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2 **INVIVO STUDY ON ORGANOMETALLIC COMPOUNDS AS**
3 **ANTICANCER AGENTS**

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ABSTRACT:

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This study aims to study the **invivo** 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, 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.

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

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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 [24] [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* 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 synergistic 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* 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-dihydroxybenzaldehyde thiosemicarbazones (3a,b) : 2,3-dihydroxybenzaldehyde; 5,6-dibromo-2,3-dihydroxybenzaldehyde; thiosemicarbazide; Copper chloride; Ammonium hydroxide (10%) and ethanol.

Ehrlich ascites carcinoma (EAC):

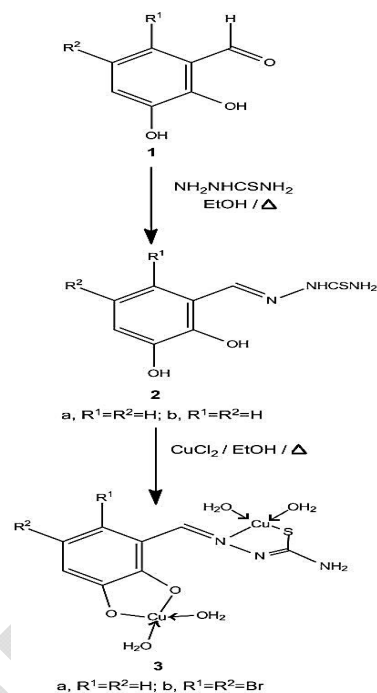
EAC cells were initially supplied from the National Cancer Institute, Cairo, Egypt (only for the first transplantation), and maintained in female Swiss albino mice through serial intraperitoneal (I.P.) injection at 8 or 10 day intervals in our laboratory in a liquid form.

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2.3 Methods:

In vivo synthesized compounds as following:

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 thiosemicarbazide 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).



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- Scheme 1: synthesis of the compounds 172a, 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].

2.2 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. The study was approved by the ethical committee of the Port Said university.

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), (2.5×10^6 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).

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At the end of the experiment, the blood samples were collected from the retro-cranial 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].

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

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Determination of catalase enzyme activity (CAT): was measured in plasma 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].

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Determination of glutathione reductase activity: This assay is based on the oxidation of NADPH to NADP^+ catalyzed by 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

285 measurement of the consumption of
 286 280 PH, as demonstrated by a decrease in
 287 285 absorbance at 340 nm (A340) as a function
 288 288 time.

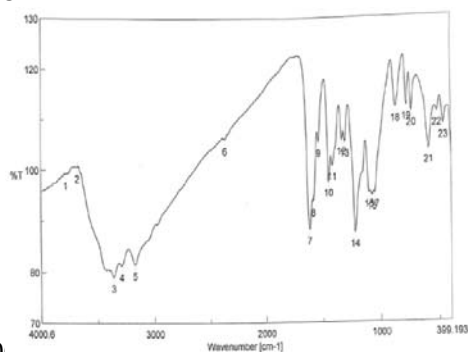
2.2.8 Statistical Analysis:

290 All statistical analyses were done by a
 291 statistical for social science package "SPSS"
 292 2.0 for Microsoft Windows, SPSS Inc and
 293 considered statistically significant at a two-
 294 sided $P < 0.05$. Numerical data were
 295 expressed as mean \pm SD. The levels of
 296 markers were analyzed by ANOVA. The
 297 correlations between serum biochemical
 298 data in different studied groups were
 299 evaluated by Pearson's correlation
 300 coefficient, to quantify the relationship
 301 between the studied parameters. P value $<$
 302 0.01 was considered significant [13].

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3.04 RESULTS:

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3.08 IR spectroscopy of compound 3a.

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311 The most effective doses were found to
 312 be "5 mg /kg" and "10 mg /kg" for
 313 compounds 3a and 3b; respectively. No
 314 mortality was observed.

315 The mean volume of EAC in the
 316 positive control group was found to be 4.2
 317 0.5 (mL), this value was significantly
 318 increased by 100%, ($p < 0.001$) for 3a & 3b
 319 treated groups; respectively as no
 320 detectable EAC cells were found in the
 321 treated groups.

322 The anti-oxidant effect of compounds
 323 (3a, 3b), was evaluated through the
 324 estimation of MDA, CAT and G-reductase
 325 activities. The mean values of MDA

326 concentration in EAC cells in positive
 327 control group were found, that found to be
 328 66 ± 5.86 (nmol/g.tissue). 3a and 3b
 329 treated groups showed a significant
 330 increase to 35.79 ± 6.58 & 35.46 ± 3.27
 331 (nmol/g.tissue) respectively ($p < 0.001$);
 332 compared to the positive control group .

333 On the other hand, CAT activity in
 334 positive control group was found to be
 335 398.14 ± 19.66 (U/g). CAT activity showed a
 336 significant increase in 3a treated group to
 337 573.16 ± 38.78 (U/g) ($p < 0.001$); and to
 338 438.47 ± 58.78 in 3b treated group,
 339 compared to positive control, ($p < 0.001$).
 340 Moreover, the mean value of G- Reductase
 341 activity in positive control group was found
 342 to be 637.19 ± 65.12 (U/g). Compounds 3a
 343 and 3b treated groups showed a significant
 344 increase to 920.1 ± 246.89 & $1442.66 \pm$
 345 136.9 (U/g) respectively; ($p < 0.001$)
 346 compared to the positive control group.

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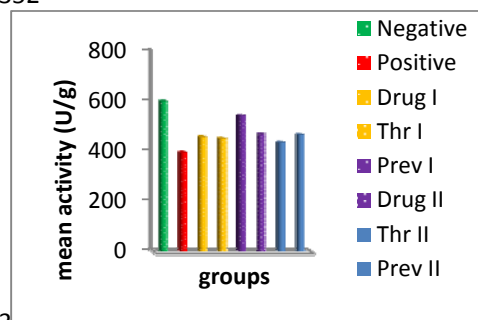
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349 **Table (1):** the effect of compounds (3a, 3b)
 350 effect on antioxidant catalase activity:

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

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

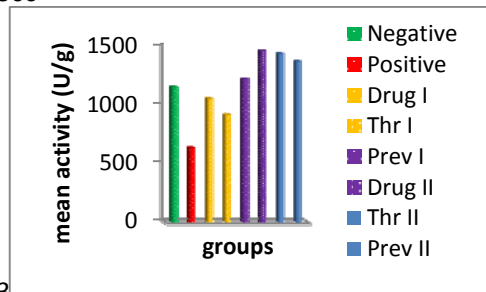
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358 **Table (2):** the effect of compounds (3a, 3b)

Effect on antioxidant G.reductase activity :

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

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Figure (2): the effect of compounds (3a, 3b)

Effect on antioxidant G.reductase activity.

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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-dihydroxybenzaldehyde thiosemi 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 4.24 \pm 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 in vivo antitumor activity against EAC of the studied compounds [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 which oxidants or free radicals are implicated such as cancer [20]. Catalase is a hemoprotein and it protects cells from the accumulation of H₂O₂ and able to prevent the tissue from reactive free oxygen and hydroxyl radicals, by catalysing the reduction of H₂O₂ to form H₂O and O₂. 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 reductase is a widely occurring enzyme and has been studied from several sources including *Plasmodium falciparum*, 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

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 derivatives 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 vivo studies. The synthesized compounds have potent antioxidant activity. These data confirm the anticancer effect of studied copper complexes and help to suppose alternatives for the present anticancer treatment.

Ethical :

The The study was approved by the ethical committee of the Port Said university.

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