Original Research Article

INVIVO STUDY ON ORGANOMETALLIC COMPOUNDS AS ANTICANCER AGENTS

ABSTRACT:

This study aims to study the invivo (ITALIC) anticancer effect of the synthesized copper complexes of 2,3-dihydroxy benzaldehyde thiosemicarbazone (3a,b), followed by evaluating their antioxidant activity. *Materials and methods:* A total number of 80 adult female swiss albino mice weighing 20-25 gm were divided into 8 groups (10 mice /each group). The acute toxicity was estimated by intraperitoneal injection of the compounds (**3a, b**). *Results:* We found that, 5 mg /kg and 10 mg /kg were considered to be the most effective dose of compounds **3a & 3b**;(REMOVE AND INSERT COMMA) respectively. The mean volume of EAC in the positive control group was found to be 4.2 ±0.5 (mL), this value was significantly decreased by 100%, ($p<0.001$) for **3a & 3b** treated groups;(REMOVE AND INSERT COMMA) respectively**.**

Keywords: anticancer, copper complexes,2,3-dihydroxy benzaldehyde thiosemicarbazone, EAC (ERHLICH ASCITES CARCINOMA),swiss albino mice

1. INTRODUCTION:

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 is due to inherited genetic mutations whereas the vast majority is 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) [1].

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Estimates are that in 2018, 18.1 million new cases of cancer and 9.6 million deaths occur globally [2]**.** Cancer is considered one of the major causes of mortality in the world. The recent advances in science, cancer have not been cured yet. It is estimated that by 2020 there will be 16 million new cancer cases every year [3]. It is, therefore, essential that new therapeutic options are needed for cancer therapy with attention to toxicity and side effects, besides the major treatment modalities including surgery, immunotherapy and radiotherapy [4].

The human genome is composed of deoxyribonucleic acid (DNA), which is the heritable macromolecule that carries the information essential for life. As a chemical, DNA is susceptible to changes that affect its capacity to perform this role. Cells use highly regulated biochemical pathways to replicate DNA, detect if it is modified, and repair modifications as they arise. Many processes are required to prevent change and to transfer the genome to daughter cells: replication must be accurate, chromosomes must be distributed correctly during cell division, and damage to DNA must be detected and repaired. The fate of a cell, be it healthy or cancerous, is dependent upon the integrity of the genome and its ability to maintain this integrity. By preventing change to DNA, healthy cells ensure their viability and the delivery of a copy of their genetic material to the next generation [5].

Anticancer activity of thiosemicarbazone complexes is mainly attributed to inhibition of RR activity, Topo- II activity and generation of ROS, but there are other possible targets as well which need to be explored. In many cases, in vitro **(ITALIC)** ribonucleotide inhibitors have been found to be poor proliferation inhibitors on whole cells. Another area which needs attention is metal/ion sequestering since thiosemicarbazones are versatile chelators, they sometimes deprive the cell of essential metal ions by forming stable chelates with them. On the other hand, the fact cannot be overruled that metal-ligand complexes are more active than pure ligands. The redox capability of transition metals like copper play an important role in activity enhancement but it can also trigger off Fenton's reactions producing significant amount of OH° radicals that can create hindrance in normal cell functions. It has also been observed that some of the ligands are more active while others are inactive for the same cell lines, hence questioning the simple diffusion hypothesis. Likewise the interaction of one metal with another can also be explored taking synergestic effect into consideration. Not only this, whether the complex acts in unison or metal and ligand act independently inside the body needs a greater depth of understanding by bridging the gap between chemistry and molecular biology [6].

This study aims to evaluate the in vivo **(ITALIC)** anticancer effect as well as the antioxidant activity of the synthesized of copper complexes of 2,3-dihydroxy benzaldehyde thiosemicarbazone (3a,b).

2. MATERIALS & METHODS:

2.1 Materials:

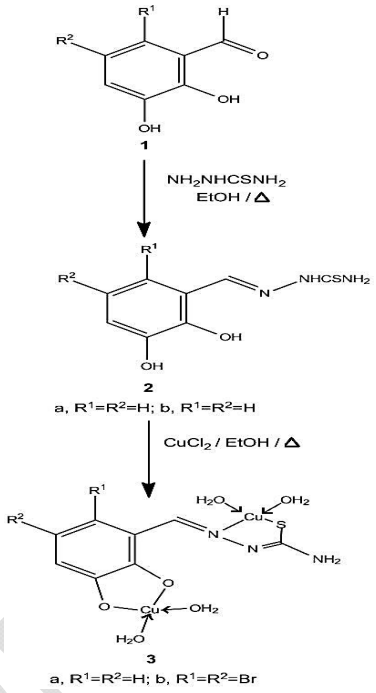
- Chemicals for synthesis of copper complexes of 2,3- dihydroxy-benzaldehyde thiosemicarbazones (3a,b) : 2,3-dihydroxybenzaldehyde; 5,6-dibromo -2,3-dihydroxybenzaldehyde; Thiosemicarbazide; Copper chloride; Ammonium hydroxide (10%) and Ethanol.

Ehrlich ascites carcinoma (EAC):

EAC cells were initially supplied from the National Cancer Institute, Cairo, Egypt (only for the first transplantation), and maintained in female Swiss albino mice through serial intraperitoneal (I.P.) injection at 8 or 10 day intervals in our laboratory in a liquid form.

2.2 Methods:

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 cupper chloride in ethanol under reflux (scheme 1).



- **Scheme 1:** synthesis of the compounds (2a, 2b, 3a & 3b).

- Determination median lethal dose (LD 50) of synthetic compounds:

Approximate LD 50 of synthetic compounds were determined according to published method [7].

- Dose response curve:

Dose response curve was determined according to published method [8]. Studies carried out for determination of the most effective dose.

- Viability and Counting of EAC cells:

The viability of EAC cells was determined by the Trypan Blue Exclusion Method [9].

2.3 Groups of the study:

Adult female Swiss albino mice weigh (20-25 g) purchased from breeding unit of the Egyptian Stock Holding Company for Biological Products of Vaccines, Sera & Drugs (VACSERA) were used throughout this study. The animals were housed in steel mesh cages (animal house, faculty of Science, Zagazig University) and maintained on a commercial pellet diet and water for one week before starting

the experiment as an acclimatization period. 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**(PLEASE MENTION WHETHER THE STUDY WAS APPROVED OR NOT. IF YES, PLEASE PROVIDE APPROVAL NUMBER)**: **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), (2.5×10^6 cells/ 0.3 ml/mouse) by (I.P) injection once at the first day**.**

Group (3): Drug group I: This group 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 (2.5×10^6 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 (2.5×10^6 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 were injected I.P. with compound 3b (10 mg/Kg) in the day before EAC injection (2.5×10^6 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 (2.5×10^6 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 [10].

- Antioxidant assays:

Plasma **malondialdehyde: (MDA)** was determined by using Biodiagnostic kit (Biodiagnostic company, Dokki, Giza, Egypt), according to the published method [11].

Determination of **catalase enzyme activity (CAT):** was measured in plasma and tissues. Catalase reacts with a known quantity of H_2O_2. The reaction is stopped after exactly one minute with catalase inhibitor. Catalase converts H_2O_2 to H_2O and O_2. According to published method [12].

Determination of **glutathione reductase activity:** This assay is based on the oxidation of NADPH to NADP+ catalyzed by a limiting concentration of glutathione reductase. One GR activity unit is defined as the amount of enzyme catalyzing the reduction of one micromole of GSSG per minute at pH 7.6 and 25°C. One molecule of NADPH is consumed for each molecule of GSSG reduced. Therefore, the reduction of GSSG is determined indirectly by the

measurement of the consumption of NADPH, as demonstrated by a decrease in absorbance at 340 nm (A340) as a function of time.

2.4 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 [13].

3. RESULTS:

The most effective doses were found to be "5 mg /kg" and "10 mg /kg" for compounds 3a and 3b; respectively.**(THE ACUTE TOXICITY STUDY SHOULD BE EXPLAINED OR DISCUSSED PROPERLY. THIS DATA IS NOT SUFFICIENT FOR READERS)**

The mean volume of EAC in the positive control group was found to be 4.2 ±0.5 (mL), this value was significantly increased by 100%, (p<0.001) for 3a & 3b treated groups; respectively as no detectable EAC cells were found in the treated groups**.**

The anti- oxidant effect of compounds (3a, 3b), was evaluated through the estimation of MDA, CAT and G-reductase activities. The mean values of MDA concentration in EAC cells in positive control group were found, that found to be 27.66 ± 5.86 (nmol/g.tissue). 3a and 3b treated groups showed a significant increase to 35.79 ± 6.58 & 35.46 ± 3.27 (nmol/g.tissue) respectively (p<0.001); compared to the positive control group .

On the other hand, CAT activity in positive control group was found to be 398.14 ± 19.66 (U/g). CAT activity showed a significant increase in 3a treated group to 453.16 ± 38.78 (U/g) (p< 0.001); and to 438.47 ± 58.78 in 3b treated group, compared to positive control, (p<0.001). Moreover, the mean value of G- Reductase activity in positive control group was found to be 637.19 ± 65.12 (U/g). Compounds 3a and 3b treated groups showed a significant increase to 920.1 ± 246.89 & 1442.66 ± 126.9 (U/g) respectively; (p<0.001) compared to the positive control group.

Table (1): the effect of compounds (3a, 3b) effect on antioxidant catalase activity:

Group	Mean ±SD
Negative	603.63 ±58.67
Positive	398.14±19.66
Drug I	458.97 ± 22.38
Thr I	453.16± 38.78
Prev I	546.58 ± 18.12
Drug II	472.23 ± 46.12
Thr II	438.47 ± 58.78
Prev II	469.82 + 62.04

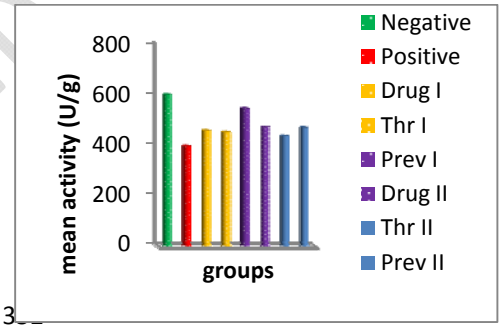


Figure (1): the effect of compounds (3a, 3b) effect on antioxidant catalase activity.

Table (2): the effect of compounds (3a, 3b) effect on antioxidant G.reductase activity :

Group	**Mean ± SD**
Negative	1158.63± 6
Positive	637.19± 65.12
Drug I	1059.15± 132.9
Thr I	920.1± 246.88
Prev I	1229.3± 143.46
Drug II	1472.12±140.01
Thr II	1442.66±126.9
Prev II	1375.66± 227.73

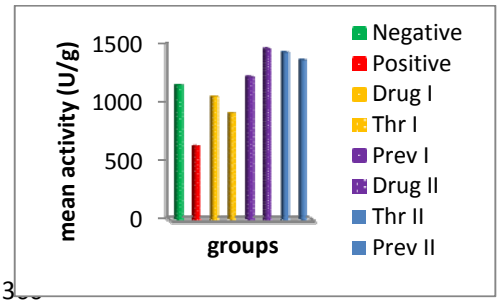


Figure (2): the effect of compounds (3a, 3b) effect on antioxidant G.reductase activity.

4. DISCUSSION:

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 ionising 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].

The synthesis of new organometallic compounds and the development of combination therapies containing organometallic components have shown significant progress in utilization of transition metal complexes as anticancer agents [15].

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 [16].

Coordination to copper increased the cytotoxic potential considerably when compared to that of free ligand. It is well-known that copper is an essential micronutrient and has important biological functions, such as cellular trafficking, redox regulation and angiogenesis modulation etc [17].

Only a limited number of in vivo studies have been done which indicate that some thiosemicarbazones show potential as chemotherapeutic agents [5].

In the present study, we aimed to evaluate the anti-tumor and antioxidant properties of recently developed synthetic copper complexes of 2,3-dihydroxy benzaldehyde thiosami carbazne (3a,b), as anticancer agents.

The acute toxicity was estimated by intraperitoneal injection of the compounds (**3a, b**) to assess the dose response curve. We found that, 5 mg /kg and 10 mg /kg were considered to be the most effective dose of compounds **3a & 3b**; respectively.

The mean volume of EAC in the positive control group was found to be 4.2 ±0.5 (mL), this value was significantly decreased by 100%, ($p<0.001$) for **3a & 3b** treated groups; respectively**.** As compared to ***Sathisha et al., 2010,*** Who studied the effect of thiosemicarbazide metal complexes on Ehrlich ascites carcinoma (EAC), the results show that the copper (II) complex showed more than 85% reduction in the growth of tumor cells.This confirm the invivo antitumor activity against EAC of the studied compunds [18].

Lipid peroxidation/oxidation process plays a key role in tumor growth invasiveness. ROS exhibit multiple functions and are involved in tumor initiation and progression. MDA, a free oxygen radical product formed during oxidative degeneration of cancerous tissues and as the end product of lipid peroxidation, is a biomarker of oxidative stress that has been reported to be exhibited at higher levels in cancer tissues than in non-diseased organs [19]. Antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated such as cancer [20]. Catalase is a hemoprotein and it protects cells from

the accumulation of H_2O_2 and able to prevent the tissue from reactive free oxygen and hydroxyl radicals, by catalysing the reduction of H_2O_2 to form H_2O and O_2. Catalase protects the tissue from highly reactive hydroxyl radicals by decomposing the hydrogen peroxide. So, reduced levels of catalase may indicate the toxic effects on the tissue [21]. Glutathione reeductase is a widely occurring enzyme and has been studied from several sources including *Plasmodium falciparum*, and most thoroughly from human erythrocytes and *E. coli*. It is one of a chain of enzymes which serves to maintain glutathione in the reduced form. It catalyzes the NADPH-driven reduction of GSSG (Oxidized glutathione) to GSH (reduced glutathione). GSH helps detoxify reactive oxygen species by donating reducing equivalents to glutathione peroxidase and detoxifies electrophilic xenobiotics with glutathione *S*-transferase [22].

The anti-oxidant effect of compounds **3a** and **3b** were evaluated in the present study, through the estimation of MDA, CAT, and G. Reductase in EAC cells.

Our results found that, the mean values of MDA concentration in EAC cells in positive control group were found to be 80.66 ± 5.86 (nmol/g.tissue). **3a** and **3b** treated groups showed a significant decrease to 35.79 ± 6.58 & 35.46 ± 3.27 (nmol/g.tissue) respectively ($p<0.001$); compared to the positive control group.

On the other hand, CAT activity in positive control group was found to be 398.14 ± 19.66 (U/g). CAT activity showed a significantly increase in **3a** treated group to 453.16 ± 38.78 (U/g) ($p< 0.001$); and to 438.47 ± 58.78 in **3b** treated group, compared to positive control, ($p<0.001$). Moreover, the mean value of G-Reductase activity in positive control group was found to be 637.19 ± 65.12 (U/g). Compounds **3a** and **3b** treated groups showed a significant increase to 920.1 ± 246.89 & 1442.66 ± 126.9 (U/g) respectively; ($p<0.001$) compared to the positive control group. All these findings ensure the anti-oxidant activity of the studied compounds, and in agreement with Thanh and Hoai, (2012) who found that some copper thiosemicarbazone complex derivaties caused inhibition of lipid peroxidation [23].

5. CONCLUSION

The compounds (3a & 3b) revealed a significant anticancer activity towards Ehrlich ascites carcinoma (EAC) cells by significant reduction of its volume and the cell count in treated groups ; respectively, compared to the positive control group. It turned out that they reduced cell viability of cancer cells in a time and concentration dependent manner in invivo studies. The synthesized compounds have potent antioxidant activity.

6. REFRENCES:

[1]: Tmamm A.S., Mohammed F.Z. and EL-Deen I.M. and (2019): Cytotoxicity of N-(P-chlorophenyl)-7- hydroxycoumarin -3-yl carboxamide and Ethyl 7-hydroxycoumarin -3-yl Ester. Asian Journal of Research in Biochemistry AJRB. 1(2): 49084.

[2]: World Health Organization (2018): Cancer Facts & Figures. Latest global cancer data, Cancer burden rises to 18.1 million new cases and 9.6 million cancer deaths in 2018. World Health Organization, Geneva, Switzerland.

[3]: Lingwood R., Boyle P., Milburn A., T. Ngoma, Arbuthnott J., McCaffrey R., Kerr S., and Kerr D.; (2008): The challenge of cancer control in Africa. *Nat. Rev. Cancer* 8: 398–403.

[4]: Kane A., and Yang I., (2010): Interferon-gamma in brain tumor immunotherapy. *Neurosurg. Clin. N. Am.* 21: 77–86.

[5]: Jackson P. and Bartek J. (2009): The DNA-damage response in human biology and disease.*Nature.*;22;461(7267):1071-1078.

[6]: Khan A ., Nazar H., Sabir S., Irshad M., , Awan S., Abbas R., Akram M., khaliq A., Rocha J., Ahmad S., farnaz malik f.(2014): antioxidant activity and inhibitory effect of some commonly used medicinal plants against lipid per-oxidation in mice brain asmatullah;11(5):83-90.

[7]: Meier J. and Theakston RD. (1986): Approximate LD50 determinations of snake venoms using eight to ten experimental animals. Toxicon 24: 395–401.

[8]: Crump S., Hoel G., Langley H., Peto R., (1976): "Fundamental Carcinogenic Processes and Their Implications for Low Dose Risk Assessment". *Cancer Research* 36: 2973–2979.

[9]: McLiman F, Dairs V., Glover L., and Rake W., (1957): The submerged culture of mammalian cells: The Spinner Culture. *J. Immunol* 79: 428-433.

[10]: Thavasu W., Longhurst S., Joel P., Slevin L., Balkwill R.(1992): Measuring cytokine levels in blood. Importance of anticoagulants, processing, and storage conditions. J Immunol Methods.;30;153(1-2):115-124.

[11]: Satoh K., (1978): Serum Lipid Peroxide in cerebrovascular disorders determined by a new colorimetric method. Clinica Chimica Acta 90: 37-43.

[12]: Aebi H. (1984): Catalase in vitro, Methods Enzymol 6:105:121.

[13]: Levesque R., SPSS. (2007): Programming and Data Management: A Guide for SPSS and SAS Users, Fourth Edition, SPSS Inc., Chicago Ill.

[14]: Helleday T ., Petermann E., Lundin C., Hodgson B., Sharma .A(2008): DNA repair pathways as targets for cancer therapy. Nat Rev Cancer.;8(3):193-204.

[15]: Jakupec A., Galanski M., Arion V., Hartinger G., Keppler K.(2008): Antitumour metal compounds: More than theme and variations. Dalton Transactions.;2:183-194.

[16]: Arora S., Agarwal S., Singhal S.(2014): Anticancer Activities of Thiosemicarbazides/Thiosemicarbazones., Int J Pharm Pharm Sci, 6, (9):34-41.

[17]: Reed G., Poyner R., Larsen T., Wedekind J., Raymenta I.(1996): Structural and mechanistic studies of enolase, Current Opinion in Structural Biology.;6(6):736-743.

[18]: Sathisha A., .Budagumpi S., , Kulkarni N., Gurunath S., Kurdekar A., Revankar A., Pai K.(2010): Synthesis, structure, electrochemistry and spectral characterization of (D-glucopyranose)-4-phenylthiosemicarbazide metal complexes and their antitumor activity against Ehrlich Ascites Carcinoma in Swiss albino mice, M.P,European Journal of Medicinal Chemistry.;45(3):106–113.

[19]: Al-Amin M., Akhter S., Hasan T., Alam T., Nageeb M., Saifullah R., Shohel M.(2015): The antioxidant effect of astaxanthin is higher in young mice than aged: a region specific study on brain. Metab Brain Dis.;30(5):1237-1246.

[20]: Hajhashemi V., Vaseghi G., Pourfarzam M. and Abdollahi A.(2010): Are antioxidants helpful for disease prevention? Res Pharm Sci.; 5(1): 1–8.

[21]: Peruma, P., Chamuah J., Rajkhowa D. and Chandan C. (2013): Effect of catalase on the liquid storage of mithun (Bos frontalis) semen. Asian Pacific Journal of Reproduction. ; 2 .(5) :209–214.

[22]: Rebecca F. and Bruce A.(2010): in Comprehensive Natural Products II, 2010.

[23]: Thanh N., * and Hoai L.(2012): Synthesis, Structure and Antioxidant Activity of (TetraO-acetyl-?-D-galactopyranosyl) thiosemicarbazones of Substituted Benzaldehydes.; Indian J. Pharm Sci., 74 (1): 54-62.