to the reported method [30-32]. One mL of fresh human red blood cells and 1 mL of fresh Hen’s egg yolk in 8 mL of phosphate buffered saline was mixed to prepare the substrate for indirect haemolytic activity. One mL of this suspension was incubated with 4-28 μg of partially purified venom for 45 min at 37°C and 9 mL ice cold sodium perborate was used to stop the reaction. The reaction mixture was centrifuged at 2000 rpm for 20 min then the released hemoglobin was read at 540 nm. For inhibition studies 10 μg of venom sample (secretory-PLA\(_2\) purchased from sigma) was incubated with various concentrations of compounds 3-8 (20-100 μM in DMSO) for 30 min at room temperature and mixed with 1 mL of substrate solution and incubated at room temperature for 30 min. The reaction was stopped by adding 9 mL of ice-cold sodium perborate and extent of hemolysis is measured at 540 nm. Aristolochic acid was used as reference drug. The percent (\%) inhibition was calculated as follows (A).

References