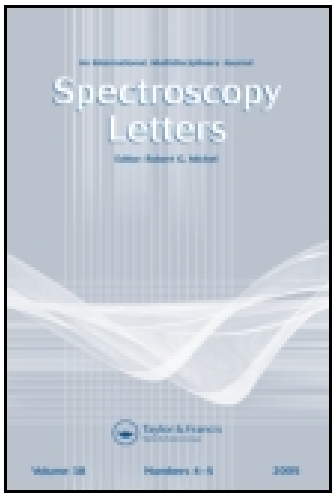

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Synthesis and Antitumor, Antioxidant Effects Studies of N-Ethylpiperazine Substitute Thiourea Ligands and Their Copper(II) Complexes

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ABSTRACT A series of N-ethylpiperazine substitute thioureas [$C_6N_2H_{13}NHCSNHR$], where R = -C_3H_5 (**L_1**), -$C_{10}H_7$ (**L_2**), and -C_7H_7 (**L_3**), and their copper (II) complexes have been synthesized. These ligands and complexes have been characterized by elemental analyses, IR, ^{1}H and ^{13}C-NMR spectra, UV-Vis, magnetic susceptibility, thermogravimetrical analyses, and MALDI-TOF MS. In vitro antitumor activity of ligands and their complexes has been screened toward several tumor cell lines. The effects on these complexes of the growth of L1210 and MCF7 were studied comparatively with that of free ligands. Antioxidant and radical scavenging activities of synthesized compounds were determined by various in vitro assays including 1,1-diphenyl-2-picryl-hydrazyl free radicals (DPPH), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid radicals (ABTS+), and ferrous ion (Fe^{2+}) chelating activities. Moreover, these activities were compared to synthetic and standard antioxidant trolox. The results showed that the synthesized compounds had effective antioxidant power.

KEYWORDS antioxidant, antitumor, spectroscopy, thiourea

INTRODUCTION

Aliphatic and aromatic thiourea, triazole, and thiadiazine compounds have been demonstrated to be antimicrobial agents able to control different microorganisms. Thioureas have strong antifungal activities comparable to the activity observed for the common antifungal antibiotic ketoconazole,[1,2] and their antimicrobial and insecticidal properties have been documented for more than 50 years.[3] Not only can thioureas be used in the control of plant pathogenic fungi,[4,5] but also they have been shown to possess antitubercular, antithyroid, anthelmintic, antibacterial, insecticidal, and rodenticidal properties.[6–9]

The finding that metal ions are capable of binding to nucleic acids brings a new, promising approach to cancer treatment using metal-based drugs.[10–13] Copper is an essential trace metal and has been used for centuries either as

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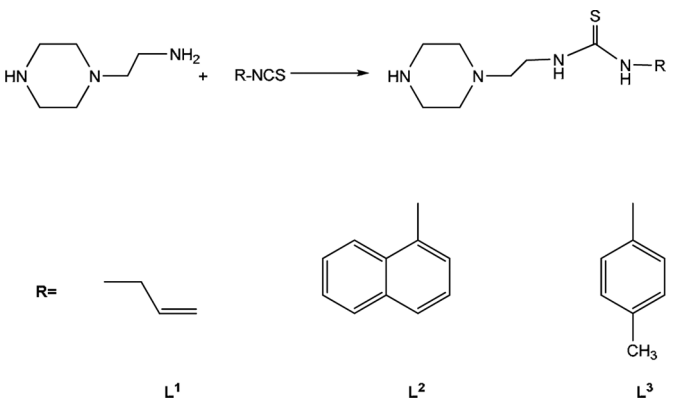
copper ions or in complexes to disinfect liquids, solids, and human tissues.[14] Accumulation of high concentrations of copper is observed to be a physiological feature of many tumor tissues and cells, as was demonstrated in many types of human cancers including breast, prostate, colon, lung, and brain.[15–17] However, some studies support the idea of copper control as an anticancer strategy.[18,19]

There are several organic metal compounds that actively and specifically inhibit the chymotrypsinlike activity of the proteasome in vitro and in human tumor-cell cultures.[20] This is because the ubiquitin/proteasome system plays an important role in the degradation of cellular proteins and organo-copper compounds inhibit the proteasome activity in tumor cells in 15 min.[21,22] The redox cycling properties between Cu^{2+} and Cu^{+} can initiate the production of highly reactive hydroxyl radicals, which can subsequently damage biomolecular species like lipids, proteins, and DNA.[23–26] DNA is one of the most affected cell components that is damaged by the metal-generated hydroxyl radical, because it contains the genetic vital material of a cell. The utilization of sulfur compounds like cysteine, glutathione, and their derivatives as antioxidants has been reported,[27] which may ameliorate oxidative damage caused by reactive oxygen species (OH, O_2^-). Copper complexes of sulphur-containing ligands, in addition to their nonbiological applications, have been tested for their cytotoxic/antiproliferative activity against various types of tumors.[28–30]

In the present work, a number of chelating thiourea derivatives, 1-allyl-3-(2-(piperazin-1-yl)ethyl)thiourea (L^1), 1-(naphthalen-1-yl)-3-(2-(piperazin-1-yl)ethyl) thiourea (L^2), and 1-(2-(piperazin-1-yl)ethyl)-3-(p-tolyl)thiourea (L^3) (Scheme 1), for complex formation with Cu(II) ions and their spectral characteristics, composition, and structure have been studied. In addition, in vitro antitumor activity and antioxidant effects of the ligands and their Cu(II) complexes have been studied.



SCHEME 1 Synthesis of N-ethylpiperazine substitute thiourea.

RESULTS AND DISCUSSION

The IR spectra of compounds (L^1, L^2, L^3) show absorption bands of $\upsilon max/cm^{-1}$ 3295–3258 (N-H stretching), 1150–1107 (C-S stretching), and 1250–1269 (HN-C=S stretching). The addition of amine to the C=N double bond takes place by attacking the carbon atom in the isothiocyanate group, resulting in the formation of the desired compounds. After complete reactions, the strong band at around 2100 cm^{-1} (N=C = S stretching) in the isothiocyanate disappears. The band around υ_{max}/cm^{-1} 2550–2610 corresponding to the -SH is not found, which indicates the absence of the N=C-SH tautomeric form in all compounds.[10,31] A comparative absorption pattern of complexes with the values of the free ligand demonstrates that the coordination of thiourea ligands to copper atoms has an insignificant effect on υ(N-H), υ(C-N-C), and υ(HNCSNH) frequencies. The stretching frequency of C=S present in the ligand and the coordinated compounds remains the same, which ruled out the involvement of the thiocarbonyl group in coordination.

The spectrum of $[Cu_2L^1(AcO)_4 \cdot (H_2O)_4] \cdot 2H_2O$ and $[Cu_2L^3_2(AcO)_4] \cdot H_2O$ shows another two bands due to υ_s and υ_{as} of an acetate group at 1385–1415 cm^{-1} and 1385–1419 cm^{-1}, respectively. The $\Delta\upsilon = 30–34\ cm^{-1}$ can be taken as evidence for the existence of a bridging bidendate acetate group in these complexes

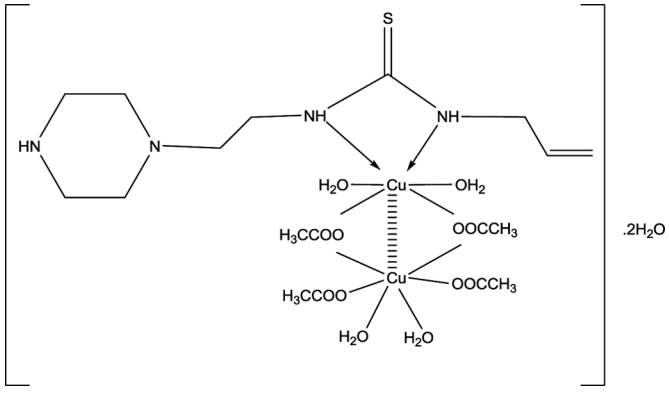


FIGURE 1 Suggested structure of the square-planar Cu(II) complex of the ligand (L^1).

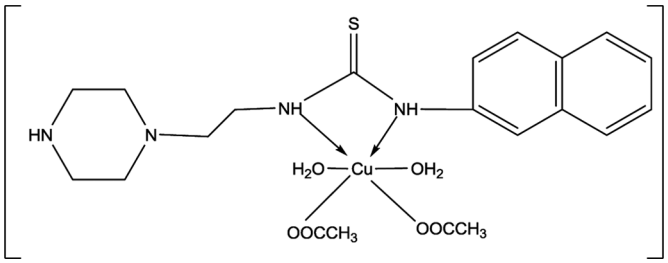


FIGURE 2 **Suggested structure of the square-planar Cu(II) complex of the ligand (L^2).**

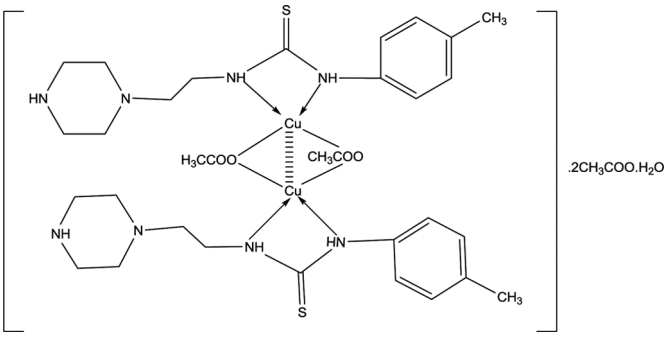


FIGURE 3 **Suggested structure of the square-planar Cu(II) complex of the ligand (L^3).**

(Fig. 1).[32,33] The spectrum of $[CuL^2(AcO)_2 \cdot (H_2O)_2]$ displays bands at 1641 and 1363 cm^{-1} due to ν_{as}(OCO) and ν_s(OCO), respectively, of the acetato ligand. The wavenumber separation value between these two bands, $\Delta\upsilon = 278\,cm^{-1}$, is characteristic of a monodentate acetato ligand in this complex (Fig. 2).[31,33,34]

The magnetic moment of the Cu(II) complexes recorded at room temperature lies in the range 1.17–1.30 BM (Table 1), corresponding to one unpaired electron, which is lower than the spin-only value, that is, 1.73 BM for one unpaired electron for L^1 and L^3 complexes (Fig. 3). This reveals that these complexes are dimeric in nature and also shows the presence of metal–metal interaction along the axial positions, whereas the L^2-type complex shows magnetic moment at 1.98 BM, which indicates the absence of metal–metal interaction.

Electronic spectra of six-coordinate Cu(II) complexes have either D_{4h} or C_{4v} symmetry and e_g and t_{2g} levels of the ^2D free ion term will split into B_{1g}, A_{1g}, B_{2g}, and E_g levels, respectively. Thus, three spin-allowed transitions are expected in the visible and near-IR region. But only a few complexes known, in which such bands are resolved by single crystal polarization studies.[35] These bands may be assigned to the following transitions: $^2B_{1g} \rightarrow {}^2A_{1g}$ $(d^2_{x-y} \rightarrow d^2_z)$, $^2B_{1g} \rightarrow {}^2B_{2g}(d^2_{x-y} \rightarrow d_{xy})$, and $^2B_{1g} \rightarrow {}^2Eg$ $(d^2_{x-y} \rightarrow d_{xz}\ d_{yz})$ in order of increasing energy.

The electronic spectra of complexes having the molecular formulas $[Cu_2L^1(AcO)_4 \cdot (H_2O)_4].\ 2H_2O$ and $[CuL^2(AcO)_2 \cdot (H_2O)_2]$ show broad bands in the range of 16.667–11.778 cm^{-1}, assigned to the $^2B_{1g} \rightarrow {}^2E_g$ transition, and a more intense band at 28.570 cm^{-1} is assigned to the LMCT transition.[30] These band positions are in agreement with those generally observed for octahedral copper (II) complexes. The electronic spectrum of $[Cu_2L^3_2(AcO)_4] \cdot H_2O$ exhibits two bands: an asymmetric broad band at 15.870 cm^{-1} is assigned to the $^3T_{2g} \rightarrow {}^2E_g$ transition and a more intense band at 28.570 cm^{-1} is assigned to the LMCT transition.[35] These band positions are in agreement with those generally observed for square-planar copper (II) complexes.[32,35]

The mass spectrum of $[Cu_2L^3_2(AcO)_4] \cdot H_2O$ showed that the molecular ion peak coincides with its molecular formula (Calcd. 936.09; found 937.08), indicating its $[M\text{-}H^+]$. On the other hand, the spectrums of $[Cu_2L^1(AcO)_4 \cdot (H_2O)_4] \cdot 2H_2O$ and $[CuL^2(AcO)_2 \cdot (H_2O)_2]$ showed peaks at 597.23 and 561.62, corresponding to $[M\text{-}2H^+]$ of the complexes' molecules.

TABLE 1 **Elemental Analyses of the Ligands and their Compounds**

Compounds	FW (g/mol)	mp (°C)	Yield (%)	μ_{eff} (BM)	Elemental analyses/ (%) calculated (found)			
					C	H	N	S
$C_{10}N_4SH_{20}$ (L^1)		228	67	—	52.63 (53.68)	8.77 (8.44)	24.56 (24.44)	14.04 (14.44)
$C_{17}N_4SH_{22}$ (L^2)		315	64	—	64.76 (65.22)	6.98 (5.99)	17.78 (17.01)	10.16 (11.01)
$C_{14}N_4SH_{22}$ (L^3)		278	56	—	60.43 (59.38)	7.91 (7.18)	20.14 (20.22)	11.51 (11.22)
$[Cu_2L^1(CH_3COO)_4 3H_2O] \cdot 2H_2O$	699.08	360<	64	1.17	30.90 (31.59)	6.29 (6.16)	8.01 (8.14)	4.58 (5.14)
$[CuL^2(CH_3COO)_2 \cdot (H_2O)_2]$	532.5	360<	42	1.30	47.36 (48.89)	6.02 (6.30)	10.52 (10.92)	6.02 (6.92)
$[Cu_2L^3_2(CH_3COO)_4] \cdot H_2O$	937.08	360<	69	1.98	38.46 (39.24)	5.89 (5.93)	11.95 (11.01)	6.83 (7.02)

The thermogravimetric results obtained are in good agreement with the theoretical formulae suggested from elemental analyses (Table 1) and mass spectra. The $[Cu_2L^1(AcO)_4 \cdot (H_2O)_4] \cdot 2H_2O$ and $[Cu_2L^3_2(AcO)_4] \cdot H_2O$ complexes have four decomposition steps. These complexes show the first mass loss step, attributed to dehydration, in the temperature range 100–122°C. In the second decomposition step of L^1 and L^2 complexes, abrupt mass losses occur at 145–220°C and 135–200°C, respectively, which may represent the new removal of coordinated water associated with the other products of the second decomposition step. The third and fourth decomposition steps are in the range of 210–701°C and 210–765°C, respectively. The fourth step ends with the formation of copper metal. In case of the copper complex or the corresponding oxides, the $[CuL^2(AcO)_2 \cdot (H_2O)_2]$ complex, there are three decomposition steps. The first mass loss step starts at 103°C, which could be due to the removal of coordination water. The second step starts at 242°C, whereas the third one is in the range of 427–670°C and comprises the formation of CuO. The amounts of residue are in good agreement with the calculated values.

Inhibition of Cell Proliferation

Cytotoxicity assays were carried out by using the trypan blue test. Growing cultures of each cell line were exposed to an increasing test compound concentration (7.5–30 μM) for 24 and 48 hr. A dose-dependent decrease in cell proliferation was clearly observed in the two cell lines studied (Tables 2 and 3). Treated samples were significantly different from the control in both MCF-7 and L1210 cells.

Ligands and their metal complexes produced dose- and time-dependent antiproliferative activity in both MCF-7 and L1210 cell cultures. It was seen that cytotoxicity of the ligands and their complexes on L1210 cells was greater than that of the MCF-7 cells.[36]

It has been reported that the structure and conformation of ligand have an influence on the redox potential of central atoms in coordination compounds. The changes (coordination sphere) in metal ions are related to the change of diverse biological function of compound. The fundamental knowledge of these laws can be used to synthesize more active complexes or contribute to our understanding of biological properties of natural biocoordinative compounds. Our results appear to confirm these explanations; the activity is affected strongly by the structure of the ligands. Furthermore, the activities of the L^2 ligand were found to be higher than that of the other ligands.[10] The L^2 Cu complex exhibits antiproliferative activity against the cancer cell lines used, comparable to that of the less active L^1 Cu and L^3 Cu complexes.

Antioxidant Activities

The antioxidant and radical scavenging effects of the synthesized compounds were determined in vitro with different bioanalytical methodologies. The antioxidant and radical scavenging activities of the compounds were compared with Trolox. These comparisons were performed using a series of in vitro tests including DPPH, ABTS radical scaveng-

TABLE 2 Dose- and Time-dependent Cell Viability Results in MCF-7 Cells after Exposure to Ligands and their Complexes

Groups (n = 7)	24 hr 7.5 μM	24 hr 15 μM	24 hr 30 μM	48 hr 7.5 μM	48 hr 15 μM	48 hr 30 μM
Control	98.33 ± 2.87	98.33 ± 2.87	98.33 ± 2.87	96.17 ± 1.83	96.17 ± 1.83	96.33 ± 2.87
L^1	80.50 ± 3.27	67.00 ± 4.24[a]	47.36 ± 2.86[c]	64.50 ± 4.23	49.00 ± 3.79[c]	37.36 ± 2.86[c]
L^1-Cu	66.00 ± 2.83	57.33 ± 5.72[c]	48.67 ± 4.27[c]	58.00 ± 4.98[b]	47.50 ± 3.21[c]	28.67 ± 4.27[c]
L^2	78.33 ± 2.80	64.17 ± 2.04	41.83 ± 7.57	54.00 ± 7.72[c]	39.83 ± 5.49[c]	10.83 ± 7.57
L^2-Cu	66.00 ± 7.72	73.17 ± 5.23	51.17 ± 6.08[c]	59.00 ± 4.34[a]	46.67 ± 5.35[c]	11.17 ± 6.08[c]
L^3	82.00 ± 8.92[c]	66.00 ± 4.69	51.33 ± 5.16[c]	63.33 ± 5.61	46.00 ± 3.79[c]	31.33 ± 5.16[c]
L^3-Cu	63.50 ± 8.19	60.00 ± 6.81[b]	46.83 ± 4.99[c]	51.00 ± 5.66[c]	47.83 ± 4.45[c]	36.83 ± 4.99[c]

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.

TABLE 3 Dose- and Time-dependent Cell Viability Results in L1210 Cells after Exposure to Ligands and their Complexes

Groups(N = 7)	24 hr 7.5 μM	24 hr 15 μM	24 hr 30 μM	48 hr 7.5 μM	48 hr 15 μM	48 hr 30 μM
Control	96.17 ± 2.14	96.17 ± 2.14	97.67 ± 1.03	96.17 ± 2.14	97.67 ± 1.03	95.33 ± 2.87
L^1	63.83 ± 7.93[a]	53.83 ± 4.99[a]	32.33 ± 2.73[a]	46.67 ± 5.61[a]	39.67 ± 14.54[a]	27.36 ± 2.86[a]
L^1-Cu	58.00 ± 2.76[a]	49.17 ± 5.95[a]	31.83 ± 5.12[a]	40.00 ± 4.89[a]	33.00 ± 3.89[a]	18.67 ± 4.27[a]
L^2	37.50 ± 6.75[a]	26.17 ± 4.83[a]	30.33 ± 6.71[a]	22.33 ± 4.59[a]	20.00 ± 7.07[a]	11.83 ± 7.57
L^2-Cu	41.00 ± 3.35[a]	20.33 ± 6.98[a]	15.17 ± 4.62[a]	16.33 ± 3.27[a]	14.00 ± 2.45[a]	7.17 ± 6.08[a]
L^3	56.67 ± 4.45[a]	46.00 ± 2.37[a]	26.33 ± 4.80[a]	38.00 ± 2.97[a]	32.67 ± 4.55[a]	11.33 ± 5.16[a]
L^3-Cu	47.00 ± 5.93[a]	37.50 ± 4.68[a]	22.00 ± 4.52[a]	13.00 ± 1.89[a]	12.17 ± 5.23[a]	16.83 ± 4.99[a]

[a]$p < 0.001$.

ing activities, and metal chelating on ferrous ion (Fe^{2+}) activities.

Ferrous ions generate the most effective pro-oxidants in biological systems. The good chelating effect is proposed to be more beneficial. Obviation of free iron ions from the cycle is a hopeful approach to prevent oxidative stress-induced diseases. By being chelated, the iron ion may lose its pro-oxidant attributes. Iron can be found as ferrous (Fe^{2+}) or ferric ions (Fe^{3+}) in nature. Ferrous chelation exposes important antioxidative effects by delaying metal-catalyzed oxidation.[37] Ferrous ion chelating activities of synthesized compounds and Trolox are shown in Table 4. The synthesized compounds also exhibited considerable Fe^{2+} chelating activity. Metal chelating activities of synthesized compounds are usually close to or higher than those used in reference antioxidants.

The free radical chain reaction is a common mechanism of lipid peroxidation. Antiradical compounds (synthetic or natural) can immediately react with radicals. They quench radicals to terminate the peroxidation chain reactions. Assays based on the use of DPPH and ABTS radicals are among the most popular spectrophotometric methods to determine the antiradical capacity of molecules. These radicals may directly react with antioxidants. Additionally, these radical scavenging assays have been used to evaluate the antioxidant activity of compounds due to their simple, rapid, and sensitive nature. These assays are standard tests in antioxidant activity studies.[37] As can be seen in Table 4, DPPH and ABTS radical scavenging activities of the synthesized compounds were evaluated. From this perspective, all synthesized compounds had marked high radical scavenging ability on DPPH and ABTS radicals. The highest DPPH scavenging effect was detected in compound P-N-Cu. The ABTS scavenging effect was more highly detected in compound P-N than Trolox.

TABLE 4 ABTS, DPPH Scavenging, and Metal Chelating Activity Results of Synthesized Compounds and Trolox

Samples	% ABTS scavenging activity (50 μg/mL)	% Metal chelating activities (250 μg/mL)	% DPPH• scavenging activity (250 μg/mL)
Control	0	0	0
L^1	93.65	51.18	30.38
L^1-Cu	70.75	94.59	79.17
L^2	95.26	95.65	9.65
L^2-Cu	93.29	85.65	89.54
L^3	92.29	89.65	7.86
L^3-Cu, P-N	73.81	78.12	86.14
Trolox	78.41	92.00	91.39

EXPERIMENTAL

Instrumentation and Chemicals

All reagents were of commercial quality and solvents were used without further purification. The human breast carcinoma MCF-7 cell line and murine leukemia L1210 cell line were obtained from the American Type Culture Collection (ATCC). Dulbecco's modified Eagle's medium (DMEM) and newborn calf serum were purchased from Hyclone (Waltham, MA, USA); trypsin, penicillin, and streptomycin were from Sigma Chemical Co. (St. Louis, MO, USA). Infrared (IR) spectrum was determined on a Perkin-Elmer Spectrum One Fourier transform-infrared (FT-IR) spectrometer. Electronic spectral studies were conducted on a Shimadzu model UV-1700 spectrophotometer in the wavelength 1000–200 nm. NMR spectra were recorded on a Bruker 300-MHz

spectrometer. Thermo analyses (TGA and DTA) of compounds were carried out in a nitrogen atmosphere with a heating rate of 20°C/min using a Shimadzu DTG-60 AH (Shimadzu DSC 60 A) thermal analyzer. MALDI-MS analyses were performed on a Bruker Micro TOF-ESI/MS system. Elemental analyses were performed on a LECO model 932 instrument. Melting point (uncorrected) was determined with the Stuart SMP 30 apparatus. The cells were incubated under 5% CO_2/air at 37°C conditions in a Nuaire humidified carbon dioxide incubator (Playmouth, MN, USA). Cell state was checked by using an inverted microscope (Soif Optical Inc., China). Results are expressed as mean ± SD. Statistical analysis and comparison between mean values for cytotoxicity were performed by Tukey variance analysis (SPSS 10.0 for Windows; Chicago, IL, USA).

The Preparations of Ligands

Preparation of 1-allyl-3-(2-(piperazin-1-yl) ethyl)thiourea (L^1) and 1-(naphthalen-1-yl)-3-(2-(piperazin-1-yl)ethyl)thiourea (L^2)

A solution of 2-aminoethyl piperazine (0.01 mol) in acetonitrile (15 mL) was added to a solution of 1-allyl isothiocyanate (4-methylphenyl isothiocyanate) (0.01 mol) in acetonitrile (15 mL) with stirring at room temperature. The reaction mixture was maintained at 50°C for 4 hr. Then the mixture was cooled and added to ice-cold water. The resulting solid was washed with water, dried, and recrystallized from ethanol.

1-Allyl-3-(2-(piperazin-1-yl)ethyl)thiourea

Colorless; mp: 234°C; IR (near): υmax: 3295–3255 (N-H), 3059 (C=C-H), 2937–2824 (aliphatic C-H), 1646 (C=C), 1555 (N-H), 1140 (C-S); ^{1}H-NMR (300 MHz, $CHCl_3$-d): 2.56 (t, 4H, J: 5.1 Hz, piperazine N-CH_2), 2.61 (t, 4H, J: 5.7 Hz piperazine NH-CH_2), 3.55 (Br, 1H, piperazine NH), 3.84 (t, 2H, J: 5.1 Hz N-CH_2), 4.08 (Br, 1H, NH), 4.34 (t, 2H, J: 6.6 Hz, HN-CH_2), 5.28 (m, 2H, C=CH_2), 5.60 (s, 2H, $_2$HC-C=), 5.93 (m,1H, HC=CH_2), 6.46 (Br, 1H, NH); ^{13}C-NMR (75 MHz, $CHCl_3$-d) 43, 45, 48, 54, 56, 115, 134, 181; Anal. calcd. for $C_{10}H_{20}N_4S$ (228.14): C, 52.63; H, 8.77; N, 24.56; S, 14.04. Found: C, 53.68; H, 8.44; N, 24.44; S, 14.44 (yield 90%).

1-(Naphthalen-1-yl)-3-(2-(piperazin-1-yl) ethyl)thiourea (L^2)

Colorless; mp: 287°C; IR (near): υmax: 3258–3160 (N-H), 3054 (aromatic C-H), 2928–2813 (aliphatic C-H), 1594 (C=C), 1520 (N-H), 1111 (C-S); ^{1}H-NMR (300 MHz, $CHCl_3$-d): 1.81 (Br, 1H, piperazine NH), 2.03 (m, 4H, piperazine N-CH_2), 2.28 (m, 6H, piperazine NH-CH_2 and CH_2), 3.57 (q, 2H, J: 4.2 Hz, N-CH_2), 6.69 (Br, 1H, NH), 7.41–7.56 (m, 4H, ArH), 7.85–8.01 (m, 3H, Ar-H), 8.35 (Br, 1H, NH); ^{13}C-NMR (75 MHz, $CHCl_3$-d): 41, 45, 53, 55, 122, 125, 126, 127, 128, 130, 132, 134, 182; Anal. calcd. for $C_{17}H_{22}N_4S$ (314.45): C, 64.76; H, 6.98; N, 17.78; S, 10.16. Found: C, 65.22; H, 5.99; N, 17.01; S, 11.01 (yield 92%).

Preparation of 1-(2-(piperazin-1-yl)ethyl)-3-(p-tolyl)thiourea (L^3)

A solution of 2-aminoethyl piperazine (0.01 mol) in THF (10 mL) was added to a solution of 1-naphthyl isothiocyanate (0.01 mol) in THF (10 mL) with stirring at room temperature. The reaction mixture was maintained at 50°C for 2 hr. Then the mixture was cooled and added to ice-cold water. The resulting solid was washed with water, dried, and recrystallized from ethanol.

1-(2-(Piperazin-1-yl)ethyl)-3-(p-tolyl)thiourea

Colorless; mp: 218°C; IR (near): υmax: 3285–3177 (N-H), 3042 (aromatic C-H), 2945–2913 (aliphatic C-H), 1595 (C=C), 1527 (N-H), 1140 (C-S); ^{1}H-NMR (300 MHz, $CHCl_3$-d): 2.28 (m, 7H, piperazine N-CH_2 and CH_3), 2.55 (m, 4H, piperazine NH-CH_2), 2.66 (s, 2H, N-CH_2), 3.60 (m, 2H, HN-CH_2), 3.86 (Br, 1H, piperazine NH), 7.29–7.09 (m, 4H, ArH), 9.25 (Br, 1H, NH), 9.67 (Br, 1H, NH); ^{13}C-NMR (75 MHz, $CHCl_3$-d): 45, 48, 54, 56, 125, 128, 129, 133, 134, 138, 181; Anal. calcd. for $C_{14}H_{22}N_4S$ (278): C, 60.43; H, 7.91; N, 20.14; S, 11.51. Found: C, 59.38; H, 7.18; N, 20.22; S, 11.22 (yield 96%).

The Preparations of Complexes

Twenty milliliters of ethanolic solution of $[Cu(CH_3COO)_2] \cdot 2H_2O$ (0.435 g, 2 mmol) acidified with 3-mL concentrate acetic acid was mixed with 20-mL hot DMF solution of ligand L^1(0.228 g, 1 mmol), L^2(0.63 g, 2 mmol), and L^3(0.556 g, 2 mmol). The

reaction mixture was refluxed for 3 hr with occasional stirring. After cooling to room temperature, the precipitated product was filtered off, washed with DMF followed by EtOH, and dried (yields: 47%, 65%, 55%).

Cell Culture and Treatment

L1210 cells were grown in RPMI 1640 medium (pH 7–8) supplemented with 10% fetal calf serum (FCS), 1% L-glutamine (200 mM), and 1% penicillin/streptomycin and incubated in an air-humidified incubator at 37°C at 5% CO_2. MCF-7 cancer cell lines were grown as a monolayer culture in a high glucose (4.5 g/L) DMEM medium supplemented with 10% fetal calf serum (FCS), 1% L-glutamine (200 mM), and 1% of penicillin/streptomycin incubated at 37°C in an air-humidified incubator at 5% CO_2.

Cell Viability

For the cytotoxicity analysis of test compounds, cells were seeded at 1×10^6 cell/mL for MCF-7 cells and 1×10^5 cell/mL for L1210 cells, per Eppendorf tubes in six-replicates.

The test compounds were dissolved in DMSO. Proliferative MCF-7 and L1210 cells were seeded in flasks and incubated for 24 hr. After preincubation, the cell-culture medium was replaced with fresh medium. Each test compound was added to the medium within the range of 7.5 and 30 μM, and the cells were incubated under 5% CO_2/air at 37°C conditions. After 24 and 48 hr of incubation, cell viability was measured using the trypan blue exclusion method.[38,39]

Vehicle-treated tubes served as controls. The final concentration of DMSO in culture medium was never >0.5%. This concentration of DMSO by itself produced no observable toxic effects. The concentration of DMSO by itself produced no observable effects on metabolic status.

ABTS·$^+$ Radical Scavenging Capacity

The spectrophotometric analysis of $ABTS^{\bullet+}$ radical scavenging capacity was determined according to the method of Re et al.[40] $ABTS^{\bullet+}$ was produced by reacting 2 mM ABTS in H_2O with 2.45 mM $K_2S_2O_8$ and stored for 12 hr at room temperature in the dark. The $ABTS^{\bullet+}$ solution was diluted to give an absorbance of 0.750 ± 0.025 at 734 nm in 0.1 M sodium phosphate buffer (pH 7.4). Then, 3 mL of $ABTS^{\bullet+}$ solution was added to 50 μL of synthesized compound solutions. The absorbance was recorded for 0.5 hr after the mixing and the percentage of radical scavenging was calculated for each concentration relative to a blank containing no scavenger. The extent of decolorization is calculated as percentage reduction of absorbance. The scavenging capability of test compounds was calculated by the following equation:[41]

$$ABTS^{\bullet+}\ \text{scavenging effect}\,(\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of control and A_1 is the absorbance in the presence of the sample of synthesized compounds or Trolox.

DPPH· Radical Scavenging Capacity

The free radical scavenging capacity of synthesized compounds was measured by 2,2-diphenyl-1-picryl-hydrazil ($DPPH^{\bullet}$).[42] Briefly, 0.1 mM solution of $DPPH^{\bullet}$ in ethanol was prepared and 4 mL of this solution was added to 0.25 mL of synthesized compound solution. Absorbance at 517 nm was determined after 0.5 hr against a blank solution containing the ethanol. Lower absorbance of the reaction mixture indicates the higher free radical scavenging activity. When a hydrogen atom or electron was transferred to the odd electron in $DPPH^{\bullet}$, the absorbance at 517 nm was decreased proportionally to the increase of nonradical forms of DPPH. The capability to scavenge the $DPPH^{\bullet}$ radical was calculated by the following equation:[41]

$$DPPH^{\bullet}\ \text{scavenging effect}\,(\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of control reaction and A_1 is the absorbance in the presence of synthesized compounds.

Metal Chelating Activity

The chelating of ferrous ions by the synthesized compounds and Trolox was estimated by the method of Dinis et al.[43] Briefly, synthesized compounds 250 μg/mL were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was started by

addition of 5 mM ferrozine (0.2 mL) and the mixture was shaken vigorously and left at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of inhibition of the ferrozine–Fe^{2+} complex formation was calculated by the following formula:[41]

$$\text{Metal chelating } (\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of control and A_1 is the absorbance in the presence of synthesized compounds or trolox.

Statistical Analysis

Results are expressed as mean ± SD. Statistical analysis and comparison between mean values for cytotoxicity were performed by Tukey variance analysis (SPSS 10.0 for Windows; Chicago, IL, USA). LSD test was used to analyze the antioxidant parameters. Level of statistical significance was set at $p < 0.05$.

CONCLUSION

A series of N-ethylpiperazine substitute thiourea derivatives were prepared by the reaction of 2-aminoethyl piperazine with 1-allyl isothiocyanate, 4-methylphenyl isothiocyanate, and 1-naphthyl isothiocyanate and their Cu(II) complexes were synthesized. All compounds were tested for their anticancer activity against MCF-7 and L1210 cell lines. In addition, synthesized compounds showed remarkable ferrous ion chelating and radical scavenging activities on DPPH and ABTS radicals. These compounds have shown higher antioxidant activity than standard antioxidant trolox.

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