Asian Journal of Research in Biochemistry

5(1): 1-9, 2019; Article no.AJRB.50401
ISSN: 2582-0516

In-vitro Biochemical Studies on Organometallic Compounds as Anticancer Agents

F. Z. Mohammed[1*], Elsherbiny H. Elsayed[2], Atef E. Abd Elbaky[3] and H.M. Shalaby[4]

[1]Department of Biochemistry, Faculty of Science, Zagazig University, Egypt.
[2]Department of Organic Chemistry, Faculty of Science, Port Said University, Egypt.
[3]Department of Biochemistry, Faculty of Pharmacy, Port Said University, Egypt.
[4]Department of Biochemistry, Faculty of Science, Port Said University, Egypt.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJRB/2019/v5i130080
Editor(s):
(1) Dr. Sulaiman Shams, Assistant Professor, Department of Biochemistry, Abdul Wali Khan University Mardan, Pakistan.
Reviewers:
(1) Rodica Olar Inorganic, University of Bucharest, Romania.
(2) Aydin Tavman, Istanbul University-Cerrahpasa, Turkey.
Complete Peer review History: http://www.sdiarticle3.com/review-history/50401

Original Research Article

Received 15 May 2019
Accepted 22 July 2019
Published 27 July 2019

ABSTRACT

This study aims to synthesis of copper complexes of 2,3-dihydroxy benzaldehyde thiosemicarbazone (3a,b), followed by evaluating their *in vitro* anticancer properties. The prepared compounds have been also evaluated for their ability to induce apoptosis. A total number of 80 adult female Swiss albino mice weighing 20-25 gm were divided randomly into 8 groups (10 mice /each group). The *in vitro* cytotoxic activities of compounds (3a, 3b) were evaluated. Minimum inhibitory concentrations of synthesized compound 3a were found to be 50 µg/mL against MCF-7, HepG2 and PC3 cell lines; also, Minimum inhibitory concentrations of synthesized compound 3b were found to be 50 µg/mL in all cell lines. The apoptotic effect of compounds 3a and 3b were evaluated by measurement Caspase-3 activity and Bcl-2 concentration. The mean values of Caspase-3 activity in positive control were found to be 2.6151 (ng/mL). On the other hand, the mean values of Bcl-2 in positive control were found to be 3.7 (ng/mL). The compounds (3a & 3b) exhibited a significant anticancer activity towards MCF-7, HEPG2 and PC3 cancer cell lines.

**Corresponding author: E-mail: mahmoud.m4206@gmail.com;*

Keywords: In vitro anticancer properties; copper complexes; 2,3-dihydroxy benzaldehyde thiosemicarbazone; adult female Swiss albino mice; MCF-7; HepG2 and PC3 cell lines.

1. INTRODUCTION

Cancer is one of the leading causes of death in the developed world. Tumour is a group of cells that have undergone un- regulated growth, and will often form a mass or lump, but may be distributed diffusely [1]. Carcinogenesis, also called oncogenesis or tumorigenesis, is the formation of a cancer, where normal cells are transformed into cancer cells. The process is characterized by changes at cellular, genetic, and epigenetic levels and abnormal cell division. Cell division is a physiological process that occurs in almost all tissues and under a variety of circumstances. Normally the balance between proliferation and programmed cell death, in the form of apoptosis, is maintained to ensure the integrity of tissues and organs [2].

Cancer is a disease characterized by failure of tissue growth regulation when the genes that regulate cell growth and differentiation are altered. Most cancers have multiple causes, only a small minority of cancer are due to inherited genetic mutations whereas the vast majority are non-hereditary epigenetic mutations that are caused by various agents (environmental factors, physical factors and hormones). Thus, although there are some genetic predispositions in a small fraction of cancers, the major fraction is due to a set of new genetic mutations (called “epigenetic” mutations) [3].

The quest for alternative drugs to the well-known cisplatin and its derivatives, which are still used in more than 50% of the treatment regimes for patients suffering from cancer, is highly needed. Despite their tremendous success, these platinum compounds suffer from two main disadvantages: they are inefficient against platinum-resistant tumors, and they have severe side effects such as nephrotoxicity. The latter drawback is the consequence of the fact that the ultimate target of these drugs is ubiquitous. In this context, organometallic compounds, which are defined as metal complexes containing at least one direct, covalent metal-carbon bond, have recently been found to be promising anticancer drug candidates [4].

Current emphasis towards the development organometallic chemotherapeutics has attracted many researchers in the search for new cancer therapeutic agents with improved activity and less toxicity [5]. For centuries, organometallic compounds have been reviewed as catalysts, but are now studied in the exploration of new potential anticancer drugs after the landmark investigation on titanocene by Kopf and Kopf-Maier. Subsequently, several titanocene-based organometallic complexes entered clinical trials and their mechanism of action was found to be different to that of the clinically approved drug cisplatin [6].

This study aims to the synthesis of copper complexes of 2,3-dihydroxy benzaldehyde thiosemicarbazone (3a,b), followed by evaluation of their in vitro anticancer properties and their ability to induce apoptosis.

2. MATERIALS AND METHODS

2.1 Materials

Denovo synthesized copper complexes.

Chemicals for synthesis of copper complexes of 2,3- dihydroxy- benzaldehyde thiosemicarbazones (3a,b) : 2,3-dihydroxybenzaldehyde; 5,6 –dibromo -2,3-dihydroxybenzaldehyde; Thiosemicarbazide; Copper chloride; Ammonium hydroxide (10%) and Ethanol.

Sigma Aldrich Chemical Co., St. Louis, Mo, U.S.A., was the source of the following chemicals: RPMI-1640 medium, Trypan blue, Fetal Bovine Serum, Penicillin/ Streptomycin antibiotic and Trypsin- EDTA.

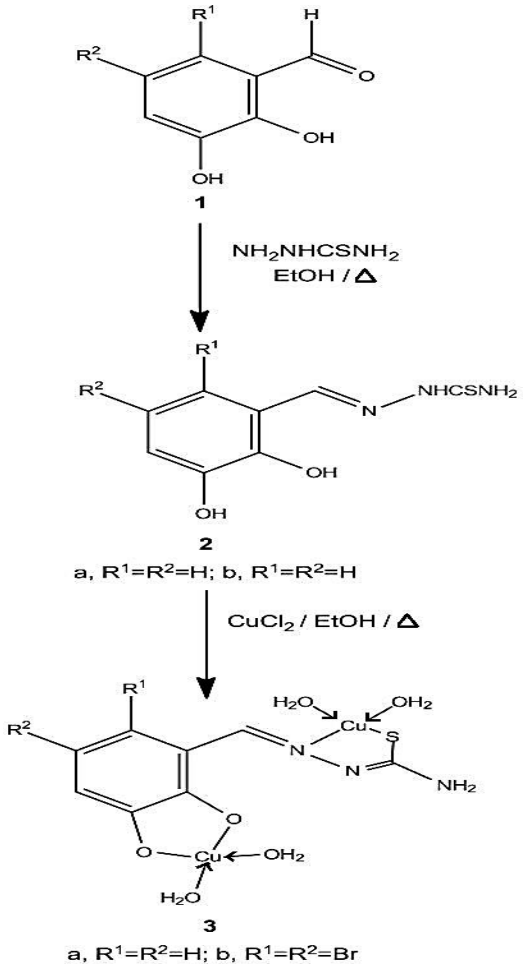
RPMI-1640 medium, Trypan blue dye, Fetal Bovine Serum (FBS), Penicillin/ Streptomycin, Trypsin- EDTA.

Human tumor cell lines MCF-7 (human breast cancer), HePG2 (Hepatocellular carcinoma), and PC3 (human prostate cancer) cell lines were used in this study obtained from the American Type Culture Collection (ATCC, Minisota, U.S.A.). The tumor cell lines were maintained at the National Cancer Institute, Cairo, Egypt, by serial sub- culturing. The study was approved by the ethical committee of Port Said University.

2.2 Methods

Synthesis of 2,3-dihydroxybenzaldehyde thiosemicarbazones derivatives (2a, b) were obtained via the condensation of aromatic

aldehydes (namely , 2,3-dihydroxybenzaldehyde & 5,6-dibromo-2,3- dihydroxybenzaldehyde) with thiosamicarbazide in ethanol under reflux. The copper complexes of 2,3-dihydroxybenzaldehyde thiosemicarbazones derivatives (3a, b) were prepared from the reaction of thiosemicarbazone derivatives (2a,b) with two mole of copper chloride in ethanol under reflux (Scheme 1).



Scheme 1. Synthesis of the compounds (2a, 2b, 3a & 3b)

Groups of the study: A total number of 80 adult female Swiss albino mice weighing 20-25 gm were divided randomly into 8 groups (10 mice /each group) as following:

Group 1: Negative Control: This group received sterile saline solution (0.9 % NaCl) day after day for 9 days.

Group 2: Positive Control: This group received Ehrlich ascites carcinoma (EAC), (2.5×106 cells/ 0.3 ml/mouse) by (I.P) injection once at the first day.

Group 3: Drug group I: This group consisted of 10 mice were injected I.P. with compound 3a (5 mg/Kg) at 1, 3, 5, 7, 9 days for 10 days (day after day).

Group 4: Preventive group I: (EAC + compound 3a): This group were injected I.P. with compound 3a (5 mg/Kg) in the day before EAC injection (2.5×106 cells/mouse), followed by I.P. injection of compound 3a at 3, 5, 7, 9 days of EAC injection for 10 days (day after day).

Group 5: Therapeutic group I: (EAC + compound 3a): This group were injected I.P. with compound 3a (5 mg/Kg) in the day after EAC injection (2.5×106 cells/mouse), followed by I.P. injection of compound 3a at 3, 5, 7, 9 days of EAC injection for 10 days (day after day).

Group 6: Drug group II: This group were injected I.P. with compound 3b (10 mg/Kg) at 1, 3, 5, 7, 9 days for 10 days (day after day).

Group 7: Preventive Group II: (EAC + compound 3b): This group were injected I.P. with compound 3b (10 mg/Kg) in the day before EAC injection (2.5×106 cells/mouse), followed by I.P. injection of compound 3b at 3, 5, 7, 9 days of EAC injection for 10 days (day after day).

Group 8: Therapeutic Group II: (EAC + compound 3b): This group were injected I.P. with compound 3b (10 mg/Kg) in the day after EAC injection (2.5×106 cells/mouse), followed by I.P. injection of compound 3b at 3, 5, 7, 9 days of EAC injection for 10 days (day after day).

Blood, EAC and tissue sampling: At the end of the experiment, the blood samples were collected from the retro-orbital venous plexus under light ether anesthesia divided to 2 parts to obtain serum and plasma. Serum was prepared by centrifuging blood at 3000 r.p.m for 10 minutes. Serum samples were aliquoted and stored at -20°C until biochemical analysis [7].

Determination and counting of viable cells: 1-50 µL of 0.05 % Trypan blue solution was added to 50 µL of the single cell suspension. The cells were examined under the inverted microscope using the haemocytometer. Non stained (viable) cells were counted and the following equation was used to calculate the cell count /ml of cell suspension.

Viable cells /mL = number of cells in 4 quarters X 2 (dilution factor) X104

The cells were then diluted to give the required cell number for each experiment.

Cryopreservation of cells: To avoid the loss of the cell line, excess cells were preserved in liquid nitrogen as follows: Equal parts of the cell suspension and freezing medium (10% DMSO In supplemented medium) were dispersed to cryotubes. The cryotubes were racked in appropriately labeled polystyrene boxes, gradually cooled till reaching -80 oC. Then the cryotubes were stored in a liquid nitrogen (-180 oC) till use [8].

Determination of potential cytotoxicity of synthetic compounds on human cancer cell line: The cytotoxicity was carried out using Sulphorhodamine-B (SRB) assay. SRB is a bright pink aminoxanthrene dye with two sulphonic groups. It is a protein stain that binds to the amino groups of intracellular proteins under mildly acidic conditions to provide a sensitive index of cellular protein content [9].

Apoptosis Assays: Colorimetric assay of caspase-3 activity [10] and Detection of Bcl2 by Enzyme Linked Immunosorbent Assay (ELISA) [11].

2.3 Statistical Analysis

All statistical analyses were done by a statistical for social science package "SPSS" 14.0 for Microsoft Windows, SPSS Inc and considered statistically significant at a two-sided $P < 0.05$. Numerical data were expressed as mean ± SD. The levels of markers were analyzed by ANOVA. The correlations between serum biochemical data in different studied groups were evaluated by Pearson's correlation coefficient, to quantify the relationship between the studied parameters. P value < 0.01 was considered significant [12].

3. RESULTS

Analysis of prepared compounds:

Copper complexes of 2,3-dihydroxyben-zaldehyde thiosemicarbazone (3a).

Orange powder, yield m.p >350ºC. IR(KBr) vmax= br-3650-2955 (H2O), 3291, 317.25 (NH2), 1617 (C=N), 1102, 1078(C-O). Calced For C8H15N3O6S CU2=C, 23.52 H,3.61,N,10.29. Found: C, 23.36, H, 3.41, N, 10.22.

Copper Complex of 5,6-dibromo- 2,3-dihydroxybenzaldehyde thiosemicarbazone (3b)

Red powder, yield 648, m.p> 350 ºC. IR (KBr): 3630-295 (br-H2O), 3288, 3183 (NH2), 1626 (C=N), 1227, 1081, 1057 (C-O) cm.-1 Calcd for (C8H13Br2N3O6SCu2): C, 17.02, H, 2.30, N, 7.45. Found: C, 16.78, H, 2.03, N, 7.03.

2,3-dihydroxybenzaldehyde thiosemicarbazone (2a):

Pale yellow crystals, yield 78%, m.p. 215ºC. IR(KBr) vmax=3365(OH), 3292, 3176(NH2), 3257(NH), 1620(C=N),1602, 1583(C=C), 1473(C=S), 1278, 1159, 1105(C-O)cm-1. 1H-NMR(DMSO-d6)S: 6.62-6.83 (dd,2H,NH2), 7.30 (d,1H,Ar-H), 7-80-8-0.7 (m,2H,Ar-H), 8.38 (S,1H,CH=N), 4.01-9.41 (br-s), 2H,2x H), 11.36 (S,1H,NH) ppm. 13CNMR (DMSO-d6) S: 178.05 (C=S), 145.99, 145-70 (2xC-O), 140.79(C==N),121.35, 119.52,117.58,116.89(C-aromatic) ppm. Calcd for C8H9N3O2S (Mwt =211): C, 45.49,H,4.26,N,19.90. Found: C,45.32, H,4.07, N,19.62.

5,6- dibromo-2,3- dihydroxybenzaldehyde thiosemicarbazone (2b):

Orange crystals, yield 7240, m.p. 236 ºC.IR (kBr). vmax= 33682(OH), 3305, 3189 (NH2) 3259 (NH), 1621, 1583 (C=C), 1472 (C=S), 1215, 1153, 1093 (C-O) cm.-1. Calcd for C8H7Br2 N3O2S (Mwt=367):C, 26.16,H, 1.91, N,11,44 Found : C,26.03,H,1:72,N,11.22.

The in vitro cytotoxic activities of compounds (3a, 3b) were evaluated. Minimum inhibitory concentrations of synthesized compound 3a were found to be 50 µg/mL against MCF-7, HepG2 and PC3 cell lines; also, Minimum inhibitory concentrations of synthesized compound 3b were found to be 50 µg/mL in all cell lines.

The apoptotic effect of compounds (3a, 3b), was evaluated through the estimation of caspase-3 and Bcl2 levels. The mean values of caspase-3 activity in positive control were found to be **2.6151** (ng/mL). Furthermore, the treatments with 3a and 3b showed a significant increase in caspase-3 activity, **4.85** & 3.66 ± (ng/ml) ($p<0.001$); in comparison to the positive control group.

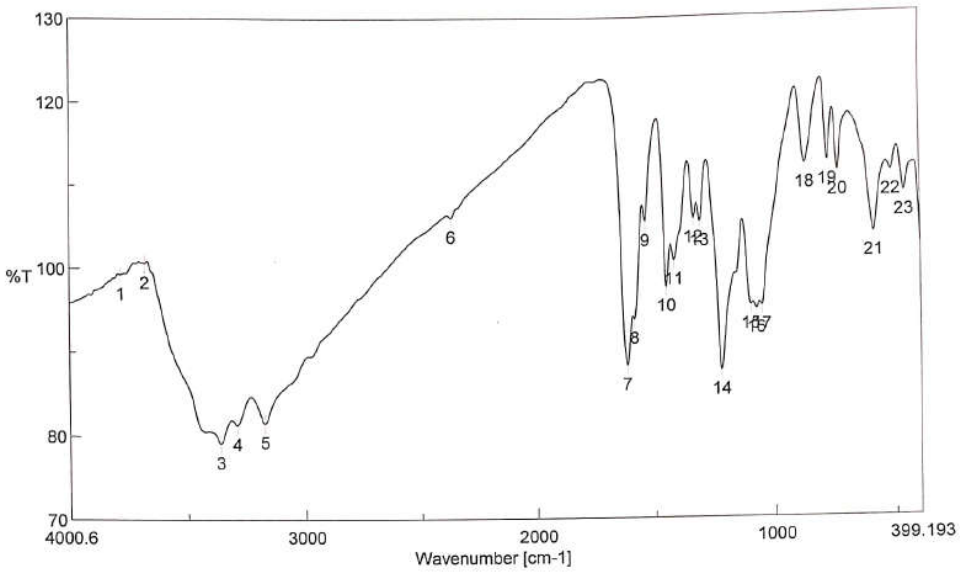


Fig. 1. IR spectroscopy of compound 3a

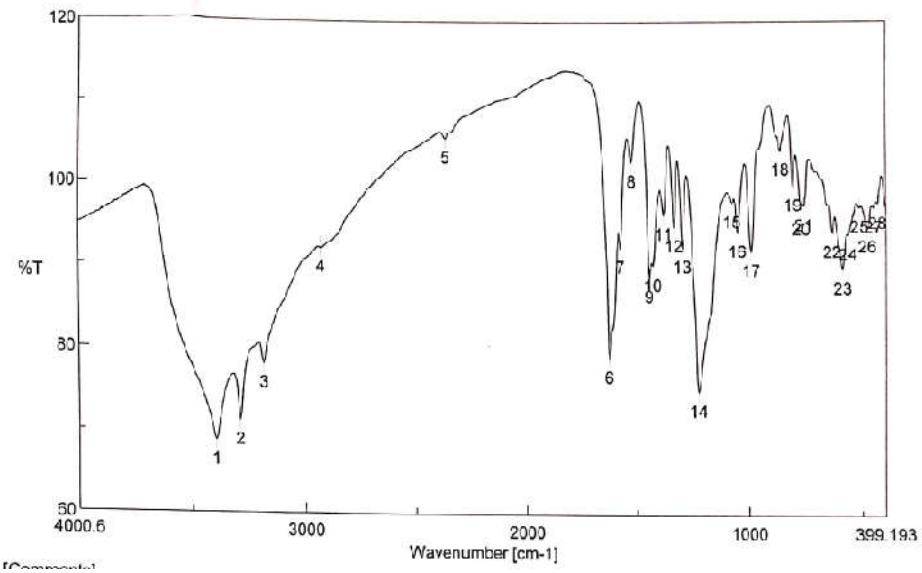


Fig. 2. IR spectroscopy of compound 3b

Table 1. Minimum inhibitory concentration of compound 3a against MCF-7 cell line

Concentration (µg/ml)	MCF7-1
0.000	1.000
5.000	0.800
12.500	0.360
25.000	0.400
50.000	0.356

Table 2. Minimum inhibitory concentration of compound 3a against HepG2 cell line

Concentration (µg /ml)	HEPG2 – 1
0.000	1.000
5.000	0.768
12.500	0.709
25.000	0.577
50.000	0.291

Table 3. Minimum inhibitory concentration of compound 3a against PC3 cell line

Concentration (µg /ml)	PC3 – 1
0.000	1.000
5.000	0.922
12.500	0.711
25.000	0.702
50.000	0.565

Table 4. Minimum inhibitory concentration of compound 3b against MCF-7 cell line

Concentration (µg /ml)	MCF7-2
0.000	1.000
5.000	0.680
12.500	0.640
25.000	0.516
50.000	0.496

Table 5. Minimum inhibitory concentration of compound 3b against PC3 cell line

Concentration (µg /ml)	PC3 – 2
0.000	1.000
5.000	0.962
12.500	0.923
25.000	0.923
50.000	0.885

Table 6. Minimum inhibitory concentration of compound 3b against HepG2 cell line

Concentration (µg/ml)	HEPG2 – 2
0.000	1.000
5.000	0.573
12.500	0.455
25.000	0.417
50.000	0.364

Table 7. The effect of compounds (3a, 3b) effect on caspase-3 activity in all studied groups (mean + SD)

Group	SD Mean + SD (ng/ml)
Negative	0.14541 +4.8988
Positive	0.37377 +2.6151
Drug I	1.24961 +7.2785
Thr I	0.35076 +4.8452
Prev I	0.31448 +5.0785
Drug II	0.37329 +7.4477
Thr II	0.22552 +3.6605
Prev II	0.37144 +4.5075

On the other hand, the mean values of Bcl-2 in positive control were found to be **3.7** (ng/mL). Furthermore, the treatments with 3a and 3b showed a significant decrease in Bcl-2 which found to be **2.9** & **2.9**2 (ng/mL) ($p<0.001$), in comparison to the positive control group.

Table 8. The effect of compounds (3a, 3b) on Bcl2 activity in all studied groups (ng/mL)

Group	Mean + SD
Negative	0.01002+ 2.4734
Positive	0.04840 +3.7013
Drug I	128360 +2.6513
Thr I	0.0717+2.9089
Prev I	0.15130+2.7310
Drug II	0.19262+3.0924
Thr II	0.08258+2.9171
Prev II	0.10419+2.7918

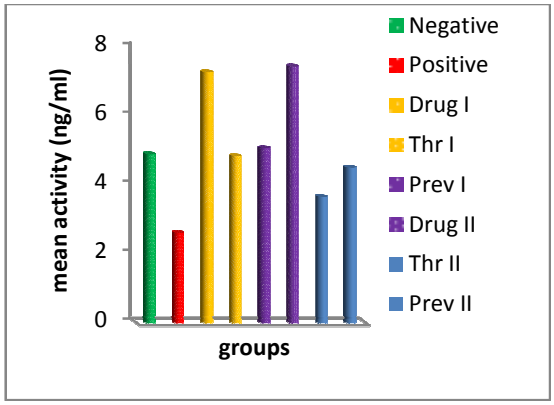


Fig. 1. The effect of compounds (3a, 3b) effect on caspase-3 activity in all studied groups

4. DISCUSSION

Cancer is a complex disease characterized by at least six hallmark characteristics. Some of these hallmarks, such as proliferation and resistance to cell death (including apoptosis) act at a cellular level and are frequently caused by changes in the genome. An increased rate of cellular proliferation is frequent, but not exclusively found in cancer cells [13]. Most cancer cells divide more often than normal cells and the process of cell division can be targeted to treat cancer patients. The aim of targeting cell proliferation is to arrest the cell cycle and/or cause cancer cell death using cytotoxic compounds (chemotherapy) or ionizing radiation (radiation therapy). DNA is one of the main targets of these therapies because DNA replication is an essential phase of the cell cycle. Many of the cytotoxic agents commonly used to treat cancer patients cause high levels of DNA damage, that initiate cell cycle checkpoints, leading to cell cycle arrest and/or cell death [14].

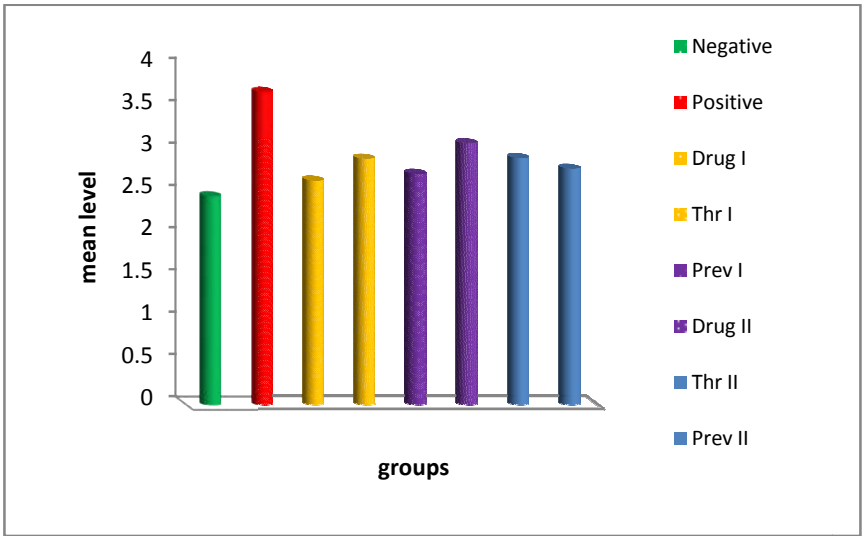


Fig. 2. The effect of compounds (3a, 3b) on Bcl2 activity in all studied groups

The search for new chemo-preventive and anti-tumor agents that are more effective and less toxic has kindled great interest [15]. Medicinal organometallic complexes consist of platinum, ruthenium, iron, titanium, and gold among other metals. Fundamental studies have been carried out on the organometallic complexes in which the mechanism of action exert their medicinal effect (e.g., induce cell death in cancer cells), the synthesis of new organometallic complexes and the development of combination therapies containing organometallic components. Research has shown significant progress in utilization of transition metal complexes as anticancer agents [16].

Thiosemicarbazones have emerged as ligands of great biological activity. The ability of thiosemicarbazones to chelate metal ions has now been recognized as a major factor in their antiproliferative effects [17].

In the present study, we aimed to evaluate the anti-tumor properties of recently developed synthetic copper complexes of 2,3-dihydroxy benzaldehyde thiosamicarbazne (3a,b), as anticancer agents, followed by evaluating for their in vitro anticancer properties against breast, liver and prostate cancer cell lines.

The in vitro cytotoxic activities of compounds (3a, 3b) were evaluated. Minimum inhibitory concentrations of synthesized compound 3a were found to be 50 µg/mL against MCF-7, HepG2 and PC3 cell lines; also, Minimum inhibitory concentrations of synthesized compound 3b were found to be 50 µg/mL in all cell lines, Compared to Koňariková et al., who studied the cytotoxicity of new copper complex derivatives against MCF-7 breast cancer cell line, and found that they have antiproliferative activity against cancer cells but not against healthy cells as they have induced autophagy in the cancer cell line MCF-7 [18].

Copper-based complexes have been investigated on the assumption that endogenous metals may be less toxic to normal cells compared to cancer cells and can induce apoptotic cell death or autophagy. Apoptotic cell death and autophagy are programmed cell deaths without inflammation of the surrounding healthy tissue [19].

Also, other authors have reported that Schiff base Cu(II) complexes exhibit significant cytotoxic effects against several cancer cell lines, such as PC3 (human prostate cancer cell line) [20], MCF-7 (human breast carcinoma cells), apoptosis in human liver cancer cell line Hep-G2 cells [21].

The apoptotic effect of compounds 3a and 3b were evaluated by measurement Caspase-3 activity and Bcl-2 in the EAC cells.

The mean values of Caspase-3 activity in positive control were found to be 2.6151 (ng/mL). Furthermore, the treatments with 3a and 3b showed a significantly increase in Caspase-3 activity, 4.85 & 3.66 ± (ng/ml) respectively ($p<0.001$); compared to the positive control

group. On the other hand, the mean values of Bcl-2 in positive control were found to be 3.7 (ng/mL). Furthermore, the treatments with 3a and 3b showed a significantly decrease in Bcl-2, 2.9 & 2.92 (ng/mL) respectively ($p<0.001$), compared to the positive control group. Our results are in concordance with previous studies which concluded the apoptotic effect of copper thiosemicarbazone complex derivatives [22].

5. CONCLUSION

The compounds (3a & 3b) exhibited a significant anticancer activity towards MCF-7, HEPG2 and PC3 cancer cell lines. The synthesized compounds are good inducer for apoptosis. This emphasize the anticancer properties of the studied copper complexes, and open new era in the anticancer treatment.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Mohammed FZ, EL-Deen IM, Tmamm AS. Biochemical studies on coumarin derivatives as antioxidant and antitumor agents. Asian Journal of Research in Biochemistry AJRB. 2019;9(2):48917.
2. Tomasetti C, Li L, Vogelstein B, et al. Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. Science. 2017;355(6331):1330–1334.
3. Alain F. Genetics, epigenetics and cancer. Canc Therapy & Oncol Int J. 2017;4(2): 555-634.
4. Gasser G, Ott I, Nils Metzler-Nolte. Organometallic anticancer compounds. J. Med. Chem. 2011;54(3):252–253.
5. Zahran F, Keshta AT, EL-Deen IM, El-behary MM. Mode of action of potassium salt of 2-thioxo-4-hydroxycoumarin [3, 4-b] pyrimidine and 9-bromo-2-thioxo-hydroxy-coumarin [3, 4-b] pyrimidine against Ehrlich Ascites carcinoma cells. Biol. Chem. Res. 2014;76-89.
6. Zaki M, Hairat S, Aazam E. Scope of organometallic compounds based on transition metal-arene systems as anti-cancer agents: Starting from the classical paradigm to targeting multiple strategies; RSC Adv. 2019;9:3239-3278.
7. Thavasu W, Longhurst S, Joel P, Slevin L, Balkwill R. Measuring cytokine levels in blood. Importance of anticoagulants, processing, and storage conditions. J Immunol Methods. 1992;153(1-2):115-124.
8. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Jonathan T. Warren, Bokesch H, Kenney S, Boyd M. New colorimetric cytotoxicity assay for anticancer-drug screening. Journal of the National Cancer Institute. 1990;82(13):4-8.
9. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc. 2006;1:1112-1116.
10. Casciola-Rosen L, Nicholson DW, Chong T, Rowan KR, Thornberry NA, Miller DK, Rosen A. Apopain/CPP32 cleaves proteins that are essential for cellular repair: A fundamental principle of apoptotic death. J. Exp. Med. 1996;183:1957-1964.
11. Reed G, Poyner R, Larsen T, Wedekind J, Raymenta I. Structural and mechanistic studies of enolase, Current Opinion in Structural Biology. 1996;6(6):736-743.
12. Levesque R SPSS. Programming and data management: A guide for SPSS and SAS Users, Fourth Edition, SPSS Inc., Chicago Ill; 2007.
13. Hanahan D, Weinberg R. The hallmarks of cancer. Cell. 2011;100(6):57–70.
14. Helleday T, Petermann E, Lundin C, Hodgson B, Sharma A. DNA repair pathways as targets for cancer therapy. Nat Rev Cancer. 2008;8(3):193-204.
15. Anto RJ, Mukhopadhyay A, Denning K, Aggarwal BB. Curcumin (diferuloyl-methane) induces apoptosis through activation of caspase-8, BID cleavage, and cytochrome c release: Its suppression by ectopic expression of Bcl-2 and Bcl-xl. Carcinogenesis. 2002;23:143–150.
16. Jakupec A, Galanski M, Arion V, Hartinger G, Keppler K. Antitumour metal com-pounds: More than theme and variations. Dalton Transactions. 2008;2:183-194.
17. Arora S, Agarwal S, Singhal S. Anticancer activities of thiosemicarbazides/ thiose-micarbazones. Int J Pharm Pharm Sci. 2014;6(9):34-41.
18. Koňariková K, Perdikaris G, Gbelcová H, Andrezálová L, Švéda M, Ruml T, Laubertová L, Režnáková S, Žitňanová I. Autophagy in MCF-7 cancer cells induced by copper complexes. Pharmacol Rep. 2016;68(6):1221-1224.
19. Liang C, Xia J, Lei D, Li X, Yao Q, Gao J. Synthesis, *in vitro* and *in vivo* antitumor

activity of symmetrical bis-Schiff base derivatives of isatin. Eur J Med Chem. 2014;74(9):742-750.

20. Vyas A, Patitungkho S, Jamadar A, Adsule S, Padhyea S, Ahmad A. ATRA-hydrazonate derivatives and their copper complexes against hormone-dependent (MCF-7), hormoneindependent (MDA-MB-231 and BT-20) breast cancer and androgen-independent (PC3) prostate cancer cell lines. Inorg Chem Com. 2012; 23(11):17-20.

21. Francisco D, Peddi P, Hair J, Flood B, Cecil A, Kalogerinis P. Induction and processing of complex DNA damage in human breast cancer cells MCF-7 and nonmalignant MCF-10A cells. Free Rad Biol Med. 2009;44(4):558-69.

22. Karlsson H, Fryknäs M, Strese S, Gullbo J, Larsson R, Sjöblom T, Pandzic T, Nygren P, Westman G, Bremberg U. Mechanistic characterization of a copper containing thiosemicarbazone with potent antitumor activity. Oncotarget. 2017;8:30217–30234.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/50401