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# INDIRUBIN-LOADED NANOLIPOSOMES MITIGATE DAUNORUBICIN-INDUCED CARDIOTOXICITY IN ALBINO WISTAR RATS: EVIDENCE FROM BIOCHEMICAL AND HISTOPATHOLOGICAL STUDIES

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## ABSTRACT

**Daunorubicin, a widely used chemotherapeutic agent, is known for its potent anticancer effects but is severely limited by its dose-dependent cardiotoxicity. This study investigated the potential of indirubin-loaded nanoliposomes (INB-NLP) as a protective strategy to mitigate this cardiotoxicity in albino Wistar rats, supported by biochemical and histopathological evidence. The effects of INB-NLP on cardiac markers, lipid profiles, inflammatory markers and enzymatic antioxidants were assessed by biochemical assays, ELISA, qRT-PCR and histo-pathology by H&E staining. The study revealed a significant rise in cardiac troponin (cTnI), total cholesterol, and triglyceride levels on administering the DNB. Post-treatment with INB-NLP significantly restored cTnI and lipid profile to normal levels. DNB caused a marked reduction in SOD, CAT, and GST levels, INB-NLP treatment significantly prevented these changes. Diminished sirt3 expression in DNB administered rats was restored by INB-NLP, while caspase-8 (casp8) expression elevated in the DNB group was significantly downregulated by INB-NLP treatment. Histopathology of aorta revealed severe damage in DNB rats, with plaque formation, foam cell infiltration, and calcium deposition. INB-NLP treatment in DNB group rats restored the aortic architecture. INB-NLP treatment significantly prevented the oxidative damage caused by DNB, indicating its antioxidative and anti-inflammatory properties. INB-NLP also efficiently ameliorated the abnormalities induced by DNB in aorta. These findings reveal the potential of INB-NLP as cardioprotective by mitigating the oxidative stress, inflammation, and tissue damage by DNB in rat myocardium.**

**Keywords:** Daunorubicin, histopathology, indirubin, *in vivo*, nanoliposomes

## INTRODUCTION

DNB is an anthracycline (ANT) aminoglycoside used to induce cardiotoxicity in this study. ANTs are a class of chemotherapy drugs and include doxorubicin (DOX), DNB, epirubicin, and idarubicin, which are commonly used to treat breast cancer, leukaemia, and many other types of malignancies. However, the use of ANT is restricted due to their cardiotoxic effects. Therefore, it is necessary to explore various damages caused by using cancer therapy and drugs to prevent this effect (Fang *et al.*, 2019; Zhang *et al.*, 2022; Tranchita *et al.*, 2022). The mechanisms of ANT induced cardiotoxicity (AIC) involve oxidative stress, inflammation, topoisomerase 2β (Top 2β) inhibition, pyroptosis, immunometabolism, autophagy, and ferroptosis. Prevention of toxicity induced by ANT in cardiomyocytes reduces AIC, which might be a novel prevention and treatment strategy for AIC (Rocca *et al.,* 2020; Zhai *et al.*, 2021).

The expanding applications of traditional Chinese medicine in disease treatment have highlighted the significant therapeutic potential of its bioactive compounds. Evidence from previous studies suggests that natural products derived from traditional Chinese medicine may exert cardioprotective effects against DOX mediated cardiotoxicity (Szponar *et al.*, 2024). Indirubin (INB) is the active ingredient of Danggui Longhui Wan, a traditional Chinese medicine, and it is present in plants such as *Indigofera tinctoria* and *Isatis tinctoria*. INB, a stable red isomer of the blue indigo, and is chemically known as 3,2′-bisindole. The planarity structure, developed hydrogen bonds, hydrophobic *π*-interactions, and rigid crystal structure make INB a poorly water-soluble compound. In efforts to improve the solubility of INB, studies have also prioritized enhancing its bioavailability (Chen *et al.,* 2012; Gaboriaud *et al.,* 2015). Nanocarrier based local drug delivery systems, such as increasing the solubility of hydrophobic drugs and improving drug permeability, were established to overcome these shortcomings of INB (He *et al.*, 2022).

Nanoliposomes (NLP) are powerful drug delivery systems consisting of phospholipids which have gained considerable attention over the past decade. They are characterized by their biocompatibility, biodegradability, and their ability to effectively entrap hydrophobic and hydrophilic drugs in addition to nucleic acid based therapeutics and imaging agents. NLP enhances the bioavailability and the biodistribution of the loaded therapeutic agent and its therapeutic performance thus causing minimal side effects to patients (Chavda *et al.,* 2022). The present study was aimed to assess the cardioprotective efficiency of INB-NLP against DNB induced cardiotoxicity in albino Wistar rats.

## MATERIALS AND METHODS

### *Chemicals*

INB (purity ≥ 98%) was purchased from Sigma Aldrich, USA. DNB was obtained from the Regional Cancer Center, Trivandrum (India). All other chemicals/biochemicals used in this study were of analytical grade.

### *Preparation of INB-NLP*

INB-NLP was prepared using L-α-lecithin (L-α-phosphatidylcholine) from soybean, cholesterol, and INB by the thin film hydration method (Torres-Flores *et al.,* 2020). The prepared INB-NLP were spherical with an average size of 82.5 ± 9 nm.

### *Animal model*

Albino Wistar rats (48 males) were purchased from Mass Biotech, Chengalpattu (India). The rats were of 6-8 weeks age and weighed between 100-120 g. The animals were housed in the Central Animal House, RMMC (Rajah Muthiah Medical College), Annamalai University. All the experiments were performed after prior approval from the Institutional Animal Ethics Committee for the Control and Supervision of Experimental Animals (vide No. AU-IAEC/1322/6/22) and the compliance with the guidelines were ensured. The rats were acclimated to the laboratory conditions, which included controlled humidity (50±10%), a temperature of 24±2˚C and a 12 h light/dark cycle. They were provided with standard feed (pellet diet- cereals, animal and vegetal proteins vitamins and minerals) and watered throughout the study as and when required.

### *Experimental design*

The rats were randomly divided into 6 equal groups, with eight rats in each group. Group 1 was completely fed with pellet diet without DNB injection, while group 2 was fed with pellet diet and intraperitoneally injected with DNB @ 3 mg $kg^{-1}$ bw in first 3 alternative days (day 1, 3 and 5) to induce cardiomyopathy. The group 3 rats were fed with pellet diet administered intraperitonially with INB-NPL from day 7 on words for 17 days. The group 4, 5 and 6 were initially fed with pellet diet, intraperitonially injected with DNB @ 3 mg kg$^{-1}$ bw on day 1, 3 and 5, and then intraperitoneally injected with INB-NPL @ 2, 4 and 8 mg kg$^{-1}$ bw, respectively. After 23 days, the rats were sacrificed by an overdose of ketamine hydrochloride (intramuscular injection) and the blood was collected, and kept on standby to separate the serum. After blood sampling, the aorta was preserved in 10% formalin for histological studies.

### *Biochemical analysis*

The biochemical analysis of cTnI, total cholesterol (TC) and triglycerides (TG), superoxide dismutase (SOD), catalase (CAT), and glutathione-S-transferase (GST) was performed in the serum and the tissue samples of the control and treated rats. The biochemical parameters (SOD, CAT, and GST) were assessed respectively by the methods of Kakkar *et al.* (1984), Sinha (1972), and Habig *et al.* (1974) and also using the ELISA kits (cTnI, TC and TG) following the manufacturer's instructions.

### *Histopathological studies*

The aorta was removed and fixed in 10% buffered formalin for 48 h. Then, it was dehydrated with a series of different ethanol concentrations, cleaned in xylene and embedded in paraffin wax. The embedded tissues were sectioned to 3-5 μM thickness using microtome and stained with hematoxylin and eosin (H&E). The specimens were evaluated with a light microscope (Zeiss PrimoStar, Germany) and photographed at 200x magnification.

### *Gene expression analysis*

The RNA was isolated from heart tissue with RNA extraction kit as per the manufacturer's instructions. The extracted RNA was preserved at -80°C. The RNA was subjected to reverse transcription using iScript™ cDNA Synthesis Kit. Gene expression analysis was then performed via real-time PCR (CFX-96 Bio-Rad PCR System) with Eagle Taq Universal Master Mix under optimal conditions for the selected primer. The obtained values for target gene expression were normalized to those of TBP (TATA-box binding protein), and the relative gene expression. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used for analysis.

### *Statistical analysis*

All the quantitative measurements were expressed as mean ± standard deviation (SD) for control and experimental groups, with three biological replications per treatment group. The data were analysed using one-way analysis of variance (ANOVA) on GraphPad Prism software. The results were considered statistically significant if the p-value was $\leq 0.05$.

## RESULTS AND DISCUSSION

The present study assessed the cardioprotective effects of INB-NLP in DNB induced cardiotoxicity in experimental rats. The results revealed significant improvement across multiple biochemical, molecular, and histopathological parameters, suggesting that INB-NLP exerts protective effects by targeting oxidative stress, dyslipidemia, inflammation, and apoptosis.

### *Effect of INB-NLP on cardiac marker*

The effect of INB-NLP (@ 2, 4, and 8 mg kg$^{-1}$ bw) in experimental rats was assessed by evaluating the cardiac marker cTnI in their serum samples (Fig. 1). Significant increases in CTnI level were noticed in DNB administered rats as compared to the control group. However, after treatment with INB-NLP in DNB induced groups, the level of cTnI was significantly reversed. The rats treated with INB-NLP alone did not show any abnormalities as compared to the control group. The elevated levels of CTnI in DNB induced rats signify extensive myocardial injury. Troponin I, a specific biomarker of cardiac damage, is released into the circulation following the degradation of sarcomeric structures during cardiomyocyte death. DNB, through its quinone group, generates ROS in cardiomyocytes, leading to the lipid peroxidation and calcium dysregulation. Excess ROS triggers mitochondrial damage, causing the release of apoptotic factors and structural protein degradation (Park *et al.*, 2017; Endale *et al.*, 2023). The reduction in CTnI level following INB-NLP treatment underscores the cardioprotective ability of INB-NLP, likely mediated by its ability to attenuate oxidative stress.

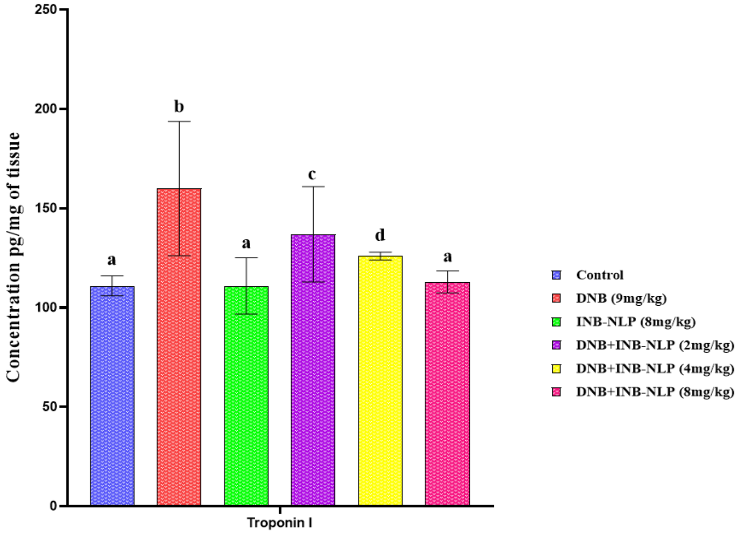

**Fig. 1: Troponin I levels in the serum of control and treated rats. The values are statistically significant at $p \leq 0.05$**

### *Effect of INB-NLP on lipid profile*

The levels of TC and TG in the serum of normal and DNB treated rats are shown in Fig. 2. Significant increase in TC and TG levels were seen in DNB administered rats as compared to the control rats. Treatment with INB-NLP brought the TC and TG levels to near normal as compared to the untreated DNB group. INB-NLP alone treated group showed no significant change as to the control group. The dyslipidemia observed in DNB induced rats, might be as a result of oxidative stress-mediated lipid peroxidation and impaired lipid metabolism. DNB may inhibit lipo-protein lipase activity and disrupt the expression of PPAR-α, a key regulator of lipid metabolism (Moon *et al.*, 2022). Treatment with INB-NLP significantly normalized TC and TG levels, probably through the activation of PPAR-α (peroxisome proliferator-activated receptor-α), which promotes fatty acid oxidation and reduces triglyceride synthesis. INB reportedly enhances PPAR-α activity, thereby improves lipid profiles and mitigating the progression of atherosclerosis (Zhang *et al.*, 2024). The restoration of lipid balance underscores the role of INB-NLP in counteracting DNB mediated metabolic disturbances.

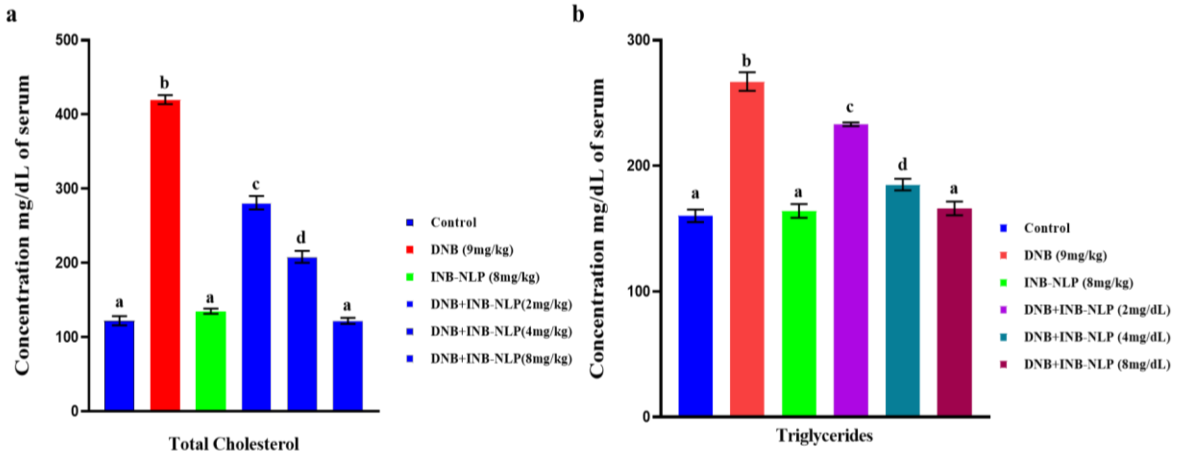

**Fig. 2: Total cholesterol (a) and triglycerides (b) in the serum of treated and control rats. The values are statistically significant at $p \leq 0.05$.**

### *Effect of INB-NLP on enzymatic antioxidants*

The activities of enzymatic antioxidants SOD, CAT, and GST in serum samples are shownt in Fig. 3. The activities of enzymatic antioxidants were significantly reduced in DNB administered rat serum as compared to the control group. Treatment with INB-NLP significantly restored their activities as compared to the untreated DNB group. INB-NLP alone treated group showed no significant changes in the antioxidant activity.

SOD is a crucial antioxidant enzyme that converts superoxide radicals into $H_2O_2$, thereby prevents the accumulation of ROS. DNB significantly reduced SOD activity in rats, consistent with previous studies indicating oxidative damage induced by anthracyclines (Wu *et al.*, 2014). ROS generated by DNB overwhelms the mitochondrial electron transport chain, leading to mitochondrial dysfunction and SOD inactivation. Treatment with INB-NLP restored SOD activity, possibly through the activation of sirt3. Sirt3 deacetylates and activates SOD2 (mitochondrial SOD), thus enhances its efficiency in neutralizing ROS and maintaining mitochondrial redox balance (Lambona *et al.*, 2024). This restoration suggests that INB-NLP prevents oxidative damage caused by DNB on mitochondria and preserves the antioxidant defence system.

CAT plays a vital role in the detoxification of hydrogen peroxide ($H_2O_2$), and converts it into water and oxygen, thereby protects the cells from oxidative stress. In DNB administered rats, the significantly reduced CAT activity induces the accumulation of $H_2O_2$ and contributes to hydroxyl radical formation, exacerbating oxidative stress (Rasheed *et al.*, 2024). The treatment with INB-NLP may regulate sirt3, which enhances CAT activity via deacetylation, promoting the efficient breakdown of $H_2O_2$. By protecting CAT activity, INB-NLP helps in preventing oxidative damage to lipids, proteins, and DNA.

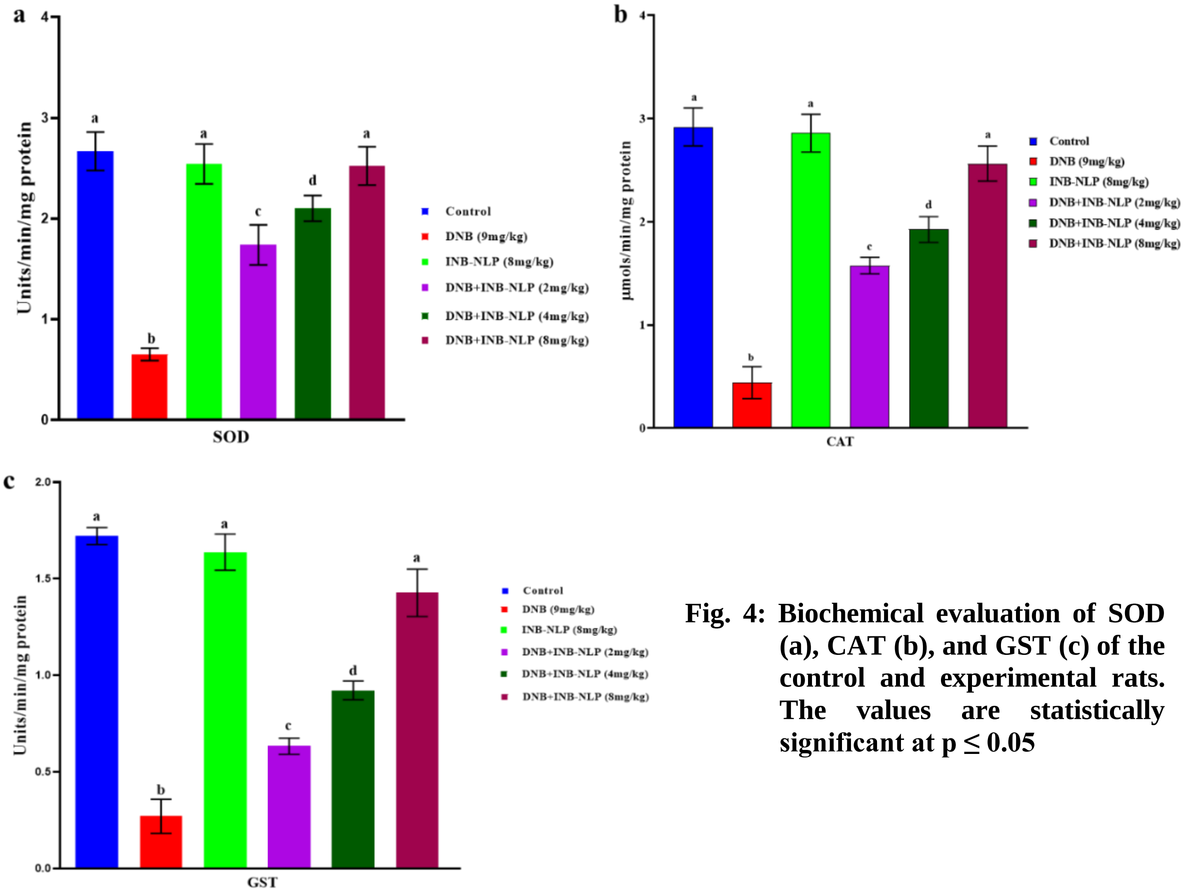

**Fig. 4: Biochemical evaluation of SOD (a), CAT (b), and GST (c) of the control and experimental rats. The values are statistically significant at $p \leq 0.05$**

Glutathione S-transferase (GST) is also an essential enzyme in cellular detoxification, and facilitates the conjugation of glutathione (GSH) to electrophilic compounds, including ROS by-products. A significant reduction in GST activity in DNB administered rats indicated the impaired detoxification capacity and oxidative stress. DNB may deplete GSH reserves, hindering GST activity and promoting the accumulation of toxic metabolites (Bélanger *et al.*, 2021). INB-NLP treatment restored GST activity, likely by replenishing intracellular GSH levels. INB is known to upregulate glutathione synthesis through the activation of Nrf2 and glutamate-cysteine ligase, the rate-limiting enzyme in GSH biosynthesis (Yang *et al.*, 2022). This restoration underscores INB-NLP's ability to enhance cellular detoxification pathways and protect the myocardium against oxidative damage.

### *Effect of INB-NLP on anti-inflammatory and inflammatory marker expression in heart tissue and serum samples*

**INB-NLP regulates the sirt3 protein expression in the DNB induced rats:** The expression level of sirt3 was assessed in the heart tissue of normal and experimental rats, and the results depicted in Fig. 5a. DNB alone treated groups showed a down-regulated expression of sirt3. However, treatment with INB-NLP in DNB induced groups expressed an upregulation in sirt3 expression in a dose dependent manner as compared to the DNB alone administered group. Also, the INB-NLP alone treated group showed a normal level of sirt3 expression. The downregulated sirt3 expression in DNB administered rats reflects the impaired mitochondrial function and oxidative stress. Sirt3 plays a pivotal role in maintaining the mitochondrial homeostasis by regulating oxidative metabolism, antioxidant defences, and apoptotic signalling (Trinh *et al.*, 2024). The upregulation of sirt3 expression in INB-NLP treated rats highlights its role in restoring mitochondrial function and enhancing resistance to oxidative damage. INB may directly activate sirt3 or upregulate its expression through indirect pathways involving AMPK activation. Upregulated sirt3 activity enhances mitochondrial biogenesis and the deacetylation of key antioxidant and metabolic enzymes, contributing to the observed cardioprotection.

**INB-NLP regulates the casp8 mRNA expression in the DNB induced rats:** The casp8 mRNA expression was assessed in heart tissue samples of control and experimental rats, and the results are depicted in Fig. 5b. A significant upregulation of casp8 mRNA expression was observed in DNB induced group. After treatment with INB-NLP, the expression of casp8 was significantly downregulated in a dose dependent manner as compared to the DNB induced group. Comparison of normal rats with INB-NLP alone treated group of rats showed no significant variation in the expression pattern of casp8.

Mammalian caspases have classically been divided into inflammatory and apoptotic caspases based on their cellular functions. Among these, casp8 is particularly interesting in terms of multiple functions in a variety of inflammatory processes (Han *et al.*, 2021). The upregulation of casp8 in DNB administered rats reflects enhanced apoptotic signalling triggered by oxidative stress and inflammation.

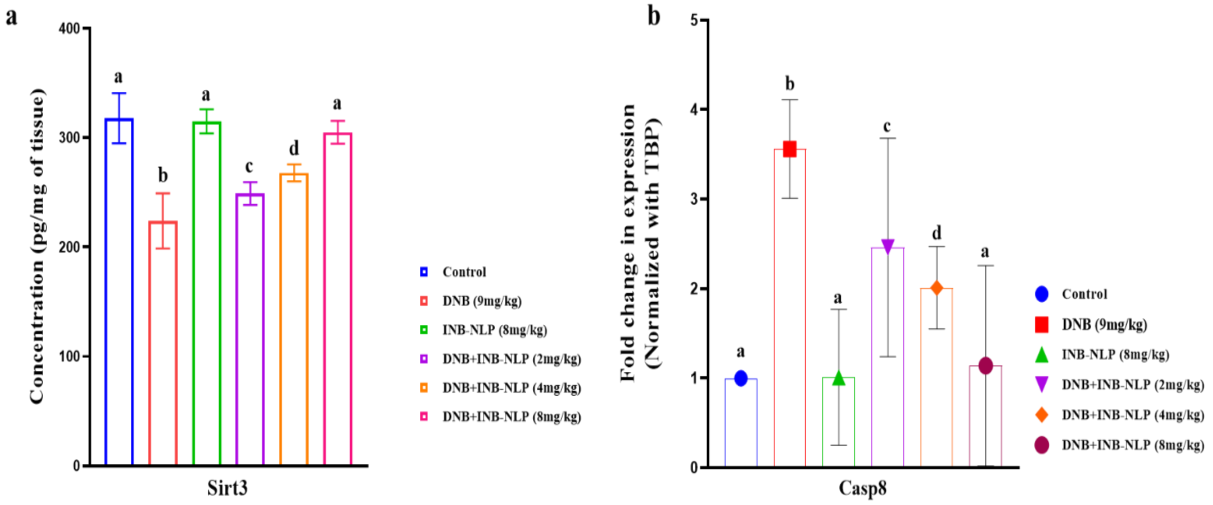

**Fig. 5: The expression of anti-inflammatory marker sirt3 (a) and inflammatory marker casp8 (b) in control and experimental rats. The values are statistically significant at $p \leq 0.05$**

DNB induces the activation of death receptors such as Fas and TNF receptors, leading to the casp8 activation (Al-Aamri *et al.*, 2021). INB-NLP treatment significantly downregulated casp8 expression, indicating reduced apoptotic activity. This effect may be attributed to INB's inhibitory effect on NF-κB signalling, which reduces the expression of pro-apoptotic and inflammatory mediators, including Fas ligand and TNF-α. By modulating these pathways, INB-NLP limits inflammation and preserves cardiomyocyte viability.

***Histopathological assessment***

The histopathological changes in the aorta of experimental rats is shown in Fig. 5. The effects of different doses of INB-NLP on control and DNB administered rats were evaluated by H & E staining.

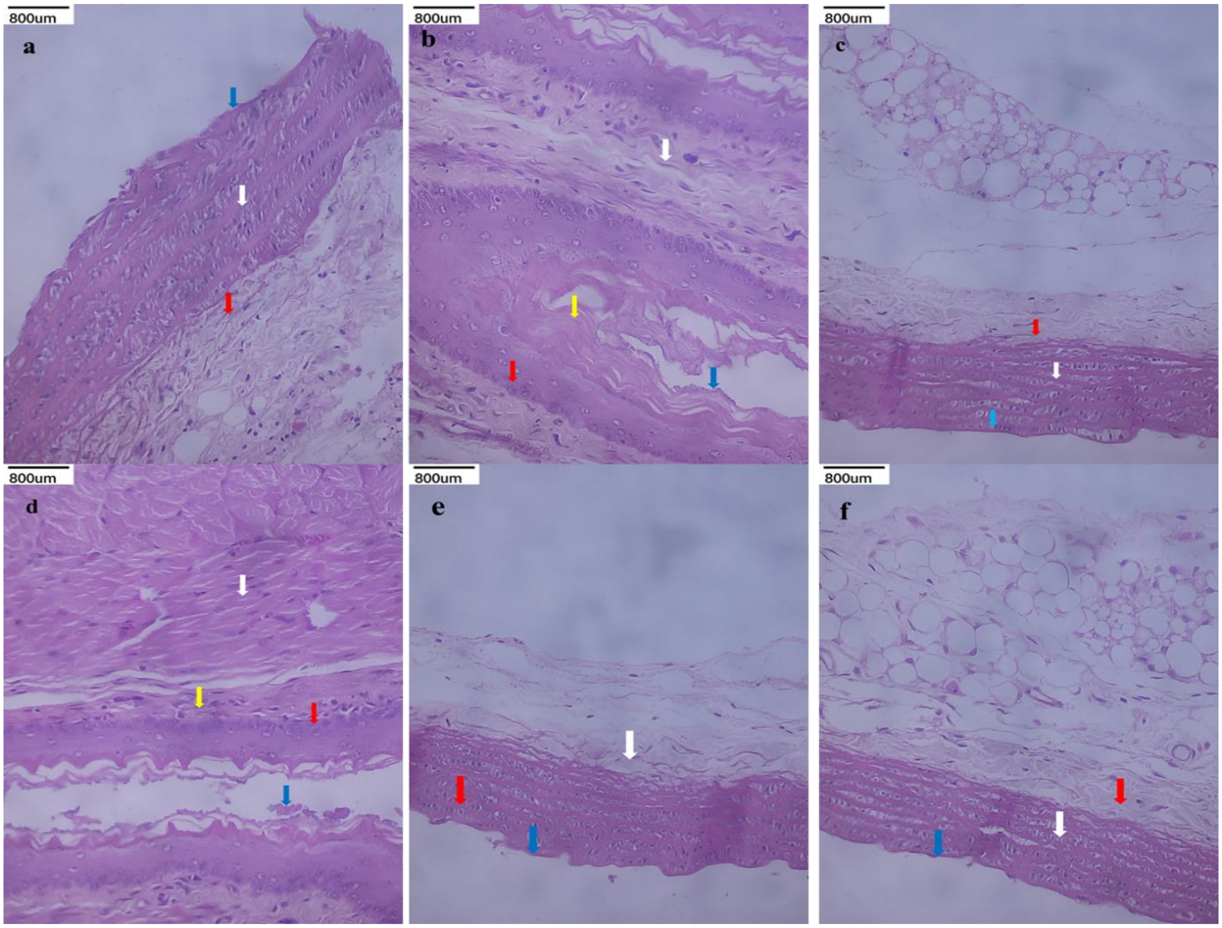

**Fig. 5: The histopathological changes in the aorta of control and experimental rats (H&E staining). Group I: (a) control, Group II: (b) DNB alone (3 mg kg$^{-1}$ bw for 3 days), Group III: (c) INB-NLP alone (8 mg kg$^{-1}$ bw), Group IV: (d) DNB + INB-NLP (2mg kg$^{-1}$ bw), Group V: (e) DNB + INB-NLP (4 mg kg$^{-1}$ bw), and Group VI: (f) DNB + INB-NLP (8 mg kg$^{-1}$ bw). (a) The arrows indicate normal tunica intima (blue), normal tunica media (white), and normal tunica adventitia (red). (b) The yellow and blue arrows indicate the atherosclerotic plaque and mild deposition of calcium. The red and white arrows indicate infiltration of foam cells in tunica media and local thickening of the aorta wall. (c) Tissue architecture in this group has no changes and is same as in control group. (d) The red and yellow arrows indicate mild foam cell infiltration in tunica media and mild fatty degeneration. The blue and white arrows indicate mild plaque formation, and tunica adventitia is noted as normal; (e) The white and blue arrows show normal tunica intima and tunica adventitia. The red arrow indicates slightly enlarged medial cells and mild fatty degeneration in myointimal cells. (f) In this group, the arrows show normal architecture of tunica intima, media and adventitia, as same as control group.**

The histopathological examination of aorta of DNB alone administered rats revealed the features of atherosclerotic plaque (yellow arrow) and the mild deposition of calcium (blue arrow). The infiltration of foam cells in tunica media (red) and local thickening of aorta wall (white arrow) were also observed. However, the treatment with INB-NLP (2 mg $kg^{-1}$ bw) in DNB administered group reduced the impact caused by DNB since foam cell infiltration in tunica media (red arrow) and fatty degeneration (yellow) were reduced in comparison to DNB alone administered group. Also, the treatment with INB-NLP significantly reduced plaque formation (blue arrow), and tunica adventitia was noted to be normal (white arrow). The DNB administered groups treated with 4 mg $kg^{-1}$ bw of INB-NLP showed normal tunica intima (white arrow) and tunica adventitia (blue arrow). However, slightly enlarged medial cells and mild fatty degeneration in myointimal cells (red arrow) were noted in this group. The DNB administered group treated with 8 mg $kg^{-1}$ bw of INB-NLP showed normal architecture of tunica intima, media and adventitia, as same as control group. The control group showed a normal tunica intima (blue), normal tunica media (white) and normal tunica adventitia (red). The INB-NLP alone group also showed normal architecture as of the control group.

The histopathological evaluation of aortic tissues revealed severe atherosclerotic changes in DNB administered rats. These findings are consistent with increased LDL oxidation and macrophage activation triggered by DNB. The foam cells, formed from lipid-laden macrophages, contribute to vascular inflammation and plaque development. INB-NLP treatment significantly restored normal aortic architecture in a dose-dependent manner. INB's lipid-lowering effects may be responsible for these improvements by activating PPAR-α, enhancing lipid metabolism and reducing oxidized LDL levels. Additionally, INB suppresses macrophage activation by modulating scavenger receptor pathways, preventing foam cell formation (Blazevic *et al.*, 2014). These histological improvements further emphasize the therapeutic potential of INB-NLP in preserving vascular integrity and preventing atherosclerosis.

The cardioprotective effect of INB-NLP is mediated through the interplay of multiple pathways. By activating sirt3, INB-NLP enhances the expression and activity of key antioxidant enzymes, neutralizing ROS and preventing oxidative damage. Simultaneously, INB-NLP also has the ability to modulate PPAR-α, thereby improving lipid metabolism and reducing dyslipidemia and foam cell formation. The suppression of NF-κB signaling further limits casp8 mediated inflammation and apoptosis, preserving cardiomyocyte viability and vascular integrity. These interconnected pathways underscore the multifaceted mechanism of INB-NLP in combating DNB mediated cardiotoxicity. Future research focusing on clinical validation and optimization of INB-NLP formulations could pave the way for its use as a novel cardioprotective agent in oncology settings.

**Conclusions:** This study demonstrated potent cardioprotective effects of INB-NLP against DNB mediated cardiotoxicity in rats by mitigating oxidative stress, dyslipidemia, and inflammation, as well as regulating apoptotic signalling. INB-NLP exhibited a multifaceted therapeutic potential. The restoration of antioxidant enzyme activities, reduction in cardiac biomarkers, normalization of lipid profiles, and attenuation of inflammatory markers by INB-NLP illustrate its efficacy in combating DNB induced damage. Furthermore, the histopathological improvement in vascular architecture and upregulation of sirt3 highlight INB-NLP ability to preserve cardiac and vascular integrity. INB-NLP was more efficient in treating DNB mediated cardiotoxicity at the dose of 8 mg $kg^{-1}$ bw than other test doses. These findings revealed the potential of INB-based nanotherapeutics as a promising strategy for reducing chemotherapy mediated cardiotoxicity.

**Ethical statement:** Animal experiments were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), and were approved by the Institutional Animal Ethics Committee (IAEC) of Rajah Muthiah Medical College, under approval number AU-IAEC/1322/6/22.

**Author's contributions:** Tani Carmel Raj (TCR) and Vennila Lakshmanan (VL) handled conceptualization and methods while TCR, Nivedha Jayaseelan, and Kanimozhi Kaliyamoorthi

conducted the experiments. TCR and VL managed the data analysis and were involved in drafting, and editing of the paper. All authors approved the final version after critically examining the article.

**Conflict of interest:** Under relevant patent laws and regulations, indirubin-loaded nanoliposomes were patented under Intellectual Property of India by T.G. Tani Carmel Raj and Vennila Lakshmanan, vide No. 202341073210 A.

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**Data availability statement:** The data shall be made available on request.

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