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## Research Article

# Synergistic Antitumor Activity of Doxorubicin and Atorvastatin Combination Loaded Nanoemulsion in Mice

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## Abstract

**Background and Objective:** Nanoemulsions (NE) have been proved to enhance the bioavailability of the therapeutic agents with reduced side effects. The objective of the study was to evaluate the antitumor potential of Doxorubicin (Doxo) and Atorvastatin (ATR) combination, either loaded in nanoemulsions (Doxo-ATR-NE) or water (Doxo-ATR-Solution) against ehrlich ascites carcinoma in Swiss albino mice. **Materials and Methods:** The DOXO and ATR loaded nanoemulsion was prepared by the successive incorporation of different constituents of the nanoemulsion. The nanoemulsion was characterized by Transmission Electron Microscopy (TEM) and further evaluated for the antitumor activity for 15 days experimental study. The antitumor effect of the preparation was assessed by the changes in the body weight, percentage of increased life span (ILS%), mean survival time, serum biochemical parameters for liver and kidney functions, hematological parameters along with the histopathological studies. **Results:** The ATR-Doxo-NE treated group produce a significant change in body weight as compared with the positive control group and ILS% of mice treated with ATR-Doxo-Sol group was greater than Doxo-Sol group. Interestingly, the ILS% of the ATR-Doxo-NE treated group was higher than the ATR-Doxo-Sol group. Doxo-NE treated mice showed the highest ILS% as compared with the rest of the other treated groups. ATR-Doxo-NE and ATR-Doxo-Solution treated mice showed a significantly normalized level of hematological parameters. Moreover, the results from the kidney and liver enzymes showed the least toxic effects of Doxo-NE and ATR-Doxo-NE in comparison with Doxo-Sol and ATR-Doxo-Sol. **Conclusion:** Overall, the Doxo-NE and ATR-Doxo-NE formulations exhibited greater anti-cancer activity with less toxic effects.

**Key words:** Antitumor, atorvastatin, carcinoma, doxorubicin, ehrlich ascites, nanoemulsion

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**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Cancer is an umbrella of several life-threatening diseases and can be scientifically defined as the uncontrolled cell division leading to the development of a tumor. The abnormal cell growth in cancer sometimes has a tendency to assault adjoining parts by invading them and spreading distant organs of the body<sup>1</sup>. According to current WHO statistics, cancer becomes the second leading cause of death throughout the world. About 1 in 6 global deaths have been reported due to cancer. A total of 9.6 million global deaths in 2018 were estimated due to cancer. It is predicted that these global cancer death figures would surge over 22 million in the coming couple of decades, particularly in developing countries<sup>2</sup>. The American Cancer Society (ACS) estimated the recent statistics on incidence, survival and mortality of cancer in the US population and reported that about 1,762,450 fresh incidents of cancer and 606,880 cancer-associated mortality are expected to take place in the United States alone in 2019<sup>3</sup>. Chemotherapy and targeted cancer therapies are two different approaches to control uncontrolled cancerous cells. Targeted cancer therapies of drugs are focused on specific molecular targets, which prevent the growth of cancerous cells and then inhibit the spread of cancer. Targeted cancer therapy basically acts by interfering with specific molecules which are known to regulate the growth of cancerous cells, including its progression and spread. Chemotherapy is a well-known *modus operandi* for the treatment of cancerous conditions. Chemotherapy utilizes one or multiple chemotherapeutic agents to treat cancer by inhibiting the mitotic cell division in the cancerous cells. However, chemotherapy has some major limitations like partial specificity of the drugs to *in vivo* cancerous cells and serious unwanted toxic effects<sup>4</sup>. Doxorubicin (Doxo) is one of the most effective antibiotics commonly employed for remitting leukemia and tumors in lymphomas and breast cancer etc<sup>5</sup>. However, its use is restricted by clinicians due to a wide spectrum of adverse effects. Cardiotoxicity, a serious and well-known adverse effect arises due to prolonged use of doxorubicin in cancer patients adversely affecting the cardiac functions. The subsequent adversity which follows doxorubicin-induced cardiotoxicity is cardiac remodeling, which alters cardiac morphology and physiology leading to cardiomyopathy and ultimately cardiac failure<sup>6</sup>. Doxorubicin-induced cardiotoxicity has been reported a leading cause of morbidity and mortality among cancer survivors<sup>7</sup>. Heart failure occurred as a consequence of doxorubicin-induced cardiac toxicity and cardiac remodeling. However, the mechanisms of Doxo-induced cardiotoxicity are still not completely

understood<sup>8,9</sup>. Besides cardiotoxicity, Doxo is also known to trigger multiple organ toxicity, including kidney, liver and sometimes brain<sup>10,11</sup>.

The anti-hyperlipidemic drugs "3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase inhibitors" are commonly known as statins are used to reduce morbidity and mortality related to cardiovascular diseases. Several statins such as; atorvastatin, simvastatin, lovastatin, pravastatin, fluvastatin, pitavastatin and rosuvastatin have been usually prescribed to control dyslipidemia and obesity. Statins are considered as first-line treatment for the prevention of cardiovascular diseases, hence used in the prophylaxis and treatment of several cardiovascular diseases. Atorvastatin is commonly prescribed for the mitigating hyperlipidemic condition and also for the prophylaxis of cardiovascular diseases. The potential anti-cancer activities of various statins as single-drug therapy and multiple drug therapy have been established against various models of cancer cell lines and animal models of the tumor, including hepatocellular, colorectal, prostate, breast and blood related malignant cancers. According to recent scientific reports, lipophilic statins such as; Atorvastatin, Simvastatin, Fluvastatin and Lovastatin elucidate anticancer effect by inhibiting cellular proliferation, pro-apoptotic or anti-angiogenic activity<sup>12-15</sup>.

Surgery, radiotherapy and chemotherapy are mainstay treatments of cancers. Multidrug chemotherapy and drug delivery system are two promising areas of treatment which involve nanoparticles and have shown remarkable results in cancer management<sup>16</sup>. There is a multitude of benefits when multidrug treatment is employed as it may produce synergistic and/or additive effects besides diminishing the risk of adverse events and side effects. Contrastingly, drug delivery systems involving nanoparticle improve the therapeutic efficiency by improving their pharmacokinetics and bio-distribution and minimize the side effects of drug payloads<sup>17</sup>.

The multidrug cancer therapy and nanoparticle drug delivery system were combined to improve cancer control management. The combination of these two approaches will enhance the therapeutic value and improve the overall quality of life for cancer patients<sup>18</sup>.

Nano emulsions (NEs) are the combination of two immiscible liquids which then form a single phase using appropriate surfactants. The resulting nano emulsion is thermodynamically stable and optically isotropic in nature<sup>19</sup>. Since the particles in NEs are very small in size, it increases the surface area for interaction and hence the drugs are absorbed uniformly in less time<sup>20</sup>. Formulating a lipophilic anticancer drug in submicron oil-in-water emulsion improves the

solubilization of the lipophilic drugs and reduces the drug's dose<sup>21</sup>. Several lipophilic anticancer agents like dacarbazine, tamoxifen, curcumin and paclitaxel have been incorporated into NEs to potentiate their toxic action against cancer cells and also to reduce the chances of development of multiple drugs resistance<sup>22,23</sup>. The given study was planned to assess the combined anti-tumor effects of ATR and Doxo loaded in a lipid-based NE in female Swiss albino mice with Ehrlich Ascites Carcinoma (EAC). The several biochemical parameters of liver and kidney function tests including body weight changes, Increase in Life Span (ILS%), Mean Survival Time (MST) and were measured to evaluate the anticancer potential of the prepared combination formula of both drugs. The histopathological investigation of both kidney and liver tissues were also performed to correlate with biochemical results.

## MATERIALS AND METHODS

**Materials:** Doxorubicin Hydrochloride (Doxo) was obtained from the US. Pharmacopeial Convention. Polyoxyethylene glycerol trihydroxy stearate 40 (EU) (Eumulgin® HRE 40), Tris (hydroxymethyl) aminomethane, soya phosphatidylcholine (SPC), hydrochloric acid (HCl) and sodium oleate were procured from the Leo Chemicals, India. Cholesterol (CHO) was procured from the chemical company Jecho Pharmacies, India. The pure 1-octanol was bought from Alfa Aesar GmbH and Co. KG Karlsruhe, Germany. Lipitor (Atorvastatin) sample was obtained as a gift sample from a pharmaceutical company "Jamjoom Pharma", Jeddah, Saudi Arabia. This study was carried out at Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia, from September, 2013 to December, 2014. Animal study was carried out at King Fahad Medical Research Center (KFMRC), King Abdulaziz University, Jeddah, Saudi Arabia.

**Animals:** A total 180 healthy, female, Swiss albino mice (average weighing about 25-30 g) and Ehrlich Ascites Carcinoma (EAC) cells were procured from the research facility of King Fahd Medical Research Centre, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia. The animals were then acclimatized for a week before being randomly segregated into different groups. The mice were given a standard marketed pellet diet and purified tap water *ad libitum* during the whole study. The research protocol was officially approved by the Institutional Medical Ethical Committee at the Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.

## Methods

**Preparation of DOXO and ATR loaded nanoemulsions:** Three unique novel combinations of NE formulations e.g., Doxo-NE, ATR-NE and ATR-Doxo-NE were prepared by adopting a modified method of Alkhatib and AlBishi<sup>24</sup>. This method included the mixing of diverse weight fractions of 1-octanol, surfactant mixture of EU/SPC/SO and Tris-HCl buffer (pH 7.24). The blank NE was prepared by mixing CHO and the surfactant mixture SPC/SO/EU. The surfactant mixture (1.25%) of SPC/SO/EU at a ratio of 3.0:3.5:3.5 was mixed with the oil phase of 0.125% of CHO (w/w). The constant percentage of 1-octanol (1%), co-surfactant (1%) and the weight percentage of Tris-HCl buffer (pH 7.22) and the aqueous phase were used at a fixed percentage (97.63%).

The Nano emulsion (NE) formulations were prepared by the successive incorporation of different constituents of the nano emulsion. First, the solid surfactants mixture (EU/SPC/SO) were triturated to obtain uniformity, CHO was then added gradually to the mixture of surfactants until the appearance of a semisolid phase. Subsequently, 1-octanol was slowly added drop by drop and the resulting mixture was then carefully diluted with warm Tris-HCl buffer (pH 7.24) and then finally vortexed. The mixture was stood for 3 h in a water bath and subsequently centrifuged carefully at 6000 rpm for 30 min to obtain a clear and transparent NE. Subsequently, the less murky layer was strained and then heated at 75°C for 4 h in a water bath. The murky layer was drained out to obtain a clear transparent NE layer of the mixture. Different NE was prepared like blank loaded-NE (Blank-NE), drug-loaded NE containing 0.3 mg/mL/150 g of Doxo loaded-NE (Doxo-NE), ATR loaded-NE (ATR-NE) and combination (2:1 ratio) of ATR to Doxo loaded-NE (ATR-Doxo-NE). Contrastingly, the distilled water-soluble drug formulations were 0.3 mg/mL/150 g of Doxo loaded-Sol (Doxo-Sol), ATR Loaded-Sol (ATR-Sol) and the combination (2:1 ratios) of ATR to Doxo loaded-Sol (ATR-Doxo-Sol).

**Characterization of the NE formulations using TEM:** The morphology and size distribution of prepared NE formulations (Blank-NE, Doxo-NE, ART-NE and Doxo-ATR-NE) were estimated with the help of TEM (JEM-1011, JEOL, Tokyo, Japan). A single droplet of the NE sample (1 mg mL<sup>-1</sup>) under study was kept on a copper electron microscope grid for the duration of 5 min. The extra sample was removed using filter paper and droplet of tri-(n-dodecyl-dimethyl-hydroxy propyl ammonium chloride) phosphate was added to the grid surface to stain it for 30 sec. Finally, the grid was cleaned by using distilled water and after removing the excess water, the sample was observed by using TEM.

Table 1: Experimental design (drug formulation)

Group	Group name	Treatments
I	Negative control	0.2 mL of 0.9% saline i.p. on alternate days (Total 3 doses)
II	Positive control	0.2 mL of 0.9% saline i.p. on alternate days (Total 3 doses)
III	Blank-NE	0.2 mL of blank NE by i.p. route on alternate days (Total 3 doses)
IV	Doxo-NE	2 mg kg <sup>-1</sup> , i.p. on alternate days (Total 3 doses)
V	ATR-NE	4 mg kg <sup>-1</sup> , i.p. on alternate days (Total 3 doses)
VI	ATR-Doxo-NE	1:2 ratio of combination on alternate days (Total 3 doses)
VII	Doxo-Sol	2 mg kg <sup>-1</sup> , i.p. on alternate days (Total 3 doses)
VIII	ATR-Sol	4 mg kg <sup>-1</sup> , i.p. on alternate days (Total 3 doses)
IX	ATR-Doxo-Sol	1:2 ratio of combination on alternate days (Total 3 doses)

**Experimental study for *in vivo* evaluation of the antitumor activity of drug formulations:** The *in vivo* method described by Alkreathy *et al.*<sup>15</sup> was adopted to evaluate the antitumor effects of prepared NE formulations (Blank-NE, Doxo-NE, ATR-NE and ATR-Doxo-NE) and aqueous NE-free-formulations (Doxo-sol, ATR-Sol and Doxo-ATR-Sol). Post acclimatization on day 0, the total 180 female Swiss albino mice were segregated into nine groups with 20 mice in each group. The details of the groups are mentioned in Table 1.

On the 15th day, ten mice were kept fasting for the duration of 24 h from each group and then sacrificed to collect blood for investigating biochemical and hematological parameters. The liver tissues were also collected separately and preserved to investigate the histopathological changes. The remaining mice in each group were kept as such for checking the Increase in Life Span (ILS%) and Mean Survival Time (MST) were estimated as described by Tagne *et al.*<sup>22</sup>:

$$\text{ILS (\%)} = \left( \frac{\text{Mean survival time of treated group}}{\text{Mean survival time of control group}} - 1 \right) \times 100$$

**Hematological parameters:** The blood was collected using heparinized micro-capillaries from retro-orbital plexus of the mice under light anesthesia. Red Blood Cells (RBCs), White Blood Cells (WBCs), platelet counts, lymphocytes, granulocytes, monocytes and hemoglobin were estimated by using haematology auto-analyzer (BC-2800 Vet, Mindray, China) at King Fahd Centre for Medical Research.

**Biochemical parameters in serum:** The blood sample was carefully centrifuged at 5000 rpm for the duration of 15 min to obtain the serum. The efficiency of the liver was evaluated by assessing serum concentration of alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), total bilirubin (BIL), albumin (ALB) and Total Protein (TP). Similarly, the kidney functions were evaluated by measuring serum levels of blood urea nitrogen (U-BUN), serum creatinine and level of electrolytes like potassium (K<sup>+</sup>), sodium (Na<sup>+</sup>),

chloride (Cl<sup>-</sup>) and carbon dioxide (CO<sub>2</sub>) in blood serum. The cholesterol (CHO), triglyceride (TAG) and High Density Lipoprotein (HDL) levels in serum were estimated to know the lipid profile. All these biochemical parameters were estimated using specific standard diagnostic kits with the help of automated modular analyzer (COBAS® 8000 series) at King Fahad Armed Forces Hospital, Jeddah, Saudi Arabia.

**Histopathological studies of liver tissues:** A small section of the liver was isolated from each group and then fixed in freshly prepared 10% formalin buffered solution and was subsequently embedded with paraffin wax to carry out histopathological investigations. Microtome was used to cut thin sections of liver tissues (5-6 μ thick), which were then stained with histological dyes known as hematoxylin and eosin dyes. The stained sections of the liver were then thoroughly investigated using a light microscope for any histopathological discrepancies in the liver cells.

**Statistical analysis:** The results of this study were presented as Mean ± Standard Deviation (X ± SD). The statistical analysis of the obtained data was carried out by ANOVA (one-way analysis of variance) by means of MegaStat software and p-value for paired t-tests. The significant difference among the obtained data was taken at p < 0.05.

## RESULTS

### Evaluation of physical characterization of NE formulation:

The physical characterization of NE formulations like morphology of the NE and its droplet size was determined by using Transmission Electron Microscopy (TEM). The images obtained by TEM revealed that the droplets of Doxo-NE (Fig. 1a), ATR-NE (Fig. 1b) and ATR-Doxo-NE (Fig. 1c) were semi-spherical, spherical and oval shapes, respectively (Fig. 1). The range of droplet diameter (nm), mean of droplet diameter (nm) and the coefficient of variation (CV%) of different formulations such as; ATR-NE, DOX-NE and ATR-Doxo-NE are

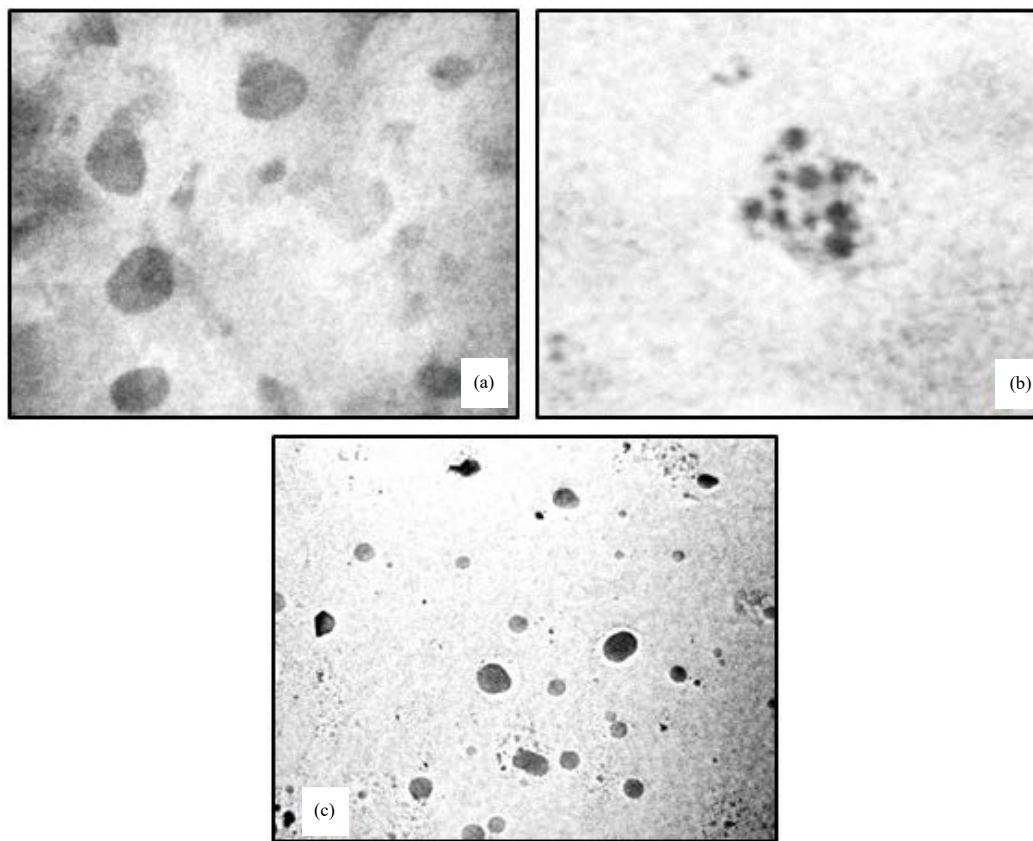


Fig. 1(a-c): TEM images of (a) DOXO-NE (0.1  $\mu$ M), (b) ATR-NE (5  $\mu$ M) and (c) ATR/DOXO-NE (0.1  $\mu$ M)

Table 2: Mean droplet diameter of NE formulation of Doxo-NE, ATR-NE and ATR-Doxo-Sol determined by TEM

Formulations	Mean of droplet diameter (nm)	Range of droplet diameter (nm)	CV%
Doxo-NE	9.48 $\pm$ 1.45	8.03-10.92	5.53
ATR-NE	67.70 $\pm$ 12.76	54.94-80.46	18.85
ATR-Doxo-Sol	5.50 $\pm$ 0.34	5.16-5.84	6.18

Data were expressed as  $\bar{X}\pm$ SD, CV is the coefficient of variation measured by dividing the standard deviation by the mean

represented in Table 2. The mean of the droplet size of the ATR-NE and Doxo-NE preparation was 67.7 $\pm$ 12.76 and 9.48 $\pm$ 1.45 nm, respectively. Contrastingly, the average diameter of ATR-Doxo-NE was found to be very low as compared to ATR-NE and Doxo-NE preparations. The mean range of droplet diameter of the Doxo-NE, ATR-NE and ATR-Doxo-NE were found to be 8.03-10.92, 54.94-80.46 and 5.16-5.84 nm, respectively.

The percentages of Coefficients of Variation (CV%) or polydispersity index were evaluated to analyze the size distribution of droplets of NE formulations. The CV% of the Doxo-NE, ATR-NE and ATR-Doxo-NE were found to be 5.53, 18.85 and 6.18%, respectively. The droplets of the NE formulations were found to be distributed homogeneously throughout the formulation, as the CV% was <25%, without

adhesion or aggregation. These findings evidently indicated the effective preparation of the new nanoemulsions encompassing Doxo-NE, ATR-NE and ATR-Doxo-NE.

**Effects of different treatment groups on the body weight, average time of survival and life span:**

A noticeable difference ( $p<0.001$ ) in the average body weight change of EAC control was found as compared with normal control. The results of the different treatment groups viz. ATR-Sol, Doxo-NE, Blank-NE and ATR-NE showed a significant reduction in the body weight of the mice when compared with normal control. Contrastingly, ATR-Doxo-NE treated group didn't produce any substantial change in body weight in comparison with the positive control group (Table 3). Furthermore, there was a substantial reduction in body weight of the mice given

Table 3: Effect of different drug formulations on body weight changes, MST and ILS% of EAC bearing mice

Groups	Average body weight changes (g)	MST (days)	ILS%
Normal	1.246±2.4559	80.0±0	*****
EAC control	14.380±2.42022 <sup>c</sup>	21.9±1.728 <sup>c</sup>	-72.625
Blank-NE	18.004±5.542 <sup>c,q</sup>	22.5±2.223 <sup>c,ns</sup>	2.739
Doxo-NE	7.806±1.605 <sup>c,r</sup>	80.0±0 <sup>ns,r</sup>	265.296
ATR-NE	13.526±3.952 <sup>c,ns</sup>	27.1667±2.483 <sup>c,ns</sup>	24.0481
ATR/ Doxo-NE	3.256±1.500 <sup>ns,r</sup>	75.7±13.597 <sup>ns,r</sup>	245.662
Doxo-Sol	-5.825±2.536 <sup>c,r</sup>	34.6±8.876 <sup>c,q</sup>	57.990
ATR-Sol	10.132±5.346 <sup>c,r</sup>	27.1667±2.483 <sup>c,ns</sup>	24.048
ATR/Doxo-Sol	-2.323±3.7522 <sup>c,r</sup>	58.2±24.352 <sup>c,r</sup>	165.753

Data were expressed as  $\bar{X} \pm SD$  for n = 10, <sup>c</sup>p<0.001 represent p-value as compared to Normal control group and <sup>q</sup>p<0.01, <sup>r</sup>p<0.001 represent p-value as compared to EAC control group, MST: Mean survival time; ILS: Increased life span; \*\*\*\*\*It was not estimated; EAC: Ehrlich ascites carcinoma, NE: Nano-emulsions, Doxo: Doxorubicin hydrochloride, ATR: Atorvastatin, Sol: Solution

Table 4: Effect of different drug formulations on haematological parameters activity of EAC bearing mice

Groups	RBC (× 10 <sup>6</sup> /uL)	WBC (× 10 <sup>3</sup> /uL)	Lymphocyte (× 10 <sup>3</sup> /uL)	Monocyte (× 10 <sup>3</sup> /uL)	Granulocyte (× 10 <sup>3</sup> /uL)	Hemoglobin (g dL <sup>-1</sup> )	Platelet (× 10 <sup>3</sup> /uL)
Normal	6.9925±0.956	7.44±2.414	3.24±1.1458	0.36±0.151	0.126±0.043	11.92±1.765	745.8±163.791
EAC control	7.138±0.445	16.54±3.144 <sup>c</sup>	12.9±1.831 <sup>c</sup>	2.92±1.420 <sup>c</sup>	0.72±0.311 <sup>c</sup>	12.56±1.289	1004.4±136.415
Blank-NE	7.138±1.371	9.84±3.160 <sup>r</sup>	9.175±2.125 <sup>c,r</sup>	1.15±0.585 <sup>ns,r</sup>	0.34±0.320 <sup>q</sup>	11.5±2.293	981.4±370.514
Doxo-NE	7.375±0.324	8.76±4.190 <sup>r</sup>	6.075±1.554 <sup>ns,r</sup>	0.625±0.109 <sup>r</sup>	0.325±0.147 <sup>q</sup>	12.42±0.828	725.4±292.920
ATR/ Doxo-NE	7.664±0.270	5.3±1.259 <sup>r</sup>	4.86±1.169 <sup>r</sup>	0.4±0.141 <sup>r</sup>	0.1±0 <sup>r</sup>	13.3±0.771	651.8±143.872 <sup>p</sup>
Doxo-Sol	7.1842±0.27	1.35±0.112 <sup>c,r</sup>	0.862±0.275 <sup>ns,r</sup>	0.384±0.042 <sup>r</sup>	0.274±0.125 <sup>r</sup>	12.8±1.826	1026.8±262.575
ATR/ Doxo-Sol	7.452±0.327	5.36±0.835 <sup>r</sup>	3.82±2.015 <sup>r</sup>	0.38±0.179 <sup>r</sup>	0.2±0.1 <sup>r</sup>	15.28±0.892 <sup>c,q</sup>	906.6±109.301

Data were expressed as  $\bar{X} \pm SD$  (n = 5), <sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 represent p-value as compared to Normal and <sup>p</sup>p<0.05, <sup>q</sup>p<0.01, <sup>r</sup>p<0.001 represent p-value as compared to EAC control. EAC: Ehrlich ascites carcinoma, NE: Nanoemulsions, Doxo: Doxorubicin hydrochloride, ATR: Atorvastatin, Sol: Solution

with combination treatments like ATR-Doxo-Sol and Doxo-Sol as compared against the positive control group. The EAC bearing mice having treatment with Doxo-Sol, Doxo-NE, ATR-Doxo-Sol and ATR-Doxo-NE showed a significant upsurge in the Mean Survival Time (MST) in comparison with the positive control group (Table 3). However, the Doxo-NE and ATR-Doxo-NE treatments didn't significantly alter the MST of the mice bearing EAC as compared against normal (Table 3). The ILS% of the EAC mice given different treatments like Doxo-NE, ATR-Doxo-NE and ATR-Doxo-Sol showed increased percentage life span (Table 3).

**Hematological parameters:** The effects of different treatment groups like Blank-NE, Doxo-NE, ATR-Doxo-NE, Doxo-Sol and ATR-Doxo-Sol on various hematological parameters are illustrated in Table 4.

It was found that the level of hemoglobin remained equivalent in all the groups, but it was significantly increased in the ATR-Doxo-Sol group as compared against the normal group (Table 4). The total WBCs level in EAC group was found to be twice of the normal group, but it was dramatically found to be far below normal in the Doxo-Sol group (Table 4). Additionally, in combination groups like ATR-Doxo-NE and ATR-Doxo-Sol, the level of leucocytes was below the normal level. The serum level of lymphocytes in the positive control was noticeably increased while, it was

significantly decreased in Blank-NE, Doxo-NE, ATR-Doxo-NE, Doxo-Sol and ATR-Doxo-Sol as compared to the positive control group.

In addition, the serum monocytes levels in the positive control (EAC) were found to be statistically significantly increased as compared to the negative control group.

The level of serum monocyte's and granulocyte counts in different studied treatment groups (viz. Blank-NE, Doxo-NE, ATR-Doxo-NE, Doxo-Sol and ATR-Doxo-Sol) were found to be significantly decreased in comparison with the positive control group (Table 4). Contrastingly, the level of serum granulocytes counts in different studied treatments groups viz Blank-NE, Doxo-NE, ATR-Doxo-NE, Doxo-Sol, ATR-Doxo-Sol were found to be significantly decreased in comparison with the positive control group. Overall, the results of all hematological studies exhibited a clear improvement in hematological parameters in different treatment groups as statistically presented in Table 4.

**Histopathological interpretation of liver tissues:** The isolated liver tissues from each of the groups were examined microscopically and it was found that normal control group (Fig. 2a) showed normal hepatic cells architecture with centrifugally organized hepatocytes around the central vein, it also had regular blood sinusoids and presence of Kupffer cell was also noted. The photomicrographs of EAC control (Fig. 2b)

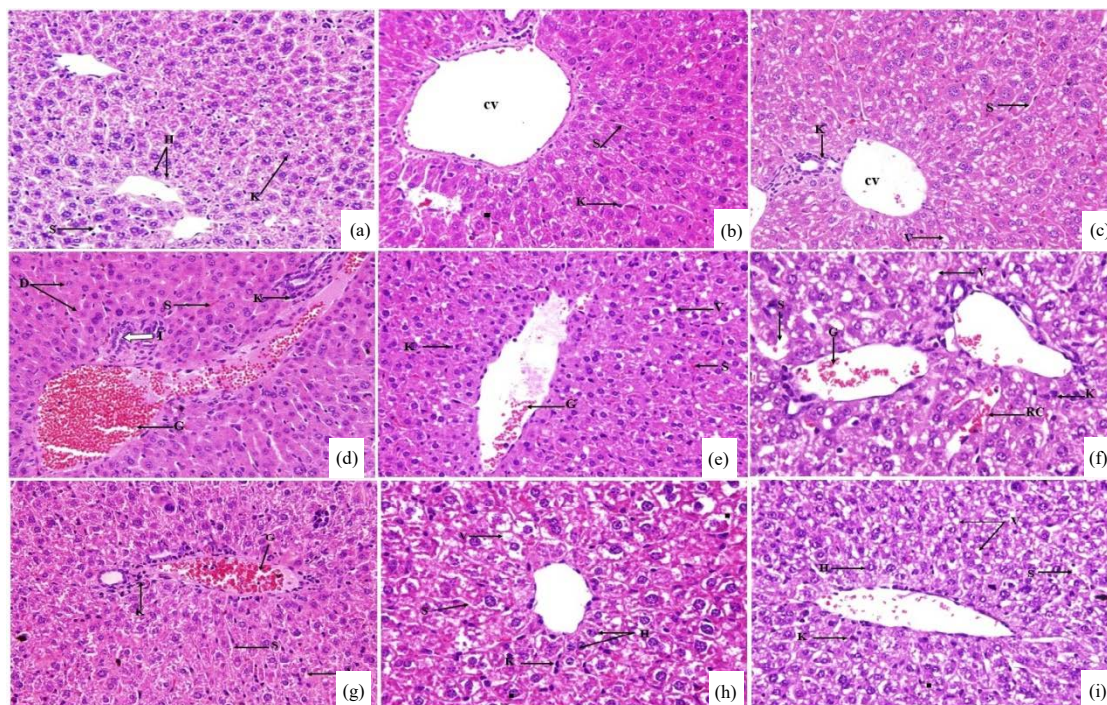


Fig.2(a-l): Various histological slides (H&E x 40) resulting from the different treatment groups, (a) Normal group showing normal hepatic architecture, radially arranged hepatocytes (H) around the central vein (cv) and blood sinusoids (S) in the presence of kupffer cells (K), (b) EAC control showing almost normal hepatic structure, radially arranged hepatocytes (H) around the central vein (cv) in the presence of Kupffer cells (K) and narrow sinusoids spaces (S), (c) Blank-NE showing mild changes in the hepatic structure in the presence of Kupffer cells (K), mild vacuolation of hepatocellular (V) and narrow blood sinusoids spaces (S), (d) Doxo-Sol showing pathological changes in the presence of more Kupffer cells (K) and dilated central vein with hemorrhage (G). Dilated blood sinusoids spaces (S), degeneration necrotic hepatocytes (D) and many inflammatory cells (I), (e) ATR-Doxo-Sol showing moderate normal hepatic structure in the presence of moderately Kupffer cells (K), moderate dilated blood sinusoids spaces (S), moderate vacuolation of hepatocellular (V) and dilated central vein with hemorrhage (G), (f) ATR-Sol showing loss of normal hepatic structure, in the presence of moderately Kupffer cells (K), wide and dilated blood sinusoids spaces (S) full by red blood corpuscles (RC), moderate vacuolation of hepatocellular (V) and dilated central vein with hemorrhage (G), (g) Doxo-NE showing relatively normal hepatocytes, in the presence of moderately Kupffer cells (K), less dilated blood sinusoids spaces (S), dilated central vein with hemorrhage (G) and moderate vacuolation of hepatocellular (V), (h) ATR-Doxo-NE showing normal hepatic structure, radially arranged hepatocytes (H) around the central vein and narrow blood sinusoids (S), less Kupffer cells (K) and large number of vacuoles (V) and (i) ATR-NE showing normal hepatic structure, radially arranged hepatocytes (H) around the central vein and blood sinusoids (S), less Kupffer cells (K) and large number of vacuoles (V)

and Blank-NE group (Fig. 2c) showed almost similar architecture of cords of hepatocytes radiating from the central vein. The hepatocytes showed well-preserved cytoplasm and distinct spherical nucleus along with a perfect configuration of Kupffer cells (Fig. 2b). The sinusoids (hepatic) were irregularly spaced with narrow gaps among them (Fig. 2b). Blank-NE group also showed mild vacuolation of hepatocytes and narrow blood sinusoidal spaces (Fig. 2c).

The liver tissue section from the Doxo-Sol group showed many histopathological alterations like the occurrence of many Kupffer cells, dilated central vein accompanied with hemorrhage, widening of blood sinusoid, degenerating necrotic liver cells and different inflammatory cells (Fig. 2d). The liver sections from mice treated with ATR-Doxo-Sol showed the presence of a moderate number of Kupffer cells, moderate vacuolation in hepatic cells, blood



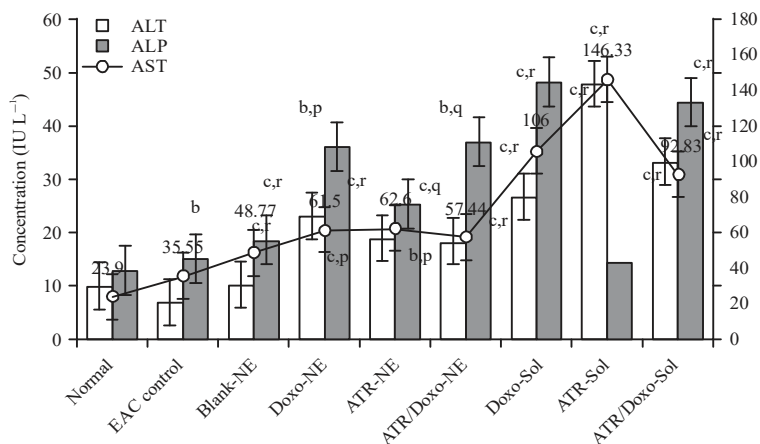


Fig. 3: Effect of different drug formulations on ALT, ALP and AST of EAC bearing mice

Data were expressed as  $\bar{X} \pm SD$  (n = 10). <sup>b</sup>p<0.01, <sup>c</sup>p<0.001 represent p-value as compared to Normal and <sup>p</sup>p<0.05, <sup>q</sup>p<0.01, <sup>r</sup>p<0.001 represent p-value as compared to EAC control, ns: Non-significant, ALT: Alanine transaminase, AST: Aspartate transaminase, ALP: Alkaline phosphate, EAC: Ehrlich ascites carcinoma, NE: Nanoemulsions, Doxo: Doxorubicin hydrochloride, ATR: Atorvastatin, Sol: Solution

sinusoids spaces were also mildly dilated along with hemorrhagic central veins (Fig. 2e). The micrographs obtained from the ATR-Sol group showed abnormal hepatic architecture e.g., occurrence of the modest number of Kupffer cells, blood sinusoids were dilated and hemorrhage was seen near the dilated central vein, also vacuolation of hepatocytes was noted (Fig. 2f). Oppositely, the group receiving Doxo-NE treatment revealed relatively normal hepatic architecture and a smaller number of Kupffer cells were present. Only mild dilatation of blood sinusoidal space and the central vein were noted with hemorrhage and moderate vacuolation of hepatocellular (Fig. 2g). Interestingly, the sections of the liver tissue of ATR-Doxo-NE (Fig. 2h) ATR-NE (Fig. 2i) treated group almost normal hepatocytes radiating outward from the central vein and fine blood sinusoids. Fewer Kupffer cells were seen and numerous vacuoles were observed.

**Analysis of liver function using different biomarkers:** Study of hepatic functions using different biomarkers in the treatment groups revealed that the serum level of AST and ALP were significantly elevated, while, ALT was reduced noticeably in the EAC group as compared against the normal group. The serum concentration of AST and ALP in each of the treatment groups, excluding the normal control, Blank-NE and EAC control were found to be outside the normal range. Factually, the levels of these biochemical parameters in NE formulations groups (Doxo-NE, ATR-NE, ATR-Doxo-NE) were remarkably lower than the solution formulations groups (Doxo-Solution, ATR-Solution and ATR-Doxo-Solution) as statistically summarized in Fig. 3. It is

remarkable to note that the AST level of the ATR-Solution group was significantly higher than the Doxo-Solution and ATR-Doxo-solution treated groups.

The concentrations of ALT of all of the studied groups, excluding the solution formulations treated groups (Doxo-Solution, ATR-Solution and ATR-Doxo-Solution) were found in the normal range. The AST serum concentrations in normal control, Blank-NE and EAC treated groups showed similar serum concentrations of ALT level, but it was significantly lower as compared with the Doxo-NE, ATR-NE, ATR-Doxo-groups as statistically represented in Fig. 3. The serum level of ALP was found to be significantly increased in different DOX formulations (Doxo-Solution, ATR-Doxo-Solution, Doxo-NE and ATR-Doxo-NE) groups as compared to EAC group (Fig. 3). However, the EAC control, Blank-NE and ART-Solution groups showed almost similar serum levels of ALP in comparison to the normal control group (Fig. 3). Interestingly, it is noted that the AST levels of all Doxo containing nanoformulations were indifferent.

The serum ALB and TP levels in among all treated groups were elevated noticeably as compared with the normal group (Fig. 4). Their serum levels were maximum in ATR-Doxo-Sol, ATR-Doxo-NE and ATR-NE groups and then Doxo-Sol and Doxo-NE treatment groups, whereas the ATR-Sol group had the lowest level of ALB and TP. Finally, the serum levels of BIL were significantly increased in every group as compared against the negative control (Fig. 4).

**Analysis of renal function using creatinine, U-BUN and ions:** The presented study revealed that creatinine level (CRE)

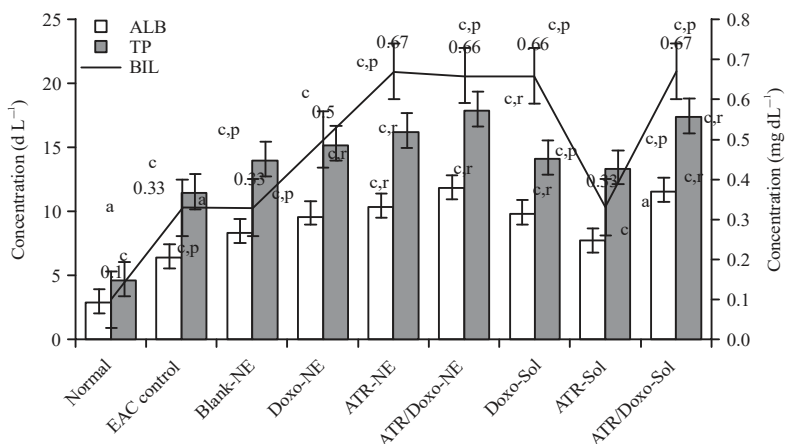


Fig. 4: Effect of different drug formulations on ALB ( $\text{g L}^{-1}$ ), TP ( $\text{g L}^{-1}$ ) and BIL ( $\text{mg dL}^{-1}$ ) of EAC bearing mice

Data were expressed as  $\bar{X} \pm \text{SD}$  ( $n = 10$ ),  $^a p < 0.05$ ,  $^c p < 0.001$  represent p-value as compared to Normal and  $^p p < 0.05$ ,  $^p p < 0.001$  represent p-value as compared to EAC control, ns: Non-significant ALB: Albumin, TP: Total protein, BIL: Bilirubin, EAC: Ehrlich ascites carcinoma, NE: Nanoemulsions, Doxo: Doxorubicin hydrochloride, ATR: Atorvastatin, Sol: Solution

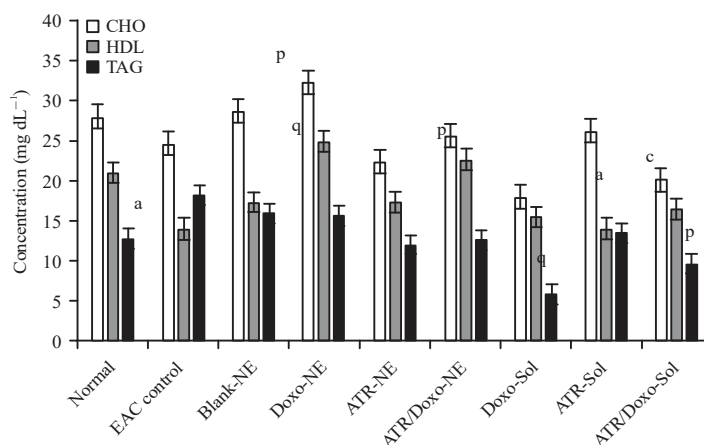


Fig. 5: Effect of treatments on the levels of cholesterol, high density lipoproteins and triglycerides of mice serum

Data are presented as Mean  $\pm$  SD ( $n = 10$ ).  $^a p < 0.05$ ,  $^c p < 0.001$  represent p-value as compared to Normal and  $^p p < 0.05$ ,  $^p p < 0.01$  represent p-value as compared to EAC control, CHO: Cholesterol, HDL: High density lipoprotein, TAG: Triglycerides, EAC: Ehrlich ascites carcinoma, NE: Nanoemulsions, Doxo: Doxorubicin hydrochloride, ATR: Atorvastatin, Sol: Solution

( $\text{mg dL}^{-1}$ ) in every group was within the normal range ( $0.3\text{-}1 \text{ mg dL}^{-1}$ ) except for Doxo-NE, ATR-Doxo-NE and ATR-Doxo-Solution groups which showed considerably higher creatinine as compared with normal control group (Table 5). On the other hand, the U-BUN level in every treatment group was found within the normal limit ( $13.9\text{-}28.3 \text{ mg dL}^{-1}$ ) excluding the ATR-NE group, which showed noticeably increased U-BUN level as compared to EAC control (Table 5).

The serum concentration of  $\text{Na}^+$  was within the normal limits ( $128\text{-}145 \text{ mEq L}^{-1}$ ) in every treatment group except groups treated with Doxo containing preparations. The Doxo-Sol and ATR-Doxo-Sol groups showed a remarkable elevation in  $\text{Na}^+$  levels as compared against the normal group.

Similarly, the serum level of  $\text{K}^+$  was in the normal limits ( $4.85\text{-}5.85 \text{ mEq L}^{-1}$ ) in every group except for ATR-Sol, which had a slight statistically insignificant elevation (Table 5). There was an insignificant elevation in the  $\text{Cl}^-$  ion and  $\text{HCO}_3^-$  concentration in EAC control, Blank-NE, Doxo-NE and ATR-Sol groups while ATR-NE, ATR-Doxo-NE, Doxo-Sol and ATR-Doxo-Sol were distinctly more than the normal limits (Table 5).

**Effect of treatments on lipid profile:** The effect of present novel treatment was carried out by estimation of the serum concentrations of TAG, cholesterol and HDL (Fig. 5). Each of the treatment groups showed a normal level of cholesterol in the serum ( $26\text{-}82.41 \text{ mg dL}^{-1}$ ) apart from the Doxo-Sol and

Table 5: Effect of different drug formulations on kidney functions of EAC bearing mice

Parameters	Creatinine (mg dL <sup>-1</sup> )	U-BUN (mg dL <sup>-1</sup> )	Na <sup>+</sup> (mEq L <sup>-1</sup> )	K <sup>+</sup> (mEq L <sup>-1</sup> )	Cl <sup>-</sup> (mEq L <sup>-1</sup> )	HCO <sub>3</sub> <sup>-</sup> (mEq L <sup>-1</sup> )
Normal	0.40±0.058	26.33±7.12	144.7±4.5	5.80±0.69	102.7±4.73	20.67±2.517
EAC control	0.53±0.08	22.44±4.35	144.0±2.00	5.64±0.25	105.7±2.89	18.00±0.00
Blank-NE	0.51±0.18	22.55±4.14	142.3±4.04	5.27±0.94	102.0±5.29	15.7±2.52 <sup>b</sup>
Doxo-NE	0.65±0.05 <sup>b</sup>	23.66±2.66	146.0±3.00	5.96±0.09	106.5±2.50	18.0±3.00
ATR-NE	0.43±0.067	38.11±4.67 <sup>r</sup>	146.0±1.00	4.49±0.45 <sup>cr</sup>	109.3±1.53 <sup>a</sup>	20.3±1.53
ATR/Doxo-NE	0.54±0.10 <sup>a</sup>	20.78±3.02	148.0±1.00	5.13±0.07	110.3±0.58 <sup>b</sup>	14.0±2.00 <sup>cp</sup>
Doxo-Sol	0.48±0.02	23.55±3.24	150.0±1.00 <sup>ap</sup>	5.30±0.28	112.0±1.00 <sup>cp</sup>	13.8±0.29 <sup>cp</sup>
ATR-Sol	0.46±0.07	19.33±3.52	146.0±2.65	6.21±0.04	109.3±1.53	18.0±1.00
ATR/Doxo-Sol	0.72±0.01 <sup>cp</sup>	23.00±2.0	155.5±0.50 <sup>cr</sup>	5.50±0.32	115.0±0.00 <sup>cr</sup>	10.5±2.50 <sup>cr</sup>

Data were expressed as  $\bar{X} \pm SD$  (n = 10), <sup>a</sup>p<0.05, <sup>b</sup>p<0.01, <sup>r</sup>p<0.001 represent p-value as compared to Normal and <sup>a</sup>p<0.05 <sup>p</sup>p<0.001 represent p-value as compared to EAC control, EAC: Ehrlich ascites carcinoma, NE: Nanoemulsions, Doxo: Doxorubicin hydrochloride, ATR: Atorvastatin, Sol: Solution

ATR-Doxo-Sol group which had significantly lower serum level of cholesterol. On the other hand, administration of Doxo-NE and ATR-Doxo-NE significantly elevated HDL serum level as compared against the normal control group (Fig. 5), whereas, ATR-Sol and EAC control groups also had a lower level of HDL. The TAG levels had almost no effect on the treatment given in present study, so no significant change was observed for it.

## DISCUSSION

The two or more chemotherapeutic agents are usually incorporated in a nano emulsion carrier for the enhancement of the drug's efficacy and to reduce their side effects. The atorvastatin and doxorubicin are the most prominent chemotherapeutic agents commonly employed for treating hyperlipidemia and several forms of cancers, respectively<sup>25</sup>. In the present study, these two drugs were incorporated in a lipid-based nanoemulsion carrier to enhance their bioavailability, efficacy with possible synergistic antitumor activity and to reduce their side-effects.

Nano emulsions (NE) are dynamic formulations that can deliver high concentrations of chemotherapeutic agents to the site of cancer leaving the other normal cells unaffected<sup>15</sup>. The characterization of the NE formulations was carried out by the determination of the morphological features and droplet size of the prepared formulations. The droplets size directly affects the absorption of the drugs. The increased absorption of the drug molecules is observed if the size of the droplet is smaller<sup>26</sup>. In this study, the morphological characterization of the prepared NE was done by the TEM. It was observed that the droplets of Doxo-NE (Fig. 1a), ATR-NE (Fig. 1b) and ATR-Doxo-NE (Fig. 1c) were semi-spherical, spherical and oval-shaped respectively (Fig. 1) with mean droplet sizes of  $9.48 \pm 1.45$ ,  $67.7 \pm 12.76$  and  $5.50 \pm 0.34$  nm, respectively. These findings were on par with other previous findings<sup>24,27-30</sup>. The smallest droplet sizes of the combination formula (ATR-Doxo-NE) indicate tight packing within the droplet due to the molecular interactions between the drugs and the NEs constituents.

The Mean Survival Time (MST), body weight and life span are considered as important safety markers of the effectiveness of various treatments of animals bearing tumor<sup>31</sup>. In the undertaken study, the Doxo-Sol and ATR-Doxo-Sol groups significantly reduced the body weight within 15 days as compared with the normal group. Several previous studies revealed that Doxo caused a reduction in the body weight of the mice<sup>30-32</sup>. In contrast, no change in the body weight was observed in the mice of Doxo-NE and ATR-Doxo-NE treated groups.

The present study revealed that the Doxo and ATR loaded NE formulations (ATR-Doxo-NE) increased the Life Span (ILS% = 245.662) of the mice with their corresponding solution combination formula (ATR-Doxo-Sol) with ILS% equal to 165.753 which was still greater than the Doxo-Sol group with ILS% equal to 57.99. The Doxo-NE, ATR-Doxo-NE and ATR-Doxo-Sol groups showed excellent antitumor activity with respect to the body weight changes, MST and ILS% which could be attributed to the anticancer effect of ATR and NEs as a better delivery system. Similar observations were reported by Patro *et al.*<sup>30</sup> who found the increased survival time of Doxo-SLN treated mice, which also confirmed enhanced efficacy and safety profile of nanoformulations.

In this study, it has been found that the hematotoxicity of Doxo was eliminated when loaded with ATR in NE implying that the Doxo effect on the bone marrow was diminished<sup>33</sup>. Additionally, the hepatotoxicity of Doxo demonstrated by many previous studies was reduced when combined with ATR in NE as indicated by the liver enzymes (ALT, AST and ALP) and histopathological studies<sup>34-39</sup>. The decreased serum ALB level in different ATR containing treatment groups might be due to the role of ATR in the prevention of Doxo-induced liver toxicity. This could help the liver to synthesize ALP and TP<sup>36</sup>. Further, similar findings were reported by El-Moselhy and El-Sheikh<sup>34</sup>, who suggested that the ATR-Doxo combination reversed the apoptotic and hepatorenal inflammatory marker expression. These findings proved the protective role of ATR against Doxo-induced toxicity by multiple mechanisms

including anti-apoptotic, antioxidant and anti-inflammatory mechanisms. Lastly, the serum bilirubin level was significantly increased in different studied groups as compared to the negative control (Fig. 4). The elevated serum level of bilirubin might be due to bile ducts blockage. This blockage might have occurred because of fibrosis and inflammation in the portal triads. Further, it might be due to the throwing up of bilirubin (conjugated) from the necrotic hepatic cells to the sinusoids<sup>39</sup>.

In the current study, the kidney functions in all studied groups were evaluated by the measurement of the biochemical parameters of creatinine, U-BUN and inorganic electrolytes. The serum creatinine level in present studied groups like Doxo-NE, ATR-Doxo-NE and ATR-Doxo-Sol treated groups were found to be significantly elevated as compared with the normal control group. This increased creatinine level indicated the Doxo toxicity, which might be due to the increased oxidative stress by the generation of reactive oxygen species<sup>32</sup>. The serum U-BUN level in different studied groups was found to be in the normal range. The results of present study were found at par with the observations of many studies who found that serum creatinine and BUN were significantly increased in the Doxo treated groups<sup>30,40</sup>. Furthermore, the Doxo treated groups in the present study had significantly elevated levels of the different electrolytes, which indicated the doxorubicin-induced renal toxicity. The electrolyte imbalance could be an indicator of the kidney's dysfunction. These findings were at par with previous study carried out by Alkreathy *et al.*<sup>15,41</sup>. In terms of the lipid profile for the experimental groups in the current study, there were no significant toxicities contrary to another previous study<sup>42</sup>.

## CONCLUSION

The combination of ATR into the Doxo-loaded-NE formulation exhibited synergistic effects against ehrlich ascites carcinoma in Swiss albino mice. This combination treatment of doxorubicin and atorvastatin loaded nano-emulsion (ATR-Doxo-NE) enhanced the efficacy along with the reduced adverse effects of doxorubicin. More detailed investigations are required to further strengthen these novel findings.

## SIGNIFICANCE STATEMENT

This study discovered the synergistic antitumor effect of novel combination of ATR and Doxorubicin loaded in nano-emulsions (Doxo-ATR-NE) against ehrlich ascites carcinoma in Swiss albino mice. This study will help the researcher to uncover the critical area of combinatorial effects of statins

with other chemotherapeutic drugs that many researchers were not able to explore. Thus, a new theory on these novel therapeutic drugs combination loaded in nano emulsions and/or other drugs delivery systems and possibly other combinations, may be arrived at. Hence, new indication of statins along with other chemotherapeutic drugs may be advised.

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