RESEARCH ARTICLE

Ruthenium(III) Thiosemicarbazone Complexes: Synthesis, Characterization, DNA Binding, Antibacterial, In vitro Anticancer and Antioxidant Studies

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ABSTRACT

Ruthenium(III) complexes of the type [RuBr2(PPh3)2L] (where L = monobasic bidentate thiosemicarbazone ligand) have been synthesized. Various physico-chemical and spectral techniques were used to determine the structural features of the complexes. Absorption spectroscopy was used to investigate DNA binding of the complexes. It indicated that the complexes bind to DNA through intercalation. The complexes have shown significant antibacterial activity than the ligands against a panel of bacteria. The efficiency of the complexes to arrest the growth of MCF-7 cancer cell line has been studied. This was done along with the cell viability test under in vitro conditions. Finally, antioxidative property of the complexes against DPPH radical was investigated.

Keywords: Ruthenium complexes, Methyl thiosemicarbazones, Antioxidant, MCF-7, DPPH radical.

1. INTRODUCTION

Metal complexes remain an important resource for the generation of chemical diversity in the search for novel therapeutic and diagnostic agents, especially in the arena of anticancer drug development. [1] The important characteristic of the complexes is lipophilicity and penetration through the lipid membrane. Also, the stereochemistry of the complexes allow a favourable tridimensional interaction with biomolecules and a high kinetic and thermodynamic stability. Moreover, the substitution in the ligands can permit the recognition by living organisms. [2] It has been demonstrated that free radicals can damage proteins, lipids and DNA of biotissues, leading to increased rates of cancer. [3] Fortunately, antioxidants can prevent this damage, due to their free radical scavenging activity. [4] Hence, it is very important to develop compounds with both strong antioxidant and DNA-binding properties for effective cancer therapy. Ruthenium complexes offer the potential of reduced toxicity, a novel mechanism of action, non-cross resistance, significant radical scavenging activity and a different spectrum of activity compared to platinum containing compounds. [5] In addition, non-nuclear targets have also been implicated in the antineoplastic activity of ruthenium complexes, particularly in the case of the clinically investigated ruthenium(III) antimitastatic drug, NAMI-A. [6] Thiosemicarbazones form an important class of compounds because of their promising pharmacological properties, such as trypanocidal activity, anti-tubercular activity, anti-oxidative activity and anti-tumor activities. [7, 8] Moreover, some thiosemicarbazones have shown to increase their biological activity by their ability to form chelates with specific metal ions. [9] In the case of pharmaceuticals, the binding capacity of thiosemicarbazones is further increased by

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condensation of the thiosemicarbazide with an aldehyde containing heteroatom. [10, 11] With this background in mind, we herein report on the synthesis and characterization of ruthenium(III) complexes of 2-chloro/nitro benzaldehyde 4-methyl-3-thiosemicarbazones. The investigation of the biological properties of the complexes focused on the binding properties with CT-DNA and was performed by UV spectroscopy. The in vitro cytotoxicity studies against a panel of bacteria and MCF-7 cancer cell line and anti-oxidative property against DPPH radicals were investigated.

2. EXPERIMENTAL

2.1. Materials and methods

The AR grade chemicals were used and are chemically pure. Solvents were purified and dried according to the standard procedure. [12] Calf-Thymus (CT-DNA) was purchased from Bangalore Genei, Bangalore, India. The metal precursor, [RuBr3(PPh3)3] and thiosemicarbazone ligands, HL1 and HL2, were prepared by literature methods. [13, 14] The C, H, N and S analysis were performed on a Vario EL III CHNS analyser at STIC, Cochin University of Science and Technology, Kerala, India. IR spectra were recorded as KBr pellets in the 400-4000 cm⁻¹ region using a Perkin Elmer FT-IR 8000 spectrophotometer. Electronic spectra were recorded in DMSO solution with a Systronics double beam UV-vis spectrophotometer 2202 in the range 200-800 nm. Magnetic susceptibility measurements of the complexes were recorded using Guoy balance. EPR spectra were recorded on a varian E-112 ESR spectrophotometer at X-band microwave frequencies for powdered samples at room temperature. EI mass spectra of the complexes were recorded on a JOEL GCMATE II mass spectrometer. Melting points were recorded with Veego VMP-DS heating table and are uncorrected.

2.2. Synthesis of ruthenium(III) thiosemicarbazone complexes

The thiosemicarbazone ligands (0.110-0.138 g, 0.5 mmol) was dissolved in methanol (20 mL) and are added to [RuBr3(PPh3)3] (0.520 g, 0.5 mmol) in benzene (20 mL). Then the solutions were refluxed for 8 h and the reaction mixture was then cooled to room temperature. The formed precipitate was filtered off and the purity of the complexes was checked by TLC. This solid was recrystallized from CH2Cl2/Hexane mixture. Our sincere effort to obtain single crystal of the complexes went unsuccessful.

[RuBr3(PPh3)L1] (1): Yield: 57 %. M.P: 268°C. Anal. calcd. for C45H35Br3ClIN2P2RuS (%): C, 53.45; H, 4.00; N, 4.22; S, 3.25. Found (%): C, 53.40; H, 3.88; N, 4.15; S, 3.17. EI-MS: Found m/z = 1012.48 (M+) (calculated m/z = 1012.15 for M+). IR (KBr, cm⁻¹): 1578 ν(C=N); 745 ν(C-S); 540 ν(Ru–N); 1433 ν(Ru–triphenylphosphine). UV-vis (DMSO), λmax (nm): 369 (ILCT), 413 (LMCT). EPR (300 K, ‘g’ value): 2.04, μ eff (300 K): 1.71 μB.  

[RuBr3(PPh3)L2] (2): Yield: 59 %. M.P: 295°C. Anal. calcd. for C45H35Br3N2O2P2RuS (%): C, 60.30; H, 4.15; N, 5.63; S, 3.22. Found (%): C, 52.94; H, 3.75; N, 5.55; S, 3.12. EI-MS: Found m/z = 1022.65 (M+) (calculated m/z = 1022.71 for M+). IR (KBr, cm⁻¹): 1521 ν(C=N); 741 ν(C-S); 523 ν(Ru–N); 1434 ν(Ru–triphenylphosphine). UV-vis (DMSO), λmax (nm): 321, 368, 407 (ILCT), 434 (LMCT). EPR (300 K, ‘g’ value): 2.17, μ eff (300 K): 1.70 μB.

2.3. DNA binding - Titration experiments

All the experiments involving the binding of complexes with CT-DNA were carried out in a doubly distilled water buffer with tris(hydroxymethyl)-aminomethane (Tris, 5 mM) and sodium chloride (50 mM) and adjusted to pH 7.2 with hydrochloric acid. A solution of CT-DNA in the buffer gave a ratio of UV absorbance of about 1.8-1.9 at 260 and 280 nm, indicating that the DNA was sufficiently free of protein. The CT-DNA concentration per nucleotide was determined spectrophotometrically by employing an extinction coefficient of 6600 M⁻¹ cm⁻¹ at 260 nm. The complexes were dissolved in a mixed solvent of 5 % DMSO and 95 % Tris-HCl buffer. Stock solutions were stored at 4°C and used within 4 days. Absorption titration experiments were performed with fixed concentrations of the complexes (25 µM) with varying concentration of DNA (0-50 µM). While measuring the absorption spectra, an equal amount of DNA was added to both the
test solution and the reference solution to eliminate the absorbance of DNA itself.

3. CYTOTOXICITY STUDIES

3.1. Antibacterial activity

The in vitro antibacterial screenings of the free ligands and its complexes were tested for their effect on certain human pathogenic bacteria by disc diffusion method. The ligands and complexes were stored dry at room temperature and dissolved in dimethyl sulfoxide. The bacteria (Escherichia coli, Staphylococcus aureus and Klebsiella pneumonia) were grown in nutrient agar medium and incubated at 37°C for 24 h followed by frequent subculture to fresh medium and were used as test bacteria. Then the petriplates were inoculated with a loop full of bacterial culture and spread throughout the petriplates uniformly with a sterile glass spreader. To each disc the test samples and reference antibiotic (Cotrimazole) were added with a sterile micropipette. The plates were then incubated at 35°C for 24 h and at 27°C for bacteria and fungus, respectively. Plates with disc containing respective solvents served as control. Inhibition was recorded by measuring the diameter of the inhibitory zone after the period of incubation.

3.2. In vitro anticancer activity

Cytotoxicity of the complexes was carried out on human breast cancer cell line (MCF-7). Cell viability was carried out using the MTT assay method. [15] The MCF-7 cells were grown in eagles minimum essential medium (EMEM) containing 10 % fetal bovine serum (FBS). For the screening experiment, the cells were seeded onto 96-well plates at a plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO₂, 95% air and 100% relative humidity for 24 h prior to the addition of the complexes. The complexes were dissolved in DMSO and diluted in the respective medium containing 1 % FBS. After 24 h the medium was replaced with the respective medium with 1% FBS containing the complexes at various concentrations and incubated at 37°C under conditions of 5% CO₂, 95% air and 100% relative humidity for 48 h. Triplication was maintained and the medium not containing the complexes served as control. After 48 h, 15 µL of MTT (5 mg mL⁻¹) in phosphate buffered saline (PBS) was added to each well and incubated at 37 °C for 4 h. The medium with MTT was then removed and the formed formazan crystals were dissolved in 100 µL of DMSO. The absorbance was then measured at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula and a graph was plotted with the percentage of cell inhibition versus concentration. From this, the IC₅₀ was calculated: % cell Inhibition = [mean OD of untreated cells (control)/mean OD of treated cells (control)] x100.

3.3. Antioxidant activity

The 2,2-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity of the complexes was measured according to the method of Elizabeth. [16] The DPPH radical is a stable free radical having a λₘₐₓ at 517 nm. A fixed concentration of the complex (100 µL) was added to a solution of DPPH in methanol (0.3 mM, 1 mL) and the final volume was made up to 4 mL with double distilled water. DPPH solution with methanol was used as a positive control and methanol alone acted as a blank. The solution was incubated at 37 °C for 30 min in dark. The decrease in absorbance of DPPH was measured at 517 nm. The tests were run in triplicate, and various concentrations (20-100 µg/ mL) of the complexes used to fix a concentration at which the complexes showed 50 % of activity. In addition, the percentage of activity was calculated using the formula, % of suppression ratio = [(A₀ - Aₐ)/A₀] X 100. A₀ and Aₐ are the absorbance in the absence and presence of the tested complexes respectively. The 50% activity (IC₅₀) can be calculated using the percentage of activity.

4. RESULTS AND DISCUSSION

In all the complexes, the analytical data and spectroscopic analysis indicates a 1:1 metal-ligand stoichiometry. The proposed structure of the complexes as well as the synthesis of the ruthenium complexes are shown in scheme B1. The complexes are mostly soluble in organic solvents like DMSO, DMF, CHCl₃, CH₂Cl₂ etc.
4.1. Infrared spectra

In order to determine the mode of coordination the most significant infrared spectral frequencies for the metal complexes are compared with free ligands. A band observed at 1585 and 1530 cm\(^{-1}\) due to the azomethine C=N stretching frequency of the free ligands HL\(^1\) and HL\(^2\) respectively was shifted to lower frequency in the spectra of the complexes at 1521-1578 cm\(^{-1}\) indicating the coordination through N atom. [17] The ligands showed band at 951 cm\(^{-1}\) for HL\(^1\) and 850 cm\(^{-1}\) for HL\(^2\) for the vibration of the C=S double bond. The C=S band was disappeared in the complexes and a new band, C=S appeared at 741-745 cm\(^{-1}\). This confirms that the other coordination of ruthenium is through thiolate sulphur. [18] The medium intensity band in the region 523-540 cm\(^{-1}\) is attributed to Ru–N. [19]

In addition, the most characteristic frequency at 1433-1434 cm\(^{-1}\) was due to triphenylphosphine. In general, monobasic NS coordinated thiosemicarbazones form an important part of the complexes.

4.2. Electronic spectra

The UV-Vis spectra of the complexes were recorded in DMSO. The complexes show three to four bands in the region 316-434 nm. The transitions in ligand, 316-369 nm was shifted when compared to free ligands (305-396 nm) which reveal that the ligands are coordinated with ruthenium. The ground state of ruthenium(III) (t\(^5\)_2g configuration) is \(^2T_2g\), while the first excited doublet levels in the order of increasing energy are \(^2A_2g\) and \(^2T_1g\), which arise from t\(^3\)_2g e\(^1\)_g configuration. [20,21]

In a d\(^5\) system, especially in ruthenium(III) whose oxidising properties are comparatively higher, the charge transfer bands of the type L\(_{2g}\)→t\(^3\)_2g are prominent in the low energy region thereby obscuring the weaker bands owing to d-d transition. Hence the conclusive assignment of ruthenium(III) complexes appearing in the visible region becomes very hard. Therefore, the bands that appear in this region (413-434 nm) have been consigned to charge transfer transitions, which are in conformity with the tasks assigned for similar ruthenium(III) complexes [17,18].

4.3. Magnetic moment and EPR spectra

The magnetic susceptibility measurements of the ruthenium(III) complexes are recorded at room temperature which shows that they are paramagnetic. The magnetic moment value, \(\mu_B = 1.70-1.71\) corresponds to single unpaired electron in a low-spin 4d\(^5\) configuration and endorses that ruthenium is in +3 oxidation state in all the complexes [19].

The magnetic moments of the complexes correspond to paramagnetic nature with one unpaired electron at room temperature (low-spin Ru(III), t\(^2\)_2g). Recording of the X-band EPR spectra of powdered samples of the complexes were carried out and the EPR spectrum of the complex 1 is shown in figure B2. The absence of any hyperfine splitting due to interactions with any other nuclei present in the complexes were revealed by the nature of the spectra. A single isotropic resonance was observed for the complexes with g values in the range 2.04-2.17. The presence of isotropic lines in the EPR spectra may be due to the occupancy of the unpaired electron in a degenerate orbital even though the complexes have some distortion in their octahedral geometries. [22] Hence, trans positions are assigned for the triphenylphosphine group. [23, 24] Moreover, the trans structure is a more favoured structure than a cis structure, due to the bulky nature of two PPh\(_3\) groups. [17] The nature of spectra obtained and previously reported ruthenium(III) complexes were in good agreement [18,24].

4.4. Mass spectral analysis

The proposed molecular structure and the mass spectrum of the complexes is in good agreement. The EI-mass spectrum of the complexes is shown in figure B3 and B4. The molecular ion peak, [M\(^+\)] appearing at m/z = 1012.48 and 1022.65 confirms the stoichiometry of the complexes, 1 and 2, respectively.

5. DNA BINDING – TITRATION EXPERIMENTS

DNA is the primary target in interactions with small molecules which reveals primary action mechanisms of antitumor activity. [25] UV-visible spectral
analysis is an effective method to find out the nature of the interaction of the metal complexes with DNA. In general, red shift and hypochromism are associated with the binding of metal complexes to DNA, owing to the intercalative mode which includes a strong contact between the base pairs of DNA and aromatic chromophore of the complexes. [26] Absorption spectra of the complexes is shown in figure B5 both in the presence and absence of CT-DNA. The complexes, 1 and 2, exhibited hypochromism of 18.65% and 15.42% with red shifts of 2 nm at 360 and 359 nm, respectively, when titrated with CT-DNA. The results suggested an intercalative interaction of the complexes with CT-DNA. After the complexes intercalate to the base pairs of DNA, the π* orbital of the intercalated complexes could couple with π orbitals of the base pairs, thus decreasing the π→π* transition energies, hence resulting in hypochromism. [27] The binding strength, the intrinsic binding constants (Kb) of the complexes with CT-DNA were determined from the following equation (5.1).

\[
[DNA]/(\varepsilon_a - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/K_b (\varepsilon_b - \varepsilon_f)
\]

where [DNA] is the concentration of DNA in base pairs and the apparent absorption coefficient εa, εf and εb correspond to Aabs/[complex], the extinction coefficient of the free complex and the extinction coefficient of the complex when fully bound to DNA, respectively. The plot of [DNA]/(εa − εf) versus [DNA] gave a slope and the intercept which are equal to 1/(εb − εf) and 1/Kb (εb − εf), respectively. Kb is the ratio of the slope to the intercept. The magnitudes of intrinsic binding constants (Kb) were calculated to be 1.5 × 10^5 M^-1 and 5.6 × 10^4 M^-1 for complexes, 1 and 2, respectively. The observed values of Kb revealed that the ruthenium(III) complexes bind strongly than the respective ligands HL1 via intercalative mode (1.7 × 10^4 M^-1) and HL2 (4.6 × 10^3 M^-1) to DNA. From this DNA binding experiment, the complex 1 bound strongly with CT-DNA to that with complex 2. The Kb values for the ruthenium(III) complexes are comparable to other known ruthenium complexes, Kb at 2.3-4.7 × 10^4 M^-1 [28].

6. CYTOTOXIC ACTIVITY

6.1. Antibacterial activity

DNA binding experiments gave an idea that the complexes did interact with DNA. Hence, it is considered worthwhile to study the cytotoxic activity of the complexes. The antibacterial activity of the complexes and ligands were tested against the human pathogens (Escherichia coli, Staphylococcus aureus and Klebsiella pneumonia). The screening data are reported in table A1. From these screening data, the complexes exhibited significant antibacterial activity than the ligands and the inhibition activity of ruthenium complexes against the bacteria suggest the chelation facilitates and the ability of a complex to cross a cell membrane which leads to subsequent injury to the cell membrane and alteration in the cell permeability. [29, 30] The mode of action of the ligands and complexes may involve the hydrogen bond through >C=N group with active centers of all cell constituents resulting in interference with normal cell process. [9] Among the various bacteria studied the complexes showed higher activity against Staphylococcus aureus. Though the complexes showed higher activity than the ligands, they could not reach the effectiveness of standard drug cotrimazole.

6.2. In vitro anticancer activity

The significant results obtained from antibacterial activity of the complexes motivated us to test their anticancer properties against MCF-7 by means of a colorimetric assay (MTT assay). The MTT assay is based on the mitochondrial reduction of the tetrazolium salt by actively growing cells to produce blue insoluble formazan crystals i.e. only live cells reduce yellow MTT to blue formazan products. The blank samples containing the same amount of DMSO are taken as controls and the test complexes were dissolved in DMSO. The effects of the complexes on the practicality of these cells were assessed with an exposure period of 48 h. The results were analyzed by means of cell viability curves and expressed with IC50 values in the studied concentration range from 0.1-100 µM. The activity of the complexes corresponds to the inhibition of cancer cell growth as shown in figure B6. When increasing the concentration of complexes, the results of MTT assays revealed that the
complex 1 (IC$_{50}$ = 75.6 µM) showed a higher cytotoxic effect than complex 2 (IC$_{50}$ = 98.8 µM) against the cancer cell line. The ligands show IC$_{50}$ value greater than 100. The only responsible factor for the observed cytotoxic property of the complexes is the chelation of the ligands with the ruthenium(III) ion. The longer incubation period may have harmful effects such as affecting non-target sites in the body when they are used for clinical purposes and can develop cellular resistance for that particular compound. Hence the complexes that show activity in shorter time are preferred. [31] Hence, the data obtained for the complexes with 48 h lower incubation period are highly significant. The cytotoxic effect of the synthesized complexes are active against the tumor cell lines under in vitro experiments. None of them could reach the effectiveness shown by the standard drug cisplatin (IC$_{50}$ = 14 µM). [32] From this, the anticancer activity of the complexes of chloro benzaldehyde thiosemicarbazone moiety exhibited higher cytotoxic effects than the complexes of nitro benzaldehyde thiosemicarbazone.

6.3. Antioxidant activity

Since the complexes exhibit significant cytotoxic activity, the study of the antioxidant activity of these complexes is considered worthwhile. In order to explore the free radical scavenging activity of the complexes experiments were carried out with the hope of developing potential antioxidants and therapeutic reagents for respiratory diseases, such as asbestosis, emphysema and asthma. [33] 2,2-Diphenyl-2-picryl-hydrazyl (DPPH) assay is widely used for assessing the ability of radical scavenging activity and it is measured in terms of IC$_{50}$ values. DPPH shows a strong absorption band at 517 nm in the visible spectrum because of the presence of odd electron. In the presence of a free radical scavenger this electron becomes paired off, absorption vanishes, and the resulting decolourization is stoichiometric with respect to the number of electrons taken up. The DPPH assay of the tested ligands and complexes is shown in figure B7. From the results, it is revealed that the complex, 1 showed significant activity compared to the standard, ascorbic acid (Aca). The IC$_{50}$ values indicated that the complexes showed antioxidant activity in the order of 1 > 2 >HL$^1$ > HL$^2$. Complex 1 showed a higher antioxidant activity than the complex 2. From these results, the scavenging effect of the ruthenium(III) complexes was found to be significantly higher than the ligands which might be due to the availability of an odd electron and d$^5$ low spin electronic configuration in ruthenium(III) complexes which increases the capacity to stabilize the unpaired electrons and thereby scavenge the free radicals [33].

7. CONCLUSION

The present contribution describes the synthesis of new ruthenium(III) complexes comprising versatile benzaldehyde 4-methyl-3-thiosemicarbazones ligands and were characterized by various physio-chemical and spectroscopic analysis. An octahedral geometry has been tentatively assigned for all the complexes. The DNA binding ability of the complexes observed by UV-Vis spectroscopy inferred an intercalative mode of binding with binding constants 1.5 X 10$^5$ M$^{-1}$ and 5.6 X 10$^4$ M$^{-1}$ for complexes 1 and 2 respectively. The complexes possess significant antibacterial activity against panel of bacteria. The in vitro anticancer activity of the complexes against MCF-7 tumour cells showed that the complex 1 has higher ability towards the inhibition of tumor cell growth than the other complex and ligands. The antioxidant activity showed that the ruthenium(III) complexes can serve as potential antioxidants than the ligands counter to DPPH radical. Many of the hopeful chemical and biological findings show that these complexes are very favourable candidates as live cell imaging reagents that could contribute to the understanding of cellular uptake of metal complexes. Further researchers are required to evaluate their pharmacological properties in vivo and to interpret the real mechanism of their biological activity.

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APPENDIX A

Table A1. Antibacterial activity of the ligands and complexes

<table>
<thead>
<tr>
<th>Ligands and Complexes</th>
<th>Diameter of inhibition zone (mm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>E. coli</td>
<td>S. aureus</td>
</tr>
<tr>
<td>HL&lt;sub&gt;1&lt;/sub&gt;</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>HL&lt;sub&gt;2&lt;/sub&gt;</td>
<td>8</td>
<td>10</td>
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<tr>
<td>1</td>
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<td>23</td>
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<tr>
<td>2</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td>DMSO</td>
<td></td>
<td>No activity</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are an average of triplicate runs.
APPENDIX B

Scheme B1. Synthetic route of the ruthenium(III) thiosemicarbazone complexes, PPh₃=triphenylphosphine

Figure B2. EPR spectrum of the complex 1
Figure B3. EI-mass spectrum of the complex 1

Figure B4. EI-mass spectrum of the complex 2
Figure B5. Electronic spectra of complexes 1 and 2 in Tris-HCl buffer upon addition of CT-DNA. [Complex] = 25 µM, [DNA] = 0-50 µM. Arrow shows the absorption intensities decrease upon increasing DNA concentrations. (Inset: Plot between [DNA] and [DNA]/(ε_a-ε_f) X 10^8).

Figure B6. % Growth inhibition of MCF-7 cell line as a function of concentration of the complexes 1 and 2.
Figure B7. Antioxidant activity of the ligands and complexes, 1 and 2, and standard, ascorbic acid (Aca) against DPPH radical