CHAPTER 5

ANTIOXIDANT ACTIVITIES

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5.1 Introduction

Free radical oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders, such as cancer, cardiovascular disease, Alzheimers, autoimmune disease, diabetes, multiple sclerosis and arthritis [83]. Free radicals are highly reactive particles with unpaired electron(s) and are produced by radiation or as by-products of metabolic processes. These particles initiate chain reactions which lead to disintegration of cell membranes and cell compounds, including lipids, proteins, and nucleic acids [84].

Reactive oxygen species (ROS) can be classified into two groups: those that contain unpaired electrons (examples, O and OH) and hence are free radicals, and those that have the ability to remove electrons from other molecules (examples, H_2O_2 and HOCl). Biological systems protect themselves against the damaging effects of activated species by several means. These include free-radical scavengers and chain reaction terminators (enzymes such as SOD and CAT system) [85]. If human disease is believed to be due to the imbalance between oxidative stress and antioxidative defense, it is possible to limit oxidative tissue damage and hence prevent disease progression by antioxidant defence supplements [86]. In other words, if the balance sways in the direction of pro-oxidants, oxidative stress can arise, which under normal circumstances is controlled by a broad range of antioxidant enzymes, proteins and antioxidants provided by the diet. The protection offered by fruits and vegetables against oxidative stress in several diseases has been attributed to various antioxidants and vitamins. Dietary phenolic compounds and flavonoids have generally been considered, as non-nutrients and their possible beneficial effect on human health have only recently been recognized. Theses compounds are known to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, neuroprotective, and anticarcinogenic activities [87].

Therefore, the search for natural or synthesized antioxidants has gained momentum in recent years. Phenolic compounds may contribute directly to the antioxidant action due to the presence of hydroxyl functional groups around the nuclear structure that are potent hydrogen donators. The phenolic compounds of plant origin show their antioxidant effect by various mechanisms, including their ability to scavenge free radicals, chelate metal ions that serve as the catalysts for production of free radicals or activate various antioxidant enzymes and inhibit oxidases [88].

Different classes of organic compounds containing rich conjugated system can be observed when these compounds contain hydroxyl groups attached to aromatic rings. Examples are compounds containing an azomethine group (-CH=N-), known as Schiff bases, formed by the condensation of primary amines with carbonyl compounds. Conjugated Schiff bases obtained from aromatic aldehydes and aromatic amines are quite stable. These bases are important as they have wide range of biological activities and industrial applications. For example, they have been shown to posses the pharmacological activities such as anticancer [89-91], antimicrobial [92-94], antifungal [95], antiviral [96-98], anti-inflammatory [99,100], antiparasitic [101], and antioxidants [102]. These Schiff bases also serve as a back bone for the synthesis of various heterocyclic compounds. It seems that the presence of azomethine group is responsible for biological activities expressed by different types of Schiff bases. However, these biological activities can be tuned, depending upon the types of substituents attached to the aromatic rings. Hence, the present work is focussed on the synthesis of different Schiff bases containing hydroxyl groups attached to the aromatic rings, and to screen these Schiff bases for their antioxidant activities using 1,1-diphenyl picrylhydrazyl (DPPH), and ferric reducing ability power (FRAP) method.

5.2 The antioxidant activities

The antioxidant activities of the synthesized Schiff bases and complexes were measured in vitro using two methods: (a) the DPPH free radical scavenging assay; and (b) the ferric reducing ability power (FRAP). All assays were carried out in triplicate and the average value was obtained. All determinations were made spectrophotometrically using the Infinite® 200 PRO plate reader (TECAN, Männedorf, Switzerland).

5.2.1 Free radical scavenging activity using DPPH method

(a) Method

Free radical scavenging activity of the test compounds was determined by DPPH assay method [103]. The concentration of each Schiff base was 1 mg/ml, using methanol as the solvent. For complexes, DMSO was used instead of methanol. These stock solutions were then diluted to 5, 10, 25, 50, and 100 μg/ml in the same solvent. Ascorbic acid (Vit. C) solution was prepared in a similar way and used as a positive control. Then, 200 μl of each sample solution or positive control were combined with 50 μl of DPPH (0.3 mml). The final concentrations of the Schiff bases and the complexes were 4, 8, 20, 40, and 80 μg/ml. The microtitre plates were incubated for 30 min at room temperature. The absorbance was then determined at 518 nm with the Infinite® 200 PRO plate reader (TECAN, Männedorf, Switzerland). The percentage inhibition was calculated according to the equation:

$$\% \text{ Inhibition} = \{(\text{Abs. blank} - \text{Abs. sample})/ \text{Abs. blank}\} \times 100$$

(b) Results and Discussion

The absorbance and percentage DPPH quenched are shown in **Table 5.1** and **Table 5.2** respectively.

Table 5.1 The absorbance (Abs.) values at 518 nm at difference concentrations

Compound	**Concentration (µg/ml)**				
	4	**8**	**20**	**40**	**80**
Blank	0.238	0.238	0.238	0.238	0.238
H_2L1	0.220	0.210	0.202	0.198	0.183
H_2L2	0.224	0.214	0.207	0.199	0.190
H_2L3	0.223	0.221	0.219	0.204	0.199
H_2L4	0.187	0.115	0.025	0.021	0.020
H_2L5	0.192	0.129	0.051	0.032	0.029
H_2L6	0.214	0.211	0.208	0.204	0.201
NiL1	0.228	0.227	0.226	0.225	0.224
NiL2	0.224	0.221	0.219	0.219	0.216
NiL3	0.222	0.221	0.220	0.217	0.216
NiL4	0.219	0.215	0.183	0.121	0.052
NiL5	0.223	0.217	0.187	0.131	0.075
NiL6	0.224	0.221	0.221	0.219	0.217
CuL1	0.236	0.236	0.233	0.232	0.231
CuL2	0.234	0.233	0.232	0.232	0.229
CuL3	0.232	0.231	0.230	0.228	0.228
CuL4	0.209	0.205	0.202	0.201	0.201
CuL5	0.212	0.210	0.210	0.209	0.207
CuL6	0.233	0.233	0.231	0.228	0.226
ZnL1	0.231	0.229	0.229	0.229	0.228
ZnL2	0.233	0.231	0.231	0.230	0.228
ZnL3	0.232	0.229	0.228	0.227	0.226
ZnL4	0.223	0.220	0.219	0.212	0.202
ZnL5	0.226	0.222	0.220	0.217	0.208
ZnL6	0.233	0.232	0.231	0.231	0.230
Vit. C	0.184	0.112	0.023	0.019	0.019

Table 5.2 Free radical scavenging activity of new Schiff bases and the complexes using DPPH method (% inhibition)

Compound	Concentration (µg/ml)				
	4	**8**	**20**	**40**	**80**
H_2L1	7.5	11.8	15.1	16.8	23.1
H_2L2	5.9	10.1	13.0	16.4	20.2
H_2L3	6.3	7.1	8.0	14.3	16.4
H_2L4	21.4	51.7	89.5	91.2	91.6
H_2L5	19.3	45.8	78.6	86.6	87.8
H_2L6	10.1	11.3	12.6	14.3	15.5
NiL1	4.2	4.6	5.0	5.5	5.9
NiL2	5.9	7.1	8.0	8.0	9.2
NiL3	6.7	7.1	7.6	8.8	9.2
NiL4	8.0	9.7	23.1	49.2	78.2
NiL5	6.3	8.8	21.4	45.0	68.5
NiL6	5.9	7.1	7.1	8.0	8.8
CuL1	0.8	0.8	2.1	2.5	2.9
CuL2	1.7	2.1	2.5	2.5	3.8
CuL3	2.5	2.9	3.4	4.2	4.2
CuL4	12.2	13.8	15.1	15.5	15.5
CuL5	10.9	11.8	11.8	12.2	13.0
CuL6	2.1	2.1	2.9	4.2	5.0
ZnL1	2.9	3.8	3.8	3.9	4.2
ZnL2	2.1	2.7	2.9	3.4	4.2
ZnL3	2.5	3.8	4.2	4.6	5.0
ZnL4	6.3	7.6	8.0	10.9	15.1
ZnL5	5.0	6.7	7.6	8.8	12.6
ZnL6	2.1	2.5	2.9	2.9	3.4
Vit. C	22.7	52.9	90.3	92.0	92.0

The value obtained from Vit. C in this work (for example, 90.4% at 20 µg/ml) is in agreement with those reported in the literature (89% at 20 µg/ml) [103]. This supports the validity of the results obtained in this work.

(i) H_2L1-H_2L6

The Schiff bases H_2L1, H_2L2, H_2L3, and H_2L6 have lower antioxidant activity, while H_2L4 and H_2L5 have similar properties compared to Vit. C, at all concentrations studied. For the latter two complexes, this may be due to a combination of two factors: (a) increase solublitiy in methanol due to the presence of additional –OH group, and (b) the phenoxy free-radicals formed as a result of H atoms (H•) abstracted from two –OH groups by DPPH• to form DPPH-H have higher stability due to increased conjugation with the aromatic ring. The proposed mechanism may be similar to that which occurs in hydroxy chalcones and hydroxy flavonoids derivatives [105,106].

It is also noted that the antioxidant activity of all of the Schiff bases increased with concentrations.

(ii) NiL1-NiL6

All nickel(II) complexes have lower antioxidant activity compared to the corresponding Schiff bases at all concentrations. This is as expected due to deprotonation of an –OH group in forming the complexes.

As similarly observed for the Schiff bases, NiL4 and NiL5 have higher antioxidant activity compared to the other complexes. This is because these two complexes still have an OH group *ortho*- and *meta*- to nickel(II) centres respectively, and hence were able to form stable free radicals after H atom abstraction by DPPH radical. Also, nickel(II) may be oxidised to nickel(III), and hence helped to reduced DPPH.

It is further noted that NiL4 and NiL5 have similar antioxidant activity at all concentrations studied. From this, it may be concluded that the different position of the –OH group has insignificant effect on these properties.

(iii) CuL1-CuL6

All copper(II) complexes have lower antioxidant activity compared to the corresponding Schiff bases and the nickel(II) complexes at all concentrations. This is probably because copper(II) is easily reduced to copper(I), and hence the complexes were less able to reduce DPPH.

(iv) ZnL1-ZnL6

All zinc(II) complexes have lower antioxidant activity compared to the corresponding Schiff bases and the nickel(II) complexes, and similar to the copper(II) complexes, at all concentrations. This is probably because zinc(II) cannot be easily reduced or oxidised. The results are consistent with the above explanation for the Schiff bases, nickel(II) complexes and copper(II) complexes.

5.2.2 Total reducing power using FRAP method

(a) Method

The total reducing power method was performed as previously described [104], but with modifications. This method is based on a redox reaction in which the antioxidants act as reductants, and an easily reduced oxidant (Fe^{3+}) is used in stoichiometric excess, resulting in a blue Fe^{2+} complex. The absorbance was then determined spectrophotometrically at 593 nm with the Infinite® 200 PRO plate reader (TECAN, Männedorf, Switzerland). For this, a Fe^{3+}–TPTZ complex solution (FRAP reagent) was freshly prepared by mixing an acetate buffer (300 mmol l^{-1}, pH 3.6), tripyridyl triazine, TPTZ (10 mmol L^{-1} in 1.0 mol L^{-1} HCl), and $FeCl_3$ (20 mmol L^{-1} in H_2O at 10:1:1 (v/v/v). Then, 200 µl of FRAP reagent solution was combined with 50 µl of Schiff base in methanol or complex in DMSO. The mixtures were shaken and incubated at 37 ºC for 30 min before absorbance reading at 593 nm. All treatments were run in triplicate. Ascorbic acid (Vit. C) were used as positive controls. The potential of the compounds as antioxidants to reduce Fe^{3+} to Fe^{2+} was expressed as Vit.C equivalent (or in µmol Fe^{2+} g^{-1} of the compound using a calibration curve of $FeSO_4 \bullet 7H_2O$ (20, 40, 60, 80 µg/ml).

The FRAP value was calculated as Fe^{2+} equivalent obtained by comparing the absorbance change in the test reaction mixture with those containing known concentrations of Fe^{2+} ion (standard). It was assumed that the higher measured FRAP value, the higher is the antioxidant activity of the compound that could reduce Fe^{3+} to Fe^{2+}[107,108].

(b) Results and discussion

The absorbance and the total reducing power using FRAP method are shown in **Table 5.3** and **Table 5.4** respectively.

Table 5.3 The absorbance (Abs.) values at 593 nm at difference concentrations

Compound	**Concentration (µg/ml)**				
	4	**8**	**20**	**40**	**80**
Blank	0.238	0.238	0.238	0.238	0.238
H_2L1	0.225	0.225	0.222	0.222	0.219
H_2L2	0.224	0.223	0.221	0.220	0.216
H_2L3	0.219	0.219	0.218	0.215	0.214
H_2L4	0.189	0.125	0.045	0.031	0.026
H_2L5	0.192	0.138	0.051	0.040	0.032
H_2L6	0.220	0.217	0.216	0.215	0.214
NiL1	0.226	0.225	0.223	0.220	0.218
NiL2	0.222	0.221	0.220	0.219	0.217
NiL3	0.224	0.223	0.220	0.220	0.216
NiL4	0.213	0.208	0.192	0.123	0.051
NiL5	0.222	0.217	0.190	0.125	0.075
NiL6	0.221	0.220	0.219	0.218	0.215
CuL1	0.225	0.224	0.222	0.220	0.219
CuL2	0.228	0.224	0.223	0.222	0.221
CuL3	0.225	0.222	0.221	0.218	0.215
CuL4	0.209	0.205	0.203	0.202	0.201
CuL5	0.212	0.211	0.209	0.208	0.206
CuL6	0.227	0.226	0.225	0.223	0.220
ZnL1	0.233	0.231	0.230	0.230	0.229
ZnL2	0.232	0.231	0.230	0.229	0.229
ZnL3	0.231	0.229	0.227	0.226	0.226
ZnL4	0.227	0.225	0.225	0.223	0.222
ZnL5	0.226	0.225	0.224	0.223	0.221
ZnL6	0.233	0.232	0.231	0.230	0.229
Vit. C	0.184	0.112	0.023	0.019	0.019

Table 5.4 Total Reducing Power of new Schiff bases and the complexes using FRAP method

Compound	Concentration (µg/ml)				
	4	**8**	**20**	**40**	**80**
H_2L1	5.5	5.5	6.7	6.7	8.0
H_2L2	5.9	6.3	7.1	7.6	9.2
H_2L3	8.0	8.0	8.4	9.7	10.1
H_2L4	20.6	47.5	81.1	86.9	89.1
H_2L5	19.3	42.0	78.6	83.2	86.6
H_2L6	7.6	8.8	9.2	9.7	10.1
NiL1	5.1	5.5	6.3	7.6	8.4
NiL2	6.7	7.1	7.6	8.0	8.8
NiL3	5.9	6.3	7.6	7.6	9.2
NiL4	10.5	12.6	19.3	48.3	78.5
NiL5	7.6	8.8	20.2	47.5	68.5
NiL6	7.1	7.6	8.0	8.4	9.7
CuL1	5.5	5.9	6.7	7.6	8.0
CuL2	4.2	5.9	6.3	6.7	7.1
CuL3	5.5	6.7	7.1	8.4	9.7
CuL4	12.2	13.9	14.7	15.1	15.5
CuL5	10.9	11.3	12.2	12.6	13.4
CuL6	4.6	5.0	5.5	6.3	7.6
ZnL1	2.1	2.9	3.4	3.4	3.8
ZnL2	2.5	2.9	3.4	3.8	3.8
ZnL3	2.9	3.8	4.6	5.0	5.0
ZnL4	4.6	5.5	5.5	6.3	7.1
ZnL5	5.0	5.5	5.9	6.3	7.1
ZnL6	2.1	2.5	2.9	3.4	3.8
Vit. C	22.7	52.9	90.3	92.0	92.0

The value obtained from Vit. C in this work (for example, 90.4% at 20 µg/ml) is in agreement with those reported in the literature (89% at 20 µg/ml) [103]. This supports the validity of the results obtained in this work.

The FRAP results (Table 5.4) show similar antioxidant activities for all Schiff bases and complexes as were found for DPPH results (Table 5.2), and may be similarly explained.

5.3 Summary

To summarise, all of the Schiff bases studied have higher antioxidant activities compared to the corresponding complexes. The Schiff bases with the highest antioxidant activities are H_2L4 and H_2L5, while the complexes with the highest antioxidant activities are NiL4 and NiL5. The antioxidant activities increase with concentration of the compounds.