Molecular and immuno-toxicological effects of Dextran coated Ferrite and Hydroxylapitite nanomaterials

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1. **Objectives as stated in the project proposal:**
   The objective of the project is to examine the influence of physical presence of nanomaterials and their effects on biological systems and underlying mechanisms. In view of this, an idea is to develop and evaluate the molecular and immune-toxicological effects of Dextran coated Ferrite and Hydroxylapitite nanomaterials which includes the cytotoxicity, acute oral toxicity, combined chronic toxicity and carcinogenicity, dermal toxicity, immunotoxicity studies, DNA damaging effects, lipid peroxidation and antioxidant enzymes. This will lead to the development of validated to be safe nanomaterials that can be used in site directed targeted drug delivery and minimizing adverse reactions.
2. **Deviation made from original objectives if any, while implementing the project and reasons thereof:**
   1. Mice was chosen as animal model, instead of rats for immunotoxicity studies because of the non availability of appropriate kits
   2. Deposition of nanomaterials was evaluated by ICP MS (Inductively Coupled Plasma Mass Spectrometry) instead of TEM

Methodology

Synthesis of Nanomaterials

Dextran-coated ferrite nanomaterials (DFNM)

Dextran-coated ferrite nanomaterials (size <25nm) were prepared using the co-precipitation method. Briefly, the stoichiometric mixtures of $FeCl_3$ and $FeCl_2.4H_2O$ (Fe 3+/Fe2+: 2: 1) were heated at 70°C. Ferrite nanoparticles were precipitated by the addition of 3M NaOH drop wise for 1h followed by hot stirring for another 1 hr. The precipitate was then washed three times with deionized water to get uniformly dispersed spherical magnetite particles. The overall reaction was carried out in N_2 atmosphere to prevent oxidation of magnetite to magnemite. Surface coating of ferrite nanoparticles with dextran was done by stirring ferrite nanomaterial in a solution of dextran of appropriate concentration for overnight (at 37°C). The precipitate was then washed and lyophilized to obtain dextran coated nanomaterials [17].

Hydroxyapatite nanoparticles (HANPs)

HANPs were synthesized by wet chemical method, where calcium phosphate was precipitated from the aqueous solution of calcium nitrate tetrahydrate (Ca (NO3)2.4H2O) and ammonium dihydrogen orthophosphate (NH4H2PO4) (Rankem, India). Precipitation was carried out at a pH of 11 and at 0ºC for 2h. After ageing for 24h, the precipitate was washed in distilled water, freeze-dried and calcined at 300ºC.

Characterization of the synthesized nanomaterials

The synthesized nanomaterials were characterized by the following techniques: Transmission electron microscopy (TEM) and Dynamic Light Scattering (DLS) for size determination. The phase purity of the nanomaterials was analyzed by X-ray Diffraction (XRD) and Fourier Transformed Infrared Spectroscopy (FTIR).

***In vitro* cell culture cytotoxicity test**

DFNM and HANPs were subjected to direct contact assays as per ISO 10993-5:1999 (E) standard to evaluate their toxicity towards mouse fibroblast L929 cells. L929 cells were seeded at a density of ~20000 cells/well in a 96-well micro plate. Different concentrations (10, 25, 50, 100, 200, 400, 600 and 800µg/mL) of DFNM and (10, 25, 50, 100, 200, 400, 600 and 700 µg/mL) of HANP were added onto the wells. The cells were incubated at 37ºC for 24 hrs. After incubation, the cells were examined microscopically for any morphological changes and the mitochondrial activity of the cells was quantified using MTT dye. . 20µL of MTT dye solution (5 mg/mL in phosphate buffer pH 7.4) was added to each well. After 4 h of incubation the MTT was removed and formazan crystals were solubilized with 200 µL of DMSO. The absorbance of each well was read on a microplate reader (ELx 808 IU ultra microplate reader, Bio- Tek instruments, USA) at 540 nm. The relative cell viability (%) with that of control wells, containing cell culture medium without nanoparticles, was calculated.

***In vitro* antioxidant assays**

For *in vitro* studies, healthy rats were sacrificed by cervical dislocation and their liver was rapidly excised, washed in normal saline, and immediately placed in an ice bath. 10% of liver tissue homogenate was prepared in phosphate buffer (0.1M, pH 7.4) using an ice-chilled glass homogenizing vessel in a rotor stator homogenizer at 900 rpm. For *in vitro* studies, the liver homogenate was incubated with increasing concentration (12.5, 25, 50 and 100µg/mL) of DFNM and HANPs for 3hour independently at 37°C in a shaking water bath and centrifuged at 3500 rpm for 10 min at 4°C in a refrigerated centrifuge. The supernatant was collected and subjected to evaluation of various antioxidant parameters.

Acute toxicity (OECD Guidelines 420)

Acute oral toxicity is to provide information on the possible health hazards likely to arise from the single or multiple exposures to a substance within a period of 24h. For oral administration, the DFNM and HANPs were suspended in aqua guard water and was loaded in a syringe after thorough agitation. A single oral administration of 300mg/kg and 2000mg/kg of both the nanomaterials were administered to 3 rats each using a gastric needle. Untreated rats were taken as control animals. All these animals were observed for 14 days for the evidence of any adverse reactions/toxicity or death [18].

Experimental Design

Dose	No. of animals	Exposure	Exposure/Duration
Control (Physiological saline)	3 each for DFNM/ HANPS	Oral	Single
DFNM (300 mg/kg body weight)	6	Oral	Single
DFNM (2000 mg/kg body weight)	6	Oral	Single
HANPs (300 mg/kg body weight)	6	Oral	Single
HANPs (2000 mg/kg body weight)	6	Oral	Single

At the end of the experimental period, all the animals were sacrificed; blood was collected in tubes with EDTA. Hematological parameters such as hemoglobin concentration (Hb), total erythrocyte count (RBC), white blood cell (WBC), hematocrit (HCT), total and differential leukocyte counts were evaluated using haematology counter. For biochemical estimation, the

serum was obtained by centrifugation of the whole blood at 3000rpm. The biochemical parameters such as ALP, SGOT, SGPT, GGT, albumin, total protein, glucose, cholesterol, urea, creatinine, total bilirubin, phosphorous etc were estimated using ERBA XL 300 Biochemical Fully automated analyzer. All the diagnostic kits were purchased from Transasia Biomedical Ltd, Mumbai. Gross necropsies of all the animals were performed which include examination of the external surface of the body and internal organs. All major organs such as heart, liver, lungs, kidneys, spleen and adrenals were observed for any gross abnormalities. Antioxidant assay was performed on liver tissue homogenates.

Combined chronic toxicity and carcinogenicity studies (OECD 453)

The effect of nanomaterials on prolonged and repeated exposure was studied by chronic oral toxicity. The animals were exposed to nanomaterials (DFNM and HANPs) for a prolonged period of 12 months. Nanomaterials were given orally by mixing with commercially available feed using aqua guard filtered water. This was prepared daily and given throughout the experimental period. The feed intake was monitored once in two weeks.

Experimental Design

Group	Dose	No. of animals		Exposure	Months of exposure
		Male	Female		
1	Control	15	15	Oral	12
2	Low	15	15	Oral	12
3	Medium	15	15	Oral	12
4	High	15	15	Oral	12
5	High Recovery	10	10	Oral	12 + 28 days

Haematological and Biochemical parameters

At the end of the experimental period, blood was collected from the orbital sinus of the animals. For hematology analysis, blood was collected in tubes with EDTA. Hematological parameters such as hemoglobin concentration (Hb), total erythrocyte count (RBC), total and differential leukocyte counts were evaluated using haematology counter.

For biochemical estimation, the serum was obtained by centrifugation of the whole blood at 3000rpm. The biochemical parameters such as ALP, SGOT, SGPT, GGT, albumin, total protein, glucose, cholesterol, urea, creatinine, total bilirubin, phosphorous etc were estimated using ERBA XL 300 Biochemical Fully automated analyzer. All the diagnostic kits were purchased from Transasia Biomedical Ltd, Mumbai.

Gross Pathology

Animals of both the DFNM and HANPs administered groups were sacrificed by cervical dislocation and gross necropsies were performed which include examination of the external surface of the body and internal organs. All major organs such as heart, liver, lungs, kidneys, spleen and adrenals were observed for any gross abnormalities.

Histopathological Analysis

The organs such as liver, kidneys, spleen, heart, lungs, gonads, uterus were collected, preserved in 10% buffered formalin and subjected to histopathological analysis. Tissues will be processed and embedded in paraffin. A 5µm thick section will be cut and stained with Haematoxylin and Eosin. Special emphasis was given to detect the neoplastic effects and determination of carcinogenic potential.

The parameters of the antioxidant assay using the liver tissue homogenate were also performed.

28days repeated dose dermal toxicity (OECD Guidelines 408)

Dermal toxicity is to provide information on the possible health hazards likely to rise from the repeated exposures by dermal route over a limited period of time usually up to 28 days. Different concentrations (25, 50, 100mg/kg) of DFNM and HANPs were exposed dermally to all animals up to 6 hours per day on a 7 day per week basis for 28 days.

Experimental design

Group	Dose	No. of Rats		Exposure route	Days of exposure
		Male	Female		
1	Control	5	5	Dermal	28
2	Low (25mg/kg)	5	5	Dermal	28
3	Medium (50mg/kg)	5	5	Dermal	28
4	High (100mg/kg)	5	5	Dermal	28

At the end of the observation period, blood was collected and subjected to haematological (WBC, RBC, Haemoglobin, hematocrit, clotting time, platelet count, MCV, MCH, MCHC) and biochemical (glucose, cholesterol, triglycerides, total bilirubin, albumin, calcium, phosphorous, chloride, total protein, creatinine, urea, SGPT, SGOT, alkaline phosphatase, GGT parameters (OECD).

All animals were sacrificed by cervical dislocation and gross necropsies were performed on all experimental animals which include examination of the external surface of the body and internal organs. All major organs (heart, liver, lungs, kidneys, spleen, adrenals, pancreas and skin) were preserved in 10% buffered formalin and subjected to histopathological studies. The fixed tissues were processed in an automated tissue processor (Leica ASP 300) and sectioned (5µm thin sections) using a rotary microtome (RM2255) and stained with hematoxylin and eosin (H&E). Stained sections were examined under a light microscope (Axio ImagerZ1, Carl Zeiss) for the evidence of tissue lesions.

Part of the liver tissues from all the rats subjected to dermal exposure were collected, homogenized and estimated for total protein, lipid peroxidation, Glutathione reductase, glutathione peroxidase, reduced glutathione, superoxide dismutase activity and oxidative stress related DNA damage analysis.

Immunological studies

The following techniques were used to evaluate the Immunotoxicity potential of nanomaterials.

Delayed hypersensitivity by Guinea pig maximization test (ISO 10993-10: 2002/Amd.1:2006(E)

The delayed hypersensitivity assay was performed as per ISO 10993-10: 2010 (E) Biological Evaluation of medical devices-Part 10: Test for irritation and skin sensitization. Clause: 7.6: Closed patch test [ISO 10993-10: 2010 (E)]. Fifteen healthy adult Guinea pigs (10 animals for test and 5 animals for control) were chosen for the study. During the Induction phase, the clipped area on the upper back skin was swabbed using 70% alcohol and air dried. A concentration of 80mg/animal of DFNM and HANPs was made into a paste with physiological saline and applied topically on the clipped upper back region of ten animals. A patch of four ply gauze (wet in physiological saline) alone was applied to other five animals. This procedure was repeated thrice a week for three weeks. The Challenge phase was fourteen days after the last application (induction period). During this stage, all the test and control animals were challenged with the DFNM and HANPs. Hairs were removed from the flank area (untested area) and swabbed with 70% alcohol DFNM and HANPs was applied as done in the induction phase. Following this the skin sensitization potential was evaluated at 24, 48 and 72hrs.

At the end of experimental period, blood was collected and subjected to haematological and biochemical analysis.

Later all guinea pigs were sacrificed, liver and brain were rapidly excised, washed in normal saline and immediately placed in an ice bath. 10% of tissue homogenate was prepared in phosphate buffer (0.1M, pH 7.4). These homogenates were analyzed for LPO, GSH, GR, GPx, and SOD. 8-OHdG estimation was performed on liver homogenates.

Immunotoxicity Study

In this study, 100mg of DFNM and HANPs were injected intraperitoneally to a total of 18 mice for immunological stimulation. Six animals for each group and in addition six animals served as control (no nanomaterials given). Animals were sacrificed at the end of 7 days, 14 days and 21 days of exposure. Control animals were sacrificed on 0 day itself. Following parameters were evaluated.

T and B lymphocytes proliferation assay

Isolation of T and B lymphocytes

Spleen was collected. Single cell suspensions of spleenocytes were isolated from the spleen using Histopaque gradient as per the standard protocol. Prepared cell suspension was separated into T cell and B cell lymphocyte population using Mouse T cell Enrichment Kit and Mouse B cell Enrichment Kit (EasySep, STEMCELL Technologies) as per the manufacturer's instruction. Briefly, normal rat serum (50µL/ml of cells) was added onto the cell suspension. 50µL of EasySep^{TM} Mouse T Cell Enrichment Cocktail for T lymphocytes and was added and the sample was incubated in refrigerator for 15 minutes. 100 µL of EasySep^{TM} Biotin Selection Cocktail was added followed by incubation in refrigerator for 15 minutes. 75 µL of EasySep^{TM} D Magentic Particles were added and the tubes were kept in refrigerator for 5 minutes. After incubation, the tubes were placed into the magnet and set aside for 5 minutes at room temperature. The magnet and the tube were inverted to pour off the selective lymphocytes. Unwanted cells will remain bound inside the tube held by the magnet.

Lymphocytes proliferation assay

Tritiated Thymidine incorporation assay

The isolated lymphocytes were seeded at a density of 2, 00,000 cells /well onto 96 well plates. The cells were cultured in complete RPMI medium with 10% FBS, at 37°C, 5%CO_2 for 48 hrs. 50 µL of ^{3}H thymidine (50µCi/well) was added onto each well and the cells were further incubated at 37°C for 24 hrs. The cells were then harvested and the amount of tritiated thymidine incorporated was counted using scintillation counter.

Assay for inflammatory cytokines

Total m-RNA was isolated from splenocytes exposed to DFNPs (200, 600, 800 µg/ml) and bare ferrite nanoparticles (600, 800 µg/ml), following the manufacturer's protocol, using Trizol reagent (Sigma, USA). 150ng of m-RNA was used for c-DNA synthesis of IL-10, IL- 1, TNF-β and β Actin in a reaction volume of 20µL using RT^2 first strand Kit (Qiagen, Germany) and the synthesis were carried out on the Eppendorf master cycler, Germany. The mouse oligo nucleotide forward and reverse primer sequence used to determine specific m-RNA gene expressions are depicted in Table 1. The real time PCR reaction was carried out with RT^2 SYBR green ROX q PCR master mix of total reaction volume of 25µl, real time PCR amplifications were done using a Chromo 4 System, Bio- Rad (MJ Research, CA) for 40 cycles as per manufacturer's protocol. The level of gene expression is reported as the ratio between the mRNA level of the target gene and the β Actin, a reference gene using the comparative $2^{\Delta\Delta Ct}$ method [21, 22].

ICP-MS Analysis

Elemental analysis of the DFNM exposed samples (liver, kidney and spleen) from chronic toxicity study and immunotoxicity study was determined by ICP-MS. The sample preparation involved digestion of the known amount of sample in Conc. Nitric acid, Concentrated Nitric acid and Perchlorica cid mixture to form a clear solution and then the samples were diluted to 50ml in

de-ionized water. The clear solution thus obtained was analyzed by ICP. The results were recorded and processed using Win Lab 32 software.

DNA Analysis

Isolation of Mitochondrial DNA (mtDNA)

100mg each of liver collected from the rats of *in vivo* study and liver collected from the fresh animals (*in vitro* study) was homogenized with 1mL of ice cold buffer supplied along with mtDNA extractor kit (mtDNA Extractor Kit, Wako, Japan), followed by centrifugation at 1000g for 1 minute. The supernatant was discarded and the pellet was subjected to mtDNA isolation as per the manufacturer's protocol. The final mtDNA pellet was dried carefully at vacuum and further suspended in TE buffer.

Purity Check

After ensuring complete solubility of mtDNA, the purity factor (260/280nm) was determined spectrophotometrically (UV-1601, Shimadzu, Japan). The concentration of 50µg/mL was taken as one unit of optical absorption for double stranded - helical DNA at 260nm with optical path of 1cm.

Agarose gel electrophoresis

The integrity of the isolated DNA was checked on 0.7% agarose gel stained with ethidium bromide (10mg/mL) by loading 12µL of DNA preparation (2µL extracted mtDNA, 2µL gel loading buffer containing 25% bromophenol blue and 30% glycerol, 8µL sterilized deionized water). The electrophoresis was carried out at 100 V using 1 X TAE buffer (2 mmol/L EDTA, 89 mmol/L Tris and 89 mmol/L boric acid; pH 8.6) for 30min. After electrophoresis, the gel was observed under a UV lamp in a gel documentation system (Alpha Innotech, USA)

Conformation of mtDNA by Polymerase Chain Reaction

The mtDNA isolated from rat liver using mtDNA Isolation Kit was confirmed by PCR using specific primer for the mitochondrial gene cytochrome C. The sequences of primers are 5' GCCAACCGTGAAAAGATGAC 3' (Forward Primer) and 5' AGCCACCAATCCACACAGAGTA 3' (Reverse Primer). Total volume (25µL) of reaction mixture, containing 1µL of forward and reverse primers (each at 0.5mM), 1µL of 10 mM dNTPs (dATP, dCTP, dGTP and dTTP), 1.52µL of MgCl2 (24mM), 0.1µL of Taq DNA polymerase, 2µL of Taq buffer, 2µL of isolated mtDNA, along with 16.4µL of sterile distilled water. All the samples were subjected to 30 cycles of amplification, consisting of denaturation at 94ºC for 30 seconds, annealing at 60ºC for 30 seconds, and extension at 72ºC for 1 minute

Sample preparation for 8-Hydroxyl-2-deoxyguanosine (8-OHdG)

Extracted mtDNA samples were hydrolyzed to deoxy ribonucleotides before performing ELISA. The mtDNA samples were heated at 95◦C for five minutes and immediately immersed in ice. Sodium hydroxide and sodium acetate were used for the purpose, and then treated with DNase 1 for 1 minute at room temperature. This was centrifuged at maximum speed for 1 min and used for assay.

8-OHdG detection by ELISA

8-OHdG in rat liver DNA digests was determined by a competitive immunosorbent assay using New 8-OHdG check (Japan Institute of Control of Aging, Japan). Samples pretreatment included dissolving 50µg of mtDNA in 100µL water and denatured into single stranded by incubating at 95°C for 5min and rapid chilling on ice. These samples were then digested into nucleosides, by incubating the denatured DNA in 0.5M sodium acetate (pH 5.1) and 1.25µL of magnesium chloride along with 1µL of DNase 1 for 10 minutes at room temperature.

Fifty microlitres of the standards (0.5, 2, 8, 20, 80, and 200 ng/mL) and the mtDNA samples were added into the wells (96-well plate, Nunc). 50µL primary antibody was added to the wells (with

shaking) and kept for overnight at 4°C after sealing with an adhesive tape. The solution from the well was removed by tapping onto an absorbent towels several times. The assay plates were then washed with 250µL of wash buffer (3 times). 100µL of the secondary antibody was then added to the wells and the assay plate was sealed tightly with an adhesive tape and incubated for 1 hr at 37°C. After the lapse of time the washing steps were followed to remove the excess antibodies. One hundred microlitre of chromatic solution (3,3,5,5-tetramethyl- benzidine) was added to the wells followed by incubation for 15 min at room temperature in dark. The chromatic solution changed the colour to blue and the reaction was stopped by adding 100µl of 2M H_2SO_4. The absorbance was measured at 450 nm (reference wavelength-620 nm). The standard curve was used to determine the amount of 8-OHdG present in the samples.

Antioxidant assays

Preparation of Liver Homogenate

For the anti oxidant studies, 10% liver tissue homogenate (Phosphate buffer, pH 7.4) was prepared from the untreated and treated animals of both the concentrations (300 and 2000mg/kg body weight). The homogenate was then centrifuged at 3500 rpm for 10 min at 4°C in a refrigerated centrifuge. The resultant supernatants were maintained in an ice bath and are used as the sample for the estimation of total protein, lipid peroxidation, glutathione reductase, reduced glutathione, glutathione peroxidase and superoxide dismutase as per the standard protocols with slight modifications.

Total Protein

Total proteins in the sample were estimated using bovine serum albumin as standard according to the protocol described [23].

Lipid Peroxidation (LPO)

The extent of lipid peroxidation in the above prepared liver homogenate was determined as described [24]. This method is based on the increase in malondialdehyde formation in response to increased peroxides within the cell. The malondialdehyde (MDA) thus produced reacts with thiobarbituric acid reactive substances (TBARS) forming a complex. The amount of MDA formed was measured spectrophotometrically at 532 nm.

Glutathione reductase (GR)

GR activity of liver homogenate was determined by measuring the reduction of GSSG in the presence of NADPH as described in [25] with slight modification. Briefly, this assay measures the rate of NADPH oxidation to NADP+, which is accompanied by a decrease in absorbance at 340nm, which can be monitored spectrophotometrically. Thus, one GR unit is defined as the reduction of one µM of GSSG per minute at 25°C and pH 7.6.

Reduced glutathione (GSH)

The concentration of reduced GSH was determined by the method [26], with slight modifications in which DTNB (5, 5'-dithiobis- (2-nitrobenzoic acid), reacts with GSH to form a spectrophotometrically detectable product that absorbs at wavelength of 412 nm. The change in absorbance at 412 nm is directly proportional to the GSH concentration in the reaction mixture. The amount of GSH present in the sample was expressed as nmol/ mg protein.

Glutathione peroxidase (GPx)

Activity of GPx in liver homogenate was assayed by the method described in [27]. This enzyme catalyzes the formation of glutathione disulfide (GSSG) from GSH. The remaining GSH after the enzyme catalyzed reaction complexed with DTNB, which absorbs at maximum wavelength of 412 nm. Enzyme activity was expressed as µg of GSH consumed /min/mg protein.

Superoxide dismutase assay (SOD)

Activity of superoxide dismutase, an enzyme that dismutates the superoxide anion radical was assessed as explained [28]. Under higher pH, Pyrogallol auto oxidizes in aqueous solution. The method is based on the inhibition of superoxide dismutase (SOD) on pyrogallol auto oxidation which was observed by decrease in absorbance at 420nm.
All measurements were carried out using UV Spectrophotometer-1601, Shimadzu, Japan.

RESULTS

Synthesis and Characterization of nanomaterials

Dextran coated ferrite nanoparticles

Ferrite nanoparticles were prepared by the standard co-precipitation method and coated with dextran to yield dextran coated ferrite nanoparticles. The synthesized nanoparticles were characterized by standard techniques. The size of the nanoparticles was determined by TEM and DLS. TEM image indicated a very uniform size distribution of DFNM particles that was found to be less than 25nm (Figure 1A). However the hydrodynamic size of these particles obtained from DLS showed that it lies between 56nm and 232nm (Figure 1B).
The XRD peaks of DFNM matches with the standard pattern of the magnetite, Fe3O4 (ICDD number-PDF01-071-6336). The refinement of XRD spectra (Figure 1C) indicated that no other phases except the magnetite are detectable. Thus the phase purity of the ferrite particles was found to be good.
The FTIR spectra of DFNM (Figure 1D) exhibited the characteristic absorption bands of polysaccharide such as 3420cm^{-1} due to the O–H stretching, 1630cm^{-1} due to water molecule bending and the absorption bands corresponding to the stretching vibration of tetrahedral iron atoms (bands below 800cm^{-1}) revealing that the coating of dextran on the iron oxide particles are effective since the spectrum of the dextran coated iron oxide matches with the pure dextran above 800cm^{-1}, while below 800 cm^{-1} it matches with the pure iron oxide spectrum.
The weight loss curve of DFNM was obtained using a thermogravimetric analyzer. For DFNM, two weight loss stages were observed in thermograms. The first weight loss (2.89%) is due to the evaporation of water which comes around 135ºC. The second stage of decomposition observed between 160ºC to 345ºC (Figure 1E), with a total loss of 6.65% (weight loss of dextran is 3.76%). The decomposition temperature of pure dextran occurs at 260ºC, while that of coated dextran starts its decomposition at 345ºC.

Hydroxy apatite nanoparticles

HANPs were synthesized by wet chemical method. Precipitation was carried out at a pH of 11 in 0°C for 2h. After ageing for 24h, the precipitate was washed in distilled water, freeze-dried and calcined at 300°C. The calcined precipitate was ball milled and sieved. The obtained HANPs were characterized by standard techniques. The average size of HANPs was found to be below 50 nm (Figure 2A) by TEM analysis. The zeta potential of the HANP in buffer was found to be high (-13.4 mV) indicating that it is stable in buffer. The HANP was found to be unstable in water (zeta potential was low 3.19 mV).
The X-ray diffraction pattern of the HANP (Figure 2B) matches with the standard pattern of the Hydroxyapatite (ICDD NO-PDF00-009-0432). Thus the phase purity of the HANP was found to be good.
The Fourier transform infrared spectral analysis of HANP showed that the hydroxyl stretching was observed at 3568cm^{-1} and the different phosphate vibrations were observed at 1090, 1036, 962 and 476 cm^{-1} (Figure 2C) and thus the presence of phosphate and hydroxyl group confirms the chemical nature of Hydroxyapatite.
The SEM/EDS of HANP in contact with the L 929 cells showed live cells with small white particles attached (Figure 2D). This portion was spot analyzed by EDS (Figure 2E) which confirmed it to be hydroxyapatite with corresponding calcium and phosphorus peaks.

In vitro **Studies**

Cytotoxicity test

Direct contact of both the DFNM and HANPs on L929 cells was performed to study the effects on cellular morphology and viability. The cells have typical spindle shaped morphology. It was observed that even after 24 h of nanomaterials exposure at varying concentration, the spindle shaped morphology was preserved (Figure 3A & B). Different concentrations (10, 25, 50, 100, 200, 400, 600 and 800µg/mL) of DFNM and HANPs (10, 25, 50, 100, 200, 400, 600 and 700 µg/mL) were tested for their cytotoxicity on L-929 fibroblast cells using MTT assay and the results indicates that, more than 80% cells were found upto a concentration of 600 µg/mL of DFNM and HANPs (Figure 4A & B).

***In vitro* antioxidant assay**

LPO

Figure 5A represents MDA production in the rat liver homogenate (*in vitro*) incubated with the different concentrations of DFNM. It was found that the level of LPO induced by the control and DFNM treated is 25.78 ± 0.976 and 35.94 ± 5.932 nmoles/mg proteins respectively. There was a significant increase in the level of LPO observed when treated with 100µg/mL of DFNM.

A slight increase in LPO was observed in HANPs exposed liver homogenate as evident by increase in malondialdehyde content with respect to the control group (24.45±1.29) (Figure 5B). LPO production at different concentration of HANPs (12.5, 25, 50 and 100µg/ml) were 25.28±1.09, 26.30±0.98, 27.79±1.72 and 28.35±2.68 respectively.

GSH

A decrease in the level of GSH was observed in the liver homogenates incubated with increasing concentration of the DFNM. The level of GSH was found to be 2.710 ± 0.018 and 2.660 ± 0.049 nmol/mg in control and 100µg/mL of DFNM respectively (Figure 6A), which was not significant. For HANPs treated liver homogenates, no changes observed in GSH level compared to control 0.872±0.15 nmol/mg protein (Figure 6B).

GR

The activity of GR was found to be slightly decreased when the liver homogenate was exposed to different concentration of DFNM *in vitro*. GR activity in control samples was found to be 0.296 ± 0.026 and 0.259 ± 0.026 at 100µg/mL of DFNM (Figure 7A).

In case of HANPs exposed liver homogenate (*in vitro)*, GR activity was similar (0.329+0.05) to the control (0.328±0.02) (Figure 7B)

GPx

It was found that there was a reduction in GPx activity when compared to control group but was not significant (0.050 ± 0.005 control and 0.036 ± 0.009 for 100µg/mL of DFNM (Figure 8A).

A concentration dependent increase in the activity of GPx was observed in liver homogenate exposed to different concentration of HANPs (Figure 8B).

SOD

A slight insignificant alternation in SOD activity was observed when the liver tissue homogenate was treated with different concentrations of DFNM under *in vitro* conditions (Figure 9A).

For HANPs treated samples, concentration dependent increase in activity was observed (Figure 9B).

***In vivo* studies**

Acute toxicity (OECD Guidelines 420)

The animals exposed to 300 and 2000 mg/kg body weight of DFNM and HANPs were observed periodically during the first 24 hours, and thereafter daily for 14 days. Appearance of skin, fur, eyes, respiratory, autonomic and central nervous system were observed during these days. It was found that all the treated animals were normal during the observation period. None of the animals

showed any abnormal behaviors like tremor, convulsion, salivation, diarrhoea, lethargy, sleep or coma. There was no loss in body weight. At the end of observation period all animals were sacrificed and subjected to gross examinations. It was observed that none of the animals' internal organs were abnormal.

Haematological and Biochemical parameters

At the end of experimental period, blood from all the animals exposed to DFNM and HANPs was collected and subjected to the analysis of haematological (WBC, RBC, Haemoglobin, hematocrit, MCV, MCH, MCHC) and biochemical (glucose, cholesterol, total bilirubin, albumin, chloride, total protein, creatinine, urea, SGPT, SGOT, alkaline phosphatise, GGT) parameters.

Table 1A indicates the haematological parameters of DFNM exposed groups. Slight fluctuation was seen when compared to control values, but the changes were not statically significant, when compared to control values. The report of biochemical parameters were given in the Table 1B. It was noted that, there was a slight changes in some of the biochemical values (statistically significant, p value <0.05) between control and treated groups**.**

Table 2A shows the hematological parameters and it was found that the hematological values of all animals exposed to 300 and 2000mg/kg of HANPs was similar to that of control values.

Table 2B indicates the report of biochemical parameters and suggests that there was no significant alternations observed in the biochemical values of treated and control animals. All the biochemical values are well under normal range except for a marked increase in alkaline phosphatase where the control value was 135.667±2.082 and that of 2000 mg/kg body weight was 207.333±17.01. A 'p value' less than '0.05' were considered significant.

Antioxidant assays

LPO

An increase in lipid peroxides level was observed in liver tissue homogenates of DFNM treated animals (Figure 10A). The mean value of LPO was found to be 4.61± 0.71 (300mg/kg), 4.82±0.07 (2000mg/kg) whereas in control group it shows only 3.42±017.

The level of lipid peroxidation was significantly higher in the liver of animals exposed orally with HANPs (300 and 2000mg/kg body weight) in comparison with the control animals. It was shown that 8.842±1.756 (nmols/mg protein) of MDA was produced when 2000mg/kg body of HANP was exposed to rats, where as the LPO induced in the control animals was 3.497±0.079 (Figure 10B) .

GSH

No change was measured in GSH concentration in the group treated with the low dose (300mg/kg) of DFNM (1.10±0.10) when compared to control non treated one (1.10±0.02), whereas a slight increase in GSH level (1.42±0.25) was obtained in high dose of DFNM (2000mg/kg) treated groups that was found to be insignificant on comparison (Figure11A).

Liver of animals, which were administered orally with HANPs, showed comparable GSH levels (1.119± 0.0448) for low dose groups (Figure 11B) in relation to control (1.104±0.021). Whereas there was a slight increase in GSH concentration observed, when 2000mg/kg (1.344±0.288) of HANPs was administered to rats.

GR

The activity of GR increased in a concentration dependent manner in DFNM treated. The GR activity increased in 2000mg/kg exposed group (0.526±0.03) with respect to control (0.286±0.02). (Figure 12A) The mean value observed in 300mg/kg treated group was 0.456±0.07. Both are statistically significant (p value <0.05).

Likewise, concentration dependent increase in GR activity was observed in the liver of HANPs treated rats (Figure 12B). The activity in control was 0.286±0.019 and that in 2000mg/kg body weight was 0.523±0.048 units/mg protein (p<0.05).

GPx

The GPx activity remains the same in both the DFNM treated as well as control group (Figure 13A). The mean values were (0.121±0.03), (0.144±0.03) and (0.112±0.001) for 300mg/kg, 2000mg/kg treated groups and the control respectively.
A noticeable increase in the activity of GPx was observed in the liver with respect to control (0.1125±0.002) in both 300 and 2000mg/kg HANPs exposed rats. The GPx activity was 0.133±0.0213 and 0.179±0.014 in 300 and 2000 mg/kg respectively (Figure 13B).

SOD

SOD also showed dose dependent increase in activity in DFNM exposed groups. The mean value for the 300mg/kg exposed group was 0.109±0.008 and for the 2000mg/kg exposed group was 0.119±0.025 (Figure 14A). Both the values were higher than the control (0.084±0.002) values.
The SOD activity was almost similar in 300 and 2000mg/kg HANPs exposed rats (300mg/kg- 0.119±0.009 and 2000mg/kg- 0.120±0.001) but there was slight increase in comparison to control (0.086±0.009) animals (Figure 14B).

28 days repeated dose dermal toxicity (OECD Guidelines 408)

Different concentration (25, 50 and 100mg/kg) of DFNM and HANPs was exposed to Wistar rats on dermal route for a period of 28 days continuously. The general physical conditions of the experimental animals were normal. The increase in body weight and feed intake were normal and none of the animals showed any abnormality or behavioral changes during the experimental period.
At the end of experimental period, blood was subjected to analyze the haematological (WBC, RBC, Haemoglobin, hematocrit, clotting time, platelet count, MCV, MCH, MCHC) and biochemical (glucose, cholesterol, triglycerides, total bilirubin, albumin, calcium, phosphorous, chloride, total protein, creatinine, urea, SGPT, SGOT, alkaline phosphatise, GGT) parameters. Table 3A showed the haematological parameters, and found that the haematological values of animals exposed to different concentration of DFNM was similar to that of control values, except a slight reduction in RBC which was under normal range (control is 8.08±0.85 and 100mg/kg DFNM is 7.14±0.88). Similarly there was no alternations observed in the biochemical values of treated and control animals Table 3B. All the biochemical values are well under normal range except one incidence on albumin at 25mg/kg of DFNM.
Gross examination of carcasses of control and different concentrations of DFNM exposed rats (male and female) did not reveal any abnormality in the organs examined.
Histopathological (heart, liver, lungs, kidneys, spleen, adrenals, pancreas and skin) examination did not reveal any abnormalities in the sections examined except three cases of bronchial associated lymphoid tissue proliferation and one case of focal changes of pneumonia in the lung in the control group (Figure 15 A & B). The carcasses of DFNM dermally exposed rats did not reveal any abnormality in the sections examined except one case of alveolar pneumonia, three cases of bronchitis, two cases of bronchitis with bronchial associated lymphoid tissue proliferation and one cases of bronchial associated lymphoid tissue proliferation in the lung. There was presence of brown particles in superficial keratohyalin layer, epidermal layers and dermis appeared normal in all animals.
Haematological values of animals (Table 4A) exposed to different concentration of HANPs was well comparable with control values, except a slight reduction in haemoglobin, platelets and MCH and is under normal range. Similarly there was no alternations observed in the biochemical values of treated and control animals. All the biochemical values are well under normal range except one incidence in chlorides, total proteins and alkaline phosphatase (Table 4B).
Gross examination of carcasses of male and female control and treated groups animals did not reveal any gross abnormality in the major organs examined, such as heart, liver, lungs, spleen, and skin. Skin revealed focal pale hairless area observed in the control group animals. Similarly, skin revealed focal pale hairless brownish stained area observed in the dorsum of treated animals.
Histopathological examinations of the skin of control group rats revealed hyperkeratosis in all cases, four cases of spinous layer with multiple basophilic pigment layers and five cases of intracytoplasmic vacuolation and edema in basal cells was observed. In dermis, mild edema and

mild infiltration was noted in all cases. Five cases were revealed focal area of hypotrichosis. Sebaceous gland was appeared normal in all cases (Figure 16).

Skin of treated group rats revealed hyperkeratosis in all cases, spinous layer with multiple basophilic pigment layers and intracytoplasmic vacuolation and edema in basal cells in all cases was observed. In dermis, mild edema and mild infiltration of mononuclear inflammatory cells (histiocytes and lymphocytes) infiltration was noted in all cases. All cases revealed focal area of hypotrichosis. Sebaceous glands were appeared normal in all cases. Seven cases of bronchial associated lymphoid tissue proliferation were noted in the lungs. All other organs examined did not reveal any abnormality in both the groups (Figure 16).

Antioxidant Assays

LPO

The level of LPO production was found to be 37.41±3.69 nmoles/mg protein in liver homogenate of rats exposed to (100mg/kg) through dermal route (Figure 17A). The induction of LPO in all concentrations (25, 50 and 100mg/kg) of DFNM was comparable with control values. Figure 17B defined the results of the LPO and shown that the LPO was slightly reduced in the 25mg/kg (9.13±0.46 nmoles/mg proteins) and 100mg/kg (10.19±5.53 nmoles/mg proteins) HANPs exposed groups. However there was an increase in LPO observed in the 50mg/kg HANPS exposed group (11.05128±2.79 nmoles/mg proteins), when compared to control (nmoles/mg proteins).

GSH

The GSH induced in liver homogenate of rats used for dermal toxicity (100mg/mL) was 38.840±3.400nmol/mg (Figure 18A), which is similar to that of control values (41.520±3.080nmol/mg) and was not significant.

The results of the GSH are shown in the Figure 18B. It was found that a decrease in the level of reduced glutathione was observed in the liver homogenates of rats exposed to HANPs dermally. The level of GSH was found to be reduced in 25mg/kg (0.85±0.17nmol/mg) and 100mg/kg (0.63±0.18nmol/mg) of HANPs exposed groups respectively, however the GSH was slightly increased in the 50mg/kg exposed group (1.06±0.48nmol/mg), when compared to control (0.98±+0.18nmol/mg) and was not significant.

GR

A slight increase in GR was observed (Figure 19A) up to a dose of 100mg/kg of DFNM and the GR values (0.369±0.016) was comparable with control values (0.335±0.026) in liver homogenate of rats used for dermal toxicity.

The level of GR was slightly reduced in 25mg/kg (0.22±0.02units/mg protein), 50mg/kg (0.19±0.0102units/mg protein) and 100mg/kg dose (0.19±0.0102units/mg protein) of HANPs exposed groups (Figure 20), when compared to control group (0.25±0.0202units/mg protein) and was not significant.

GPx

Dose dependent increase in GPx activity was observed in the liver homogenates of rats exposed to DFNM via dermal route. The values are found to be 0.100±0.015 for 25mg/kg, 0.129±0.012 for 50mg/kg and 0.131±0.016 for 100mg/kg (Figure 19B). These values were not significant when compared to control values.

It was also found that the level of GPx was found to reduce in low, medium and high dose groups respectively, when compared to control (Figure 20). The results indicated that there was a dose dependant decrease in the level of GPx observed at 25mg/kg (0.042±0.001), 50mg/kg (0.032±0.001) and 100mg/kg (0.035±0.014) of HANPs. These values were not significant when compared to control values.

SOD

The SOD activity seems to be decreased (0.232±0.046) in DFNM exposed rats compared to control (0.301±0.017) but was not significant (Figure 19C).

The result of the SOD production indicated that, there was a slight decreased when 25, 50 and 100 mg/kg body weight of HANPs exposed groups (0.086±0.011, 0.084±0.003, 0.077±0.007), (Figure 20) when compared to control group (0.088±0.001) and was not significant.

Immunological Studies

Delayed hypersensitivity by Guinea pig maximization test (ISO 10993-10: 2002/Amd.1:2006(E)

The appearance of the challenge skin sites of both the test (DFNM and HANPs) and control G. pigs were observed at 24h, 48h and 72h after removal of dressings and patches after challenge dose. The animals used for the study did not show any abnormalities during the experimental period. Result of the study (Table 5A) indicated that the DFNM fails to elicit any skin sensitization potential in G. pigs. The skin irritation score designated at DFNM treated and control areas are '0'. The skin reactions for erythema and edema were scored and recorded the numerical grading as per ISO 10993-10: 2010 (E).

Also, Guinea pigs exposed to HANPs did not show any abnormalities during the experimental period. The appearance of the challenge skin sites of test and control Guinea pigs were observed at 24 h, 48 h and 72 h after removal of dressings and patches. The result of the delayed hypersensitivity assay is shown in table 6A and it was found that the HANP did not elicit any skin sensitization in Guinea pigs when compared to control animals. The skin irritation score designated for the HANP treated and control areas are '0'. The skin reactions for erythema and oedema were scored and the numerical grading was recorded as per ISO 10993-10 [23].

Hematological parameters

The hematological values were indicated in the Table 5B. It was noted that all hematological values were under normal range in DFNM challenged animals. Whereas in case of HANPs challenged animals, there was a significant increase in RBC count, hemoglobin and hematocrit level and a significant decrease in platelet count when compared to control animals.

Table 6B indicates that the WBC count of HANP treated animals were well comparable with the control and was within the normal range. There was a significant increase in RBC count, hemoglobin and hematocrit level and a significant decrease in platelet count was observed in HANP treated group when compared to control animals.

Biochemical parameters

No significant difference in the biochemical values were observed in DFNM challenged groups on comparison to control (Table 5C). The fluctuations in glucose (Test: 179.54±46.29 and control: 160.12±41.35) and ALP (Test: 116.80±18.54 and control: 131.60±15.81) values were also under normal range. Animals treated with HANP showed a slight decrease in SGOT, glucose and urea than that of control animals. The variations observed in SGPT and ALP values were within the normal range. Cholesterol value in test and control groups were lower than the normal range and are not significant. All other biochemical parameters were found to be comparable with control.

The animals treated with HANP showed a slight decrease in SGOT, GLU and Urea than that of control animals. The variations observed in SGPT and ALP values were within the normal range. Cholesterol value in test and control groups were lower than the normal range and are not significant. All other biochemical parameters were found to be comparable with control (Table 6C).

Antioxidant Assays

LPO

The amount of LPO production was found to be 22.69±4.12 and 33.61±5.14 in liver and brain homogenates of DFNM challenged G. pigs (Figure 21A, B) compared to control 21.66±0.48 (liver) and 48.57±9.94 (brain). The level of MDA in HANP challenged animals (liver: 17.01 ± 3.36, brain: 17.67 ± 1.32) treated group was similar to that of control (liver: 16.38±1.06, brain: 18.69±1.87) group (Figure 21C) respectively.

GSH

The level of GSH is given in the Figure 22A & B. A slight increase in GSH level was observed in liver homogenates of DFNM treated animals (0.824±0.337 nmol/mg) when compared to control (0.782±0.121 nmol/mg). The level of GSH in brain homogenates was also increased (control-0.966±0.290 and Test: 1.586±0.553 nmol/mg) in DFNM exposed group, but is not significant.
The level of GSH in HANP treated animal depicted in Figure 22C was comparable to control animals. Following are the GSH values in control (liver: 1.82±0.49 and brain: 1.46±0.27) and HANP treated (liver: 1.52±0.38, brain: 1.82±0.14) groups.

GR

For DFNM exposed G. pigs, a slight decrease in GR activity was observed (Test: 0.852±0.274 and Control: 0.974±0.087) in the liver homogenates, whereas significant increase in GR activity was observed in brain tissue homogenate (Test: 1.064±0.097 and Control: 0.492±0.344) (Figure 23A & B).
For HANPs exposed G. pigs, the activity of GR was well comparable to control groups. The values are (Test: 0.526±0.180 and Control: 0.371±0.021) in the liver homogenate and (Test: 0.586±0.071 and Control: 0.665±0.160) in the brain homogenate (Figure 23C).

GPx

The results (Figure 24 A & B) indicated that there was a slight reduction in the level of GPx in liver (control: 0.025±0.001 and Test: 0.021±0.003) and brain (control: 0.039±0.008 and Test: 0.028±0.003) homogenate of DFNM treated G. pigs. These values were not significantly differs from control values.
There was no alternations in the level of GPx (Figure 24C) in liver and brain tissues of HANP treated group when compared to control group in liver (Test: 0.034±0.007 and Control: 0.047±0.004) and brain (Test: 0.111±0.047 and Control: 0.103±0.036) homogenates.

SOD

Figure 25A and B represents SOD activity in liver and brain homogenate of G. Pigs exposed to 80mg/animal of DFNM repeatedly. The activity of SOD was slightly reduced in both the liver (Test: 0.456±0.018 and Control: 0.480±0.005) and brain (Test: 0.538 ±0.014 and Control: 0.670±0.130) tissues.
The SOD activity analyzed in liver and brain tissues of G. pigs treated with HANPs is depicted in Figure 25C. The values are well comparable with the control both in liver (Test: 0.251 ± 0.073 and Control: 0.276 ± 0.070) and brain tissues (Test: 0.640 ± 0.069 and Control: 0.528 ± 0.025).

Immunotoxicity Study

The general physical conditions of the experimental animals were normal. The increase in body weight and feed intake was normal and none of the animals showed any abnormality or behavioral changes during the experimental period. Gross examination of carcasses of control and DFNPs exposed mice did not reveal any abnormality in the organs examined.

T and B lymphocyte proliferation assay

The proliferation assay was evaluated in both T and B lymphocytes isolated from spleen of animals exposed to DFNPs and HANPs at various time periods. The result of the proliferation assay in cells exposed to DFNM is mentioned in Figure 26 A& B. It was observed that at 7 days after exposure, a slight increase in thymidine incorporation with no significant difference was observed when compared to control. T cell showed significantly decreased proliferation only after 21 days of exposure, whereas B cells showed significantly reduced thymidine incorporation after 14 and 21 days of exposure on comparison to control.
In case of HANPs treated animals, the proliferation activity of T lymphocytes changes with time. For 7 days group, the proliferation increases (Figure 27) which subsequently decreases in 14 days followed by increased proliferation on 21 days. The proliferation of B lymphocytes seems to be decreased on all the three groups (7 days, 14 days and 21 days) compared to control.

Real time PCR analysis for determining m-RNA of specific cytokines

As evident from Figure 28A, the m-RNA expression for IL-10 in 600µg DFNPs is increased by 3 fold when compared with the similar concentration of bare ferrite particles. mRNA expression for IL-10 in 800 µg DFNPs is increased by 2 fold when compared with the similar concentration of

bare ferrite particles. It was also found that the DFNPs did not exhibit any change in the m-RNA expression of IL-1 when compared to bare ferrite particles (Figure 28B). Eventually there was a dramatic decrease in m-RNA expression of TNF-β in DFNPs particles in a concentration dependent manner when compared with bare ferrite particles. 800µg DFNPs showed decreased m-RNA expression of TNF-β when compared with its counterpart 800µg bare Ferrite particles (Figure 28C). The values are expressed in mean ± SE.

Combined chronic toxicity and carcinogenicity studies (OECD 453)

Different concentration of DFNM and HANPs were orally exposed to the animals for a prolonged period of 12 months. The nanomaterials were mixed with the commercially available feed using aqua guard filtered water and orally fed to animals daily. During the experimental period, the increase in body weight and feed intake were normal and none of the animals showed any abnormality or behavioural changes.

At the end of experimental period, blood was collected and subjected to analyze the haematological (WBC, RBC, Haemoglobin, hematocrit, clotting time, platelet count, MCV, MCH, MCHC) and biochemical (glucose, cholesterol, triglycerides, total bilirubin, albumin, calcium, phosphorous, chloride, total protein, creatinine, urea, SGPT, SGOT, alkaline phosphatise, GGT) parameters. Table 7A (i,ii) shows the haematological parameters of the rats exposed to DFNM. Biochemical parameters were noted in table 7B (i,ii). All the parameters were normal on comparison to control. Very few changes were observed in biochemical parameters of the treated group.

For HANPs treated rats, both the biochemical and the hematological values were normal except in few cases where statistical differences were observed. The values of the all the parameters were listed in the table 8A (i,ii) and 8B (i,ii).

Gross examinations of the carcasses of DFNM test group (low group, medium group, high group, and high recovery group) and control rats did not reveal any gross abnormality in the organs examined.

Histopathological examinations of carcasses of control groups did not reveal any abnormality in the sections examined except 14 cases of bronchial associated lymphoid proliferation, 2 cases each of collapsed alveoli and alveolar hemorrhages, 7 cases of thickened alveolar septa, 3 cases of compensatory emphysema seen as bulla, 4 cases of consolidation and one case of congestion. The tubular and glomerular hemorrhages were observed in about four cases and liver showed sinusoidal congestion and three cases and focal mononuclear cell infiltration in one case. One case each of cystic degeneration of endometrium and focal mononuclear cell infiltration in urinary bladder was observed. Similarly histopathological examinations of carcasses of test groups did not reveal any abnormality in the sections examined except seventeen cases of bronchial associated lymphoid tissue proliferation, one case of collapsed alveoli, sixteen cases of thickened alveolar septa, two cases of compensatory emphysema seen as bulla, three cases of consolidation and three cases of alveolar hemorrhage. The tubular and glomerular hemorrhages were observed in about five cases and cloudy swelling of renal tubules was observed in one case. The liver showed sinusoidal congestion in two cases and mild fatty changes in two cases. Meningeal congestion was observed in one case. The images were depicted in (Figure 29 A&B).

Gross examinations of the carcasses of HANPs test group (low group, medium group, high recovery group) and control rats also did not reveal any gross abnormality in the organs examined. However the gross examinations of high dose group did not reveal any abnormality in the organs except one rat showed a fibrous fluctuating mass at the base of the left ear and was considered as not pertaining to the test material.

Histopathological examinations of carcasses of all groups showed mild to moderate bronchial associated lymphoid tissue proliferation (BALT) proliferation in lungs, heamosiderosis in spleen and mononuclear cells infiltration in trachea. One case of control and high dose showed follicular hyperplasia of the mammary gland. Hyperplasia of C cells of thyroid gland and congestion of spleen was observed each in one control group animal. Lung of one control animal showed pneumonic lesions, pleurisy and consolidated alveoli whereas abscess was observed in two

animals. Liver in one control group animal showed a cyst and appeared fibrosed. kidney in one each case of control and high dose groups showed cystic tubular degeneration and fibrosis whereas mono nuclear cell collection was observed in four high dose group animals. Ovaries were found to be cystic in one animal of control as well as high dose groups. An abscess was observed in prepuce in one high dose group animal. Three cases of sub mucosal mono nuclear cell infiltration in intestinal tract were observed in high dose group (Figure 30 A& B).

Antioxidant Assays

LPO

Both the DFNM and HANPs did not induce lipid peroxides formation at the three different concentrations tested. The amount of peroxides formed in the rat liver after exposure to DFNM was much lower when compared to control (36.41±7.84). Both in male and female rats no significant increase in peroxide formation was observed (Figure 31A (i, ii)).

In case of HANPs treated male rats showed increased lipid peroxides formation compared to control and is not significant. Dose dependent increase was observed in male rats with the values of 16.97±2.91for low groups; 18.97±6.99 for medium groups. Similar increase in lipid peroxides was observed in female rats treated with HANPs which is not significant. The amount of lipid peroxides observed in the low groups was higher (14.69±3.12) than the medium (13.31±2.48) and high groups (8.24±1.36). In contrast to male rats, dose dependent decrease in peroxide formation (Figure 31B (i,ii)) was clearly visible in female rats exposed to HANPs.

GSH

Normal cellular glutathione level was maintained in the DFNM treated group. When compared to the control group (Male- 0.76±0.11; Female- 1.01±0.08), the GSH level remains the same in both the male and female groups except in case of medium groups of female rats which show significant increase in GSH level (0.39±0.02). The results are depicted in the Figure 32A (i,ii).

Cellular glutathione level seems to increases in a dose dependent manner in male rats exposed to HANPs. The values are depicted in the figure 32B (i,ii). HANPs did not influence any changes in GSH level in female rats. The total cellular level is maintained under normal range in the treated groups (low-1.88±0.01; medium- 2.07±0.09; high- 1.69±0.30) as similar to control (1.79±0.17).

GR

Both the increase and decrease in GR activity was observed in male rats exposed to DFNM (Figure 33A(i,ii)). The activity was found to be decreased in low group (0.44±0.03) where as increases or similar in medium (0.59±0.13) and high group (0.57±0.12) respectively on comparison to control (0.571±0.06). Female rats showed increased GR activity when compared to control (0.28±0.03) with significant increases observed in medium groups (0.39±0.02).

GR activity increases in male rats on exposure to HANPs with respect to increased GSH production observed. But the increase is not significant compared to control, whereas in female rats significant decrease in GR activity was observed in low group (0.51±0.03). Comparable activity was monitored in medium (0.61±0.07) and high group (0.61±0.06) relating to control (Figure 33B (i,ii)).

GPx

An obvious decrease in GPx activity was observed in male rats on exposure to DFNM (Figure 34A (i,ii)). The value was found to be 0.35±0.07 for low groups; 0.29±0.008 for medium and 0.52±0.02 for high groups. Significant increase in GPx activity was observed in female rats when exposed to DFNM (low- 0.22±0.01; medium- 0.22±0.02; high-0.17±0.03) compared to control (male- 0.50±0.03; female-0.14±0.02).

Significant changes were observed in GPx activity in male rats exposed to HANPs for a long period. The activity decreases in low (0.26±0.00) and medium groups (0.32±0.01) compared to control (0.41±0.01) whereas in high group, the activity seems to increases (0.44±0.01) but is not significant. Likewise, GPx activity was found to be decreased in female rats on comparison to control. The changes are illustrated in figure 34B (i,ii).

SOD

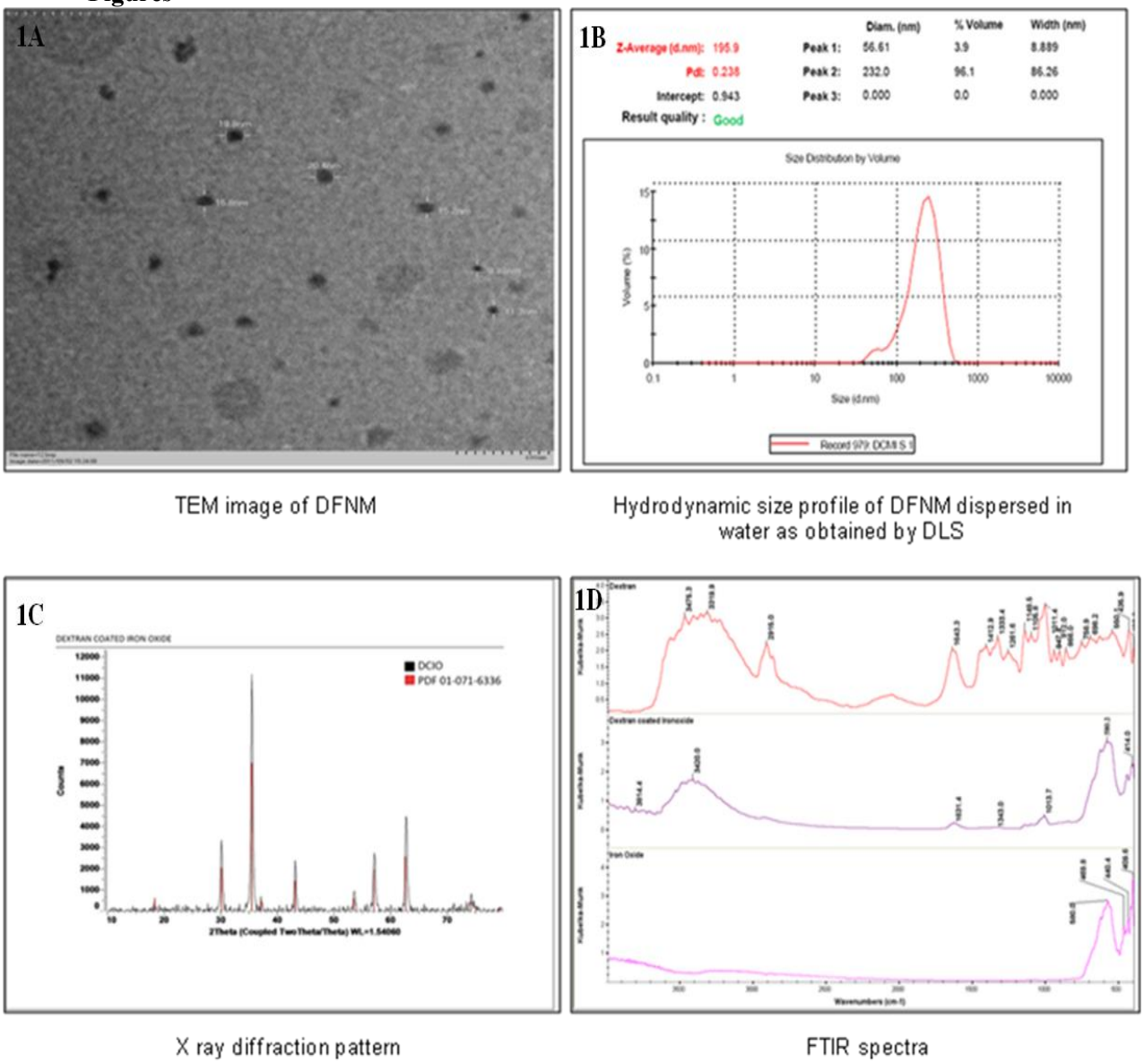
Increased SOD activity was observed in both the male and female rats exposed to DFNM. Significant increase was observed in medium (male-0.20±0.003; female-0.16±0.00) and high group (male-0.20±0.00; female-0.16±0.00) of both the cases. The graphs are demonstrated in figure 35A (i,ii).
Activity of SOD increases in high group of male rats (0.39±0.00) exposed to HANPs which is significant when compared to control (0.05±0.00). The activities in low and medium groups were almost comparable to control. Similarly in female rats, increased activity was observed in high groups (0.64±0.08). For low and medium groups the values were less (low- 0.13±0.02; medium-0.14±0.00) than that of control (0.47±0.05) (Figure 35B (i,ii)).

***In vitro* 8OHdG Analysis**
Oxidative DNA damage in mitochondrial DNA of liver samples of rats exposed to different concentration of DFNM (*in vitro*) was analyzed by detecting 8-OHdG concentration using competitive ELISA. Its concentration was calculated from the standard values of known concentration of 8OHdG. Figure 36A shows the level of 8OHdG induced in liver samples. It was found that the level of 8OHdG in 100µg/mL of DFNM (1.756± 0.010) exposed group was similar to that of control values (1.726± 0.010) and was not significant.
Figure 36B shows the level of 8OHdG formed on genomic DNA in rat liver exposed to different concentration of HANPs. It was found that the level of 8-OHdG in 100 µg/mL of HANP (22.48±1.23) exposed group was comparable to control values (16.58± 2.10) in *in vitro* conditions.
***In vivo* 8OHdG Analysis**
Genomic DNA was isolated from the liver samples of G. pigs exposed to both DFNM and HANPs. The level of 8OHdG observed on exposure to DFNM was slightly lower than the control values (9.00±1.16) (Figure 37A).
It was also found that the level of 8-OHdG on exposure to HANP (liver - 8.09±0.62) was slightly increased when compared to control (liver - 7.62±0.97) (Figure 37B) and is not significant, indicating that no oxidative stress was developed in liver of Guinea pigs.

ICP-MS analysis
The results obtained from the ICP-MS analysis indicated the time dependent increase in DFNM accumulation in all the three samples (spleen, kidney and liver) of immunotoxicity study. The deposition was found to be higher in liver and spleen samples compared to kidney. Similarly, increased amount of Fe content was observed in liver samples of chronic toxicity study than the control. Fe content was high in both the liver and kidney samples of high dose when compared to control. The values are listed in the Table 9 and 10.

Conclusions summarising the achievements and indication of scope for future work:
The synthesized nanomaterials DFNM of size <25 nm and HANPs of size below 50nm was found to be non-cytotoxic to L929 fibroblasts cells under culture conditions. Both the nanoparticles were characterized by standard characterization techniques. The nanoparticles were non-toxic when exposed to laboratory animals under different exposure conditions. The nanoparticles do not seem to induce oxidative stress mediated toxicity and no alteration in the normal physiological processes or any pathological lesions examined. All the animals exposed were normal and no behavioral changes were observed. No allergic or delayed hypersensitive reactions were observed. Thus it can be conclude that both the nanomaterials were non-irritant, non-toxic and biocompatible under laboratory conditions. These nanoparticles can be considered as unclassified compounds as per Globally Harmonized System for Classification (Category 5) for chemical substances and mixtures [39]. Absence of any molecular toxicity authenticates the development of safe nanomaterials which can be used for various biomedical applications. The use of safe and biocompatible nanomaterials is highly beneficial to the mankind as it greatly reduces the risk associated with their toxicity or adverse side effects.

Figures



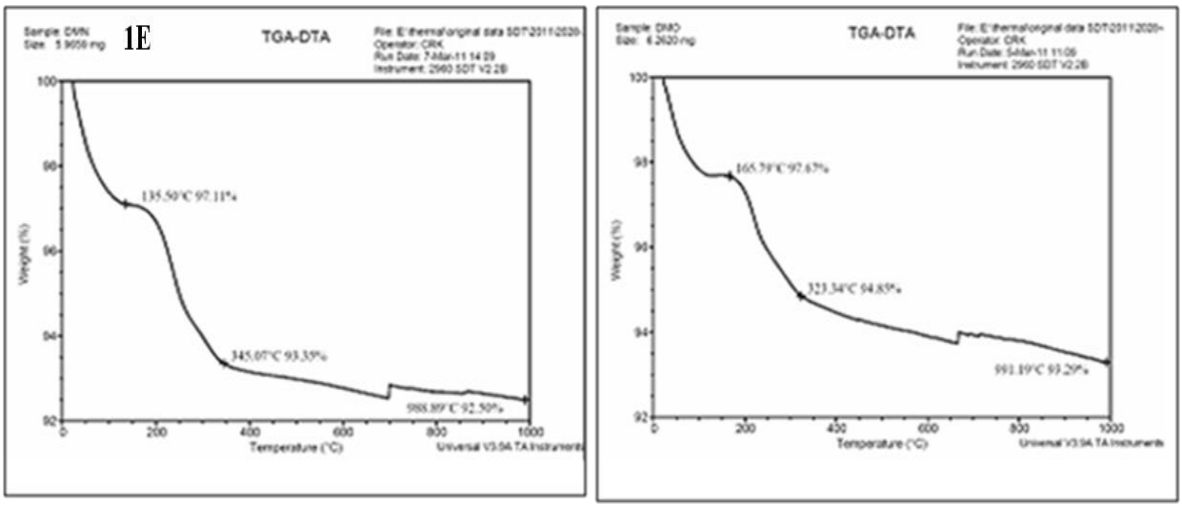
TEM image of DFNM

Hydrodynamic size profile of DFNM dispersed in water as obtained by DLS

X ray diffraction pattern

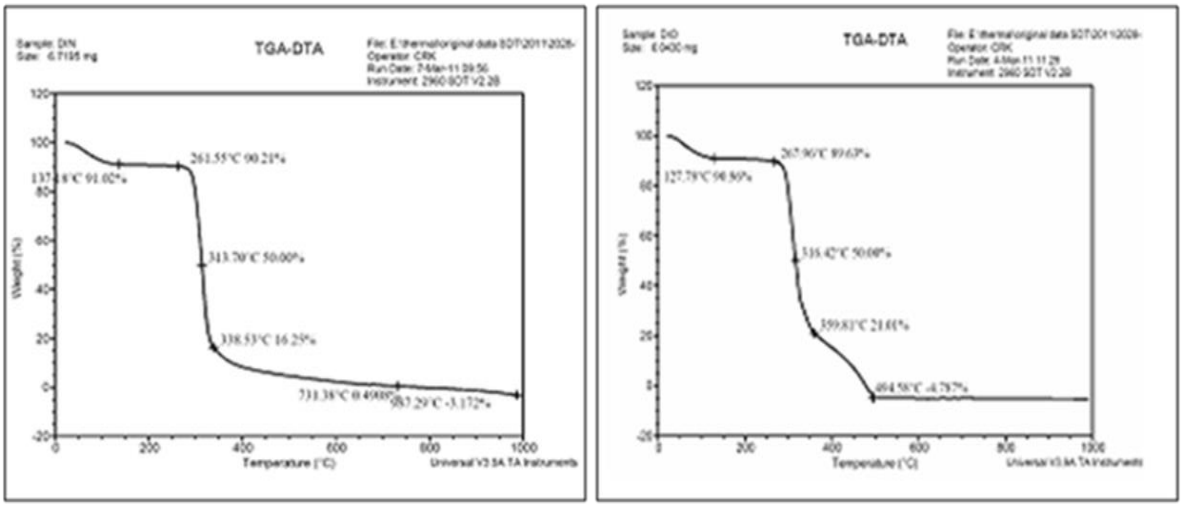
FTIR spectra

Fig 1: Characterization of DFNM nanomaterials by standard techniques



Dextran coated ferrite in Nitrogen atmosphere

Dextran coated ferrite in Oxygen atmosphere

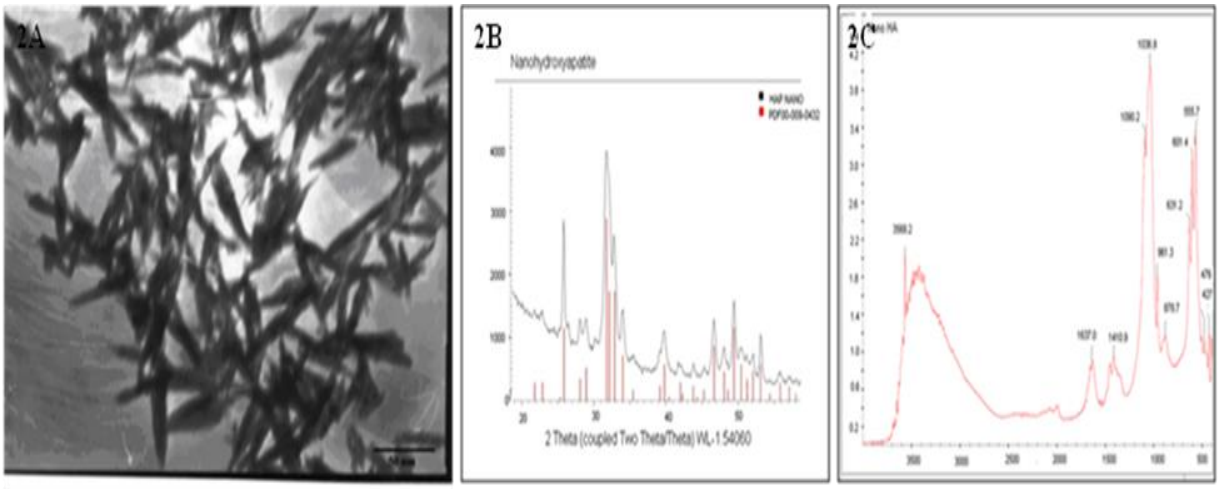


Pure dextran in nitrogen atmosphere

Pure dextran in oxygen atmosphere

Thermo gravimetric analysis of DFNM

Fig 1A: Characterization of DFNM nanomaterials by standard techniques



TEM micrograph of HANPs | XRD pattern of HANPs | FTIR Spectra of HANPs

SEM analysis of cells with HANP | Spot analysis (EDS) of HANP in cell suspension

Fig 2A: Characterization of HANPs by standard techniques

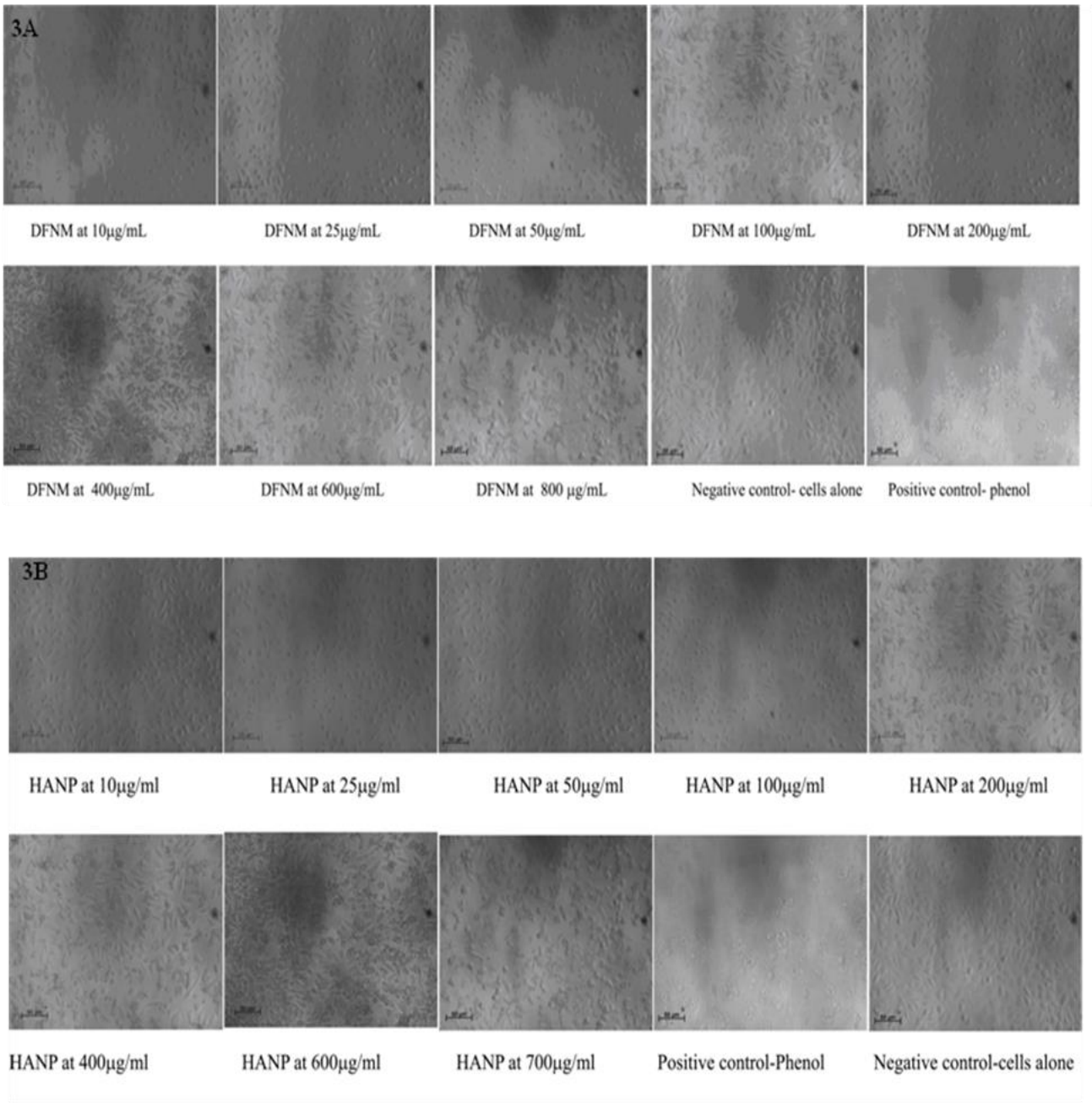


Figure 3: Morphological analysis of cells exposed to different concentration of nanomaterials for a period of 24 hrs.

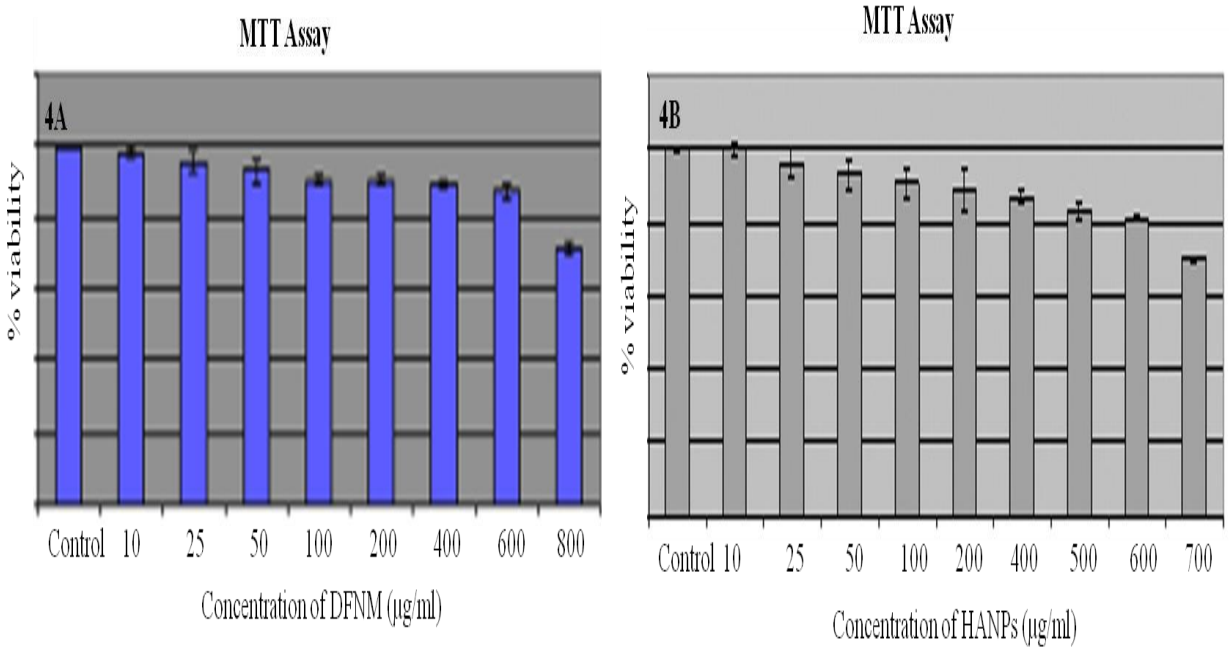


Figure 4: *In vitro* cytotoxicity assay of L929 cells exposed to different concentration of nanomaterials for a period of 24 hrs.

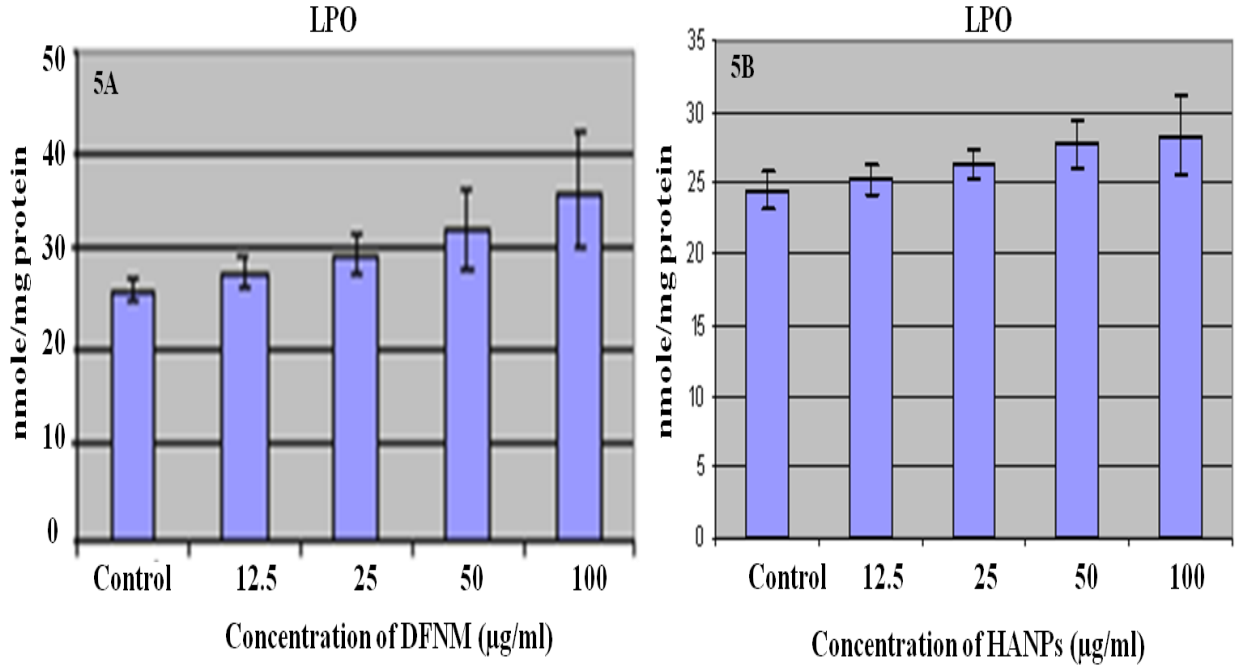


Figure 5: Amount of lipid peroxides observed in rat liver after nanomaterials exposure (*in vitro*)

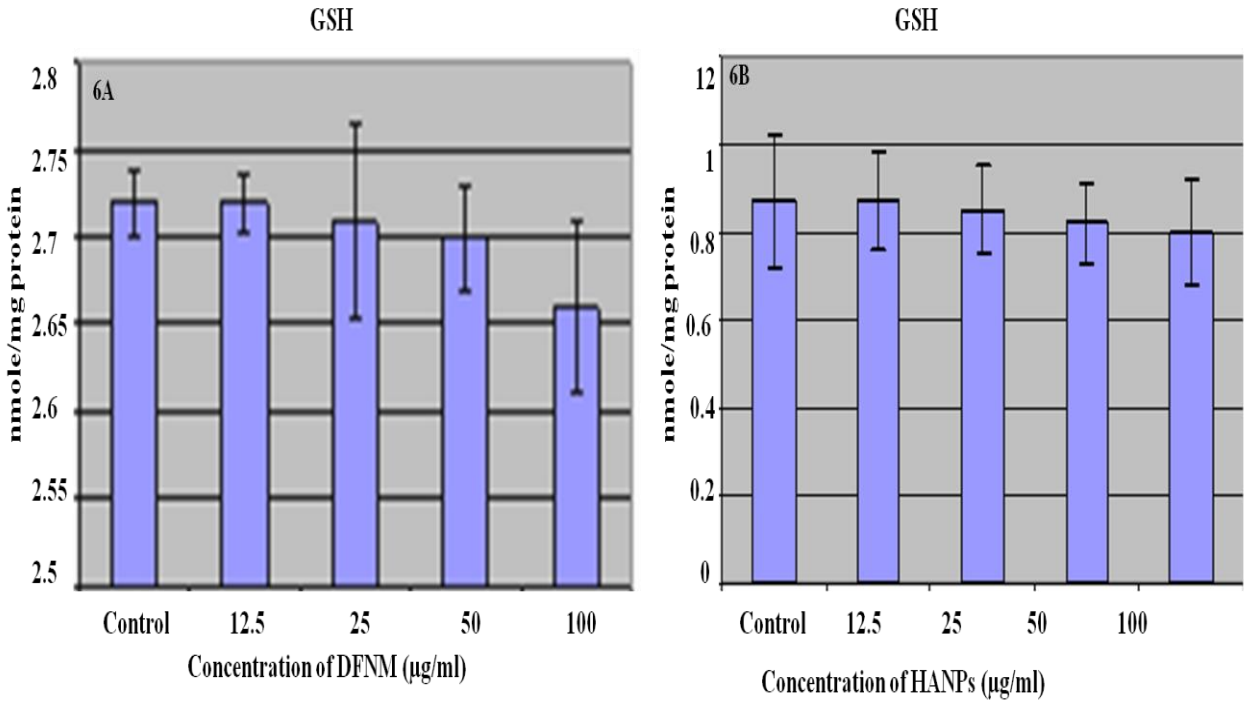


Figure 6: Amount of GSH observed in rat liver after nanomaterials exposure (*in vitro*)

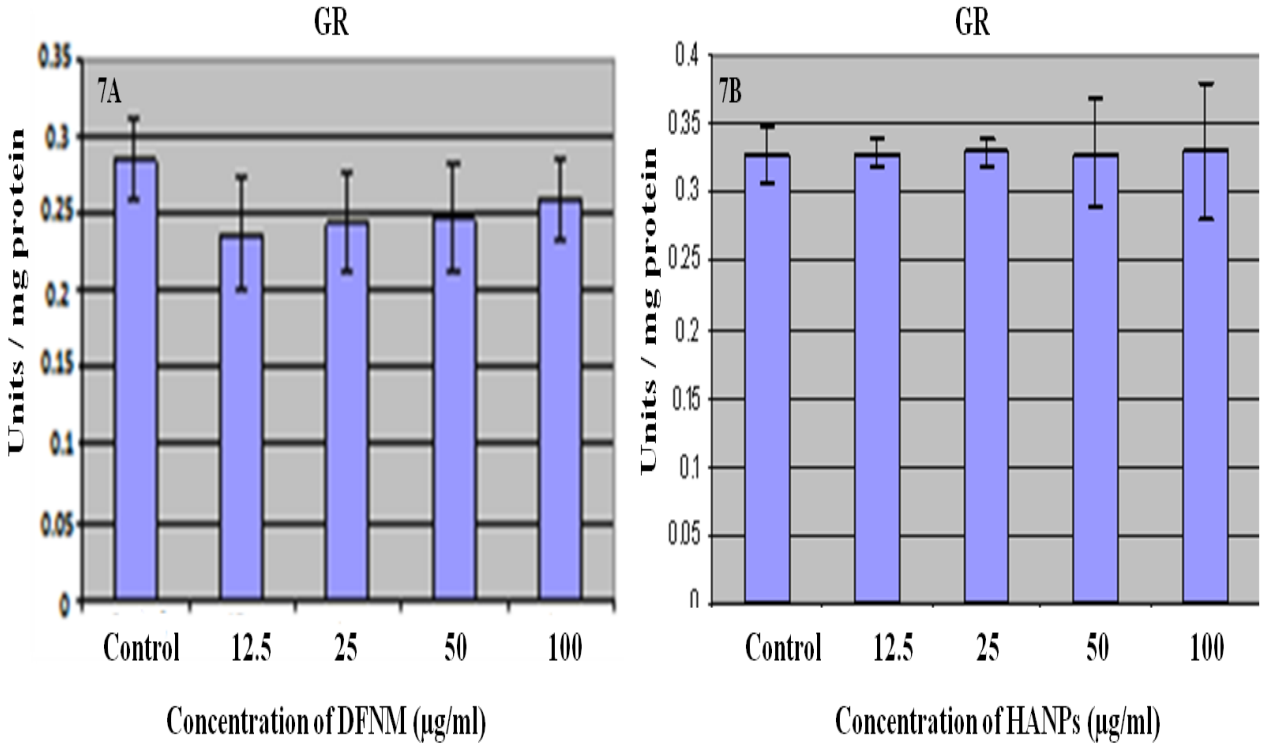


Figure 7: GR activity observed in rat liver after nanomaterials exposure (*in vitro*)

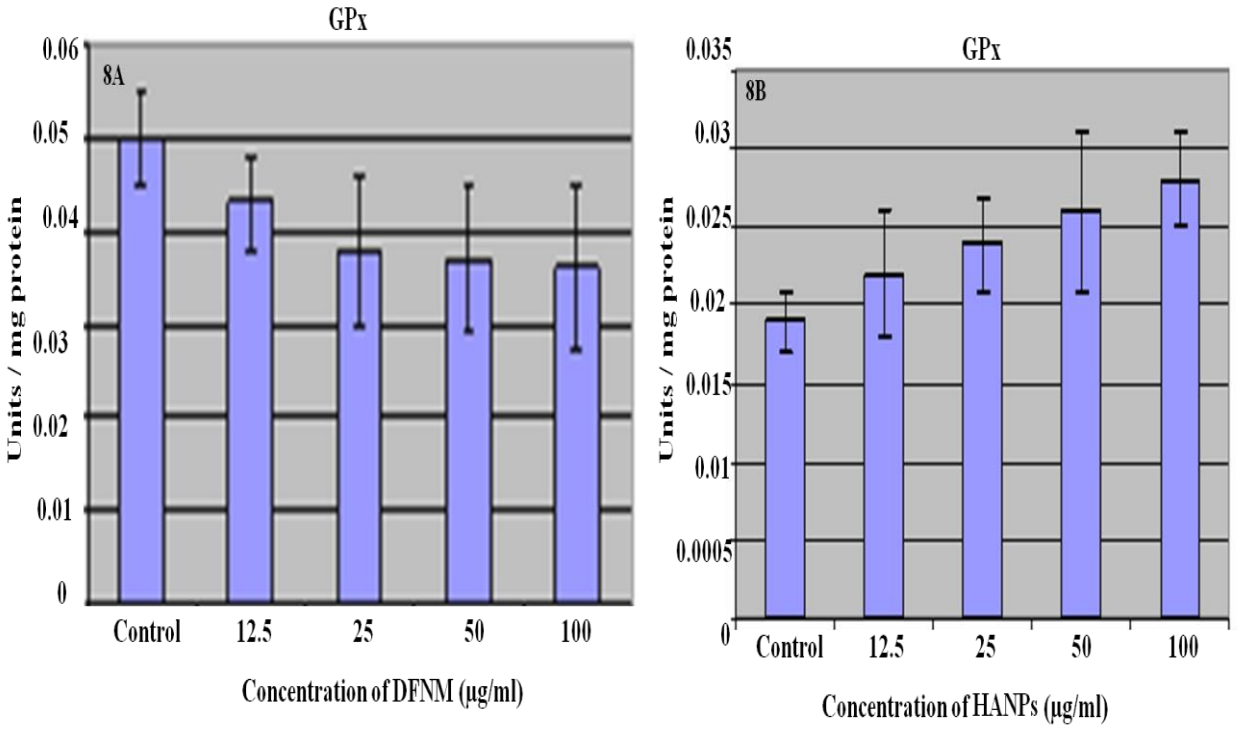


Figure 8: GPx activity observed in rat liver after nanomaterials exposure (*in vitro*)

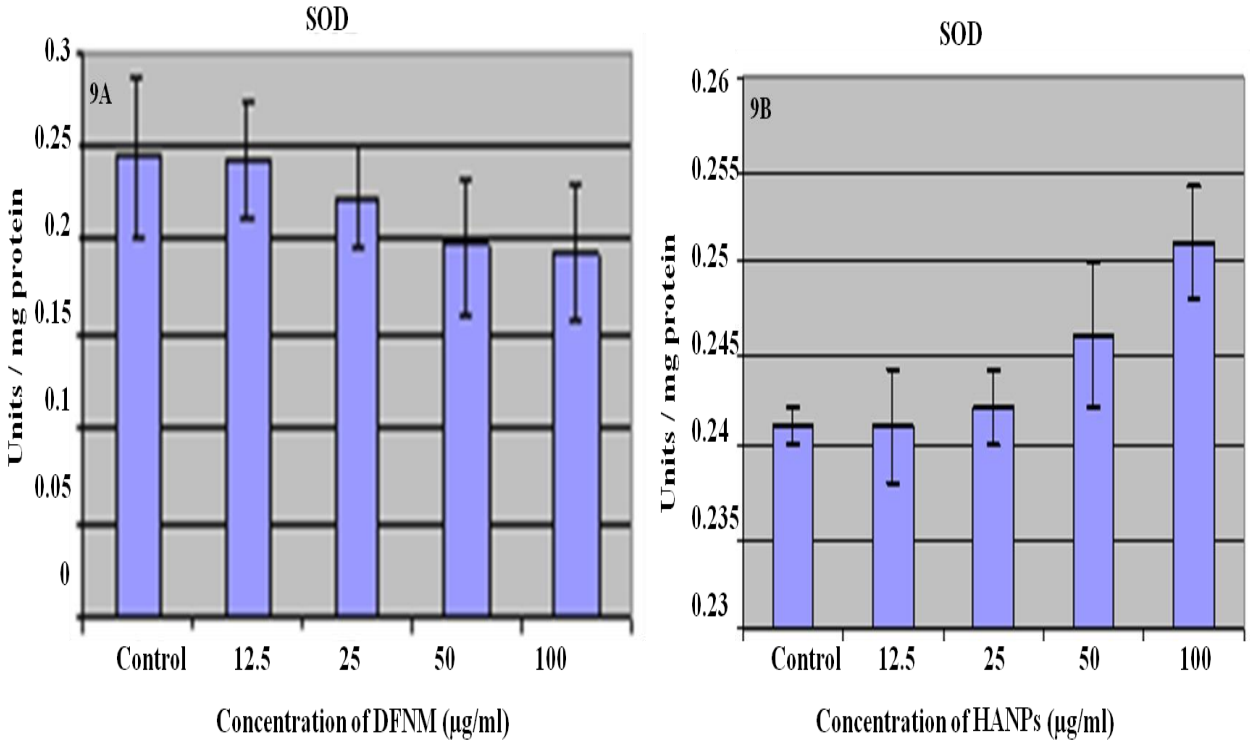


Figure 9: SOD activity observed in rat liver after nanomaterials exposure (*in vitro*)

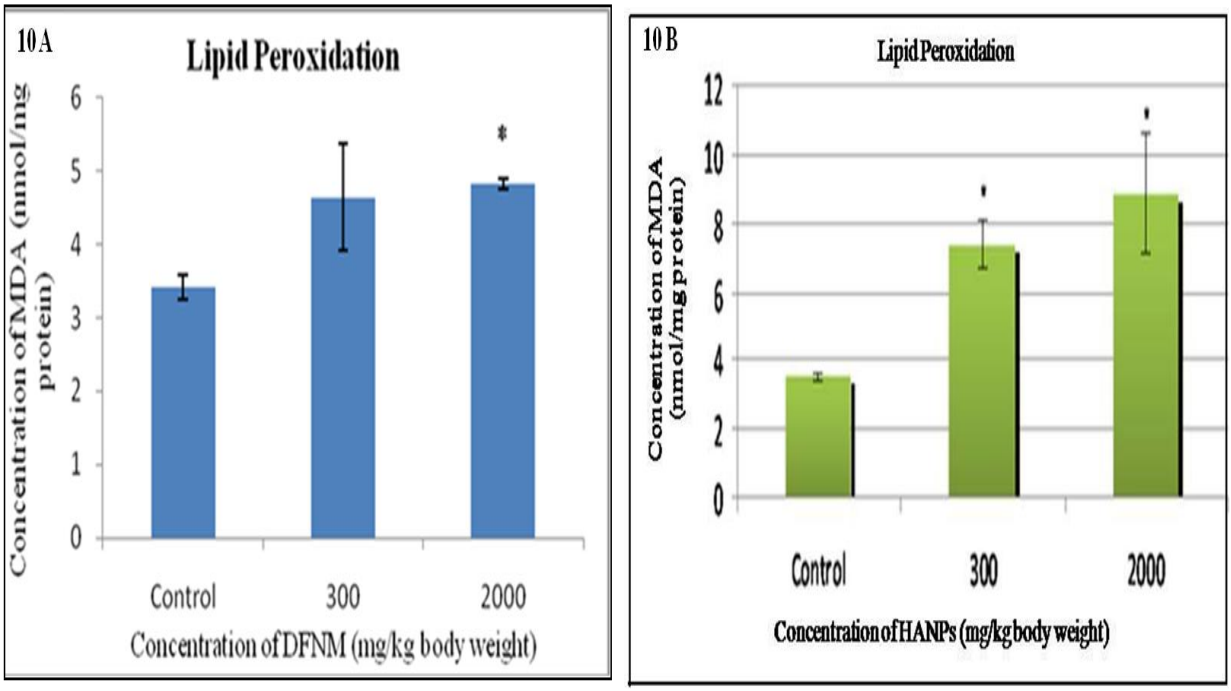


Figure 10: Extent of lipid peroxidation in liver tissue homogenates of rats administered with A) DFNM B) HANPs (acute toxicity study)

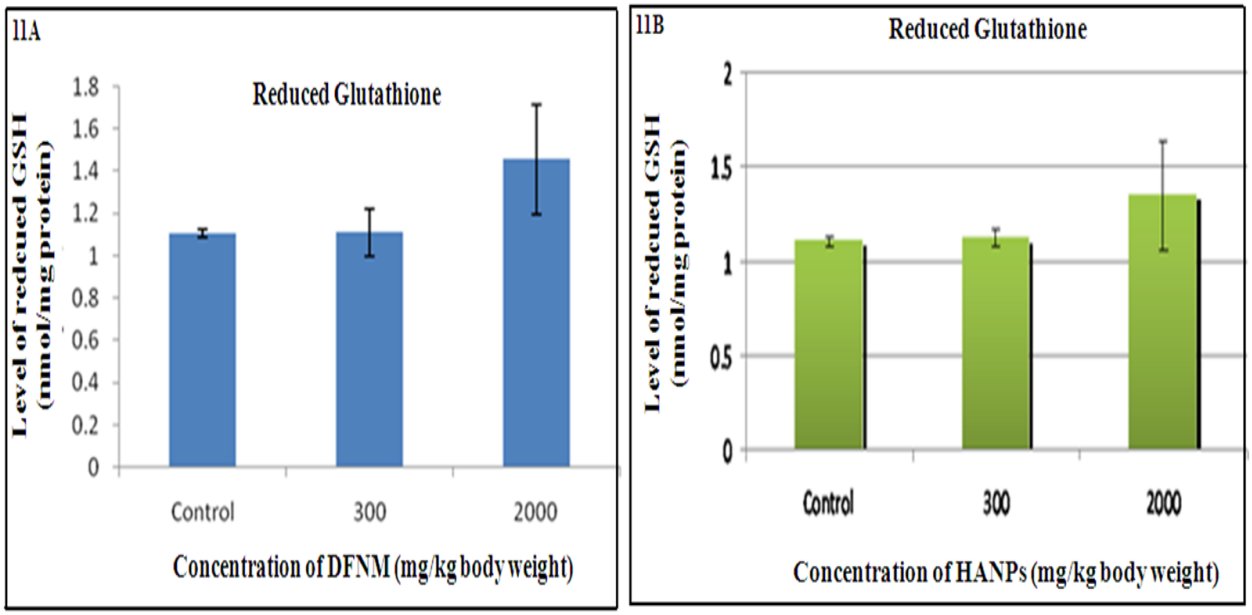


Figure 11: Concentration of reduced glutathione in liver tissue homogenates of rats administered with A) DFNM B) HANPs (acute toxicity study)

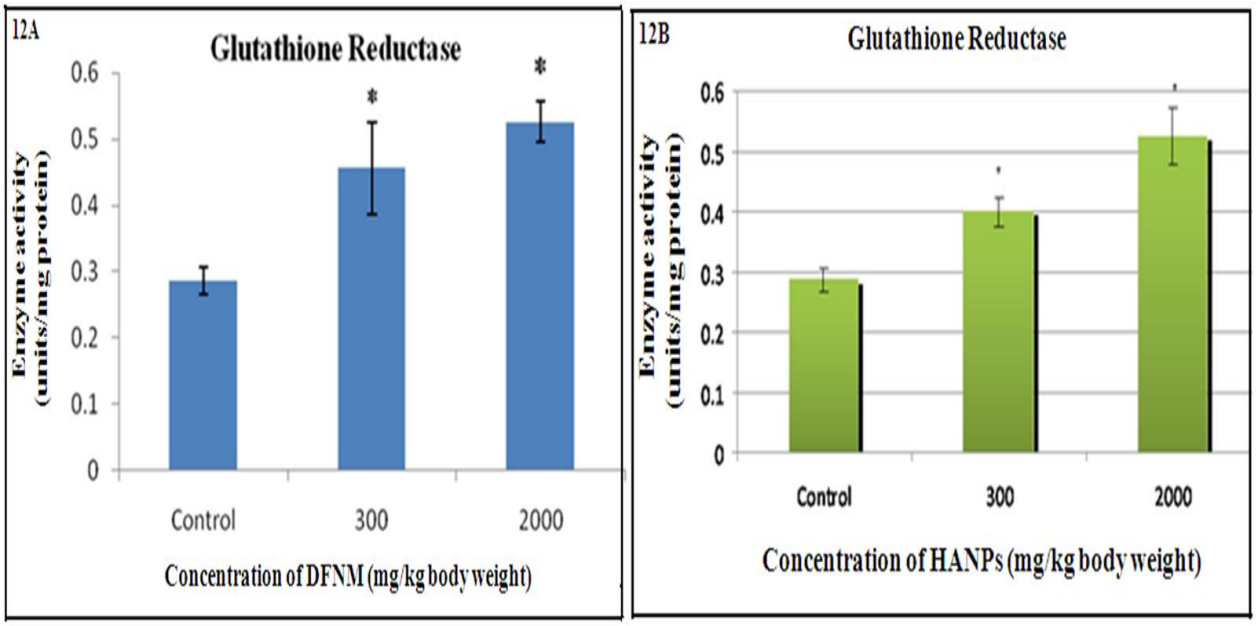


Figure 12: Activity of glutathione reductase in liver tissue homogenates of rats administered with A) DFNM B) HANPs (acute toxicity study)

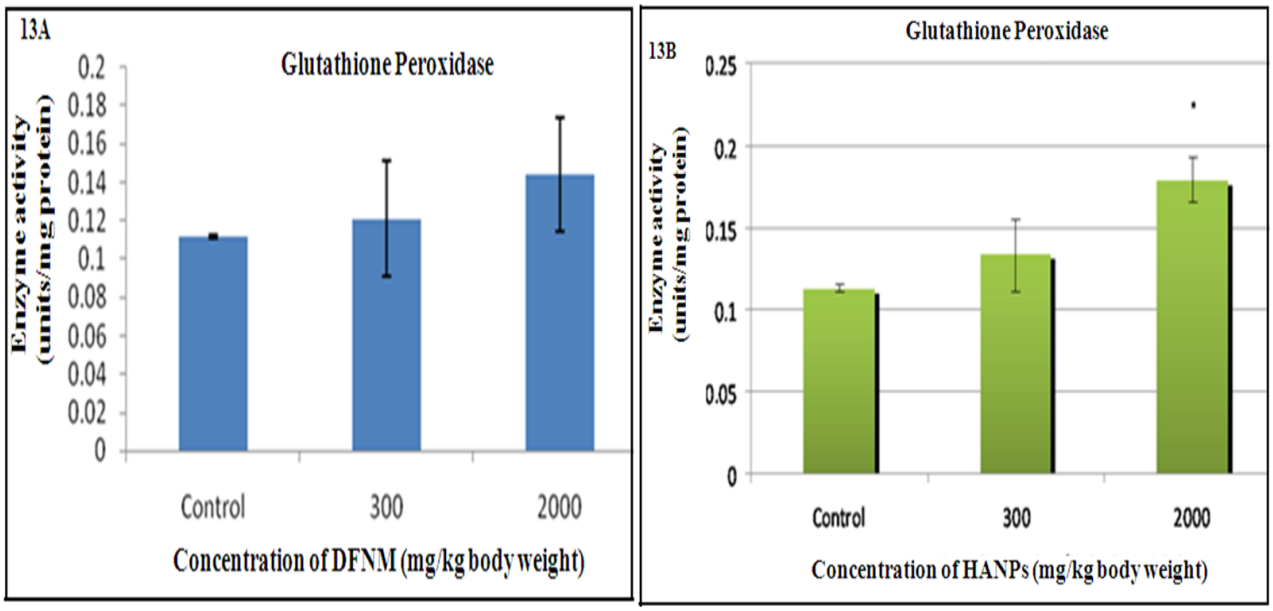


Figure 13: Activity of glutathione peroxidase in liver tissue homogenates of rats administered with
A) DFNM B) HANPs (acute toxicity study)

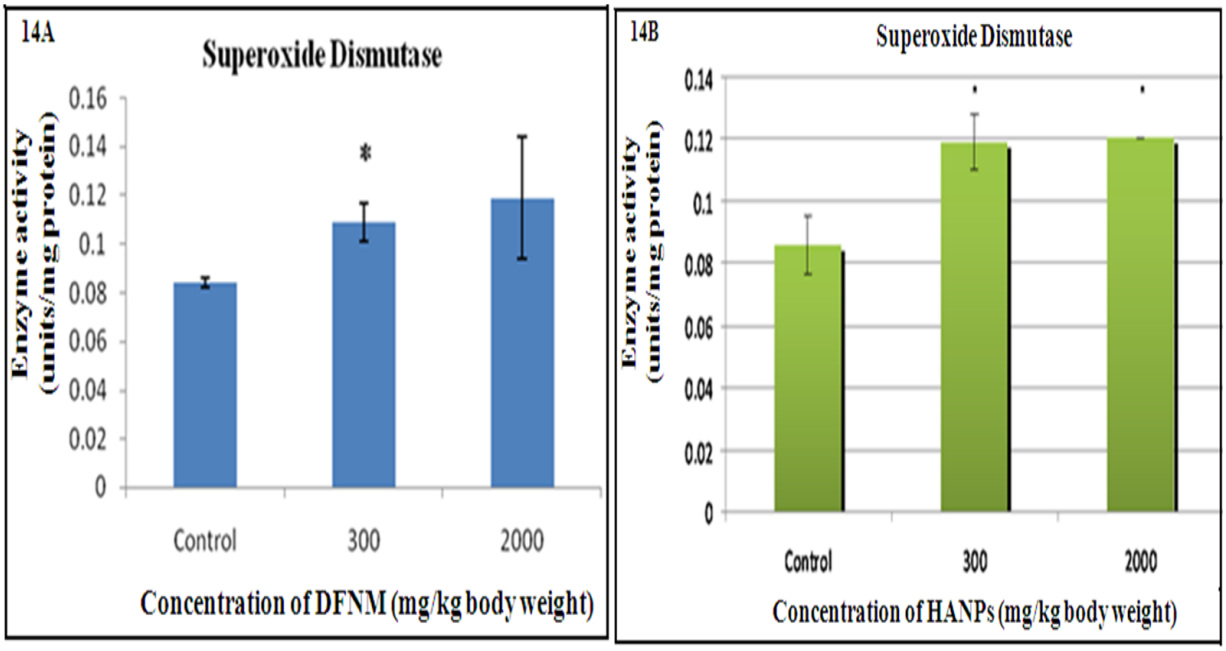


Figure 14: Activity of superoxide dismutase in liver tissue homogenates of rats administered with
A) DFNM B) HANPs (acute toxicity study)

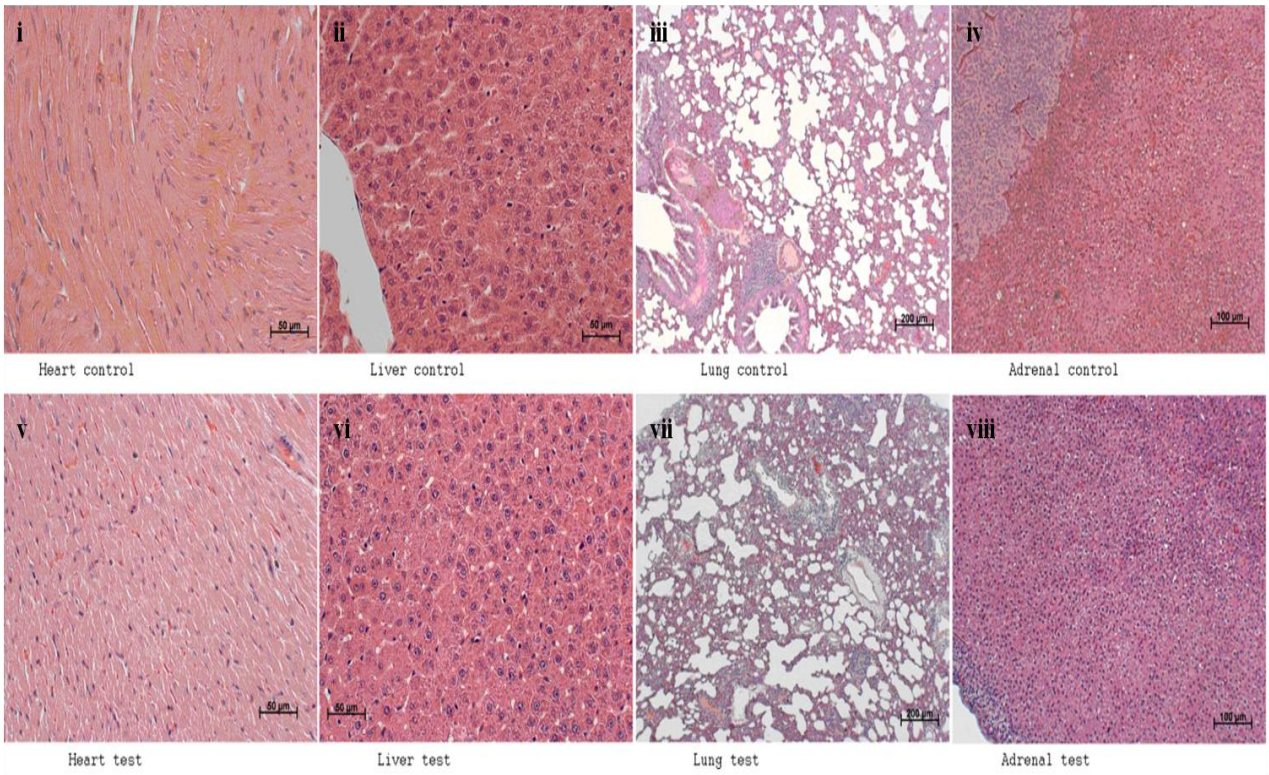


Figure 15 A: Photomicrographs of Histopathology of heart, liver, lungs, adrenal
(i-iv) Control; (v-viii) DFNM treated

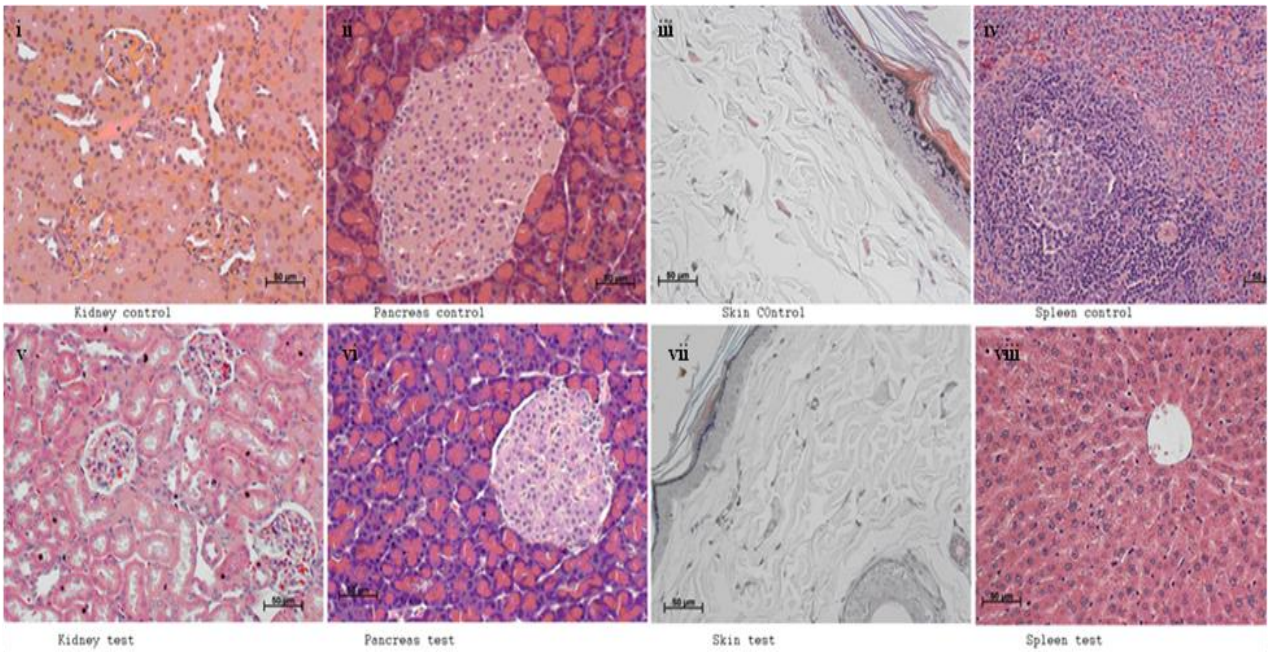


Figure 15 B: Photomicrographs of Histopathology of kidney, pancreas, skin, spleen
(i-iv) Control; (v-viii) DFNM treated

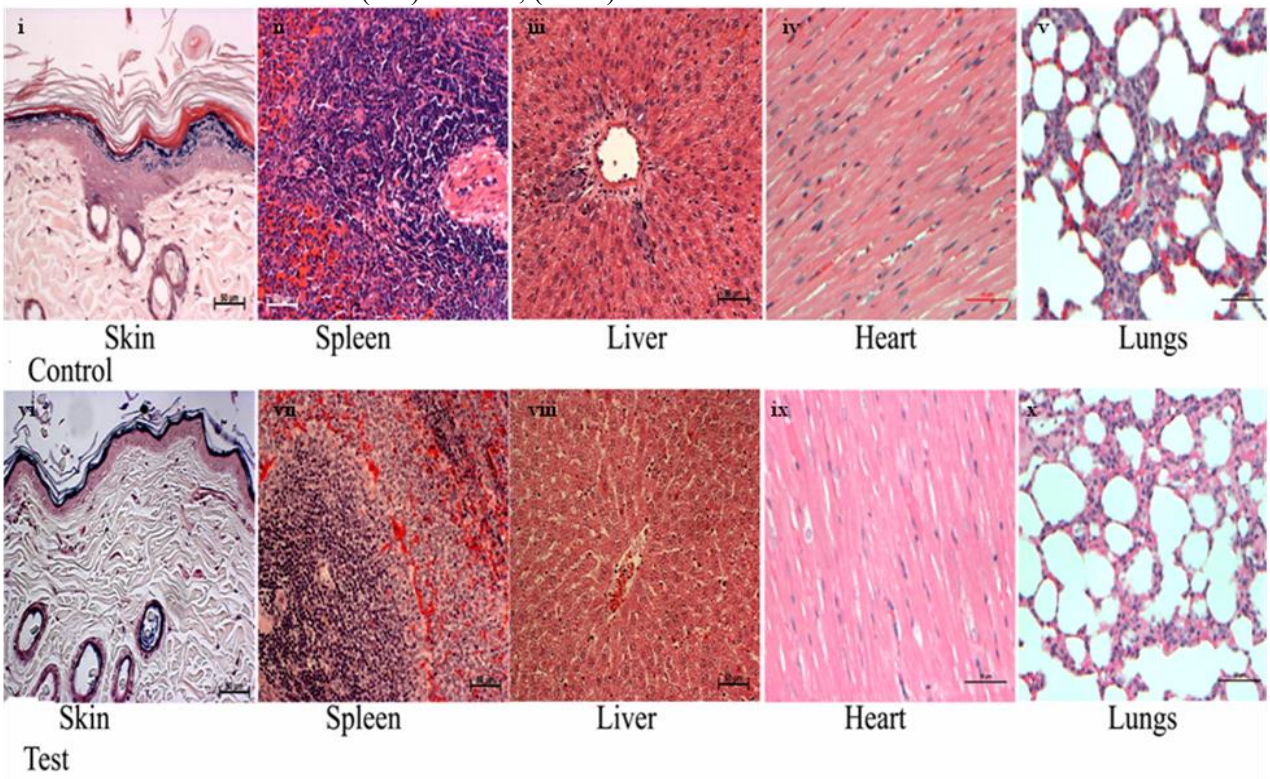


Figure 16: Photomicrographs of Histopathology of skin, spleen, liver, heart, lungs
(i-v) Control; (vi-x) HANPs treated

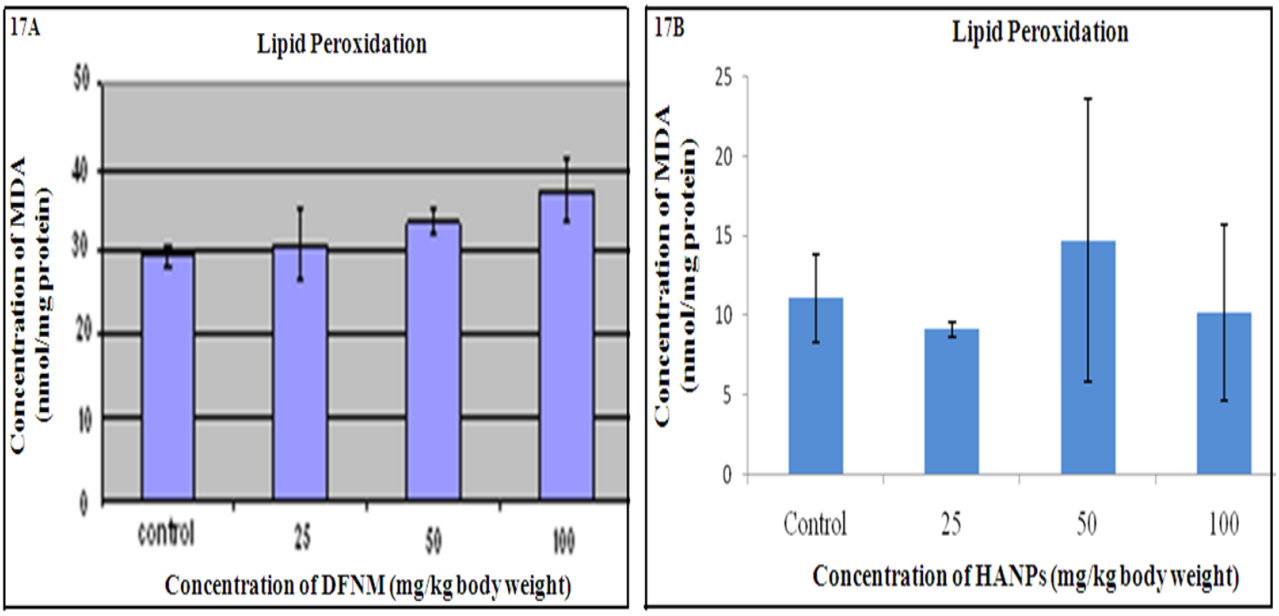


Figure 17: Amount of lipid peroxides in the liver of rat dermally exposed to A) DFNM B) HANPs (Mean ± SD, n=5)

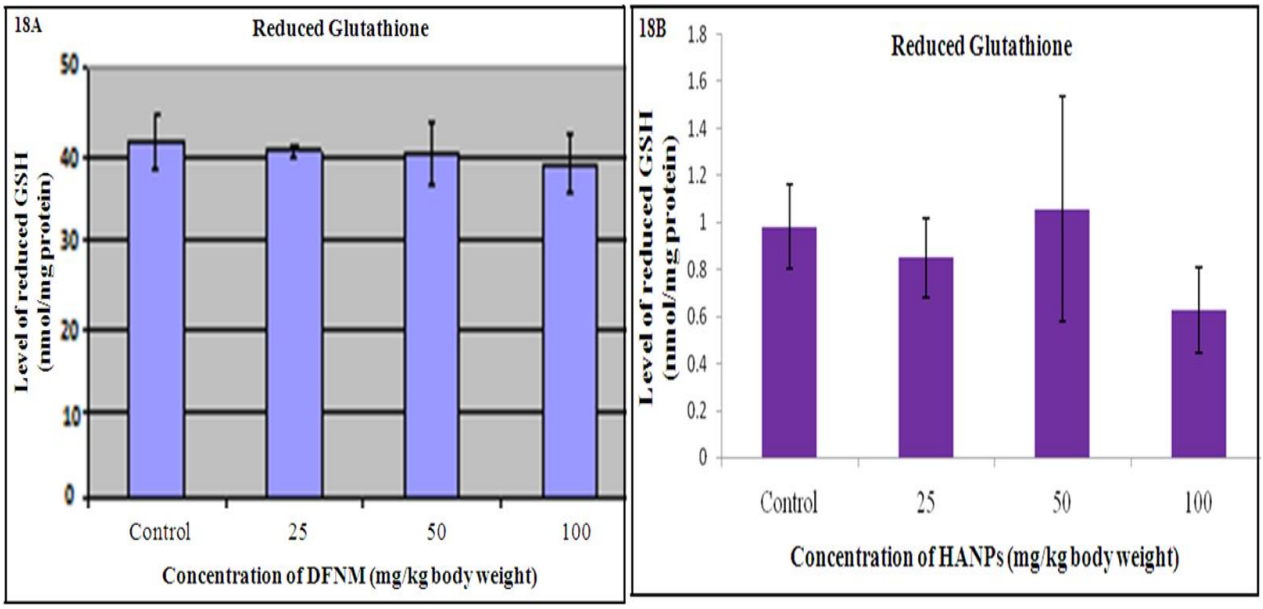


Figure 18: Concentration of reduced glutathione in the liver of rats dermally exposed to A) DFNM B) HANPs (Mean ± SD, n=5)

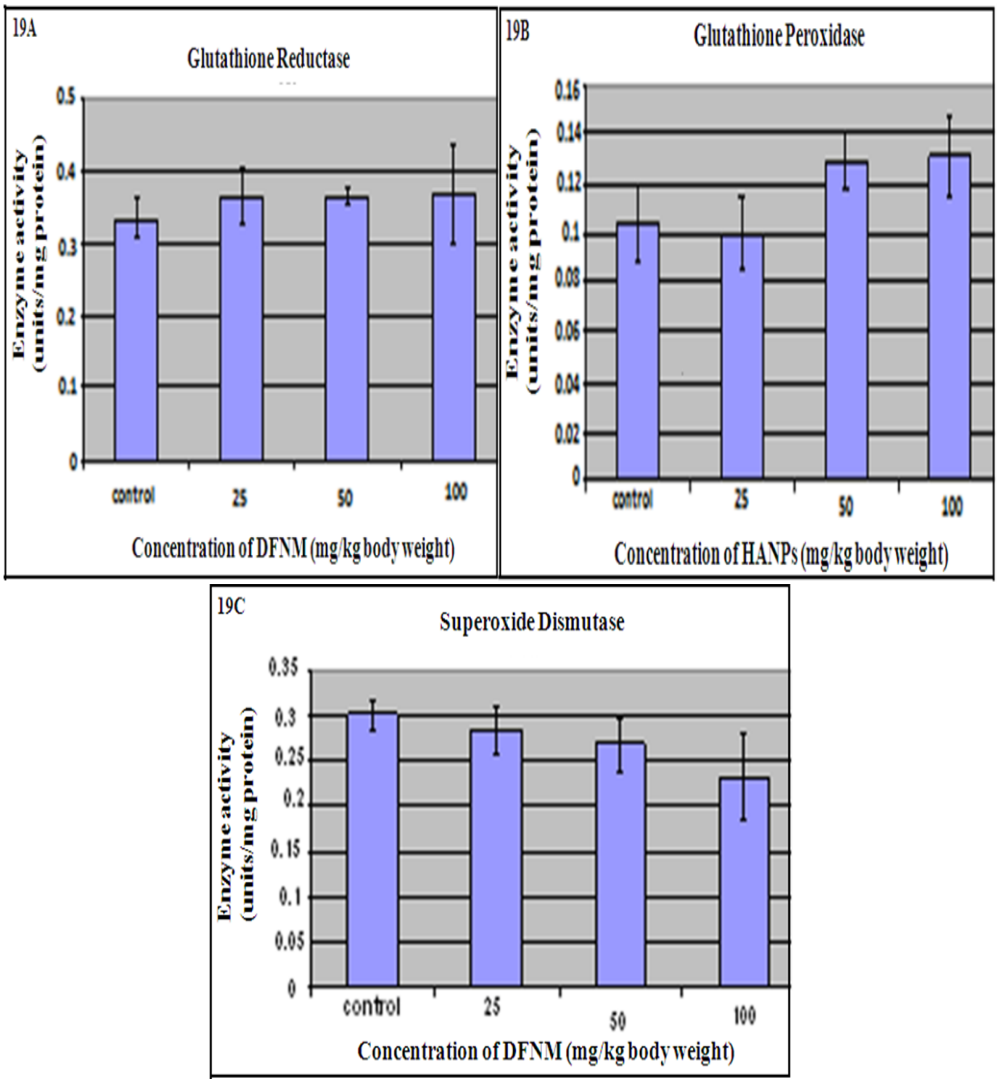


Figure 19: Activity of antioxidant enzymes in the liver of rat exposed to DFNM (Mean ± SD, n=5)

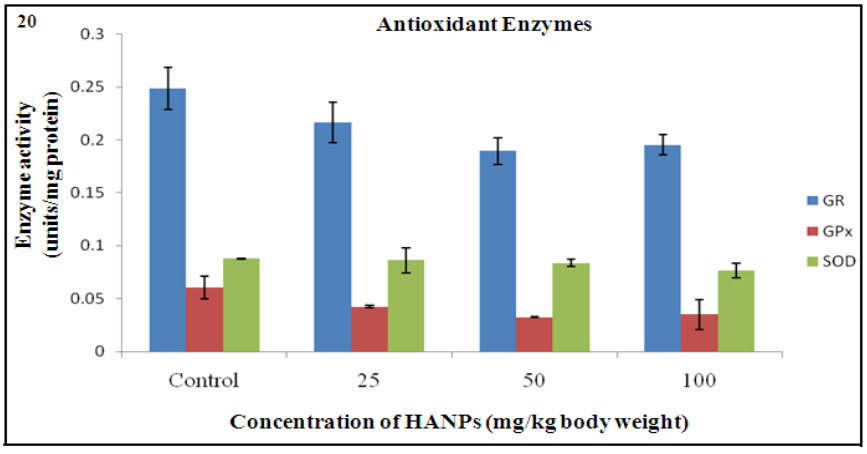


Figure 20: Activity of antioxidant enzymes in the liver of rat exposed to HANPs (Mean ± SD, n=5)

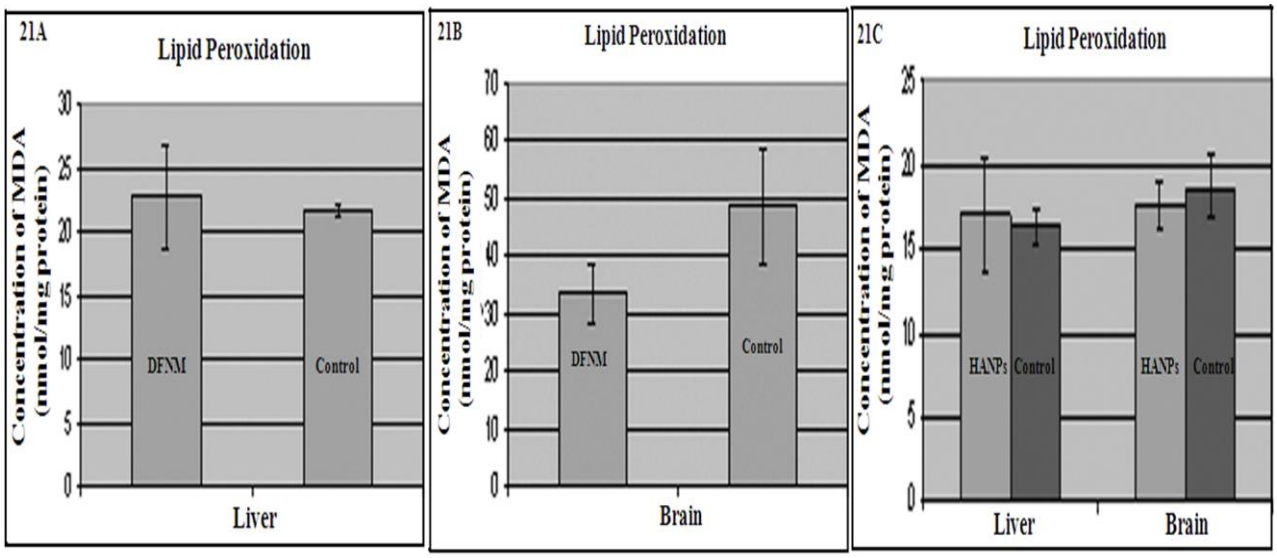


Figure 21: Amount of lipid peroxides formed in the tissue sample of G. pigs after exposure to nanomaterials

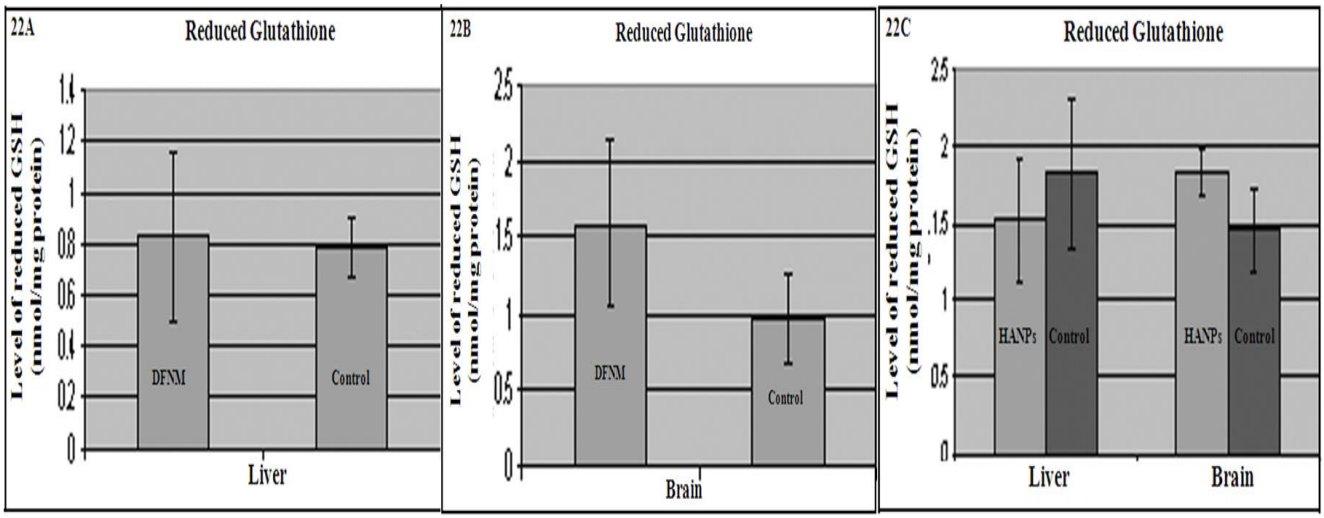


Figure 22: Concentration of reduced glutathione in tissue homogenates of G.pigs exposed to nanomaterials

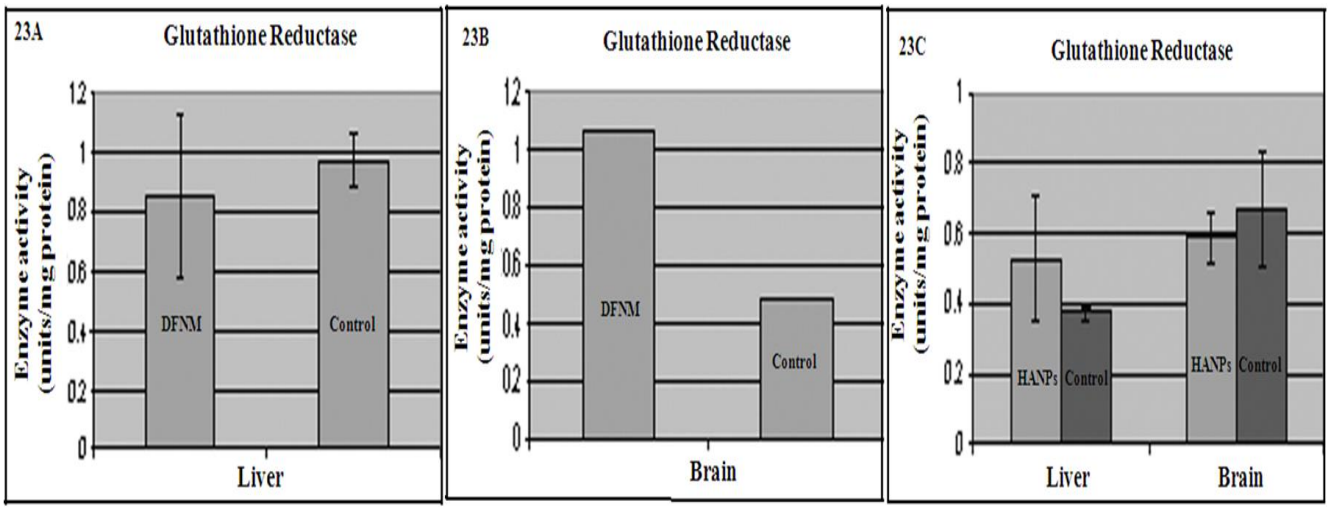


Figure 23: Glutathione reductase activity in tissue homogenates of G. pigs exposed to nanomaterials

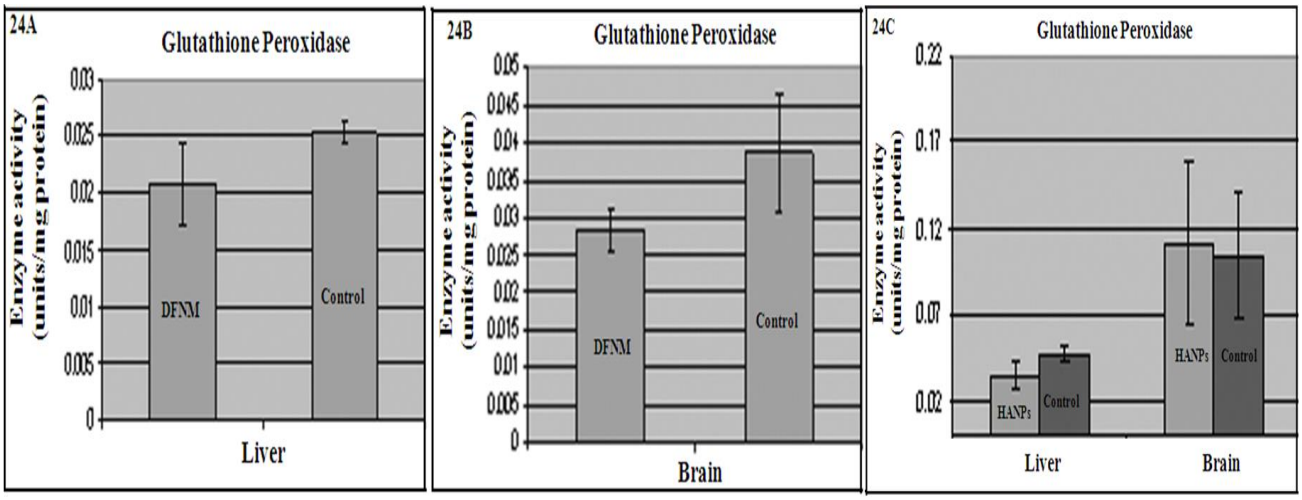


Figure 24: Glutathione peroxidase activity in tissue homogenates of G. pigs exposed to nanomaterials

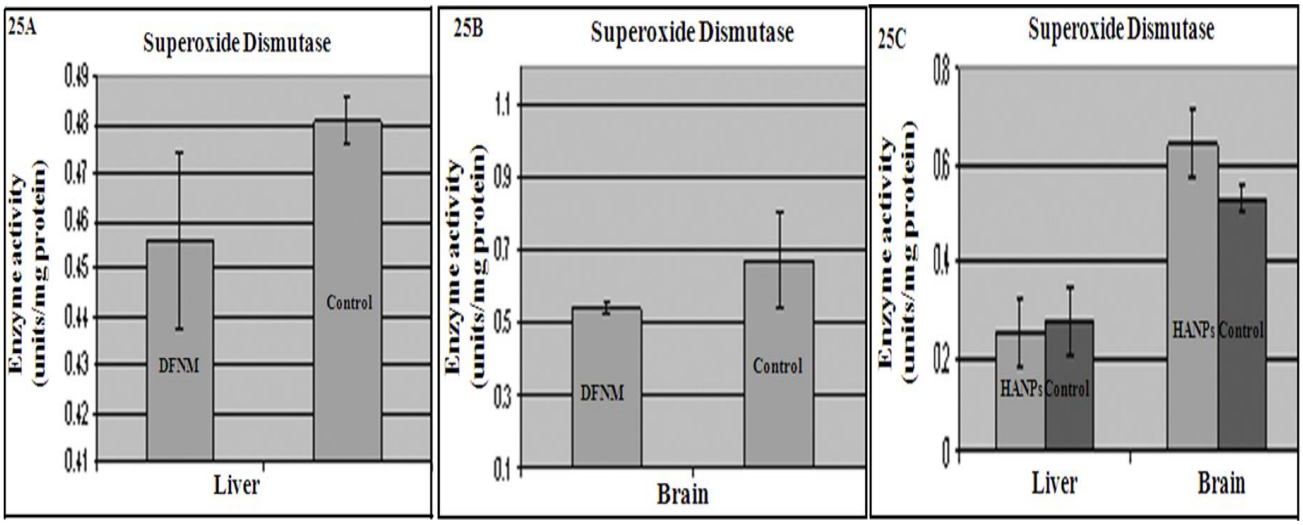


Figure 25: Superoxide dismutase activity in tissue homogenates of G. pigs exposed to nanomaterials

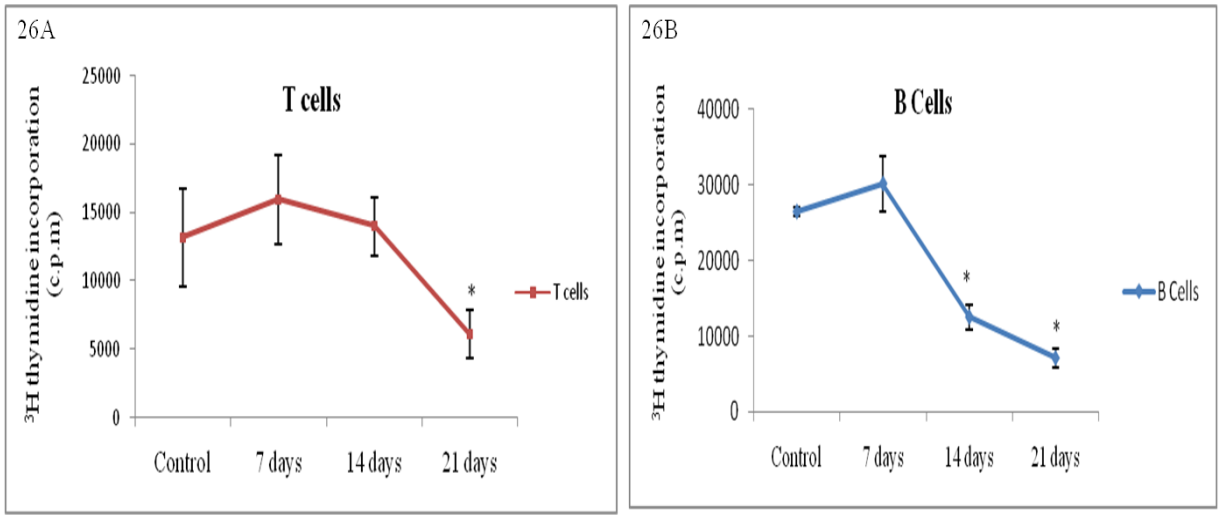


Figure 26: Proliferative activity in spleen cells exposed to DFNPs. (values are Mean ± SD, n=5), *Statistically Significant P<0.05

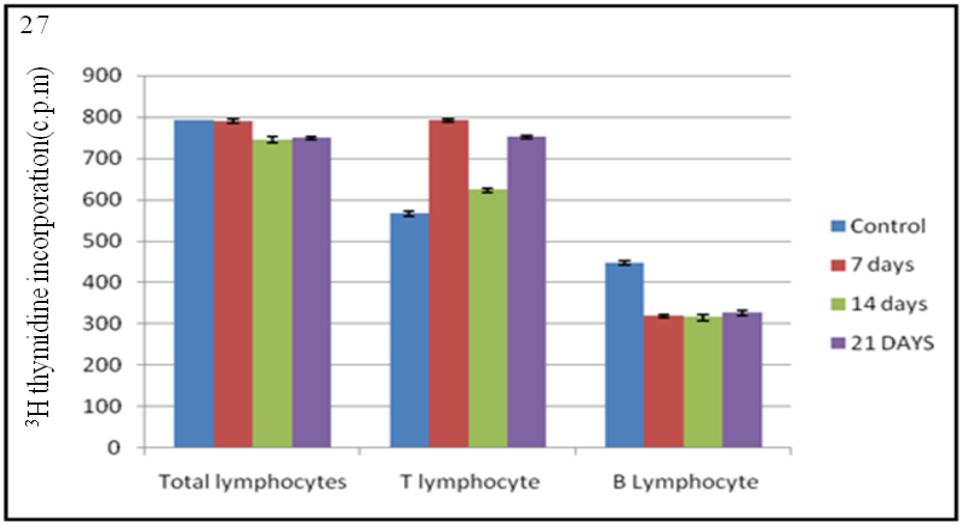


Figure 27: Proliferative activity in spleen cells exposed to HANPs (Values are given as mean±SD)

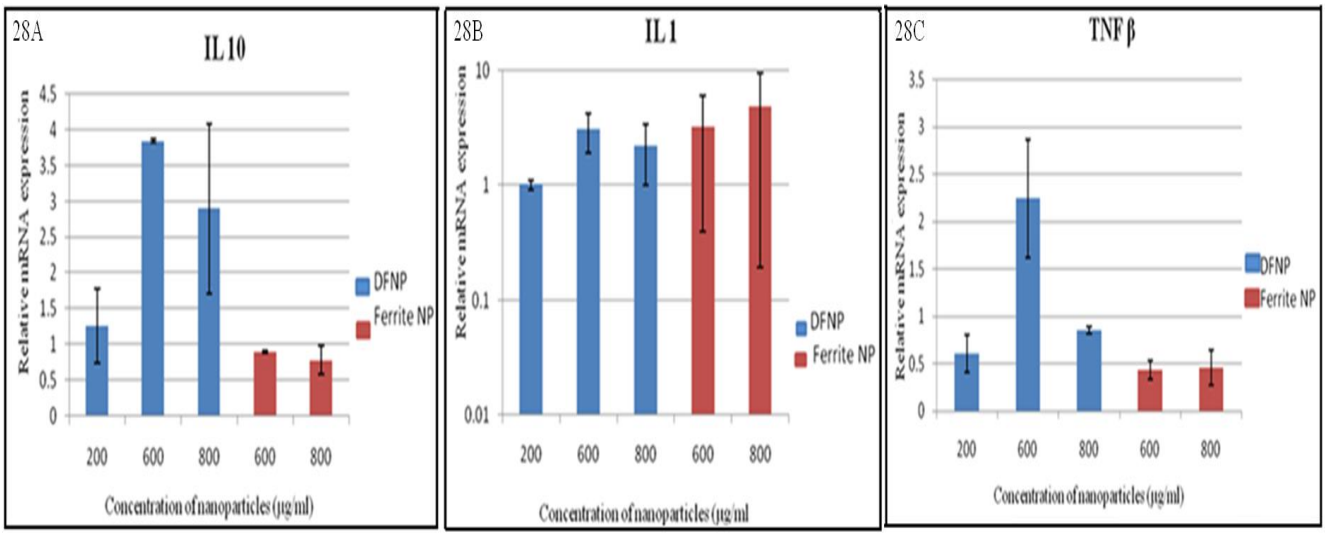


Figure 28: A- Relative mRNA expression (IL 10) when splenocytes were exposed to different concentrations of DFNPs; B- Relative mRNA expression (IL 1) when splenocytes were exposed to different concentrations of DFNPs; C- Relative mRNA expression (TNF β) when splenocytes were exposed to different concentrations of DFNPs. (All values are given as Mean ± SE, n=3).

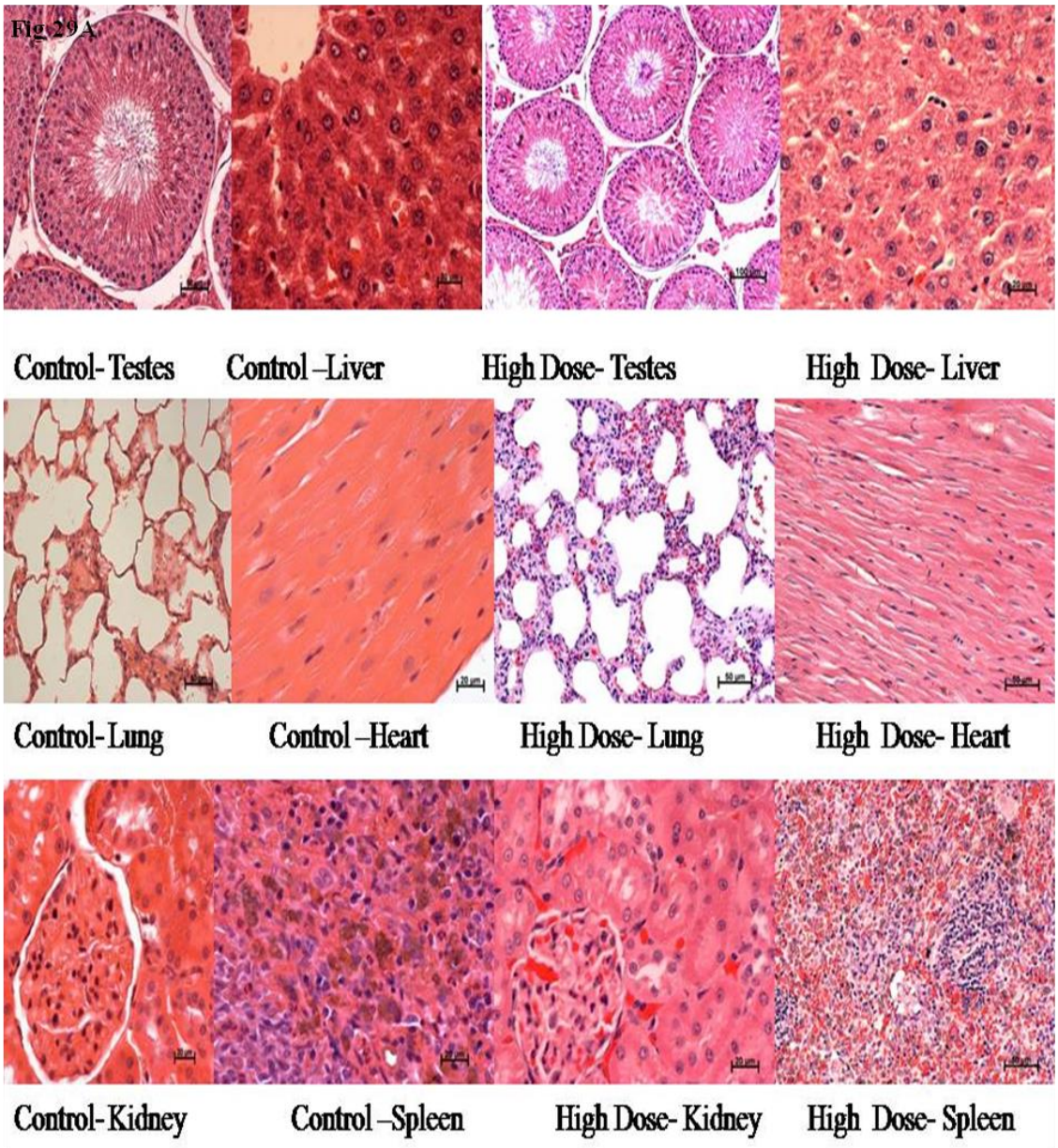


Figure 29A: Photomicrographs of histopathology of male rats exposed to DFNM (Chronic toxicity study)

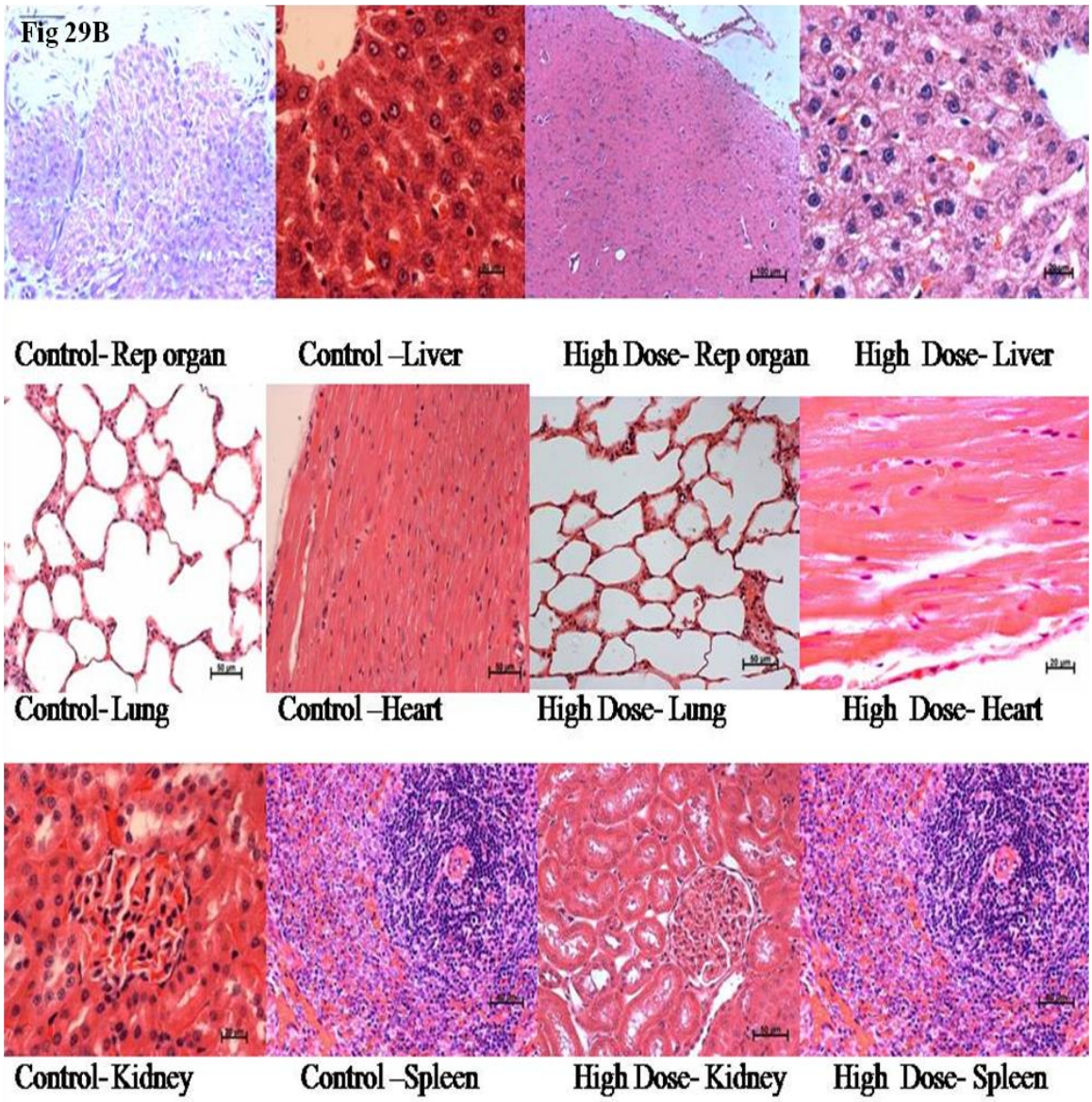


Figure 29 B: Photomicrographs of histopathology of female rats exposed to DFNM (Chronic toxicity study)

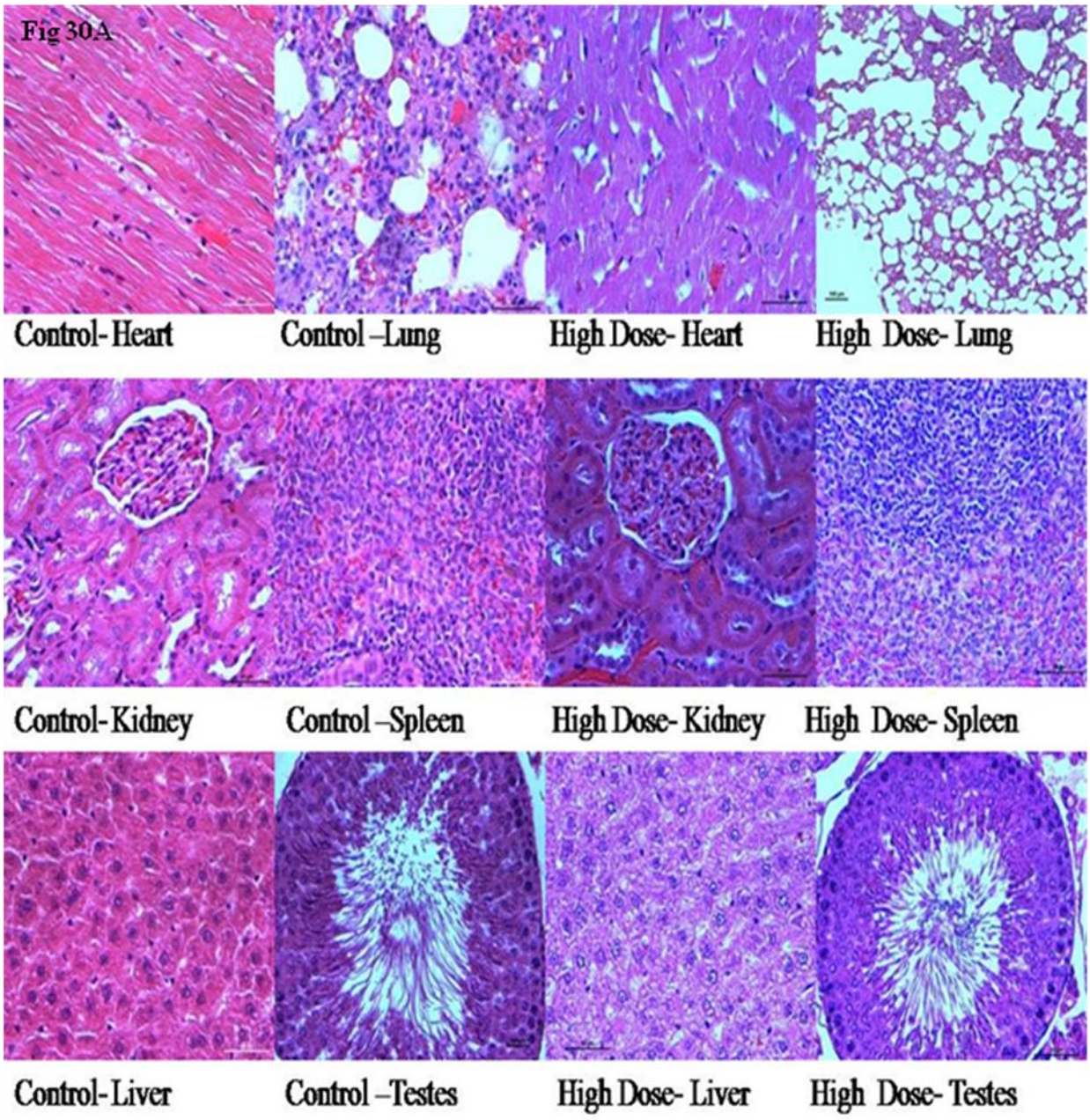


Figure 30A: Photomicrographs of histopathology of male rats exposed to HANPs (Chronic toxicity study)

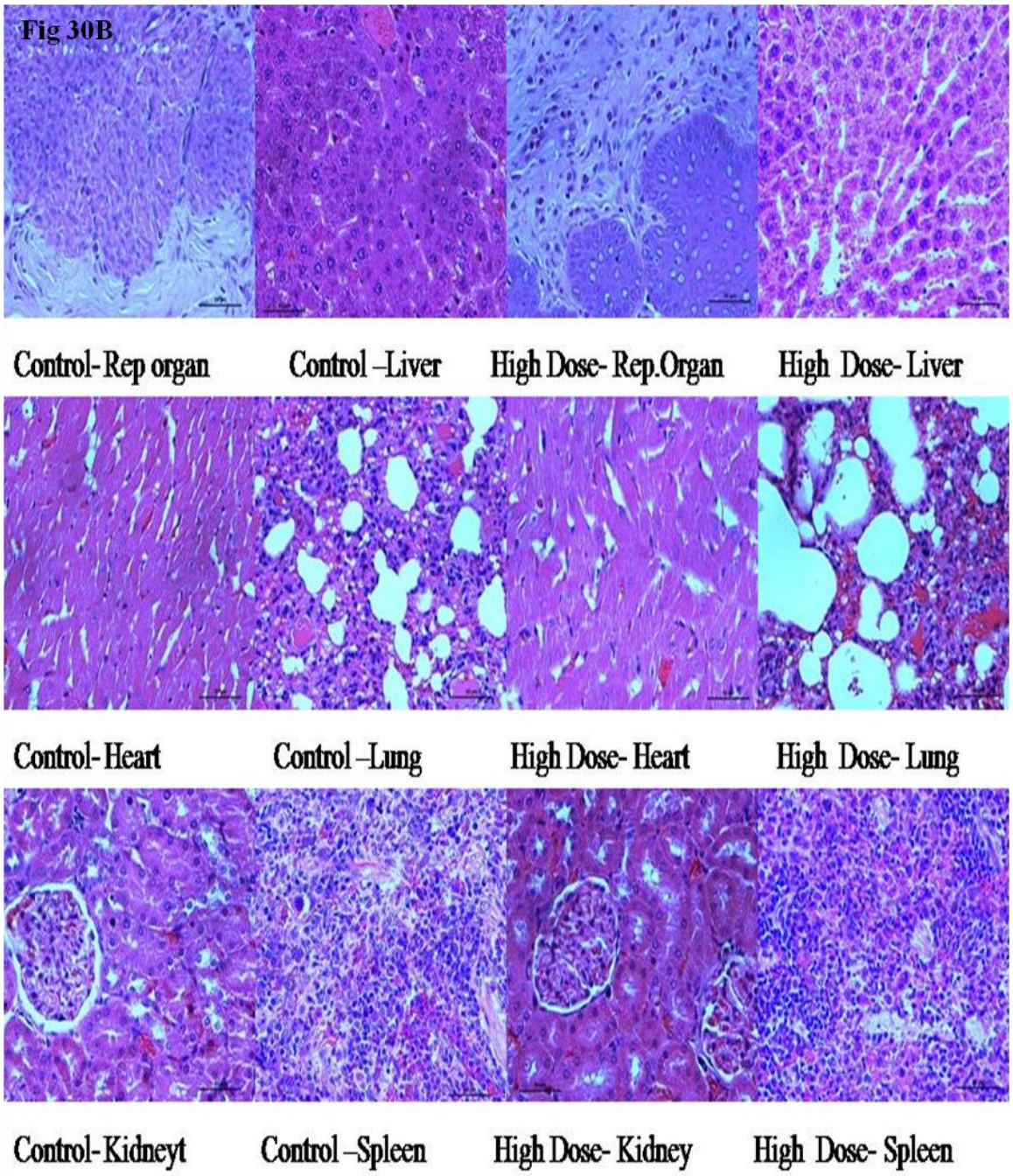


Figure 30B: Photomicrographs of histopathology of female rats exposed to HANPs (Chronic toxicity study)

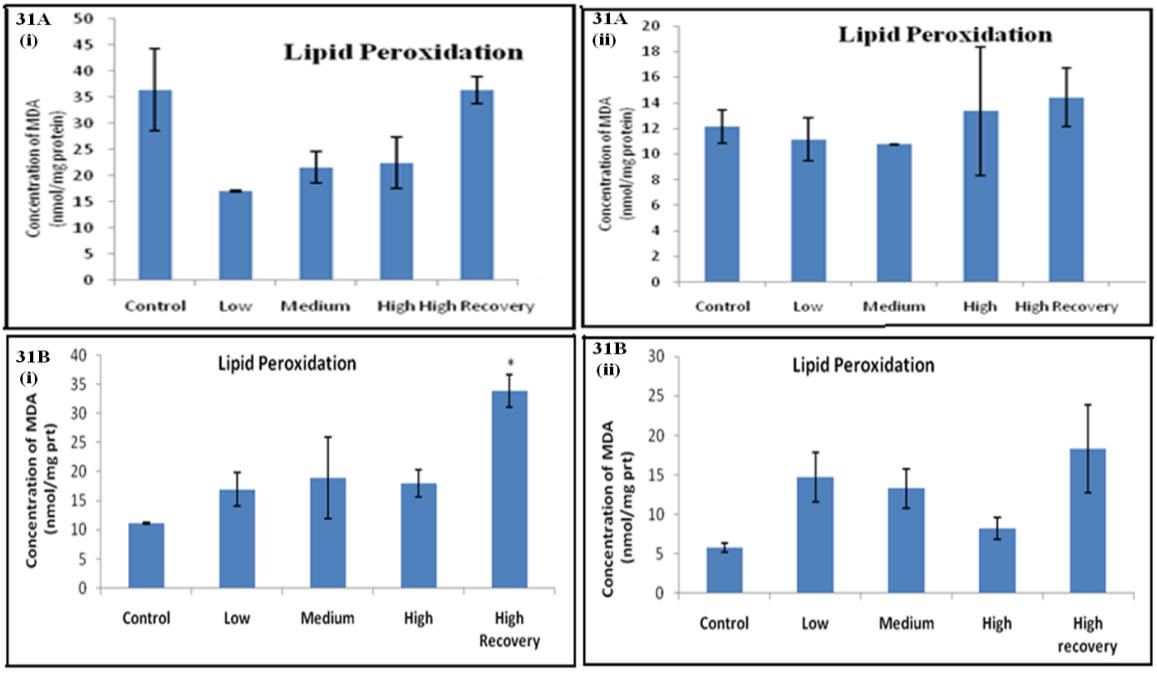


Figure 31: Amount of malonaldehyde formed in rats after chronic exposure to nanomaterials
A) DFNM B) HANPs

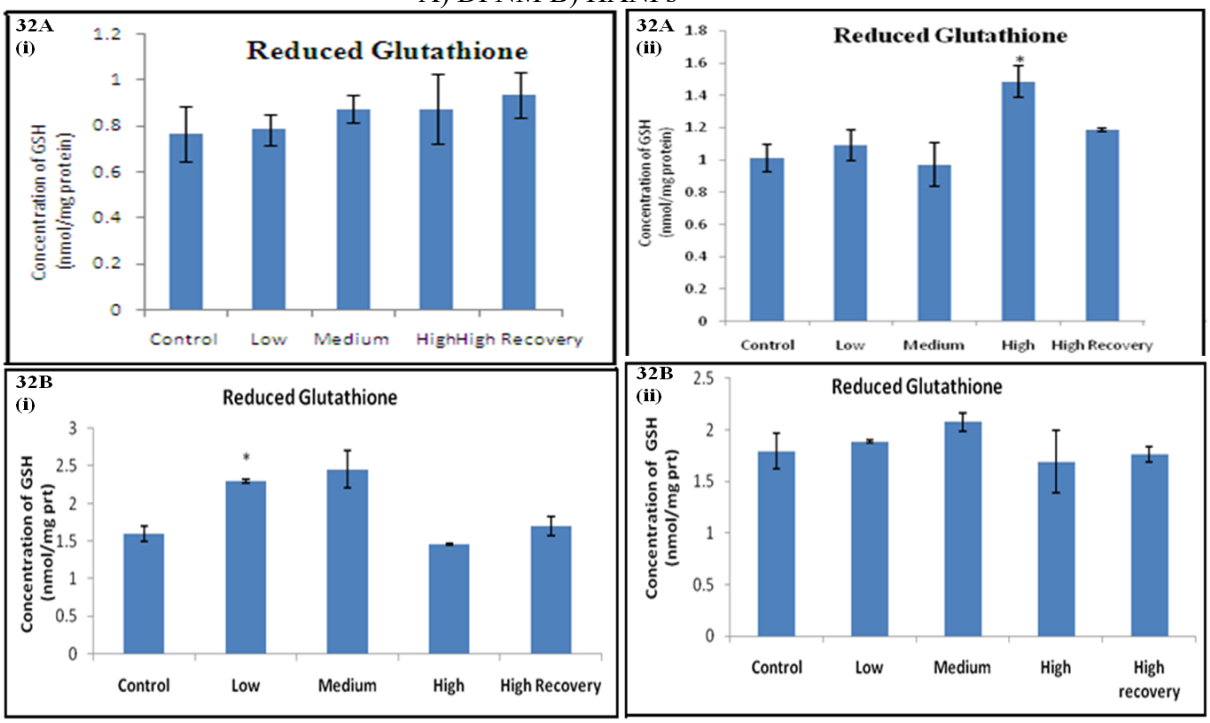


Figure 32: Total amount of intracellular GSH found in rats after exposure to nanomaterials
A) DFNM B) HANPs

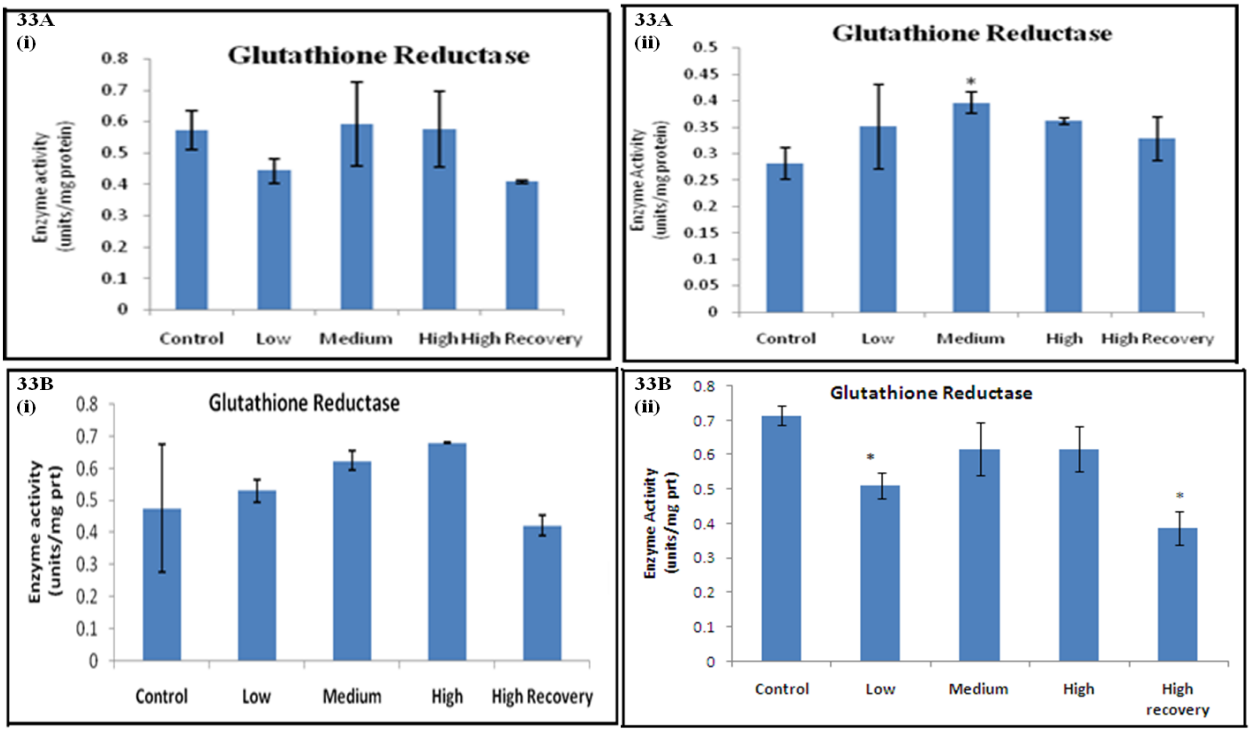


Figure 33: Enzymatic activity of glutathione reductase observed in rats after chronic exposure to nanomaterials A) DFNM B) HANPs

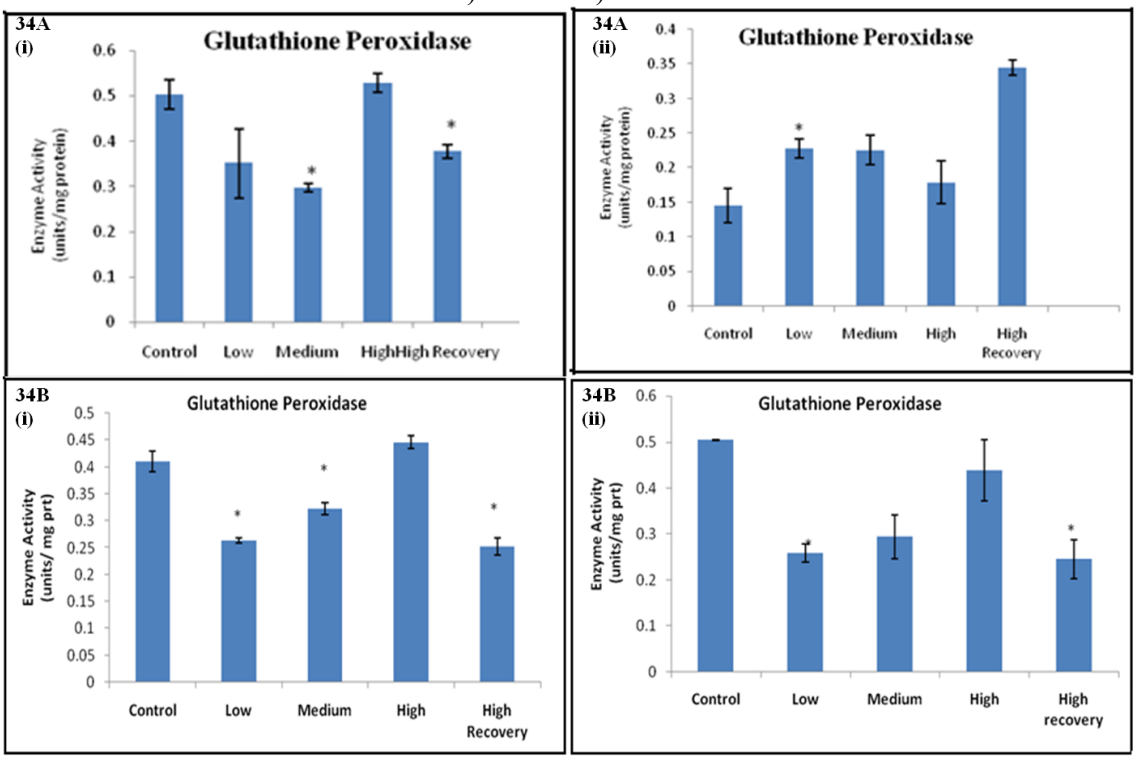


Figure 34: Activity of glutathione peroxidase observed in rats after chronic exposure to nanomaterials A) DFNM B) HANPs

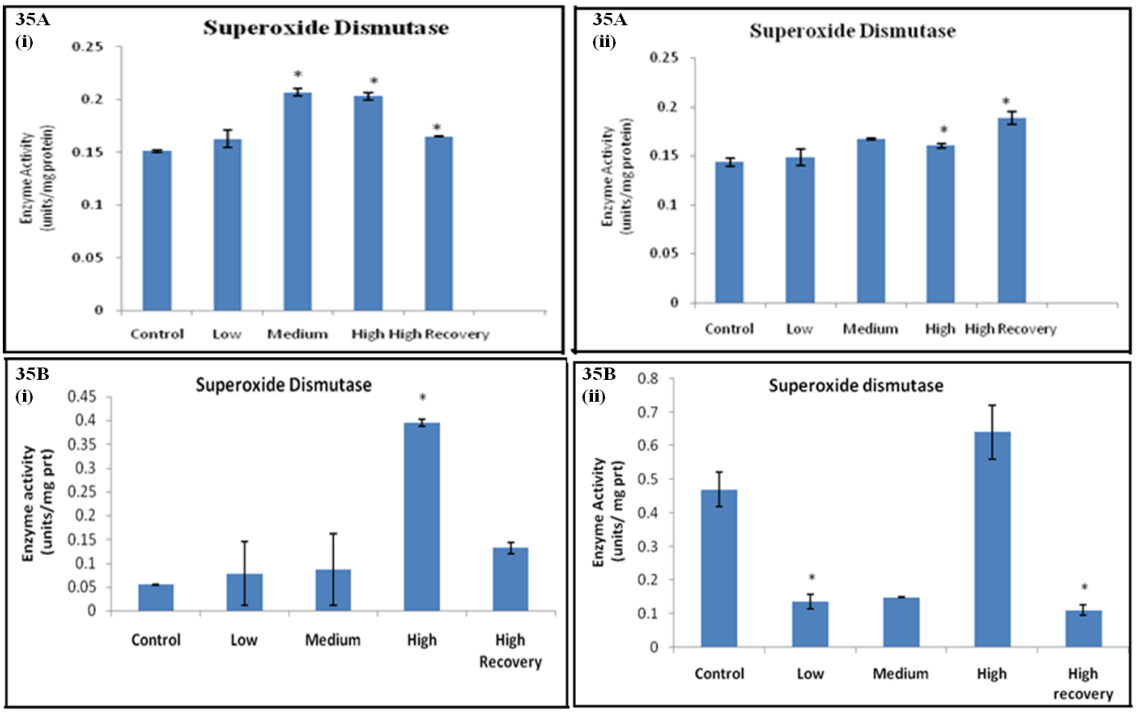


Figure 35: Activity of superoxide dismutase observed in rats after chronic exposure to nanomaterials
A) DFNM B) HANPs

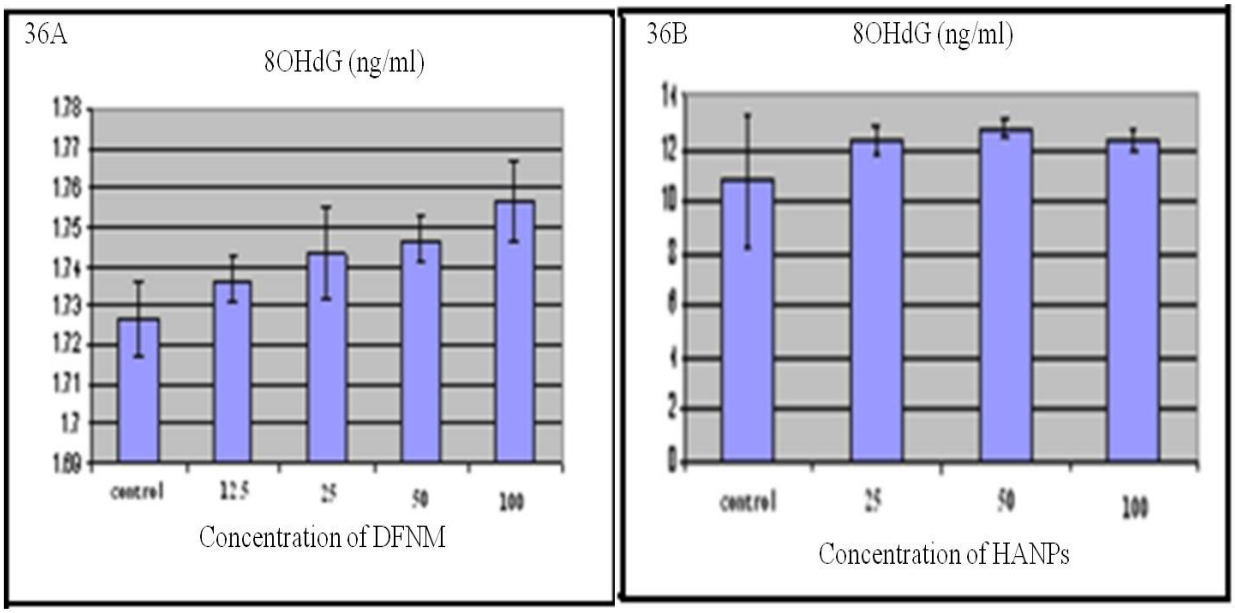


Figure 36: Amount of 8OHdG observed in rat liver homogenates after exposure to nanomaterials (*in vitro*)

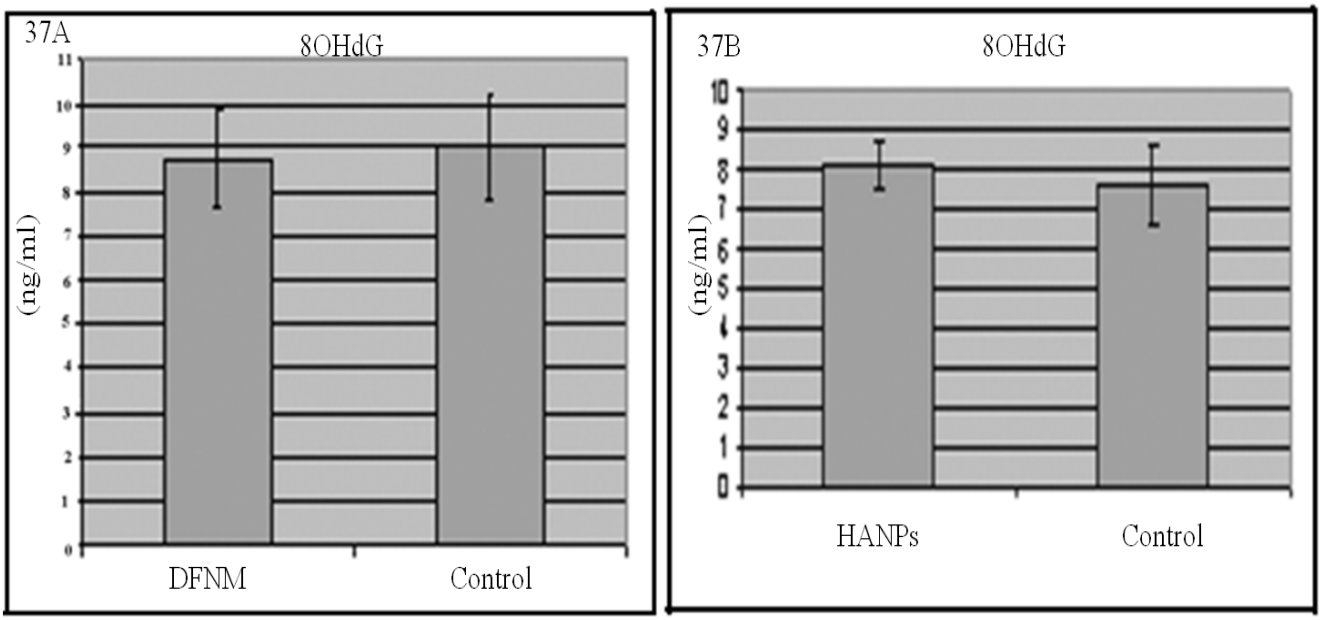


Figure 37: Amount of 8OHdG observed in liver homogenates of G.pigs after exposure to nanomaterials (*in vivo*)

Tables

Acute Toxicity of DFNM

Table 1A: Haematological parameters of DFNM administered animals

Parameters	Control	300 mg/ kg body weight	2000mg/kg body weight
	Mean ± SD		
WBC (10^3/mm^3)	6.93 ±1.46	7.17 ±1.62	6.83 ±1.01
RBC (10^6/mm^3)	6.71 ±0.18	6.80 ±0.48	6.54 ±0.41
HGB (g/dL)	15.27±0.31	14.83±0.81	14.9±0.95
HCT (%)	36.6±0.49	35.30±2.44	35.53±1.62
MCV	54±1.00	53.67±1.53	54.33±1.15
MCH	22.4±0.35	22.77±0.93	22.8±0.7
MCHC	41.13±0.35	42.4±0.66	41.93±1.00

(Student's 't' test. For all comparisons $p<0.05$ was considered significant, n=3)

Table 1B: Biochemical parameters of DFNM administered animals

Biochemical Parameters	Mean±SD		
	Control	300 mg/ kg body weight	2000mg/kg body weight
Total Protein (g/dL)	7.23±0.21	7.7±0.2*	8.23±0.87
Creatinine (mg/dL)	0.89±0.02	0.98±0.11	0.79±0.09
Urea (mg/dL)	30.77±2.04	34.6±2.36	33.53±3.88
SGPT (U/L)	76.6±10.86	89.95±18.31	54.75±2.89
SGOT (U/L)	142.2±7.3	129.1±0.14	106.63±9.15*
Alkaline Phosphatase (U/L)	135.1±2.08	177±16.97	165±4.24*
Glucose (mg/dL)	101.05±0.49	87.1±2.82	117.75±2.05*

Cholesterol (mg/dL)	98±1.00	78.33±10.16	87.67±6.81
Bilirubin Total (mg/dL)	0.08±0.01	0.36±0.25	0.22±0.25
Albumin (BCG) (g/dL)	4.46±0.59	4.3±0.1	4.13±0.15
Phosphorus (mg/dL)	5.63±0.14	7.6±3.32	6.72±1.35
Chlorides (mEq/L)	97.13±2.35	103.1±0.56*	101.3±0.82*
GGT (U/L)	1.47±0.99	1.77±1.59	2.33±1.30

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant), *(p value <0.05, n=3)

Table 2A: Hematological parameters of HANPs administered animals

Parameters	Control (Mean±SD)	300mg/kg Body weight(Mean±SD)	2000mg/kg body weight(Mean±SD)
WBC $x10^3/mm^3$	6.25±1.679	7.167±1.202	7.2±0.885
RBC $x10^6/mm^3$	6.615±0.021	6.59±0.438	6.31±0.0849
Hb (g/dL)	15.1±0.141	15.2±0.707	15.4±0.566
Hct (%)	36.4±0.424	36.1±2.263	36.25±1.485
MCV (μm^3)	54±1	53.667±1.528	54.333±1.155
MCH (pg)	22.4±0.346	22.767±0.929	22.8±0.7
MCHC (g/dL)	41.3±0.351	42.4±0.656	42.5±0.283

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=3)

Table 2B: Biochemical parameters of HANPs administered animals

Parameters	Control (Mean±SD)	300mg/kg Body weight(Mean±SD)	2000mg/kg body weight(Mean±SD)
Glucose (mg/dL)	100.6±0.854	101.85±0.354	106.2±5.657
Cholesterol (mg/dL)	98±1	101±5.657	104.5±6.364
Bilirubin (mg/dL)	0.08±0.01	0.125±0.078	0.213±0.248
Albumin(g/dL)	4.25±0.636	4.3±0.283	4.35±0.212
Phophorous (mg/dL)	5.633±0.405	6.105±1.138	7.01±0.453
Chlorides (mmol/L)	97.133±2.346	102.35±1.485	103.05±3.606
Total proteins (g/dL)	7.233±0.208	7.467±0.321	7.45±0.071
Creatinin (mg/dL)	0.887±0.021	0.885±0.049	0.955±0.007
Urea (mg/dL)	30.767±3.47	35±2.043	35.6±3.27
SGPT (IU/L)	76.6±10.857	77.55±10.253	77.7±0.566
SGOT (IU/L)	142.2±2.051	143.75±2.051	144.05±2.475
AP (IU/L)	135.667±2.082	203±8.485*	207.333±17.01*
GGT (IU/L)	1.467±0.987	1.9±1.706	2.633±0.252

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=3)

Dermal Toxicity of DFNM

Table 3A: Haematological parameters of rats exposed dermally to DFNM

Parameters	Concentrations of DFNM			
	Control (Mean±SD)	25mg/kg (Mean ±SD)	50mg/kg (Mean±SD)	100mg/kg (Mean±SD)
WBC ($10^3/mm^3$)	7.76±1.90	7.27±1.81	8.01±1.58	7.82±2.34
RBC ($10^6/mm^3$)	8.08±0.85	7.65±0.66	7.59±0.52	7.14±0.88*
Hb (g%)	15.71±0.69	16.47±0.88	15.95±0.65	15.25±1.83
Hct (%)	41.47±4.55	42.41±3.13	40.45±2.62	38.18±4.74
PLT ($10^3/mm^3$)	841±158.26	866±123.82	782.9±112.78	774.9±117.69
MCV (μm^3)	53.9±1.10	54.8±1.03	53.4±0.97	54.1±1.20
MCH (pg)	21.1±0.84	21.32±0.80	21.03±0.91	21.58±0.96
MCHC (g/dl)	39.06±1.16	38.79±1.50	39.41±1.62	39.99±1.56
Clotting time (seconds)	258±49	252±68	234±66	234±53

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=7)

Table 3B: Biochemical parameters of DFNM dermally exposed to rats

Parameters	Concentrations of DFNM			
	Control (Mean±SD)	25mg/kg (Mean ±SD)	50mg/kg (Mean±SD)	100mg/kg (Mean±SD)
Glucose (mg/dl)	104.84±22.09	102.86± 6.37	111.73±37.19	104.97±33.58
SGPT (IU/L)	117.71±14.69	118.09±14.21	120.20±35.38	115.46±14.07
SGOT (IU/L)	153.40±50.57	127.36±20.42	160.14±73.49	166.31±61.69
Alk. Phosphatase (IU/L)	242.86±52.46	289.43±77.94	249.00±63.99	210.57±48.00
Chloride (mmol/L)	110.90± 3.09	113.56± 1.78	111.14± 1.2	112.31± 1.71
Triglycerides (mg/dl)	105.28±19.59	90.43 ±33.39	104.43±16.17	98.14 ±26.60
Cholesterol (mg/dl)	59.43±5.97	61.14±9.41	61.28±3.90	60.28±4.42
Calcium (mg/dl)	10.53±0.33	10.41±0.41	10.10±0.19	10.54±0.21
Urea (mg/dl)	44.2±5.5	47.83±4.24	42.26±3.9	41.83±2.66
Total protein (g/dl)	7.53±0.28	7.59±0.15	7.21±0.30	7.47±0.28
Albumin (g/dl)	4.39±0.18	4.73±0.18**	4.49±0.21	4.29±0.21
Phosphorous (mg/dl)	8.03±0.68	8.34±1.02	8.45±1.37	8.62±1.14
GGT (IU/L)	5.17±4.55	6.41±5.86	3.79±2.52	3.9±2.57
Total bilirubin (mg/dl)	0.22±0.15	0.13±0.08	0.23±0.23	0.20±0.10
Creatinine (mg/dl)	0.88±0.07	0.86±0.02	0.89±0.04	0.83±0.05

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=7)

Table 4A: Haematological values of rats when exposed to HANPs (mean ± SD, n=5).

Parameters	Control	HANPs		
		(25mg/kg)	(50mg/kg)	(100mg/kg)
WBC	5.64±2.20	6.25±1.51	5.85±1.46	7.62±2.22
RBC	6.09±0.39	6.35±0.48	6.14±0.63	6.02±0.63
Hb	15.77±0.81	15.95±0.92	15.46±0.10	14.66±0.91*
Hct	33.03±1.82	34.58±2.83	33.31±3.18	32.12±3.20
PLT	705.0±106.66	702.7±54.49	690.1±85.74	600.9±71.45*
MCV	54.5±1.35	54.5±1.08	54.4±1.51	53.5±0.71
MCH	25.91±0.92	25.14±0.76	25.3±1.46	24.52±1.33*
MCHC	47.75*±1.39	46.2±1.57	46.55±2.17	45.89±2.42

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=10)

Table 4B: Biochemical values of rats when exposed to HANPs (mean ± SD, n=5).

Parameters	Control	HANPs		
		(25mg/kg)	(50mg/kg)	(100mg/kg)
Glucose	110.8±35.47	88.6±13.81	91.6±15.78	91.2±14.46
Cholesterol	60.2±6.06	59.4±4.56	63.0±5.478	57.6±5.73
Triglycerides	106.0±18.37	122.0±0.65	123.0±0.65	112.6±0.62
Bilirubin	0.176±0.10	0.128±0.04	0.094±0.01	0.120±0.044
Albumin	4.08±0.32	4.36±0.27	4.40±0.25	4.14±0.25
Calcium	10.68±0.50	10.92±0.38	10.68±0.24	10.46±0.47
Phosphorus	9.05±2.23	8.13±1.71	7.94±1.94	8.86±1.83
Chlorides	108.6±2.43	106.5±1.65	104.1±0.99*	109.5±1.65
Total proteins	7.56±0.27	7.54±0.21	7.54±0.41	7.52±0.51
Creatinine	0.95±0.09	0.86±0.06	0.87±0.07	0.89±0.08
Urea	37.1±2.62	38.6±2.93	37.8±3.30	38.4±3.30
SGPT	37.1±28.54	151.9±34.61	107.5±13.26	113.1±21.82
SGOT	188.7±79.28	123.2±20.65	127.7±8.62	153.0±36.23
ALP	170.8±24.01	195.4±28.11	206.8±38.52	204.6±19.28*
GGT	8.28±9.11	2.52±1.74	4.34±7.16	1.38±0.29

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=10)

Delayed Hypersensitivity

Table 5A: Average skin reactions in Guinea pigs exposed to DFNM

Animal group	Erythema			Oedema		
	24h	48h	72h	24h	48h	72h
Test (10 animals)	0	0	0	0	0	0
Control (5 animals)	0	0	0	0	0	0

Table 5B: Hematological values when Guinea pigs were exposed to DFNM

Parameter	Test	Control
	Mean ± SD	Mean ± SD
WBC ($10^3/mm^3$)	7.71±0.17	7.89±0.15
RBC ($10^6/mm^3$)	5.06±0.11	4.95±0.066
Haemoglobin (g/dl)	14.30±0.23	14.19±0.23
HCT (%)	39.08±0.52	40.09±1.92
Platelet ($10^3/mm^3$)	425.40±2.88	428.80±1.92
MCV (μg^3)	79.20±1.30	80.00±1.73
MCH (pg)	36.30±1.03	36.68±0.99
MCHC (g/dl)	45.88±1.05	46.02±0.65

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=5)

Table 5C: Biochemical values when Guinea pigs were exposed to DFNM

Parameter	Test	Control
	Mean ± SD	Mean ± SD
Glucose (mg/dl)	179.54±46.29	160.12±41.35
Cholesterol (mg/dl)	27.00±6.12	31.00±6.96
Triglyceride (mg/dl)	127.60±43.01	111.80±15.74
Bilirubin (mg/dl)	0.04±0.23	0.022±0.02
Albumin (g/dl)	3.16±0.14	3.26±0.18
Calcium (mg/dl)	10.00±0.53	10.46±0.54
Phosphorus (mg/dl)	6.96±0.79	6.542±0.55
Chloride (mmol/dl)	107.60±0.80	106.50±0.70
Total protein(g/dl)	5.56±0.19	5.76±0.28
Creatinine(mg/dl)	0.73±0.03	0.73±0.04
Urea(mg/dl)	47.04±3.80	44.38±7.03
SGOT(IU/L)	99.52±36.82	102.72±15.26

SGPT(IU/L)	78.10±2.52	70.88±6.54
ALP(IU/L)	116.80±18.54	131.60±15.81
GGT(IU/L)	18.84±5.41	19±20.25

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=5)

Table 6A: Average skin reactions in Guinea pigs exposed to HANPs

Animal group	Erythema			Oedema		
	24h	48h	72h	24h	48h	72h
Test (10 animals)	0	0	0	0	0	0
Control (5 animals)	0	0	0	0	0	0

Table 6B: Haematological parameters in Guinea pigs exposed to HANPs

Parameters	**Test**	**Control**
WBC ($10^3/mm^3$)	7.88±2.23	8.18±1.19
RBC ($10^6/mm^3$)	7.67±0.39*	4.66±0.28
Hb (g/dl)	23.04±1.13*	14.18±0.58
HCT %	62.68±3.14*	39.62±1.38
PLT ($10^3/mm^3$)	189.2±43.04*	426.6±60.33

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=5)

Table 6C: Biochemical parameters in Guinea pigs exposed to HANPs

Parameters	**Test**	**Control**
Urea (mg/dL)	55.4±9.83	65±4.74
SGOT (IU/L)	116.4±5.85	132.2±25.72
SGPT (IU/L)	81.8±13.68*	63.8±9.67
ALP (IU/L)	121.6±15.3*	80±22
GGT (IU/L)	21±5.7	16.4±2.07
GLU (mg/dL)	145.4±6	187.4±32.21
CHO (mg/dL)	27.6±4.39	25.6±2.45
TG (mg/dL)	105.4±32.8	87.8±14.7
TP (g/dL)	5.5±0.46	5.3±0.22
ALB (g/dL)	3.12±0.04	3.16±0.32
CAL (mg/dL)	10.46±0.49	10.3±1.03
PHO(mg/dL)	6.9±1.29	6.9±1.35
CHLO (mmol/L)	105±0.7	105±2
BIL. T (mg/dL)	0.03±0.02	0.06±0.02
CRE (mg/dL)	0.77±0.05	0.73±0.09

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=5)

Chronic Oral Toxicity

Table 7A (i): Hematological parameters of male rats fed with DFNM (Combined chronic toxicity and carcinogenicity)

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=6)

Parameters	Control	Low	Medium	High	High Recovery
RBC ($10^6/mm^3$)	8.38±0.32	7.84±0.41*	8.61±0.36	8.36±0.17	6.59±0.60*
HGB (g%)	14.60±0.54	14.03±0.57	15.32±0.84	14.55±0.31	15.67±0.95*
PLT ($10^6/mm^3$)	6.49±0.68	7.18±2.13	7.87±0.77*	6.43±0.27	6.44±0.66
MCV (μm^3)	56.57±1.48	57.13±4.29	56.07±3.31	56.22±1.87	54.33±1.75*
MCH (pg)	17.35±0.31	17.95±1.20	17.80±0.56	17.42±0.57	23.92±2.03*
MCHC (g/dl)	30.73±0.48	31.42±0.68	31.23±0.33	31.02±0.08	43.98±2.46*

Table 7A (ii): Hematological parameters of female rats fed with DFNM (Combined chronic toxicity and carcinogenicity)

Parameters	Control	Low	Medium	High	High Recovery
RBC ($10^6/mm^3$)	7.76±0.23	7.61±0.45	7.74±0.38	7.81±0.28	5.60±0.19*
HGB (g%)	14.27±0.37	14.08±0.63	14.32±0.40	14.35±0.76	15.37±0.43*
PLT ($10^6/mm^3$)	8.56±0.62	7.87±0.82	7.49±1.21*	6.80±0.46*	6.62±0.45*
MCV (μm^3)	58.30±1.65	57.93±2.64	58.40±2.00	58.10±1.99	56.00±1.26*
MCH (pg)	18.40±0.51	18.53±0.94	18.52±0.71	18.35±0.30	27.48±0.46*
MCHC (g/dl)	31.57±0.29	32.03±0.37*	31.67±0.50	31.62±0.54	49.07±0.96*

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=6)

Table 7B(i) : Biochemical Parameters in male rats orally fed with DFNM (Combined chronic toxicity and carcinogenicity)

Biochemical Parameters		Mean±SD			
	Control	Low	Medium	High	High Recovery
Total Protein (g/dL)	8.30 ± 0.85	8.05 ± 0.22	8.18 ± 0.49	8.24 ± 0.39	8.50 ± 0.37
Creatinine (mg/dL)	0.99 ± 0.06	0.91 ± 0.07*	0.99 ± 0.07	0.95 ± 0.05	0.87 ± 0.06*
Urea (mg/dL)	41.23 ± 4.73	43.15 ± 3.46	42.12 ± 3.15	40.69 ± 1.92	37.15 ± 1.67*
SGPT (U/L)	90.09 ± 16.53	83.55 ± 12.83	104.03 ± 11.82	80.39 ± 10.45*	99.55 ± 9.49
SGOT (U/L)	109.55 ± 31.77	97.30 ± 17.68	181.48 ± 114.55	95.15 ± 33.06	128.72 ± 18.62
Alkaline Phosphatase (U/L)	355.79 ± 93.18	285.00 ±48.35*	284.00 ± 59.51	298.40 ± 94.19	338.33 ± 66.03
Glucose (mg/dL)	98.04 ± 12.93	89.07 ± 11.25	94.82 ± 31.97	96.67 ± 15.00	114.67 ± 7.27*
Cholesterol (mg/dL)	55.21 ± 8.40	66.17 ± 7.78*	76.00 ± 7.59*	67.60 ± 11.83*	80.67 ± 8.43*
Bilirubin Total (mg/dL)	0.13 ± 0.03	0.15 ± 0.04	0.15 ± 0.02	0.17 ± 0.04*	0.14 ± 0.03
Albumin (BCG) (g/dL)	4.42 ± 0.83	4.45 ± 0.29	4.55 ± 0.42	4.55 ± 0.24	4.66 ± 0.21
Phosphorus (mg/dL)	7.80 ± 1.48	6.37 ± 1.07*	7.60 ± 0.57	6.64 ± 1.27*	4.99 ± 2.26*
Chlorides (mEq/L)	110.99 ± 1.54	112.03 ± 2.74	112.38 ± 1.72	113.45 ± 2.20*	110.07 ± 2.36
GGT (U/L)	4.05 ± 3.28	1.80 ± 1.42*	3.87 ± 1.65	2.64 ± 1.40	2.13 ± 1.55

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=6)

Table 7B (ii) : Biochemical Parameters in female rats orally fed with DFNM (Combined chronic toxicity and carcinogenicity)

Biochemical Parameters		Mean±SD			
	Control	Low	Medium	High	High Recovery
Total Protein (g/dL)	9.12±1.03	8.02±1.02*	8.58±0.32	8.69±1.21	8.73±0.37
Creatinine (mg/dL)	0.96±0.08	0.91±0.04	0.92±0.05	0.92±0.07	0.87±0.04*
Urea (mg/dL)	43.94 ± 5.75	46.90 ± 4.94	45.12 ± 13.55	45.63 ± 8.55	36.93 ± 5.55*
SGPT (U/L)	78.22 ± 9.48	82.18 ± 11.93	73.30 ± 12.49	84.88 ± 11.18	85.52 ± 8.92
SGOT (U/L)	100.58 ± 26.66	110.13 ± 14.13	111.10 ± 13.01	128.87 ± 25.77*	96.43 ± 16.63
Alkaline Phosphatase (U/L)	265.13 ± 90.92	211.67 ± 46.11	208.83 ± 48.31	256.87 ± 75.55	191.33 ± 38.98*
Glucose (mg/dL)	107.08 ± 15.02	122.62 ± 26.56	100.47 ± 19.90	109.40 ± 17.38	117.17 ± 9.35
Cholesterol (mg/dL)	71.60±13.09	63.50±6.69	77.17±13.98	68.00±16.90	92.67±13.53*
Bilirubin Total (mg/dL)	0.18±0.04	0.14±0.02*	0.16±0.02	0.16±0.05	0.16±0.02
Albumin (BCG) (g/dL)	5.24±0.66	4.90±0.32	5.13±0.16	4.73±0.89	5.44±0.28
Phosphorus (mg/dL)	5.49±0.92	4.49±0.59*	4.42±0.89*	5.05±1.21	4.19±0.43*
Chlorides (mEq/L)	113.35±2.74	111.90±1.85	112.28±1.45	114.68±2.73	110.22±1.71*
GGT (U/L)	2.26 ± 1.48	2.00 ± 1.58	1.65 ± 0.69	2.61 ± 2.96	1.42 ± 1.20

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=15)

Table 8A (i): Haematological parameters of male rats exposed to HANPs orally (Combined chronic toxicity and carcinogenicity)

Parameters	Control	Low	Medium	High	High Recovery
RBC ($10^6/mm^3$)	8.93±0.63	9.04±0.33	8.84±0.26	8.91±0.39	9.09±0.26
HGB (g%)	15.15±1.06	15.52±0.73	15.32±0.24	15.35±0.48	15.50±0.53
PLT ($10^6/mm^3$)	8.39±1.12	8.54±0.83	8.95±1.33	8.16±1.12	9.28±0.95
MCV (μm^3)	52.67±1.38	54.55±1.32*	54.13±1.56	54.48±1.23*	53.38±1.74
MCH (pg)	16.98±0.35	17.13±0.33	17.35±0.49	17.25±0.45	17.08±0.38
MCHC (g/dl)	32.25±0.45	31.43±0.33*	32.03±0.25	31.65±0.52	31.98±0.50

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=6)

Table 8A (ii): Haematological parameters of female rats exposed to HANPs orally (Combined chronic toxicity and carcinogenicity)

Parameters	Control	Low	Medium	High	High Recovery
RBC (10^6/mm^3)	7.78±0.33	8.05±0.27	7.92±0.15	7.90±0.37	7.80±0.35
HGB (g%)	14.68±0.61	14.95±0.47	14.68±0.37	14.72±0.39	14.33±0.38
PLT (10^6/mm^3)	7.69±0.68	9.05±0.67*	9.38±0.35*	8.28±1.25	10.04±1.29*
MCV (μm^3)	57.17±1.00	57.58±0.74	56.78±1.77	57.20±1.67	55.95±1.25
MCH (pg)	18.85±0.27	18.60±0.24	18.53±0.48	18.65±0.60	18.37±0.40*
MCHC (g/dl)	32.98±0.55	32.28±0.12*	32.68±0.42	32.60±0.45	32.83±0.12

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=6)

Table 8B (i): Biochemical parameters of male rats exposed to HANPs orally (Combined chronic toxicity and carcinogenicity)

Biochemical Parameters		**Mean±SD**			
	Control	**Low**	**Medium**	**High**	**High Recovery**
Total Protein (g/dL)	8.27±0.87	8.87±0.27	8.35±0.33	9.42±0.75*	8.25±0.27
Creatinine (mg/dL)	0.74±0.06	0.76±0.09	0.72±0.07	0.78±0.12	0.71±0.04
Urea (mg/dL)	38.85±5.35	35.72±1.24	34.07±2.93	35.92±2.27	31.47±4.25*
SGPT (U/L)	162.13±80.78	148.75±36.66	132.75±15.26	132.62±29.84	108.90±17.21
SGOT (U/L)	150.22±40.99	140.03±36.91	124.58±10.82	135.77±22.96	139.88±27.03
Alkaline Phosphatase (U/L)	274.17±44.26	275.67±42.38	289.33±26.15	337.33±27.3*	261.17±64.13
Glucose (mg/dL)	124.95±27.18	100.38±9.00	100.97±13.21	118.57±13.07	100.43±8.46
Cholesterol (mg/dL)	56.17±11.36	75.33±13.95*	64.20±13.92	59.17±14.96	62.50±9.35
Triglycerides (mg/dL)	171.33±76.79	160.00±23.62	140.17±32.29	154.00±33.02	137.67±24.28
Bilirubin Total (mg/dL)	0.13±0.03	0.16±0.03	0.15±0.02	0.14±0.03	0.14±0.02
Albumin (BCG) (g/dL)	4.72±0.22	4.86±0.17	4.82±0.38	4.92±0.23	4.65±0.24
Phosphorus (mg/dL)	8.68±2.63	8.38±1.76	7.18±0.82	7.64±1.92	6.63±0.86
Chlorides (mEq/L)	103.37±26.75	111.83±1.84	112.00±1.66	117.85±6.48	111.62±1.96
GGT (U/L)	2.90±0.69	1.17±0.59*	1.93±0.46	1.50±0.34	1.63±0.70
Calcium (mg/dL)	12.27±3.67	14.07±0.41	13.82±0.58	14.18±0.96	11.25±0.43

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=6)

Table 8B (ii): Biochemical parameters of male rats exposed to HANPs orally (Combined chronic toxicity and carcinogenicity)

Biochemical Parameters	Mean±SD				
	Control	Low	Medium	High	High Recovery
Total Protein (g/dL)	8.23±0.25	8.68±0.65	8.40±0.48	8.58±0.20*	8.05±0.34
Creatinine (mg/dL)	0.71±0.05	0.71±0.03	0.70±0.08	0.67±0.07	0.67±0.06
Urea (mg/dL)	32.58±2.13	38.76±4.66*	36.02±3.79	37.60±3.24*	37.73±2.86*
SGPT (U/L)	95.58±8.73	117.62±17.34*	108.30±21.90	99.38±23.16	98.82±14.54
SGOT (U/L)	128.77±17.75	138.95±42.05	135.77±16.84	161.73±77.41	144.50±51.38
Alkaline Phosphatase (U/L)	199.50±33.35	216.50±37.36	207.40±63.03	232.50±58.63	298.40±159.57
Glucose (mg/dL)	117.63±22.37	104.10±24.91	95.13±22.63	105.85±14.60	112.97±19.01
Cholesterol (mg/dL)	38.50±7.89	49.67±6.22*	37.67±6.68	39.83±9.30	39.50±13.55
Triglycerides (mg/dL)	223.83±99.32	160.50±28.80	160.00±69.61	173.83±50.75	210.50±48.28
Bilirubin Total (mg/dL)	0.14±0.02	1.98±3.71	0.10±0.06	0.14±0.02	0.16±0.06
Albumin (BCG) (g/dL)	4.93±0.22	4.97±0.99	5.08±0.28	5.03±0.20	4.98±0.16
Phosphorus (mg/dL)	4.99±0.65	6.45±1.11*	5.20±1.18	5.45±1.39	5.32±0.25
Chlorides (mEq/L)	110.47±1.32	104.56±16.04	112.58±2.20	110.85±2.92	112.88±1.85*
GGT (U/L)	2.60±1.12	2.80±2.27	2.17±1.45	3.45±2.23	3.90±2.65
Calcium (mg/dL)	13.18±0.27	10.55±1.15*	12.43±1.41	13.40±0.30	10.82±0.17*

(Student's 't' test. For all comparisons, $p<0.05$ was considered significant),
*(p value <0.05, n=6)

Table 9: ICP analysis of samples from immunotoxicity study of DFNM

Sample	Liver (µg/g of tissue)	Spleen (µg/g of tissue)	Kidney (µg/g of tissue)
Control	33.19	119.73	14.48
7 days exposure	57.19	164.34	27.08
14 days exposure	204.65	274.96	15.76
21 days exposure	564.85	656.87	49.72

Table 10: ICP analysis of samples from chronic toxicity study of DFNM

Sample	Liver (µg/g of tissue)	Kidney (µg/g of tissue)
Control	108.9	55.17
High Group	195.22	104.31

Publications

S No	Authors	Title of paper	Name of the Journal	Volume	Pages	Year
1	Nirmal RS Syama S Gayathri V Varma HK Mohanan PV	An in vitro study on the interaction of hydroxyapatite nanoparticles and bonemarrow mesenchymal stem cells for assessing the toxicological behavior	Colloids Surf B and Biointerfaces	28 (117C)	389-397	2014
2	Syama S Reshma SC Leji B Anju M Sreekanth PJ Varma HK Mohanan PV	Toxicity evaluation of dextran coated ferrite nanomaterials after acute oral exposure to Wistar rats	J Allergy Ther doi:10.4172/2155-6121.1000166	5(2)		2014
3	Syama S Reshma SC Gayathri V Varma HK Mohanan PV	Assessment of hydroxyapatitenanoparticles induced oxidative stress- An in vitro study	J Free Rad Antioxidants	140	286-293	2014
4	Reshma SC Syama S Leji B Anju M	Determination of antioxidant defense mechanism after acute oral administration of	J Free Rad Antioxidants	140	318-327	2014

	Sreekanth PJ Varma HK Mohanan PV	hydroxyapatite nanoparticles in rats.				
5	Mohanan PV Syama S Sabareeswaran A Sreekanth PJ Varma HK	Molecular toxicity of dextran coated ferrite nanoparticles after dermal exposure to Wistar rats	J of Toxicology and Health	104	406-422	2014
6	Mohanan PV Geetha CS Syama S Varma HK	Interfacing of dextran coated ferrite nanomaterials with cellular system and delayed hypersensitivity on Guinea Pigs	Colloids and Surfaces B: Biointerfaces Doi-10.1016/J.colsufb.2013.10.033.	116	633-642	2013
7	Geetha CS Remya NS Leji B Syama S Reshma SC Sreekanth PJ Varma HK Mohanan PV	Cells-nano interactions and molecular toxicity after delayed hypersensitivity in Guinea pigs on exposure to Hydroxyapatite nanoparticles	Colloids and Surfaces B: Biointerfaces	112	204-212	2013