Biochemical study on the effect of Metallo-Surfactant and its loaded nano-analogue as anticancer drug

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Abstract: *In vivo* antitumor activity, liver function, hematological and antioxidant status of copper cetyl trimethyl ammonium bromide (Cu-CTAB) loaded cyclodextrin nano-analogue was evaluated against Ehrlich ascites carcinoma (EAC) tumor in mice. Mice were then sacrificed for estimation of simultaneous alterations in the hematological profile, liver biochemical parameters, antioxidant status and histopathological changes. The cardiotoxic effect represents in measuring lactate dehydrogenase and creatine phosphokinase enzymes of both compounds and Doxorubicin were investigated in rats. The antibacterial and antifungal effect of copper cetyl trimethyl ammonium bromide (Cu-CTAB) loaded cyclodextrin nano-analogue was evaluated against *Desulfonamonas pigra, Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. The synthesized compounds showed a potential activity comparable to the parent compound cetyl trimethyl ammonium bromide (CTAB).
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Keywords: Antitumor agents, *in vivo* anticancer activity nano-analog, surfactant complex.

1. Introduction

Cancer (medical term: malignant neoplasm) is a diverse class of diseases in which a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on/and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body *via* lymph or blood).

Cancers are caused by abnormalities in the genetic material of the transformed cells **(Kinzler *et al*, 2002)**. These abnormalities may be due to the effects of carcinogens, such as tobacco smoke, radiation, chemicals, or infectious agents. Other cancer-promoting genetic abnormalities may randomly occur through errors in DNA replication, or are inherited, and thus present in all cells from birth. The heritability of cancers is usually affected by complex interactions between carcinogens and the host's genome.

One of the main side effects of anthracyclines is that it can damage cells of heart muscle along with the DNA of cancer cell leading to cardiac toxicity. Anthracyclines (such as Doxorubicin, Daunorubicin, etc) consider one of the antitumor antibiotics used in treatment of many types of cancers. These compounds are cell-cycle nonspecific and are used to treat a large number of cancers including lymphomas, leukemia, uterine, ovarian, lung and breast cancers **(Ewer *et al*., 2011).**

Surface-active agents have been used for years as an aid to the study of membrane structure and function **(Helenius and Simons, 1975; Gonenne and Ernst, 1978)**. Some of these compounds have been found to have an effect on cell proliferation at concentrations well below those required for lysis of the plasma membrane **(Kato *et al*., 1971; Lotan and Nicolson, 1977; Tarnowski *et al*., 1978)**. The results of these *in vitro* investigations have shown an increased sensitivity of certain transformed cells to some anionic, cationic, non-ionic, and zwitter ionic surfactants. *In vivo* studies have revealed that some of these surfactants have antitumor activity **(Nishikawa *et al*., 1976)** and inhibit the development of tumor metastases **(Franchi *et al*., 1971; Silk and Sigman, 1972)**. The action of one halogenic quaternary ammonium compound on the *in vitro* proliferation of different lines of human cancer cells, indicate that halogenic quaternary ammonium present a potent growth inhibitory activity of different cancer cells lines. The presence of quaternary ammonium group,

responsible for some alkylating effect **(Gastaud *et al*., 1998).**

2. Materials and Methods

Materials

Experimental animals

Experimental animals were obtained from the animal house of National Cancer Institute (NCI), Cairo University. These include the following:

Rats

Mature Swiss albino rats weighing 120-150 g body weight were used to study the cardiotoxic effect of the compounds under investigation.

Mice

Swiss albino female mice weighing 20-25 g body weight each were used to study the anticarcinogenic effect of the compounds under investigation.

Reagent and kits

Superoxide dismutase enzyme from bovine erythrocyte (4520 unite/mg), Nutrient agar medium (Mueller-Hinton agar) were obtained from Sigma Chemical (St Louis, MO, USA). Doxorubicin hydrochloride (10mg/vial) was purchased from Pharmacia (Italy). The cardiac enzymes estimation kits (LDH and CK-MM) were performed using kits purchased from Biosystem Company. The liver enzymes estimation kits were performed using kits (total protein, AST, ALT and ALK) purchased from Diamond Company. The hemoglobin level was measured using kit purchased from Biodiagnostic Company.

Methods

A- Evaluation of the cardiotoxic effect *in vivo.*

Thirty two male albino rats weighing 120-150g were obtained from the National Cancer Institute, Cairo University. The animals were housed in appropriate cages under standard laboratory conditions of temperature, humidity and light-dark cycle. The animals were supplied with special diet and water *ad libitum* **(Ola *et al*., 2000)**. They were divided into the following groups.

"Control group"

Eight male rats were injected with sterilized saline. The dose was administered daily for a period of one-week.

"Cu-CTAB group"

Eight male rats were i.p injected with 0.5 ml of 6.5 mg/Kg body weight Cu-CTAB. The dose was administered daily for a period of one-week.

"CD-CTAB group"

Eight male rats i.p injected with 0.5 ml of 28 mg/Kg body weight CD-CTAB. The dose was administered daily for a period of one-week.

"Doxorubicin group"

Eight male rats were i.p injected with 0.5 ml of 1.7 mg/Kg body weight doxorubicin hydrochloride. The dose was administered daily for a period of one-week. After the observation period was terminated, rats were sacrificed and blood samples were collected into tubes without anticoagulant to separate the sera by centrifugation, sera were used for the determination of AST, LDH and CK-MM.

B- Determination of LD_{50} using female albino mice

Female Swiss albino mice weighing (18-20 g) obtained from the breeding unit of the National Cancer Institute, Cairo University, were used through these experiments. They were maintained on a standard pellet diet and tap water. The animals were housed in suitable cages in conditioned atmosphere (20-22^{o}C) and kept on a standard diet. The diet consisted of not less than 20 % protein, 5 % fibers, 3.5 % fats and 6.5 % ash and supplied with vitamin A and water *ad libitum*. The LD_{50} of the studied compounds were determined as described by **Kärber, (1931).**

C- Investigation of the antitumor activity

Two hundred and forty female albino mice were divided into 8 groups each of which contains thirty female albino mice divided into twenty mice for evaluation of the antitumor effect by measuring the tumor volume and body weight for each group and the remaining ten mice were left for determination the mean survival time. The animals were grouped as follow:-

Group I: "Control group"

Untreated female mice were maintained on a standard diet, drinking water *ad libitum* and injected with 0.5 ml sterilized saline.

Group II: "EAC group"

IIa- Twenty mice were injected intraperitonially (i.p.) with 2.5 x 10^6 EAC cells and six doses day after day of saline.

IIb- Ten mice were injected (s.c.) in the right thigh of the lower limb with 2.5 x 10^6 EAC cells and six doses day after day of saline.

Group III: "Cu-CTAB group"

IIIa- Twenty mice were injected (i.p.) with 0.5 ml of 6.5 mg/Kg body weight Cu-CTAB and six doses day after day.

IIIb- Ten mice were injected (s.c.) in the right thigh of the lower limb with 0.5 ml of 6.5 mg/Kg body weight Cu-CTAB and six doses day after day.

Group IV: "CD-CTAB group"

IVa- Twenty mice were injected (i.p.) with 0.5 ml of 28 mg/Kg body weight CD-CTAB and six doses day after day.

IVb- Ten mice were injected (s.c.) in the right thigh of the lower limb with 0.5 ml of 6.5 mg/Kg body weight. CD-CTAB and six doses day after day.

Group V: "Doxorubicin group"

Va- Twenty mice were injected (i.p.) with 0.5 ml of 1.7 mg/Kg body weight doxorubicin and six doses day after day. Ten of them were left till end of experimentation period to study the survival rate.

Vb- Ten mice were injected (s.c.) in the right thigh of the lower limb with 0.5 ml of 6.5 mg/Kg body weight doxorubicin and six doses day after day.

Group VI: "Cu-CTAB + EAC group"

VIa- Twenty mice were injected (i.p.) with 2.5 x 10^6 EAC. Twenty-four hours post EAC inoculation, they were injected with 0.5 ml of 6.5 mg/Kg body weight Cu-CTAB and six doses day after day.

VIb- Ten mice were injected (s.c.) in the right thigh of the lower limb with 2.5 x 10^6 EAC. Twenty-four hours post EAC inoculation, they were injected with 0.5 ml of 6.5 mg/Kg body weight Cu-CTAB and six doses day after day.

Group VII: "CD-CTAB + EAC group"

VIIa- Twenty mice were injected (i.p.) with 2.5 x 10^6 EAC. Twenty-four hours post EAC inoculation, they were injected with 0.5 ml of 28 mg/Kg body weight CD-CTAB and six doses day after day.

VIIb- Ten mice were injected (s.c.) in the right thigh of the lower limb with 2.5 x 10^6 EAC. Twenty-four hours post EAC inoculation, they were injected with 0.5 ml of 28 mg/Kg body weight CD-CTAB and six doses day after day.

Group VIII: "Doxorubicin group + EAC group"

VIIIa- Twenty mice were injected (i.p.) with 2.5 x 10^6 EAC. Twenty-four hours post EAC inoculation, they were injected with 0.5 ml of 1.7 mg/Kg body weight doxorubicin and six doses day after day. Ten of them were left till the end of experimentation period to study the survival rate.

VIIIb- Ten mice were injected (s.c.) in the right thigh of the lower limb with 2.5 x 10^6 EAC. Twenty-four hours post EAC inoculation, they were injected with 0.5 ml of 1.7 mg/Kg body weight doxorubicin and six doses day after day**.**

The body weights

It was determined every two days until the 19th day. Data were presented as percent of increase in body weights of treated animals that compared with the controls treated with saline (Group I) and those i.p inoculated with EAC (Groups IIIa, IVa, Va, VIa, VIIa and VIIIa). The percent of change in body weight was determined using the following formula **(Kuttan *et al.*, 1985)**:

$$\textbf{Percentage change in weight} = \frac{W_2 - W_1 \times 100}{W_1}$$

Where W_1 is the average body weight of animals at the start of the experiment and W_2 is the average body weight of animals at the specific day of observation.

The tumor volume

The change in tumor volume as response to the treatment with or without the Cu-CTAB, CD-CTAB and doxorubicin were measured by vernir caliper starting from the 7th day. The tumor volume was measured in groups IIb, IIIb, IVb, Vb, VIb, VIIb and VIIIb by the following formula **(Kuttan *et al.*, 1985)**:

$$\textbf{Tumor volume (mm}^3\textbf{)} = \frac{4\,\pi \quad (A/2)^2 - (B/2)}{3}$$

Where

$\pi = 3.14$

A = the tumor length.

B = the tumor width.

Survival rate

The survival of animals was recorded daily, until the observation period was terminated. Data were presented in days. The life span of animals treated with either of the investigated compounds was compared with the controls treated with saline and those treated with EAC alone. The mean survival time (M.S.T) was also determined and it was defined as the sum of survival days of all mice in each group divided by the total number of animals in this group (10 mice) **(Malaya *et al.*, 2004)**.

III- Biochemical analyses

a- Liver function test

As the observation period was terminated, female albino mice were sacrificed and blood samples were collected into two portions, the first in 10 mmol/L EDTA tubes, for estimation the hemoglobin concentration, white blood cells count and white blood cells differential count. The second portion of the blood was transferred to another tubes free from EDTA, the sera were obtained by centrifugation and used for the determination of total proteins, aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase enzyme (ALK).

b- Determination of oxidative stress

Superoxide dismutases (SOD) are a family of metallo-enzymes which are known to catalyze dismutation of superoxide radical to hydrogen peroxide (H_2O_2) and molecular oxygen.

The method, involves generation of superoxide by pyrogallol autoxidation and the inhibition of superoxide dependent reduction of the tetrazolium dye MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to its formazan.. The reaction was terminated by the addition of dimethyl sulfoxide (DMSO). The yellow color produced was measured at 570 nm formed and was stable for many hours **(Madesh and Balasubramanian, 1998)**.

c- Blood parameters

Haemoglobin level was estimated using Drabkin method. WBCs and differential leucocytes counts were estimated using methods described by **Monica, (2000).**

d- Histopathology

Livers and other organs were removed from mice after the day 21 from the injection of the investigated compounds. Photographs of the livers were taken using a digital camera. Subsequently, the organs were fixed in 10% formalin and stained with haematoxylin and eosin. Histological changes were observed under a microscope and photographs were taken using the Fujix digital camera HC-2500 under an Olympus microscope. The digital images were processed in brightness using computer software.

Evaluation of the Antimicrobial activity

Antimicrobial activity of the Cu-CTAB was determined using a modified Kirby-Bauer disc diffusion method **(Bauer *et al.*, 1966).**

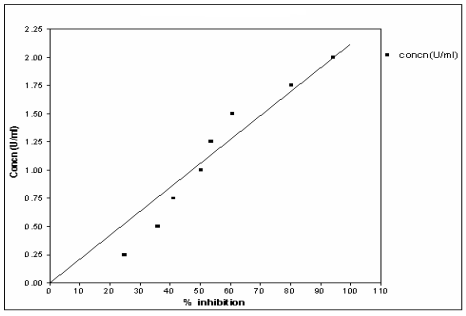

Fig. (1):- The standard curve of superoxide dismutase (SOD).

3. Results and Discussion

a- Investigation of cardiotoxicity using rats

Effect of doxorubicin, Cu-CTAB and CD-CTAB on sera AST, LDH and CPK activities were represented in table (1) and graphically in figs. (2, 3 and 4) showed that, repeated treatment with doxorubicin (3 mg/kg b.wt) for two weeks resulted in a highly significant increase (two-fold) in sera AST, LDH and CPK levels. Treatment with Cu-CTAB and the CD-CTAB has a significant effect of the serum enzymes studied but less than the effect of doxorubicin. In previous studies copper complexes were found to be had a little effect on the myocytes through decreasing the cellular contents of ATP and phosphocreatine **(Hernández-Esquivel *et al.*, 2006).** Anthracyclines such as doxorubicin induce membrane damage *via* lipid peroxidation in all tissues, including the heart. Whereas formation of reactive oxygen species are induced by the quinone moiety of anthracyclines **(Pacher** et al*., 2003)*.

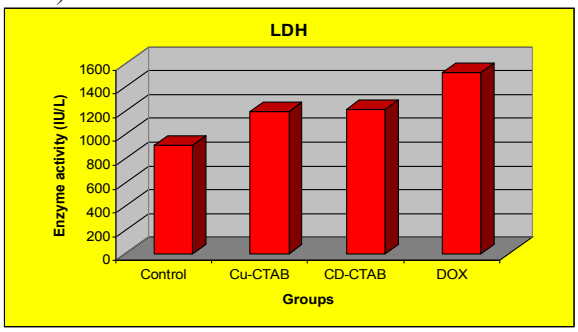

Fig. (2):- The activity of the AST in sera of rats in the different studied groups.

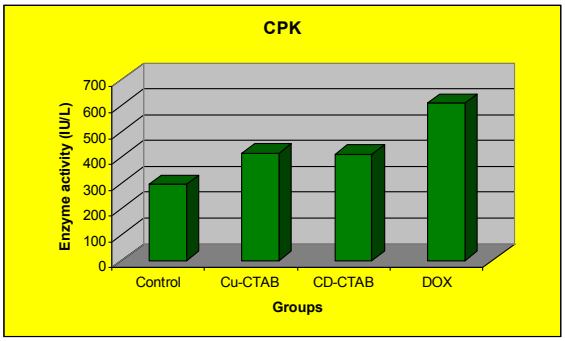

Fig. (3):- The activity of the LDH in sera of rats in the different studied groups.

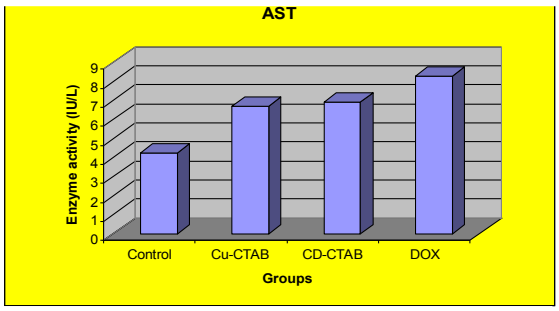

Fig. (4):- The activity of the CPK in sera of rats in the different investigated groups.

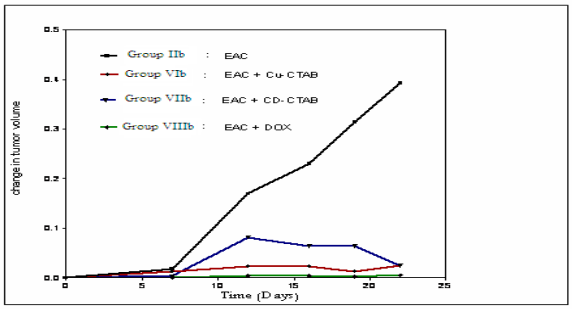

Fig.(5):-The change of body weight of the mice in different studied groups.

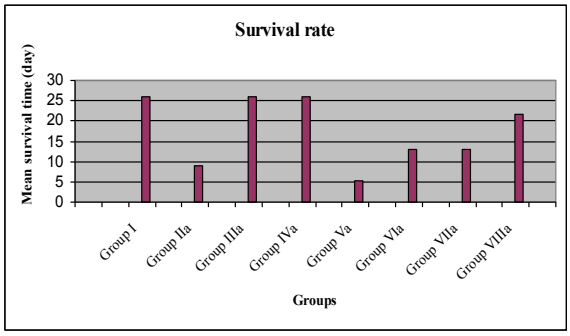

Fig. (6):- The change in tumor volume of the mice in different studied groups.

Investigation for the anticancer effect of the Cu-CTAB and CD-CTAB.

The anticancer effect represented in determination the percentage change in body weight, tumor volume and animal survival rate for the investigated compounds were studied. The untreated EAC inoculated group showed an increase in the percentage change in body weight and tumor volumes compared with the Cu-CTAB and CD-CTAB which recorded a promised anticancer effect through decreasing the body weight and tumor volumes leading to raising the mean survival rate (13 days) compared with the untreated EAC inoculated group (9 days). The doxorubicin inoculated EAC group recorded the best decrease in body weight and tumor volume leading to the longer mean survival rate (21.5 days) compared with the other groups specially the doxorubicin group which suffered from the lowest mean survival rate (5.3 days) (Figs. 5-7).

f- Liver functions

The Cu-CTAB, Cu-CTAB inoculated EAC, CD-CTAB and CD-CTAB inoculated EAC groups showed a moderate elevation of serum total protein and liver function enzymes which indicates presence of a moderate damage of liver. Doxorubicin treated and doxorubicin inoculated EAC groups showed great elevation of total proteins and liver enzymes as compared with the EAC group, Cu-CTAB, Cu-CTAB inoculated EAC, CD-CTAB and CD-CTAB inoculated EAC groups table (5) and fig. (8). The lipid peroxidation, protein oxidation and oxidative alterations to nucleic acids leads to hepatocytes damage and liver injury **(Luza and Hernan, 1996)** and **(Hossam *et al*, 2005).**

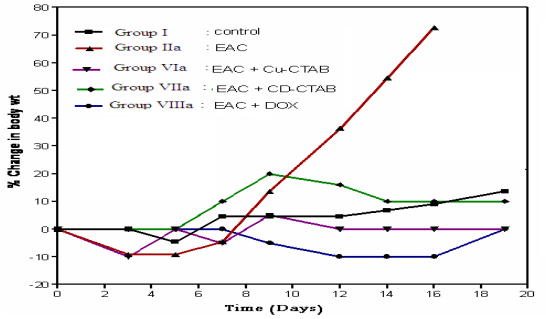

Fig (7):- Mean survival rate of mice in the different studied groups.

g- Hemoglobin concentration measurement (Hb)

Hematological parameters of tumor bearing mice showed significant changes when compared with normal control. The hemoglobin concentrations in EAC, Cu-CTAB, Cu-CTAB injected EAC bearing mice, CD-CTAB and CD-CTAB injected EAC bearing mice groups were normal compared with the control group. This indicates the safety of Cu-CTAB and CD-CTAB. The doxorubicin and doxorubicin injected EAC bearing mice groups showed blood hemoglobin concentration lower than other groups which indicate the effect of doxorubicin on the hemoglobin level Fig.(9).

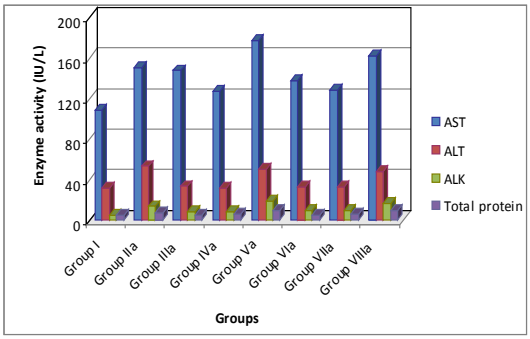

Fig. (8):- The liver functions (AST, ALT, ALK and total protein) in sera of the mice in different studied groups.

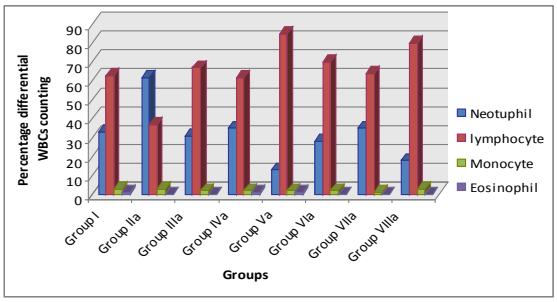

Fig. (9):- The blood hemoglobin concentration of the mice in all different studied groups

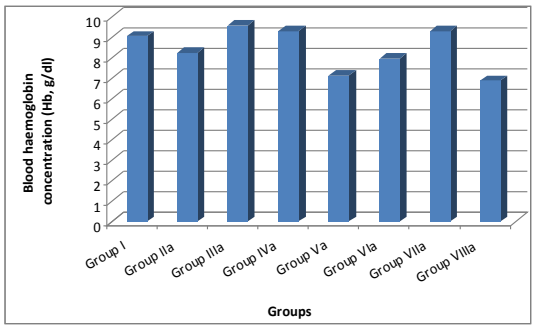

Fig. (10):- The white blood cells counting of the mice in all different studied groups.

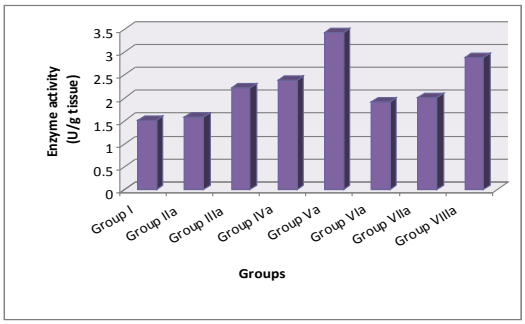

Fig. (11):- The differential white blood cells counting the mice in different studied groups.

h- White blood cells (WBCs) and white blood cells differential counts

In the present study it was found that doxorubicin treated and doxorubicin EAC bearing mice groups showed the lowest white blood cells and neutrophils count and the highest lymphocytes count. This indicates the presence of sever neutropenia compared with the other groups Figs. (10, 11).

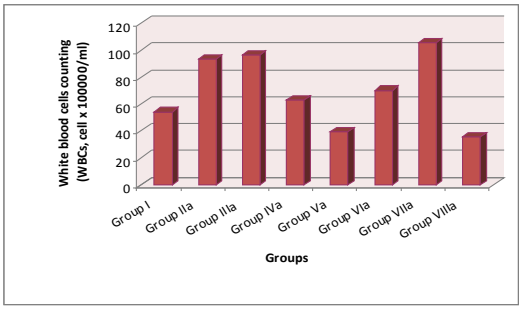

Fig. (12):- The content of Superoxide dismutase enzyme (SOD) in the liver tissues of the mice in different studied groups.

j- Oxidative stress

The level of super oxide dismutase enzyme (SOD) in the liver showed an increase in SOD levels in doxorubicin + EAC bearing mice and doxorubicin alone. This may indicate an elevation of ROS in liver tissues. Besides, Cu-CTAB and CD-CTAB with and without EAC tumor cells recorded a moderate elevation of SOD levels if compared with the normal control group and EAC bearing mice group. The EAC group did not show significant difference when compared with control untreated mice. The results were illustrated in
Fig. (12).

Microscopic examination

E-Histopathological examination:

Macroscopical examination of livers excised from the mice treated with doxorubicin and doxorubicin injected EAC bearing mice were white in colour after doxorubicin treatment (Figs. 13 and 14). On the other hand, livers excised from control, EAC, CD-CTAB treated mice, Cu-CTAB and CD-CTAB treated EAC bearing mice were reddish and normal.

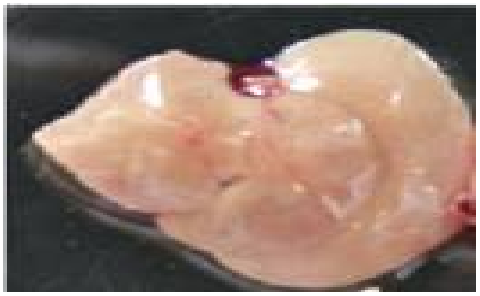

Fig. (13):- The figure shows macroscopic changes in livers of mice receiving none, EAC, Doxorubicin, Cu-CTAB, and CD-CTAB alone and with EAC.

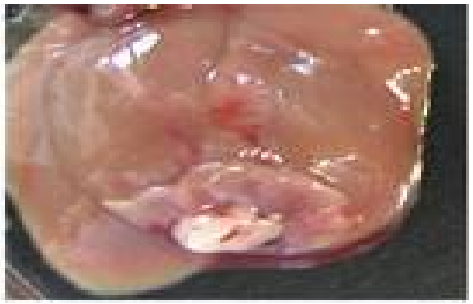

Fig. (14):- The figure shows macroscopic changes in livers of mice receiving doxorubicin and doxorubicin + EAC.

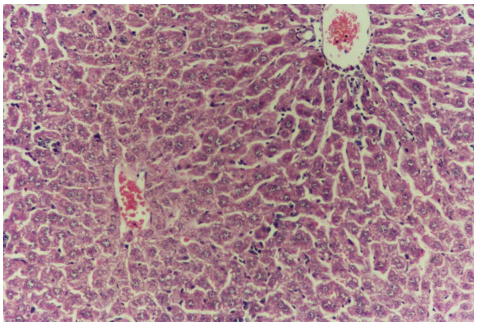

Fig. (15):- Histopathological sections from livers of control untreated mouse showing preserved hepatic architecture. Hepatocytes are arranged in one to two thick cords. There is mild interstitial inflammation. Hepatocytes are polygonal with central round nuclei demonstrated occasional nucleoli. The hepatic arteries and portal veins are in the normal limits.

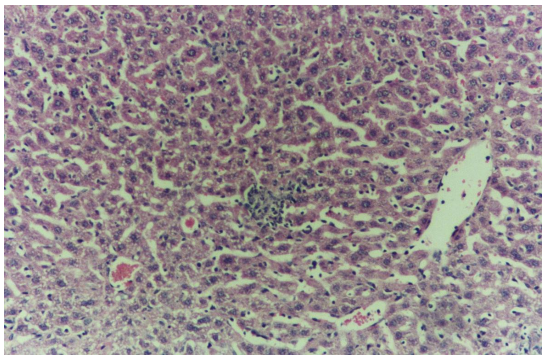

Fig. (16):-Histopathological sections from mouse inoculated with Ehrlich asites carcinoma (EAC) cells showed an inflammatory effects represented by a mono-nuclear infiltrate affecting the portal tract which revealed the presence of increased amount of Eosinophiles and Neutrophiles. Normal lobular architecture with spaces between hepatic cords, no fatty changes and no signs of any tumors.

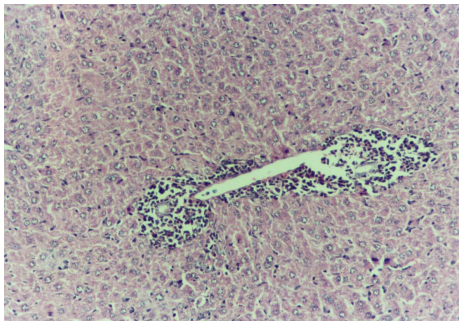

Fig. (17):-Histopathological sections (10X) from mouse inoculated with doxorubicin showed a mild focal parenchymal inflammatory effects represented by a polymorphal nucleated leucocytes infiltrate affecting the portal tract which revealed presence of increased amount of monocytes, lymphocytes and a remarked degree of cytoplasmic degeneration. Normal lobular architecture with spaces between hepatic cords, no fatty changes and no signe of any tumors.

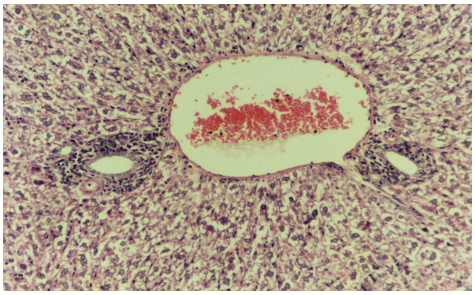

Fig. (18):-Histopathological sections (10X) from mouse inoculated with doxorubicin + EAC showed dense prevascular and focal parenchymal inflammatory effects represented by polymorphal nucleated leucocytes infiltrate affecting the portal tract which revealed the presence of increased amount of monocytes, lymphocytes, a sever massive focal cytoplasmic degeneration and presence of giant cells. Normal lobular architecture with spaces between hepatic cords, no fatty changes and no signs of any tumors.

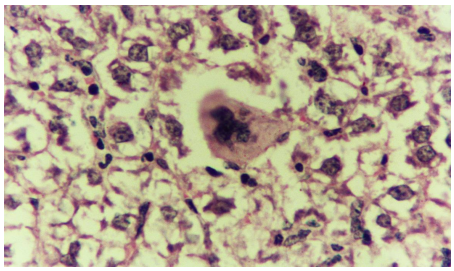

Fig. (19):-Histopathological sections (40X) from mouse inoculated with doxorubicin + EAC showed sever massive focal cytoplasmic degeneration and presence of giant cells.

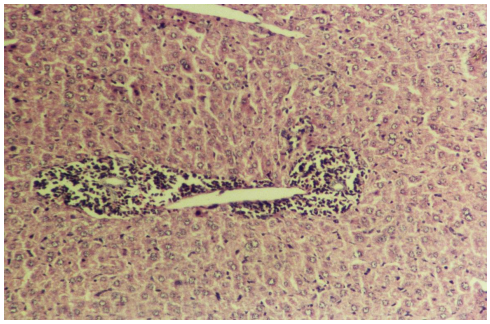

Fig. (20):-Histopathological sections (10X) from mouse inoculated with Cu-CTAB showed a mild to moderate prevascular and focal parenchymal inflammatory effects represented by a polymorphal nucleated leucocytes infiltrate affecting the portal tract. Normal lobular architecture with spaces between hepatic cords, no fatty changes and no signs of any tumors.

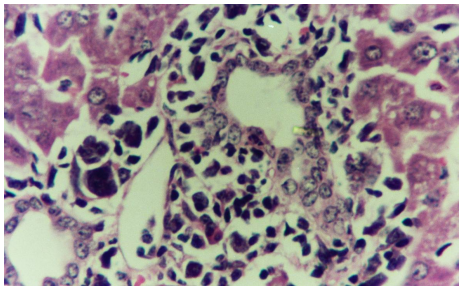

Fig. (21):-Histopathological sections (40X) from mouse inoculated with Cu-CTAB + EAC showed a moderate prevascular and focal parenchymal inflammatory effects represented by polymorphal nucleated leucocytes infiltrate affecting the portal tract. Hepatocytes showed a mild focal cytoplasmic degeneration and focally active mitotic figures indicated focal liver regeneration were evident Normal lobular architecture with spaces between hepatic cords, no fatty changes and no signs of any tumors.

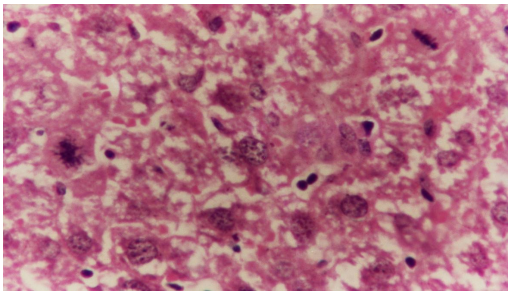

Fig. (22):-Histopathological sections (40X) from mouse inoculated with Cu-CTAB + EAC showed a mild focal cytoplasmic degeneration and focally active mitotic figures indicateed focal liver regeneration.

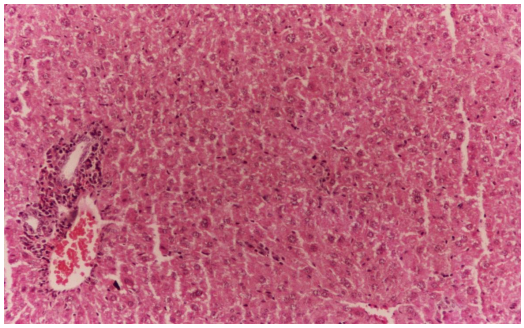

Fig. (23):-Histopathological sections (10X) from mouse inoculated with CD-CTAB showed a mild prevascular and focal parenchymal inflammatory effects represented by a polymorphal nucleated leucocytes infiltrate affecting the portal tract. Normal lobular architecture with spaces between hepatic cords, no fatty changes and no signs of any tumors.

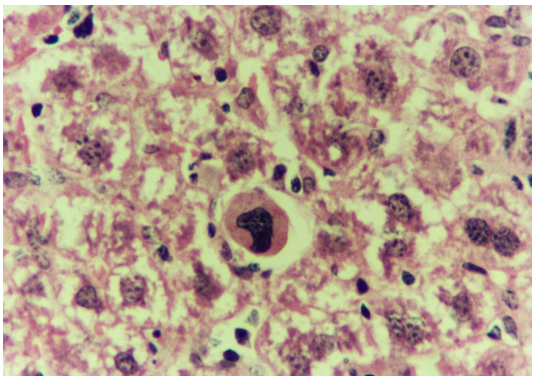

Fig. (24):-Histopathological sections (40X) from mouse inoculated with CD-CTAB + EAC showed a mild prevascular and focal parenchymal inflammatory effects represented by a polymorphal nucleated leucocytes infiltrate affecting the portal tract, a remarked degree of cytoplasmic degeneration and presence of giant cells. Normal lobular architecture with spaces between hepatic cords, no fatty changes and no signs of any tumors.

The antimicrobial activity

The results given in table (15) showed antimicrobial and antifungal activity of the target compounds. A highly potential antimicrobial and antifungal effect than the parent cationic surfactant cetyl trimethyl ammonium bromide was recorded where the activity increased by 35% after copper complexation. The antimicrobial and antifungal activity of CD-CTAB was decreased by 73% than the non-loaded Cu-CTAB where the concentration of Cu-CTAB into the loaded nano-analogue was 25%.

Table (9). The antibacterial and antifungus activity of Cetyltrimethyl ammonium bromide (CTAB), Cu-CTAB and CD-CTAB

Microorganism	Gram staining reaction	Inhibition zone diameter (Ø, mm)			
		Control water	CTAB	Cu-CTAB	CD-CTAB
Desulfonamonas pigra (SRB)	(G^-)	0.0	15	23	6
Escherichia coli	(G^-)	0.0	15	23	6
Staphylococcus aureus	(G^+)	0.0	15	24	6.25
Candida Albicans	Fungus	0.0	14	20	5

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