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Effects of Doxorubicin and Nanoparticle Zinc oxide on DNA damage and Hepatotoxicity induced by Carbon tetrachloride in rats.

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Abstract

The purpose of this study is to determine the effects of doxorubcin and nanoparticle Zinc oxide in normal and hepatotoxicity induced by Carbon tetrachloride(CCl4) in rats. The nanoparticle of ZnO (ZnO-NPs) were prepared by wet reaction of Zn acetate and sodium hydroxide. The prepared ZnO NPs were identified and characterized by Scanning Electron Microscope (SEM) with Energy Dispersire X-ray spectrometer (EDX) Fourier Transmitter Infra Red (FTIR) and UV visible , the structure of particles was 100 nm. The investigated parameters were Liver enzymes, Protein electrophoresis and DNA gel electrophoresis in normal and injected rats by CCl4. Atotal of forty eight adult male albino rats (130-165 g) were divided into 8 groups, each of 6 rats. Group one was kept as a control –ve, second and third groups were injected intraperitoneally with DOX(6mg/kgb.wt) and nZnO(5mg/kgb.wt) respectively for 3 successive days. While fourth group was injected intraperitoneally nZnO followed by DOX for the same period. On the other hand, other four groups were injected subcutaneously with CCL4 50% (0.1 ml/100g.b.wt.twice/week for two weeks)to induce DNA damge and hepatotoxicity. One of this groups was kept as a control +ve (CCL4 groups). Sexth and seventh groups were injected intraperitoneally with DOX (6 mg/kgb.wt) and nZnO (5 mg/kgb.wt) respectively for the same period. While eighth group was injected intraperitoneally nZnO followed by DOX for the same period. At the end of experimental period, blood samples were collected from each rat for biochemical analysis and the rats were sacrificed to illustrate DNA damage in hepatocytes. Subcutaneous injection of CCL4 caused significant increase in serum levels of aspartateaminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphate (ALP) while the levels of malondialdhyde (MDA), Total Antioxidant Capacity (TAC), albumin and globulin were significantly decreased .The smear on agarose gel had been observed in CCL4 injected groups indicating random DNA fragmentation. Rats were injected DOX showed similar changes. NZnO significantly restored the serum levels of the biochemical parameters and DNA damage directed toward to normal as compared with the control +ve group (injected with CCl4). Thus, it can be concluded that zinc oxide nanoparticles followed doxorubicin intraperitoneally

injection improved the alteration of some serum biochemical parameters and DNA damge induced by CCL4.

Introduction

Recent years have witnessed unprecedented growth of research and applications in the area of nanoscience and nanotechnology. There is increasing optimism that nanotechnology, as applied to medicine, will bring significant advances in the diagnosis and treatment of disease. Anticipated applications in medicine include drug delivery, both in vitro and in vivo diagnostics, nutraceuticals and production of improved biocompatible materials **(Duncan 2003; De Jong et al 2005; ESF 2005; European Technology Platform on Nanomedicine 2005 and Ferrari 2005)**. Nano-Zinc oxide (nZnO) is a new product with particle diameter between 1-100 nm **(Dawei, et al., 2009 ; Medina, et al., 2007).** Recently, nZnO has been considered as an important factor in the area of animal science **(Dawei, et al., 2009).** It appears that nano materials hold excessive potential to pass some of the barriers to efficient targeting of cells and molecules in many diseases **(Said et al., 2012 ; Singh Suri, et al., 2007).**

Doxorubicin was one of the early anthracyclines, isolated from Streptomyces peucetius almost four decades ago**(Kin Tam, 2013)**. Doxorubicin (DOX) is a widely used chemotherapeutic agent. Chemotherapy is often used to slow the progress of advanced liver cancer **(Simmonds, 2000)**. The action mechanisms of DOX include: DOX interacts with DNA by intercalation and inhibition of macromolecular biosynthesis, which inhibits the progression of the enzyme topoisomerase II, thus blocking the process of replication**(Fornari, et al ., 1994 ; Momparler, et al., 1976)** .

However, owing to its severe toxic side effects,its clinical use has been limited **(Taveira, et al., 2012).**

The liver is essential for metabolism of drugs and exogenous toxins **(Grünhage et al., 2003)**. In earlier work we have shown, that metabolomics allows for the identification of hepatotoxic effects of compounds known to have this property in rats as well as humans. This was demonstrated for “classical” chemicals such as carbon tetrachloride, or pharmaceuticals such as paracetamol **(van Ravenzwaay et al., 2010)**. Carbon tetrachloride (CCl4) is a toxin that was used extensively to induce liver toxicity **(Calin et al., 2014)**. CCl4 administered to rats induces histologically proven severe hepatopathology, and an increase of serum concentrations of liver enzymes such as aspartate aminotransferase (AST), alanine aminotransferase(ALT),that are indicators of liver tissue damage **(Teocharis et al., 2001)**. CCl4 is activated by cytochrome enzymes to form the trichloromethyl radical, CCl3*. Adduct formation between CCl3* and DNA is thought to function as initiator of hepatic cancer **(Weber et al., 2003)**.

The goal of this study is to elucidate the effects of Doxorubcin and Zinc oxide nanoparticle on DNA damage and hepatotoxicity induced by CCl4 . The investigated

parameters were Liver enzymes, Protein electrophoresis and DNA gel electrophoresis in normal and injected rats by CCl4.

Materials and methods

Chemicals

-Doxorubicin :

DOX (EbewePharma co. Austria)2 mg/ml concentratefor solution for infusion (vial of 25 ml contains: 50 mg Doxorubicin HCl) The chemical name of doxorubicin HCl is (8S,10S)-10-[(3-amino-2,3,6-trideoxy-α-L-lyxohexopyranosyl)oxy]-8-glycolyl-7,8,9,10-tetrahydro-6,8,11-trihydroxy-1-methoxy-5,12naphthacenedione hydrochloride. The molecular formula is C27-H29 -NO11•HCl; its molecular weight is 579.99(**PuranBadkoobeh et al.,2013).**

-Synthesis of Zinc oxide NPs :

0.02M aqueous Zinc acetate dihydrate was dissolved in50 ml distilled . At room temperature, aqueous 2.0M NaOH was added drop by drop to reach pH 12. Which was then placed in a magnetic stirrer for 2hr. The white precipitate formed was washed thoroughly with distilled water followed by ethanol to remove the impurities. The precipitate was dried in a hot air oven for overnight at 60˚C. Complete conversion of Zn (OH) 2 into ZnO NPs took place during drying.

The reactions involved in the synthesis process are as follows:

Zn (CH3COO)2 + 2 CH3OHZnOH + 2 CH3COOCH3

ZnOH +2NaOH ZnO + Na2O + 2H2O

The prepared ZnO NPs were identified and characterized by Scanning Electron Microscope (SEM) with Energy Dispersire X-ray spectrometer (EDX) Fourier Transmitter Infra Red (FTIR) and UV visible , the structure of particles was 100 nm.

-Carbon tetrachloride (CCl4):

Carbon tetrachloride (99.9 purity) was purchased from Sigma chemical company. It was used as 50% in propylene glycol to rats at dose (0.1 ml /100g.b.wt)twice/week for tow weaks subcutaneously according to **Borah et al (2004).**

Animals

Fourty eight white Albino rats (Sprague Dawley strain) of an average body weight 130-165 g. They were obtained from the laboratory of colony,Ministry of Health and population, Helwan, Cairo,Egypt. Animals were acclimatized to laboratory condition before being used. Rats were fed on standard diet supplying the essential vitamins,trace elements and water supply was given adlibitum.

Experimental design

This was planned to study the effects of Doxorubicin and Nanoparticle Zinc oxide in normal and hepatotoxicity induced by CCl4 in rats. For this purpose 48 adult rats were divided into 8 equal groups of 6 rats each for 28 successive days.

Group 1 : Kept as control negative.

Group 2 : Injected intraperitonily with DOX (6 mg/kgb.wt).
Group 3 : Injected intraperitonily with**n**ZnO (5 mg/kg/day) .
Group 4 : Injected intraperitonily with**n**ZnO (5 mg/kg/day) followed by DOX (6 mg/kgb.wt) one day before.
Group 5 : Kept as control positive, it was injected subcutaneously by CCl4 (0.1ml/100gb.wt.) twice/week for tow weeks.
Group 6 (CCL4 group) : Injected intraperitoneally with DOX (6 mg/kgb.wt).
Group 7 (CCL4 group) : Injected intraperitoneally with**n**ZnO (5 mg/kg/day) .
Group 8 (CCL4 group): Injected intraperitoneally with**n**ZnO (5 mg/kg/day) followed by DOX (6 mg/kgb.wt) one day before.
All groups were treated for 3 days.

Sampling:

Blood samples were obtained from the orbital plexuses of each animal and received into clean dry tubes. Samples were left to clot at room temperature for about 2 hours, stored overnight in a refrigerator at 4^{o}C and centrifuged at 3000r.p.m. for 15 min. Serum samples were drawn in dry clean capped bottles and kept in a deep freeze for estimation of some biochemical analysis.

DNA gel electrophoresis.

The extent of DNA fragmentation (DNA ladder) has been assayed by electrophoresing genomic DNA samples,isolated from normal as well as experimental rat liver,on1% agarose/ethydium bromide gel by the procedure described by **Sellins and Cohen(1987)**. DNA extraction: Principle: High quality genomic DNA was extracted from (-80˚C preserved liver samples of all treated and control groups) by precipitation of protein and other contaminants and further precipitation of high molecular weight genomic DNA by absolute ethanol**(Sambrook et al., (1989)**.

Biochemical analysis

The activities of serum aspartateaminotransferase (AST) and alanine aminotransferase (ALT) were assayed by the method of **Reitman and Frankel (1957)**;alkaline phosphate (ALP) **(Dumas et al., 1971)**;Catalase(CAT) activity; lipid peroxidation as malondialdhyde (MDA) and reduced glutathione (GSH) (**Aebi , 1974; Okhawa et al., 1979)** and**(Ellman, 1959),** respectively**;**TAC(**Koracevic et al., 2001)**and estimation of serum total protein and electrophoretic pattern **(SonnenWirth and Jareet, 1980; Davis, 1964;** 55**Julia Zaias et al., 2009)**.

Statistical analysis

The statistical interpretation of the results was performedwith One-Way ANOVA test.The results were given as mean ± standard error using **SPSS 14 (2006)**.
The value of $p<0.05$ was considered significant.

Result and Discussion

The present study was carried out to elucidate the Effects of Doxorubcin and Nanoparticle Zinc oxide in normal and hepatotoxicity induced by CCl4 in rats. The investigated parameters were Liver enzymes, Protein electrophoresis and DNA gel electrophoresis in normal and injected rats by CCl4.

Subcutaneous injection of CCl4 induce DNA fragmentation(Figure 2). These results are in agreement with **Weber et al., (2003)** who stated that CCl4 is activated by cytochrome enzymes to form the trichloromethyl radical, CCl3*. Adduct formation between CCl3* and DNA is thought to function as initiator of hepatic cancer .Intraperitoneally injection of doxorubicin showed DNA fragmentation Figure (3,4). Our results are in agreement with **King et al., (2001); Kalender et al., (2005); Ichihara et al.,(2007) and Saalu et al., (2010)** who stated that anthracyclines like DOX exert their antitumor properties as well as other organ toxicity by intracellular producing of free radicals and ROS accompanied by intercalation with DNA and consequent inhibition of topoisomerase. While rats injected Doxorubicin and NZnO showed less DNA fragmentation Figure (5,6). This may be due to NZnO is able to protect cell membrane integrity against oxidative stress damage, increase antioxidant enzyme levels and decrease MDA level**(Dawei et al., 2009).** Diffraction (XRD) is a rapid methodical technique primarily used for phase identification of crystalin material and provide information on unite cell dimensions structure and peak purity of ZnO nanoparticle from XRD as in fig.(1). That the obtained products are composed of near flower shape morphology with the average size in the range of 0.5µm **(Gnanasangeetha and SaralaThambavani, 2013;Theodore, 2006).**

Our results are in agreement with **Sharma et al., (2012)**who stated NZnO can improve antioxidant activity, enhance the activities of antioxidases and decrease the levels of free radicals. And disagreement with **Xiong et al., (2011)** who stated awell documented link between NPs and oxidative stress, one of the possible modes that can be suggested for ZnO-NPs induced DNA damage may be lipid peroxidation and oxidative stress. **Marnett et al.,(2002); Martinez et al.(2003) and Niedernhofer et al.,(2003)** mentioned that ROS are known to react with DNA molecule causing damage to both purine and pyrimidine bases as well as DNA backbone. Another important outcome of ROS production is lipid peroxidation which generates a variety of products reactive towards cellular macromolecules including DNA. **Marnett (2000)** who stated that the products of lipid peroxidation react with DNA to form adducts MIG, the mutagenic pirimedopurinone adduct of deoxyguanosine. Like other macromolecules such as lipids and proteins, nucleic acids are also attacked by free radicals to cause oxidative DNA damage.

Subcutaneous injection of CCl4 and intraperitoneally injection of doxorubicin showed a significant increase, compared with the control negative group, in the hepatic levels of

ALT, AST, and ALP enzymes(Table 1). These results are in accordance with **Rahman et al. (2001)**; **Injac et al. (2008)**who attributed the liver marker enzyme, including ALT,AST and ALP level has been shown to increase leakage from damaged and necrotic hepatocytes as a result of toxicity. Due to Trichloromethyl as a peroxyl radicals, peroxidation of lipids, tissue damage, and liver injury were produced in hepatotoxic rats which treated with CCL4 **(Lee, 2004)**. This result indicates potent role of DOX administration in induce oxidative stress. Doxorubicin is an anthracyclin antibiotic that is considered as one of the most effective antitumor agents. The clinical use of DOX is limited by its toxicity to normal tissues, such as the heart and liver **(Ibrahim, 2010; Wang et al., 2012)**.

Intraperitoneally injection of Nanoparticle Zinc oxide in normal rats showed no significant change in serum ALT, AST, and ALP activities as compared to normal control, and restored the level of ALT, AST, and ALP activities into the normal in serum of rats injected with CCl4 (table1). Intraperitoneally injection of nanoparticle Zinc oxide followed by DOX significantly improved serum ALT, AST, and ALP activities as compared to normal control, and restored the level of ALT, AST, and ALP activities into to the normal in serum of rats injected with CCl4(table 1). These results are in accordance with **Sharma et al. (2012)** who attributed that nZnO can improve antioxidant activity, enhance the activities of antioxidases and decrease the levels of free radicals. And disagree with **Wang et al.(2008)** mentioned that nZnO induce liver damage documented by the elevation of serum ALT compared to control group, implying cellular leakage and loss of the functional integrity of cell membranes in liver.
Intraperitoneally injection of Nanoparticle Zinc oxide followed by DOX showed a significant increase in the antioxidant enzymes CAT more than Doxorubicin alone or NZnO alone, no significant change in the antioxidant enzymes GSH , compared with the control positive group. There was a significant decrease , compared with the negative control group, in the antioxidant enzymes MDA and TAC enzymes following subcutaneous injection of CCl4.
These results are in agreement with many other authors **(AbdEl-Aziz et al., 2001; Kalender et al., 2005;Yagmurcaa et al., 2007)** who stated that one of the most prevailing hypothesis of hepatic damage from doxorubicin administration is the ability of the drug to produce reactive oxygen species (ROS) and suppress antioxidant defense mechanism. They also revealed that the increased lipid peroxidation play a critical role in liver injury. **Injury.Yeh et al. (2009)** reported that rats administrated with DOX showed increase in the level of lipid peroxidation (MDA) and depressed antioxidant enzymes activities (SOD, glutathione peroxidase and glutathione) and elevate apoptotic index. **Mohan et al.(2006)**reported that DOX might cause excessive consumption, reduced production or chemical deactivation of these enzymes. But NZnO can improve antioxidant activity, enhance the activities of antioxidases and decrease the levels of

free radicals **(Sharma V et al 2012)**. These results are in agreement with **Dawei et al.(2009)** who reported that nZnO is able to protect cell membrane integrity against oxidative stress damage, increase antioxidant enzyme levels and decrease MDA level .
Subcutaneous injection of CCl4 showed a significant decrease in the Albumin, total alpha globulin and Gamma 1 globulin, compared with the control negative group (table 3). This decrease is improve after Doxorubicin and NZnO(table 3). These results are in agreement with **El-Maraghy et al. (2009)** who stated that alteration in serum protein to changes in protein and free amino acids and their synthesis in the injured liver cells and increased protein degradation. Due to The chemical structure of doxorubicin causes the generation of free radicals and the induction of oxidative stress that correlates with cellular injury **(Saad et al., 2001)**. Doxorubicin causes an imbalance between free oxygen radicals (ROS) and antioxidants. The disturbance in oxidant–antioxidant systems results in tissue injury that is demonstrated with lipid peroxidation and protein oxidation in tissue **(Karaman et al., 2006)**.

Conclusion:

It can be concluded that zinc oxide nanoparticles followed doxorubicin intraperitoneally injection improved the alteration of some serum biochemical parameters and DNA damge induced by CCL4.

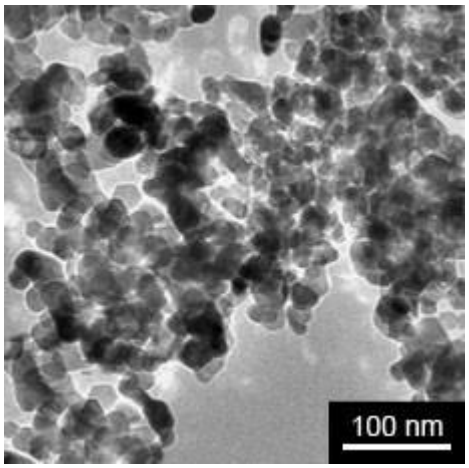
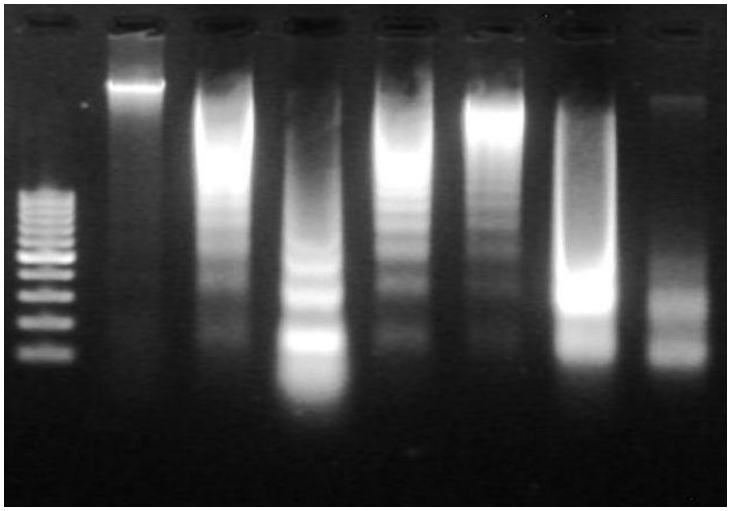
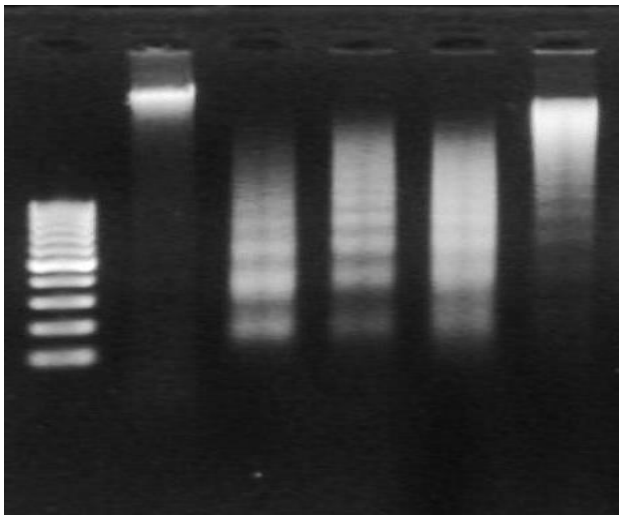


Fig.(1)showing the structure and peak purity of ZNO nanoparticle from XRD .



L C 21 22 23 24 25 26

Figure (2):DNA fragmentation on agarose / ethydium bromide gel. DNA isolated from experimental liver tissues was loaded into 1% (w/v) agarose gels. L:100-bp ladder. Lane C represents DNA isolated from control rats (G1) ; lane 21,22,23,24,25,26: DNA isolated from rats control positive Group 5 .



L C 6 7 8

Figure (3):DNA fragmentation on agarose / ethydium bromide gel. DNA isolated from experimental liver tissues was loaded into 1% (w/v) agarose gels. L:100-bp ladder. Lane C represents DNA isolated from control rats (G1) ; lane 6,7,8,9: DNA isolated from rats injected DOX (6 mg/kg/day) Group 2 .

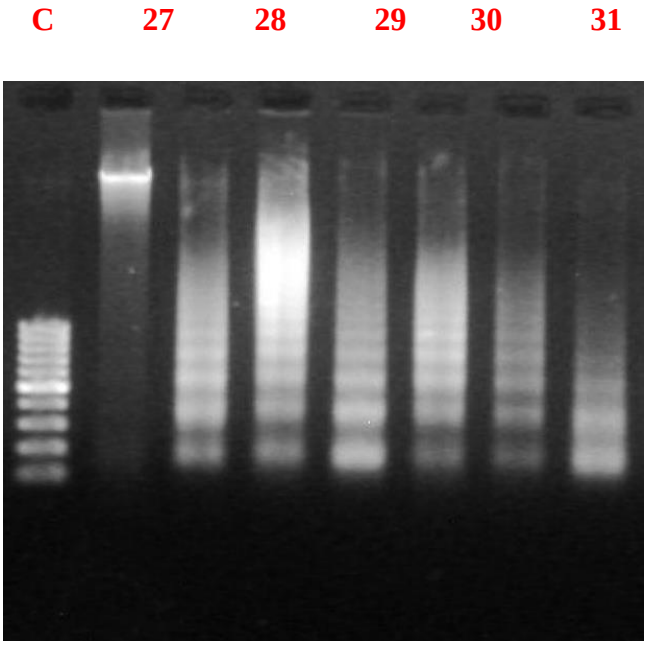


Figure (4):DNA fragmentation on agarose / ethydium bromide gel. DNA isolated from experimental liver tissues was loaded into 1% (w/v) agarose gels. L:100-bp ladder. Lane C represents DNA isolated from control rats (G1) ; lane 27,28,29,30,31 ,32: DNA isolated from rats received DOX (6 mg/kg/day) Group 6 (CCl4 group)

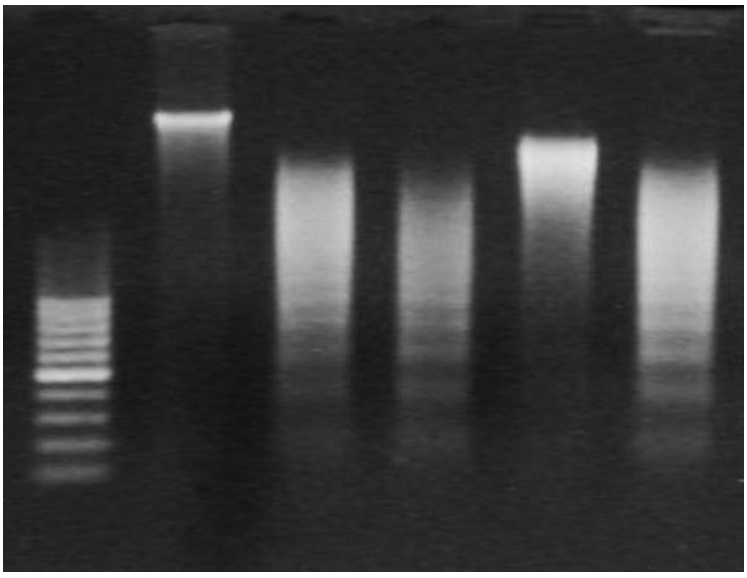


Figure (5):DNA fragmentation on agarose / ethydium bromide gel. DNA isolated from experimental liver tissues was loaded into 1% (w/v) agarose gels. L:100-bp ladder. Lane C represents DNA isolated from control rats (G1) ; lane 39,40,41,42: DNA isolated from rats received DOX (6 mg/kg/day) and nZnO (5 mg/kg/day) Group 8 (CCL4 group)

L C 6 10 15 21 27 33

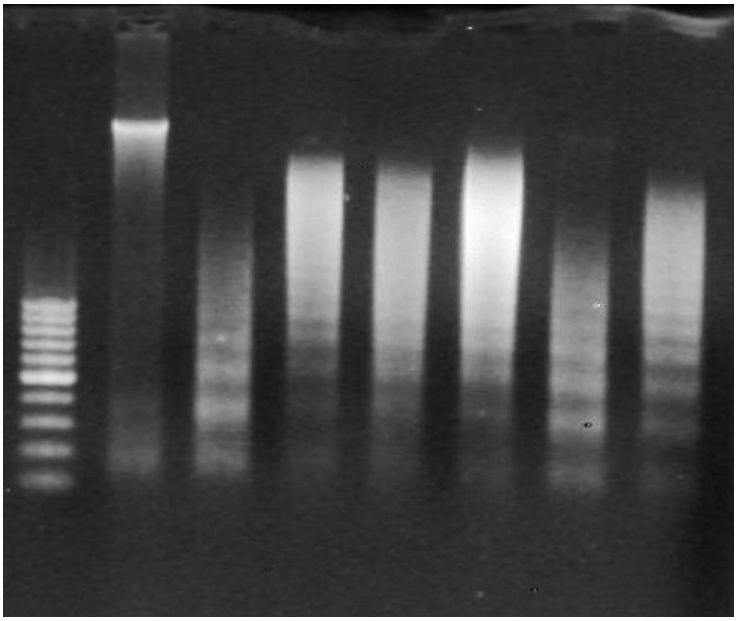


Figure (6):DNA fragmentation on agarose / ethydium bromide gel. DNA isolated from experimental liver tissues was loaded into 1% (w/v) agarose gels. L:100-bp ladder. Lane C represents DNA isolated from control rats (G1) ; lane 6: received DOX (6 mg/kg/day) Group 2;Lane 10: received nZnO (5 mg/kg/day) Group 3 ;Lane 15:received DOX (6 mg/kg/day) and nZnO (5 mg/kg/day) Group 4; Lane 21:represents DNA isolated from CCL4 group +ve control group Group 5; Lane27:DNA isolated from rats received DOX (6 mg/kg/day) Group 6 (CCL4group);
Lane33:represents DNA isolated from received nZnO (5 mg/kg/day) Group 7 (CCL4 group).

-Table (1)the Effects of Doxorubcin and Nanoparticle Zinc oxide on normal and injected with CCl4 on serum liver enzymes levelscomparing with control rats (n= 6).

Groups	AST (U/L)	ALT (U/L)	ALP(U/L)
Group 1 -ve control	176±21.73^{cd}	37.8±3.42^{e}	235.6±11.18^{c}
Group 2 -veDox.	143.75±13.12^{d}	44.75±2.94^{de}	338.75±37.54^{b}
Group 3 -ve N ZnO	185±14.31^{bcd}	53.40 ± 5.42^{cd}	319.40±30.41^{bc}
Group 4 -veDox+NZnO	154.33±23.36^{cd}	38.50± 0.96^{e}	101.50±25.74^{d}
Group 5 +ve control	240.67±21.36^{ab}	93.50±2.81^{a}	446.50 ± 50.31^{a}
Group 6 +veDox	254±17.48^{a}	65±4.90^{b}	414.33±26.96^{ab}
Group 7 +veNZnO	206.67±9.45^{abc}	56.67±2.58^{bc}	375.83±47.76^{ab}
Group 8 +veDox+NZnO	160.75±24.78^{cd}	51.25±1.40^{cd}	105.25 ± 11.50^{d}
p-value	.0008	0.000	0.000

#Significant at P<0.05 using **ANOVA** test.
a,b.c,d,eInsignificant difference between similar letter using Duncan´smultiple range test at p<0.05 . AST: aspartateaminotransferase.ALT: alanine aminotransferase.ALP:alkalinephosphatase.

Table (2)the Effects of Doxorubcin and Nanoparticle Zinc oxide on normal and injected with CCl4 on serum antioxidant enzymes levels of rats comparing with control rats(n= 6).

Groups	MDA (nM/ml)	TCA (mM/l)	CAT (u/ml)	GSH (mM/L)
Group 1 -ve control	2.84±0.17^{abc}	1.71±0.01^{d}	56.78±2.67^{b}	7.42±0.73^{a}
Group 2 -veDox.	3.83±0.48^{a}	3.72 ±0.12^{ab}	51.44 ±1.84^{b}	5.72 ±1.58^{abc}
Group 3 -ve N ZnO	3.39±.72^{ab}	3.104±0.21^{bc}	48.07±4.06^{b}	3.407 ±0.469^{c}
Group 4 -veDox+NZnO	3.095±0.25^{abc}	4.37±0.45^{a}	60.99 ±1.29^{ab}	6.37±0.95^{ab}
Group 5 +ve control	2.701±0.28^{abc}	2.54± 0.26^{c}	53.128 ±3.46^{b}	5.103± 0.43^{abc}
Group 6 +veDox	2.2633+0.44^{bc}	1.71± 0.34^{d}	60.38±9.49^{b}	6.25 ± 0.57^{ab}
Group 7 +veNZnO	1.83±0.29^{c}	1.48± 0.22^{d}	61.52±7.79^{ab}	4.57±0.76^{bc}
Group 8 +veDox+NZnO	2.135±0.37^{bc}	1.83 ±0.03^{d}	74.38±2.67^{a}	4.761±0.71^{abc}
p-value	0.023	0.000	0.024	0.057

#Significant at P<0.05 using **ANOVA** test.
a,b.c,d,eInsignificant difference between similar letter using Duncan´smultiple range test at p<0.05 .

Table (3):the Effects of Doxorubcin and Nanoparticle Zinc oxide on normal and injected with CCl4 on serum protein and its fractions ofrats comparing with control rats.(n= 6)

Groups	T.Protein (g/dl)	Alb (g/dl)	T.alpha (g/dl)	alpha1 (g/dl)	alpha2 (g/dl)	T.beta (g/dl)	beta 1 (g/dl)	beta 2 (g/dl)	T.gamma (g/dl)	gamma 1 (g/dl)	Gamma2 (g/dl)	T.globulin (g/dl)	AG.ratio
-ve control	6.32 ± 0.37^{ab}	1.62 ± 0.13^{ab}	1.22 ± 0.05^{a}	0.70 ± 0.08^{a}	0.53 ± 0.05^{a}	1.97 ± 0.165^{a}	1.32 ± 0.13^{a}	0.64± 0.03^{ab}	1.55 ± 0.07^{ab}	0.80 ± 0.03 b^{cd}	0.74 ± 0.05^{a}	4.69± 0.25$^{a b}$	0.34 ±0.01^{c}
-veDox.	5.57± .09^{b}	1.486 ± .02^{abc}	0.846 ± 0.04$^{a b}$	0.47 ± 0.47^{ab}	0.37 ± 0.03^{ab}	2.04 ± 0.05^{a}	1.33 ± 0.06^{a}	0.71± 0.05^{ab}	1.19 ± 0.10^{b}	0.57 ± 0.04$^{c d}$	0.65 ± 0.05^{a}	4.15± 0.12$^{b c}$	0.36 ± 0.01^{c}
-ve NZnO	7.07 ± 0.41^{a}	1.80 ± 0.13^{ab}	1.16 ± 0.15$^{a b}$	0.71 ± 0.11^{a}	0.45 ± 0.05^{ab}	2.08 ± 0.08^{a}	1.33 ± 0.07^{a}	0.77 ± 0.03^{ab}	2.01 ± 0.05^{a}	1.28 ± 0.06^{a}	0.73 ± 0.04^{a}	5.27± 0.28^{a}	0.34 ± 0.01^{c}
-ve Dox +NZO	6.17 ± 0.35^{ab}	1.41 ± 0.13^{bc}	.76 ± 0.09^{bc}	0.36 ± .091^{b}	0.40 ± 0.03^{ab}	2.13 ± 0.15^{a}	1.31 ± 0.17^{a}	0.81 ± 0.11^{a}	1.86 ± 0.21^{a}	1.11 ± 0.20$^{a b}$	0.75 ± 0.10^{a}	4.76± 0.25$^{a b}$	0.296± 0.02^{cd}
+ve control	6.39 ± 0.36^{ab}	1.55 ± 0.13^{abc}	0.87 ± 0.14$^{a b}$	0.50 ± 0.10^{ab}	0.36 ± 0.04^{b}	2.13 ± 0.14^{a}	1.21 ± 0.06^{a}	0.92 ± 0.11^{a}	1.83 ± 0.14^{a}	1.08 ± 0.15$^{a b}$	0.74 ± 0.05^{a}	4.8376± 0.25$^{a b}$	0.31 ± 0.01^{cd}
+veDox	5.75 ± 0.87^{ab}	1.10 ± 0.29^{c}	1.047 ± 0.24$^{a b}$	0.71 ± 0.20^{a}	0.33 ± 0.05^{bc}	1.88 ± 0.28^{a}	1.16 ± 0.18^{a}	0.71 ± 0.11^{ab}	1.72 ± 0.32^{a}	0.97 ± 0.20$^{a bc}$	0.75 ± 0.13^{a}	4.65± 0.72$^{a b}$	0.25 ± 0.04^{d}
+veNZnO	6.18 ± 0.41^{ab}	1.92 ± 0.15^{ab}	0.94 ± 0.12$^{a b}$	0.46 ± 0.04^{ab}	0.48 ± 0.08^{ab}	2.11 ± 0.19^{a}	1.37± 0.13^{a}	0.74 ± 0.11^{ab}	1.16 ± 0.18^{b}	0.62 ± 0.14$^{c d}$	0.54 ± 0.04^{a}	4.23± 0.33$^{a bc}$	0.46 ± 0.03^{b}
+veDox+ NZnO	5.20 ± 0.36^{b}	1.95 ± 0.16^{a}	0.45 ± .02^{c}	0.24 ± 0.01^{b}	0.21 ± 0.01^{c}	1.73 ± 0.14^{a}	1.23 ± 0.10^{a}	0.50 ± 0.05^{b}	1.05 ± 0.05^{b}	0.55 ± 0.02^{d}	0.50 ± 0.06^{a}	3.24± 0.21^{c}	0.59 ± 0.02^{a}
p-value	.159	0.010	0.005	0.018	0.003	0.665	0.939	0.096	0.001	0.001	.129	.011	0.000

#Significant at P<0.05 using **ANOVA** test.
a,b.c,d,eInsignificant difference between similar letter using Duncan´smultiple range test at p<0.05 .

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