Antioxidative properties of recently synthesised D–*Π*–D type Schiff-base ligands

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Two recently synthesised D–π–D type Schiff base ligands have been screened for their antioxidant capacity using several tests; reducing power, chelating ability on metal ions, scavenging capacity against the radicals 2,2-diphenyl-1-picrylhydrazyl, superoxide, hydroxyl, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and N,N-dimethyl-p-phenylenediamine dihydrochloride. The results were compared with synthetic antioxidants *e.g.* butylated hydroxytoluene, butylated hydroxyanisole, α-tocopherol, trolox or ascorbic acid. The results indicate that the ligands have significant hydroxyl and free radical scavenging activity, metal chelating effect and reducing power against various antioxidant systems *in vitro*. One ligand (L_2) showed excellent activity on scavenging the ABTS radical and superoxide radical. Therefore, these ligands may be a new kind of effective scavengers of reactive oxygen species.

Keywords: Schiff base, antioxidant activity, radical scavenging ability, reducing power, metal chelating effect

Schiff bases form an interesting class of chelating ligands that has enjoyed popular use in the coordination chemistry. As is known, Schiff bases are reagents which are becoming increasingly important in the medicinal and pharmaceutical fields as well as dye, plastic and liquid crystal technology.[1] Some Schiff bases and their derivatives represent an interesting class of compounds possessing a wide spectrum of biological activities, such as analgesic, antiviral, antifungal and anticancer activities.[2–5] Schiff base ligands and their complexes have been a fascinating area of research, due to their biological relevance. These compounds can act as pro- or anti-oxidants. The published opinions on the structure and antioxidant activity relationships are, however, quite inconsistent. It can be assumed from several scientific works that the quality of antioxidant action depends on the type of ligands forming the bioactive complexes.[6]

Antioxidants are important inhibitors against oxidative damage. Antioxidant supplements are used to help the human body reduce oxidative damage from free radicals and active oxygen species.[7] Reactive oxygen species (ROS) are chemical entities that include oxygen free radicals, and they can be generated from metabolic pathways within body tissues, and they can also be introduced by external sources such as drugs, food, UV radiation and environmental pollution. In vivo, such species are securely coupled at their site of generation or are detoxified by endogenous antioxidative defences so as to preserve optimal cellular function. In pathological conditions, however, the detoxifying mechanisms are often inadequate as excessive quantities of ROS can be generated. This resulting pro-oxidant shift, a process known as oxidative stress, can result in the degradation of cellular components such as DNA, carbohydrates, polyunsaturated lipids and proteins, or precipitate enzyme inactivation, irreversible cellular dysfunction and ultimately cell death if the pro-oxidant-antioxidant balance is not restored.[7,8].

The aim of this study was to investigate the antioxidant effect of recently synthesised D–π–D type Schiff base ligands[1] and to elucidate their antioxidative action. For this purpose, the ligands (L_1 and L_2) have been screened for reducing power, chelating ability on metal ions, scavenging capacity against the radicals 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide, hydroxyl, and those derived from 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) and N,N-dimethyl-p-phenylenediamine dihydrochloride ($DMPD^{\bullet+}$). The results were compared with synthetic antioxidants *e.g.* butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), α-tocopherol, trolox or ascorbic acid.

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Experimental

6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD), and trichloroacetic acid (TCA) were obtained from Sigma (Sigma-Aldrich GmbH, Germany). 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were obtained from from Fluka (Buchs, Switzerland). All other chemicals used were of analytical grade and obtained from either Sigma-Aldrich, Fluka or Merck.

Two Schiff bases of the D–π–D type were prepared according to our previously reported procedures and characterised by comparing their spectroscopic data to those reported earlier (Scheme 1)[1]. The ligands were stable in room temperature but hygroscopic, soluble in common polar organic solvents and partially soluble in non-polar organic solvents.

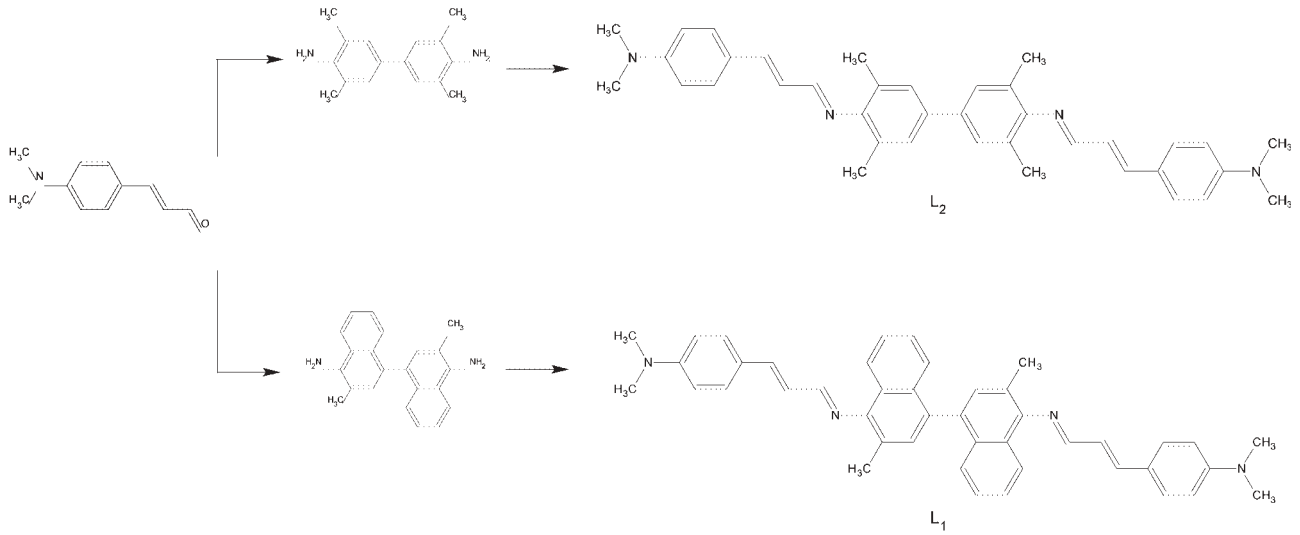
Reducing power: The reducing powers of the ligands (L_1 and L_2) were measured according to the method of Oyaizu.[9] Various amounts of the samples (10–50 μg) were mixed with phosphate buffer (2.5 mL of 0.2 M, pH 6.6) and aqueous potassium ferricyanide [$K_3Fe(CN)_6$] solution (2.5 mL of 1%, w/v), and the mixture was incubated at 50 °C for 30 min. Afterwards, aqueous trichloroacetic acid (2.5 mL of 10%, w/v) was added to the mixture which was then centrifuged at 3000 rpm for 10 min. Finally, some of the upper-layer solution (2.5 mL) was mixed with distilled water (2.5 mL) and aqueous $FeCl_3$ solution (0.5 mL of 0.1%, w/v), and the absorbance was measured at 700 nm. α-Tocopherol, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were used as standard antioxidants. The higher the absorbance of the reaction mixture the greater the reducing power.

Chelating activity towards ferrous ion: The chelating activity of the recently synthesised compounds towards ferrous ions (Fe^{2+}) was measured according to the method of Decker and Welch.[10] Aliquots (1 mL) of different concentrations of the samples were mixed with deionized water (3.7 mL). The mixture was incubated with aqueous $FeCl_2$ (0.1 mL of 2 mM) for 30 min. After incubation the reaction was initiated by addition of ferrozine (0.2 mL of 5 mM) for 10 min at room temperature and then the absorbance was measured at 562 nm. A lower absorbance indicates a higher chelating power. The chelating activity of the samples owards Fe^{2+} was compared with that of EDTA at the same concentrations. Chelating activity was calculated using the following formula:

$$\text{Chelating activity (\%)} = [1 - (\text{Absorbance of sample/Absorbance of control})] \times 100$$

The control test was performed without addition of the sample.

Free radical scavenging activity: The free radical scavenging activity of the L_1 and L_2 ligands was measured with 1,1-diphenyl-2-picrylhydrazil (DPPH•) using slightly modified methods of Brand-Williams *et al*.[11]. Briefly, DPPH• solution (20 mg L^{-1}) in methanol was prepared and some of this solution (1.5 mL) was added to the sample, BHA, BHT or α-tocopherol (0.75 mL of 10–50 μg mL^{-1}). The mixture



Scheme 1 Formation reaction of the Schiff base ligands.

was shaken vigorously and the decrease in absorbance at 517 nm was measured after 30 min. Water (0.75 mL) in place of the sample was used as control. The inhibition activity was calculated using the following equation:

$$\text{Inhibition activity (\%)} = [(A_0 - A_1)/A_0 \times 100],$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample solution.

Superoxide radical scavenging activity: Measurements of superoxide anion scavenging activity of the ligands were based on the method described by Liu *et al.*[12] Superoxide anions were generated in a non-enzymatic phenazine methosulfate–nicotinamide adenine dinucleotide (PMS–NADH) system by oxidation of NADH and assayed by reduction of nitroblue tetrazolium (NBT). In this experiment, the superoxide anion was generated in 3 mL of tris-HCl buffer (16 mM, pH 8.0) containing 1 mL of NBT (50 μM) solution, 1 mL of NADH (78 μM) solution and different concentrations (0.1–1.0 mg mL^{-1}) of sample solution. The reaction was started by adding PMS solution (1 mL, 10 μM) to the mixture. The reaction mixture was incubated at 25 °C for 5 min and the absorbance at 560 nm was recorded against blank samples. Epicatechin, BHA, trolox and ascorbic acid were used as standard samples (0.1–1.0 mg mL^{-1}). The inhibition of superoxide radical generation (%) was calculated by the following equation:

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

where A_0 is the absorbance of the control reaction and A1 is the absorbance in the presence of the extract sample or standards.

Hydroxyl radical scavenging activity: The effect on the hydroxyl radical was assayed by using the 2-deoxyribose oxidation method.[13] 2-Deoxyribose is oxidised by the hydroxyl radical that is formed by the Fenton reaction and degraded to malondialdehyde. The reaction mixture contained sodium phosphate (0.45 mL of 0.2 M, pH 7.4), 2-deoxyribose (0.15 mL of 10 mM), $FeSO_4$-EDTA (0.15 mL of 10 mM), hydrogen peroxide (0.15 mL of 10 mM), distilled water (0.525 mL) and (0.075 mL of 20–100 μg mL^{-1}) of sample solution. The reaction was started by the addition of hydrogen peroxide to the mixture. After incubation at 37 °C for 4 h, the reaction was stopped by adding (w/v) trichloroacetic acid (0.75 mL of 2.8%) and (w/v) of thiobarbituric acid (0.75 mL of 1.0%). The mixture was boiled for 10 min, cooled in ice and then the absorbance was measured at 520 nm. The reaction mixture not containing a test sample was used as control. The scavenging activity on hydroxyl radicals (HRSA) was expressed as:

$$\text{HRSA} = [(A_0 - A_1)/A_0 \times 100],$$

where A_0 is the absorbance of the control reaction and A1 is the absorbance in the presence of the sample.

$ABTS^{\bullet+}$ scavenging assay: For the $ABTS^{\bullet+}$ cation radical scavenging assay, the procedure followed the method of Arnao *et al.*[14] with some modifications. The stock solutions included ABTS solution (7.4 mM) and potassium persulfate solution (2.6 mM). The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing $ABTS^{\bullet+}$ solution (1 mL) with methanol (60 mL) to obtain an absorbance of 1.1 ± 0.02 units at 734 nm. Fresh $ABTS^{\bullet+}$ was prepared for each assay. The ligand samples, BHT, α-tocopherol or ascorbic acid (150 μL) were allowed to react with the $ABTS^{\bullet+}$ solution (2850 μL) for 2 h in the dark. Then the absorbance was measured at 734 nm using. The inhibition activity was calculated using the following equation:

$$\text{Inhibition activity (\%)} = [(A_0 - A_1)/A_0 \times 100],$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the sample solution.

$DMPD^{\bullet+}$ scavenging assay: The $DMPD^{\bullet+}$ radical scavenging ability of the ligands was measured according to the method of Fogliano *et al.*[15]. DMPD (100 mM) was prepared in deionised water and this solution (1 mL) was added to acetate buffer (100 mL of 0.1 M, pH 5.3), and the coloured radical cation ($DMPD^{\bullet+}$) was obtained by adding a solution $FeCI_3$ (0.2 mL of 0.05 M). The absorbance of this solution, which was freshly prepared daily, was constant up to 12 h at room temperature. Different concentrations of standard antioxidants or ligands were added in test tubes and the total volume was adjusted with distilled water to 0.5 mL. After 10 minutes, the absorbance was measured at 505 nm. $DMPD^{\bullet+}$ solution (1 mL) was added directly to the reaction mixture and the absorbance of the mixture was measured at 505 nm. The buffer solution was used as a blank sample. The scavenging capability of $DMPD^{\bullet+}$ radical was calculated using the following equation:

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

where A_0 is the absorbance of the initial concentration of $DMPD^{\bullet+}$ and A_1 is the absorbance of the remaining concentration of $DMPD^{\bullet+}$ in the presence of the sample solution.

Results and discussion

Reducing power: Figure 1 shows the reducing power of the L_1 and L_2 ligands. The reducing power has been used as one of the antioxidant capability indicators.[16] In the reducing power assay, the presence of reductants (antioxidants) in the tested samples results in the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). The amount of Fe^{2+} complex can therefore be monitored by measuring the formation of Perl's Prussian Blue at 700 nm.[13] Based on a comparison of the absorbance at 700 nm, the ligand L_1 showed higher reducing power than ligand L_2. The reducing power of L_2 was quite similar to α-tocopherol for all concentrations tested. However, ligand L_2 exhibited quite similar activity to BHT at lower concentration (10–30 mg mL^{-1}). These results revealed that these recently synthesised ligands were good electron and hydrogen donors and could terminate the radical chain reaction, converting free radicals into more stable products.

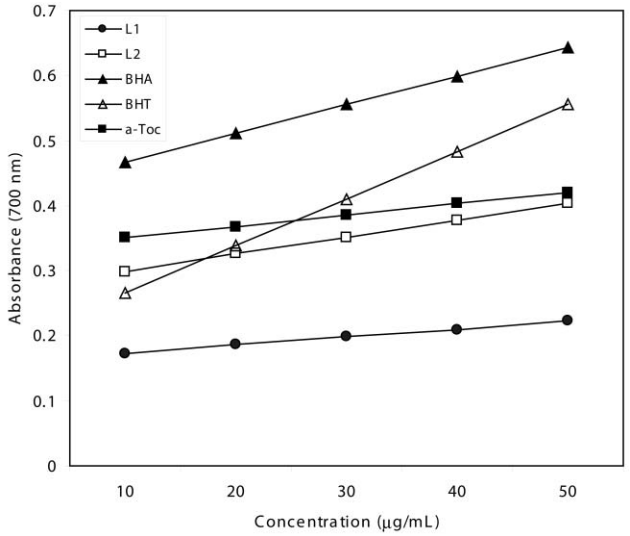


Fig. 1 Reducing power of D-π-D type Schiff base ligands L_1 and L_2.

Chelating activity towards ferrous ion: Chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilising the oxidised form of the metal ion. Iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. In particular, Fe^{2+} ion is the most powerful pro-oxidant among the various species of metal ions. The ferrous ion (Fe^{2+}) chelating effect of the ligands is presented in Figure 2. Ligands L_1 and L_2 at 20 µg mL^{-1} concentration showed 2.41% and 8.56% chelating effect respectively towards ferrous ions at an incubation time of 30 min,. The results were compared with EDTA at the same concentrations. At 20 µg mL^{-1} concentration, EDTA showed a 15.71% chelating effect towards ferrous ions at an incubation time of 30 min. The data obtained from our study reveal that the ligand L_2 has a moderate capacity for iron binding, suggesting that its action as an antioxidant might be related to its iron binding capacity.

Free radical-scavenging activity: The DPPH· radical scavenging effects of the ligands are presented in Fig. 3. 1,1-diphenyl-2-picrylhydrazil (DPPH·) is used as a free radical to evaluate the antioxidative activity of some new sources.[17] The ligand L_1 and ligand L_2 exhibited low free radical scavenging activity for all the concentrations tested. The activity was not concentration dependent. The scavenging activity of BHA, BHT and α-tocopherol, known antioxidants, were much higher than those of the samples. From these results, it can be stated that the samples tested have only a moderate ability to scavenge free radicals but could serve as a free radical inhibitors or scavengers.

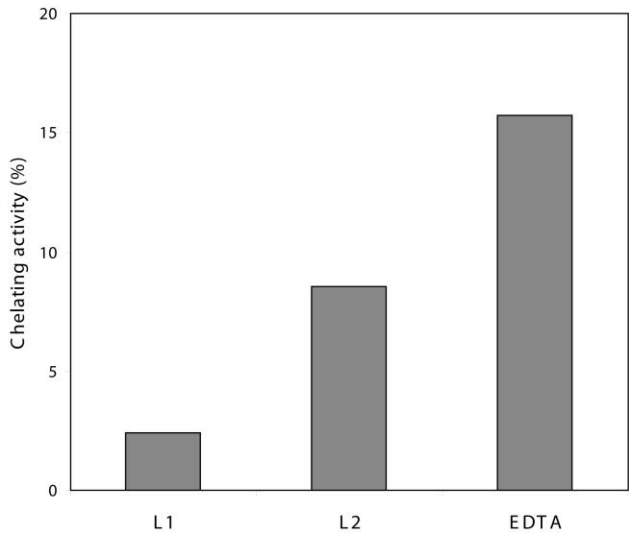


Fig. 2 Chelating effect of L_1, L_2 and EDTA at 20 µg mL^{-1} concentration for 30 min of incubation time.

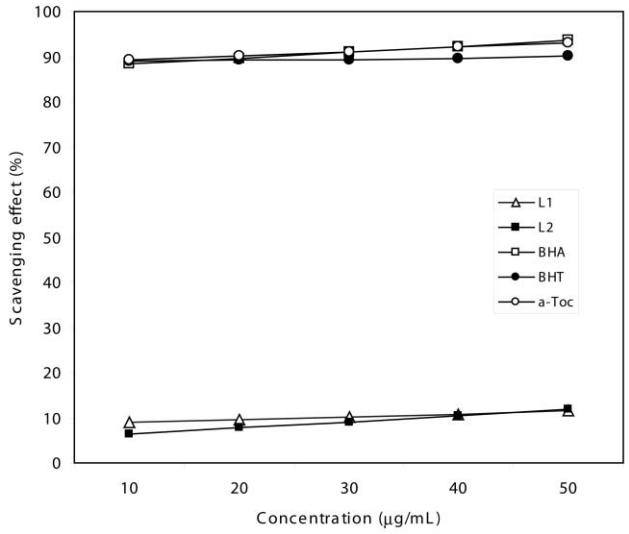


Fig. 3 Scavenging activities of D-π-D type Schiff base ligands L_1 and L_2 against the 1,1-diphenyl-2-picryl-hydrazil (DPPH.) radical.

Superoxide radical scavenging activity: The superoxide radical is known to be very harmful to cellular components as a precursor of the more reactive oxygen species, contributing to tissue damage and various diseases. In a biological system, its toxic role can be eliminated by superoxide dismutase[17]. In the PMS–NADH–NBT system, superoxide anion derived from dissolved oxygen by the PMS–NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of the superoxide anion in the reaction mixture. Figure 4 shows the superoxide radical scavenging activity by 100 µg mL^{-1} of L_1 and L_2 in comparison to the same amount of BHA, trolox, epicatechin and ascorbic acid. L_2 showed higher superoxide radical scavenging activity than BHA while it showed the similar activity when compared to trolox. The superoxide radical scavenging activities of the samples were in the following order: Ascorbic acid > epicatechin > trolox > L_2 > BHA > L_1. These results showed that L_1 and L_2 have a scavenging effect on superoxide radicals.

Hydroxyl radical scavenging activity: Among the oxygen radicals, the hydroxyl radical is the most reactive chemical species known. The hydroxyl radical induces oxidative damage to biomolecules such as all proteins, DNA, nucleic acid, and almost any biological molecule it touches and this damage causes aging, cancer and several diseases[18] Figure 5 shows the hydroxyl radical scavenging effects determined

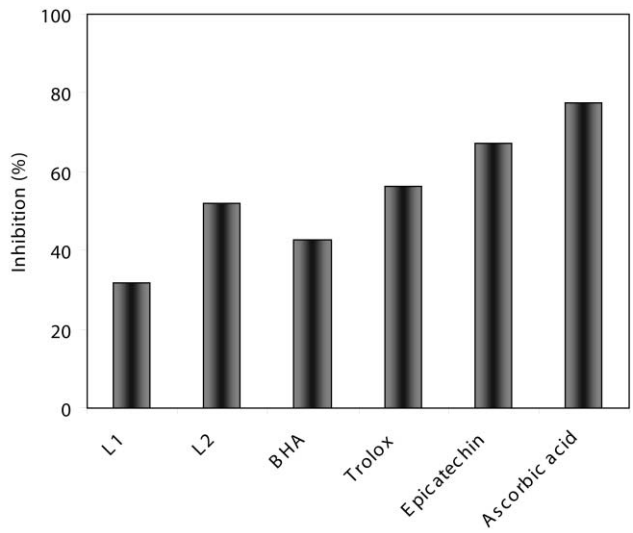


Fig. 4 Superoxide radical scavenging activity of L_1, L_2, BHA, trolox, epicatechin and ascorbic acid at 100 µg mL^{-1} concentration.

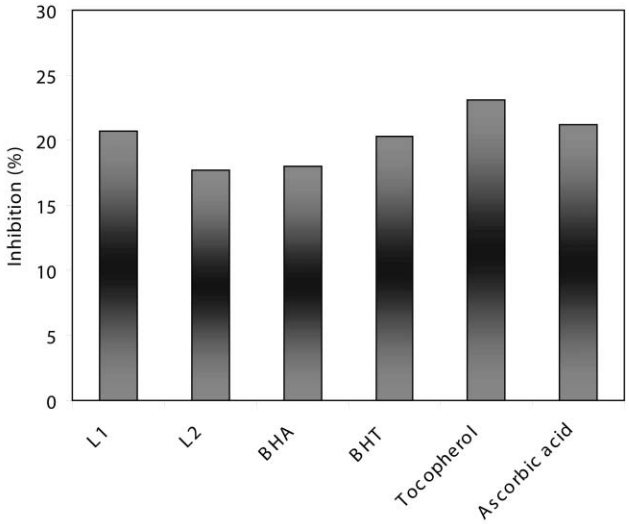


Fig. 5 Hydroxyl radical scavenging activity of L_1, L_2, BHA, BHT, α-tocopherol and ascorbic acid at 20 μg mL^{-1} concentration.

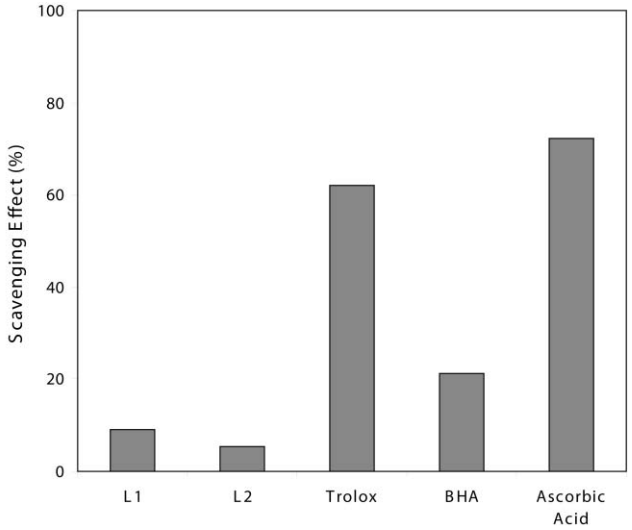


Fig. 7 DMPD radical -scavenging activities of D–π–D type Schiff base ligands L_1, L_2, BHA, trolox and ascorbic acid at 20 μg mL^{-1} concentration.

by the 2-deoxyribose oxidation method. At 20 μg mL^{-1} concentration ligands L_1 and L_2 exhibited similar hydroxyl radical scavenging activity to BHT and BHA, respectively. The hydroxyl radical scavenging activity of those samples were in the following order: α-Tocopherol > ascorbic acid > BHT = L_1 > BHA = L_2.

$ABTS^{\bullet+}$ scavenging assay: One of the most commonly used organic radicals for the evaluation of antioxidant efficiency of pure compounds and complex mixtures is the radical cation derived from 2,2′-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$). These radical cations can be generated by enzymatic, chemical, and electrochemical means. The extent and the rapidity with which polyphenols quench the $ABTS^{\bullet+}$ radical cation chromophores are the criteria used to assess their relative antioxidant capacity as compared with other standard antioxidants. In these $ABTS^{\bullet+}$ -based methods it is assumed that the antioxidants simply reduce the radicals back to the parent compound, ABTS.[19] The antioxidant activity measurements of ligands, expressed as scavenging inhibition %, are presented in Fig. 6. L_1 ligand exhibited moderately activity for all concentrations and activity was not concentration dependent. However, L_2 ligand showed higher activity than L_1 and similar activity to α-tocopherol.

$DMPD^{\bullet+}$ scavenging assay: As shown in Fig. 7, the ligands were moderately effective DMPD radical scavengers in all concentrations tested. The scavenging ability of ligands was not concentration dependent. The scavenging effect of L_1 and L_2 and standards on

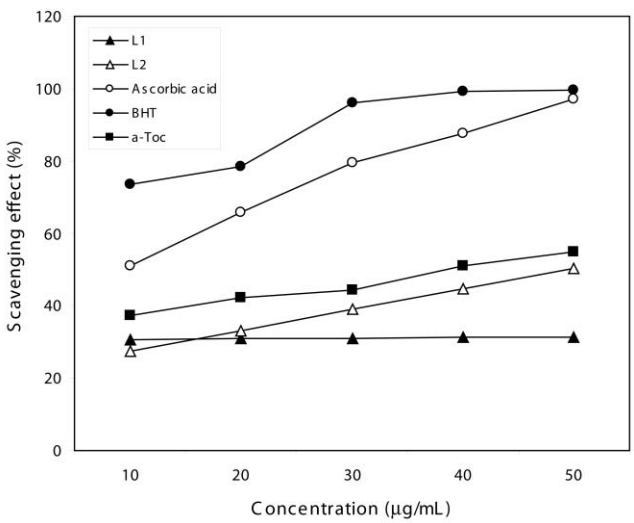


Fig. 6 ABTS radical -scavenging activities of D–π–D type Schiff base ligands L_1 and L_2.

$DMPD^{\bullet+}$ decreased in the order: ascorbic acid > trolox > BHA > L_2 > L_1, which was at the concentration of 20 μg mL^{-1}, respectively.

Conclusions

This study showed that the recently synthesised D–π–D type ligands L_1 and L_2 exhibited different levels of antioxidant activity in all tests. The results indicate that the ligands have a significant hydroxyl and free radical scavenging activity, metal chelating effect and reducing power against various antioxidant systems in vitro. Ligand L_2 showed excellent activity on scavenging the ABTS radical and the superoxide radical. Therefore, these ligands may be a new kind of effective scavenger of ROS.

Received 4 May 2010; accepted 16 May 2010
Paper 1000102 *doi: 10.3184/030823410X12759843433884*
Published online: 2 July 2010

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