Research Article

Synthesis and Antimicrobial and Antioxidant Activities of Some New 5-(2-Methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-amine Derivatives

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A series of 5-(2-methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-amine derivatives (3–5) were synthesized. These previously unknown compounds were characterized by spectral studies and elemental analysis. These compounds were evaluated for their antimicrobial and antioxidant activities. Among all the compounds tested 5d exhibited promising antibacterial, antifungal, radical scavenging, and ferric ions (Fe³⁺) reducing antioxidant power (FRAP) activities, whereas the compounds 3b, 4c, and 5e exhibited good FRAP and metal chelating activities. In general compounds containing chloro and methyl substituent exhibited better antimicrobial and antioxidant activities.

1. Introduction

Indole and its analogs display remarkable pharmacological activities like Hepatitis C Virus NS5B protein inhibitors [1–3] peroxisome proliferator-activated receptor γ (PPAR-γ) agonist [4], antiproliferative against human tumor cells [5, 6], antibacterial, antifungal [7, 8], and anticoccidal [9] activities. On the other hand, oxadiazoles possess desirable electronic and charge transport properties into which various functional groups can be easily introduced. Considerable evidence has been accumulated during the last decades demonstrating the various pharmacological properties of its isomer 1,3,4-oxadiazole which includes anticonvulsant [10], tuberculostic [11], analgesic and ulcerogenicity [12], antibacterial, antifungal [13, 14], anti-inflammatory [15], anti-HIV [16], nonpeptide angiotensin II receptor antagonists [17], tyrosinase inhibition [18], nematocidal, insecticidal, and herbicidal [19] activities. All of which can be attributed to its characteristic properties of electrophilic substitution, and nucleophilic substitution, thermal and photochemical reactions due to the presence of -N=C-O- group. The importance of substituted indoloyxadiazoles is well known for various applications. Indolyl-1,3,4-oxadiazoles, triazoles, and pyrazoles have been reported as antimicrobials [20, 21]. Indole substituted with oxadiazoles or triazoles at positions 1 and/or 3 were found to exhibit enhanced biological activities [22]. In the perspective of bioactivities of the abovementioned structures and in search of novel antimicrobials and antioxidants, we herein report synthesis of novel bis (indolyl) analogues.

2. Results and Discussion

The synthetic strategy was planned as depicted in Scheme 1. The requisite starting materials 5-(2-methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-amine (I) were prepared by the cyclo-condensation of 2-methyl-1H-indol-3-carboxaldehyde with cyanoacrylamide in ethanol under reflux conditions [23] and 5-substituted 2-phenyl-1H-indol-3-carboxaldehydes 2a–c were prepared by the previously reported method [24]. Condensation of compound I with 2a–c in methanol, under reflux temperature afforded the key intermediate N-[[(5-substituted 2-phenyl-1H-indol-3-yl) methylene]-5-(2-methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-amines (3a–c).

Compounds 3a–c on cyclocondensation with easily available and economically viable reagents such as thioglycolic acid, phenyl acetyl chloride, and chloro acetyl chloride...
yielded 2-(5-substituted 2-phenyl-1H-indol-3-yl)-3-[5-[(2-methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-yl] thiazolidin-4-ones (4a–c), 4-(5-substituted 2-phenyl-1H-indol-3-yl)-1-[5-(2-methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-yl]-3-phenylazetidin-2-ones (5a–c), and 3-chloro-4-(5-substituted 2-phenyl-1H-indol-3-yl)-1-[5-(2-methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-yl]azetidin-2-ones (5d–f), respectively.

2.2. Antioxidant Activities

2.2.1. 1,1-Diphenyl-2-picryl Hydrazyl (DPPH) Radical Scavenging Activity (RSA). Investigation of the RSA of the test compounds (1 and 3–5) was conducted as described by Hatano’s and colleagues [26] and the results were compared with the standards. The structure-activity relationship (SAR) revealed that the compounds with chlorine substituent at C-5 position of indole and in azetidine system are important for enhancing the antimicrobial activity, whereas methyl substituent at C-5 position of indole is found to reduce the antimicrobial activity. Thus, compound 5d has been found to exhibit maximum zone of inhibition against all the bacteria and fungi. Replacement of azetidine nucleus by thiazolidinone system leads to formation of compounds 4a–c with decreased antimicrobial activity. On the other hand, compounds 3a and 4a exhibited less zone of inhibition compared to 5d against all bacterial strains and the fungus A. niger. Removal of chlorine substituent at C-5 position of indole (compound 5f) leads to decrease in antibacterial activity against B. subtilis. Replacement of chlorine at C-5 position of indole by methyl group and azetidine system by thiazolidinone ring (compound 4b) exhibited same zone of inhibition as that of 5d against bacterial strain K. pneumonia and showed slight decrease in antifungal activity against A. flavus. Substitution of chlorine by phenyl group in 5d (compound 5a) leads to decrease in antifungal activity against A. flavus and A. fumigates. On the same line, when chlorine at C-5 position of indole in 5d was replaced by methyl group (compound 5e) slight decrease in antifungal activity against A. fumigates was noticed. However, none of the compounds exhibited better antimicrobial activity compared with standards.
### Table 1: Antimicrobial activities of the compounds (1 and 3-5).

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>R</th>
<th>R’</th>
<th>Diameter of zone of inhibition in mm&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>E. coli</td>
</tr>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
<td>14 ± 1.63</td>
</tr>
<tr>
<td>3a</td>
<td>Cl</td>
<td>—</td>
<td>10 ± 2.29</td>
</tr>
<tr>
<td>3b</td>
<td>CH₃</td>
<td>—</td>
<td>05 ± 1.83</td>
</tr>
<tr>
<td>3c</td>
<td>H</td>
<td>—</td>
<td>02 ± 1.01</td>
</tr>
<tr>
<td>4a</td>
<td>Cl</td>
<td>—</td>
<td>14 ± 2.00</td>
</tr>
<tr>
<td>4b</td>
<td>CH₃</td>
<td>—</td>
<td>05 ± 1.83</td>
</tr>
<tr>
<td>4c</td>
<td>H</td>
<td>—</td>
<td>10 ± 2.05</td>
</tr>
<tr>
<td>5a</td>
<td>Cl</td>
<td>Ph</td>
<td>03 ± 1.57</td>
</tr>
<tr>
<td>5b</td>
<td>CH₃</td>
<td>Ph</td>
<td>13 ± 2.15</td>
</tr>
<tr>
<td>5c</td>
<td>H</td>
<td>Ph</td>
<td>07 ± 1.24</td>
</tr>
<tr>
<td>5d</td>
<td>Cl</td>
<td>Cl</td>
<td>16 ± 2.23</td>
</tr>
<tr>
<td>5e</td>
<td>CH₃</td>
<td>Cl</td>
<td>02 ± 1.23</td>
</tr>
<tr>
<td>5f</td>
<td>H</td>
<td>Cl</td>
<td>10 ± 1.60</td>
</tr>
<tr>
<td>Std-1</td>
<td>—</td>
<td>—</td>
<td>17 ± 1.80</td>
</tr>
<tr>
<td>Std-2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Including diameter of well#, control (DMF) = no activity, Std-1 = streptomycin, Std-2 = fluconazole.

### Results and Discussion

The antimicrobial activities of the test compounds were evaluated by measuring the diameter of the zone of inhibition around each well. The results are summarized in Table 1. The compounds exhibited varying levels of antibacterial and antifungal activity against the tested pathogens.

From the RSA results of test compounds (Figures 1 and 2), it could be seen that the introduction of chlorine substitution at C-5 position of indole ring (compound 3a) enhances the RSA (72.35% at 100 µg/mL), whereas the introduction of methyl group at the same position of indole (compound 3b) leads to a decrease in RSA (55.28% at 100 µg/mL). Replacement of the azomethine group in compounds 3 by thiazolidinone nucleus (compounds 4) resulted in a decrease in RSA. However, replacement of azomethine group by azetidinone ring with chlorine substitution at C-5 of indole (compound 5d) was found to enhance the RSA (74.15% at 100 µg/mL). Replacement of chlorine substituent by phenyl group in azetidinone system (compound 5a) or replacement of chlorine substitution by phenyl group in azetidinone ring and methyl group at C-5 position of indole (compound 5b) was found to decrease RSA (62.12% at 100 µg/mL).

2.2.2. Ferric Ions (Fe<sup>3+</sup>) Reducing Antioxidant Power (FRAP). The ferric ion (Fe<sup>3+</sup>) is a relatively biologically inactive form of iron. However, it can be reduced to active Fe<sup>2+</sup>, depending on the condition, particularly pH [27] and oxidized back through Fenton type reaction with production of hydroxyl radical or Haber-Weiss reaction with the generation of superoxide anions. Reducing power is to measure the reductive ability of antioxidant. It is evaluated by the transformation of Fe<sup>3+</sup> to Fe<sup>2+</sup> by donation of an electron, in the presence of test compounds. Therefore, the Fe<sup>2+</sup> can be monitored by measuring the formation of Perl’s Prussian blue at 700 nm.

2.2.2.1. Ferric ion (Fe<sup>3+</sup>) reducing power of test compounds (1 and 3-5) at different concentrations (25, 50, 75, and 100 µg/mL) was conducted by Oyaizu method [28] using BHA, TBHQ, and AA as standards. The FRAP results (Figures 3 and 4) revealed that methyl substitution at C-5 position of indole (compound 3b) leads to increase in FRAP, whereas introduction of chlorine substituent at C-5 of indole (compound 3d) leads to decrease in FRAP. Replacement of azomethine group by thiazolidinone ring (compound 4a) increases the FRAP, but introduction of either chlorine or methyl substituent (compound 4a and 4b) leads to decrease in FRAP, whereas replacement of azomethine group by azetidinone ring with chloro substitution (compound 5e) exhibited promising FRAP. Introduction of chlorine or...
methyl substituent at C-5 position of indole and replacement of chlorine by phenyl group in azetidinone ring (compounds 5a and 5b) lead to decrease in FRAP.

2.2.3. Ferrous (Fe^{2+}) Metal Ion Chelating Activity. The chelating effect of ferrous ions (Fe^{2+}) towards the test compounds (1 and 3–5) and standards were determined by following Dinis method [29] and the results were compared with standards BHA, TBHQ, and AA. Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red colored) formation is interrupted, as a result the red color of the complex is decreased. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction.

In metal chelating activity (Figures 5 and 6), the SAR study revealed that introduction of chloro or methyl substitution at C-5 position of indole (compounds 3a and 3b) along with azomethine group leads to improved metal chelating activity (71.24 and 72.51% at 100 μg/mL, resp.). Replacement of azomethine group by thiazolidinone ring (compound 4c) showed higher activity (75.48% at 100 μg/mL). Introduction of methyl group (compound 4b) leads to slight decrease in metal chelating activity, whereas introduction of chlorine at C-5 position of indole (compound 4a) drastically reduced the metal chelating activity. Introduction of chlorine substituent at C-5 position of indole (compound 5d) was found to increase metal chelating activity (66.25% at 75 μg/mL), whereas the introduction of either chloro or methyl substituent at C-5 position of indole and phenyl substitution in
3. Conclusion

We have reported the synthesis and antimicrobial and antioxidant activities of novel bis (indolyl) compounds 1, 3a–c, 4a–c, and 5a–f. Compounds bearing chloro substitution exhibited significant antimicrobial and antioxidant activities. Among the synthesized compounds, 5d was found to be most active against all the microorganisms tested and also exhibited promising RSA and FRAP suggesting that the presence of chloro substituent and the azetidine nucleus are responsible for activities among the synthesized compounds.

4. Experimental Protocol

All the chemicals and reagents were commercially obtained and used by further purification. Melting points were determined by an open capillary method and are uncorrected. Purity of the compounds was checked by TLC using silica gel-G coated aluminium plates (Merck) and spots were visualized by exposing the dry plates in iodine vapours. The IR (KBr) spectra were recorded with a Perkin-Elmer spectrometer one FT-IR spectrometer. The $^1$H NMR (DMSO-$d_6$) spectra were acquired with a Bruker NMR (500 MHz) and the chemical shifts are expressed in ppm ($\delta$ scale) downfield from TMS. Mass spectra were obtained with a JEOL GCMATE II GC-MS mass spectrometer. Elemental analysis was carried out using Flash EA 1112 series elemental analyzer.

4.1. General Procedure for the Synthesis of 5-(2-Methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-amine (1). A mixture of 2-methyl-1H-indol-3-carboxylic acid (0.01 mol) and cyanogen bromide (0.01 mol) in EtOH was refluxed with stirring for 90 min. The reaction mixture was then cooled and neutralized by sodium bicarbonate solution. The product separated was filtered, dried and recrystallized in ethanol to afford pure 1. Yield: 75%; mp 285-86°C; $R_f$ 0.65 ethyl acetate : methanol (6:4) mixture; FTIR (KBr) cm$^{-1}$: 3441 (NH), 3289 (NH$_2$), 1610 (C=N), 1174 (C–O–C); $^1$H NMR (DMSO-$d_6$, $\delta$, ppm): 12.01 (s, 1H, indole NH), 7.00–8.05 (m, 4H, Ar-H), 5.85 (s, 2H, NH$_2$), 2.82 (s, 3H, CH$_3$); MS (EI): $m/z$ 214 (M$^+$). Anal. % C$_{11}$H$_9$N$_2$O: C, 61.67; H, 4.71; N, 26.15. Found: C, 61.64; H, 4.69; N, 26.14.

4.2. General Procedure for the Synthesis of N-[(5-Substituted 2-phenyl-1H-indol-3-yl)methylene]-5-(2-methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-amines (3a–3c). Compound 1 (0.01 mol) and 5-substituted 2-phenyl-3-carbaldehydes (2a–2c) (0.01 mol) were refluxed in methanol (25 mL) on water bath for 8 hrs. Excess of solvent was removed under reduced pressure. After cooling the reaction mixture to room temperature light yellowish crystalline product separated out was collected by filtration and recrystallized in alcohol.

N-[(5-Chloro-2-phenyl-1H-indol-3-yl)methylene]-5-(2-methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-amine (3a). Yield: 75%, mp 228-29°C; $R_f$ 0.77 ethyl acetate : acetone (6:4) mixture; FTIR (KBr) cm$^{-1}$: 3310 (NH), 3289 (NH$_2$), 1626 (C=N), 1096 (C–O–C); $^1$H NMR (DMSO-$d_6$, $\delta$, ppm): 12.00, 11.75 (s, 2H, indole NH), 8.52 (s, 1H, N=CH), 6.92–7.98 (m, 12H, Ar-H), 7.22 (s, 3H, CH$_3$); $^1$C-NMR (DMSO-$d_6$, 125 MHz, $\delta$): 160.3, 158.1, 156.4, 142.5, 134.9, 134.0, 133.0, 130.1, 129.3, 128.5, 127.2, 126.8, 126.1, 125.8, 124.3, 123.0, 122.6, 122.1, 121.1, 121.4, 119.8, 119.5, 15.4; MS (EI): $m/z$ 451 (M$^+$); 452 (M$^+$ + 2). Anal. % C$_{26}$H$_{18}$N$_2$O: C, 69.10; H, 4.01; N, 15.50. Found: C, 68.64; H, 3.99; N, 15.24.

5-(2-Methyl-1H-indol-3-yl)-N-[(5-Substituted 2-phenyl-1H-indol-3-yl)methylene]-1,3,4-oxadiazol-2-amine (3b). Yield: 76%, mp 240-41°C; $R_f$ 0.65 ethyl acetate : acetone (6:4) mixture; FTIR (KBr) cm$^{-1}$: 3210 (NH), 3200 (NH), 1620 (C=N), 1085 (C–O–C); $^1$H NMR (DMSO-$d_6$, $\delta$, ppm): 11.93, 11.52 (s, 2H, indole NH), 8.45 (s, 1H, N=CH), 6.98–7.95 (m, 12H, Ar-H), 3.13 (s, 3H, CH$_3$); $^1$C-NMR (DMSO-$d_6$, 125 MHz, $\delta$): 160.3, 158.2, 156.4, 142.5, 134.9, 134.0, 133.0, 130.1, 129.3, 128.5, 127.2, 126.8, 126.1, 125.8, 124.3, 123.0, 122.6, 122.1, 121.6, 121.4, 119.8, 119.5, 15.4; MS (EI): $m/z$ 451 (M$^+$); 452 (M$^+$ + 2). Anal. % C$_{26}$H$_{18}$N$_2$O: C, 75.16; H, 4.91; N, 16.23. Found: C, 75.14; H, 4.79; N, 15.65.

5-(2-Methyl-1H-indol-3-yl)-N-[(2-phenyl-1H-indol-3-yl)methylene]-1,3,4-oxadiazol-2-amine (3c). Yield: 78%, mp 212-13°C; $R_f$ 0.58 ethyl acetate : acetone (6:4) mixture; FTIR (KBr) cm$^{-1}$: 3215 (NH), 3200 (NH$_2$), 1620 (C=N), 1085 (C–O–C); $^1$H NMR (DMSO-$d_6$, $\delta$, ppm): 11.93, 11.52 (s, 2H, indole NH), 8.45 (s, 1H, N=CH), 6.98–7.95 (m, 12H, Ar-H), 3.13 (s, 3H, CH$_3$); $^1$C-NMR (DMSO-$d_6$, 125 MHz, $\delta$): 160.3, 158.2, 156.4, 142.5, 134.9, 134.0, 133.0, 130.1, 129.3, 128.5, 127.2, 126.8, 126.1, 125.8, 124.3, 123.0, 122.6, 122.1, 121.6, 121.4, 119.8, 119.5, 15.4; MS (EI): $m/z$ 451 (M$^+$); 452 (M$^+$ + 2). Anal. % C$_{26}$H$_{18}$N$_2$O: C, 75.16; H, 4.91; N, 16.23. Found: C, 75.14; H, 4.79; N, 15.65.

azetidine ring (compound 5a and 5b) lead to decrease in metal chelating activity. Replacement of azetidine ring with methyl substituent at C-5 position of indole and chloro substitution in azetidine ring (compound 5e) exhibited higher metal chelating activity (75.84% at 100 µg/mL). However, none of the compounds showed activity better than the standards.
4. General Procedure for the Synthesis of 2-(5-Substituted 2-phenyl-1H-indol-3-yl)-3-[5-(2-methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-yl]thiazolidin-4-ones (4a–4c).

To the solution of compounds 3a, 3b, and 3c (0.02 mol) in DMF (45 mL), thioglycolic acid (0.02 mol) and anhydrous zinc chloride (0.02 mol) were added and refluxed for 6 hrs. After the completion of reaction, excess of solvent was removed under reduced pressure, cooled to room temperature, and poured onto crushed ice. The solid product thus separated was filtered, washed with cold water, and recrystallized in alcohol to afford (4a–4c).

4-(5-Chloro-2-phenyl-1H-indol-3-yl)-1-[5-(2-methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-yl]phenylazetidin-2-one (5a).

Yield: 71%, mp 158-59°C; R_f 0.59 ethyl acetate: acetone (6:4) mixture; FTIR (KBr) cm⁻¹: 3430 (NH), 3387 (NH), 1765 (C=O), 1626 (C=N), 1084 (C-O-C); ¹H NMR (DMSO-d₆, δ, ppm): 11.93, 11.58 (s, 2H, indole NH), 8.43 (d, 1H, CH-N), 7.00–8.06 (m, 17H, Ar-H), 5.58 (d, 1H, CH-Ph), 2.21 (s, 3H, CH₃); 13C-NMR (DMSO-d₆, ppm): 157.2, 159.3, 156.4, 127.6, 126.8, 126.3, 125.1, 123.0, 122.8, 122.0, 121.8, 119.0, 55.9, 35.2, 15.5; MS (EI): 525 (M⁺+2), 527 (M⁺+2).

4.5. General Procedure for the Synthesis of 3-Chloro-4-(5-substituted-2-phenyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-yl]azetidin-2-ones (5d–5f).

To the solution of compounds (3a–3c) (0.02 mol) in dioxane, chloroacetyl chloride (0.04 mol) and triethyl amine (0.04 mol) were added with simultaneous stirring at 0–5°C temperature during 10 mins. After the addition was over, the reaction mixtures were refluxed for 8–10 hrs and the excess of solvent was removed under reduced pressure. After cooling at room temperature the product obtained was filtered, washed with petroleum ether (40:60), and recrystallized in aqueous ethanol.

4-(5-Chloro-2-phenyl-1H-indol-3-yl)-1-[5-(2-methyl-1H-indol-3-yl)-1,3,4-oxadiazol-2-yl]azetidin-2-one (5d).

Yield: 74%, mp 273-74°C; R_f 0.71 ethyl acetate: benzene (6:4) mixture; FTIR (KBr) cm⁻¹: 3411(NH), 3357(NH), 1716 (C=O), 1628 (C=N), 1073 (C-O-C); ¹H NMR (DMSO-d₆, δ, ppm): 12.02, 11.89 (s, 2H, indole NH), 8.75 (d, 1H, CH-Ph), 7.07–8.06 (m, 12H, Ar-H), 5.31 (d, 1H, N-CH), 2.23 (s, 3H, CH₃); 13C-NMR (DMSO-d₆, 125 MHz, δ): 167.2, 159.3, 156.4,
5. Biological Activities

5.1. Antimicrobial Activities. The in vitro biological screening of the synthesized compounds (1 and 3–5) was carried out against bacterial species, *E. coli, B. subtilis*, and *K. pneumonia* and fungal species *A. niger, A. flavus*, and *A. fumigates* by cup-plate method [28] using nutrient agar and PDA medium for antibacterial and antifungal activities, respectively. The holes of 6 mm diameter were punched carefully using a sterile cork borer and these were filled with test solution (1 mg/mL in DMF), standard solution (1 mg/mL in DMF), and DMF as control. The plates were incubated at 37°C for 24 hrs and 72 hrs for the evaluation of antibacterial and antifungal activities, respectively. The diameter of the inhibition zones for all the test compounds was measured (in mm) and the results were compared with the results obtained by using streptomycin and fluconazole as positive standard for antibacterial and antifungal activities, respectively.

5.2. Antioxidant Activity Assay

5.2.1. 1,1-Diphenyl-2-picryl Hydrazyl (DPPH) Radical Scavenging Activity (RSA). The radical scavenging activity (RSA) of test compounds (1 and 3–5) in methanol at different concentrations (25, 50, 75, 100 μg/mL) containing freshly prepared DPPH in methanol (0.004% w/v) was carried out and the results were compared with the results obtained by using standards (BHA, TBHQ, and AA) by Hatano’s method [26]. All analyses were performed in three replicates and results were reported as averages of three replicates. The results in percentage are expressed as the ratio of absorbance of DPPH solutions measured at 517 nm in the presence and the absence of test compounds by using ELICO SL171 mini spectrophotometer. The results are shown in Figures 1 and 2. The percentages of DPPH free radical scavenging activity of the samples were determined using the following equation:

\[
\% \text{ DPPH radical scavenging} = \frac{A_c - A_s}{A_c} \times 100, \tag{1}
\]

where \( A_c \) = absorbance of control; \( A_s \) = absorbance of test sample.

5.2.2. Ferric Ions (Fe\(^{3+}\)) Reducing Antioxidant Power (FRAP). The reducing power of the synthesized compounds (1 and 3–5) was determined according to the Oyaizu method [28]. Different concentrations of samples (25, 50, 75, and 100 μg/mL) in DMSO (1 mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1 w/v). The mixture was incubated at 50°C for 20 min. A portion of trichloroacetic acid (2.5 mL, 10% w/v) was then added to the mixture and the mixture was centrifuged for 10 min at 1000 xg. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 0.1 w/v). Absorbance at 700 nm was then measured in spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power. The results are shown in Figures 3 and 4.

5.2.3. Ferrrous Ions (Fe\(^{2+}\)) Metal Chelating Activity. The ferrous ion chelating activities of synthesized compounds (1 and 3–4) and standards were estimated using the method reported by Dinis and colleagues [29]. The test samples (25, 50, 75, and 100 μg/mL) in ethanol (0.4 mL) were added to ferrous chloride (0.05 mL, 2 mM) prepared in ethanol. The reaction was initiated by the addition of ferrozine (0.2 mL, 5 mM) and the volume was adjusted to 3.5 mL with ethanol and 0.5 mL water so as to make the final total volume 4.0 mL. Ferrozine reacts with the divalent iron to forms stable magenta complex species that were very soluble in water. The mixture was shaken vigorously and kept at room temperature for 10 min. Then the absorbance of the solution was measured spectrophotometrically at 562 nm. All analyses were run in three triplicates and results are reported as the averages of three replicates. The results are shown in Figures 5 and 6. The percent inhibition of the ferrozine-Fe\(^{2+}\) complex formation was calculated using the following formula:

\[
\% \text{ Ferrous ion chelating effect} = \frac{A_c - A_s}{A_c} \times 100, \tag{2}
\]

where \( A_c \) = absorbance of control; \( A_s \) = absorbance of test sample.

Conflict of Interests

Since the authors have procured the IR, NMR, and mass spectra of the synthesized compounds from the National Research Centre, namely, The Indian Institute of Technology, Madras, Chennai, India, as per the condition of institution authors should acknowledge their services in the research paper while
publishing the work, which includes the data provided by them in the research paper. The same has been acknowledged in the acknowledgement section. The authors do not have any agreement, financial assistance, or sponsorship from Perkin-Elmer spectrum, Brucker NMR, ..., and so forth. These names are mentioned in the experimental protocol as these are the instrument models, and it is mandatory for authors to mention the instrument models used to scan the spectra of unknown compounds. Otherwise the corresponding author or coauthors have no direct financial relationship with the commercial identity mentioned in our paper in any form.

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