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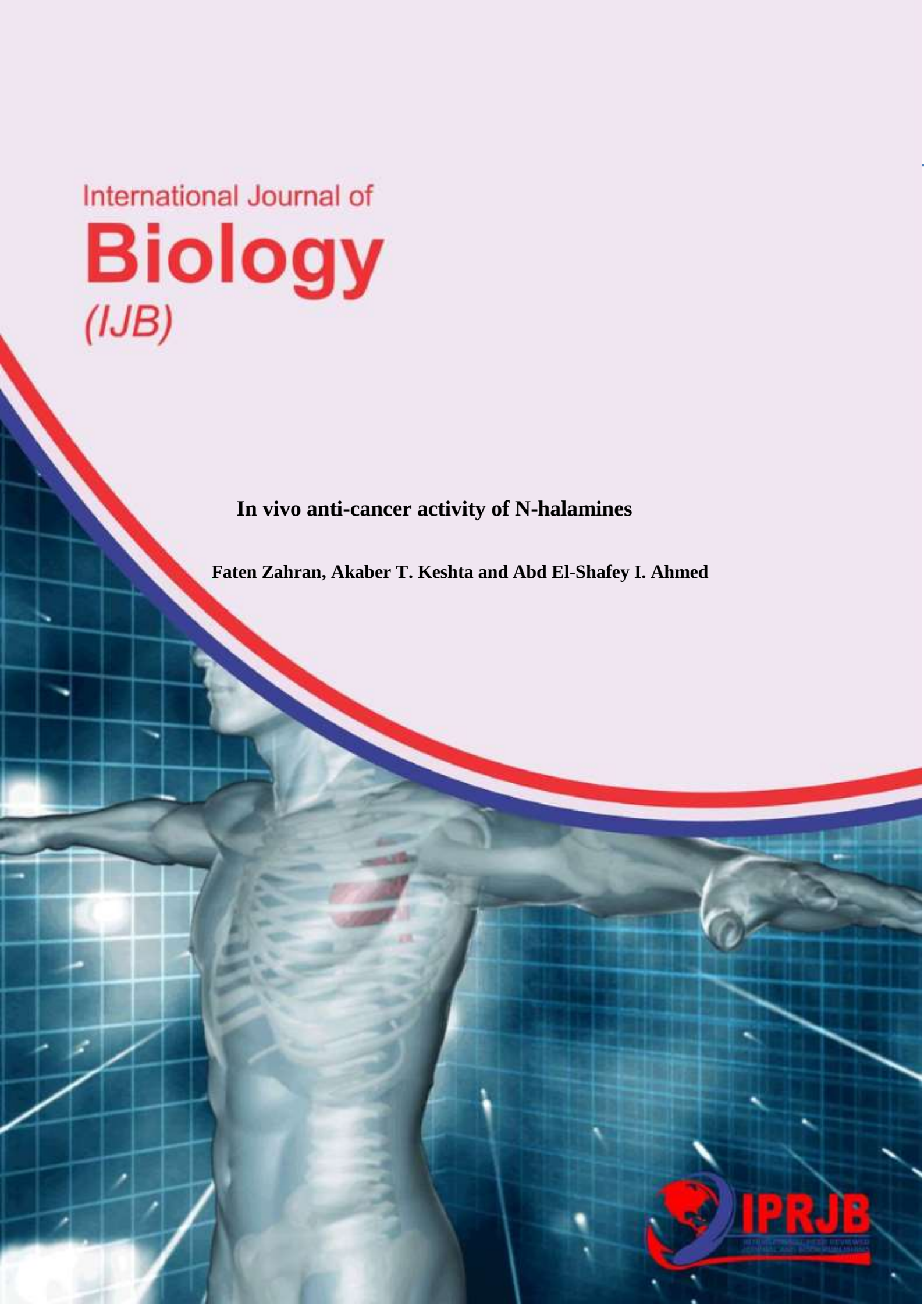

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In vivo anti-cancer activity of N-halamines

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ABSTRACT

Purpose: N-halamines were known for their antimicrobial action due to the presence of halogen in their structure. In our search for new anti-cancer agents, we have evaluated the anti-cancer and anti-oxidant properties of some synthetic N-Halamines with high and low molecular weight in comparison with their non-halogenated forms. Urea epichlorohydrin copolymer (1), 4(1H, 3H-2, 6-dioxo-1, 3, 5-trizenyl)-Oiminomethylpolyethylene (3) and cynuric acid (5) in addition to their halogenated forms 2, 4 and 6 were selected for this study.

Methodology: the toxicity for the synthesized compounds was determined. The anticancer and anti-oxidant activities were studied by evaluation the viability of tumor cells, life span prolongation, and estimation of antioxidants, and effects of these compounds on liver histology.

Findings: Doses up to 2000 mg/kg indicated good safety in all investigated compounds. In Vivo antitumor activity results against Ehrlich ascites carcinoma (EAC) cells for the investigated compounds revealed that, the volume of ascites was significantly decreased in compounds 1, 2, 3, 4, 5 and 6 treated groups. EAC cell count was significantly reduced for similar groups, respectively, compared to the positive control group. Malonadildehyde, and nitric oxide showed a significant reduction in their levels in the treated groups, while catalase showed a significant elevation in its activity in the same groups compared to positive control group.

Conclusion: Halogenated compounds showed good anti-oxidant behavior while compound 6 showed the best anti-tumor effect.

Keywords: *N-halamines; Polymers; Ehrlich ascites carcinoma cells; Cancer.*

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1. INTRODUCTION

Cancer is a group of diseases that cause cells in the body to change and grow out of control (American Cancer Society, 2016). Cancer is considered one of the major causes of mortality in the world. Despite the recent advances in science, cancer has not been cured yet. It is estimated that by 2020 there will be 16 million new cancer cases every year (Devegowda *et al.*, 2010). Some significant progress has been made to understand the pharmacological and chemical properties in this approach to provide more details which may improve and establish proper strategies to prevent cancer. However, the non-selectivity and acute toxicity of many antitumor agents have been the major deterrent in their usage for treating human cancer, prompting the search for new chemo-preventive and antitumor agents with improved tumor selectivity, efficiency and safety (Musa *et al.*, 2011).

Chlorinated compounds have been used for disinfection purposes since the early 1900s due to their effective bactericidal action (Ahmed *et al.*, 2009) and (Dakin et al., 1916). They can act either as free small species, such as HOCl and NaOCl (which are considered to be the most effective disinfectants for water and clinical purposes) (Sen et al., 1999) or attached to amino containing compounds (N-halamines). The latter can be present as low molecular weight compounds such as halogenated isoxazoles and imidazoles (Worley *et al.*, 1992) or high molecular weight such as N-halamine polymers (Chen *et al.*, 2004) (Ahmed *et al.*, 2008).

These polymers are an important type of biocidal polymers which was prepared by incorporating heterocyclic rings on a polymer skeleton followed by halogenation (Ahmed *et al.*, 2009). They were used successfully in water treatment systems and other disinfection purposes. N-halamines may contain one or more nitrogen-halogen covalent bonds. They have antimicrobial efficacy similar to hypochlorite bleach but they are more stable, less corrosive, and have much less tendency to generate halogenated hydrocarbons. Therefore, N-halamines have found wide applications as food and water disinfectants (Cao, and Sun, 2009), (Luo, and Sun, 2006 and 2008) (Lauten *et al.*, 1992). Their mode of action against bacterial cells was believed to be a combination of three different ways; contact, release and changing the medium nature around the cells (Ahmed *et al.*, 2009). As soon as the polymer delivers halogen to cells it restores its original status before halogenation so it can be recycled by recharging with halogen which increasing its commercial value especially in water filters.

Reducing the dose of N-halamine may result only in changing the internal metabolic structure of the cells without killing it as reported by Ahmed and his coworkers (Ahmed et al., 2011). Taking this fact into consideration we thought about the possibility of applying such compounds to tumor cells. No trails were reported in literature about investigating the effect of N-halamines against such cells. The halogen in N-halamines is attached to nitrogen atom and not to carbon atoms which enables the separation of halogen ion positively charged which may have different mechanism in dealing with tumor cells. A comparison will be conducted as well between different types of N-halamines which have different molecular weight and structure.

In order to substantiate this fact, implanted tumor Ehrlich carcinoma cells were assessed in vivo to Swiss mice strain. A group of N-halamines, Scheme 1, were selected based on: molecular weight and structure to see their effect on reactivity. Monomeric and polymeric N-halamines were selected to test the effect of using low and high molecular weight species on reactivity. Linear polymeric N-halamines contain halogen in cyclic (4) and acyclic (2) formulation was compared to see the effect of halogen presence in stable cyclic structure8 on its reactivity in comparison with acyclic ones.

2. MATERIALS AND METHODS

2.1.Materials

2.1.1. Chemicals: Cynuric acid (compound 5, Scheme 1), trichloroisocynuric acid (compound 6, Scheme 1), polyacrylonitrile and epichlorhydrin were obtained from Sigma Aldrich, UK. Urea, sodium hydroxide, ethanol absolute and sodium hypochlorite were obtained from El-Nasr Chemical Co. Egypt. All chemicals were used as received without extra purification.

2.1.2. Animals: Female Swiss albino mice of 8 weeks of age, weighed 22 to 25 g body weight were housed at the experimental animal house, Faculty of Science, Zagazig University. The animals were maintained in controlled environment of temperature, humidity and light. They were feed on a commercial standard diet and tap water *ad libitum*.

2.1.3. Tumors: Ehrlich ascites carcinoma (EAC) was initially supplied by the National Cancer Institute, Cairo, Egypt, and maintained in female Swiss albino mice through serial intraperitoneal (I.P) inoculation in an ascites form. **2.2.Methods**

Urea epichlorohydrin copolymerization

Urea, epichlorohydrin and sodium hydroxide were heated in a round bottom flask fitted with a reflux condenser in ratio of 1:1:1. After complete dissolving, a vigorous reaction was happened followed by the formation of a white precipitate. The refluxing continued until all the liquid converted to solid white material. The prepared material (1) was washed with water, diluted HCl and methanol followed by drying in air over night, (compound 1, Scheme 1).15 The prepared polymer (1) has shown the following analysis: FTIR υ (cm-1): a broad band at 3400-3500 corresponding to NH and OH, a band at 2995 corresponding to CH aliphatic, a band at 1672 corresponding to C=O imide, and a band at 1115 corresponding to C-O. Solid state C13NMR (ppm): 40, 60, 70 and 160 (Ahmed *et al.*, 2014).

2.2.1. Preparation of 4(1H,3H-2,6-dioxo-1,3,5-trizenyl)-O-iminomethylpolyethylene (3)

Cyanuric acid (2.58 g) was added to sodium ethoxide solution (prepared by dissolving 1.38 g of sodium in 40 ml absolute ethanol). The reaction mixture was heated at 80oC for 30 min to form the required sodium salt. Polyacrylonitrile (0.53 g) was added and the mixture was refluxed for 48 hrs. The reaction mixture was cooled, introduced into an ice/hydrochloric acid mixture and pH was adjusted to 7 using diluted HCl solution. The solid red product formed was filtered, washed copiously with hot water and dried (compound 3, Scheme 1) (El-Masry et al., 2004).

2.2.3. Chlorination:

Prepared polymers (1 and 3, Scheme 1) were halogenated by soaking 1g of each sample in sodium hypochlorite (10 ml, 10%) for 30 min at ambient temperature. The resulting halogenated polymers (2, 4) were filtered, washed copiously with distilled water and dried at 40oC overnight. The halogen content was determined using iodometric titration (Ahmed *et al.*, 2008), (Chen, and Sun, 2006). The halogenation conditions were changed until similar dose was loaded to both polymers (Ahmed *et al.*, 2009). **2.2.4. Determination median lethal dose (LD 50) of the synthesized compounds**

Approximate LD50 of compounds 1, 2, 3, 4, 5, and 6 in mice were determined according to the method described by Meier and Theakston (1986).

2.2.5. Dose response curve

Dose response curve of compounds 1, 2, 3, 4, 5, and 6 in mice was determined according to the method of (Crump *et al.*, 1976).

2.2.6. Experimental design

Female albino Swiss albino mice were divided into 7 groups each one contains of 10 mice: Group 0 "served as positive control; injected with $2.5x10^6$ of Ehrlich ascites carcinoma "EAC" cells. Group I "compound 1 therapeutic group", injected with 10 mg/kg one day after EAC injection and repeated dose twice during the experiment; Group II "compound 2 therapeutic group", injected with 10 mg/kg one day after EAC injection and repeated dose twice during the experiment; Group III "compound 3 therapeutic group", injected with 5 mg/kg one day after EAC injection and repeated dose twice during the experiment. Group IV "compound 4 therapeutic group", injected with 5 mg/kg one day after EAC injection and repeated dose twice during the experiment; Group V "compound 5 therapeutic group", injected with 10 mg/kg one day after EAC injection and repeated dose twice during the experiment; and Group VI "compound 6 therapeutic group", injected with 5 mg/kg one day after EAC injection and repeated dose twice during the experiment.

At the end of the experiment, EAC cells were harvest from each mouse in centrifuge tube containing heparinized saline. Note the volume of ascetic fluid in each mouse in each group. Each sample of cells was undergoing viability test of EAC cells and antioxidants assays were performed. Also, liver tissue from each group was preserved in 10% neutral formalin solution for histological examination.

2.2.7. Viability of EAC cells and life span prolongation

The viability of EAC cells was determined by the Trypan Blue Exclusion Method described by McLiman et al., (1957). Life span was calculated according to the method described by Mazumdar et al., (1997).

2.2.8. Antioxidant assays

Malondialdehyde (MDA), Nitric Oxide (NO) levels, and Catalase (CAT) activity were estimated according to methods of described by (Satoh,1978); Montgomery and Dymock, (1961), and Aebi, (1984); respectively.

2.2.9. Histological study

Liver specimen was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. After de-paraffinization and dehydration, the paraffin blokes were stained with hematoxylin and eosin for microscopic examination (Lillie, 1976). Light microscopy was used to evaluate the pathological changes in the liver tissues.

2.2.10. Statistical analysis

Statistical analysis was performed using SPSS software II version 14 (Levesque, 2007). The effect of each parameter was assessed using the one way analysis of variance. Individual differences between groups were examined using Dunnett's test and those at $p < 0.05$ were considered statistically significant.

3. RESULTS

3.1. Determination of median lethal dose (LD_{50})

The acute toxicity LD_{50} was estimated by I.P. injection of compounds 1, 2, 3, 4, 5 and 6; doses up to 2000 mg /kg indicated safety in all polymers.

3.2. Dose response curve of polymer tested (N-halamines) compounds

It was cleared that 10 mg/kg was found to be the most effective dose of compounds 1, 2, and 5; while; 5 mg/kg was found to be the most effective dose of compounds 3, 4, and 6; as this dose reduced the number of EAC cells compared to positive control group at twice I.P. injection, Figure 1.

3.3. Effect of polymer tested (N-halamines) compounds on volume, EAC cell count

Table I summarized the effect of compounds 1, 2, 3, 4, 5 and 6 on EAC cells volume and count. The mean volume of EAC in the positive control group was found to be 5.1±0.21 (ml) as reported by Faten *et al.,* (2013). This value was significantly decreased by 13.7%, 52.9%, 39.2%, 15.6%, 62.7 and 82.3% ($p<0.01$) in compounds 1, 2, 3, 4, 5 and 6 treated groups; respectively, Figure 2. Also, the mean count of EAC cells in the positive control group was found to be 250.3±11.4 ($\times 10^6$), which significantly decreased to 213.5 ±33.7, 125.5 ±6.0, 107.7 ±8.5, 160.8 ±7.9, 82.3 ±12.3, and 43.3 ± 9.5 by -14.7%, -49.8%, -56.9%, -35.7%, -67.1% and -82.7%, ($p<0.01$) in compounds 1, 2, 3, 4, 5 and 6 treated groups; respectively, compared to the positive control group, Figure 3.

3.4. Effect of polymer tested (N-halamines) compounds on life span in all studied groups

Table II illustrated the life span prolongation of the studied tested compounds. The mean life span prolongation in the positive control group (EAC bearing tumor group) was found to be 15 days. Compounds 1, 2, 3, 4, 5, and 6 treated groups showed a significant increase in the life span prolongation to 19 days by 26.6% (T/ C ratio = 126.6%), 22 days by 46.6% (T/ C ratio = 140.0%), 25 days by 66.6% (T/ C ratio = 166.6%), 21 days by 40.0% (T/ C ratio = 140.0%), 28 days by 86.6% (T/ C ratio = 186.6%) and 33 days by 120.0% (T/ C ratio = 220.0%); respectively compared to the positive control group.

3.5. Effect of polymer tested (N-halamines) compounds on antioxidants in all studied groups

Table III and figures 4 and 5 illustrated the anti-oxidant activity of the investigated compounds. The mean MDA level was found to be 22.6±.058 (nmol/ml) in positive control group. Groups I, II, III, IV, V and VI showed a reduction in MDA levels to 21.2 ±1.0, 18.0 ±0.8, 15.9 ±0.6, 19.4 ±0.7, 13.2 ±0.8, and 9.9 ±0.7 by 6.1%, 20.3%, 29.6%, 14.1%, 41.5% and 56.1%, respectively ($p<0.01$), compared to positive control group. Also, there was a decrease in NO levels from 95.9 ±2.3 in group 0 to 89.3 ±5.5, 64.5 ±4.1, 50.5 ±3.7, 72.7 ±3.9, 40.7 ±1.8, and 27.4 ±4.2 in groups I, II, III, IV, V and VI; respectively by 6.8%, 32.7%, 47.3%, 24.1%, 57.5% and 71.4%; respectively, ($p<0.01$) compared to group 0.

Meanwhile; CAT activity was significantly increased in all treated groups by from 0.08 ±0.01 in group 0 to 0.09 ±0.004, 0.1 ±0.03, 0.21 ±0.01, 0.1 ±0.003, 0.3 ±0.02, and 0.6 ±0.1; in groups I, II, III, IV, V and VI; respectively by 12.5%, 25.0%, 162.5%, 25.1%, 275.0% and 650.0%; respectively, compared to group 0, ($p<0.01$).

3.6. Effect of pyrazoline derivatives tested compounds on liver tissues in all studied groups

The histological examinations of liver tissues with Hematoxylin and Eosin stain in the different studied groups to confirm the effect of these compounds on other tissues such as liver as it is the site of most metabolisms. As illustrated in Fig. (6), Histopathological examination of liver sections from control animals' revealed normal healthy parenchyma; while in EAC group, liver showed focal large area of necrotic cells infiltrated with mononuclear cells. Different compounds treatment showed different alterations in liver tissues, where compound 6 showed

congested central vein with necrosed hepatocytes, compared with compound 4 that showed area of hepatic necrosis with mononuclear cells infiltration.

4. DISCUSSION

Cancer is now one of the world's most pressing health challenges. Research continues to deliver new and improved treatment options for thousands of people living with cancer (ASC, 2016). Organic N-halamines are known for their biocidal activity due to the covalently bonded halogen attached to the nitrogen atom in their structure. These halogen atoms are the main reason for their mode of action due to their exchange with cells (Chen *et al.,* 2003). The exchange can happen by direct contact between the particles and cells, halogen releasing to water and then to cells and changing the nature of medium around the cells by halogenating the medium constituents (Ahmed *et al.,* 2009). Lower doses of halogen on the polymer may retard cells growth and affect the metabolomic structure rather than killing them (Ahmed *et al.,* 2011). N-halamines can be present as low molecular weight compounds such as triclorocynuric acid or high molecular weight polymeric materials (N-halamine biocidal polymers). The unique property of N-halamine polymers is that they can be repeatedly charged with halogen by simply reacting them with sodium hypochlorite solution (household bleach) which increases their commercial value. The high molecular weight N-halamines (N-halamine polymers) were prepared by incorporating heterocyclic rings on the polymer followed by halogenation or by direct halogenation to linear polymers. The structure of the polymer should contain amide or imide function group to support the halogenation process. Presence of halogen in a cyclic structure increases its stability. In addition presence of electron donating groups attached to the rings can further increase this stability (Ahmed *et al.,* 2008).

Halogenated heterocyclic rings have shown potent antioxidant activity as compare with some standard drugs due to the presence of halogen atom (Chen *et al.,* 2003). Presence of halogen has increased the penetration of molecules into the lipid membrane so that they increase the antioxidant activity by combining with the reactive oxygen species, which is generated by the different disease conditions (Bano *et al.,* 2012). But what was reported in literature for halogenated heterocyclic rings was based on halogen attached to carbon atom. In this work it is the first time to evaluate halogen attached to nitrogen atoms.

Based on the previous facts we were thinking that N-halamines may have anti-cancer and anti-oxidant properties. For this purpose we have designed the protocol of this paper to cover different structures of N-halamines starting from low to high molecular weight species and from linear to cyclic frame containing compounds. For example polymer 2 is a linear shape polymer while 4 is a linear polymer contains the halogen in a cyclic structure rather than in an open form such as 2. At the same time compound 6 is a low molecular weight biocidal compound which considered as another chance to compare the effect of low and high molecular weight halogenated biocidal compounds. We believe that this selection may help in the evaluation of anticancer and anti-oxidant properties of such family of compounds and would indicate a clear bath to which design can be used.

In vivo antitumor activity results against Ehrlich ascites carcinoma cells for compounds 1, 2, 3, 4, 5 and 6 revealed that, the investigated compounds had anti-tumor activity by decreasing the volume of ascites, EAC count, and increased the life span. Also, the investigated compounds had anti-oxidant activity by decreasing MDA and NO levels and increasing CAT activity. this is due to the substitution in the aromatic ring system with halogens like chlorine sharply enhanced the antioxidant potency, as chlorine atom has lone pair electron as well as its electronegative power enhanced the formation and subsequent stabilization of the nitrogen-

ring radical through intervening aromatic system property, it might have enhanced the power to absorb free radicals, especially reactive oxygen and reactive nitrogen species (ROS and RNS) (Kalpana *et al.*, 2014). In general, (substitution by a halogen atom particularly, a chlorine atom) in the 'meta' position of the phenyl ring led to a better anti-tumor effect than that observed in the absence of any substituent (Kempen *et al.*, 2003).

This indicates that compound 6 has in vivo antitumor activity against EAC more than other compounds; this may be attributed to the presence of chlorine atom and its low molecular weight. Reducing the molecular weight can increase the action of the compound as this can enables its diffusion through the cell wall. N-halamine structures possess several useful features including good stability for long-term use, storage over a wide temperature range and the ability to be regenerated in a chlorine solution repeatedly (Sun and Sun, 2004). Compared with inorganic halogens, Nhalamine polymers are very stable, less corrosive, and do not decompose in water to form toxic products, or release halogen until contact with the bacteria (Kenawy et al., 2007). In addition, presence of halogen atom can increase this action due to its effect on cell wall and internal metabolic structure (Ahmed *et al.*, 2014). This can be noticed from the effect of halogenated compounds mainly 2 and 6 which is higher than that of 1 and 5.

It has been suggested that halogenated compounds can denature bacterial DNA by pyrimidine base halogenation which reduces the ATP level leading to immediate cell death (Ahmed *et al.*, 2014), (Camper, and Mcfeters, 1979). Some other opinions were suggesting that treating cells with halogenated compounds may result in amino acid neutralization and protein denaturation due to halogen transfer. It was reported also in the literature that halogen containing compounds can result in the oxidation of SH groups of essential enzymes and change the intracellular pH, ultimately leading to global disruption of cell metabolism (Ahmed *et al.*, 2014), (Estrela *et al.*, 2002). The low effect of compound 4 may be explained on the fact that presence of halogen in cyclic frame may retard the release of halogen. Presence of halogen in cyclic frame increases its stability and decreases its exchange possibilities. Presence of electron donating groups attached to such cyclic system may increase this stability to a higher level. In general, we believe that the mode of action of N-halamines against tumor cells is similar to that against bacterial cells. This action mainly depends on the attached halogen as stated above which transferred to the cells by contact, release and through the medium constituents around the cells. Presence of any component around the cells that can be halogenated can act as intermediate to transfer the halogen to the required cells.

Moreover from the results it can be seen that linear polymeric compounds are showing better results than that of the others, which contains heterocyclic rings (halogenated and non-halogenated forms). This could be explained on the base that linear structures are able to diffuse through the cell walls better than other structures with cyclic frames. But still this leaner polymer is not able to beat the power of the low molecular weight structure of compound 6. Further efforts will be conducted to explain and study the mechanism and the effect of such compounds on living cells in comparison with bacterial cells .

The histological examinations of liver tissues with Hematoxylin and Eosin stain in the different studied groups were performed to confirm the effect of these compounds on other tissues such as liver. As, liver is a predominant organ for the metabolism, and also is the center detoxifying any foreign compounds entering the body (Marrs, 2012). As illustrated in figure 6, Histopathological examination of liver sections from control animals revealed normal healthy parenchyma while in EAC group, liver showed focal large area of necrotic cells infiltrated with mononuclear cells. Different compounds treatment showed different alterations in liver tissues.

From this study we can concluded that compound 6 has the highest anti-tumor activity with slightly alterations in liver tissues compared to other compounds.
Most of the investigated compounds have shown anti-oxidant characters. Halogenated species effect was more than that of non-halogenated ones. This can be explained on the base that N-halamines are able to produce halogen ions which can interact with the moieties generated by oxidant reducing their effect .

The previous results could be used as a base for further efforts to investigate the potential effect of applying such family (N-halamines) as anticancer and antioxidant. In addition, the mechanism of their effect of healthy and sick cells is another issue to be studied. We are working on more trails in this direction and results will be published in due courses.

5. CONCLUSIONS

The previous results indicated that most of the investigated halogenated compounds are showing better anticancer and antioxidant properties than that of non-halogenated ones. Linear polymers with acyclic structure showing better behavior than others contain halogen in cyclic frames. The lower the molecular weight of the compounds, the higher of its activity. The histological study has indicated that compound 6 has the highest anti-tumor activity with slightly alterations in liver tissues compared to other compounds.

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Table (1): Effect of N-halamines compounds (1, 2, 3, 4, 5, and 6) on the volume and count of EAC in all studied groups:

Group	Group 0	Group I	Group II	Group III	Group IV	Group V	Group VI
Volume of Ascites Fluid (ml)	5.2 ±0.2	4.4 ±0.3	2.4 ±0.4	3.1 ±0.2	4.3 ±0.3	2 ±0.5	0.9 ±0.3
Count of EAC cells ($\times 10^6$)	250.3 ±11.4	213.5 ±33.7	125.5 ±6.0	107.7 ±8.5	160.8 ±7.9	82.4 ±12.3	43.4 ± 9.5

The significant difference: $P^{**} < 0.01 \rightarrow$high significant $P^{*} < 0.05 \rightarrow$ significant

Table (2): Effect of N-halamines compounds (1, 2, 3, 4, 5, and 6) on the on life span prolongation in all studied groups:

Group	Group 0	Group I	Group II	Group III	Group IV	Group V	Group VI
Days	15	19	22	25	21	28	33
% Change	----	26.6	46.6	66.6	40.0	86.6	120.0

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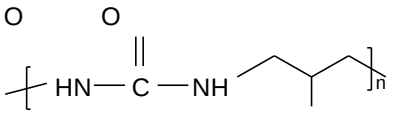
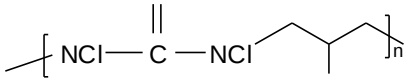
Table (3): Effect of N-halamines compounds (1, 2, 3, 4, 5, and 6) on anti-oxidants in all studied groups:

Variables	Group 0	Group I	Group II	Group III	Group IV	Group V	Group VI
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
MDA (nmol/ml)	22.6 ±0.06	21.2 ±1.0*	18.0 ±0.8**	15.9 ±0.6**	19.4 ±0.7**	13.2 ±0.8***	9.9 ±0.7***
% change	------	-6.1%	20.3%-	-42.1%	-16.4	-41.4	-56.1 %
NO (µmol / l)	95.9 ±2.3	89.3 ±5.5*	64.5 ±4.1**	50.5 ±3.7**	72.7 ±3.9**	40.7 ±1.8***	27.4 ±4.2***
% change	------	-6.8%	32.7%-	-47.3%	-24.1	57.5%-	71.4%-
CAT (U/g tissue)	0.08 ±0.01	0.09 ±0.004*	0.1 ±0.03**	0.21 ±0.01**	0.1 ±0.003**	0.3 ±0.02***	0.6 ±0.1***
% change	------	12.5%	25.9%	42.1%	162.5	275.0%	650.0 %

*Significance at *P < 0.05, **P < 0.01, ***P < 0.001





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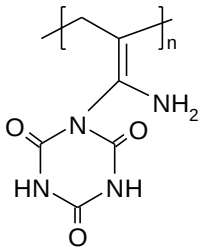
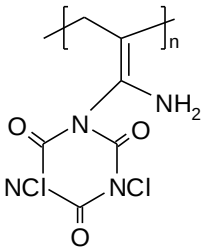
2





3 **4**

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Scheme 1: Chemical structure of investigated compounds.

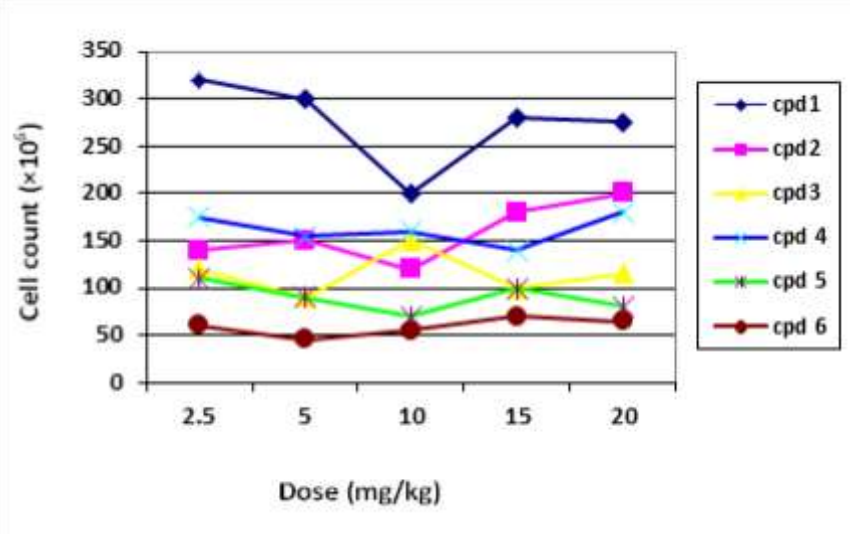

Figure 1: Dose response curves for compounds

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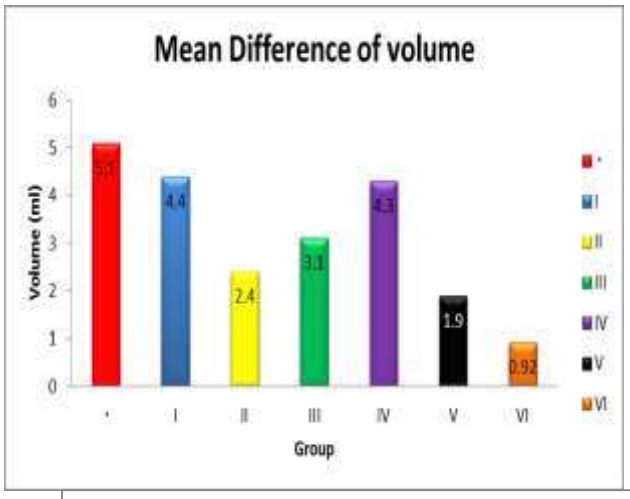

igure 2: Effect of compounds 1, 2, 3, 4, 5, and 6 EAC volume

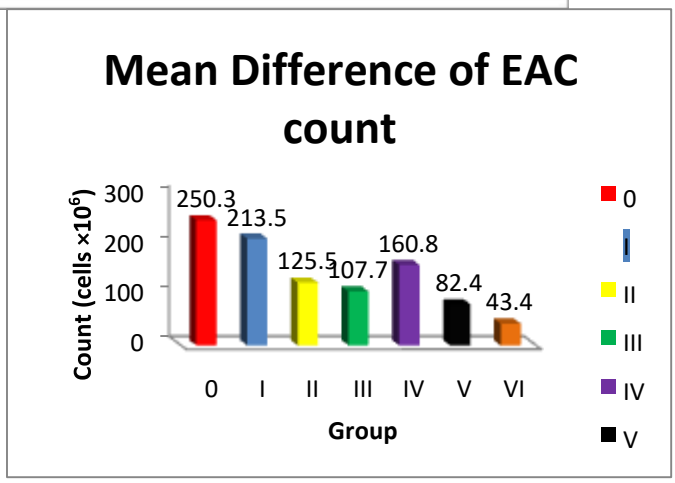

Figure 3: Effect of compounds 1, 2, 3, 4, 5, and 6 on EAC count.

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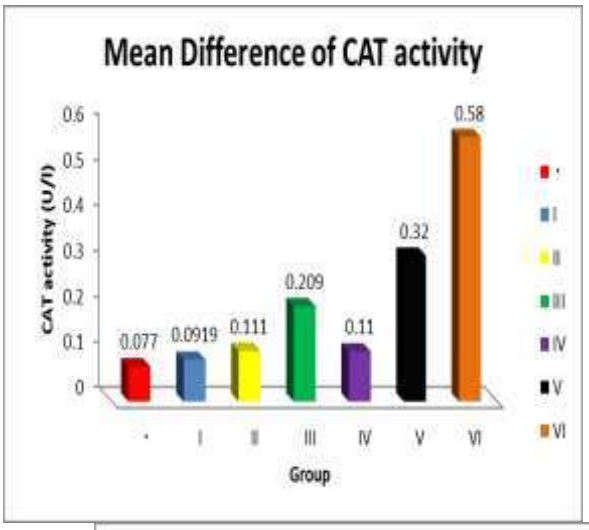

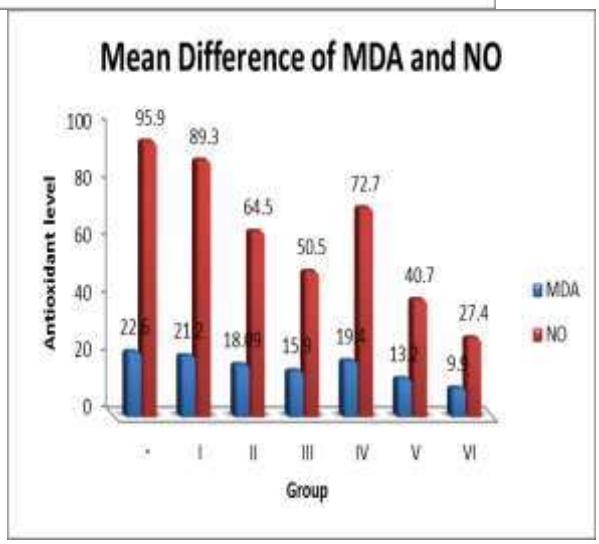

Figure 4: Effect of compounds 1, 2, 3, 4, 5, and 6 on MDA and NO levels in EAC cells. **Figure 5:** Effect of compounds 1, 2, 3, 4, 5, and 6 on CAT activity in EAC cells. photomicrograph of negative control showing healthy hepatic parenchyma classical liver sinusoids radiated from the central vein, ***G(0):*** photomicrograph of positive control showing a focal large area of necrotic cells infiltrated with mononuclear cells, ***G(I):*** photomicrograph of compound 1 treated group showing congested central vein and necrosed hepatocytes with large vesiculated nucleus, ***G(II):*** photomicrograph of compound 2 treated group showing dilated hepatoportal blood vessel which permeated with leucocytic cells, ***G(III):*** showed massive hepatoportal infiltration with mononuclear cells, ***G(IV)***: showed multiple areas of necrotic areas infiltrated with

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inflammatory cells, ***G(V):*** showed congested central vein, G (VI) showed congested central vein.

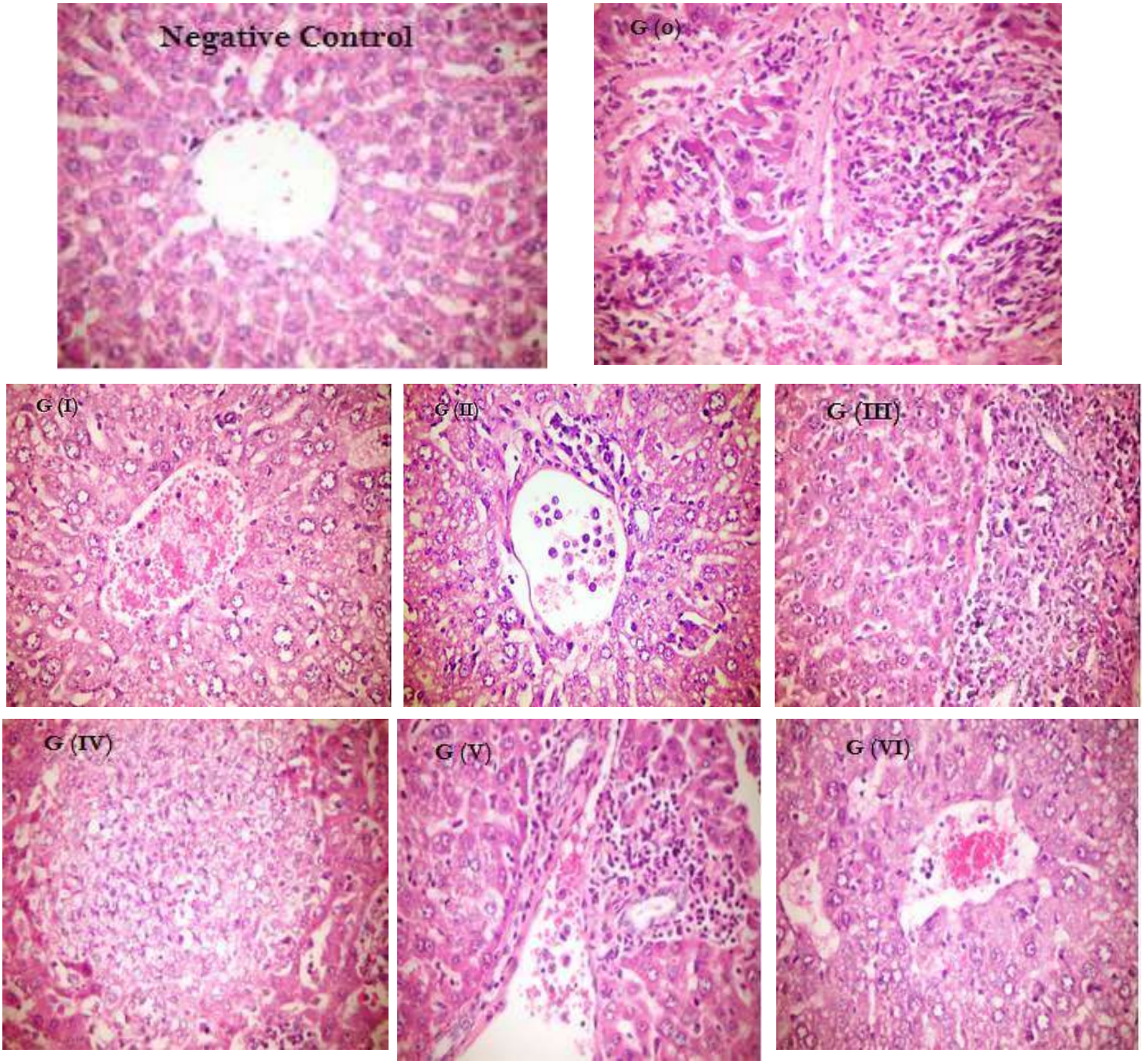

Figure 6: Light photomicrograph of mice liver in the studied groups Hx & E X400;