**SUPPLEMENTARY MATERIAL**

# Unveiling the nano-particle enabled synergistic mitigation of Bcl2/Cyt c/CYP1A1 signaling axis as protective therapeutic route in mitochondrial dysfunction associated diabetes

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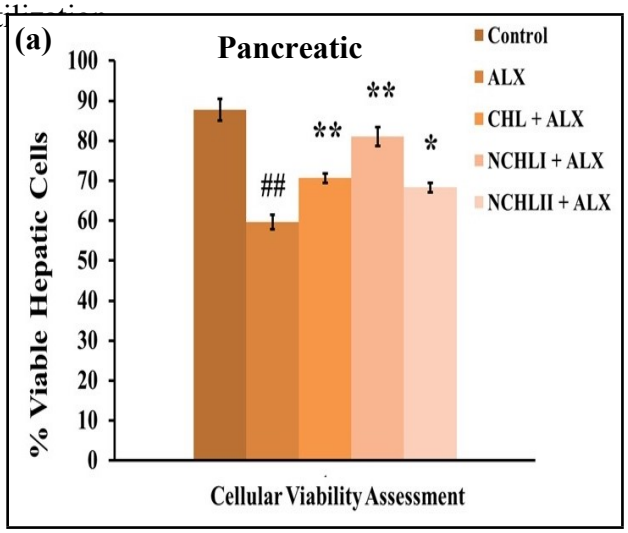
## Results and discussion

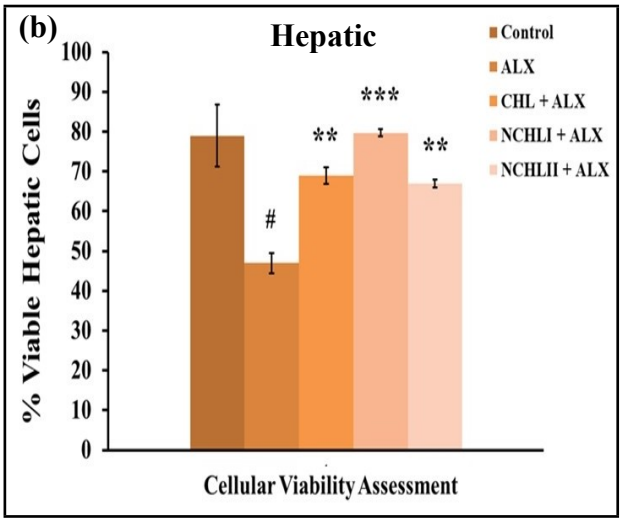
### CHL and NCHL alleviating ALX-induced cellular disruption

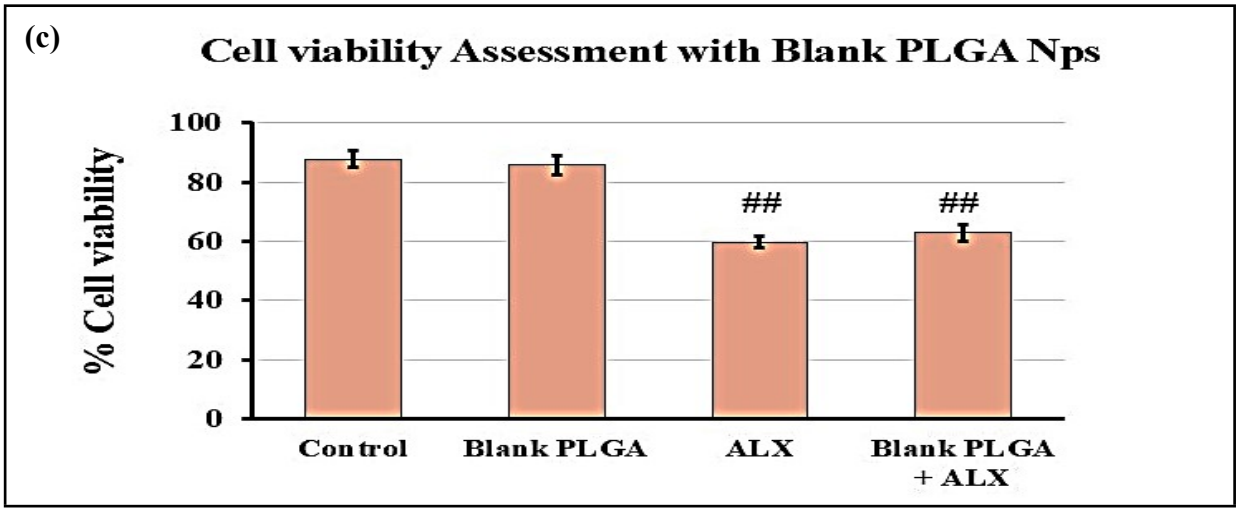
Mice treated with ALX showed a reduced percentage of viable cells in pancreatic (**Fig. S1a**) and hepatic (**Fig. S1b**) tissues compared to controls. As a cytotoxic glucose analogue, ALX enters β-cells *via* GLUT2 transporters, inhibiting insulin secretion by inactivating glucokinase and generating reactive oxygen species (ROS) that damage cellular components and lead to hepatic and pancreatic β-cell death. Trial experiments using blank PLGA nanoparticles (without any original drug component) showed no significant changes in cellular viability of pancreatic tissue compared to that of control whereas observations in the PLGA nanoparticle + ALX treated group was close to that of ALX treated group indicating no harmful effects of PLGA on diabetic mice (**Fig. S1c**). Therefore, this group was deliberately obliterated from further experiments to minimize the loss of animal lives and cost of the experiments. Our result also corroborated with previous studies of our laboratory as well. CHL pre-treatment limited cell loss, but NCHL pre-treatment resulted in a significant increase in viable cells in both tissues, suggesting a protective role against ALX-induced cytotoxicity. Compared to CHL, NCHLI dose demonstrated enhanced protective effects even at a 10-fold reduced dosage, effectively mitigating cytotoxicity. Moreover, maintenance of glucose

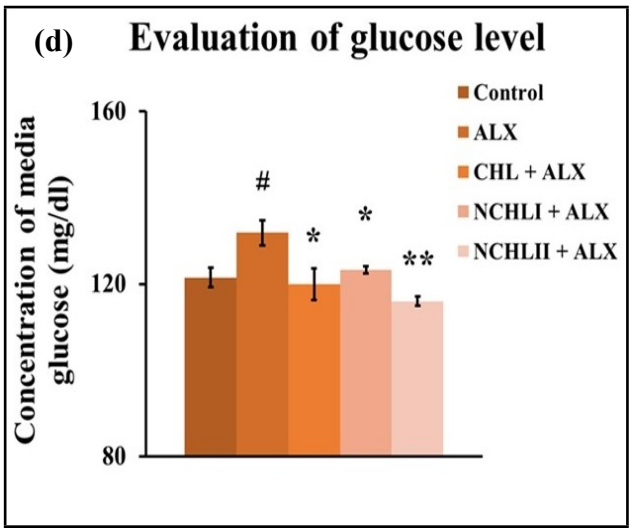
levels in both L6 cell lines and mice were ascertained by estimating the media glucose level as well as the blood glucose levels of different experimental groups in *in vitro* and *in vivo* models, respectively. Higher glucose uptake was observed in L6 cells pre-incubated with NCHL than in CHL pre-incubated cells prior to administering ALX, suggesting that NCHL was effective in lowering hyperglycemic conditions in L6 cells. Consequently, NCHL functions more effectively than CHL in restoring the decreased glucose absorption caused by ALX in L6 cells (**Fig. S1d**). The *in vitro* studies represented the impaired glucose uptake by skeletal muscle cells that contributes to hyperglycemic stress and diabetes, making ALX a critical focus for investigating its effects on glucose metabolism. Similar study was replicated in mice model showing that on the 5th day post-ALX injection, blood glucose levels in ALX-treated mice significantly increased compared to controls. However, mice pre-treated with CHL, NCHLI, and NCHLII before ALX exposure showed a significant delay in hyperglycemia progression. Notably, NCHLI pre-treatment resulted in blood glucose levels closer to normal compared to ALX-only mice, highlighting its superior efficacy in mitigating ALX-induced hyperglycemia over the CHL dose (**Fig. S1e**). Additionally, to confirm the ALX-induced tissue hamper, histopathological changes were determined in pancreatic tissue using H&E staining. H&E staining showed that pancreatic islets in the control group were surrounded by compact acinar cells and thin collagen fibers. In contrast, ALX-treated diabetic mice exhibited reduced tissue integrity, with fewer islet cells and uneven collagen lining. NCHL pre-treatment significantly reduced the loss of collagen fibers and islet cells, indicating its protective role against ALX-induced tissue damage and suggesting it may help prevent diabetes onset by regulating insulin production affected by β cell loss (**Fig. S1f**). Moreover, morphometric analyses of pancreatic Langerhans confirmed a decline in the mean number of islet cells in the ALX-treated group, reflecting toxicity. However, NCHL pre-treatment preserved islet cell counts closer to control levels, indicating superior protection of pancreatic architecture against ALX-induced diabetes compared to CHL (**Fig. S1g**). The restoration of the percentage of viable β cells and muscle cells might be the outcome of the overall protective efficacy of NCHL doses against ALX-induced cytochrome c (Cyt c) release from the mitochondria to the cytosolic region of the cells. This, mediated the down-regulation of Bcl2 protein, which is a mitochondrial membrane associated protein, inhibiting the initiation of mitochondria dependent apoptotic pathway. Moreover, the blood glucose levels of drug and nano-drug treated groups following oral glucose administration are represented in **Fig. S1h.** The elevation of blood glucose was minimised in the groups of CHL and NCHL treated group significantly as compared with ALX treated mice group. This oral glucose tolerance test (OGTT) further confirmed that NCHL has a significant role in glucose homeostasis. Thus, NCHL doses help maintain glucose homeostasis by tightly regulating mitochondrial signaling. This prevents dysfunction that could disrupt glucose uptake and utilization in peripheral

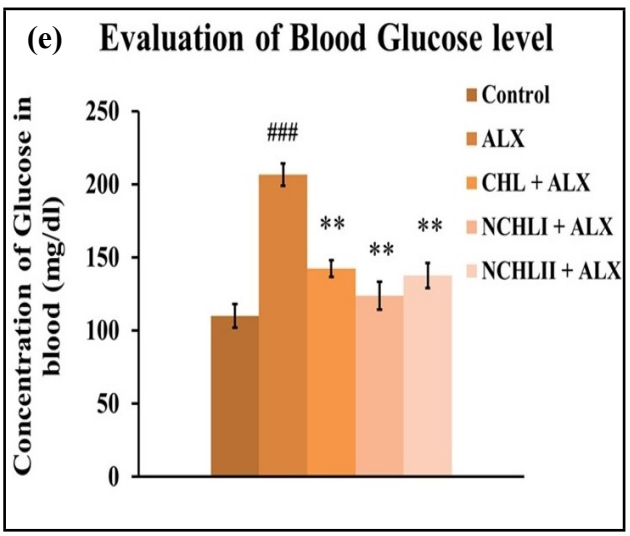
cells, thereby reducing the risk of hyperglycemia and diabetes. Moreover, the positive effects of CHL against ALX-induced toxicity, bring forth a new set of challenges including cost-effectiveness, bioavailability, and targeted delivery of drug that ultimately demanded the exploration of nanotechnology by solvent displacement method using PLGA as a biodegradable carrier. This aided in achieving the of enhanced drug efficacy at lower doses while improving solubilization, absorption, and distribution, particularly in tissues markedly responsible for glucose utilization.



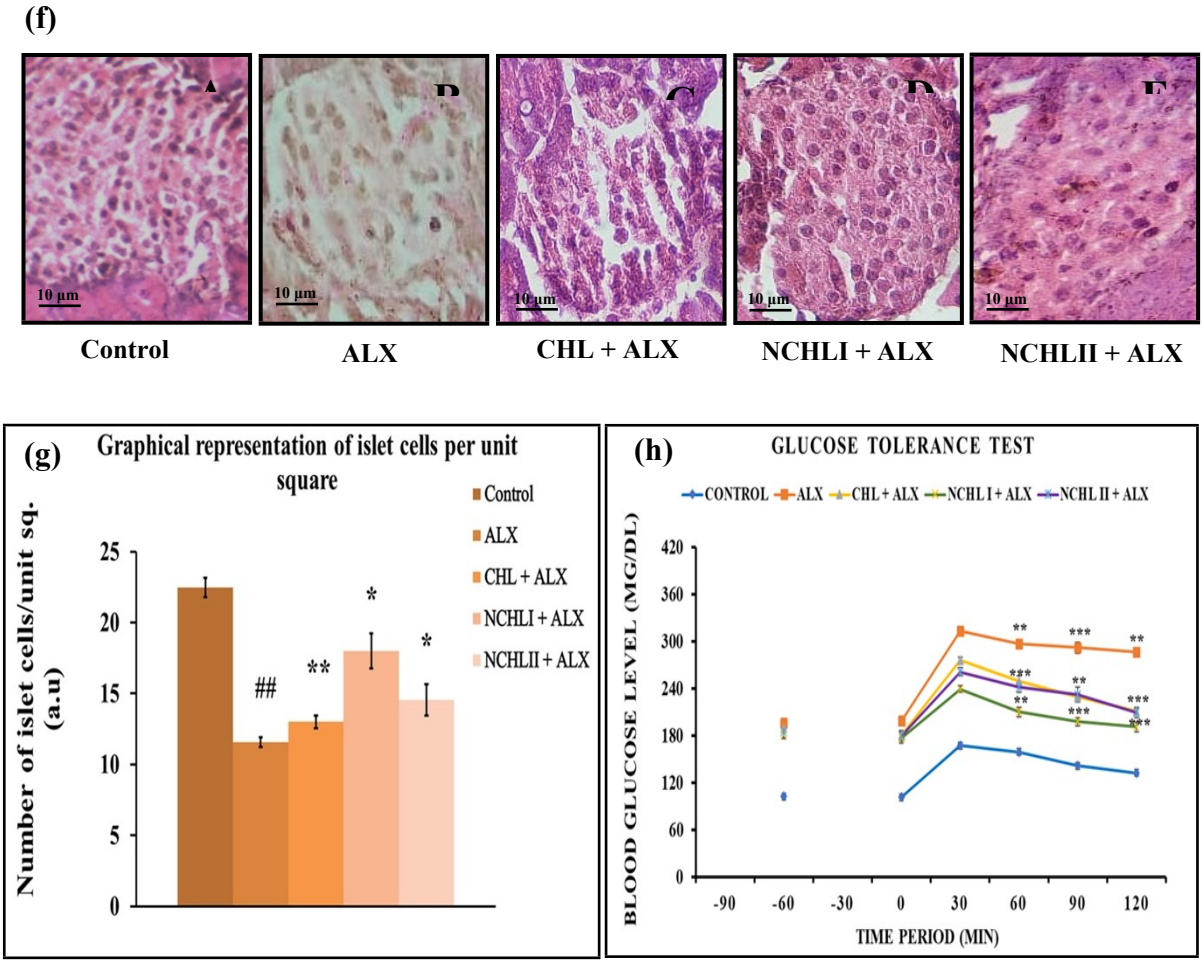



**Fig. S1. (a)** Graphical representation of % cell-viability in pancreatic tissue, **(b)** Graphical representation of % cell-viability in hepatic tissue of different experimental groups of mice. ##$p < 0.01$ *vs.* Control; #$p < 0.05$ *vs.* Control, ***$p < 0.001$ *vs.* ALX, **$p < 0.01$ *vs.* ALX, *$p < 0.05$ *vs.* ALX; were considered significant for Student's t-test, **(c)** Graphical representation of % cell-viability in pancreatic tissue using Blank PLGA Nanoparticles as positive control, ##$p < 0.01$ *vs.* Control, was considered significant for Student's t-test, **(d)** Graphical representation of concentration of media glucose level in control and experimental L6 cells, **(e)** Graphical representation of modulation of blood glucose level in the experimental groups of mice. ###$p < 0.001$ *vs.* Control, #$p < 0.05$ *vs.* Control, **$p < 0.01$ *vs.* ALX, *$p < 0.05$ *vs.* ALX, were considered significant for Student's t-test **(f)** Histological changes in islets of Langerhans of mice pancreas in control and different experimental mice groups, **(g)** Morphometric analyses of pancreatic islets focussing the islet cell count per unit square area. ##$p < 0.01$ *vs.* Control, **$p < 0.01$ *vs.* ALX, *$p < 0.05$ *vs.* ALX, were considered significant for Student's t-test, (**h**) Glucose tolerance test in experimental mice groups.

**CHL and NCHL role in optimum hepatic function**

Total cholesterol levels were determined from the hepatic tissues, the increased levels of which indicates a significant hepatic injury. A significant elevation in the levels of total cholesterol was observed in the ALX treated mice group when compared to the control mice which were somehow retained to near normal level in mice pre-treated with CHL and NCHL, where the NCHL doses showed better results in restricting the whole process altogether (**Fig. S2a**). The optimum levels of the metabolic end products, such as aspartate aminotransferase (AST) (**Fig. S2b**) and alanine transaminase (ALT) (**Fig. S2c**) levels indicates status of functional liver. An elevation of both the parameters of AST and ALT in the diabetic mice was observed which was significantly inhibited by both CHL and NCHL pre-treatments, with the best result seen in case of the NCHL doses, which resulted almost similar to the control group. In *in vivo* experimental model, pre-treatment with NCHL before ALX exposure protected tissue structure and cell integrity in mice, similar to the control group. ALX causes poor liver conditions as indicated by increased liver cholesterol levels. Moreover, the liver enzymes like AST and ALT, which are essential for normal liver metabolism and functioning gets affected by hepatotoxicity ensued by ALX. Thus, the NCHL drug doses reduced AST and ALT levels, alleviating ALX-induced liver damage.

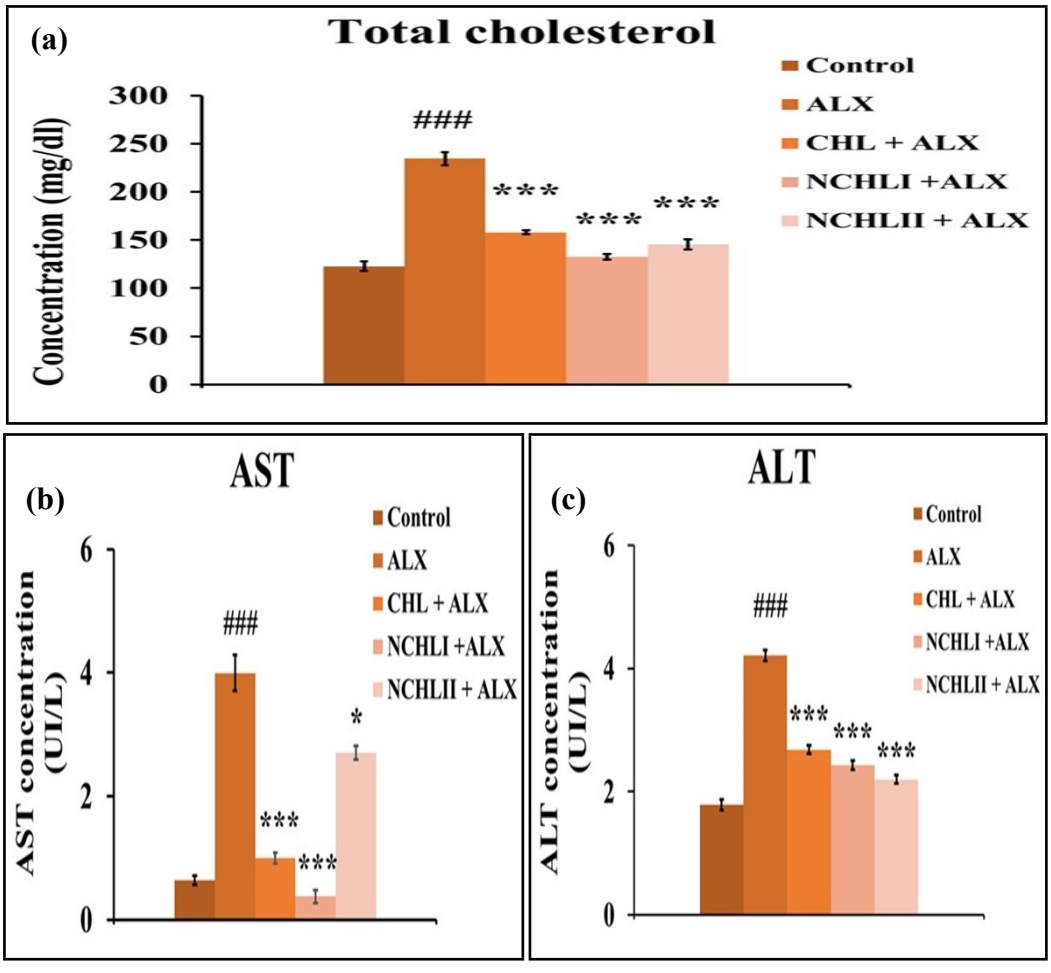


**Fig. S2.** Graphical representation of liver function tests: **(a)** Assessing the total cholesterol levels in hepatic tissue, **(b)** AST levels **(c)** ALT levels from control and different experimental groups. ###p < 0.001 *vs.* Control; ***p < 0.001 *vs.* ALX, *p < 0.05 *vs.* ALX; were considered significant for Student's t-test.

**CHL and NCHL role in mitigating oxidative stress**

In order to determine the score of oxidative stress induced in cells due to ALX toxicity, the anti-oxidative enzyme profiles were evaluated from the pancreatic tissues of control and different experimental groups of mice. The findings with anti-oxidative enzymes in pancreatic tissues revealed that nano-drug pre-treatments maintained enzymatic levels near normal. The NCHL pre-treated groups showed a significant increase in catalase activity compared to the CHL + ALX group (**Fig. S3a**). Additionally, NCHL + ALX treatment resulted in higher glutathione levels than the CHL pre-treated group (**Fig. S3b**). Superoxide dismutase (SOD) levels and activity were also greater in NCHL pre-treated mice compared to CHL pre-treated ones (**Fig. S3c**). Moreover, lipid peroxidase (LPO) levels were reduced in both pre-treated groups, with NCHL maintaining levels closer to the control group (**Fig. S3d**). Additionally, another study was performed to analyse the role of CHL and NCHL in preventing ROS (Reactive Oxygen Species) in pancreatic tissue. ROS production in pancreatic tissues was assessed using $H_2$DCFDA dye and spectrofluorimetric analysis (λexi = 485 nm; λemi = 526 nm). ALX treatment resulted in increased reactive oxygen species (ROS), promoting cytotoxicity due to oxidative stress, as indicated by increased emission spectra. In contrast, NCHL doses effectively suppressed ALX-induced ROS production in pancreatic tissues more successfully than CHL doses (**Fig. S3e**). From the overall scenario, it might be suggested that NCHL pre-treatment doses inhibited the oxidative stress conditions in the mitochondria of pancreatic cells triggered due to ROS generation due to the redox reactions in the cells. Alongside restricted production of ROS, NCHL pre-treatment further boosted the antioxidant enzyme activities, specifically controlling lipid peroxidation and increasing superoxide dismutase (SOD), total thiol and catalase levels maintaining the oxidative state of mitochondria.

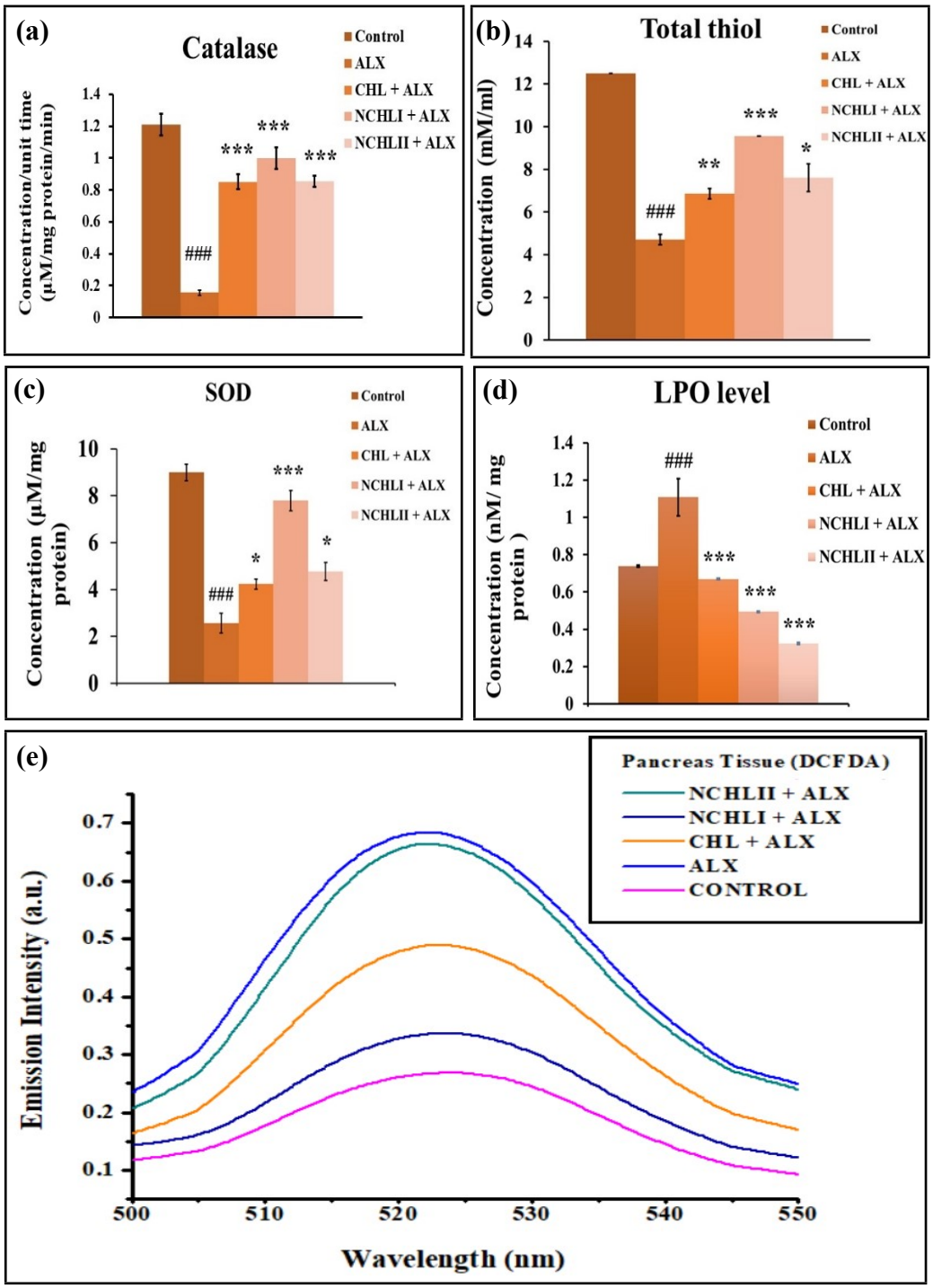



**Fig. S3.** Analysis of anti-oxidative enzyme levels and their activity **(a)** Catalase activity, **(b)** Total thiol content**, (c)** SOD levels, **(d)** LPO levels, in control and different experimental groups of mice. ###p < 0.001 *vs.* Control, ##p < 0.01 *vs.* Control; ***p < 0.001 *vs.* ALX, **p < 0.01 *vs.* ALX, *p < 0.05 *vs.* ALX; were considered significant for Student's t-test, **(e)** Assessment of intracellular ROS generation in pancreatic tissue using $H_2$DCFDA dye.