Int. J. Bio-Inorg. Hybr. Nanomater., 9(1): 37-43, Spring 2020

Anti-tumor activity of Fe (III), Co(II) and Pd(II) complexes of N3-{phenyl [(4-pyridylcarbonyl)amino]methyl}

F. Shabani[1], S. Jamehbozorgi[2,*], M. Sheikhpour[3]

[1] Department of Chemistry, Science faculty, Arak Branch, Islamic Azad University, Arak, Iran
[2] Department of Chemistry, Science faculty, Hamedan Branch, Islamic Azad University, Hamedan, Iran
[3] Department of Mycobacteriology and Pulmonary Research, Microbiology Research Center (MRC), Pasteur Institute of Iran, Tehran, Iran

Received: 17 November 2019; Accepted: 19 January 2020

ABSTRACT: An anti-tumor compound as N3-{phenyl [(4-pyridylcarbonyl) amino] methyl} were synthesized and identified (NPPA). Fe (III), Co(II) and Pd(II) metal complexes of this ligand prepared by reaction of chloride salt of Fe (III), Co(II) and Pd(II) with NPPA in dry acetonitrile. Identification and Characterization of the ligand was performed by FT-IR, ^{1}H-NMR spectroscopy and elemental analysis and all complexes were characterized by several techniques using elemental analyze (C, H, N), FT-IR, ^{1}H-NMR (except Co complex), electronic spectroscopy and molar conductance measurements. These new compounds were used to biological studies; and its anticancer properties against the two types of cancer cells such as k562 (human chronic myeloid leukemia) and Jurkat (human T lymphocyte carcinoma) was revealed. The results of in vitro activities were very interesting and promising. These results showed that complexes offer a new outlook for chemotherapy. It should be noted that the results suggest that complex showed greater effects than ligand.

Keywords: *Anti-tumor; Anticancer; In vitro activities; N3-{phenyl[(4 pyridylcarbonyl)amino]methyl}; Fe(III), Co(II) and Pd(II) complexes; Synthesis.*

INTRODUCTION

Most therapeutic agents and drugs will first be tested in tissue culture on a suitable model system. For prospective anticancer drugs, for example, in vitro data obtained by proliferation or colony formation on the cytotoxicity of the agents (Farrell, 1989). Nicotinamide (nia) is a water soluble vitamin better known as Niacin. One of its coenzymes is NAD+ which, for example, converts alcohols to aldehydes and ketones when acting as an oxidising agent. Deficiency of this vitamin results in the disease Pellagra. Papaverine (pap) is an alkaloid derived from Papaver Somniferum and Rauwolfia Serpentine. It is a smooth muscle relaxant, vasodilator and antiasthmatic agent (Williams, *et al.*, 2003). The medicinal uses and applications of metals complexes are of increasing clinical and commercial importance (Williams, *et al.*, 2003). Transition metal complexes with potential biological activity are the focus of extensive investigations (Georgieva, *et al.*, 2006). Presently, there

(*) Corresponding Author - e-mail: sjamehbozorgi@gmail.com

is a growing interest in the coordination chemistry of structurally modified bio-ligands. Transition metal complexes with potential biological activity are the focus of extensive investigations (Singh, *et al.*, 2007). The modes of action of metal ions are often complex but are believed to involve bonding to the heteroatoms of the heterocyclic residues of biological molecules, i.e., proteins, enzymes, nucleic acids, etc. In ligand type compound, substituted amine compounds take special place in particular processes. The mechanism of their interaction with surrounding molecules in condensed medium in most cases is determined by hydrogen bonds, providing flexibility, speed and variety of biochemical reactions (Saydam and Yilmaz, 2006, Borisenko, *et al.*, 2008). Chemotherapy with Pt complexes is one of the main pillars in the treatment of cancer today. Out of thousands of synthesized and evaluated PtII complexes, only three cisplatin, carboplatin, and oxaliplatin have been approved for worldwide clinical practice (in 1978, 1993, and 2002, respectively), while nedaplatin, lobaplatin and heptaplatin have been approved as anticancer agents only in Japan, China and South Korea, respectively (Galanski, el al., 2003, Jakupec, *et al.*, 2003, Galanski, *et al.*, 2005). However, severe side effects and activity in a restricted spectrum of tumors as well as acquired or intrinsic resistance limit their successful therapeutic use. Therefore, alternative metal compounds are presently being evaluated in clinical trials (Clarke, 2003, Clarke *et al.*, 1999).

On this basis, studying different kinds of complexes of transition metals from among the ligands with biological properties and activities seems interesting. In this paper the synthesis, characterization and antitumor properties of a number of the first row transition metal complexes have been studied.

EXPERIMENTAL SECTION

MATERIALS AND METHODS

Iron(III) chloride hexahydrate, cobalt chloride hexahydrate and palladium chloride, were either Merck chemicals and were used without further purification. Organic solvents were reagent grade. All organic solvents were reagent grade. Infrared (IR) spectra were recorded as KBr pellets by using the Bruker Tensor 27-Model 420 IR spectrofotometer. The electronic spectra of all combinations have been recorded by model 350 UV/Visible machines of Camspec and Wpa bio Wave S2 100. Elemental analyses were performed by Heraeus CHNO-Rapid elemental analyzer. ^{1}H-NMR, ^{13}C-NMR and ^{19}F-NMR spectra were recorded on the Bruker-AVANCE DRX 500 machine; All the chemical shifts are quoted in ppm using the high-frequency positive convention; ^{1}H-NMR and ^{19}F- NMR spectra were referenced to external SiMe4 and $CFCl_3$ respectively, and finally for the purpose of obtaining the melting point Electro-thermal 9200 machine was used. Elemental analyses were performed by Heraeus CHNO-Rapid elemental analyzer.

CELL CULTURE ASSAY

The human chronic myeloid leukemia: K562 cell line and the human T lymphocyte carcinoma: Jurkat cell line, used for treatment with the compounds, was provided. K562 and Jurkat cells were grown at 37 °C in an atmosphere containing 5% CO_2, with RPMI-1640 MEDIUM HEPES Modification with L-glutamine and 25 mM HEPES (SIGMA-ALDRICH CHEMIE GmbH) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 2.7% sodium bicarbonate and 500 mg/L ampicillin.

SYNTHESIS OF THE NPPA LIGAND

To a magnetically stirred mixture of Nicotineamide (2.371 g, 1mmol) in hot methanol (20 mL) was added to the benzaldehyde (0.58 g,1mmol) via a syringe and refluxed for 24h at 20°C. After cooling to room temperature, the resulting white precipitate was filtered and washed with hexane (20 mL) (Fig. 1).

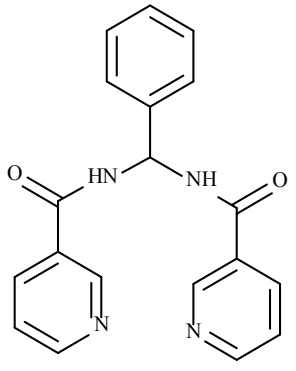


Fig.1. Structure of the ligand, NPPA.

^{1}HNMR (δ ppm $CDCl_3$, 300MHz): 914, 8.89, 8.84, 8.32, 8.22, 7.63 [q, 2d, s, 4H, pyridine), 7.21, 7.12 and 7.08 [3q, 2d, 5H, arom]; 8.1 [d, 1H, NH]; 6.08 [m, 1H, CH]; 3.03 [d, 2H, CH_2]. IR absorptions (cm^{-1} KBr): 3147.14 ν(N-H), 1665.47 ν(C=N), 1626 ν(C=O), 1466.33 ν(C=C), 1345.37 ν(C-N), 623-911.7 ν(C-H). Anal. Calc. for $C_{19}H_{16}N_4O_2$: theory: C, 68.62; H, 4.08; N, 16.85; found: C, 68.94; H, 4.33; N, 17.12. UV- vis (MeCN): λ_{max} 218 nm (e 200), λ 261 nm (e 62).

SYNTHESIS OF THE METAL COMPLEXES; GENERAL METHOD

A solution of metal salt dissolved in acetonitrile added gradually to a stirred acetonitrile solution of the ligand (NPPA), in the molar ratio 1:1 (metal: ligand). Total compound was mixed at room temperature; and in order to ensure the completeness of the reaction stirring was continued for 2–3 hour. The precipitated solid complexes were filtered, washed several times with 50% (v/v) ethanol–water to remove any traces of the unreacted starting materials. Together with the increase in sediment the mixture became colorful at the time of reaction; and finally colorful precipitation of the formed complex was rinsed using Hexane and diethyl ether and dried in vacuum desiccators over anhydrous $CaCl_2$ for the purpose of subsequent reviews.

ANALYSIS OF $[Fe(C_{19}H_{14}N_4O_2)]Cl_2$, NPPAIC

^{1}HNMR (δ ppm $CDCl_3$, 300MHz): 9.1-10.9 [q, 2d, s, 4H, pyridine), 7.1-7.95 [3q, 2d, 5H, arom]; 5.51 [t, 1H, CH]; 3.6 [d, 2H, CH_2]. IR absorptions (cm^{-1} KBr): 1716.19 ν(C=N), 1615.16 ν(C=O), 1459.88 ν(C=C), 1232.97 ν(C-N), 642-909 ν(C-H), 529.5 ν(Fe-N). Anal. Calc. for $[Fe(C_{19}H_{14}N_4O_2)]Cl_2$: theory: C, 46.31; H, 2.84; N, 11.37; found: C, 46.65; H, 3.08; N, 11.74. UV- vis (MeCN): λ_{max} 243 nm (ε 157), l 312 nm (ε 198), l 362 nm (ε 198).

ANALYSIS OF $[Co(C_{19}H_{14}N_4O_2)]Cl_2$, NPPACC

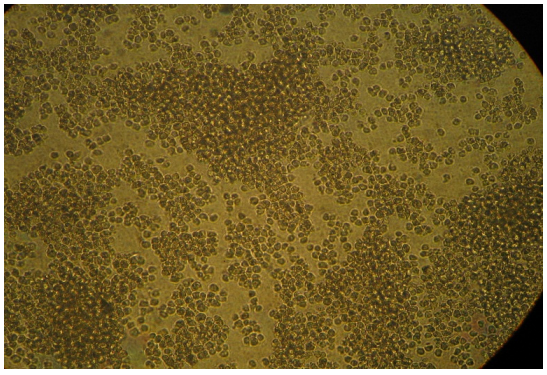
IR absorptions (cm^{-1} KBr): 1798.51 ν(C=N), 1690.4 ν(C=O), 1538.7 ν(C=C), 1410.6 ν(C-N), 673-904 ν(C-H), 521.7 ν(Co-N). Anal. Calc. for $[Co(C_{19}H_{14}N_4O_2)]Cl_2$: theory: C, 71.7; H, 4.88; N, 7.6 ; found: C, 72.05; H, 5.13; N, 7.95. UV- vis (MeCN): λ 214 nm (ε 220), λ_{max} 255 nm (ε 240), λ 590 nm (ε 20), λ 680 nm (ε 38).

ANALYSIS OF $[Pd(C_{19}H_{14}N_4O_2)]Cl_2$, NPPAPC

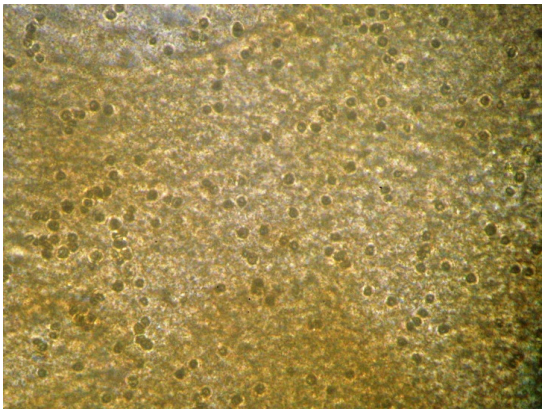
^{1}HNMR (d ppm $CDCl_3$, 300MHz): 8.2-8.4 [q, 2d, s, 4H, pyridine), 7.02-7.87 [3q, 2d, 5H, arom]; 5.91 [t, ^{1}H, CH]; 2.5 [d, 2H, CH_2]. IR absorptions (cm^{-1} KBr): 1714.9 ν(C=N), 1664.9 ν(C=O), 1520 ν(C=C), 1395 ν(C-N), 680-948 ν(C-H), 463.4 ν(Pd-N). Anal. Calc. for $[Co(C_{19}H_{14}N_4O_2)]Cl_2$: theory: C, 71.7; H, 4.88; N, 7.6; found: C, 72.05; H, 5.13; N, 7.95. UV- vis (MeCN): λ_{max} 260nm (ε 170).

IN VITRO ACTIVITIES

The two cell lines were provided by the Pastour Instutiute (Iranian Type Culture Collection (NCBI Code; C122 & C121). The procedure for cytotoxicity studies was similar to that reported earlier (Zhao, *et al.*, 1998). Briefly, K562 and Jurkat cell lines in (SIGMA) RPMI culture medium containing 10% FBS, 2 mmol L-Glotamine, 2gr/liter Sodium Bicarbonate, 100 Iu/ml penicillin and 100 micrograms Streptomycin was cultivated into the flask with 50 ml capacity. After the cell culture, when the number of cells reached to an appropriate level, transferring the cell into the plate was



(a)



(b)

Fig. 2. (a) Tumor cell after 72h without NPPAPC compound (b) Tumor cell after 72h with NPPAPC compound

done with 96 parts special for the Nunc culture. So that to each vial of the culture plate, a volume of K562 cell suspension and another volume of Jurkat cell suspension containing 5×104 cells in milliliter were added. Then desired ligand and complexes with specified dilutions are added to it; and using the Neobar Lam and Trypan blue method cellular fatality and cytotoxicity rate of materials and percentage of dead cells are investigated. The final concentration of DMSO in the growth medium was 2%(v/v) or lower, concentrations without effect on cell replication (Ishida, *et al.*, 1999, son, *et al.*, 2007). After incubation periods 72 h for all cell lines, the cell concentrations were determined both in control and in compound-treated cultures. All experiments were carried out in six times and series (Fig. 2).

RESULTS AND DISCUSSION

Preparation for Ligand, NPPA and Fe (III), Co(II) and Pd(II) complexes

The reaction of Fe (III), Co(II) and Pd(II) salts with the ligand, NPPA, results in the formation of [ML] for M= Fe (III), Co(II) and Pd(II). All complexes are quite stable and could be stored without any appreciable change. All complexes were characterized by several techniques using elemental analyze (C, H, N), FT-IR, electronic spectra and molar conductance measurements. The elemental analysis data suggest the stoichiometry to be 1:1 [M: L] ratio formation. The molar conductance measurements reveal the presence of 1:1 electrolytic nature complexes. The NPPA ligand have 215-218°C melting point and the $[Pd(C_{19}H_{14}N_4O_2)]Cl_2$ complex do not have sharp melting point but decompose above 350°C, but $[Fe(C_{19}H_{14}N_4O_2)]Cl_2$ and $[Co(C_{19}H_{14}N_4O_2)]Cl_2$ complexes have 59-62°C and 214-216.4 °C melting point respectively. They are insoluble in common organic solvents, such as ethanol, methanol, chloroform or acetone. However, they are soluble in DMSO and DMF. Their structures were characterized by elemental analysis, ^{1}H-NMR (except Co Complex), and IR. Their elemental analyses are in accord with their proposed formula. The spectral data of the complexes have good relationship with the literature data.

Cytotoxicity studies

In this research, two cell lines concerning the blood cancer named with K562 (human chronic myeloid leukemia) and Jurkat (human T lymphocyte carcinoma) were selected. After culturing the cells and reaching to the desired value, complexes and ligand were prepared in six different concentrations from 0.1-300μM for NPPA, 0.1-100μM for $[Fe(C_{19}H_{14}N_4O_2)]Cl_2$, 0.1-480μM for $[Co(C_{19}H_{14}N_4O_2)]Cl_2$ and 0.1-160μM for $[Pd(C_{19}H_{14}N_4O_2)]Cl_2$ in DMSO; and after filtering by filter (0.2 plastic) 1λ was added to the micro plates of 96 parts which are present in each one of the wells containing 5×10^4 cells. Every 24 hours micro plates were monitored. The under the hood laminar was filtered by 0.2 plastic filter. This takes place to remove the possible contaminations from bacteria and mushroom. The number of cells into the flask containing K562 and Jurkat cells was calculated using Neobar Lam. Given that the number of cells in each one of the plate vials should be 96 parts of 5×10^4,185/18μL of cell line of K562 was transferred to each one of the vials of the plate by sampler from the fresh environment. This was repeated for six times in 6 rows of the plate for the purpose of reducing the errors. In each row in order to control the effects, complex of the two vials was used as control. These two vials do not contain any concentration of the complexes or ligand. The mentioned plate was transferred to a 37°C and 5% CO_2 incubator. Checking the status of the cells was performed every 24 hours. After 72 hours, the number of cells in each row and any concentration was counted by Neobar Lam. For this purpose, at first the number of control vials and then the numbers of vials beginning from the most dilute to the most concentrated ones were counted. The IC_{50} cytotoxicity values of the complexes were compared to those found for the starting organic bases as well as for some of the anticancer agents used nowadays, that are cisplatin and oxaplatin compounds (Kim, *et al.*, 2004). The corresponding 50% and 90% inhibitory doses (IC_{50} and IC_{90}) values are shown in Table 1.

The results were very interesting and promising; and in the following we deal with this part of the study. It is clear from the above discussion Fe (III), Co(II) and Pd(II) complexes offer a new outlook for chemotherapy. It should be noted that the results suggest that

Table 1. 72 hour IC_{50} and IC_{90} values (μM) obtained for four compounds.

Compounds	IC_{50} for Cell line		IC_{90} for Cell line	
	K562	Jurkat	K562	Jurkat
NPPA	>25	>22	-	-
NPPAIC	>10	>10	>70	>68
NPPACC	> 8.5	>8	>40	>35
NPPAPC	>9	>9	>75	>62

complex showed greater effects than ligand. These results are concerned with the effect of the metal on complex; and this, causes increase in its Cytotoxic and Inhibitory effects.

The mechanism by which these complexes act as antitumor agents is apoptosis. It has been proposed that there is close relationship between concentration and inhabitation, so that concentration plays a vital role in increasing the degree of inhabitation. (Shabani, *et al.*, 2008, Germi, *et al.*, 2014, Shabani, *et al.*, 2015, Shabani, *et al.*, 2010a, Shabani, *et al.*, 2009a, Shabani, *et al.*, 2009b, Shabani, *et al.*, 2010b, Shabani, *et al.*, 2010c, Shabani, *et al.*, 2012). Although apoptosis may be a natural physiological occurrence, excessive apoptosis results in tissue damage. Alternatively, apoptosis may be viewed as the response to hyper proliferation in an attempt to reduce tissue growth (Peek, *et al.*, 1999).

ACKNOWLEDGMENT

We gratefully acknowledge the financial support from the Research Council of Arak Islamic Azad University and many technical supports that provided by Tehran University.

REFERENCES

Borisenko, V. E.; Krekov, S. A.; Fomenko, M. Yu.,Koll, A.; Lipkovski, P.; (2008) Influence of methoxy-and nitro-substitutions in the aromatic ring on proton donation ability in hydrogen bond and on theamino group parameters of free and H-bonded molecules of 2-aminopyrimidine, J. M. Stru., 882: 9-23.

Clarke, M.J.; Zhu, F.; Frasca, D.R.; (1999). Non-platinum chemotherapeutic metallo pharmaceuticals. Chem. Rev., 99: 2511–2533.

Clarke, M.J., (2003). Ruthenium metallopharmaceuticals, Coord. Chem. Rev. 236: 209–233.

Farrell, N.; (1989). Metal Complexes as Drugs and Chemotherapeutic Agents, Springer Netherlands.

Galanski, M.;Arion, V.B.; Jakupec, M.A.; Keppler, B.K.; (2003). Recent developments in the field of tumor-inhibiting metal complexes, Curr. Pharm. Des., 9: 2078–2089.

Galanski, M.; Jakupec, M.A.; Keppler, B.K.; (2005). Update of the Preclinical Situation of Anticancer Platinum Complexes: Novel Design Strategies and Innovative Analytical Approaches, Curr. Med. Chem., 12: 2075–2094.

3Georgieva, I.; Tredafilova, N.; Bauer, G.;(2006). Spectroscopic and theoretical study of Cu(II), Zn(II), Ni(II),Co(II) and Cd(II) complexes of glyoxilic acid oxime. Spectrochim. ActaA, 63(2): 403-415.

Germi, K. G.h.; Shabani, F.; Khodayari, A.; Azizian, Y.; (2014). Structural and biological properties of CuO nanoparticles prepared under ultrasonic irradiation, Synthesis and Reactivity in Inorganic, Metal- Organic, and Nano-Metal Chemistry, 44: 1286–1290.

Ishida, J.; Wang H.K.; Bastow, K.F., Hu, C.Q., Lee, K.H.; (1999). .Antitumor agents 201. cytotoxicity of harmine and β-carboline analogs. Bioorganic & Medicinal Chemistry Letters, 9: 3319-3329.

Jakupec, M.A.; Galanski, M.; Keppler, B.K.; (2003). Tumour-inhibiting platinum complexes--state of

the art and future perspectives, Rev. Physiol. Biochem. Pharmacol., 146: 1–53.

Kim, Y.S.; Song. R.; Chung, H.C.; Jun, M.J.; Sohn, Y.S.; (2004). Coordination modes vs. antitumor activity: synthesis and antitumor activity of novel platinum(II) complexes of N-substituted amino dicarboxylic acids, Journal of Inorganic Biochemistry, 98: 98–104.

Peek, R.M.Jr.; Blaser, M.J.; Mays D.J.; Forsyth, M.H.; Cover, T.L.; Song, S.Y.; Krishna, U.; (1999). Pietenpol, Helicobacter pylori strain-specific genotypes and modulation of the gastric epithelial cell cycle, J.A. Cancer Res., 59: 6131–6124.

Saydam, S.; Yilmaz, E.;(2006).Synthesis, Characterization and Thermal Behavior of 4- chloromethyl-2-(2- hydroxybenzilidenehydrazino) thiazole and its complexes with Cr(III), Co(II), Ni(II) and Cu(II), M. and Biom. Spect.; 63(2): 506-516.

Shabani, F.; Ghammamy, S.; Mehrani, K.; (2008). Anti-tumor activity of 6-(cyclohexylamino)-1,3-dimethyl-5(2-pyridyl)furo[2,3-d] pyrimidine-2,4(1H,3H)-dione and its Ti (IV), Zn(II), Fe (III) and Pd(II) complexes on K562 and Jurkat cell lines, Bioorg. Chem. Appl., 10: 501021-5.

Shabani, F.; Ghammamy, S.h.; (2009). Synthesis, characterization and anti-tumor activity of Ti (IV) and Mn(II) complexes of N4[(E,2E)-3-Phenyl-2-propenylidene] isonicotinohydrazide, Der Pharma Chemica, 1: 30–36.

Shabani, F.; Ghammamy, S.h.; (2009). Synthesis, characterization and anti-tumour activity of VOF3 and Cu (II) porphyrine complexes, Der Pharma Chemica, 1: 124–129.

Shabani, F.; Ghammamy, S.h.; (2010). Anti-tumor Activity of N4[(E)-1-(2-hydroxyphenyl) methylidene] isonicotinohydrazide and Its Ti (IV) and Cu (II) complexes on K562 and Jurkat, Journal of Chemical and Pharmaceutical Research, Res., 2: 1-6.

Shabani, F.; Saghatforoush, L.A.; Ghammamy, S.; (2010). Synthesis, characterization and Anti-tumor activity of Iron (III) Schiff base complexes with unsymmetric tetradentate ligands, Bull. Chem. Soc. Ethiopia, 24: 1–7.

Shabani, F.; Ghammamy, Sh.; (2010). Anti-tumor activity of N4 [(E)-1-(2-hydroxyphenyl) methylidene], N4-[(E)-2-Phenylethylidene], N4 [(E,2E)-3-Phenyl-2-propenylidene] and N4[(E)ethylidene] isonicotinohydrazide on K562 and Jurkat cell lines, J. Young Pharm., 2: 399–402.

Shabani, F.; Germi, K.G., Khodayari, A.; (2012). Biological(anti-tumor and anti-bacterial) studies of new Halo complexes, World Appl. J., 18: 186–190.

Shabani, F.; Khodayari, A.; Azizian, Y.; (2015). Structural, compositional and biological characterization of Fe3O4 nanoparticles synthesized by hydrothermal method, Synthesis and Reactivity in Inorganic, Metal-Organic, and Nano-Metal Chemistry, 45: 356–362.

Singh, B.K.; Jetley, U.K.; Sharma, R.K.; Garg, B.S.; (2007). Synthesis, characterization and biological activity of complexes of 2-hydroxy-3,5-dimethylacetophenoneoxime (HDMAOX) with copper(II), cobalt(II), nickel(II) andpalladium(II), Spectrochimica Acta Part A, 68: 63–73.

Son, J.K.; Zhao L.X.; Basnet, A.; Thapa, P.; Karki, R.; Na Y.; Jahng, Y.; Jeong, T.C.; Jeong, B.S.; Lee, C.S.; Lee, E.S.; (2007). Synthesis of 2, 6 –diaryl-substituted pyridines and their antitumor activities, European Journal of Medicinal Chemistry, 12: 1-8.

Williams, C.J.;Morris, H.; Svorec, J.; Valkov, M.; Valkob, M.; Moncol, J.; Mazur, M.; Valach, F.; Melnikv, M.; (2003). A study of copper(II) carboxylato complexes with the biological ligands nicotinamide and papaverine. Journal of Molecular Structure, 659: 53–60.

Zhao, G.; Lin, H.; Zhu, S.; Sun, H.; Chen, Y.; (1998) Dinuclear palladium(II) complexes containing two monofunctional [Pd(en)(pyridine)Cl]+ units bridged by Se or S. Synthesis, characterization, cytotoxicity and kinetic studies of DNA-binding, J. Inorg. Biochem., 70: 219-26.

AUTHOR (S) BIOSKETCHES

Fahmideh Shabani, Ph.D., Department of Chemistry, Science faculty, Arak Branch, Islamic Azad University, Arak, Iran

Saeed Jamehbozorgi, Associate Professor, Department of Chemistry, Science faculty, Hamedan Branch, Islamic Azad University, Hamedan, Iran, *Email: sjamehbozorgi@gmail.com*

Mojgan Sheikhpour, Assistant Professor, Department of Mycobacteriology and Pulmonary Research, Microbiology Research Center (MRC), Pasteur Institute of Iran, Tehran, Iran