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Synthesis, Characterization, and the Antioxidative Activity of 4-{[(3,4-Dimethyl pyrrole-2-carbonyl)hydrazono](phenyl)methyl}-3-methyl-1-phenylpyrazol-5-ol and Its Zinc(II), Copper(II), Nickel(II) Complexes

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A new ligand H_3L, 4-{[(3,4-dimethylpyrrole-2-carbonyl)hydrazono](phenyl)methyl}-3-methyl-1-phenylpyrazol-5-ol and its three transition metal complexes, $M(H_2L)OAc \cdot H_2O$ (M=Cu, Ni), $Zn(H_2L)_2$ have been synthesized and fully characterized on the basis of ESI-MS, elemental analyses, molar conductivities, IR spectra and ^1H-NMR. In addition, their antioxidative activities have been investigated by spectrophotometer measurements. The experimental results show that the complexes have higher activity in the suppression of $O_2^{\cdot-}$ and $^{\cdot}OH$ than the free ligand.

Key words antioxidative activity; synthesis; pyrrole-based heterocyclic ring; 1-phenyl-3-methyl-5-hydroxy-4-pyrazolyl phenyl ketone; transition metal complex

Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$), are generated by all aerobic cells during normal oxygen metabolism.[1] Cumulative information has been proved that the oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury and cardiovascular disease.[2,3] Catalase, superoxide dismutase, glutathione and uric acid are examples of antioxidants produced by organisms under normal conditions as parts of a defense system against ROS-mediated cellular injury. However, if this defense system is challenged or overwhelmed by excessive generation of ROS, redox imbalance or oxidative stress may occur.[4] Numerous natural or synthetic antioxidants against ROS have been tested with success in various disease models.[5] Among the antioxidants, the enzyme superoxide dismutase (SOD) plays a pivotal role in clinics, particularly in patients with inflammatory joint disease. SOD neutralizes $O_2^{\cdot-}$ by transforming it into hydrogen peroxide (H_2O_2), thereby preventing the formation of highly aggressive compounds such as peroxynitrite ($ONOO^-$) and hydroxyl radical ($^{\cdot}OH$).[6] But, except for some concerns about price, as a treatment, SOD was found inadequate as it was unstable, did not penetrate into cells, and provoked an immune response. So searching for new types of antioxidants has come into focus.

Some researches have shown that several compounds with related heterocyclic ring and their Schiff base possess biochemical and pharmacological properties, which are used widely to support reaction chemistry and catalysis by transition metal cations.[7,8] In addition, the Schiff bases derived from 1-phenyl-3-methyl-5-hydroxy-4-pyrazolyl phenyl ketone (PMBP) and their transition metal complexes have great characters in the fields of antibacterial and antioxidative activities.[9,10] As a continuation of our research, in this paper, a new ligand (H_3L) both bearing pyrrole and PMBP unit, 4-{[(3,4-dimethylpyrrole-2-carbonyl)hydrazono](phenyl)methyl}-3-methyl-1-phenylpyrazol-5-ol, and its three transition metals complexes have been synthesized and characterized. Additionally, the antioxidative activities (scavenging effects on $O_2^{\cdot-}$ and $^{\cdot}OH$) of the ligand and its complexes have also been studied. The information obtained from this study will be helpful to develop some new antioxidants.

Experimental

Instrumentation and Materials Melting points of the compounds were determined on an XT4-100x microscopic melting point apparatus. Elemental analyses (C, H, N) were carried out on an Elemental Vario EL analyzer. IR spectra were obtained in KBr discs on a Thermo Nicolet FT-IR spectra 4000—400 cm^{-1} region. ^1H-NMR spectra were recorded on a Bruker AM200 Hz spectrometer with TMS as an internal standard. All conductivity measurements were performed in DMF with a DDS-11A conductometer at 25 °C. FAB-MS (fast atom bombardment mass spectrometry) was obtained on a VG ZAB-HS mass spectrometer. Electrospray ionization (ESI) mass spectrometry was recorded on APEX II FT-ICR MS using methanol as mobile phase. The antioxidative activities were performed in DMF with a 721E spectrophotometer. (Shanghai Analytical Instrument Factory China).

Nitroblue tetrazolium (NBT), methionine (MET), and vitamin B_2 ($VitB_2$) were purchased from Sigma Chemical Co. Safranin, EDTA, PMBP, $M(OAc)_2 \cdot nH_2O$ [M=Cu(II), n=1; M=Zn(II), n=2; M=Ni(II), n=4] were produced in China. All the chemicals used were of analytical grade. EDTA–Fe(II) and KH_2PO_4–Na_2HPO_4 buffers were prepared with deionized water.

Preparation of Ligand and Its Transition Metal Complexes. Preparation of 3,4-Dimethylpyrryl-2-carboxylic Acid Hydrazide (Compound 2) The compound, ethyl 3,4-dimethylpyrrole-2-carboxylate (compound **1**) (Fig. 1) was prepared according to the literature.[11] Synthesis of the 3,4-dimethylpyrryl-2-carboxylic acid hydrazide (compound **2**) was in accordance with the following method: an ethanol solution (2 ml) which contained compound **1** (3.0 g, 0.02 mol) and hydrazine hydrate (15 ml) were refluxing for 20 h, then part of the solvent was removed on a rotary evaporator. After cooling to room temperature, a white solid precipitate was formed then separated by filtration. Recrystallized from methanol and water (1 : 1). Yield: 70%. mp: 218—220 °C. ^1H-NMR (DMSO-d_6) δ: 10.62 (1H, s, NH of pyrrolic ring), 8.51 (1H, s, NH), 6.57—6.58 (1H, d, CH pyrrolic ring), 4.27 (2H, s, NH_2), 2.13 (3H, s, CH_3), 1.90 (3H, s, CH_3).

Preparation of Ligand H_3L As shown in Fig. 1, compound **2** (0.46 g, 3 mmol) and PMBP (0.84 g, 3 mmol) were added to a methanol solution (10 ml). After refluxing for 15 h, a yellow precipitate was filtered and recrystallized from DMF and methanol (1 : 1). Yield: 65%. mp: 252—253 °C. ^1H-NMR ($CDCl_3$) δ: 9.21—9.01 (1H, d, NH of pyrrole, disappear after D_2O exchange), 8.12 (1H, s, OH of PMBP, disappear after D_2O exchange), 7.15—8.05 (10H, m, H of phenyl), 7.54 (1H, s, NH, disappear after D_2O exchange), 6.77—6.60 (1H, d , CH of pyrrole), 2.33 (3H, s , CH_3), 2.05 (3H, s, CH_3), 1.60 (3H, s, CH_3). IR (KBr) cm^{-1}: 3372, 3408, 1629, 1585, 1569. FAB-MS: 414 $[M+H]^+$. *Anal.* Calcd for $C_{24}H_{23}N_5O_2$: C, 69.72; H, 5.61; N, 16.94. Found: C, 69.62; H, 5.36; N, 16.54.

Preparation of Complexes The complexes were synthesized by reac-

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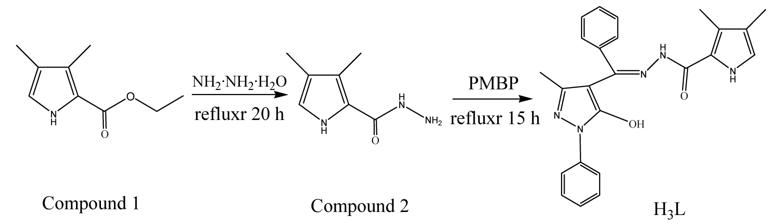


Compound 1 Compound 2 H_3L

Fig. 1. The Preparation of the Ligand

tion of the ligand and $M(OAc)_2 \cdot nH_2O$ (M=Cu, n=1; M=Zn, n=2; M=Ni, n=4) in methanol. After stirring for 24 h at room temperature, the precipitate was separated by suction filtration, purified by washing three times with methanol, and dried for 48 h.

[$Zn(H_2L)_2$]: Yield: 47%. IR (KBr) cm^{-1}: 3369, 1599, 1576, 1449, 495. Λ_m ($S\,cm^2\,mol^{-1}$): 1.8. *Anal.* Calcd for $C_{48}H_{44}N_{10}O_4Zn$: C, 64.75; H, 4.98; N, 15.73. Found: C, 64.58; H, 4.72; N, 15.41.

[$Cu(H_2L)OAc \cdot H_2O$]: Yield: 50%. IR (KBr) cm^{-1}: 3440, 3396, 1652, 1592, 1569, 1502, 1436, 490. Λ_m ($S\,cm^2\,mol^{-1}$): 0.5. *Anal.* Calcd for $C_{26}H_{27}N_5O_5Cu$: C, 56.46; H, 4.92; N, 12.66. Found: C, 57.08; H, 4.22; N, 12.40.

[$Ni(H_2L)OAc \cdot H_2O$]: Yield: 48%. IR (KBr) cm^{-1}: 3455, 3419, 1652, 1599, 1559, 1526, 1436, 486. Λ_m ($S\,cm^2\,mol^{-1}$): 0.9. *Anal.* Calcd for $C_{26}H_{27}N_5O_5Ni$: C, 56.96; H, 4.96; N, 12.77. Found: C, 56.24; H, 5.26; N, 12.47.

Scavenger Measurements of $O_2^{\cdot-}$ and $^{\cdot}OH$ The superoxide radicals ($O_2^{\cdot-}$) were produced by the system of MET/VitB$_2$/NBT[12] and determined spectrophotometrically by nitroblue tetrazolium (NBT) photoreduction method with a little modification in the method adopted elsewhere.[13,14] The amount of $O_2^{\cdot-}$ and suppression ratio for $O_2^{\cdot-}$ can be calculated by measuring the absorbance at 560 nm. Solution of VitB$_2$ and NBT were prepared under the condition of avoiding light. The tested compounds were dissolved in DMF (*N*,*N*-dimethylformamide). The assay mixture, in a total volume of 5 ml, contained MET (10 mM), NBT (46 μM), VitB$_2$ (3.3 μM), the tested compound (0.4—2.0 μM) and a phosphate buffer (67 mM, pH=7.8). After illuminating with a fluorescent lamp at 30 °C for 10 min, the absorbance of the samples (A_i) was measured at 560 nm. The sample without the tested compound was used as control and its absorbance was A_0. All experimental results were expressed as the mean and (±) standard deviation (S.D.) of triplicate determinations. The suppression ratio for $O_2^{\cdot-}$ was calculated from the following expression. The suppression ratio $\eta_a=(A_0-A_i)/A_0\times100\%$.

The hydroxyl radicals ($^{\cdot}OH$) in aqueous media were generated through the Fenton system.[15] The solution of the tested compound was prepared with DMF. The 5 ml assay mixture contained following reagents: safranin (11.4 μM), EDTA–Fe(II) (40 μM), H_2O_2 (17.6 μM), the tested compound (1.0—5.0 μM) and a phosphate buffer (67 mM, pH=7.4). The assay mixtures were incubated at 37 °C for 30 min in a waterbath. After which, the absorbance was measured at 520 nm. All the tests were run in triplicate and expressed as the mean and (±) standard deviation (S.D.). A_i is the absorbance in the presence of the tested compound; A_0 is the absorbance in the absence of the tested compound; A_c is the absorbance in the absence of the tested compound, EDTA–Fe(II), H_2O_2. The suppression ratio (η_a) was calculated on the basis of $(A_i-A_0)/(A_c-A_0)\times100\%$.

Results and Discussion

Characterization of the Compounds. Properties of the Complexes The complexes are soluble in DMSO and DMF, slightly soluble in ethanol and methanol, insoluble in benzene, water and diethyl ether. The molar conductivity of the complexes in DMF is 0.5—1.8, indicating that the complexes are all nonelectrolytes.[16] The elemental analyses and molar conductivity data show that the formulas of the complexes conform to $M(H_2L)OAc \cdot H_2O$ (M=Cu, Ni) and $Zn(H_2L)_2$.

IR Spectra As shown in Table 1, in the Cu(II), Zn(II), Ni(II) complexes, the ν(C=O) and ν(C=N) shift by *ca.* 30 (from 1629 to 1599) and 16 (from 1585 to 1569) cm^{-1} towards lower wave numbers, thus indicating that the O atom of C=O and the N atom of C=N take part in coordination to the metal ion.[17—19] The ν(C=O) (PMBP unit) of the ligand appears at 1569 cm^{-1}, but in the complexes, ν (C=O) (PMBP unit) shift by *ca.* 120 cm^{-1} towards lower wave numbers, thus indicating that the C=O take part in coordination with an enolic format.[20] Weak bands at 490 cm^{-1} are assigned to ν (M–O).[10,18,20] For the Cu(II) complex, the absorption at 3396 cm^{-1} can be assigned to the aqueous ν(OH) bands, which shows that there is crystal water in the complex.[17] Besides, there are two new other absorptions at 1652 and 1502 cm^{-1} in the Cu(II) complex, which are assigned to ν_{as} (CO_2^-) and ν_s (CO_2^-) of the CH_3COO^- ion, and the stretching frequency difference [ν ($\nu_{as}-\nu_s$)=150 cm^{-1}] is smaller than that of CH_3COONa (*ca.* 195 cm^{-1}), indicating that the coordinated of the CH_3COO^- ion is bidentate.[19] The characteristic absorption peaks of Ni(II) and Cu(II) complexes are very similar. So the structure of Ni(II) complex can be speculated in the same way. Whereas, the relevant aqueous ν(OH), ν_{as} (CO_2^-) and ν_s (CO_2^-) can not be found in the IR spectra of Zn(II) complex. In order to make sure the structure of Zn(II) complex, ESI-MS spectrum of the Zn(II) complex has been analyzed as follows.

Table 1. Some Main IR Data of the Ligand and Its Complexes

Compounds	H_3L	$Zn(H_2L)_2$	$Cu(H_2L)$-$OAc \cdot H_2O$	$Ni(H_2L)$-$OAc \cdot H_2O$
ν(OH)			3440 s	3455 m
ν(NH)	3372 m			
ν(NNH)	3408 m	3369 s	3396 s	3419 s
ν(C=O)	1629 s	1599 s	1592 s	1599 s
ν(C=N)	1585 s	1576 s	1569 s	1559 s
ν_{as} (CO_2^-)			1652 m	1652 m
ν_s (CO_2^-)			1502 m	1526 m
ν(C=O) (PMBP unit)	1569 m	1449 m	1436 m	1436 m
ν(M–O)		495 w	490 w	486 w

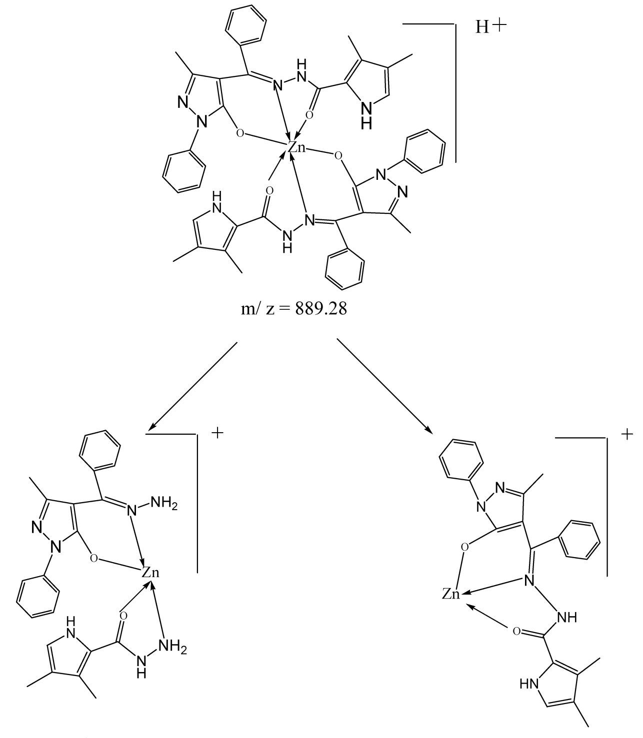
s=strong; m=medium; w=weak.

ESI-MS Spectra of the Zn(II) Complex Figure 2 demonstrates the proposed fragments in Zn(II) complex. The mass spectrum of the Zn(II) complex has three significant peaks *m/z* of 889.2542, 508.1242 and 476.0990, which can be assigned to fragments $[ZnL_2+H]^+$, F_1^+, F_2^+, respectively.

Antioxidative Activity. Hydroxy Radical ($^{\cdot}OH$) Scavenging Activity The hydroxyl radical ($^{\cdot}OH$) is the most reactive product of ROS formed by successive 1-electron reductions of molecular oxygen (O_2) in cell metabolism, and is primarily responsible for the cytotoxic effects observed in aerobic organisms extending from bacteria to plants and animals.[21] In order to obtain an efficient scavenger, in this paper, the ligand (H_3L) and its transition metal complexes have been studied by Fenton system.

The experimental data were collected in Table 2. It can be seen that the inhibitory effect of the tested compounds on $^{\cdot}$OH is concentration related and the suppression ratio increases with the increasing of sample concentration in the range of 1.0—8.0 μM (Fig. 3). The antioxidant activities of these compounds are expressed as 50% inhibitory concentration (IC_{50} in μM). The IC_{50} values of Cu(II) and Zn(II) complexes are 5.26, 3.23 μM respectively, Although the IC_{50} values of Ni(II) complexes, the ligand, compound (**1**) and compound (**2**) can not be read in Fig. 3, it can be seen clearly Zn(II) complex is the most efficient scavenger against $^{\cdot}$OH, and the suppression ration take the order of Zn(II) complex>Cu(II) complex>Ni(II) complex>ligand>compound (**2**)>compound (**1**) at the same concentration.

Superoxide Radical ($O_2^{\cdot -}$) Scavenging Activity The scavenging effect of the Cu(II) complex on $O_2^{\cdot -}$ has been shown in Fig. 4. As shown in Table 3, the Cu(II) complex has great superoxide radical scavenging activity, while other compounds, such as the ligand, the Zn(II) complex, the Ni(II) complex, compound (**1**) and compound (**2**) have nearly no scavenging effect under the same experimental conditions. Furthermore, the inhibitory effect of the Cu(II) complex on superoxide radical was also concentration related, and at a concentration from 0.4 to 2.0 μM, the suppression ratio valued from 41.97±1.37% to 56.99±1.47%. IC_{50} value of Cu(II) complex is 1.22 μM, which is significantly higher than



F1 m/z = 508.14

F2 m/z = 476.11

Fig. 2. The Proposed Degradation of the Zn(II) Complex

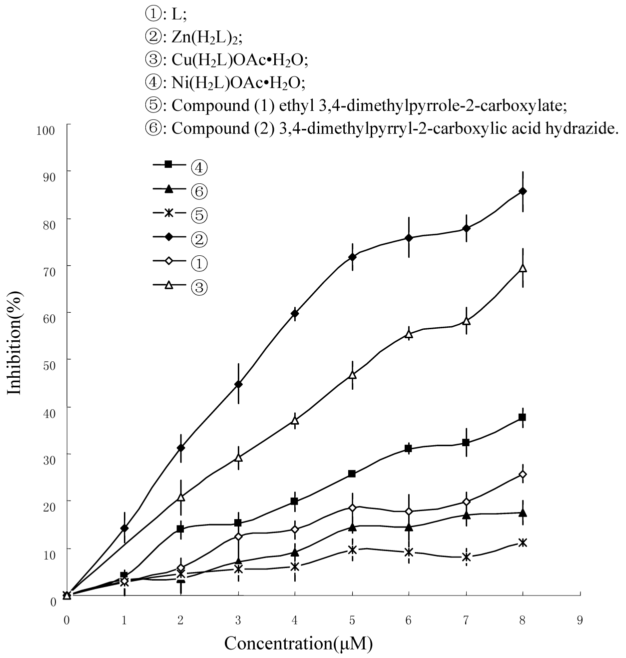


Fig. 3. Effect of the Tested Compounds on $^{\cdot}$OH

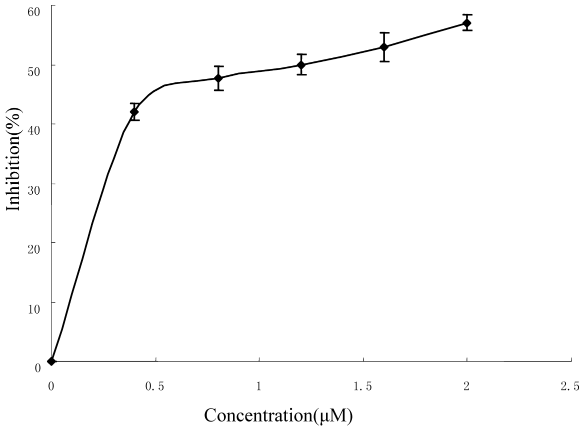


Fig. 4. Effect of the Cu(II) Complex on $O_2^{\cdot -}$

Table 2. The Influence of Investigated Compounds for $^{\cdot}$OH

Compounds	①	②	③	④	⑤	⑥
η_a%	2.98±1.40	0.69±1.39	14.35±3.21	3.97±0.00	2.69±2.58	3.43±1.06
η_a%	5.96±1.99	20.83±3.67	31.25±2.89	13.90±1.99	4.68±3.11	3.68±3.11
η_a%	12.58±2.29	29.17±2.40	44.91±4.24	15.23±2.29	5.67±2.58	7.16±2.98
η_a%	13.90±1.99	41.67±2.40	59.72±1.39	19.86±1.99	6.17±3.11	9.15±1.72
η_a%	18.54±3.03	47.22±2.78	67.69±2.85	25.82±0.00	9.65±2.28	14.63±1.49
η_a%	17.87±3.44	55.56±1.39	71.76±2.89	31.12±1.45	9.15±2.28	14.63±2.98
η_a%	19.86±1.99	58.33±2.78	77.78±2.78	32.44±3.03	8.15±1.72	17.11±2.28
η_a%	25.82±1.99	69.44±4.17	85.65±4.24	37.74±1.99	11.14±0.86	17.61±2.58
IC_{50}(μM)	—	3.23	5.26	—	—	—

①: L; ②: $Zn(H_2L)_2$; ③: $Cu(H_2L)OAc\cdot H_2O$; ④: $Ni(H_2L)OAc\cdot H_2O$; ⑤: compound (**1**) ethyl 3,4-dimethylpyrrole-2-carboxylate; ⑥: compound (**2**) 3,4-dimethylpyrryl-2-carboxylic acid hydrazide.

Table 3. The Influence of Investigated Compounds for $O_2^{\cdot-}$

Compounds	①	②	③	④	⑤	⑥
η_a%	2.52±1.30	1.13±1.19	41.97±1.37	3.21±1.03	1.57±1.04	2.03±1.83
η_a%	3.55±0.79	4.42±0.79	47.72±2.07	5.11±1.19	2.94±0.80	3.64±0.41
η_a%	4.24±0.52	3.04±2.60	49.97±1.66	3.04±1.82	2.71±1.19	2.97±2.27
η_a%	4.93±1.96	4.42±1.96	52.90±2.37	4.24±0.52	4.83±0.73	3.88±0.82
η_a%	5.82±1.87	5.45±2.09	56.99±1.47	3.04±1.82	4.84±1.24	5.80±0.85
IC_{50}(μM)	—	—	1.22	—	—	—

①: L; ②: $Zn(H_2L)_2$; ③: $Cu(H_2L)OAc\cdot H_2O$; ④: $Ni(H_2L)OAc\cdot H_2O$; ⑤: compound (**1**) ethyl 3,4-dimethylpyrrole-2-carboxylate; ⑥: compound (**2**) 3,4-dimethylpyrryl-2-carboxylic acid hydrazide.

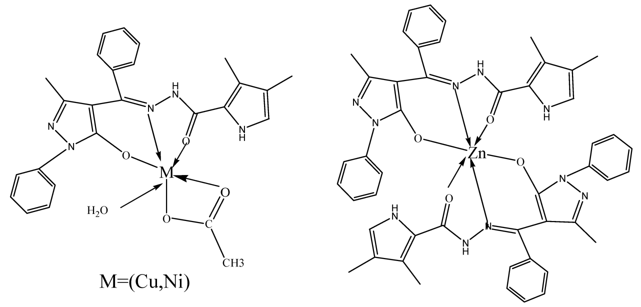


Fig. 5. The Suggested Structure of the Complexes

that of nitroxide Tempo ($IC_{50}=60\pm3.1$ μM) used in biological system for its capacity to mimic superoxide dismutase.[12)]

Conclusions

On the whole, we have synthesized and characterized a new H_3L, 4-{[(3,4-dimethyl pyrrole-2-carbonyl)hydrazono]-(phenyl)methyl}-3-methyl-1-phenylpyrazol-5-ol, and its three transition metal complexes. The possible structures of the complexes are shown in Fig. 5. In addition, their antioxdative activities have been studied and they are clearly shown that the complexes effectively scavenged $O_2^{\cdot-}$ and $^{\cdot}OH$ in a concentration-dependent manner and the transition metal ions such as Cu(II), Zn(II) and Ni(II) have differential and selective nature for scavenging $O_2^{\cdot-}$ and $^{\cdot}OH$. Furthermore, the scavenging effect on $O_2^{\cdot-}$ and $^{\cdot}OH$ can be enhanced by the formation of metal–ligand coordination complexes and the effect on scavenging $^{\cdot}OH$ of the Zn(II) and Cu(II) complexes are more remarkable while the Cu(II) complex is found to exert super activity of scavenging $O_2^{\cdot-}$ in comparison to the remaining compounds. It is believed that the information obtained from the present work would ultimately be helpful to develop new potent antioxidants and new therapeutic reagents for some diseases.

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