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To Evaluate the Anti-cancer Effect of Doxorubicin When Given in Combination With Cardioprotective Agents like N-acetlycysteine, Enalapril and Vitamin C in Ehrlich Ascites Tumor Induced Adult Wistar Rats

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ABSTRACT:

Background: The anthracycline antibiotic, Doxorubicin has beenproved very effective in a broad range of haematogenous and solid human malignancies. Its use is limited by its irreversible degenerative cardiomyopathy. This has driven us to find novel treatment modalities to reduce its cardiac side effects without conceding its anti-cancer effect.

Objective: To evaluate the anti cancer effect of Doxorubicin when given in combination with cardioprotective agents like N-acetlycysteine, Enalapril and Vitamin c in Ehrlich ascites tumor inducedwistar rats.

Methodology: The male adult wistar rats selected for investigate, divided into six groups with six rats in each group. Rats bearing Ehrlich ascites tumor were selected for the study. The rats in the control group and toxic control were administered sterile water and Doxorubicin respectively on day 5. N-Acetylcysteine, Enalapril and Vitamin c administered orally for seven days as dual drug and triple drug combinations. On day seven, the rats were sacrificed and blood was collected for estimation of cardiac stress markers and cardiac tissue was sent for histopathological estimation. The Peritoneal fluid was aspirated and the volume and viable tumor cell count assessed, using trypan blue exclusion technique. Statistical Analysis: Student 't' test and one way ANOVA followed by Bonferroni test is applied.

Result and conclusion: The tumor volume and cell count at the end of the experiment was measured and it was found out that the anti-cancer effect of doxorubicin is not compromised on instilling various cardioprotective drugs in various combinations to treat doxorubicin induced cardiotoxicity.

KEYWORDS: Doxorubicin, Ehrlich ascites tumor, Trypan blue, N-Acetylcysteine, Enalapril, Vitamin C.

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INTRODUCTION

Combination therapy along with anti-cancer agents to prevent its toxicity is a usual practice and plenty of novel therapies are tried to overcome the toxicity of anti-cancer agents.

The anthracycline antibiotic, Doxorubicin has been proved very effective in a wide range of haematogenous and solid malignancies in human. Doxorubicin use is limited by its irreversible degenerative cardiomyopathy[1-3]. This has driven us to find treatment modalities to reduce its cardiac side effects without conceding its anti-cancer effect.

Experimental tumors have great significance for the purposes of modelling, and Ehrlichascites carcinoma (EAC) is commonest among them. It appeared as a spontaneous type of breast cancer in female mouse[4].

EAC is a highly undifferentiated type of carcinoma, and is characteristically hyperdiploid. It has high transplant able capacity, non-regressive and rapidly proliferating. EAC has a short lifespan and a classical 100% malignancy rate and it lacks tumor specific transplantation antigen which helps in the rapid proliferation.The effusion which gets collected into the peritoneal cavity after injection of the tumor cells intraperitoneally is referred to as “ascites”. Frequently, tumor virulence surges via repetitious passage of the tumor cell containing ascitic fluid and there is also a gradual increase in the proliferation rate of the tumor cells[4].

However, differentiation of the tumor cells gradually disappears, while the cells get free growth control mechanisms, gain heterotransplantability nature and thereby it is getting converted into the ascites form.EAC cells can be used either as a solid tumor form or as ascites form due to these purposes[4].

Following the inoculation of the EAC cells into the peritoneal cavity of mice or rat, EAC cells continues to grow in two phases. The first one is a proliferating phase, in which the number of tumor cells shows an exponential increase and the second one is a plateau phase in which number of tumor cells will almost stay constant, which is further followed by a resting phase. The EAC tumor cells has a resemblance with the human tumors and also very sensitive to chemotherapy as it is a highly undifferentiated and rapid growing tumor[4].

The basic principle of chemotherapy, which serves as the most widely applied mode of treatment for cancer therapy, is to check the progression and growth of the tumor cells or to destroy them completely without affecting the normal human cell, i.e. without side effect or with a minimal side effect. Hence in this study we have used a Novel combination of N-acetylcysteine, Enalapril and Vitamin c to overcome the cardiotoxicity induced by Doxorubicin[5] and also to evaluate its anti-cancer activity on the EAC tumor cells.

MATERIALS AND METHODS

STUDY DESIGN

Experimental animal study. All the procedures in the study were reviewed and approved by the Institutional animal ethical Committee. The animals were taken care as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

SAMPLE SIZE

36 adult male albino wistar rats of weight (180-250gm) were selected for the experiment. Rats were divided randomly into six groups of six rats in each group.

ANIMALS

Laboratory bred adult male albino Wistar rats (10–12 weeks old) having body weight range of 180–250 gm was used for this study. They were kept in the animal house (SMVMCH, Pondicherry , India) for one week for proper acclimatization before starting the experiment under controlled conditions of illumination (12 h light/12 h darkness) and temperature 20–25°C(air conditioned room). They were maintained on standard pellet diet and water ad libitum throughout the experimental period.

DRUGS AND CHEMICALS

Doxorubicin Hcl was obtained from Samarth Life Sciences Pvt. Ltd. N-Acetylcysteine, Enalapril and Vitamin C were obtained from Cipla Pharmaceutical Ltd. LDH assay kit was procured from Clinqa Corporation, USA and CK MB assay kit was procured from Beacon Diagnostics Pvt. Ltd., INDIA. All other chemicals were of analytical

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grade and chemicals required for sensitive biochemical assay were purchased from sigma and Hi Media Chemicals Ltd. EAC tumor cell lines were procured from National centre for cell science (NCCS), Pune, India.

EXPERIMENTAL PROTOCOL

In this experiment, 36 adult male albino wistar rats of weight (180-250gm) were selected. Rats were divided randomly into six groups of six rats in each group.

To closely model the clinical scenario, only rats bearing Ehrlich Ascites Carcinoma (EAC)[4] were used for the study. The tumor cells acquired from NCCS, were allowed to grow in peritoneum of rats and then inoculated from one animal to the other by means of ascitic fluid aspirate, which contains the active EAC tumor cells (collected on 5th day of growth).The EAC cells (1*107) were inoculated intraperitoneally (I.P.)in the rats of all the groups by means of continuous i.p. innoculation of ascitic fluid from one rat to the other and 5 days after inoculation the following procedures were done;

In Group A, the control group, rats were administered sterile Normal Saline 0.5 ml per orally for 7 days and as intra peritoneal injection on day 5. On the 7th day 5ml of rat blood was collected from retro orbital sinus and sent for laboratory estimation of cardiac markers and oxidative stress markers and then sacrificed by pentobarbitone overdose and the cardiac tissue was sent for histopathological examination.

Similarly the ascitic fluid was removed from the peritoneal cavity with a heparinized syringe and needle and then it was carefully drained into a heparinized flask. The use of heparin was necessary to inhibit the clotting which often occurs at the death of the animal. After removal of fluid, the peritoneal cavity was washed twice, with2 ml. of isotonic saline. For counting, the cells were diluted in a blood pipette first by about a two-thirds volume of saline, then the cells were stained with Trypan blue.[6] The viability of the tumor cells was calculated based on Trypan blue exclusion technique. The dead tumor cells took up the stain while the live ones didn't. Photographs were taken using a camera attached to the microscope. A Neubauer ruled hemacytometer was used to obtain cell counts for each pipette using the formula[7].

$$\text{Cell count} = \frac{\text{Number of cells} \times \text{dilution factor}}{\text{Area} \times \text{thickness of liquid film}}$$

The results obtained were kept as the baseline standard for the study.

In Group B ,the toxic control group rats were administered DOX(15mg/kg stat dose)[8] intra peritoneal(i.p.)on the 5th day , calculated 5 days after inoculation of EAC cells and normal saline 0.5 ml was given per orally from day 1-7.

Then NAC (200mg/kg/day)[9] was administered per orally(p.o.)for 7 days and Enalapril (2mg/kg/day p.o.)[10] was administered for 7 days and Vitamin C (250mg/kg/day p.o)[11] was administered for 7 days respectively in different combinations in the following groups, as follows;

Group C - DOX+ENAL+NAC,
Group D - DOX+ ENAL +VITC,
Group E- DOX+NAC+VITC,
Group F - DOX+ ENAL +NAC+VITC respectively and DOX was given on the 5th day as i.p. injection in groups C, D, E & F.

After 48 hrs of DOX treatment on day 7, the same procedure was repeated as mentioned in group A.

The tumor cell concentration per ml was calculated using the below formula:

Average number of cells in one large square*dilution factor*10^4 (Dilution factor is usually 2)

The percentage of viable tumor cells = No. of viable cells counted/Total cells counted (viable + dead)*100.

PARAMETERS MEASURED:

1. Lactate dehydrogenase (LDH)

LDH activity was measured by reagent kits supplied by CLINQA corporation, USA adapted to accentuated clinical chemistry analyser[12].

2. Creatine Kinase MB (CK MB)

CK MB activity was measured by reagent kits supplied by BEACON DIAGNOSTICS Pvt. Ltd., INDIA, adapted to accentuated clinical chemistry analyser[13].

3. Glutathione (GSH) determination

GSH concentration of blood was determined by a standard enzymatic recycling procedure[14].

4. Thiobarbituric acid reactive substance (TBARS)

The TBARs level was estimated as per the spectrophotometric method described by Ohkawa et al.[15]

5. Histopathological analysis

Tissue samples from both the ventricles of three rats from each group was processed and

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embedded in paraffin and sectioned at 4 µm. The Section was stained with hematoxylin and eosin for routine histological microscopic analysis and myocardial degenerative (necrotic) changes were evaluated using a scale of 0 (no change) to 3 (severe lesion) [5].

6. Viable/Nonviable Tumor Cell Count

The viability and nonviability of the tumor cells was evaluated using trypan blue exclusion assay.The cells were stained with trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable, and those that took up the dye were nonviable[6]. These viable and nonviable cells were counted using the formula as mentioned above.

STATISTICAL ANALYSIS

All results are expressed as mean ± standard deviation (SD). Comparison of cardiac enzymes, stress parameters and tumor cell counts between controls and toxic controls was done by unpaired student't' test. Comparison among different treatment groups was done using one-way ANOVA with Post-hoc Bonferroni correction. Correlation analysis was performed with Pearson analysis. All statistical analysis was performed by Epi Info version 3.4.3. A p value <0.05 was considered statistically significant.

RESULTS

On comparing the biochemical parameters of the control and toxic control groups there is a significant increase (*p=0.000*) in the levels of the cardiac markers like LDH and CK MB and also an increase in the cardiac oxidative stress marker like TBARS and a decrease in the GSH levels which explains the cardiotoxicity and oxidative stress damage caused by Doxorubicin (table 1)[5]. This was overturned on giving treatment with NAC, ENAL & VIT C in different combinations. This reversal was appreciated well in the triple drug combination group (table 2)[5] (*p=0.000*).

There is a significant increase in the levels of TBARS in the toxic control group which was reversed on giving the above mentioned drugs (table 2.). And the decrease in the levels of GSH in the toxic control group was also reversed in the treatment groups significantly (table 2). This explains the increased oxidative stress caused by Doxorubicin and the protective effect of anti-oxidants used as treatment. There is also a significant correlation between the cardiac markers and the stress markers, which explain the oxidative stress, induced cardiotoxicity by Doxorubicin[5].

Table 1. Shows the comparison of cardiac marker enzymes and oxidative stress markers between control and toxic control group.

Parameters±SEM	Control±SEM	Toxic Control±SEM	*P value*
CK-MB (IU/L	20.65 ± 2.13	45.12 ± 6.50	*0.001*
LDH (IU/L)	135.48 ± 7.49	278.56 ± 19.42	*0.001*
TBARS (nmol/ml plasma)	1.82 ± 0.07	1.01± 0.06	*0.001*
GSH (mg/gm of H)	1.82 ± 0.07	1.01± 0.06	*0.001*

- All values are expressed as mean± SEM, n=6 in each group.
- Student't' test applied to calculate p value and p value of less than 0.05 is considered statistically significant.
- There is a significant cardio toxicity which is shown by the cardiac markers and stress markers in the above table.
- CTR-Control, CK-MB–Creatine kinase, LDH-Lactate dehydrogenase, GSH-glutathione, TBARS-Thiobarbituric acid reactive substance.

Table 2. Shows the comparison of cardiac marker enzymes and oxidative stress markers between toxic control and different treatment groups.

Parameters	TOXIC CTR ± SEM	*DOX+ENAL+NAC*		*DOX+ENA+VITC*		*DOX+NAC+VITC*		*DOX+ ENAL+ NAC+VITC*	
		Value± SEM	*P*	Value± SEM	P	Value± SEM	*P*	Value± SEM	*P*
CK-MB (IU/L)	45.12±6.50	27.08±3.38	*0.001*	30.22±2.46	0.004	29.86 ±4.74	*0.001*	23.14±2.20	*0.001*

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LDH (IU/L)	278.56±19.42	236.13±19.42	*0.005*	238.65±32.71	0.03	220.83±18.37	*0.001*	207.33±17.46	*0.001*
TBARS (nmol/ml plasma)	1.34±0.03	1.06 ±0.05	*0.001*	1.15 ±0.12	0.004	0.94±0.04	*0.001*	0.90 ±0.03	*0.001*
GSH (mg/gm of Hb)	1.01±0.06	1.39 ±0.14	*0.001*	1.10 ±0.06	0.03	1.22±0.05	*0.001*	1.50 ±0.06	*0.001*

All values are expressed as mean± SEM, n=6 in each group.
P<0.05 is considered statistically significant. ANOVA followed by Bonferroni test was applied.
This table shows the significant cardio protective effect of different combinations of drugs used against Doxorubicin induced cardio toxicity.
CTR-Control, CK-MB–Creatine kinase, LDH-Lactate dehydrogenase, GSH-glutathione, TBARS-Thiobarbituric acid reactive substance, DOX-Doxorubicin, VIT C-Vitamin C, ENAL-Enalapril, NAC-N-Acetylcysteine.

The histopathological examination of the rat heart tissue between the different groups showed a significant difference in the cardiac damage and the triple drug combination group showed the least cardiac damage when compared with the other treatment groups[5]. The EAC tumor cell count (viable cells) at the end of the experiment was measured (figure 1 & 2)and it was found out that there was no significant difference (chart 1 & 2) in the tumor cell count and tumor volume, when compared with the toxic control group and all the other treatment groups (table 3). This gives us a clear evidence that the anti-cancer effect of doxorubicin is not compromised on instilling theabove mentioned cardioprotective drugs to treat doxorubicin induced cardiotoxicity.

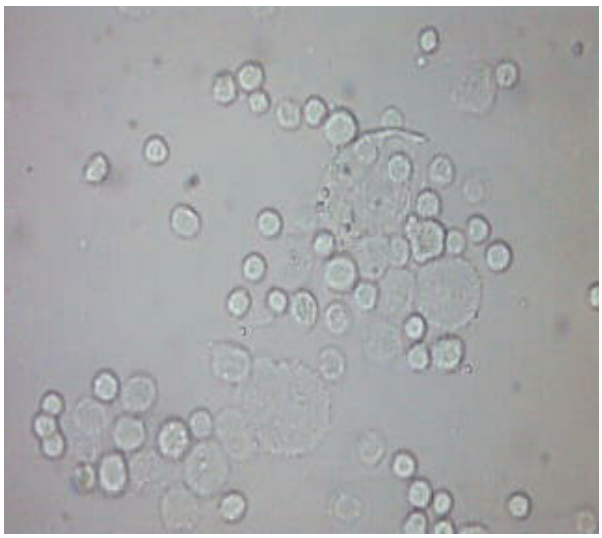

Figure 1. Trypan blue Staining – EAC tumor cells.
CONTROL GROUP: This group consists of many live tumor cell which has not taken up the Trypan blue stain.

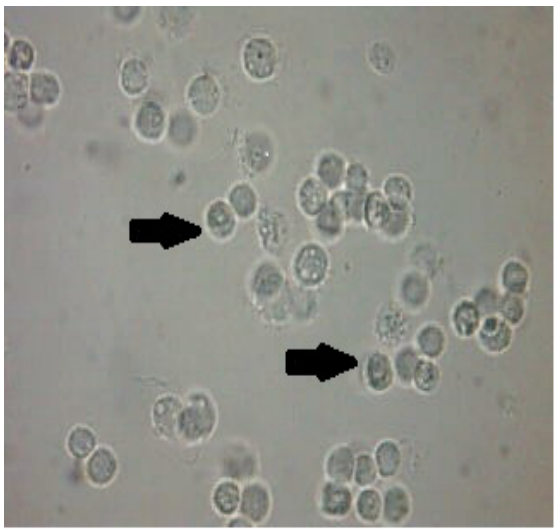

Figure 2. TOXIC CONTROL GROUP (DOX): The arrow denotes the dead cells, which has taken up the stain of trypan blue. The viability of the tumor cells after treatmentis measured by trypan blue exclusion technique. The viable cells will not pick up the stain, but the non-viable cells will pick up the stain

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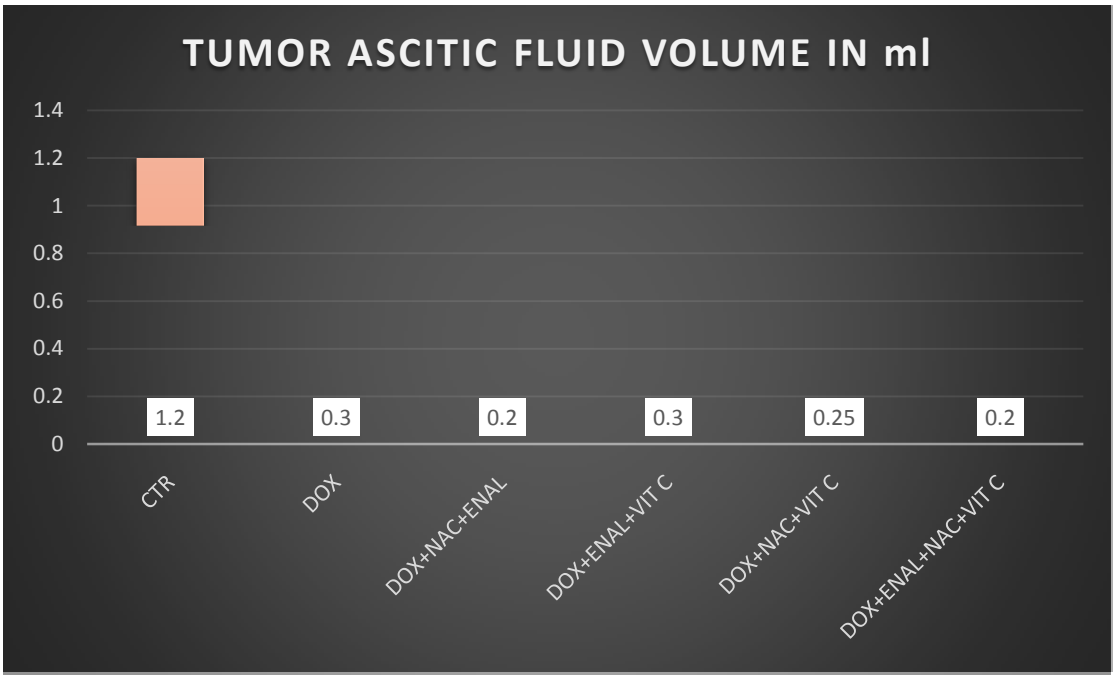

Chart 1.Tumor ascetic fluid (ml).

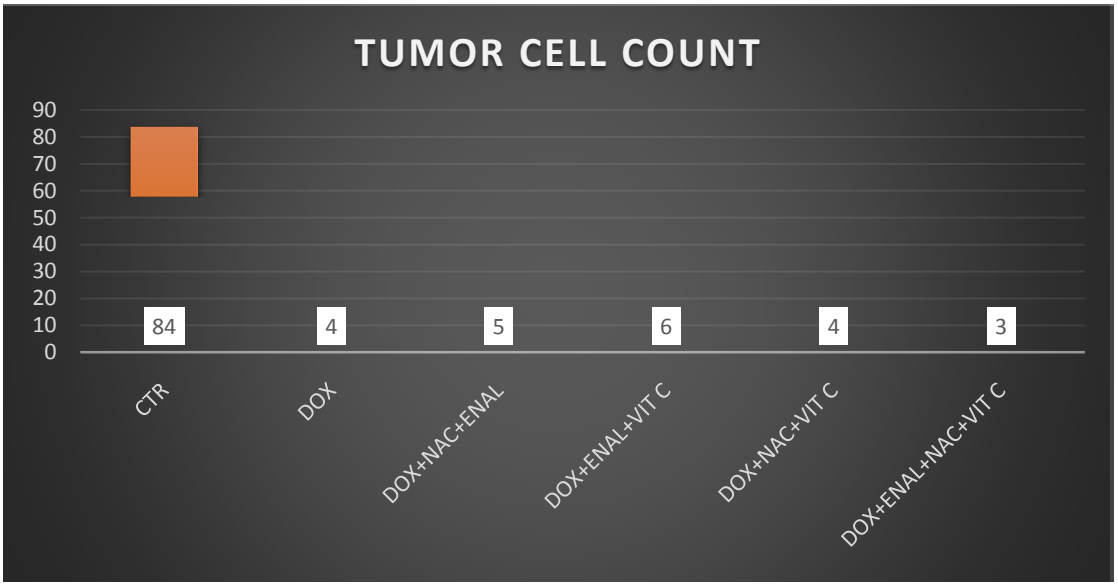

Chart 2.Tumor cell count.

Table 3. Shows the percentage of Ehrlich ascites tumor cell inhibition at the end of the study.

Groups	Ascitic fluid volume (ml)	Avg. number of tumor cells	% of tumor cell growth	% of tumor cell growth inhibition
CTR	1.2 ml	84	100.00	00.00
DOX	0.3 ml	4	4.76	95.24
DOX+NAC+ENAL	0.2 ml	5	5.95	94.05

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DOX+ENAL+VIT C	0.3 ml	6	7.14	92.86
DOX+NAC+VIT C	0.25ml	4	4.76	95.24
DOX+ENAL+NAC+VITC	0.2 ml	3	3.57	96.43

Tumor cell conc. /ml = Avg. no. of cell in 1 large square×Dilution factor ×10 4 (Using 0.4 % trypan blue stain). (Figure: 1& 2)
There is no significant difference in the % of tumor cell inhibition between the different treatment groups.

DISCUSSION

The results suggest that the proposed combination of drugs vetoed DOX induced cardiotoxicity. As suggested by a number of studies it states that DOX administration is associated with a decrease in endogenous antioxidants and increase in oxygen free radicals resulting in increased oxidative stress, which is followed by cardio toxicity.[16]Our results are evident that the different combinations of treatment drugs reverse the increase in TBARS, decrease in GSH, the elevated cardiac enzyme markers and the histopathological changes in the heart tissue. Out of which the triple drug combination seems to be more effective[5].

The Histopathological report suggests that pre-treatment with NAC+VIT C + ENAL effectively inhibited DOX induced cardiac damage by reversal of infiltration of inflammatory cells and fragmentation of myofibrils. Out of the different combinations, the triple drug combination is showing the least cardiac damage both biochemically and histopathologically[5].

The EAC tumor cell volume and the tumor cell count at the end of the experiment was measured and it was found out that there was no significant difference in the tumor cell count when compared with the toxic control group and all the other treatment groups (table 3). Also there was no significant difference in the volume of the ascitic fluid when compared with the toxic control group and all other treatment groups. This gives us evidence that the anti-cancer effect of doxorubicin is not compromised (Chart 1 & 2) on instilling various cardioprotective drugs to treat doxorubicin induced cardiotoxicity.

Thus, the different combinations of drugs used in this study as a cardioprotective agent against Doxorubicin induced cardiotoxicity can be used without depriving the anti-cancer effect of Doxorubicin.

CONCLUSION

Based on the results we conclude that DOX induce cardiotoxicity is in correlation with oxidative stress and the different drug combinations used in the experiment were able to reverse the cardio toxicity[5], out of which the triple drug combination proved to be more fruitful.

The tumor volume and the tumor cell count at the end of the experiment was measured and it was found out that there was no significant difference between the different treatment groups.

Thus, it is conclusive that the anti-cancer effect of Doxorubicin is not compromised on giving different combination of drugs to protect the heart from Doxorubicin induced cardiotoxicity.

Hence the triple combination of N-Acetylcysteine, Enalapril and Vitamin C can be used as an active treatment along with Doxorubicin to reverse its free radical mediated cardiotoxicity without compromising the anti-cancer effect of Doxorubicin. Thus, the above drug combinations can be used in further clinical trials in patients of Doxorubicin induced cardiotoxicity.

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