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The Effect of Zn-Hydrazone Complex Against Solid Tumor In Mice.

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Abstract: Chemotherapeutic treatment of cancer is commonly used even through it has detrimental side effects, specially the adverse effect on normal cells, therefore, The extension of the life span of tumor-bearing animals is a reliable criterion for judging the value of any novel anticancer drug. In the current study the effect of Zn-hydrazone complex (ZHC) against experimentally induced solid tumor was investigated. The solid tumor was induced by injecting sc.injection of 2×10^6 EAC cells into each experimental animal's right hind limb. After 24hrs of the tumor cell inoculation, mice were treated at a dose 160 mg/kg (i.p) Once daily for month , according to their respective groups. Results showed significant increase in survival rate and a significant decrease in tumor volume after treatment. In addition, ZHC induced cell cycle arrest in G0/G1 and apoptosis. These effects were supported by inhibition of Ki-67 and PCNA, activation of P53 and Bax and down regulation in Bcl-2 levels in solid tumor cells. SOD, Gpx, GSH, and TAC levels increased significantly, while MDA levels decreased significantly

keywords: Ehrlich ascites carcinoma, Zn-hydrazone complex, Apoptosis, Antioxidants, Cell cycle , Chemoprevention.

1.Introduction

Cancer is the second most popular disease in the world after cardiovascular disease accountable for human deaths [1].

EAC is a transplantable, poorly differentiated, a malignant tumor that began as a spontaneous murine mammary adenocarcinoma [2]. It can stimulate respectively solid and ascetic tumor types in almost all strains of mice, because of these issues; it has been commonly used for several years to research the antitumor effect of many natural and synthetic compounds [3-5].

Chemoprevention is the use of natural, synthetic, or biological agents to reverse, suppress, or prevent either the early stages of carcinogenesis or the progression of premalignant cells to invasive disease [6].

To date, the cancer problem, and also the failure of conventional chemotherapy to decrease mortality rates for common epithelial malignancies like lung, colon, breast, prostate, and pancreas carcinomas, suggests an important need for new cancer control approaches [7].

Several naturally occurring compounds in foods, especially antioxidative compounds found in plants, have shown promise as potential chemopreventive agents [8]. The recently discovered yellow, orange, and red carotenoid pigments are among these phytonutrients. In epidemiological studies, consumption of vegetables and fruits has consistently been linked to a lower incidence of a variety of cancers [9], Additionally, dietary carotenoid intake from all these sources has been associated with a lower cancer risk [10].

A chemistry and biology of hydrazone complexes is a significant branch of chemistry that is connected to theoretical and computational work on metal ion-donor ligand bonding [11-13].

Antibacterial properties of transition metal complexes of quinoline hydrazone derivatives are gaining popularity [14-16]. Quinoline hydrazones and their Zn (II) complexes have recently been reported to have anti-tuberculosis and fluorescence properties [17-20].

Metals and metal complexes have been crucial in the development of modern

chemotherapy. Metals can play an important role in modifying the pharmacological properties of known drugs after coordinating to a metal. Metal-based drugs are also used to treat diabetes, rheumatoid arthritis, inflammatory, and cardiovascular diseases, as well as diagnostics [21].

Zinc has been the most abundant trace intracellular element, and there is mounting evidence that zinc is essential for genetic stability and function [22].

Zinc's role in cancer is receiving more attention. Studies on humans, animals, and cell cultures have all found a link between zinc deficiency and cancer. When compared to healthy controls, cancer patients have lower zinc levels [23]. Oxidative DNA damage is caused by zinc deficiency [24], and In zinc-deficient animals, chromosome breaks have been observed [25]. When rats are exposed to carcinogenic compounds, dietary zinc deficiency increases their susceptibility to tumour development [26].

Chemotherapeutic treatment of cancer is commonly used even through it has detrimental side effects, specially the adverse effect on normal cells, therefore, The extension of the life span of tumor-bearing animals is a reliable criterion for judging the value of any novel anticancer drug. The current study found that the protective effect of ZHC against experimentally induced solid tumor was investigated.

2. Materials and methods

All reagents were purchased from Sigma-Aldrich company Ltd and were of the highest purity available. (Gillingham, United Kingdom). EA was freshly prepared by dissolving in 5% polyethylene glycol 400.

1.Cell line:

The National Cancer Institute at Cairo University in Egypt generously provided EAC lines. EAC cells were kept alive through weekly intraperitoneal (i.p.) transplantation of 2.5 x 10^6 (in a volume of 0.5 ml saline) cells in female adult BALB/c mice. Nine-day-old EAC cells were used in this study.

2.Experimental animals:

Female BALB/c mice with average body weight 25±2g were purchased from National institute of cancer for experimental animals, Cairo, Egypt. They were housed in the zoology department's animal house at Mansoura University's Faculty of Science. The animals were housed in cages with groups of mice in each cage in a controlled environment (25 ± 2 C, 50-60% relative humidity, and a 12-hour light-dark cycle). The animals were supply by a rodent diet and allowed water for a week, for acclimatization.

3. Induction of solid tumor:

The solid tumor was induced by injection of 2×10^6 EAC cells into each experimental animal's right hind limb. After 24 hours of tumor cell inoculation, the animals were divided into groups and treated once daily for month.

4. Design of Experiment:

After 24 hours of tumor cell inoculation mice were classified into four groups. The first group considered untreated control mice. The second group was ZHC administered group injected intraperitoneal (i.p.) with 160 mg/kg once daily for month. The third group was solid tumor group, injected with 2×10^6 EAC cells and served as positive (tumor) control. The fourth group was solid tumor incorporated mice administered with ZHC. During the experimental period body weight and survival rate was monitored.

5. Collection of samples:

At the end of the experiment period tumor volume was obtained and measured by the caliper. Tissue samples (Tumor and Liver) were separated and stored for different analysis.

Methods:

1.body weight changes

Body weight was measured in all groups day after day for a month.

2.Survival rate

The mortality rate was recorded every day for a month .

3. Calculating the volume of the solid tumor

Tumors were extracted from the leg and measured with a vernier caliper. The following formula was used to calculate the tumor volume:

Tumor volume (ml) = $4/3\pi r_1^2 r_2$ (cm^3)

Where, r_1 and r_2 are two perpendicular solid tumor radii measured with a vernier caliper [27].

During the experiment, the tumor size and body weight of each mouse were measured every other day, and mouse survival was monitored daily.

4.Antioxidants analysis:

Biodiagnostic colorimetric kits were used to determine the activity of superoxide dismutase (SOD) and glutathione peroxidase (Gpx), as well as the concentrations of glutathione (GSH), malondialdehyde (MDA), and total antioxidant capacity (TAC) [28-32] respectively.

5. Molecular analysis:

a. Flow cytometric analysis of cell cycle progression by propidium iodide (PI)

Flow cytometric analysis was performed using a FACS Caliber Flow Cytometer (Becton Dickinson, Sunnyvale, CA, USA) equipped with a compact air-cooled low-power 15 mW argon ion laser beam (488 nm). Briefly, after sample preparation, the cells were incubated with 200 µl of a solution containing 20 µg/ml of PI (Sigma, St. Louis, MO, USA), 200 µg/ml RNase A (Invitrogen Biotechnology, Carlsbad, CA, USA) and 0.1% Triton v/v in PBS. After 30 min of incubation in the dark, flow cytometric analysis was performed, and samples were analyzed by Flow Jo 7.2.2 software (Cell Quest, USA). The results are expressed as a percent of cells in G0/G1, S or G2/M phases [33].

b. Flow cytometric analysis of P53, Bcl-2, Bax, PCNA and Ki67 expression in EAC

Antibodies against P53, Bcl-2, Bax, PCNA and Ki67 proteins and other reagents were purchased from Santa Cruz Biotechnology, Inc. (10410 Finnell Street Dallas, Texas, 75220, USA). Each vial contained 200 µg IgG in 1.0 ml of PBS with <0.1% sodium azide and 0.1 gelatin that was conjugated to fluorescein (FITC) for flow cytometry. Cells were prepared appropriately. First, 100 µl of cell suspension was pipetted into test tubes. Then, appropriately labeled antibody (FITC-conjugated antibody) was added at the recommended dilution, mixed well and incubated at room temperature for 30 min. Cells were washed with 2 ml of PBS/BSA and centrifuged at 400 for 5 min, and the resulting supernatant was discarded. Cells were resuspended in 0.2 ml of PBS/BSA or with 0.2 ml of 0.5% paraformaldehyde in PBS/BSA. A total of 20,000 cells were acquired for analysis using Cell Quest software, and a histogram plot of conjugate- fluorescence (x-axis) versus counts (y-axis) is shown with a logarithmic fluorescence intensity.

6. In vitro study;

Cytotoxic activity (Deteremination of viability by MTT assay):

Breast cell lines (MCF7) were obtained from VACSERA-Egypt specially the cell culture department. MCF7 were plated in 96 well plates (5×10^3 cells/well), cells had been incubated using drug (ZHC) then, Serially dilution was performed and treatment was for 48&72 hrs and cytotoxic effect was detected using inverted microscope (Hund –- Germany). As a negative control, untreated cells were used. Following incubation, the detached cells were washed with phosphate buffered saline

(PBS). Residual live cells were stained using MTT stain as 0.05 ml/well (0.5 mg/ml) in PBS to all wells. Plates were incubated at 37°C for 4 hours before adding (50µL) of DMSO to dissolve the formed formazan crystals. ELISA plate reader (ELX-800, BIOTEC-USA) was used to read the treated plates at 570 nm, and the absorbance cell viability was calculated using the equation below:

$Viability\% = \frac{Mean\ OD\ of\ test\ dilution \times 100}{Mean\ OD\ of\ cell\ control}$Using the Master plex 2010 software, the inhibitory concentrations or (IC50)values of cell viability were determined.

7.Statistical analysis:

The results were presented as means ±SE. Statistical significance was determined using one-way ANOVA, followed by post hoc tests for multiple comparisons. The Graph pad prism was used to perform all statistical analyses. P ≤0.05 was used to determine whether or not a difference was significant.

P ≤0.05 was used to determine whether or not a difference was significant.

3.Results and discussion

1-Cytoxicity assay:

The cytotoxic effects of ZHC on MCF-7 cell line viability was measured using MTT assay. The graph was plotted as percentage of inhibition against concentration of ZHC. The data revealed that the mean viability percentage of ZHC treated cells decreased when compared to control cells as ZHC concentration increased from 2.5µg/ml to 5000µg/ml. The IC50 value of ZHC was 388µg/ml post 48hrs treatment and 160µg/ml post 72hrs treatment.

2.Body weight changes:

Fig(3) shows body weight changes(g) and body weight gain percent(%) of control and different treated mice groups. The data clearly show that the weight of group (3) mice which inoculated with solid tumor significantly increased compared with the other treated group which shows non-significant changes.

3.Survival rate (%) of treated and non-treated solid tumor experimentally induced in female mice:

Fig (4) shows survival percent of treated and non-treated solid tumor experimentally induced in female mice groups , the illustrated data clearly showssignificant decrease in gp (3), which inoculated mice with solid tumor without any treatment specially in the 3rd and 4th weeks. Although, all treated groups shows no changes in the survival (%).

4. Tumor volume (mm^3) of treated and non-treated solid tumor experimentally induced in female mice:

Figure(5) shows the weekly changes in tumor volume (mm^3) in mice group (3) which have inoculated tumor treated with ZHC with non-treated tumor mice group, the data shows that the volume increase gradually until the 4th week but the mice group which treated with the ZHC shows a significant decrease especially at the end of the 3rd week and 4th week competing with the non-treated mice.

5.Analysis of antioxidants and oxidative stress markers:

Figures depict the levels of SOD, Gpx, GSH, MDA, and TAC in tumor and liver tissues (6,7,8,9&10). The data revealed a significant increase ($p < 0.01$) in the levels of SOD, Gpx, GSH, and TAC in the treated group with Zn-hydrazone complex when compared to the control and other animal groups, but a significant decrease in the level of MDA.

6.cell cycle analysis:

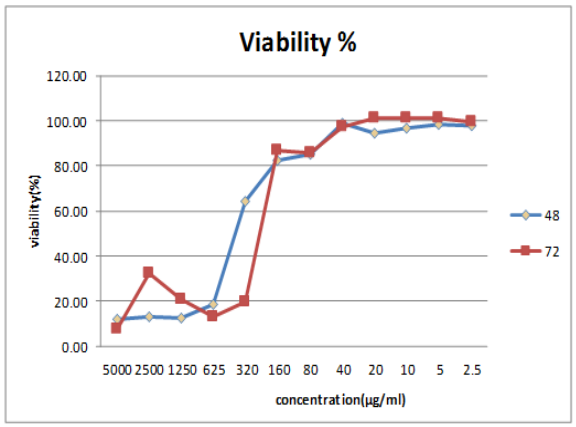
Figures (11a,11b) show cell cycle analysis for a mouse solid tumor that was experimentally induced. The distribution of cell cycle phase of experimentally induced solid tumor groups had been illusterated by flow cytometer to discussed the decrease in tumor volume by ZHC inhibition of the progression of cell cycle. The Ehrlich ascites-induced solid tumour in female mice results in a significant decrease in tumor tissue G0/G1 % when compared with treated group with ZHC which showed improvement in tumor tissue. The data displayed that the adminstration of ZHC as a protective agent against experimentally induced solid tumor showed reduction in tumor tissue S% when compared to the solid tumor group which results in a significant increase in tumor tissue S%. The solid tumor induced by Ehrlich ascites in female mice causes a significant increase in tumor tissue G2/M% and the treated group with ZHC showed improvement in tumor tissue G2/M%. The solid tumor induced by Ehrlich ascites in female mice results in a significant increase in tumor tissue Apop% and the treated group with ZHC showed reduction in tumor tissue Apop% when compared with tumor group.

7. Apoptotic markers (P53,Bax and Bcl-2):

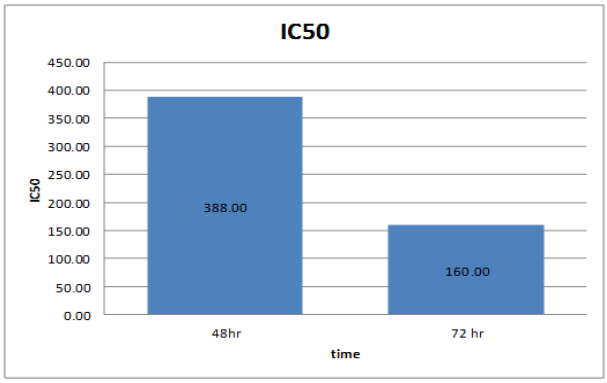
Figure(12) illustrate P53 experession in solid tumor in different groups.The treatment group with ZHC display a significant increase in P53 level in compared with solid tumor group. figure(13) illustrate Zn-hydrazone compound effect on the percentage of Bax expression in solid tumor in different mice group. The data described a significant increase in the percentage of apoptotic Bax protein level of treatment with ZHC compared with solid tumor group. figure(14) illustrate Bcl-2 experession on solid tumor in different groups. The data revealed a significant decrease in the percentage of Bcl-2 in the treated group when compared to the solid tumour group.

8. Proliferative markers (Ki-67 and PCNA):

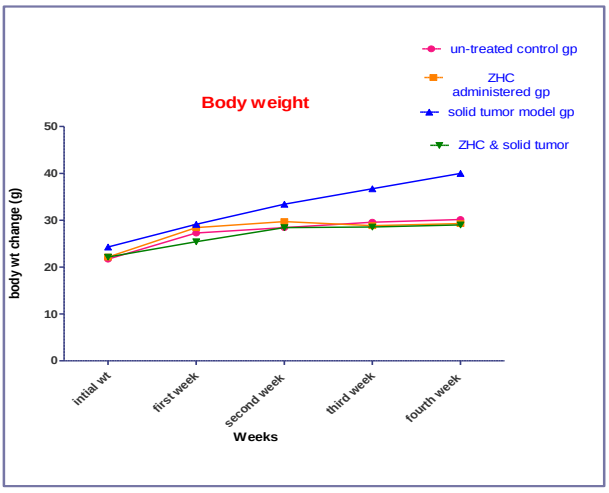
Figures (15,16) show the effect of ZHC on proliferative markers (Ki-67 and PCNA) in treated and non-treated solid tumor groups in female mice. When compared to the solid tumour group, the treatment group had a significant decrease in Ki-67 and PCNA



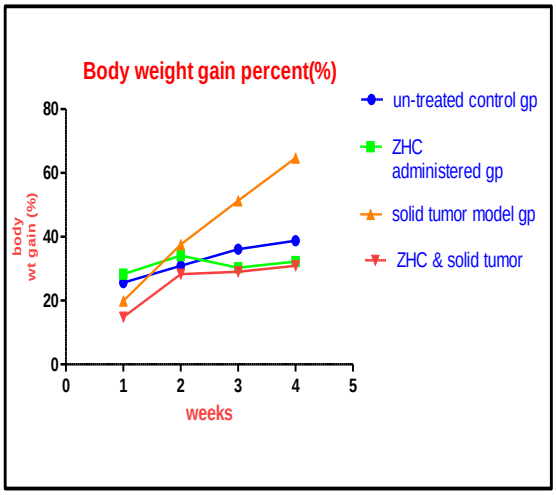
Fig(1) Evaluation of viability percentage of breast cancer cell line (MCF-7) post treatment with different concentration of ZHC for 48&72hrs



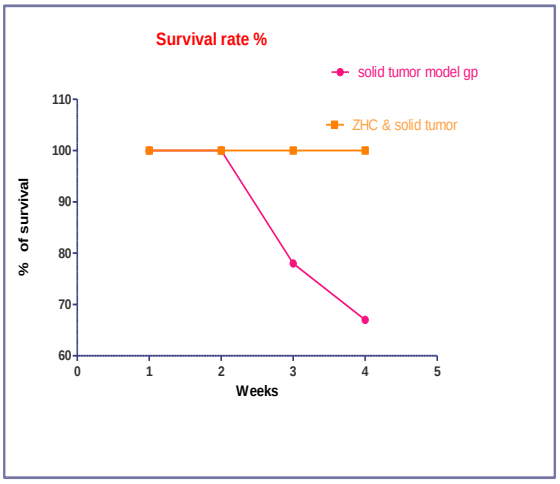
Fig(2) IC50 values of ZHC in(48hrs and 72hrs)



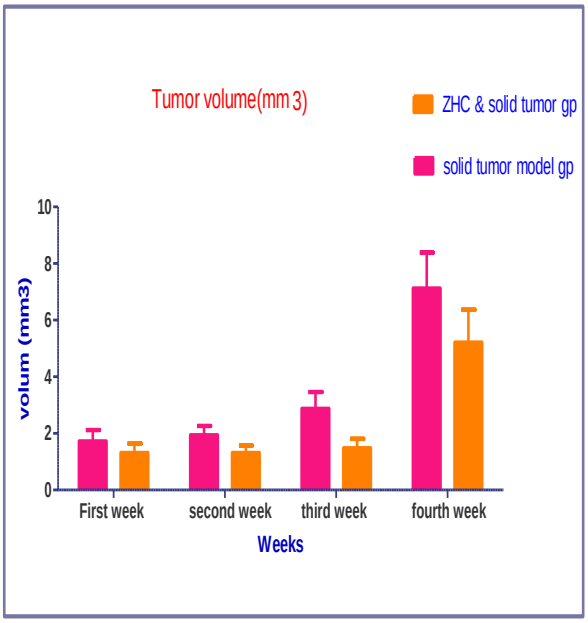
Fig(3a) Body weight (g) in control and different treated mice group

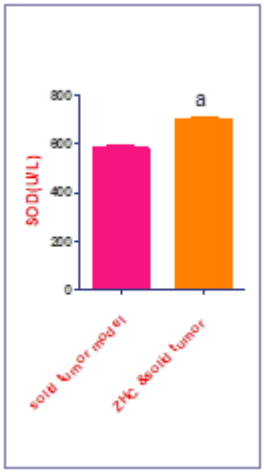


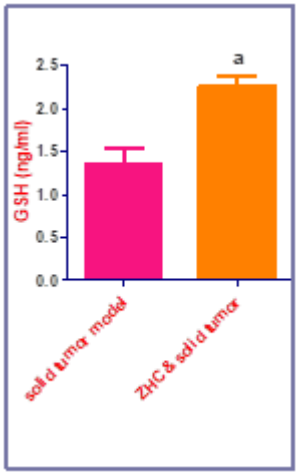
Fig(3b) Body weight gain (%) in control mice and different mice group

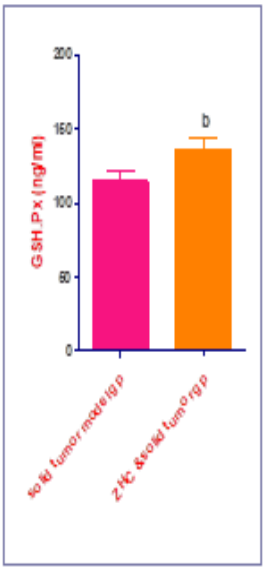


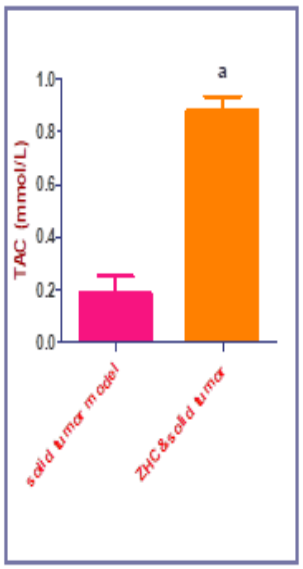
Fig(4) survival percent of control and different treated mice groups

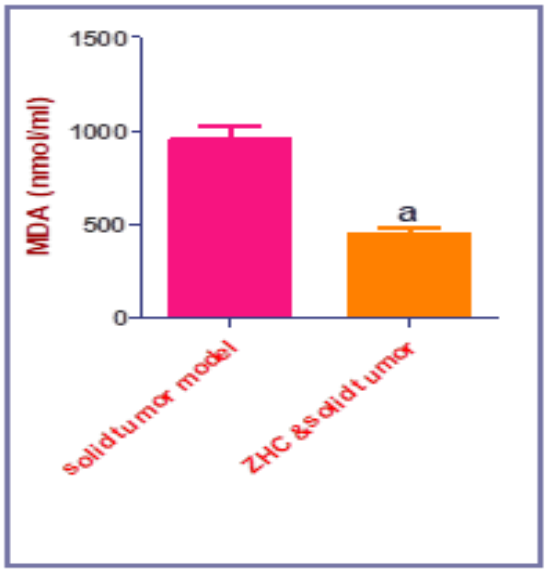


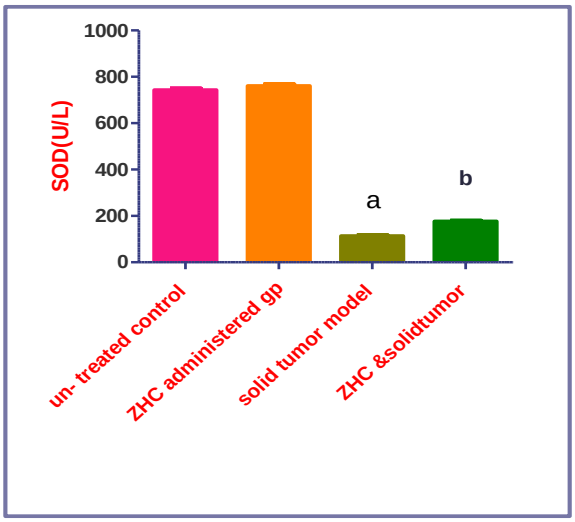


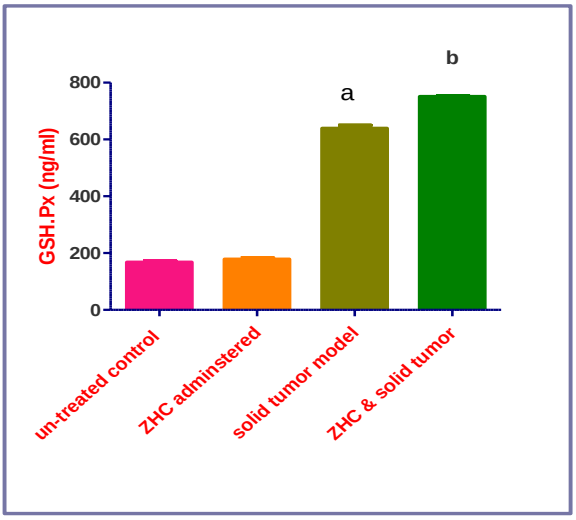


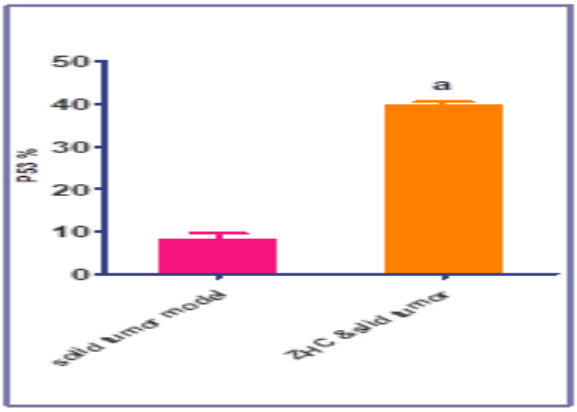


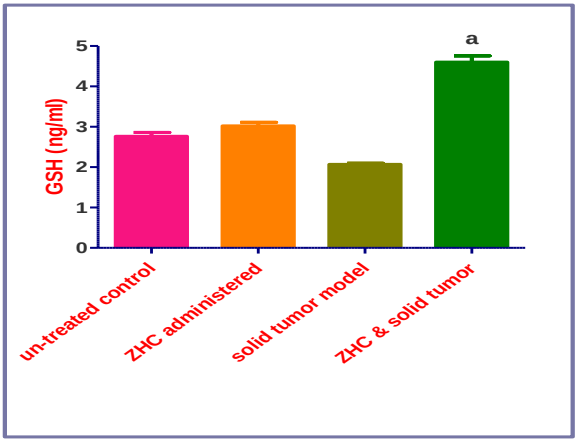






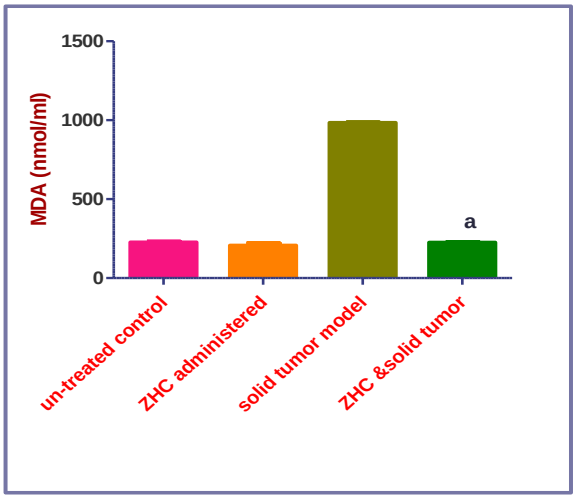


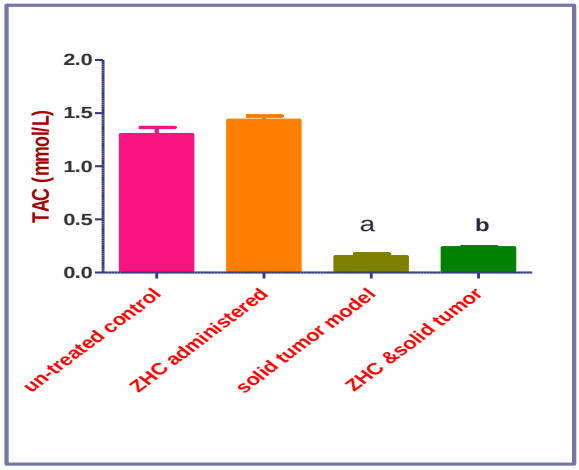




Figs (6,7,8,9&10 a) Effect of ZHC on SOD,GPx,GSH,TAC and MDA in tumor tissue in solid tumor and treated mice group.

a, Significant at (p< 0. 01) from a solid tumor group . b, Significant at (p< 0.05) from a solid tumor group . Values are means ±SE (n=5).





Fig(6,7,8,9&10 b) Effect of ZHC on SOD,GPx,GSH,TAC and MDA in liver tissue in control an different mice groups

a, significant at ($p< 0.01$) from control group .b, Significant at ($p< 0.05$) from solid tumor group. Values are means ±SE (n=5)

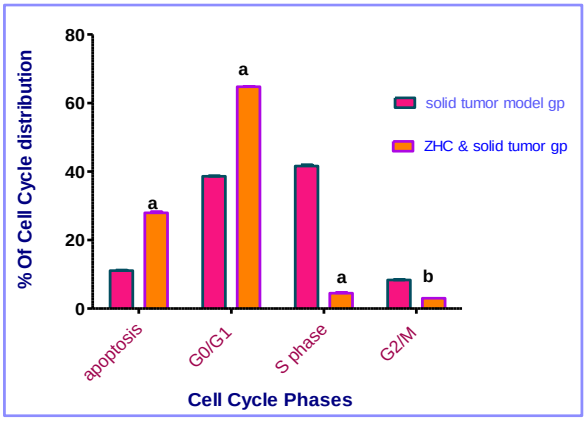
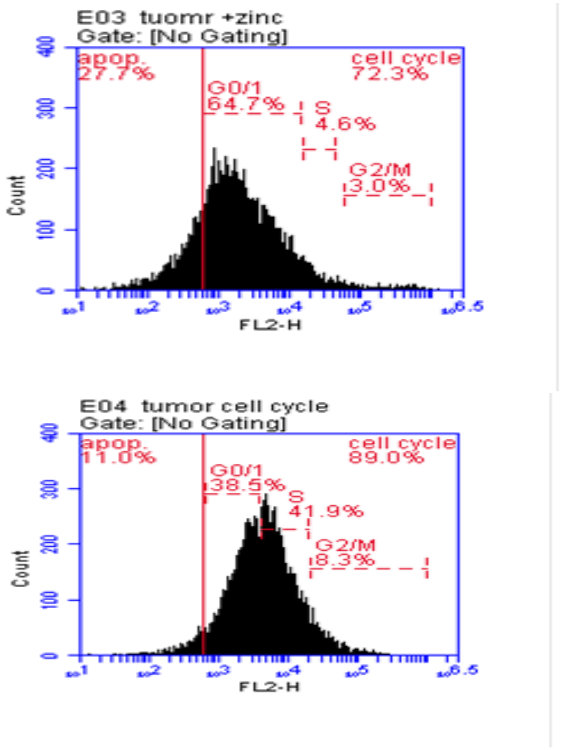
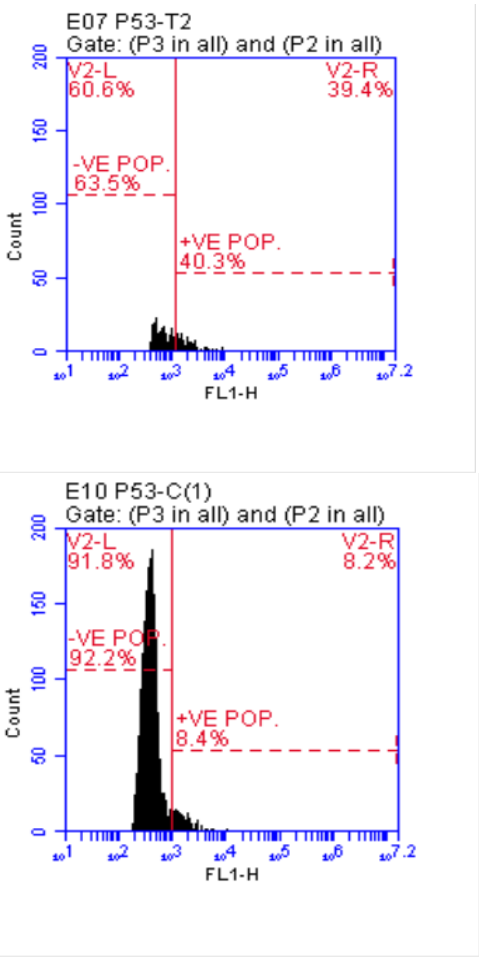


Fig (11a)Effect of ZHC on cell cycle progression and apoptosis in experimentally induced solid tumor mice.

Values are means ± SE.a, Significant different ($p<0.01$) from tumor group. b, Significant different ($p<0.05$) from tumor group

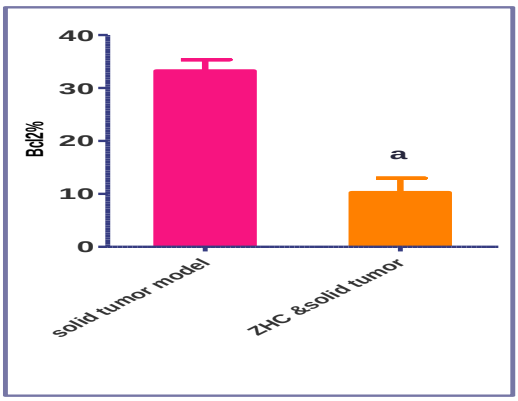


Fig(11b) Flow cytometric histogram of P53 in experimentally solid tumor in mice.

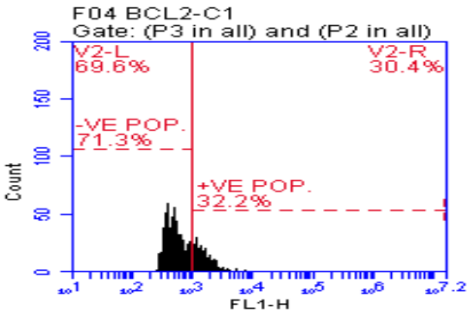


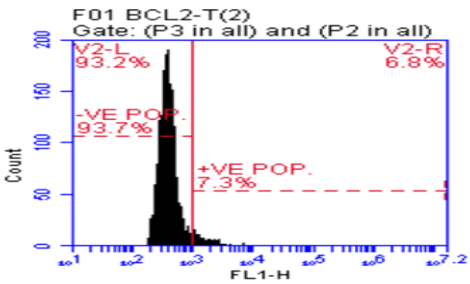
Fig(12a) ZHC effect on P53 in experimentally induced solid tumor in mice.

a,Significant at ($p< 0.01$) from solid tumor group.



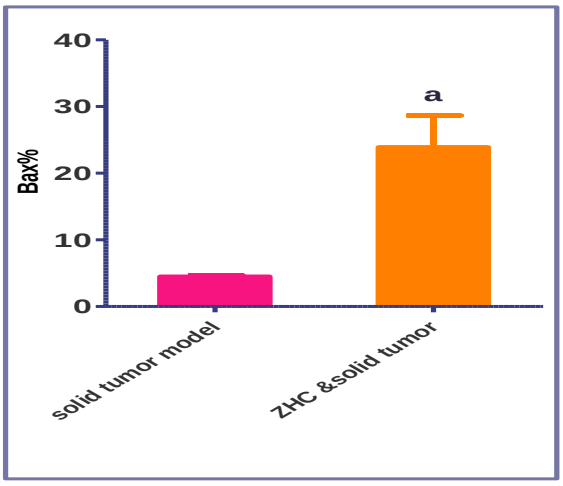
Figure(12b) Flow cytometric histogram of P53 in experimentally solid tumor in mice.



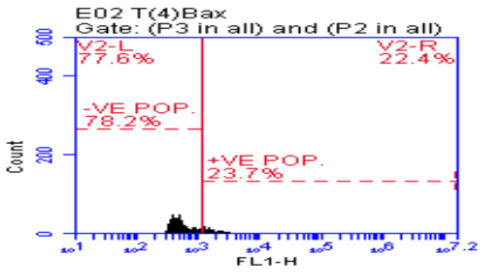


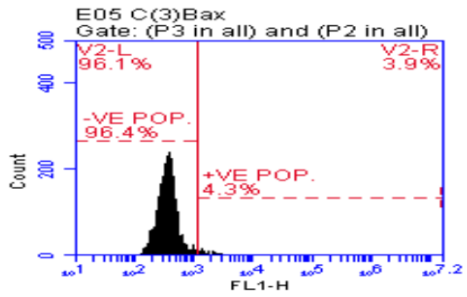
Fig(13a) ZHC effect on Bcl-2 in experimentally induced solid tumor in mice.

a,Significant at ($p< 0.01$) from solid tumor group.



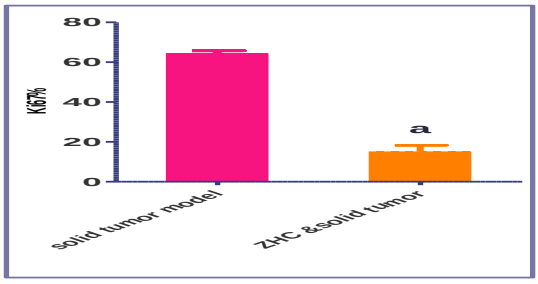
Figure(13b) Flow cytometric histogram of Bcl-2 in experimentally solid tumor in mice.



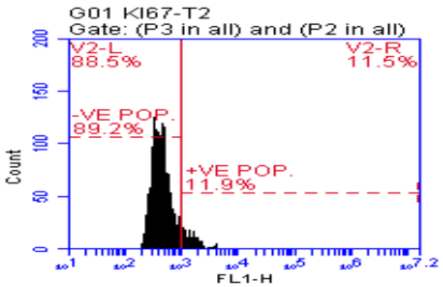


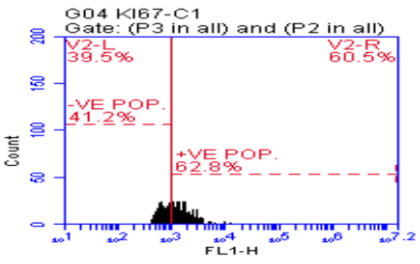
Fig(14a) ZHC effect on Bax in experimentally induced solid tumor in mice.

a,Significant at ($p< 0.01$) from solid tumor group.



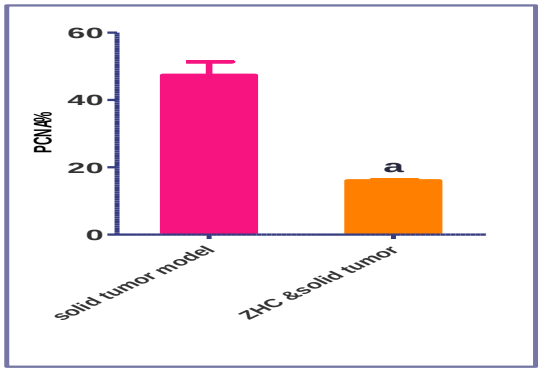
Fig(15a) ZHC effect on Ki-67 in experimentally induced solid tumor in mice.



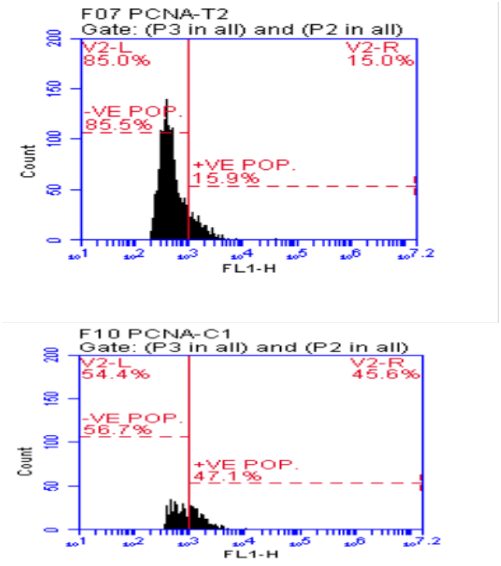


Figure(15b) Flow cytometric histogram of Ki-67 in experimentally solid tumor in mice.

a,Significant at ($p< 0.01$) from solid tumor group.



Fig(16a) ZHC effect on PCNA in experimentally induced solid tumor in mice.



Figure(16b) Flow cytometric histogram of PCNA in experimentally solid tumor in mice.

Discussion:

According to research, hydrazones have pharmacological properties and play an important role in the regression of many cancers [34]. Chemotherapeutic cancer treatment is widely used, despite the fact that it has negative side effects, particularly on normal cells. As a result, extending the life span of tumor-bearing animals is a reliable criterion for determining the value of any novel anti-cancer drug. Schiff base hydrazones have recently piqued the interest of researchers due to their anti-tumor, antimicrobial, and anti-tubercular properties. Because they form stable coordination complexes with the majority of transition metal ions, Schiff base hydrazones are important in inorganic chemistry [35-37]. Several studies have found that solid tumor models are a useful tool for studying biological activities in cancer and evaluating the efficacy of potential cancer drugs and chemicals [38]. The current study sought to investigate the anti-tumor activity of the zinc-hydrazone complex in female mice with EAC solid tumors. Modern metal-based anti-tumor drugs are being developed at a rapid pace in search of new chemotherapeutic alternatives that may be more effective with fewer or no side effects. According to research, zinc is essential for normal human physiology. It has also been linked to pathophysiological conditions such as many cancers [39]. As a result, their derivatives may be biologically active agents.

The current study showed that when compared to the solid tumor group, the zinc-hydrazone complex significantly increased survival and suppressed solid tumor formation. Increased animal survival and health quality is a reliable criterion for assessing the efficacy of anticancer agents. The ZHC may exert its anticancer effect by inhibiting the viability of solid tumour cells, decreasing tumor volume, and extending the survival time of solid tumor-bearing mice. Initially, we noticed that the complex administration reduced both mouse mortality and tumor size. The mean body weight of the tumor-non-treated mice group increased at the end of the study when compared to the ZHC treated group. Body weight loss resulted in stunned growth not only due to reduced food consumption, but also due to the tumor burden and its rapid growth rate. The criteria for success of any antitumor drug are a reduction in body weight gain and a decrease in tumor volume [40]. The findings explained a decline in tumor weight in EAC solid tumor bearing mice treated with ZHC, which was consistent with previous findings that ZHC' cytotoxic activity against tumor cells has the potential to be used as anticancer therapy by targeting cancerous cells, enhancing cytotoxicity, and cell death [41], When used as an anticancer agent, ZHC showed no cytotoxicity in liver tissues, confirming previous findings which zinc complex did not show any signs of systemic toxicity [42]. Our data reported a reduction in tumor volume and survival rate, in the same line with previous study who noticed that the tumor volume and survival rate decreased significantly with different monotherapy treatment regimens

compared to the non-treated group, demonstrating that combined therapy had the best antitumor activity [43]. Based on these results, we hypothesized that ZHC had an anticancer effect.

The present study demonstrated that ZHC was effective in inducing growth inhibition, cell cycle deregulation, and apoptosis in solid tumor cells. After ZHC treatment, the cell cycle was significantly arrested at the G0/G1 phase, with an increasing percentage of apoptotic solid tumor cells. Our result discovered that solid tumor inoculated mice exhibited remarkable alterations in cell cycle analysis, with a significant accumulation at the S percent phase as well as the G2/M phase, and a significant decrease in apoptosis percent. Because the tumor tissue is a rapidly growing carcinoma, the cells continuously increase their nuclear materials at the expense of their normal apoptotic rate [44]. The tumor tissue of mice treated with ZHC, on the other hand, showed an accumulation of sub-G1 phase cells arrested in the G1/G0 phase of the cell cycle, as well as a significant decrease in cells at the S and G2/M phases of the cell cycle. These findings demonstrate that the complex induces antiproliferative and apoptotic effects. In solid tumor cells, cell cycle arrest is associated with a significant decrease in the expression of the proliferation markers Ki-67 and PCNA. This finding is consistent with previous research that found zinc complex inhibits colon cancer cell proliferation [45].

Apoptotic pathways are governed by a balance of proteins that mediate decreased cell division with cell death, such as P53, P21, and Bax, and proteins that promote cell viability, such as Bcl-2 [46]. Among these proteins, the tumor suppressor gene P53 is essential for the activation of apoptotic machinery in damaged cells as well as autologous tumor cell apoptosis in response to chemotherapy [47]. According to the current findings, ZHC induces apoptosis and increases P53 levels, which is consistent with previous findings [48] who noted that zinc induces apoptosis of melanoma cells is similarly associated with increase of intracellular ROS , as well as P53. Apoptosis is a type of cell death that is regulated by several proteins produced by specific genes and occurs as a result of the activation of a pre-programmed pathway of biochemical reactions that directly lead to cell death. Bax is a Bcl-2 family member that promotes apoptosis [49]. In addition, Bcl-2's cell survival effect is inhibited [50]. In the present ZHC elevated the level of Bax that is in agree with previous study which discovered that zinc accumulation induced apoptosis in choricarcinoma cells and human epithelial ovarian cancer cells, as well as an increase in the cellular level of Bax but a decrease in the level of Bcl-2 [51].

Antioxidant enzymes plays a great role against cancer to prevent the damge of oxidative DNA& risk of cancer cells [52]. The role of antioxidant are decreasing oxidative stress, malignant transformation,DNA damage & other parameters of cell damage in vitro [53]. Reactive oxygen species are crucial in tumor proliferation and development [54]. Our findings show that zinc hydrazone complex significantly increases SOD, Gpx, GSH, and TAC levels while decreasing MDA levels. These findings are consistent with previous research, which found that zinc supplementation increased SOD, Gpx, GSH, and TAC levels while decreasing MDA levels [55].

In conclusion, the current findings show that zinc hydrazone complex reduces cell proliferation, stimulates apoptosis, attenuates expression of key tumor markers and suppresses carcinogenesis, which is confirmed by suppressing the growth of EAC solid tumor in female mice, thus it may play a beneficial role in cancer management.[1]. Ali I, Wani WA, Saleem K. Cancer scenario in India with future perspectives. Cancer therapy. 2011;8(2):56-70.

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