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ORIGINAL PAPER

Synthesis, characterization, antioxidant and anticancer activities of a new Schiff base and its M(II) complexes derived from 5-fluorouracil

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Abstract
In this study, Schiff base ligand was obtained from the condensation reaction of benzene-1,2-diamine and 5-fluoropyrimidine-2,4(1H,3H)-dione (5-FU). Metal(II) complexes were synthesized with Fe(II), Co(II) and Ni(II) chloride salts. The synthesized ligand and metal complexes were characterized by FT-IR, UV–vis, ^{1}H-^{13}C NMR, elemental analyses, mass spectroscopy, magnetic moments, molar conductivity and thermogravimetric analysis studies. With the help of different techniques reveal Fe(II), Co(II) and Ni(II) complexes have exhibited tetrahedral and octahedral geometry. Ligand acted as bidentate and it binds metal(II) ions through deprotonated-NH, imine-N atom and carbonyl-O atom, respectively. DPPH, ABTS, FRAP, CUPRAC and total antioxidant activity methods were used to determine the antioxidant properties of ligand and metal complexes. According to the results, the synthesized compounds showed very high antioxidant activity compared to 5-FU. The cytotoxicities of the synthesized compounds were performed on MCF-7 (human breast cancer) and L-929 (fibroblast) cell lines using the MTT assay. In addition, the effect of electroporation (EP) on the cytotoxicity of the compounds was investigated. Our results demonstrated that novel Co(II) and Ni(II) complexes show potential as new anticancer agents and ECT may be a viable treatment option for breast cancer.

Keywords Anticancer · Antioxidant · Electrochemotherapy · 5-Fluorouracil · Metal complexes

Introduction

Schiff bases or ketimines are versatile imine (C=N) containing compounds possessing a broad spectrum of biological activity and are widely used for industrial purposes. They are usually two, three, or tetradentate chelate ligands that form stable complexes with metal ions. In contrast to 4d and 5d transition metal complexes, 3d transition metal complexes reveal valuable properties as low toxicity and easily enter to the cell membrane of microbes [1]. This character causes the special structures and unique properties of Schiff base-complexes, we used, which make them appropriate option for doing extensively investigation and vastly application in different medical and industrial usages [1–3]. Schiff bases and their metal complexes have been extensively studied for their antiviral, anticancer, herbicidal, antifungal and antibacterial activities [4–6]. The addition of metal ions to the structure of the compounds has increased their current biological activities. Therefore, an attempt has been made to study the interaction of the ligand with the transition metals of biological activity and to examine the coordination chemistry of such interactions.

The main source of free radicals, which are formed as a result of various metabolic reactions in the organism, is the oxygen molecule. It is known that very reactive free radicals cause various diseases in the body, including cancer [7, 8]. Therefore, the presence of endogenous and exogenous antioxidants that neutralize free radicals is vital to prevent

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oxidative damage [9]. Schiff bases, one of the exogenous antioxidants, neutralize free radicals by providing electrons. It has also been reported that metal complexes derived from Schiff bases increase the activity of the ligand and impart activities such as antioxidant, antimicrobial and anticancer to the compound [10, 11].

Despite advancements in surgical and radiotherapy procedures, as well as the discovery of modern systemic medications in recent years, the outcomes of current treatments are still unsatisfactory [12]. In addition, since the structure and weight of many newly synthesized molecules that can be anticancer agents are large, they either pass little or not through the cell membrane, and therefore cannot show their cytotoxic effects fully. For these reasons, innovative treatment strategies such as electrochemotherapy (ECT) are needed [13]. ECT is a successful treatment technique in which electroporation is used to enhance the cytotoxicity of anticancer agents by increasing their uptake into cancer cells [14, 15]. In electroporation (EP), temporary nanometer-sized pores are created in cell membranes by applying high strength (< 1500 V cm^{-1}) and short duration (100 µs) electrical pulses to tissues or cells [16]. However, EP pulses also contribute to the efficacy of ECT through both immunological and vascular immunological mechanisms [17]. Local application of electrical pulses to the tumor causes temporary vasoconstriction and reduction of blood flow in the tumor, thereby enhancing the effect of drugs by keeping them in the tumor for a long time [18]. In the clinic, ECT has a high treatment efficiency, attaining up to 80% local tumor suppression with no systemic side effects.

The aim of this study is to synthesis and characterization a new Schiff Base and its Fe(II), Co(II) and Ni(II) complexes, then to examine their antioxidant and anticancer activities and to contribute to the development of biologically effective compounds. For this purpose, ligand derived from 5-FU and its Fe(II), Co(II) and Ni(II) complexes were synthesized and characterized. Antioxidant properties of synthesized compounds were investigated using different methods. In addition, the cytotoxic effects of the compounds were compared with the results of 5-FU.

Experimental

Materials and equipment

The fluoropyrimidine-2,4(1H,3H)-dione, benzene-1,2-diamine, $CoCl_2{\cdot}6H_2O$, $NiCl_2{\cdot}6H_2O$, $FeCl_2{\cdot}4H_2O$ and used solvents were purchased from Sigma Aldrich and used without further purification.

Melting point (m.p.) was performed on the Gallenkamp apparatus in open glass capillaries. C, H, and N analyses were recorded on a Leco CHN analyzer. Perkin Elmer 65 spectrometer was used to obtain IR spectra in the range 4000–400 cm^{-1} by the KBr technique. ^{1}H-^{13}C NMR spectra were carried out in d_6-DMSO on a Bruker 400 MHz spectrometer using TMS as the internal standard. Magnetic susceptibilities were determined on a Gouy balance using $Hg[Co(NCS)_4]$ as the calibrant. Electronic absorption spectra were performed with an Agilent Cary-60 spectrophotometer. Thermal analysis was recorded in a nitrogen atmosphere with a heating rate of 10 °C min^{-1} using a Shimadzu analyzer model 60 H. Molar conductivity was measured on Jenway 4010 conductivity bridge in dry DMF ca. 10^{-3} M solution.

Synthesis of ligand

The 10 mL ethanol solution of benzene-1,2-diamine (1 g 1.00 mmol) was added to 5-fluoropyrimidine-2,4(1H,3H)-dione (2.4 g 2 mmol) in ethanol (15 mL) and refluxed until the reactant was completely converted into the product as monitored with the help of TLC. The resulting precipitate was filtered, washed with diethyl ether and crystallized from methanol. The synthetic route of the ligand is shown in Fig. 1.

Ligand (L): Yield: 86%; m.p.: 230 °C; reddish brown solid, Anal. calc. for $C_{14}H_{10}F_2N_6O_6$ (%): C, 50.61; H, 3.03; N, 25.29. Found: C, 51.00; H, 3.50; N, 25.86; FT-IR (KBr pellet, cm^{-1}): 3385, 3363, 3282 ν(NH), 3062, 3024 ν(C–H), 1723, 1660 ν(C=O), 1634 ν(C=N), 1591, 1500 ν(C=C); ^{1}H NMR (400 MHz, d_6-DMSO, δ (ppm)): 11.51 (s, H, NH),

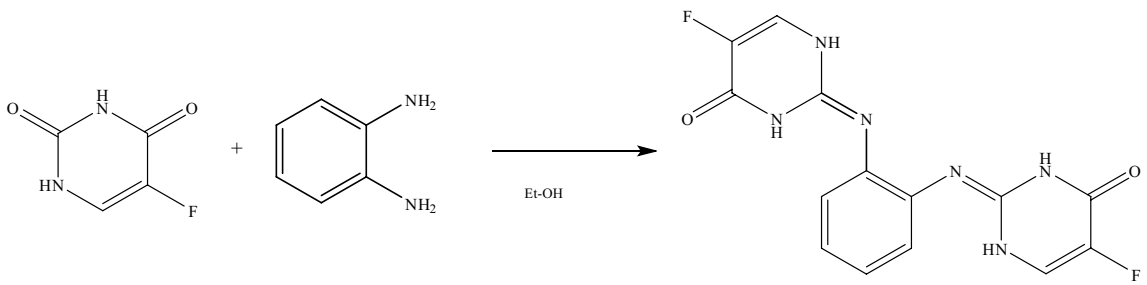

Fig. 1 The synthesis scheme of ligand

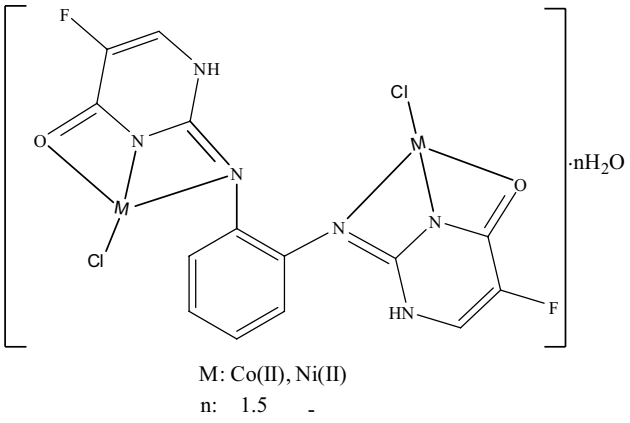

Fig. 2 Structure of Co(II) and Ni(II) complexes

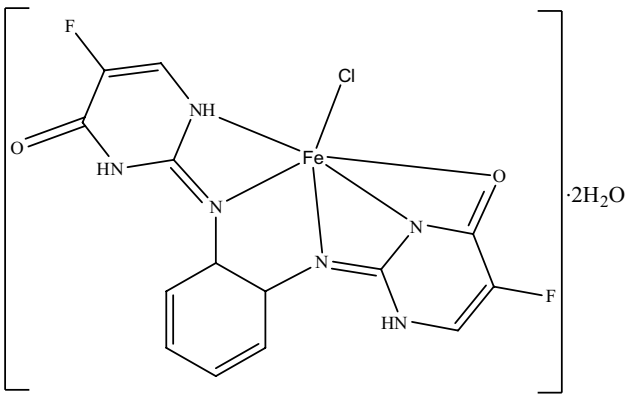

Fig. 3 Structure of Fe(II) complex

8.23 (d, H, NH), 7.79–6.36 (m, 6H, Ar–H); ^{13}C NMR (100 MHz, d_6-DMSO, δ (ppm)): 158.56, 157.74 (C=O), 150.57 (C=N), 144.39–102.40 (Ar–C); UV–visible (λmax (nm) (ε, M^{-1} cm^{-1})): 208 (2249), 242 (390), 293 (211), 342 (3), 350 (4), 358 (5).

Synthesis of metal complexes

A solution of metal chlorides (0.60 g (2.50 mmol) of $CoCl_2{\cdot}6H_2O$; 0.60 g (2.50 mmol) of $NiCl_2{\cdot}6H_2O$; 0.24 g (1.25 mmol) of $FeCl_2{\cdot}4H_2O$) in ethanol was added dropwise to an ethanol solution (25 mL) of (2*E*,2′*Z*)-2,2′-(1,2-phenylenebis(azan-1-yl-1-ylidene))bis(5-fluoro-2,3dihydropyrimidin-4(1*H*)-one) (0.41 g, 1.25 mmol) with constant stirring at 25 °C. The mixture was refluxed for about 4–7 h. The synthesized products were washed with ethanol and crystallized from the chloroform–methanol mixture (1:1) (Figs. 2, 3).

Co(II) complex: Yield: 87%; FW: 545.89 g mol^{-1}; m.p.: >250 °C; black, Anal. calc. for $C_{14}H_{11}F_2N_6O_{3.5}Co_2Cl_2$(%): C, 30.80; H, 2.01; N, 15.38. Found: C, 31.00; H, 2.10; N, 15.47; FT-IR (KBr pellet, cm^{-1}): 3551 (OH), 3369, 3230 ν(NH), 3130 ν(C–H), 1691 ν(C=O), 1620 ν(C=N), 1571, 1500 ν(C=C), 599, 585 (M–O), 430 (M–N); UV–visible (λmax (nm) (ε, M^{-1} cm^{-1})): 255 (3367), 266 (3495), 367 (567), 433 (1442), 564 (150), 662 (24); Mass spectrum [ESI], m/z = 547.89 (calc. mass) 547.00 $(M+2H)^{2+}$ (found mass).

Ni(II) complex: Yield: 85%; FW: 518.59 g mol^{-1}; m.p.: >250 °C; light brown, Anal. calc. for $C_{14}H_8F_2N_6O_2Ni_2Cl_2$(%): C, 32.42; H, 1.54; N, 16.19. Found: C, 32.50; H, 1.56; N, 16.32; FT-IR (KBr pellet, cm^{-1}): 3398, 3301, 3218 ν(NH), 3092 ν(C–H), 1689, 1651 ν(C=O), 1617 ν(C=N), 1572, 1545, 1500 ν(C=C), 588 (M–O), 478 (M–N); UV–visible (λmax (nm) (ε, M^{-1} cm^{-1})): 258 (2460), 280 (1355), 342 (194), 434 (802), 659 (37); Mass spectrum [ESI], m/z = 519.59 (calc. mass) 519.10 $(M+H)^{+}$ (found mass).

Fe(II) complex: Yield: 78%; FW: 458.44 g mol^{-1}; m.p.: >250 °C; black, Anal. calc. for $C_{14}H_{13}F_2N_6O_4FeCl$ (%): C, 36.67; H, 2.83; N, 18.32. Found: C, 36.70; H, 2.88; N, 18.34; FT-IR (KBr pellet, cm^{-1}): 3502 (OH), 3294 ν(NH), 3108 ν(C–H), 1677 ν(C=O), 1602 ν(C=N), 1577, 1500 ν(C=C), 582, 544 (M–O), 463 (M–N); UV–visible (λmax (nm) (ε, M^{-1} cm^{-1})): 264 (2673), 275 (2665), 345 (170), 362 (169), 456 (1411), 611 (18); Mass spectrum [ESI], m/z = 456.44 (calc. mass) 456.20 $(M-2H)^{2-}$ (found mass).

Antioxidant activity assays

Total antioxidant activity assay

In this study, in which the thiocyanate method [19] was used, samples at different concentrations were placed in test tubes. After completing their total volume to 500 µL with buffer solution, 500 µL of linoleic acid was added and incubated. 20 µL of samples were taken every 8 h and transferred to test tubes containing 470 µL of ethyl alcohol. After adding $FeCl_2$ and NH_4SCN, their absorbance was taken at 500 nm. The experiment was terminated as soon as the absorbance of the control began to decrease.

Determination of Fe^{3+} reduction power (FRAP)

The total volumes of the samples at 5, 10 and 20 µg mL^{-1} concentrations were made up to 200 µL with distilled water. 500 µL of phosphate buffer and potassium ferrocyanide $[K_3Fe(CN)_6]$ were added on them. After 20 min of incubation, 500 µL of TCA was added and 500 µL of the supernatant was taken. 500 µL of distilled water and 100 µL $FeCl_3$ were added to the mixture and absorbance was taken at 700 nm [20].

Determination of Cu^{2+} reduction power (CUPRAC)

After taking samples at different concentrations into test tubes, their total volume was completed to 1 mL with distilled water. 0.25 mL of 0.01 M $CuCl_2$, 7.5×10^{-3} M ethanolic neocuproin and 1 M ammonium acetate buffer were added to them. The absorbances of the samples incubated for half an hour were read at 450 nm [21].

1,1-Difenil 2-picril hydrazil ($DPPH^{\cdot}$) radical scavenging assay

In order to measure the radical scavenging activity of the samples, the volumes of the samples at different concentrations (5, 10, and 20 µg μL^{-1}) were made up to 600 µL with ethyl alcohol. 200 µL of 1 mM DPPH radical solution was added to them. After 25 min of incubation, absorbance was taken at 517 nm [22].

2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) radical scavenging assay

ABTS radical scavenging activities of the samples were performed according to the Re method [23]. First, solutions of 2.45 mM $K_2S_2O_8$ and 7 mM ABTS were mixed in a 1:1 ratio. It was incubated overnight at room temperature and in the dark. After incubation, the absorbance of the solution was taken at 734 nm and diluted with ethanol until an absorbance of 1.660 ± 0.02 was reached. After adding samples at different concentrations to the test tubes, they were filled to 200 µL with ethanol. After adding 800 µL of ABTS radical solution, it was left to incubate for approximately 2 h. The absorbance of the samples was recorded at 734 nm.

Anti-cancer activity

Cell cultures

The cytotoxicities of the synthesized compounds were performed on two cell lines, one human breast cancer cell line (MCF-7) and the other healthy fibroblast cell line (L-929). Breast cancer is one of the most common causes of cancer-related death in the world [24]. Because of its poor response to treatment and increased rate of metastasis to other tissues, this cancer is a global health concern. 5-FU is one of the chemotherapy drugs to treat breast cancer, but it is associated with several side effects and in long-term treatments, cancer cells show resistance to this drug [25]. For these reasons, we selected breast (MCF-7) cancer cells for anti-cancer activity studies. L-929 was used for the biocompatibility of the tested compounds. The cell lines used in the study were provided by Muş Alparslan University Application and Research Center. At 37 °C and 5% CO_2, the cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) mixed with 10% fetal bovine serum (FBS, Sigma-Aldrich, USA) and 1% pen-strep. Cells incubated for proliferation were fed every two or three days. When they reached approximately 80–90% confluency, they were taken out from flasks by trypsinization and used in experiments.

Chemotherapy with compounds

To prepare the main stock solution at 1 mM concentration, newly synthesized compounds and 5-FU were dissolved in DMEM. Then, the desired concentrations (0, 1, 5, 10, 50, 100 and 200 µM) were obtained by taking the appropriate amount of solution from the main stock and mixing it with DMEM. The main and other stock solutions were kept at 4 °C until use.

When cells reached 80–90% confluence, they were taken out from flasks, centrifuged and seeded into 96 well microculture plates at 10,000 cells per well and left in an incubator with 5% CO_2 at 37 °C for 24 h to attach. Then, DMEM in the wells was decanted and different concentrations (0, 1, 5, 10, 50, 100 and 200 µM) of 5-FU or synthesized compounds were added and allowed to incubate again for 24 h. After the incubation period, the viability of the cells was examined with the MTT analysis method and the cytotoxicity of the substances was evaluated.

Electrochemotherapy (ECT)

We examined the effects of EP in the MCF-7 cancer cells alone or in combination with 5-FU or newly synthesized Co(II) and Ni(II) complexes (These complexes demonstrated good cytotoxicity in chemotherapy). Firstly, cells were removed from the flask surface with trypsin, centrifuged (5 min, 1300 rpm) and suspended in DMEM. 400 µL of cell solution (1×106 cells mL^{-1}) were placed in EP cuvettes (4 mm) with a final concentration of 10 µg mL^{-1} for each substance. After that, the cells were exposed to 320 V square wave electrical pulses (100 µs, 1 Hz, 8 pulses). The procedure used has already been optimized in previous studies [26, 27]. ECT applications were performed using Gemini X2 EP device (BTX Harvard Apparatus, USA). The cells were then incubated at room temperature for 15 min. After 15 min incubation, the cells were seeded into 96-well plates (10.000 cells per well) and allow to incubate for another 24 h. The MTT assay was used to assess cell viability after the incubation period.

Cell viability assay (MTT)

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test can be used for the assessment of cell

viability, proliferation, and cytotoxicity [28]. MTT test was used in this study to evaluate the cytotoxicities of the newly synthesized compounds. Following a 24 h incubation period, the media was withdrawn from the wells and 10 µL of MTT solution (5 mg mL^{-1} in PBS), and 90 µL of DMEM was put in its place, after that, it was incubated for 4 h at 37 °C. The MTT-containing media was discarded after incubation, and 100 µL of dimethylsulphoxide (DMSO) was placed in per well to dissolve the formazan crystals generated as a result of MTT reduction. Subsequently, a spectrophotometer was used to detect the absorbance at 570 nm wavelength. The % of cell viability was stated as a percentage of treated cells compared to the control cells. The IC50 concentration (50% inhibiting concentration) of a substance was calculated using a graph showing the percentage of inhibition plotted versus the concentration of the substance.

Statistical analysis

All of the tests were carried out in triplicates, and the findings were determined as the average of three independent experimental results ± standard error. Analysis of data was performed with an Independent test, Student's *T* test, and ANOVA using SPSS software (20.0, SPSS, Inc., USA). A statistically significant difference was defined as $p < 0.05$.

Results and discussion

Structure elucidation of ligand and its metal complexes

The elemental analyses result for C, H and N in metal complexes indicated good agreement between calculated and found values, approving the proposed formula for Fe(II), Co(II) and Ni(II) complexes. Molar conductance values of the all complexes were too low in DMF (15–24 Ω^{-1} cm^2 $mole^{-1}$), demonstrating metal complexes' non-electrolytic behavior. The ligand has some characteristic stretching frequencies of the ν(NH), ν(C=O) and ν(C=N) groups at 3385, 3363, 3282 cm^{-1}, 1723, 1660 cm^{-1} and 1634 cm^{-1}, respectively. The characteristic ν(C=N) shifted to lower frequencies in complexes (1602 cm^{-1} for Fe(II) complex, 1620 cm^{-1} for Co(II) complex, 1617 cm^{-1} for Ni(II) complex) due to bonding. The carbonyl ν(C=O) shifted to lower frequencies and was seen in the region 1691–1651 cm^{-1} confirming the participation of the oxygen atom of carbonyl in complexation with the metal ions as such without undergoing enolization. The coordination of metal ions with ligand was supported through the appearance of two new peaks in the Fe(II), Co(II) and Ni(II) complexes, one range from 490 to 463 cm^{-1} corresponding to (M–N) and another in the range from 599 to 544 cm^{-1} assigned to (M–O) stretching vibrations respectively [29]. The 3551–3502 cm^{-1} broad bands in Co(II) and Fe(II) complexes were thought to be related to ν(OH) vibrations of lattice water (H_2O) molecules [30, 31].

Proton (^1H) NMR spectra of ligand, the signals in the 11.51 ppm and 8.23 ppm attributed to the NH protons. The multiplet observed in the region 6.36–7.79 ppm were assigned to Ar–H. The ^1H NMR spectra for metal complexes were pointless because of their paramagnetic nature [32].

The ^{13}CNMR spectra of ligand showed peaks at 144.39–102.40 ppm which corresponds to the Ar.–C ring. The imine carbons (C=N) were seen at 150.57 ppm. The carbon of (C=O) group was seen at 158.56, 157.74 ppm [33].

Mass spectra of metal complexes exhibited molecular ion peaks at *m/z* at 456.20, 547.00 and 519.10, respectively. The proposed molecular formula of the complexes was verified by comparing their molecular weights with *m/z* values [34].

The thermal degradation of complexes indicates loss of coordinated water molecules in the first step followed by degradation of organic moieties. The Fe(II) complex showed a weight loss in three-stage degradation between 50 and 800 °C. The first stage corresponds to the loss of two lattice water molecules (calc. 7.89%, found 6.42%) in a temperature range of 50–160 °C [35]. The second degradation step in the temperature range 180–275 °C with mass loss of 4.78% (calc. 4.16%) corresponding to loss of a fluoride. A peak corresponding to mass loss 24.94% (calc. 24.22%) at 275–360 °C was due to the loss of organic molecule C_2H_2NOFCl in the third stage. The fourth degradation stage in the temperature range 360–405 °C with mass loss of 29.00% (calc. 28.71%) corresponding to loss of organic molecule $C_6H_3N_4$ and as a final step, it leaves C_3H_2 as residue (calc. 8.11%, found 8.62%). The remaining product was the organic molecule C_3H_2NOFe. Similar decomposition was seen in the TGA curve of Co(II) and Ni(II) complexes.

Magnetic measurements showed that the complexes have a paramagnetic character. The electronic spectra of the ligand and its metal complexes showed absorption bands in the range 208–358 and 255–662 nm, respectively. The lower wavelength for the ligand (208–293 nm) is assigned to $\pi \rightarrow \pi^*$ transitions and lower energy absorption bands at 342–358 nm were attributed to $n \rightarrow \pi^*$ transitions of non-bonded electrons available in C=N group. In Co(II) and Ni(II) complexes the position of these bands was changed and a new band was observed at 433 and 434 nm related to LMCT transitions. The absorption band at 564 nm in the Co(II) complex corresponds to $^4A_{2g} \rightarrow {}^4T_{1g}$ transition, which is characteristic of a tetrahedral geometry around the Co(II) ion. This is supported by the magnetic susceptibility value of the Co(II) complex ($\mu_{eff} = 4.28$ B.M) [36, 37].

The UV–vis spectra of the Ni(II) complex indicated band at 659 nm that were assigned to $^3A_{2g}(F) \rightarrow {}^3T_{1g}$ (P), $^3A_{2g}(F) \rightarrow {}^3T_{2g}$ (F), $^3A_{2g}(F) \rightarrow {}^3T_{1g}$ (F) transitions. The Ni(II)

complex has a μ_{eff} value of 3.40 B.M., indicating tetrahedral geometry [37, 38].

The UV–vis spectrum of the Fe(II) complex showed d-d transition bands at 456 and 611 nm assigned to $^5T_{2g} \rightarrow {}^5E_g$ transition. The Fe(II) complex has an octahedral geometry and a μ_{eff} value of 5.09 B.M [39, 40].

Antioxidant activities

Antioxidants are considered potential research methods in the treatment of diseases. In vitro, antioxidant methods are still preferred by researchers in terms of ease of application and rapid results [41–43]. In this study, the total antioxidant activities of the compounds were investigated. According to this method, the ability of the compounds to remove lipid peroxides in the environment was compared. DPPH and ABTS methods were preferred as radical scavenging methods. These artificial radicals are the most preferred compounds in research and have very high reactivity. The FRAP method was used for the determination of the removal of ferric ions, and the CUPRAC method was used for the reduction of cupric ions. DPPH, ABTS, and total antioxidant activity results were expressed as IC_{50} (Table 1). According to the FRAP and CUPRAC results, the reducing capacities of the compounds for ferric and cupric ions are also shown in Fig. 4. The antioxidant results of Schiff Base and its Ni(II), Fe(II) and Co(II) complexes were compared with the results of 5-FU in the previous study [44]. In addition to the previous study [44] in this study, the ABTS radical scavenging activity of 5-FU was studied and is shown in Table 1.

Table 1 Total antioxidant, DPPH and ABTS scavenging activities of synthesized compounds, BHA, BHT and 5-FU

Samples	Lipid peroxide inhibition (IC_{50})	DPPH radical scavenging (IC_{50})	ABTS radical scavenging (IC_{50})
Ligand	100.85 ± 5.93	96.21 ± 2.67	84.5 ± 11.82
Fe(II) complex	84.9 ± 5.89	162.87 ± 2.29	50.97 ± 1.13
Co(II) complex	83.62 ± 9.04	231.27 ± 1.53	54.76 ± 2.50
Ni(II) complex	74.69 ± 0.36	61.05 ± 2.30	54.53 ± 2.49
5-FU	226.14 ± 0.99	1666.67 ± 6.57	116.28 ± 0.16
BHA	66.21 ± 4.51	57.89 ± 7.28	43.5 ± 0.65
BHT	62.08 ± 1.72	96.77 ± 7.29	43.68 ± 0.64

The antioxidant results showed that ligand and metal complexes exhibited better activities than 5-FU. The percentages of lipid peroxide removal of the compounds were calculated according to Eq. (1). According to the results of total antioxidant activity, the percentages of lipid peroxide removal of the synthesized compounds, 5-FU and standard antioxidants (BHA and BHT) at 20 µg mL^{-1} were as follows: BHT (70.24%) > Ni(II) complex (64.20%) > Fe(II) complex (62.41%) > BHA (61.95%) > Co(II) complex (61.78%) > L (51.82%). According to the results, while the metal complexes showed higher activity than the ligand, 5-FU showed very low activity. It is known that transition metal(II) complexes prevent lipid peroxidation and play an important role in curing related diseases. In addition, Schiff bases and their metal complexes exhibit important biological activities due to the electron-donating atoms such as oxygen, sulfur and nitrogen [45, 46]. However, it has been reported in previous studies that 5-FU causes lipid peroxidation [47, 48].

$$\textit{Lipid peroxide inhibition}\ (\%) = \left[\frac{(\mathrm{Ac} - \mathrm{As})}{\mathrm{Ac}}\right] \times 100] \quad (1)$$

Ac: Absorbance value of control, As: Absorbance value of samples.

Antiradical capacities of synthesized compounds, standard antioxidants and 5-FU were measured by DPPH and ABTS methods. According to the results, it was seen that the compounds had similar activities on radicals. It was determined that Ni(II) complex showed the best activity in scavenging radicals, while 5-FU had the lowest activity. The scavenging percentages of the compounds for DPPH radicals were calculated according to the formula:

$$\textit{DPPH radical scavenging activity}\ (\%) = \left[\frac{(\mathrm{Ac} - \mathrm{As})}{\mathrm{Ac}}\right] \times 100] \quad (2)$$

The DPPH radical scavenging percentages of the compounds at 20 µg mL^{-1} were listed as follows: BHA (70.19%)

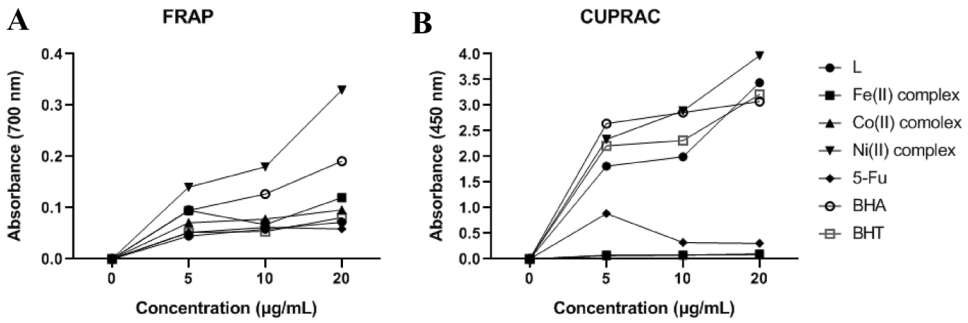

Fig. 4 **A** FRAP and **B** CUPRAC results of synthesized compounds, BHA, BHT and 5-FU

> Ni(II) complex (62.59%) > BHT (44.71%) > L (33.87%) > Co(II) complex (10.56%) > Fe(II) complex (10.02%).

The following formula was used to calculate the percentages of ABTS radicals:

$$ABTS\ radicals\ cavenging\ activity\ (\%) = \left[1 - \frac{As}{Ac}\right] \times 100] \quad (3)$$

The percentages of the compounds scavenging ABTS radicals at 20 µg mL^{-1} were listed as follows: BHT (85.16%) > BHA (84.70%) > Ni(II) complex (81.85%) > Fe(II) complex (70.43%) > Co(II) complex (62.78%) > L (60.50%) > 5-FU (34.82%).

It has been reported in previous studies that Schiff base and metal complexes strongly scavenge DPPH and ABTS radicals [7, 49]. The radical scavenging capacity of the metal complexes attached to the Schiff base varies according to the presence of electron-donating groups. In some cases, they show lower activity than the ligand, and sometimes their activity is increased [49]. According to the literature, it is seen that the 5-FU does not show good activity in scavenging DPPH radicals [33] and this is in line with the results of our study.

According to the results of the FRAP method, the compound with the highest ferric ion reducing capacity was Ni(II) complex, while 5-FU exhibited the lowest activity. Similar activities were observed according to the CUPRAC results. Ni(II) complex reduced cupric ions best, while Co(II) and Fe(II) complexes performed moderately. However, according to the results of both methods, it was determined that the activities of the compounds increased depending on the increase in concentration. The presence of free Fe^{2+} and Cu^{+} ions in the organism is $OH^{\cdot}$, the most important free radical, through Fenton and Haber–Weiss reactions. It causes the formation of radicals [50].

When the results were evaluated in general, it was observed that the Ni(II) complex had very high activity, while the 5-FU drug exhibited very low activities compared to the synthesized compounds and standard antioxidants. The results obtained show that Schiff base and metal complexes exhibit significant antioxidant activities [9, 10] and 5-FU causes lipid peroxidation [48, 49] and creates a toxic effect [51, 52] supporting previous studies reporting it.

In vitro anticancer activity of compounds

The anticancer properties of the newly synthesized ligand and its Fe(II), Co(II), Ni(II) complexes were investigated against cancer (MCF-7) and healthy (L-929) cell lines with MTT assay. The MTT method was used to determine the cytotoxicity of compounds [53]. Table 2 summarizes the IC_{50} values for the produced compounds as well as the reference drug 5-FU. Three times, each experiment was repeated independently.

Table 2 IC_{50} values of 5-FU and newly synthesized compounds against cancer (MCF-7) and healthy (L-929) cell lines

Compounds	IC_{50} (µM) of MCF-7	IC_{50} (µM) of L-929
5-FU	45.52	62.89
Ligand	83.77	204.43
Fe(II) complex	102.47	169.78
Co(II) complex	46.43	85.16
Ni(II) complex	9.43	231.44

As shown in Table 2, Co(II) and Ni(II) complexes demonstrated a strong cytotoxic effect on MCF-7 cancer cells. The new Ni(II) complex was observed to have the highest cytotoxicity effect on MCF-7 cancer cells with an IC_{50} value of 9.43 µM. Ligand (IC_{50} = 83.77) and Fe(II) complex (IC_{50} = 102,47) showed lower cytotoxic effect on MCF-7 cancer cells. The IC_{50} values of 5-FU (reference drug), ligand, Fe(II), Co(II) and Ni(II) against L-929 healthy cells as follows: Ni(II) complex (231.44 µM) > ligand (204.43 µM) > Fe(II) complex (296.78 µM) > Co(II) complex (85.16 µM) > 5-FU (62.89 µM). While the Ni(II) complex showed higher cytotoxicity in MCF-7 cancer cells than the reference drug 5-FU, it showed lower cytotoxicity in healthy L-929 cells. On the other hand, the Co(II) complex exhibited a cytotoxic effect close to the 5-FU reference drug. Many previous studies have shown that Ni(II) and Co(II) complexes exhibit good cytotoxicity in various cancer cells and tissues [27, 54–57]. Our findings suggest that novel Co(II) and Ni(II) complexes may be potential anticancer agents for MCF-7 breast cancer cells. Therefore, our ECT studies were continued with these two compounds.

Electrochemotherapy treatment efficiency

In ECT of MCF-7 cancer cells, 320 V square wave electrical pulses (100 µs, 1 Hz, 8 pulses) and 10 µg mL^{-1} of 5-FU and new compounds were used. Figure 5 demonstrates the viability of MCF-7 cancer cells subjected to different agents (5-FU, Co(II), and Ni(II)) with or without EP.

In Fig. 5, it is seen that the cytotoxic effects of all compounds are highly increased with EP. As in chemotherapy, Ni(II) complex showed the best cytotoxicity in ECT. In ECT treatment, reference drug 5-FU reduced MCF-7 cell viability to 68.55%, while Co(II) and Ni(II) complexes decreased to 55.8% and 45.24%, respectively. When the viability of ECT group cells was compared with the viability of control, EP and CT group cells, a statistically significant decrease was observed in all three compounds (5-FU, Co(II) and Ni(II)) ($p < 0.05$).

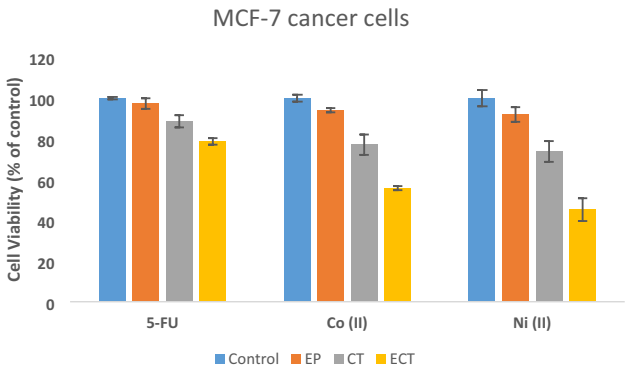

Fig. 5 Cytotoxic effects of 5-FU, Co(II) and Ni(II) with or without electroporation (EP) in MCF-7 human breast cancer cells (CT: compound-alone; ECT: compound + EP)

The ECT technique allows for a significant rise in cytostatic absorption by cancer cells and, as a result, an escalation of their effect. Electroporation forms temporary pores in the cancer cell membrane enabling the hydrophilic molecules (large structure molecules, cytotoxic drugs, nucleic acids, etc.) to pass into the cell [58]. Some previous studies have shown that ECT is effective in patients who have failed to react to traditional chemotherapy treatment [59, 60]. ECT can be utilized to treat cutaneous and subcutaneous metastatic breast tumors on a local level [61]. Wichtowski et al. [62] stated that ECT has a major success rate in the treatment of breast cancer metastases. In addition, according to Esmekaya et al. [63], EP applications increased tamoxifen absorption into MCF-7 cells and thus lowering the minimum tamoxifen dosage necessary for the treatment of breast cancer. The results obtained in this study are in parallel with the results of these studies.

In our study, EP significantly increased the cytotoxicity of 5-FU and newly synthesized compounds ($p < 0.05$). When 5-FU + EP, Co(II) + EP, and Ni(II) + EP treatment groups were compared to the compounds alone and control groups, a statistically significant reduction in cell viability was observed ($p < 0.05$). The electric current (electroporation) used may have increased the cytotoxic effect of the compounds by increasing the electrical conductivity and permeability of MCF-7 cancer cells [27, 64]. Thus, compounds with heavy molecular structure, especially newly synthesized from 5-FU, can enter MCF-7 cancer cells and increase their anticancer effects. In addition, local electroporation application causes temporary vasoconstriction and reduction of blood flow in the tumor, thereby enhancing the effect of drugs by keeping them in the tumor for a long time [65].

Conclusion

Three dentate Schiff base and its cobalt(II), iron(II) and nickel(II) complexes were successfully synthesized and characterized using physicochemical and spectral techniques. It was found that the ligand behaved like tridentate chelation coordinates to the M(II) ion upon complexation and exhibited a tetrahedral and octahedral geometry structure. Spectroscopic and analytical data of metal complexes suggest that the ligand to metal ratio of the complexes is 1:2 stoichiometry of the type $[M_2L(Cl_2)]nH_2O$ ($n = 1,5$ and 0) for Co(II) and Ni(II) complexes and 1:1 stoichiometry of the type $[MLCl]nH_2O$ ($n = 2$) for Fe(II) complex. Five different antioxidant methods were used to determine the antioxidant capacity of the synthesized ligand and its metal complexes. BHA and BHT were preferred for standard antioxidants. The results obtained were compared with the results of 5-FU. The antioxidant study results showed that ligand and metal complexes have very high activities compared to 5-FU. It was determined that especially Ni(II) complex showed activity close to standard antioxidants. In addition, it was determined that ligand, Fe(II) and Co(II) metal complexes showed better activity than 5-FU. The new Co(II) and Ni(II) complexes show potential as new anticancer agents because they are highly cytotoxic to cancer cells (MCF-7) and have a low affinity for healthy cells (L-929). When the test compounds were used in combination with electroporation (ECT), their anticancer properties were significantly increased and MCF-7 cancer cell viability was greatly reduced. Our findings showed that novel Co(II) and Ni(II) complexes show potential as new anticancer agents and ECT may be a viable treatment option for breast cancer, particularly when systemic treatments have failed to control it.

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Declarations

Conflict of interest The authors have disclosed no conflict of interest. The authors have no relevant financial or non-financial interests to disclose.

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