

Synthesis, Characterization and Antioxidant Activity of Naringenin Schiff Base and Its Cu(II), Ni(II), Zn(II) Complexes

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A new ligand, naringenin-2-hydroxy benzoyl hydrazone (H₃L), was prepared by condensation of naringenin with 2-hydroxy benzoyl hydrazine. Its Cu(II), Ni(II), Zn(II) complexes have also been synthesized and characterized on the basis of ¹H-NMR, IR, UV-Vis spectra, elemental analyses, molar conductivity and thermal analyses. The general formula of these complexes was M(H₃L) [M=Cu(II), Ni(II) and Zn(II)]. In addition, the antioxidant activities (superoxide and hydroxyl radical) of the free ligand and its complexes were determined *in vitro*. These compounds were found to possess potent antioxidant activity and be better than standard antioxidants like vitamin C and mannitol. In particular, the Cu(II) complex displayed excellent activity on the superoxide radical.

Key words naringenin schiff base; transition metal complex; characterization; antioxidant activity

It has been proposed that reactive oxygen species (ROS) are involved in the pathogenesis of various diseases, such as lifestyle-related diseases including hypertension and photoaging due to exposure to ultraviolet radiation.¹⁾ A predominant cellular free radical is an oxygen-derived species, superoxide anion (O₂⁻), which is formed by the leakage of high-energy electrons along mitochondrial electron transport chains and by a variety of cytosolic and membrane-bound enzymes, including xanthine oxidase and cytochrome P450 complexes. O₂⁻ mediates direct cell damage and reacts with hydrogen peroxide (H₂O₂) and nitric oxide radicals to generate the extremely reactive species hydroxyl radical (OH[·]) and peroxynitrite (ONOO⁻), respectively.²⁾ Superoxide dismutase (SOD) is a natural scavenger for O₂⁻. It often can catalyze the dismutation of superoxide anions into hydrogen peroxide and dioxygen, to keep the free radical density in the body at a normal level. SOD activity in mammalian cells, including those of man, tends to decrease with age. Increasing intracellular levels of SOD or administering exogenous SOD has been investigated for use in protection against oxidative injury. However, the use of the SOD protein as a therapeutic is confined by its price, size, charge and rapid clearance.³⁾ Likewise, a variety of OH[·] scavengers are known, but their application is also limited.⁴⁾ Many researchers have been working hard to develop metal complexes in order to achieve efficient antioxidants.^{5,6)}

During development of SOD-mimetic metalloelement complexes, it shows the complexes that prepared with pharmacologically active ligands often have better SOD-mimetic activity.¹⁾ Naringenin (4',5,7-trihydroxyflavanone), a predominant flavanone, are widely spread in nature and easily extracted from a lot of different plants. Its protective effect against lipid peroxidation of membranes, involved in several physiological and pathological disorders, as aging, inflammation, atherosclerosis, ischemia, toxicity of oxygen and chemical substances has been largely studied.⁷⁾ Our previous work showed that the rare earth complexes of naringenin benzoyl hydrazone have certain antioxidative and cytotoxic activities.^{8,9)}

As a continuation of our research, a ligand, naringenin-2-hydroxy benzoyl hydrazone (Fig. 1), and its three transition metal complexes have been synthesized and characterized.

The antioxidant activities (scavenging effects on O₂⁻ and OH[·]) of the ligand and its complexes have also been studied. Information obtained from this study will be helpful to develop some new antioxidants.

Experimental

Materials Nitroblue tetrazolium (NBT), methionine (MET), vitamin B₂ (VitB₂) were purchased from Sigma Chemical Co. Naringenin was produced in Hui Ke Co. (P.R.C.). 2-Hydroxy benzoyl hydrazine, vitamin C, safranin, ethylenediaminetetraacetic acid disodium salt (EDTA), FeSO₄·7H₂O and M(NO₃)₂·nH₂O [M=Cu(II), n=3; M=Ni(II), Zn(II), n=6] were produced by Shang Hai Reagent Co. (P.R.C.). All materials and solvents were of analytical grade. EDTA-Fe(II) and Na₂HPO₄-KH₂PO₄ buffers were prepared with twice distilled water.

Physical Measurements Melting points were determined on an XT4-100x microscopic melting point apparatus (Beijing Electrooptical Instrument Factory, China). Elemental analyses (C, H, N) were carried out with an Elemental Vario EL analyzer. Metal contents of the complexes were determined by titration with EDTA. Infrared spectra (4000–400 cm⁻¹) were determined with KBr disks on a Thermo Nicolet FTIR spectrometer. UV-Vis spectra were recorded on a Varian Cary 100 Conc spectrophotometer. Thermal behavior was monitored on a WCT-2A differential thermal analyzer (Beijing Optics Instrument Factory, China). Molar conductance measurements were made at 25 °C using a DDS-11C conductivity meter (Shanghai Leici Factory, China). ¹H-NMR spectra were recorded in DMSO-*d*₆ on a Varian VR 300-MHz spectrometer. Chemical shifts were shown as a δ-value (ppm) with tetramethyl silane (TMS) as an internal standard. Mass spectrum was performed on a VG ZABHS (FAB) instrument. The antioxidant activities were tested on a 721E spectrophotometer (Shanghai Analytical Instrument Factory, China).

Preparation of Ligand Naringenin (2.72 g, 10 mmol) and 2-hydroxy benzoyl hydrazine (1.33 g, 9 mmol) were dissolved in ethanol (40 ml). Acetic acid (1.0 ml) was added to this solution. The solution was refluxed on an oil bath for 1 d with stirring and a yellow precipitate formed (Fig. 1). The precipitate was filtered and washed with water and ethanol. Yield: 60%. Melting point: 292–293 °C. ¹H-NMR (300 MHz, DMSO-*d*₆) δ: 3.1 (2H, d, *J*=10.5 Hz, 3-H), 5.1 (1H, d, *J*=10.5 Hz, 2-H), 5.9–7.9 (10H, m, Ar-H), 9.6–11.7 (4H, s, -OH, D₂O exchangeable), 12.9 (1H, s, N-H, D₂O exchangeable). IR (KBr) cm⁻¹: 3212, 1638, 1607, 1517. UV-Vis λ_{max} (DMF) nm (log ε): 331 (5.29), 356 (5.26), 382 (5.10). Fast atom bombardment mass spectrometry (FAB-MS), *m/z* 407 [M+H]⁺. Anal. Calcd for C₂₂H₁₈N₂O₆: C, 65.02; H, 4.46; N, 6.89. Found: C, 65.26; H, 4.21; N, 7.17.

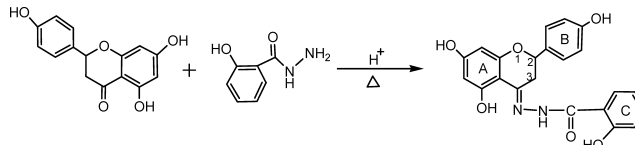


Fig. 1. Preparation Route of the Ligand

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Preparation of Complexes The ligand (0.20 g, 0.5 mmol) was dissolved in acetone (14 ml) and triethylamine (0.10 g, 1.0 mmol) was then added. After 5 min, $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ (0.12 g, 0.5 mmol) was added. Immediately, there was a green precipitate, the Cu(II) complex: $\text{Cu}(\text{H}_3\text{L})$, in the solution. The precipitate was separated by suction filtration, purified by washing several times with acetone, and dried in a vacuum. Ni(II) and Zn(II) complexes were prepared by the same way. $\text{Cu}(\text{H}_3\text{L})$: yield, 90%; IR (KBr) cm^{-1} : 3388, 1601, 1561, 1520, 505, 456. UV-Vis λ_{max} (DMF) nm (log ϵ): 354 (5.28), 380 (5.31), 399 (5.23). *Anal.* Calcd for $\text{C}_{22}\text{H}_{16}\text{N}_2\text{O}_6\text{Cu}$: C, 56.47; H, 3.45; N, 5.99; Cu, 13.58. Found: C, 56.37; H, 3.51; N, 6.16; Cu, 13.74. $\text{Ni}(\text{H}_3\text{L})$: yield, 44%; IR (KBr) cm^{-1} : 3395, 1611, 1538, 1519, 528, 466. UV-Vis λ_{max} (DMF) nm (log ϵ): 331 (5.18), 365 (5.31), 400 (5.13). *Anal.* Calcd for $\text{C}_{22}\text{H}_{16}\text{N}_2\text{O}_6\text{Ni}$: C, 57.06; H, 3.48; N, 6.05; Ni, 12.67. Found: C, 57.31; H, 3.22; N, 6.27; Ni, 12.42. $\text{Zn}(\text{H}_3\text{L})$: yield, 47%; IR (KBr) cm^{-1} : 3399, 1607, 1554, 1523, 503, 445. UV-Vis λ_{max} (DMF) nm (log ϵ): 346 (5.28), 384 (5.29), 403 (5.17). *Anal.* Calcd for $\text{C}_{22}\text{H}_{16}\text{N}_2\text{O}_6\text{Zn}$: C, 56.25; H, 3.43; N, 5.96; Zn, 13.92. Found: C, 56.09; H, 3.51; N, 6.27; Zn, 13.86. $^1\text{H-NMR}$ (300 MHz, DMSO- d_6), δ : 2.9 (2H, d, $J=12$ Hz, 3-H), 5.0 (1H, d, $J=12$ Hz, 2-H), 5.6–7.8 (10H, m, Ar-H), 9.3–9.5 (2H, s, -OH), 14.0 (1H, s, N-H).

Superoxide Radical Scavenging Assay The superoxide radicals ($\text{O}_2^{\cdot-}$) were generated *in vitro* by non-enzymatic system and determined spectrophotometrically by nitro blue tetrazolium (NBT) photoreduction method with a little modification in the method adopted elsewhere.^{10,11} The amount of $\text{O}_2^{\cdot-}$ and suppression ratio for $\text{O}_2^{\cdot-}$ can be calculated by measuring the absorbance at 560 nm. Solution of MET, VitB₂ and NBT were prepared at 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), VitB₂ (3.3 μM), the tested compound (2–20 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_1) 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 \pm standard deviation (S.D.) of triplicate determinations. The suppression ratio for $\text{O}_2^{\cdot-}$ was calculated from the following expression:

$$\text{suppression ratio (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

Drug activity was expressed as the 50% inhibitory concentration (IC_{50}). IC_{50} values were calculated from regression lines where: x was the tested compound concentration in μM and y was percent inhibition of the tested compounds.

Hydroxyl Radical Scavenging Assay The hydroxyl radicals (OH^{\cdot}) in aqueous media were generated through the Fenton system.¹² 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 (1.76 mM), the tested compound (2–20 μM) and a phosphate buffer (67 mM, pH 7.4). The assay mixtures were incubated at 37 °C for 30 min in a water-bath. After which, the absorbance was measured at 520 nm. All the tests were run in triplicate and expressed as the mean \pm standard deviation (S.D.). The suppression ratio for OH^{\cdot} was calculated from the following expression:

$$\text{suppression ratio (\%)} = \frac{A_1 - A_0}{A_c - A_0} \times 100 \quad (2)$$

(Where A_1 =the absorbance in the presence of the tested compound; A_0 =the absorbance in the absence of the tested compound; A_c =the absorbance in the absence of the tested compound, EDTA-Fe(II), H_2O_2 .)

Results and Discussion

All of the complexes are air stable for extended periods and soluble in DMSO (dimethylsulphoxide) and DMF; slightly soluble in ethanol, methanol, chloroform and acetone; insoluble in water. The molar conductivities of the Cu(II), Ni(II) and Zn(II) complexes are 5.8, 4.0 and 0.8 S $\text{cm}^2 \text{mol}^{-1}$, respectively in DMF, showing that all complexes are non-electrolytes.¹³ The elemental analyses and molar conductivities show that the formulas of the complexes conform to $\text{M}(\text{H}_3\text{L})$ [where M=Cu(II), Ni(II) and Zn(II)].

$^1\text{H-NMR}$ Spectra Compared with that of the ligand, it can be seen two hydrogen of -OH groups disappear in $^1\text{H-NMR}$ spectra of $\text{Zn}(\text{H}_3\text{L})$. The disappearance of H indicates

Table 1. IR Spectral Data of the Ligand and Its Complexes

Compound	$\nu(\text{O-H})$	$\nu(\text{C=O})$	$\nu(\text{C=N})$	$\delta_{(\text{NHNH})}$	$\nu(\text{M-O})$	$\nu(\text{M-N})$
H_3L	3212 b	1638 s	1607 s	1517 m		
$\text{Cu}(\text{H}_3\text{L})$	3388 b	1601 s	1561 s	1520 m	505 w	456 w
$\text{Ni}(\text{H}_3\text{L})$	3395 b	1611 s	1538 s	1519 m	528 w	466 w
$\text{Zn}(\text{H}_3\text{L})$	3399 b	1607 s	1554 s	1523 m	503 w	445 w

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

that these two hydroxy H atoms are substituted so the hydroxy groups take part in coordination. It also can be seen that the hydrogen of -NHN= group still exists, and this conclusion is also supported by IR spectra.

IR Spectra The most significant IR band assignments are given in Table 1. The $\nu(\text{O-H, N-H})$ for the ligand appears at 3212 cm^{-1} , and this peak for the complexes shifts to 3395 cm^{-1} or so. The $\nu(\text{C=O})$ vibration of the free ligand is at 1638 cm^{-1} ; for the complexes the peak shifts to 1601–1611 cm^{-1} , $\Delta\nu_{(\text{ligand-complex})}$ is equal to 27–37 cm^{-1} . In the complexes, the band at 503 cm^{-1} or so is assigned to $\nu(\text{M-O})$. These demonstrate that the oxygen of carbonyl has formed a coordinative bond with the metal ions.¹⁴ δ_{NHNH} vibrations of the free ligand (1517 cm^{-1}) and of the complexes (1519–1523 cm^{-1}) show that the active hydrogen of the -NHN= group still exists in the complexes. The band at 1607 cm^{-1} for the free ligand is assigned to the $\nu(\text{C=N})$ stretch. It shifts to 1554 cm^{-1} or so for the complexes. In the complexes, weak bands at 456 cm^{-1} or so are assigned to $\nu(\text{M-N})$. These further confirm that the nitrogen of the imino-group bonds to metal ion.¹⁵

Thermal Analyses $\text{Cu}(\text{H}_3\text{L})$, $\text{Ni}(\text{H}_3\text{L})$ and $\text{Zn}(\text{H}_3\text{L})$ begin to decompose at 329 °C, 382 °C and 323 °C, respectively. While being heated to 500 °C or so, the decomposition process finished. In this process, one exothermic peak appears. The corresponding TG curves show a series of weight loss. Finally, the complexes become their corresponding oxides. The residues are in accordance with calculation. Under 200 °C, there are no endothermic peak and no weight loss on corresponding TG curves. It indicates that there are no crystal or coordinate solvent molecules.

UV-Vis Spectra The free ligand exhibits three absorption band at 331 (band I), 356 (band II) and 382 (band III) nm (DMF was used as solvent). Band I is related to ring A, band II to ring C and band III to ring B.¹⁶ The complexes also have three peaks at approximately 346 (band I), 380 (band II) and 399 (band III) nm. The large red-shifts at band I and II suggest that ring A and C are involved in coordination.¹⁷

According to the elemental analyses, molar conductivity, $^1\text{H-NMR}$, IR, UV-Vis spectra and thermal analyses, the possible structure of these complexes is shown in Fig. 2.

Stability of the Complexes in Solution The stability of these complexes in aqueous solution has been studied by observing the UV-Vis spectrums and estimating the molar conductivities at different time intervals for any possible change.¹⁸ The tested compounds were prepared in DMF and for experiments freshly diluted in phosphate buffer system (pH 7.4, 7.8). Then, the UV-Vis spectrums and molar conductivities were researched at different time intervals.

The investigations revealed that the UV-Vis spectrums

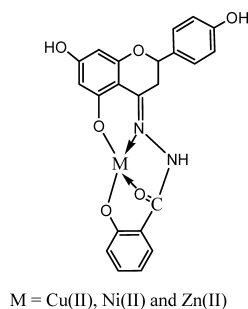
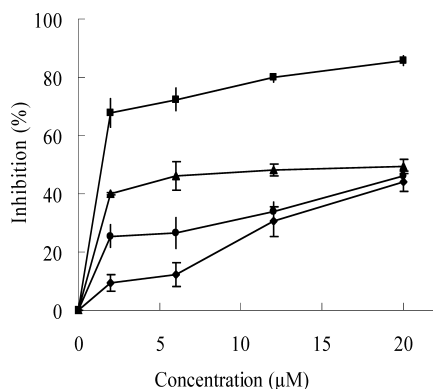


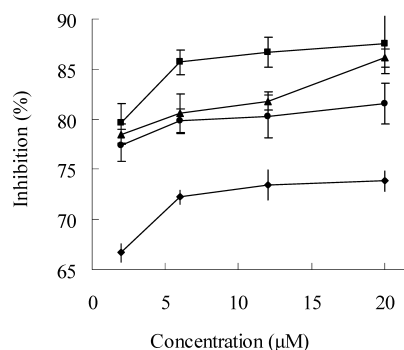
Fig. 2. Possible Structure of the Complexes

Fig. 3. Effect of the Tested Compounds on $O_2^{\cdot -}$. ■, Cu(H₃L); ▲, ligand; ●, Zn(H₃L); ◆, Ni(H₃L). Experiments were performed in triplicate. Values are expressed as mean ± standard deviation ($n=3$).

had no obvious change for the solutions and their molar conductance values have remained unaltered for very freshly prepared and for over the whole experiment (5 h). It indicates these three complexes are quite stable in phosphate buffer.

Superoxide Radical Scavenging Activity It can be seen that the inhibitory effect of the tested compounds on $O_2^{\cdot -}$ are concentration related and the suppression ratio increases with the increasing of sample concentration in the range of the tested concentration (Fig. 3). The antioxidant activities of these compounds are expressed as 50% inhibitory concentration (IC_{50} in μM). IC_{50} values of Cu(H₃L), Ni(H₃L), Zn(H₃L) and the ligand are 1.5, 29, 20 and 17 μM , respectively. These compounds clearly show higher superoxide dismutase activity compared with that of standard antioxidant like vitamin C (IC_{50} : 852 μM). In comparison with the complexes searched by Serbest *et al.*¹⁹) and other compounds studied in this paper, the Cu(H₃L) shows higher several fold potent activity, and its SOD-like activity is similar to that of porcine brain Cu₄Zn₃MT-III and rabbit liver Cd₅Zn₂MT-II searched by Shi *et al.*²⁰)

Hydroxy Radical Scavenging Activity We can find that all compounds scavenge OH \cdot also in a concentration-dependent manner (Fig. 4). Usually, mannitol and vitamin C are used as standard hydroxy radical scavenging reagent. When arrive at similar inhibitory value, it can be seen IC_{50} values of the ligand and its complexes are far less than that of mannitol and vitamin C. The ligand and its complexes show highly active scavenging effect on OH \cdot . Moreover, it also indicates that, as standard hydroxy radical scavenging reagent, mannitol is better than vitamin C. Among these compounds, the order of the suppression ratio for OH \cdot is H₃L > Zn(H₃L) >

Fig. 4. Effect of the Tested Compounds on OH \cdot

■, Cu(H₃L); ▲, ligand; ●, Zn(H₃L); ◆, Ni(H₃L). Experiments were performed in triplicate. Value are expressed as mean ± standard deviation ($n=3$).

Ni(H₃L) at different concentrations. The hydroxyl radical scavenging activity of the Cu(H₃L) is better than that of the ligand at 6 and 12 μM , but similar to that of the ligand at 2 and 20 μM .

Taken together, these compounds are found to possess potent antioxidant activity and be better than standard antioxidants like vitamin C and mannitol. The Cu(H₃L) complex displays excellent activity on scavenging the superoxide radical, deserves to be further researched.

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