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Manganese Complexes of Curcumin Analogues: Evaluation of Hydroxyl Radical Scavenging Ability, Superoxide Dismutase Activity and Stability towards Hydrolysis

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In order to improve the antioxidant property of curcumin and its analogue, diacetylcurcumin, manganese was incorporated into the structures in order to enhance superoxide dismutase (SOD) activity. Manganese (Mn) complexes of curcumin (CpCpx) and diacetylcurcumin (AcylCpCpx) were synthesized and firstly investigated for SOD activity and hydroxyl radical (HO·) scavenging ability. SOD activity was evaluated by both the nitroblue tetrazolium (NBT) reduction assay and electron paramagnetic resonance (EPR) with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as a spin trapping agent. CpCpx and AcylCpCpx inhibited the NBT reduction and decreased the DMPO/OOH adduct much greater than corresponding antioxidants or ligands, with IC_{50} values of 29.9 and 24.7 μM (NBT), and 1.09 and 2.40 mM (EPR), respectively. For EPR, potassium superoxide (KO_2) was used as a source of $O_2^{-\cdot}$ where qualitative results suggested that CpCpx and AcylCpCpx were SOD mimics, which catalyze the conversion of $O_2^{-\cdot}$ to dioxygen and hydrogen peroxide (H_2O_2). Additionally, CpCpx and AcylCpCpx exhibited the great inhibition of DMPO/OH adduct formation with an IC_{50} of 0.57 and 0.37 mM, respectively, which were comparable to that of curcumin (IC_{50} of 0.64 mM), indicating that both Mn complexes are also an effective HO· scavenger. The stability against hydrolysis in water, various buffers and human blood/serum was carried out *in vitro*. It was found that both Mn complexes were pH and salt concentration dependent, being more stable in basic pH. In the human blood/serum test, CpCpx was more stable against hydrolysis than AcylCpCpx with about 10 and 20% of free Mn^{2+} releasing, respectively.

Keywords: Curcumin; Manganese; Complex; Antioxidants; Lipid peroxidation; Free radical

Abbrevations: SOD, superoxide dismutase; AcylCp, diacetyl Curcumin; CpCpx, curcumin manganese (II) complex; AcylCpCpx, diacetylcurcumin manganese (II) complex; FTIR, fourier transform infrared spectroscopy; ^{1}H-NMR, hydrogen-1 nuclear magnetic resonance spectroscopy; ^{13}C-NMR, carbon-13 nuclear magnetic resonance spectroscopy; KBr, potassium bromide; NBT, nitroblue tetrazolium; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DPTA, diethylenetriaminepentaacetic acid; EPR, electron paramagnetic resonance; PPB, phosphate buffer; IC_{50}, fifty percent inhibition concentration

INTRODUCTION

Reactive oxygen species (ROS), namely superoxide anion ($O_2^{-\cdot}$), perhydroxyl radical ($HO_2^{\cdot}$), hydroxyl radical (HO·), hydrogen peroxide (H_2O_2) and nitric oxide ($^{\cdot}NO$), are produced during normal cellular function. $O_2^{-\cdot}$, a predominant cellular free radical, is involved in a large number of degenerative changes, often associated with an increase in peroxidative processes and linked to low antioxidant concentration. In addition to the direct reaction with biological targets, it is now clear that $O_2^{-\cdot}$ can react with the H_2O_2 and $^{\cdot}NO$ radical to generate the extremely reactive species $HO^{\cdot}$ and peroxynitrite ($ONOO^-$), respectively.[1] Under normal circumstances, the formation of $O_2^{-\cdot}$ is tightly controlled by superoxide dismutase (SOD) enzymes, the metalloenzyme that catalyzes the conversion of $O_2^{-\cdot}$ to H_2O_2, which can in turn be reduced to H_2O by catalase and/or peroxidase enzymes. Native SOD has been used as a therapeutic agent to attenuate

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reactive oxygen species-induced injuries. However, applications of exogenous SOD still have many problems including short clearance ($t_{1/2}$=6 min), brain inaccessibility, chemical instability, oral bio-availability, specific tissue targeting and immunogenicity.[1,2] An increasing number of low-molecular-weight SOD mimics have been developed to overcome these limitations. Most SOD catalytic mimics are designed with a redox active metal center, similar to the active site metals of the native SODs, i.e. Cu, Fe or Mn. It seemed safer to base such mimics upon Mn because Cu and Fe have the potential to participate in Fenton chemistry with the production of highly reactive hydroxyl radical. Many manganese based SOD mimics have been developed such as Mn porphyrins,[3] Mn macrocyclics,[4,5] salen Mn complexes[6] and Mn desferroxamines.[7]

Curcuma longa Linn. or turmeric (Zingiberaceae) is a medicinal plant widely cultivated in tropical regions of Asia. Turmeric extract from the rhizomes, commonly called curcumin, are mainly composed of about 75–95% curcumin and a small amount of demethoxycurcumin and bisdemethoxycurcumin.[8] Curcumin is well-known for having a potent antioxidant activity and various related activities in biological systems have been extensively investigated.[9–14] Moreover, curcumin has been shown to exhibit anti-inflammatory[11] and anticarcinogenic[12] activities in addition to anti HIV-1 integrase activity.[14] Although the specific molecular mechanisms of these effects of curcumin are not completely understood, its antioxidant properties through scavenging of free radical species are well documented.[25–29] Curcumin is a potent scavenger of methyl,[15] alkoxyl/phenoxyl,[16] peroxyl,[16] hydroxyl[17–19] and nitrogen dioxide radicals.[20] However, recent studies have demonstrated that curcumin is not an effective superoxide radical scavenger.[19] Structurally, curcumin consists of two *ortho* methoxylated phenols and a β-diketoned moiety, and they are all conjugated providing an electron-rich donor structure.

In this study, we were interested in improving the antioxidant properties of curcumin and its derivative, diacetylcurcumin, by complexation with manganese. The complexes were characterized using elemental analyses, infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy and electron paramagnetic resonance (EPR). In order to investigate the effect of manganese complexes and related compounds on $O_2^{-\cdot}$, SOD activity was conducted using the nitroblue tetrazolium (NBT) reduction method[21] and EPR spin-trapping.[22–24] The hydroxyl free radical scavenging ability of the prepared manganese complexes was also examined using EPR-spin trapping method.[24] In addition, the stability against hydrolysis of complexes in buffers of various pH and in human blood/serum was also evaluated.

MATERIALS AND METHODS

Chemicals

Turmeric extract was obtained from TCFF, Thailand. Manganese acetate, dimethyl sulfoxide (DMSO) and hydrogen peroxide (H_2O_2) were from E. Merck. Gelatin was from Fluka. Cupric sulfate ($CuSO_4$) was from Mallinckrodt. Potassium dihydrogen phosphate (KH_2PO_2) and manganese chloride ($MnCl_2$) were obtained from Baker. Diethylene-triaminepentaacetic acid (DPTA) was obtained from Aldrich. NBT, SOD, xanthine, xanthine oxidase, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), potassium superoxide (KO_2), [N-(2-hydroxylethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES) and 2-[morpholino]ethanesulfonic acid (MES) were obtained from Sigma. The DMPO was purified by stirring an aqueous solution with activated charcoal and filtering through 0.45 μm filter paper. The purified DMPO did not give any EPR signals when scanned at 100 mM concentration.

Synthesis

The compounds were characterized by FTIR, ^{1}H NMR, ^{13}C NMR, and elemental analyses. The metal (Mn) content of these complexes were determined after decomposing the complexes in acid, using EPR techniques. Spectral data were consistent with the assigned structures in all cases.

Curcumin (purified curcumin, Cp) was separated from the commercial curcuminoids or turmeric extract using silica gel column chromatography (eluent: chloroform/methanol/acetic acid, 98:5:2), mp 185–187°C. IR(KBr)(cm^{-1}): 3420 (O–H), 2952 (alkane C–H), 1632 (C=O ketone), 1590 (C=C), 1282-1118 (C–O,C–N), 861-792 (aromatic C–H). ^{1}HNMR (d-DMSO): δ 3.82 (s, 6H, O–C*H*$_3$), 6.05 (s, 1H, –CO–C*H*=COH–), 6.75 (d, J 15.87 Hz, 2H, Ph-CH=C*H*–CO–, Ph-CH=C*H*–COH–), 6.81 (d, J 7.93 Hz, 2H, Ph–*H3*, Ph–*H3′*), 7.14 (dd, J 1.53 and 8.24 Hz, 2H, Ph–*H2*, Ph–*H2′*), 7.31 (d, J 1.53 Hz, 2H, Ph–*H1*, Ph–*H1′*), 7.53 (d, J 15.87 Hz, 2H, Ph–C*H*=CH–), 9.67 (br, 1H, exchangeable with D_2O, Ph–CO*H*=CH). TLC: stationary phase-silica gel GF 254, mobile phase-chloroform/ethanol/acetic acid (98:5:2): R_f of curcumin = 0.70, R_f of demethoxycurcumin = 0.63 and R_f of bisdemethoxycurcumin = 0.52.

Diacetylcurcumin, AcylCp

Curcumin (0.368 g, 1 mmol) was dissolved in (molecular sieve) dried pyridine (2 ml). Acetic

anhydride (0.48 ml) was then added dropwise to this solution and the reaction mixture was stirred for 2 h at room temperature. Saturated sodium bicarbonate solution was added to the reaction mixture to neutralize excess acetic anhydride. The mixture was extracted with ethyl acetate (3 × 30 ml). The combined ethyl acetate layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The resulting solid was collected and recrystallised with ethyl acetate/hexane to give diacetylcurcumin 420 mg (92.92% yield), mp = 166–167°C. IR (KBr) (cm^{-1}): 3460 (O–H), 3039 (alkene C–H), 2940 (alkane C–H), 1762 (C=O ester), 1637 (C=O ketone), 1604-1512 (C=C), 1452 (–CH_3), 1189-1123 (C–O). ^{1}H NMR (d-DMSO): δ 2.27 (s, 6H, Ph–O–CO–CH_3), 3.85 (s, 6H, Ph–O–CH_3), 6.20 (s, 1H, –COH=C*H*–CO–), 7.003 (d, J 15.89 Hz, 1H, Ph–CH=C*H*–COH–), 7.16 (d, J 8.07 Hz, 2H, Ph-*H*6), 7.33 (dd, J 1.23 and 8.24 Hz, 2H, Ph–*H*5), 7.52 (d, J 1.20 Hz, 2H, Ph-*H*3), 7.65 (d, J 16.09 Hz, 2H, Ph–C*H*=CH–COH–). ^{13}C NMR (d-DMSO): δ20.57 (2C), 56.08 (2C), 81.81 (1C), 112.15 (2C), 121.60 (2C), 123.50 (2C), 124.72 (2C), 133.83 (2C), 140.03 (2C), 141.03 (2C), 151.33 (2C), 168.60 (1C), 183.37 (1C), 183.98 (2C).

Curcumin Manganese Complex, CpCpx

Manganese acetate (0.165 g, 0.65 mmol) was dissolved in ethanol (1 ml) and heated at 60°C under nitrogen gas. Curcumin (0.240 g, 0.65 mmol) in ethanol (15 ml) was added dropwise to the solution of manganese acetate and the reaction mixture was allowed to reflux for 3 h under nitrogen gas. The solid obtained was collected by filtration, washed with cold ethanol and dried *in vacuo* at room temperature over silica gel to afford a red powder (0.150 g, 54.55% yield). mp > 200°C. IR(KBr)(cm^{-1}): 3342 (O–H), 3032 (alkene C–H), 2937 (alkane C–H), 1621 (C=O ketone), 1592 (aromatic C=C), 1281-1123 (C–O) 971-814 (aromatic C–H). ^{1}H NMR (d-DMSO): broad spectrum. *Anal. Calc.* for $C_{21}H_{19}O_6Mn.OCH_2CH_3.H_2O$ (M.W. 485.39): C, 56.88; H, 5.35; Mn, 11.32. Found: C, 56.55; H, 4.96; Mn, 11.06.

Diacetylcurcumin Manganese Complex, AcylCpCpx

AcylCpCpx was prepared in the same manner as CpCpx but starting with AcylCp. A yellow-red powder of AcylCpCpx (0.135 g, 48.44% yield) was obtained, mp > 200°C. IR(KBr) (cm^{-1}): 3012 (alkene C–H), 2940 (alkane C–H), 1759 (C=O ester), 1643 (C=O ketone), 1597-1508 (C=C), 1196-1112 (C–O) 906-827 (aromatic C–H). ^{1}H NMR (d-DMSO): broad spectrum. *Anal. Calc.* for $C_{50}H_{46}O_{16}Mn$(M.W 957.22): C, 62.70; H, 4.84; Mn, 5.74. Found: C, 62.54; H, 4.76; Mn, 5.66.

Effect on Superoxide Anion

Nitroblue Tetrazolium (NBT) Asssay[21]

The SOD assay was carried out 2.0 ml of an assay mixture containing 1100 μl of 0.1 M phosphate buffer (PPB) pH 7.4, 1% of 500 μl of gelatin in PPB, 300 μl of catalase (6 units ml^{-1}) and 100 μl of 4 mg ml^{-1} of NBT (final concentration of 195 μM) in PPB, in a 3 ml cuvette, followed by 250 μl of xanthine oxidase solution and 250 μl of test compound. After 30 s, the reaction was started by adding 500 μl of 1 mM xanthine and monitored at 540 nm for 7 min. For a control, the test compound was replaced by its vehicle (DMSO). SOD activity was calculated based on the rate of absorbance change per minute (ΔA/min) and expressed in terms of the IC_{50} of NBT reduction obtained from a linear regression plot of percent SOD activity vs. test compound concentrations. Rate constants for the reaction of test compounds with $O_2^{-\cdot}$ were determined based upon the competition with 195 μM NBT, $K_o = 5.8 \times 10^4\ M^{-1}\ s^{-1}$.[26] Possible interference through inhibition of xanthine oxidase by test compounds was examined by following the rate of the urate accumulation at 295 nm in the absence of NBT.

EPR Spin-trapping with DMPO[22,23]

The reaction mixture consisted of 50 μM xanthine, 1 mM DPTA, various concentrations of test compound in DMSO, 250 mM DMPO and 0.5 mIU ml^{-1} xanthine oxidase in 40 mM of phosphate buffer, pH 7.4. For a control, the test compound was replaced by DMSO (10% v/v). The reaction was started with the addition of xanthine oxidase and recorded after mixing on a Varian E-4 EPR spectrometer at the following settings: magnetic center field 3395 G, frequency 9.530 GHz, power 20 mW, scan width 80 G, modulation amplitude 1 G, gain 5000, time constant 0.128 s and scan time 2 min.

In order to study the mechanism of how manganese complexes and curcumin react with $O_2^{-\cdot}$, KO_2 powder (final concentration ~20 mM) was added directly to the test compound solution in phosphate buffer pH 7.4 containing 10% DMSO. DMPO was added after 30 s; then, the mixture was immediately recorded within 20 s.

Effect on Hydroxyl Radical

HO· scavenging ability was examined using EPR spin-trapping.[24] Hydroxyl radicals were generated from the Cu^{2+}/H_2O_2 Fenton reaction system. The reaction mixture contained 0.25 mM $CuSO_4$, various concentrations of test compound (dissolved in DMSO, 10% v/v), 100 mM DMPO and 1 mM H_2O_2, in 40 mM phosphate buffer pH 7.4. For a control, the test compound was replaced by DMSO

(10% v/v). The reaction was initiated by the addition of H_2O_2 and EPR spectra were recorded 5 min after mixing the solution. EPR conditions were similar to that described for the detection of $O_2^{-\cdot}$ adduct above.

Stability Evaluation

A 10 mM stock solution of manganese complex was prepared in DMSO, then diluted to 1 mM concentration in water, 0.1 M HEPES, pH 8.6, 7.4 or 6, and 0.1 M MES buffer, pH 2, at 25°C. The hydrolysis was monitored by detecting released free manganese (Mn^{2+}). The amount of free Mn^{2+}in each test solution was measured up to 72 h by EPR at the following conditions: magnetic center field 3295 G, frequency 9.272 GHz, power 10 mW, scan width 1000 G, modulation amplitude 10 G, gain 5,000, time constant 0.5 s and scan time 4 min. Standard curves of free Mn^{2+}at different buffer pH were calibrated daily.

To determine the stability of synthesized manganese complexes in a biological system, the *in vitro* stability in human blood/serum (freshly prepared) was also measured. The whole blood was collected from an asian healthy volunteer, 29 years male. The solution of complexes in DMSO was diluted to 3 mM final concentration (contained 10% DMSO finally) in human blood/serum and then kept at 25°C during the experiment. The amount of free Mn^{2+}in each test solution was measured up to 72 h with the conditions as similar as that described for the detection of free Mn^{2+} in buffer.

RESULT AND DISCUSSION

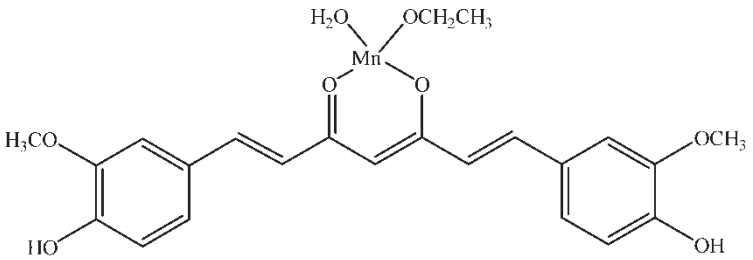
Synthesis of Manganese Complexes

The manganese complexes were prepared by complexation of MnOAc with curcumin and diacetylcurcumin in ethanolic solution. The complexes are powder-like and stable to atmospheric oxygen. Both complexes are insoluble in both water and alcoholic solvents, but soluble in DMSO and DMF. The analytical results indicated a 1:1 and 1:2 (metal/ligand) stoichiometry of CpCpx and AcylCpCpx, respectively. The different stoichiometry is probably due to the higher nucleophilicity of carbonyl carbons in curcumin than that in diacetylcurcumin, which provides more extensive $p\pi-d\pi$ cycloconjugation in approaching the aromatic system.[25] The coordination geometry of both manganese complexes is tetrahedral as shown in Fig. 1. The infrared (IR) absorption frequencies are listed in the Experimental section. The C=O (ketone) stretching frequency observed at 1632 cm^{-1} for curcumin was much weaker intensity and shifted to lower frequencies of 1621 cm^{-1} in the complex, CpCpx. The IR spectra of AcylCpCpx showed a weak C=O (ketone) signal at 1643 cm^{-1}, which was weaker intensity and at higher frequency than found in the ligand, diacetylcurcumin (1637 cm^{-1}), whereas the intensity and frequency of the C=O (ester) signal of AcylCpCpx and diacetylcurcumin were identical. The IR data confirm the interaction of the metal ion at the keto-enol function in both complexes. Moreover, the ^1H NMR spectra of both complexes were found to be broad due to paramagnetic relaxation broading from the manganese ion.

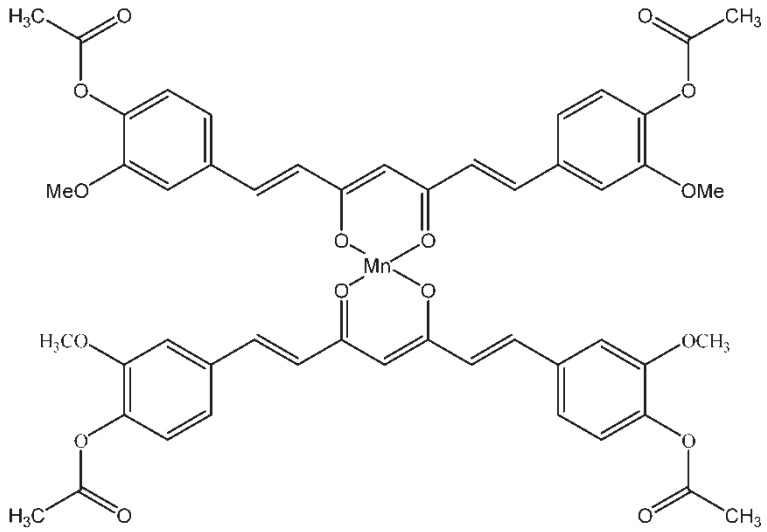
Effect on Superoxide Anion

The protective effect of these complexes against $O_2^{-\cdot}$ was tested by the NBT assay and EPR spin-trapping. SOD activity was examined spectrophotometrically by NBT reduction to blue formazan.[21] Percent SOD activity was calculated from the rate of absorbance change per minute (ΔOD/min) when compared with the absence of test compound. The IC_{50} values were calculated from the regression plots of the percent SOD activity vs. the concentrations of test compound, which was linear with $r^2 > 0.98$ in all compounds. The relationship of percent SOD activity and concentration of test compounds were dose dependent as was the SOD enzyme. The activity of the test compounds was also expressed as a rate constant, according to the equation $V_o/\nu - 1 = K_i[T]/K_o[D]$, where V_o and ν are the rates of NBT reduction in the absence and presence of test compound, K_i and K_o are the rate constants for the reaction of free radical with test compound and NBT, [T] and [D] refer to the test compound and NBT concentrations.[26–28] Rate constants for the reaction of test compounds with $O_2^{-\cdot}$ were based upon competition with NBT, K_{o_NBT} at pH 7.4 $= 5.8 \times 10^4\ M^{-1}\ s^{-1}$.[26] The rate constant of dismutation catalyzed by SOD was $2.1 \times 10^8\ M^{-1}\ s^{-1}$, which is relevant to the previously reported, $k_{cat} = 2.0 \times 10^9\ M^{-1}\ s^{-1}$.[27] The IC_{50} values of CpCpx and AcylCpCpx were 29.9 ($k = 3.8 \times 10^5\ M^{-1}\ s^{-1}$) and 24.7 μM ($k = 4.6 \times 10^5\ M^{-1}\ s^{-1}$), respectively, whereas curcumin and diacetylcurcumin showed only limited inhibition of NBT reduction at 270 μM with 18.6 and 0% inhibition, respectively (Table I).

The complexes were also examined by EPR spin-trapping. In these experiments, DMPO concentration was sufficiently high (250 mM) to trap almost all of the $O_2^{-\cdot}$. After adding xanthine oxidase to mixture solution, the EPR signals of DMPO/OOH adduct appeared immediately and were measured as soon as possible (within 30 s) after mixing. A characteristic 2:4:4:2 pattern (12 lines) of the DMPO/OOH adduct with hyperfine splitting constants $a_N = 13.89$ G, $^{\beta}a_H = 11.61$ G and $^{\gamma}a_H$ 1.28 G, and



CpCpx



AcylCpCpx

FIGURE 1 Structure of curcumin and diacetylcurcumin manganese complexes (CpCpx and AcylCpCpx).

$g = 2.0062$ were obtained (Fig. 2A). The relative intensity of the DMPO/OOH adduct from $O_2^{-\cdot}$ generated in the xanthine/xanthine oxidase system decreased in the presence of manganese complexes. The concentrations of CpCpx and AcylCpCpx to inhibit 50% of the DMPO/OOH adduct signal were found to be 1.09 ($k = 6.9 \times 10^3\ M^{-1}\ s^{-1}$) and 2.40 mM ($k = 3.1 \times 10^3\ M^{-1}\ s^{-1}$), respectively, much more

TABLE I Effect on superoxide anion and hydroxyl free radical

Compounds	NBT assay (IC_{50}, μM)	EPR spin-trapping method (IC_{50}, mM)		Rate constant ($M^{-1}s^{-1}$)*		
		Inhibition of DMPO/OOH	Inhibition of DMPO/OH	$K_{SP(NBT)}$†	$K_{SP(DMPO)}$‡	$K_{OH(DMPO)}$¶
Curcumin	18.67%§	29.9%‖	0.64	–	–	3.3×10^{11}
Diacetylcurcumin	NA#	4.7%‖	8.34	–	–	2.5×10^{10}
CpCpx	29.9	1.09	0.50	3.8×10^5	6.9×10^3	4.2×10^{11}
AcylCpCpx	24.7	2.40	0.37	4.6×10^5	3.1×10^3	5.7×10^{11}
EtOH	–	–	3007.06	–	–	6.9×10^7
Trolox	2821.2	–	–	3.9×10^3	–	–
SOD	5.19 unit ml^{-1}	–	–	2.1×10^8	–	–

*The rate constant was calculated according to the equation $V_0/\nu - 1 = K_i[T]/K_o[D]$, where V_o and ν are the rates of NBT reduction(or radical trapping by DMPO) in the absence and presence of test compound, K_i and K_o are the rate constants for the reaction of free radical with test compound and NBT (or DMPO), [T] and [D] refer to the test compound and NBT (or DMPO) concentrations. When plotted as $(V_0/\nu - 1)$ vs. concentration of test compound yielded a straight line, IC_{50} was found at $V_0/\nu - 1 = 1$. † $K_{SP(NBT)}$ is the rate constant for the reaction of $O_2^{-\cdot}$ and test compound in the NBT reduction assay. The rate constant of NBT reduction by $O_2^{-\cdot}$, K_o at pH 7.4 = $5.8 \times 10^4\ M^{-1}\ s^{-1}$, was used for calculation.[26] ‡ $K_{SP(DMPO)}$ is the rate constant for the reaction of $O_2^{-\cdot}$ and test compound in the EPR spin trapping assay. The rate constant of the reaction of DMPO and $O_2^{-\cdot}$, K_o at pH 7.4 = $30\ M^{-1}\ s^{-1}$, was used for calculation.[27] ¶ $K_{OH(DMPO)}$ is the rate constant for the reaction of ·OH and test compound in the EPR spin trapping method. The rate constant of the reaction of DMPO with ·OH, K_o at pH 7.4 = $2.1 \times 10^9\ M^{-1}\ s^{-1}$, is used for calculation.[28] § % inhibition of NBT reduction at the concentration of 270 μM. ‖ % inhibition of DMPO/OOH adduct formation at the concentration of 10 mM. # NA = not significantly suppressed at 270 μM.

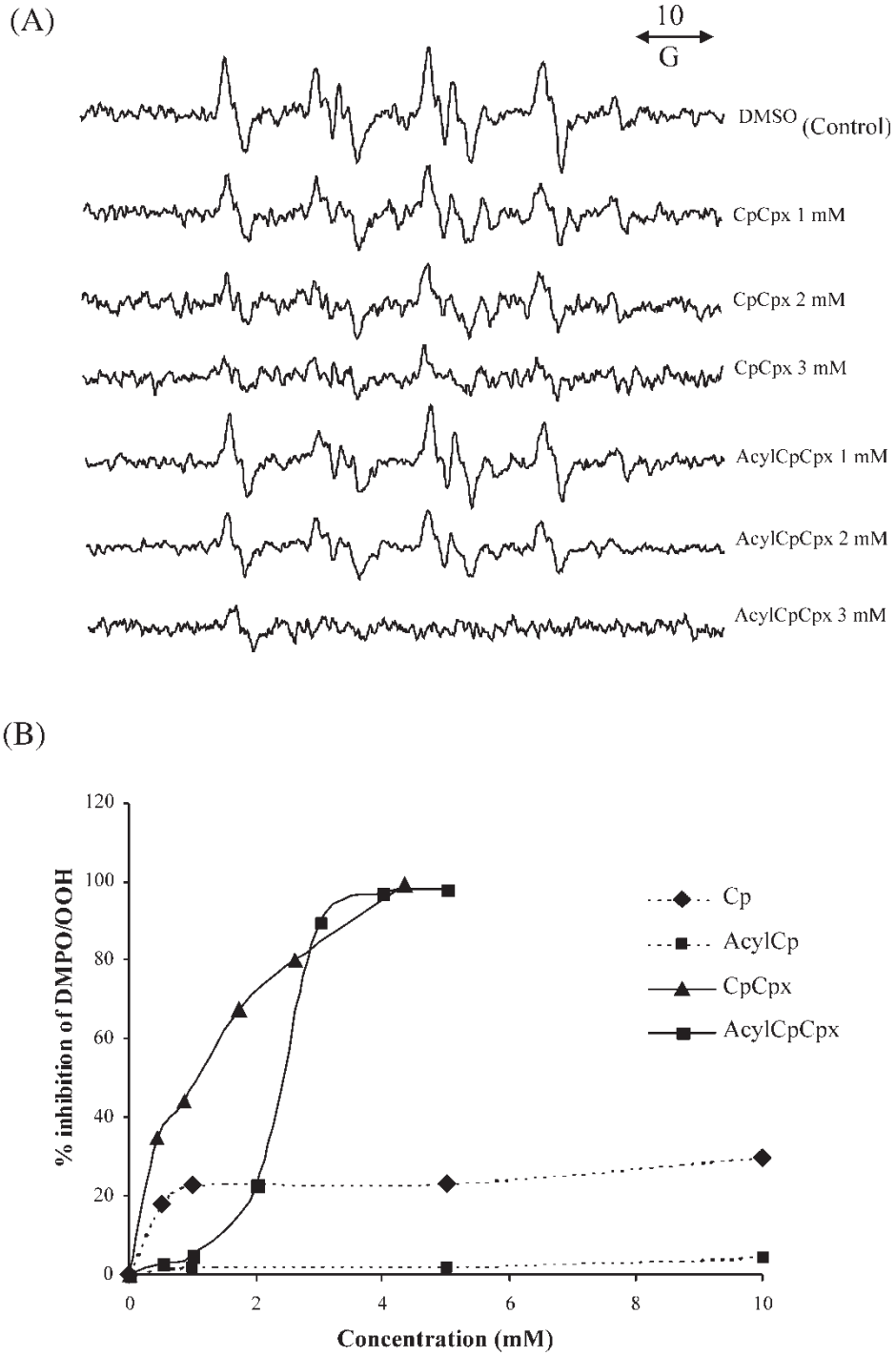


FIGURE 2 Effect of AcylCpCpx, CpCpx, curcumin (Cp) and diacetylcurcumin (AcylCp) on the intensity of DMPO/OOH adduct formed from DMPO and superoxide anion generated by the xanthine/xanthine oxidase system. (A) EPR signal of DMPO/OOH adduct in the presence of manganese complexes as described in Experimental section. (B) The percent inhibition was calculated from the peak height of the EPR signal of the first and last peaks.

potent than corresponding ligands, curcumin and diacetylcurcumin, which were able to inhibit only 29.9 and 4.7% at 10 mM concentration, respectively (Fig. 2B, Table I).

As the manganese complexes might inhibit xanthine oxidase or NBT reduction directly leading to the lower amount of liberated superoxide anion, the activity of XO was determined by monitoring the produced uric acid, the production of uric acid by XO in the presence of Mn-cpxes were not significantly different from those without the complex. This indicated that the complexes did not directly inhibit xanthine oxidase nor the NBT reduction. In addition, the experiments with

changing the order of adding xanthine oxidase and the manganese complexes and the experiment using KO_2 to generate $O_2^{-\cdot}$ were conducted. It was found from both the NBT assay and the DMPO/OOH EPR signals that the results were not significantly different neither xanthine oxidase was added before nor after adding the manganese complexes. When $O_2^{-\cdot}$ was generated directly by KO_2 in the presence of manganese complexes, the EPR signals decreased within a dose dependent manner as same as those found in the xanthine/xanthine oxidase system. To rule out interference with the assay by direct reaction with the DMPO adduct in the KO_2 experiment, Mn complexes were added after allowing DMPO to react completely with radical. It was found that the adduct signal was not changed.

The demonstrated activity may possibly be due to the free manganese released from the complex or other contaminated metals. Therefore, the stability of manganese complexes in the presence of the chelating agents was determined in order to rule out an effect of free manganese or the contaminated metal. When albumin (1.6%, final concentration) or EDTA in excess (5% or 134 μM, final concentration) was added to the reaction mixture, the results from the NBT assay were not significantly different (data not shown). This suggested that the manganese complexes were substantially stable and the activity was not due to traces of manganese dissociating from the complexes.

Curcumin was earlier shown to scavenge $O_2^{-\cdot}$.[17–19] However, the effect of curcumin on $O_2^{-\cdot}$ could only observed at very high concentrations. Reddy *et al.* reported a 39% inhibition of reduction of NBT by 54 μM curcumin.[17] In other studies, a maximum inhibition of 24–40% in the reduction of ferricytochrome C was observed by 80 μM curcumin.[18,19]. In the present study, we observed only 18.6% decrease in the rate or NBT reduction by 270 μM curcumin and 29.9% inhibition of DMPO/OOH adduct by 10 mM curcumin, respectively. These results suggest that curcumin is not an effective $O_2^{-\cdot}$ scavenger, it can only partially scavenge $O_2^{-\cdot}$ at very high concentration. In our present report, we have shown that both curcumin manganese complexes had much better effect on $O_2^{-\cdot}$ than curcumin in both NBT and EPR spin-trapping assays. It appears that manganese is essential for activity against $O_2^{-\cdot}$.

Next, the mechanism of CpCpx and AcylCpCpx against $O_2^{-\cdot}$ was elucidated. An excess of $O_2^{-\cdot}$ radical (~20 mM) was generated directly using KO_2. The powder of KO_2 was added to a solution containing various concentrations of test compound. After 30 s of reaction, DMPO was added to the solution mixture to trap excess free radicals. In the presence of DMSO, an EPR signal of the DMPO/OOH adduct was obtained (Fig. 3). In the presence of low concentration of AcylCpCpx (250 μM), combined signals from both DMPO/OOH and DMPO/OH were found while only DMPO/OH adduct was obtained in higher concentration of AcylCpCpx. As expected, the DMPO/OH adduct signal disappeared with the addition of excess AcylCpCpx as shown in Fig. 3. CpCpx also suppressed $O_2^{-\cdot}$ in almost the same manner as AcylCpCpx. In the presence of curcumin, a combination of DMPO/OOH and DMPO/OH signals was observed although higher concentrations were used. SOD enzyme and free Mn^{2+}(manganese acetate) was also tested in the experiment; it was found that both SOD and free Mn^{2+} decreased the EPR signals with the same manner as the complexes did. Interestingly, when H_2O_2 was added instead of test compound, only DMPO/OH was observed. Because the high concentration of $O_2^{-\cdot}$ was used in this assay, the conversion of DMPO/OOH to DMPO/OH was possible. However, the observation of a DMPO/OH adduct in the presence of H_2O_2 confirmed that the HO· radical in the system was probably generated from the reaction between $O_2^{-\cdot}$ and H_2O_2 (Weiss-Haber reaction), which was catalyzed by metal in buffer. These results suggested that CpCpx and AcylCpCpx may catalyze the conversion of $O_2^{-\cdot}$ to dioxygen and H_2O_2. AcylCpCpx and CpCpx could decrease the EPR signal completely at 12.5 and 10 mM, respectively, while free Mn^{2+}at 30 mM could not inhibit the adduct completely. Interestingly, the DMPO/OH signals found in the presence of AcylCpCpx disappeared within 10 min after mixing, while the signals from CpCpx was much more stable (up to 20 min) (data not shown). Regarding the effects on the DMPO/OH signal, there might be additional mechanisms of AcylCpCpx in attenuating the spin adduct, which were different from those of CpCpx. This explains why AcylCpCpx had more effect on $O_2^{-\cdot}$ with kinetics of NBT reduction, but less effect than CpCpx from EPR spin-trapping, where the signals at 1 min after mixing were compared.

Effect on Hydroxyl Radical

Hydroxyl free radicals were generated by the Fenton reaction (H_2O_2/Cu^{2+}). Although it was found from UV data that curcumin and diacetylcurcumin could react with Cu^{2+}; but only a trace of Cu-cpx was found when the curcumin or Mn-cpx was added. The excess of Cu^{2+} was used in the assay so that the binding of complex and Cu^{2+} would not interfere with the assay used to generate HO·. As presented in Fig. 4A, a characteristic 1:2:2:1 pattern of DMPO/OH with $a_N = a_H = 14.82$ was obtained when H_2O_2 was added to a $CuSO_4$ solution containing DMPO and test compound.

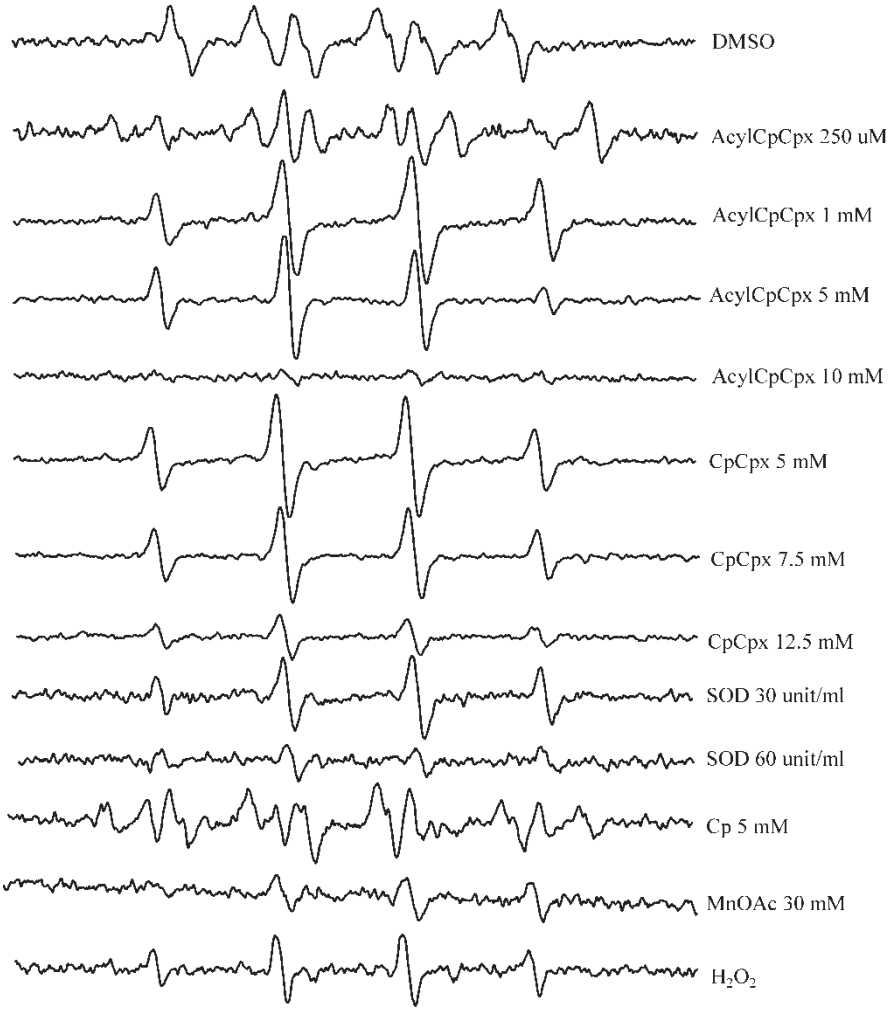


FIGURE 3 EPR signals of DMPO adduct formed from the addition of DMPO to a solution of KO_2 (~20 mM) in the presence of only vehicle (DMSO), AcylCpCpx, CpCpx, curcumin (Cp) or H_2O_2.

In this study, ethanol was used as the reference HO· scavenger, which inhibited the DMPO/OH signal with an IC_{50} of 3.01 M (Table I). In the presence of CpCpx, AcylCpCpx, curcumin or diacetylcurcumin, the intensity of DMPO/OH decreased in a dose-dependent manner as demonstrated in Fig. 4. Curcumin showed inhibitory action against DMPO/OH adduct at 0.64 mM, whereas diacetylcurcumin were less potent than curcumin with an IC_{50} of 8.34 mM, presumably because the phenolic hydroxyl groups were blocked. The manganese complexes, CpCpx and AcylCpCpx, exhibited a great capacity to inhibit the DMPO/OH adduct with an IC_{50} of 0.57 ($k = 4.2 \times 10^{11}\,M^{-1}\,s^{-1}$) and 0.37 mM ($k = 5.7 \times 10^{11}\,M^{-1}\,s^{-1}$), respectively, values comparable to that of curcumin (Table I). This data demonstrated that CpCpx and AcylCpCpx are also effective HO· scavengers as is curcumin. However, the manganese complexes have superior antioxidant properties by additionally exerting SOD action.

Stability Evaluation

The kinetics of demetallation the manganese complexes was carried out in various pH buffers. In acid, the complexes decompose via hydrolysis reaction (1).

$$MnL + 2H^+ \rightarrow Mn^{2+} + H_2L \qquad (1)$$

As demonstrated in Fig. 5, both CpCpx and AcylCpCpx gave broad spectra with a *g*-values of 2.1535 and 2.6102, respectively, while a free Mn^{2+} shows the well characterized six-line signal ($g = 2.0037$) (Fig. 5). Because the broad spectra of the complexes overlaps the first three peaks of free Mn^{2+} signal, the average peak height of the two high field peaks were compared in each experiment. It was found that both complexes were very stable in purified water (pH 6.5) up to 72 h at 25°C (Fig. 6). Both complexes, rapidly decomposed at acidic pH 2 and the dissociation of complexes was decreased in higher (basic) pH, indicating that the stability of

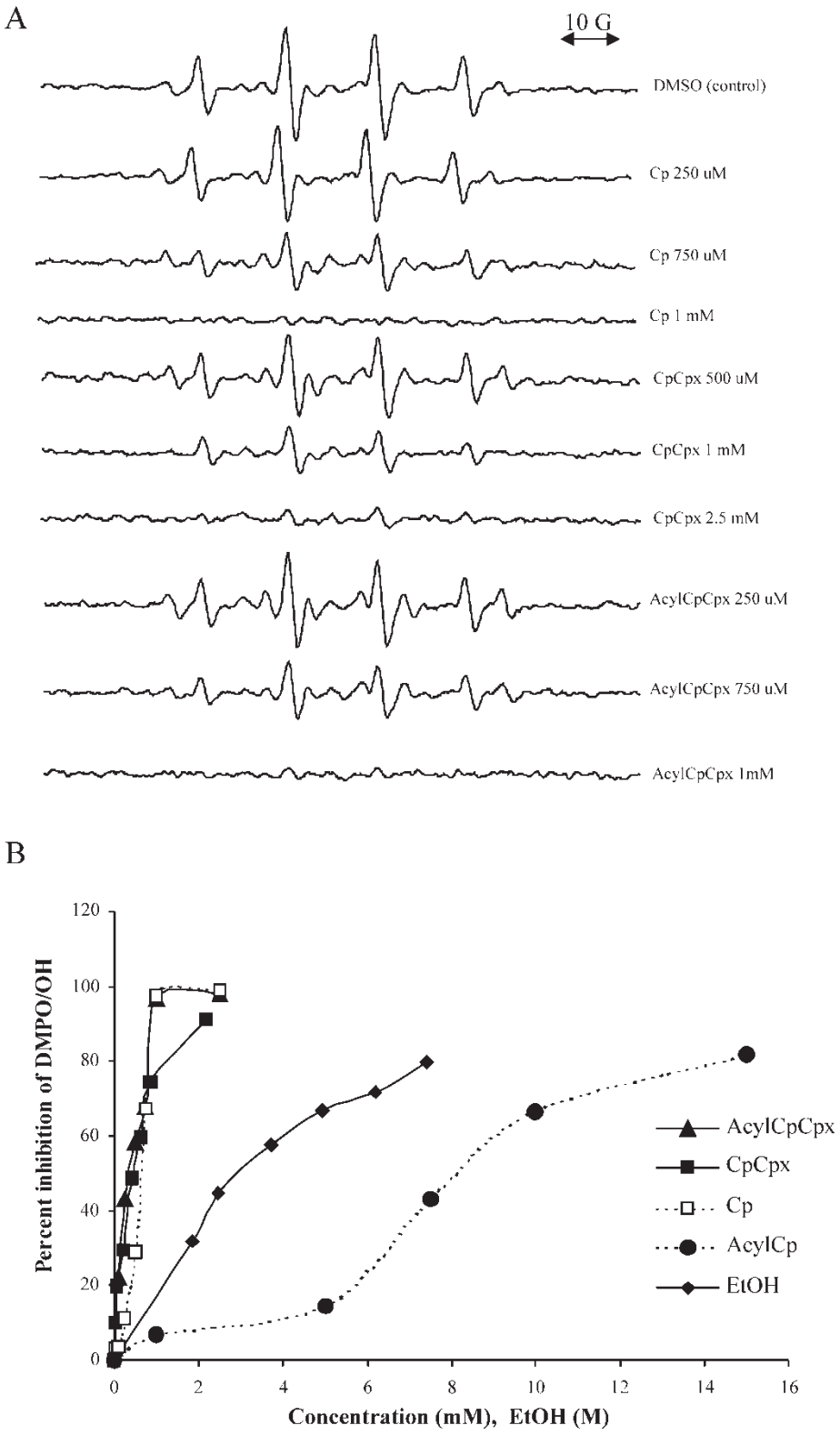


FIGURE 4 Effect of AcylCpCpx, CpCpx, curcumin (Cp), diacetylcurcumin (AcylCp) and ethanol on DMPO/OH adduct formed from hydroxyl radical generated using H_2O_2/Cu^{2+}system and DMPO. (A) EPR signal of DMPO/OH adduct in the presence of various concentrations of curcumin and complexes. (B) The percent inhibition was calculated from the peak height of EPR signal of all peaks.

CpCpx and AcylCpCpx was dependent on proton concentration. As presented in Fig. 6, the dissociation of complexes was in equilibrium by releasing of 15% free Mn^{2+} in buffer pH 8.6, and the released free Mn^{2+}, increased to 40% and 60–75% at pH 7.4 and 6, respectively. The dissociation was found to be 100% in acidic buffer (pH 2). However, more than a 40% decrease in free Mn^{2+} was obtained by further

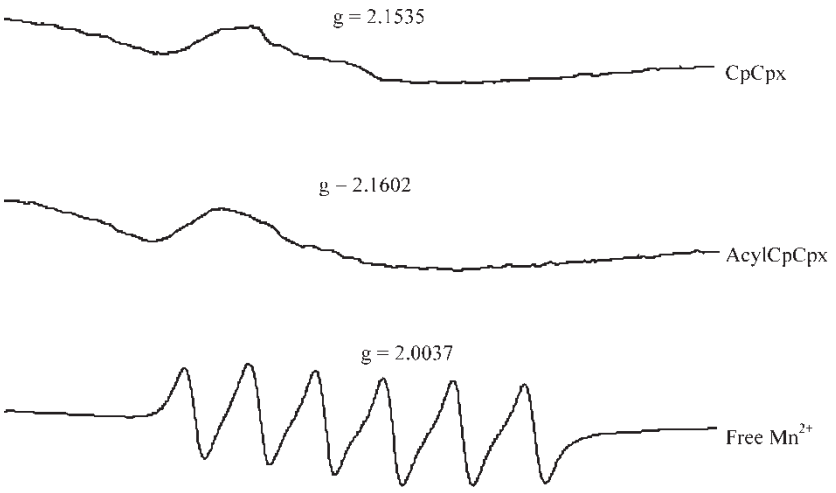


FIGURE 5 EPR spectra of 1 mM of CpCpx, AcylCpCpx and $MnCl_2$ in water contained 10% DMSO.

addition of HEPES buffer and at pH 7.4, suggesting that the 15–40% free Mn^{2+} found at pH 8.6 and 7.4 was because they were in equilibrium. CpCpx released free Mn^{2+} reaching an equilibrium constant level after 24 h in almost all buffer pH (Fig. 6A), whereas the level of free Mn^{2+} found in AcylCpCpx solution was constant after 12 h (Fig. 6B). Thus CpCpx had more stability than AcylCpCpx supporting the fact that the more nucleophilic group attached to the carbonyl carbon of the diketone would provide a more stable complex due to the stabilization of $p\pi-d\pi$ cycloconjugation.

The experiments done in human blood/serum found that the lowest concentration of free Mn^{2+} in blood/serum that could be detected by our EPR setup was about 500 μM, while that in HEPES buffer was less than 50 μM. The concentration of the complexes in this experiment needed to be higher than 3 mM. Figure 7 shows the stability of CpCpx and AcylCpCpx in human blood/serum. Free Mn^{2+} found in human serum was less than that in human blood at the beginning (before 18 h) of the experiment, indicating that the protein components in blood, which were removed in serum, might effect on the complex stability. However, the free Mn^{2+} found in human blood was comparable to that in human serum after 24 h. With both complexes, the free Mn^{2+} increased with time reaching to a maximum level at 9 h (in blood) and 18 h (in serum), and it was decreased to constant level after 24 h. This result indicated that the released free Mn^{2+} might react or form complexes with some chelates (e.g. albumin or other proteins) in human blood/serum. The dissociation of the complex was found for both CpCpx and AcylCpCpx with percentage of free Mn^{2+} released at about 10 and 20%, respectively,

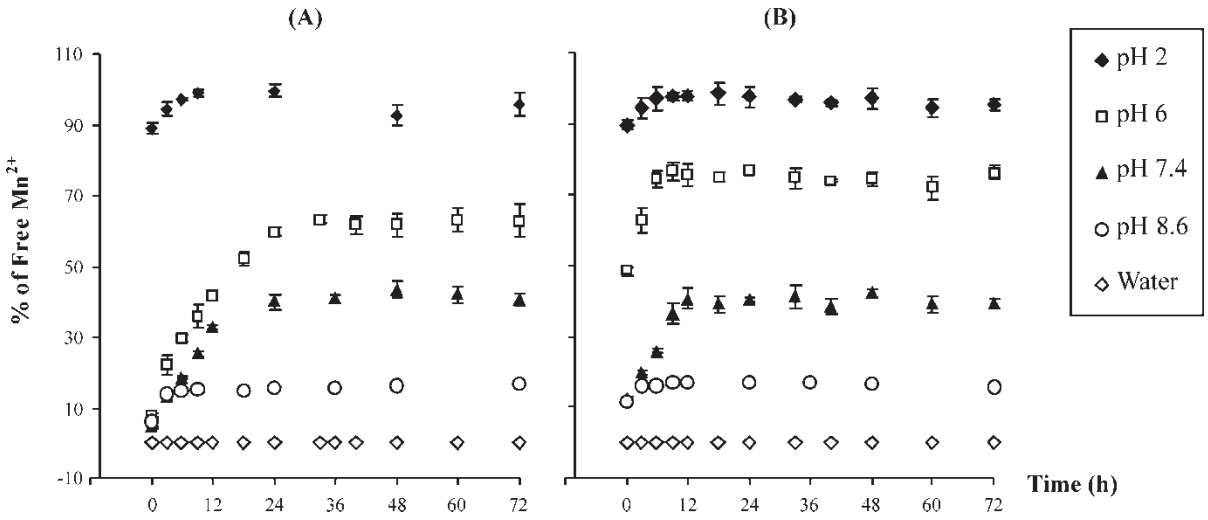


FIGURE 6 Stability of 1 mM of CpCpx (A) and AcylCpCpx (B) in water and various pH of 0.1 M buffer at 25°C. Data are expressed as mean ± SD ($n = 2–3$).

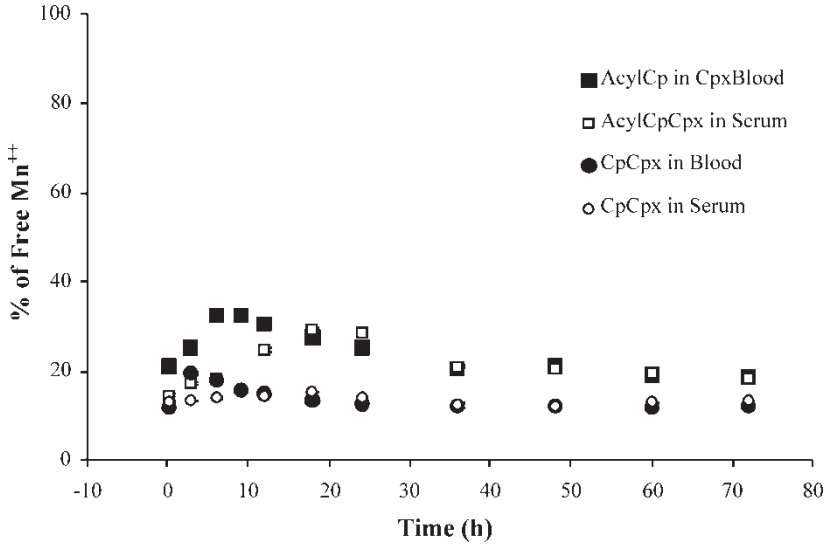


FIGURE 7 Stability of CpCpx and AcylCpCpx in human blood/serum at 25°C. Data are expressed as mean ± SD ($n = 2–3$).

showing that CpCpx had greater stability than AcylCpCpx.

CONCLUSION

From this study, we found that by incorporating manganese into the structures of these antioxidants were able to improve their activity toward superoxide anion. Both complexes, CpCpx and AcylCpCpx, may catalyze the conversion of superoxide to hydrogen peroxide and dioxygen. While the antioxidant properties of these curcumin complexes is promising, their stability may not good enough for therapeutic purposes. The data suggest that the stability is dependent on the nucleophilic substituting group at the carbonyl carbon of the diketone (or keto-enol) group. It is of interest to further develop the analogues of curcumin, which are more effective to form complex with metalloelement.

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