THERAPEUTIC POTENTIAL OF DOXORUBICIN LOADED POLY (D, L-LACTIC-CO-GLYCOLIC ACID) AGAINST p-DIMETHYLAMINOAZOBENZENE INDUCED LIVER CANCER

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ABSTRACT

The anticancer effect of doxorubicin loaded PLGA nanoparticles on p-Dimethylaminoazobenzene (p-DAB) induced liver cancer in male albino rats has been studied. Nanoparticles of poly (D, L-Lactic-co-glycolic acid) (PLGA) were developed by nanoprecipitation method as a delivery system for doxorubicin. Doxorubicin was chemically conjugated to a terminal end group of PLGA. Group I animals were fed with feed and water *ad libitum* and served as control. Group II-IV animals were received p-DAB intraperitoneal injection at 20mg/kg body weight once in a week for two months. Then group III animals received an intravenous injection of doxorubicin (240µg/kg body weight) in a tail vein daily. Group IV animals were received intravenous injection of PLGA nanoencapsulated doxorubicin (420µg of equivalent doxorubicin/kg body weight) in tail vein daily and stopped to the conclusion of the experiment. Doxorubicin loaded PLGA nanoparticles were characterized by X-ray diffractometer (XRD), Transmission Electron Microscopy (TEM) and Fourier Transmission Infra-Red microscopy (FTIR). The elevated levels of protein, SGOT, SGPT in the p-DAB administered group were significantly reduced by doxorubicin loaded PLGA nanoparticles than free doxorubicin. In vivo anticancer activity showed that administration of doxorubicin loaded PLGA nanoparticles had superior activity over free doxorubicin.

KEY WORDS: p-Dimethylaminoazobenzene, doxorubicin, nanoparticles, liver cancer, poly (D, L-Lactic-co-glycolic acid)

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the major health problem of the world. Its incidence is increasing in both developing and developed countries. HCC represents the third cause of cancer-related deaths[3]. p-Dimethylaminoazobenzene (p-DAB) is reported to induce damage in liver[22] and caused tumors in several species of experimental animals by different routes of administration[10]. Current cancer treatments can only prolong the life of the patients, but do not cure, because of the high toxicity and poor specificity of currently used drugs. Chemotherapeutic drugs were systemically active and cannot target cancer cells. Increasing the chemotherapeutic dosages is not possible. High dosage of chemotherapeutic drugs causes severe side effects like destruction of bone marrow cells, which impairs the erythrocyte production, cardiotoxicity, nephrotoxicity, hepatotoxicity and hematotoxicity. To overcome these problems nanporticles are used in the cancer treatment. A nanoparticle-mediated drug delivery system can eliminate drug or drug carrier side effects significantly[11]. The major advantage of nanotechnology targets drug delivery to the site of the disease. This can be achieved either by passive targeting of drugs to the site of action or by active targeting of the drug[19]. Passive targeting exploits the anatomical differences between normal and diseased tissues to deliver the drugs to the required site, because the physiology of diseased tissues may be altered in a variety of physiological conditions through the enhanced permeability and retention (EPR) effect. The thermoplastic aliphatic polyesters like poly (lactide-co-glycolide) (PLGA), the copolymer of lactide and glycolide have generated tremendous interest due to their favorable properties such as good biocompatibility, biodegradability and mechanical strength[12]. They are easy to formulate into different devices for carrying a variety drug classes such as vaccines, peptides, proteins and micromolecules. Also, they have been approved by the Food and Drug Administration (FDA) for drug delivery[15].

Doxorubicin (Adriamycin) is a potent anti-neoplastic agent isolated from *Streptomyces peuceticus.* Doxorubicin was chemically conjugated to a terminal end group of PLGA by an ester linkage and the doxorubicin–PLGA conjugate was formulated into nanoparticles. A carboxylic acid end group of PLGA was conjugated to a primary hydroxyl group of doxorubicin. The primary amine group of doxorubicin was protected during the conjugation process and then deprotected. The nanoparticle contains the conjugate exhibited sustained doxorubicin release profiles over a month period[8]. Hence, the present work has been carried out to study the effect of doxorubicin-loaded PLGA on p-DAB induced liver cancer in rats.

MATERIALS AND METHODS

Wister male albino rats weighing 150-200 gm, reared and maintained under the supervision of IAEC in the animal house of the Bharathidasan University (BDU/IAEC/17/2013/09.04.2013), were used for the study. p-Dimethylaminoazobenzene (DAB) was purchased from LOBA Chemie Pvt. Ltd, Mumbai-400 005. PLGA polymer was purchased from Sigma Aldrich Co., 3050 Spruce Street, St.Louis, MO 63103 USA 314-771-5765. Polyvinyl alcohol (PVA) was purchased from Hi Media Laboratories Pvt. Ltd, Mumbai-400 086. Doxorubicin hydrochloride was purchased from Fresenius Kabi Oncology Ltd, HP-173 205, India.

(i) Experimental Design
All animals were acclimatized for 15 days and fed *ad libitum* with rat feed and water. After acclimatization, animals were divided into four groups consisting of 5 rats each. Group I animals were served as normal control and received food and water for 90 days. Group II animals were served as disease control and received DAB (20 mg/Kg body weight) once in a week, intraperitoneally for 2 months[20]. Group III animals were received DAB (20mg/kg body weight) intraperitoneally, once in a week for 2 months and after received intravenous injections of doxorubicin hydrochloride

(240µg/kg body weight) in a tail vein daily and stopped to the conclusion of the experiment[8]. Group IV animals were received DAB (20mg/kg body weight) intraperitoneally, once in a week for 2 months and after received intravenous injection of PLGA nanoencapsulated doxorubicin hydrochloride (420µg of equivalent doxorubicin/kg body weight) in tail vein daily and stopped to the conclusion of the experiment.

(ii)Preparation of nanoparticles and encapsulation

The modified method of Derakhshandeh[5,6] was used for nanoencapsulation. The nanoprecipitation method was used for the formation of drug-encapsulated PLGA nanoparticles. PLGA polymer (40 mg) was dissolved in acetone. Then the polymer solution contains the same amount of drug was added drop-wise into PVA aqueous solution and stirred magnetically at room temperature until complete evaporation of the organic solvent.

Then, the nanoparticle suspension was centrifuged by cooling centrifuge at 12,000 rpm for 1 hour. The separated nanoparticles were redispersed and centrifuged three times in distilled water to remove free drug completely[18]. Finally, nanoparticles were dried via vaccum drier at room temperature for 5 minutes, and then were characterized.

RESULTS AND DISCUSSION

1. Evaluation of Doxorubicin Encapsulation

Nanoparticles were centrifuged at 12,000 g for 30 min in cooling centrifuge, and then supernatants of Doxorubicin solutions were measured by UV-spectrophotometer at 473 nm. Figure 1 showed the OD values of total drug (485.57) and free drug (473.10). Calculations were performed by using the calibration curve, and encapsulation efficiency was calculated as described by Aydın and Mehlika[2].

$$\text{Doxorubicin encapsulation efficiency (\%)} = \frac{\text{Total Doxorubucin} - \text{Free Doxorubicin}}{\text{Total Doxorubucin}} \times 100$$

In the present study, Doxorubicin encapsulation efficiency was as 57.5 for 20 mg of PLGA polymer. However, that the encapsulation efficiency of chitosan nanoparticles (5.0mg/mL 5-FU) was 29.98 ± 0.8.[2]

UV-Visible analysis

Figure 1
UV-Vis analysis of free drug and total drug shows peaks at 485.57 and 473.10 respectively


Instrument Model: Lambda 35
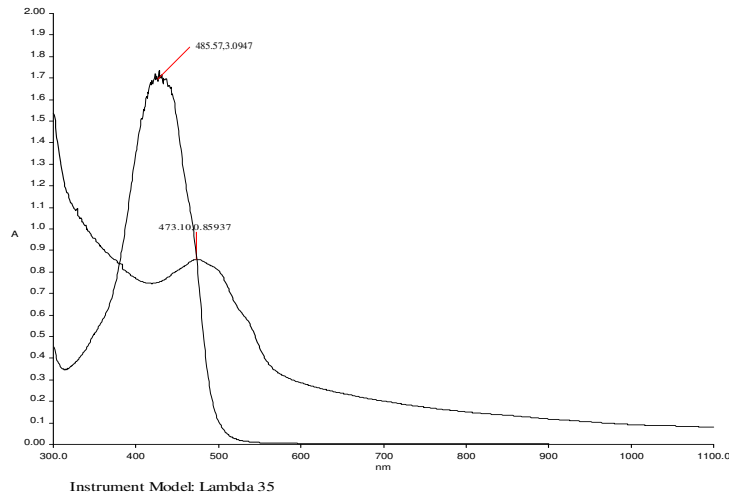
2. FTIR analysis
Infrared spectroscopy was used to study the interactions between the drug and the polymers. In order to investigate possible molecular interaction between drug and the polymer, FTIR spectroscopy (Perkin-Elmer, Spectrum RXI) of the Doxorubicin, PLGA polymer and Doxorubicin-loaded PLGA nanoparticles (NPs) was carried out. The FTIR spectra were collected in the range of 4000–400 cm^{-1} at room temperature[1].

FTIR analysis

Figure 2
FTIR analysis shows the various functional groups of doxorubicin, PLGA and doxorubicin loaded PLGA nanopartices
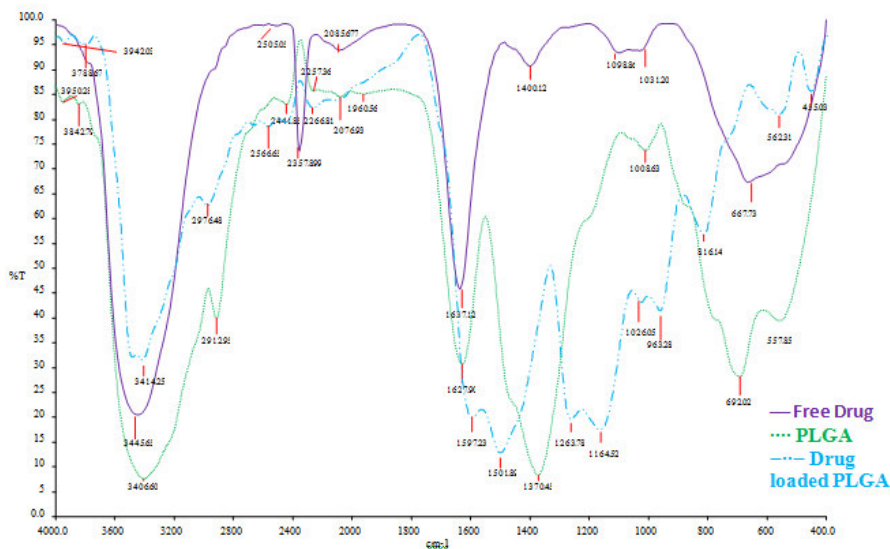
FTIR analysis of free drug (Figure 2) showed distinct peaks at wave numbers 667.73 cm^{-1}, 1031.20 cm^{-1}, 1098.86 cm^{-1}, 1400.12 cm^{-1}, 1637.12 cm^{-1}, 2085.67 cm^{-1}, 2357.89 cm^{-1}, 3445.65 cm^{-1}. The peaks corresponding to Alkyne C-H, Aromatic C-H, Aromatic C-H, Carboxylic acids, Alkenyl C=C stretch, NH$_3$ structure, Charged amines C=NH$^+$, H-bonded OH stretch. FTIR analysis of PLGA polymer showed distinct peaks at wave numbers 692.02 cm^{-1}, 1008.63 cm^{-1}, 1370.45 cm^{-1}, 1627.90 cm^{-1}, 1960.56 cm^{-1}, 2257.36 cm^{-1}, 2441.85 cm^{-1}, 3406.60 cm^{-1}. The peaks corresponding to thioether CH$_2$-S, cyclohexane ring, methyl C-H bend, alkenyl C=C stretch, aromatic combination bands, saturated nitriles C≡N, charged amines C=NH$^+$, OH stretch. FTIR analysis of Doxorubicin loaded PLGA showed distinct peaks 963.28 cm^{-1}, 1026.05 cm^{-1}, 1164.52 cm^{-1}, 1263.78 cm^{-1}, 1501.89 cm^{-1}, 1597.23 cm^{-1}, 2266.65 cm^{-1}, 2451.92 cm^{-1}, 2566.65 cm^{-1}, 2976.48 cm^{-1}, 3414.25 cm^{-1}. The peaks corresponding to trans C-H stretch, cyclohexane ring vibrations, aromatic C-H, esters of aromatic acid, aromatic ring stretch, aromatic ring stretch, diazonium salts (R-C=N=N)$^+$, charged amines C=NH$^+$, thiols S-H stretch, alkanes –CH$_3$, H bonded OH stretch. Doxorubicin was chemically conjugated to a terminal end group of poly (D, L-lactic-co-glycolic acid) [PLGA] by an ester linkage. 1263.78 cm^{-1} showed that ester linkage. Arjmand et al.,[1] reported that the spectra of the PLGA NPs and Alpha 1-antitrypsin-loaded PLGA NPs showed two distinct peaks at wave numbers 1300-1450 cm-1 and 1720-1800 cm-1, which were assigned to δCH and V(C=O).

3. XRD analysis

Figure 3 revealed the diffraction lines, which were observed at 2θ angle 16.780°, 20.440° and 21.100° and 23.060°. By using 2θ angle, the size of the nanoparticle was calculated. Debye-Scherrer's formula was used to calculate the size of the nanoparticle. XRD patterns obtained for drug loaded PLGA nanoparticle in figure 3 shows characteristic peaks (at $2\theta = 44°$), which may be indexed based on the face-centred cubic structure. The XRD pattern thus clearly shows that the drug loaded nanoparticles were crystalline in nature.

XRD analysis

Figure 3
XRD pattern of doxorubicin loaded PLGA nanoparticle display peaks at 16.780°, 20.440° and 21.100° and 23.060°
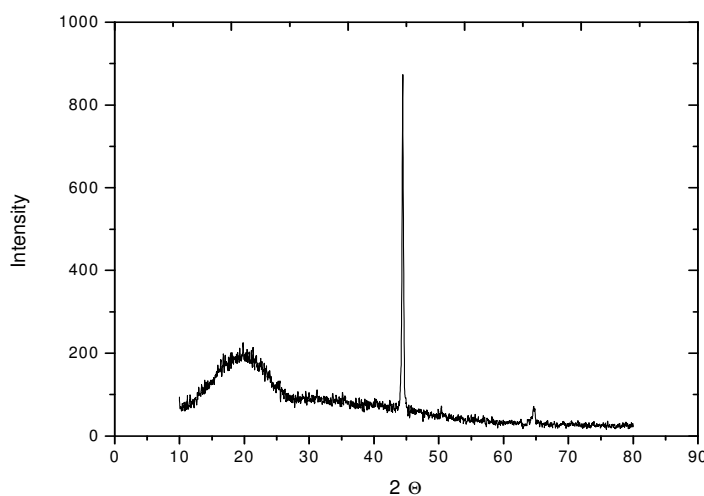
Doxorubicin Loaded PLGA Nanoparticles size were measured by using Debye-Scherrer's equation. d=0.9λ/βcosθ where *d* is the mean diameter of the nanoparticles, λ is wavelength of X-ray radiation source, β is the angular FWHM of the XRD peak at the diffraction angle θ. The size of the drug loaded PLGA nanocrystallites as estimated from the FWHM of the peak using the Scherrer formula is 57.48 - 68.92 nm.

4. TEM analysis

Microphotographs of the Doxorubicin loaded PLGA were obtained by transmission electron microscopy (Zeiss). Figure 4 showed the Transmission Electron Microscope (TEM) image of the Doxorubicin loaded PLGA nanoparticles. It revealed that the NPs have a spherical shape. The resulting NPs were predominantly spherical and of uniform size and shape.

Figure 4
TEM analysis of doxorubicin loaded PLGA nanoparticles revealed the Spherical shape of the nanoparticles
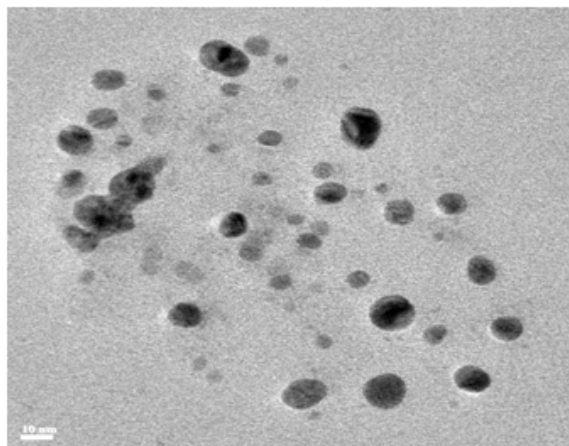
Ciofani *et al.*,[4] reported that the imaging of the GC–BTNPs by TEM revealed well-dispersed structures. Watnasirichaikul *et al.*,[21] reported that the TEM of freeze- fractured samples showed that the particles had a central cavity surrounded by a polymer wall.

5. Histopathological appearance of rat liver

Normal rats show more compact and well-distributed junctional complexes (Figure 5 *a*). In DAB-administered rats, hepatic cells exhibit damaged cell structure (Figure 5 b). However, animals treated with doxorubicin (Figure 5 *c*) show high cell density with compact junctional complexes than DAB-administered animals. The animals treated with doxorubicin loaded PLGA show higher cell density with compact junctional complexes (Figure 5 d) than doxorubicin treated animals. Velanganni and Balasundaram[20] reported that hepatic cells exhibit loss of contact inhibition (polarity) and damaged central vein of liver lobules in DAB-administered rats.

Histopatology Slides

Figure 5
Histopathological appearance of rat liver

a) Normal

b) p-DAB treated

c) p-DAB+Free Doxorubicin

d) p-DAB+ PLGA encapsulated Doxorubicin
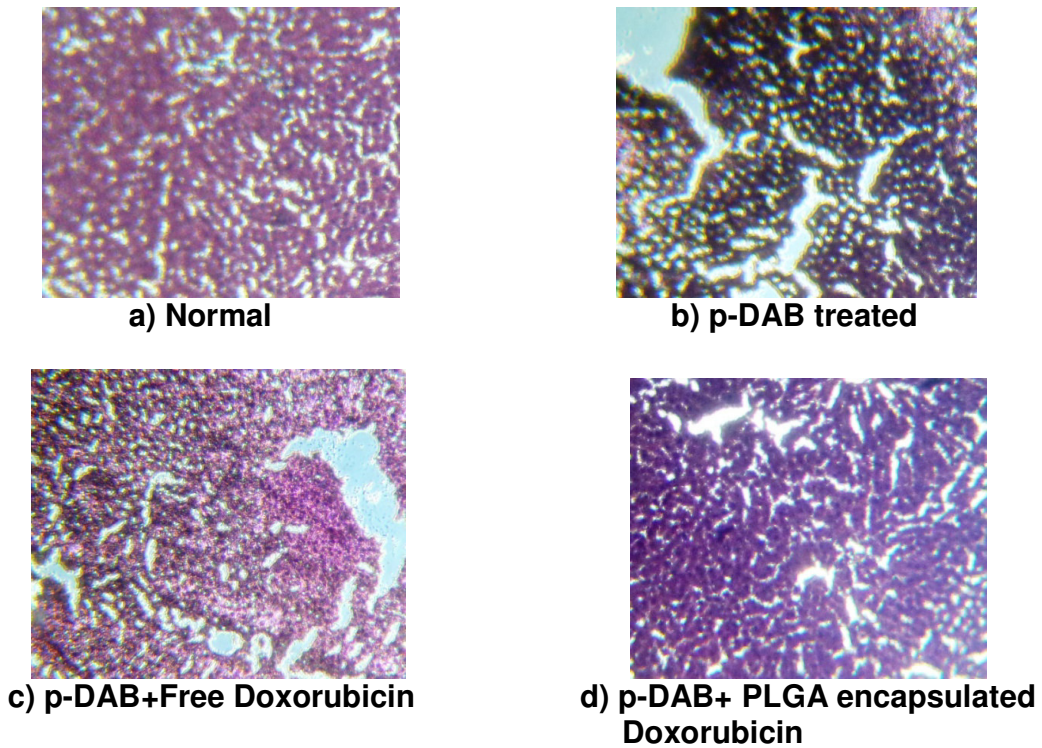
3.6. Biochemical analysis
The graph 1 showed the effect of doxorubicin on protein levels of rats administered with p-DAB. Protein level was estimated by Lowry's method[13]. Protein level, which increased after DAB administration, was found to be lowered significantly ($P < 0.001$) by Doxorubucin loaded PLGA when compared to free Doxorubicin (Table 1).

Graph 1
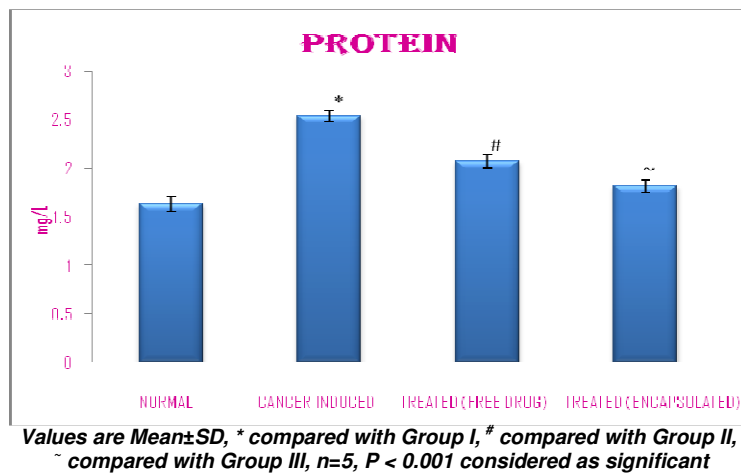*Effect of Doxorubicin on Protein Levels of Rats Administered With p-DAB*

*Values are Mean±SD, * compared with Group I, # compared with Group II,*
~ compared with Group III, n=5, P < 0.001 considered as significant

Table 1
***Effect of Doxorubicin on Protein Levels of Rats
Administered with p-DAB***

Groups	Status	Protein Levels
I	Normal Rats	1.632±0.073
II	DAB alone	2.540±0.060
III	DAB+Doxorubicin (Free Drug)	2.072±0.068*
IV	DAB+ PLGA Encapsulated Doxorubicin	1.816±0.0650*

$P < 0.001$ vs. Group II; Values are mean ± SD; n = 5.

Graph 2 showed the effect of doxorubicin on SOD and catalase levels of rats were administered with p-DAB respectively. SOD was estimated by Markland & Markland's method[14]. Catalase level was estimated by Sinha's method[17]. Antioxidants SOD and catalase, which were decreased after DAB administration and found to be increased after the administration of Doxorubicin and Doxorubicin loaded PLGA. Doxorubicin loaded PLGA increased the antioxidants levels significantly ($P < 0.05$) when compared to free doxorubicin (Table 2).

Graph 2
***Effect of Doxorubicin on Super Oxide Dismutase and Catalase
Levels of Rats Administered with p- DAB***
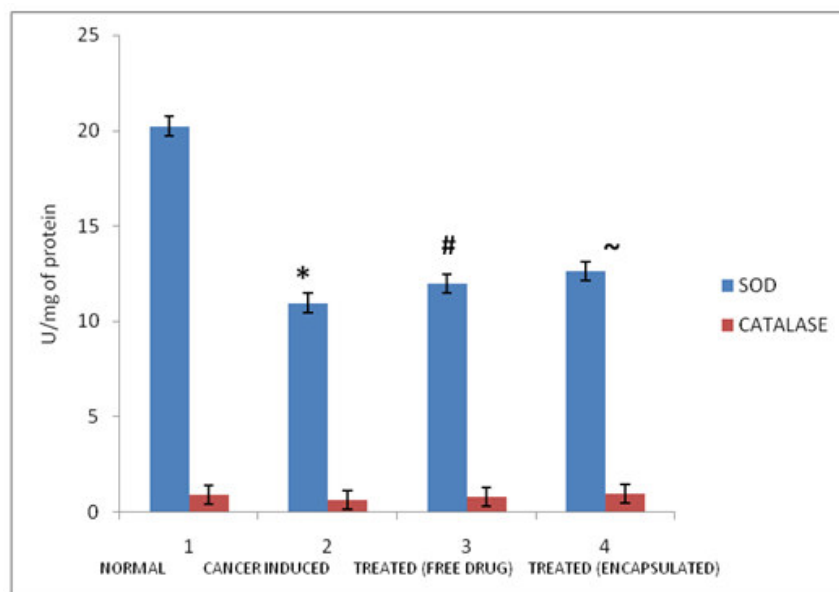
*Values are Mean±SD, * compared with Group I, # compared with Group II,
~ compared with Group III, n=5, P < 0.05 considered as significant.*

Table 2
***Effect of doxorubicin on super oxide dismutase and Catalase
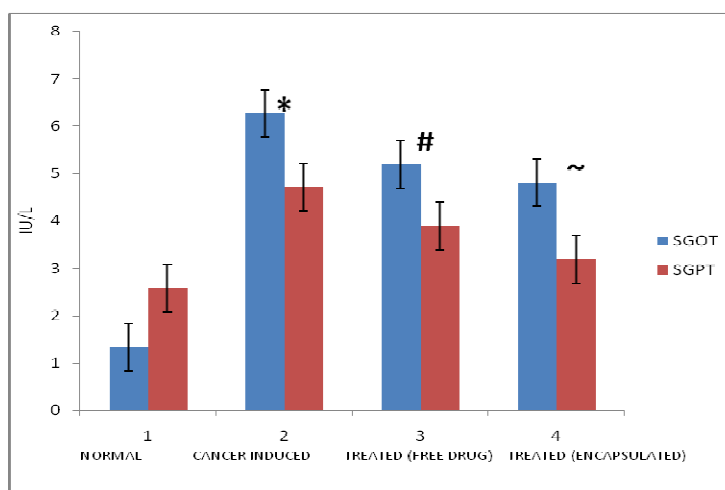levels of rats administered with p-DAB***

Groups	Status	SOD Levels	Catalase Levels
I	Normal Rats	20.230±0.080	0.92±0.076
II	DAB alone	10.966±0.099	0.636±0.070
III	DAB+Doxorubicin (Free Drug)	11.964±0.211*	0.89±0.043*
IV	DAB+PLGA Encapsulated Doxorubicin	18.058±1.636*	0.954±0.030*

$P < 0.05$ vs. Group II; Values are mean ± SD; n = 5.

Graph 3 showed the effect of doxorubicin on SGOT and SGPT levels of rats administered with p-DAB respectively. SGOT and SGPT levels were estimated by Reitman's method[16]. SGOT and SGPT levels, which increased after DAB administration, were found to be lowered significantly ($P < 0.05$) by Doxorubicin loaded PLGA when compared to free Doxorubicin. Velanganni and Balasundaram[20], reported that the levels of GSH, ALP, GST and bilirubin were increased after DAB administration and these were found to be lowered by vitamins A, C and E (Table 3).

Graph 3
Effect of doxorubicin on SGOT and SGPT levels
of rats administered with p-DAB

Values are Mean±SD, * compared with Group I, # compared with Group II, n=5, ~ compared with Group III, P < 0.05 considered as significant.

Table 3
Effect of Doxorubicin on SGOT and SGPT Levels
of Rats Administered with p-DAB

Groups	Status	SGOT Levels	SGPT Levels
I	Normal Rats	1.334±0.096	2.578±0.072
II	DAB alone	6.266±0.110	4.706±0.055
III	DAB+Doxorubicin (Free Drug)	5.144±0.262*	3.888±0.064*
IV	DAB+PLGA Encapsulated Doxorubicin	3.804±0.105*	3.088±0.123*

*P < 0.05 vs. Group II; Values are mean ± SD; n = 5.

CONCLUSION

The *in vivo* anti-tumor activity of the PLGA loaded doxorubicin nanoparticles was higher than the free doxorubicin. Hence, Doxorubicin loaded PLGA nanoparticles can be used to improve the therapeutic efficacy of Doxorubicin in the treatment of cancer.

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