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Original Article

CYTOTOXICITY EVALUATION OF TITANIUM AND ZINC OXIDE NANOPARTICLES ON HUMAN CELL LINES

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ABSTRACT

Objective: *In vitro* cytotoxicity evaluation of titanium dioxide, 20 nm (TNP 20) and zinc oxide, 20 nm (ZNP 20) nanoparticles (NP) were tested on different types of human skin (HaCat), lung (A549), liver (Hep G2) and colon (Caco-2) cell cultures in relevance to human risk assessment

Methods: The different concentrations of test TNP 20 and ZNP 20 1-300 µg/ml were exposed to determine the cell viability reduction on four human cell lines after 48 h post exposure using 3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The mitochondrial membrane activities of the viable cells were determined with intensity of formazon formation by interpreting ELISA absorbance values at 470 nm.

Results: The percent of cytotoxicity was determined by comparing percentage of cell viability reduction of test with that of control. The ZNP 20 produced higher cytotoxicity at the doses 100 ($p<0.05$) and 300 ($p<0.001$) µg/ml significantly on tested four human skin (HaCaT), lung (A549), liver (Hep G2) and colon (Caco-2) cells compared to TNP 20. The tested NP induced lesser cytotoxicity at lower concentrations with 1 and 3µg/ml in all the tested four cell lines. The induced cytotoxicity was an indicator for increased intracellular reactive oxygen species which further cause's major cell damage and cell death.

Conclusion: The tested NP were induced greater cytotoxicity in the colon, Liver, lung and skin cells at higher concentrations 100 and 300 µg/ml significantly. The cytotoxicity order of TNP 20 and ZNP 20 at the highest dose (300µg/ml) were concluded as Caco-2>Hep G2>A549>HaCaT for 48 h post exposed cells.

Keywords: TNP 20, ZNP 20, TEM characterization, Cytotoxicity, MTT assay

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INTRODUCTION

Nanotechnology is considered as one of the key technologies of the 21st century and promises revolutionizing the world by the application. Nanotechnology has gained a great deal of public interest due to the needs and applications of NP in many areas of human endeavors including industry, agriculture, business, medicine and public health [1]. Recently, TNP 20 and ZNP 20 have gained much importance due to their wide spread use of applications in nanodermatology and nanocosmetology [2]. Due to their extensive applications in many commercial products was increased exposure to human beings [3]. More than 150 items nanotechnology based consumer products that would have enhanced long-term dermal contact of these NP in the present nanoworld. TNP 20 and ZNP 20 were the most commonly used NP found in dermally applicable consumer products such as tooth paste, food colorants and nutritional supplements [4]. According to a recent study, candies, sweets and chewing gums have a higher amount of TNP and ZNP (<100 nm) which can enters into human body through dermal, inhalation and oral routes of exposure in relevance to toxicology [5]. The size of the NP matters a lot which will influence the cell-particle interaction [6, 7]. The cytotoxicity in variety of cells types includes HaCaT, A549, HepG2, and caco-2 [8-12]. As of now very few authors reported the toxicity effect of TNP and ZNP with different sizes. So the cell-particle type interaction or nanotoxicology is gaining much importance in the nanoworld due to their high volume of production, extensive applications and unknown health complications after exposure to human beings and animals. So, in the present study the TNP 20 and ZNP 20 were evaluated for possible cytotoxicity on human four cell lines using mitochondrial membrane activity assay according to the risk exposure in human populations by *in vitro*.

MATERIALS AND METHODS

Particles and chemicals

The TNP 20 and ZNP 20 NP were purchased from Sigma Aldrich, Mumbai, India. Nano Quartz (NQTZ) particles (<100 nm; 99.94% purity) were purchased from Berkely Springs, West Virginia, USA used as a positive control. The Dulbecco's modified eagles medium (DMEM), Roswell Park Memorial Institute (RPMI 1640) culture media, 1% L-glutamine, 1% penicillin-streptomycin antibiotic solution, trypsin-EDTA, cell culture flasks, 96 well plates and phosphate buffer saline (PBS) were purchased from Himedia, Mumbai, India. MTT was purchased from Sigma Chemicals Co. Ltd. (St. Louis, MO, USA).

Particles treatment

Human skin (HaCaT), lung (A549) liver (Hep G2) and Caco-2 cells were purchased from National Center for Cell Sciences (NCCS), Pune, India. They were adherent, suspension and mixed type. They have been received with job number 1610 from the cell repository. Cell culture flasks were found to be free from bacterial and fungal contamination. They should be endotoxin free. These cells were sub cultured with passages number 18-23 depending on the cell type.

Cells were allowed to grow in suitable culture media (DMEM/RPMI 1640) supplemented with 10% FBS, antibiotic solution. The cell confluency 1.0 x 10^4cells/0.1 ml were loaded into the 96 well plates after trypsinisation. The test TNP 20 and ZNP 20 were prepared as suspensions in PBS as a solvent with<0.1% sodium citrate as stabilizer. TNP 20, ZNP 20, NQTZ (1-300 µg/ml) in serum free culture medium were freshly prepared and used for cytotoxic activity [13].

Cytotoxicity assessment by MTT assay

The effect of test nanomaterials on the cellular proliferation and viability was determined by using MTT assay method [13]. MTT assay was performed to determine the dose response of TNP 20, ZNP 20 and NQTZ. The test nanoparticles concentrations with 1, 3, 10,100, and 300 µg/ml were exposed. Viable cells in presence of dye impart the colour and cell numbers were counted with the help of neubauer chamber. The cell media was discarded and culture flask

was washed with 2-3 ml of PBS. The cells were trypsinised and transferred to 15 ml torson tube. The cell pellet was obtained after centrifugation. The pellet was resuspended in DMEM/RPMI 1640 in tissue reagent trough. All the cells were seeded at a density of 3 × 10^4cells/well 100 µl volume (triplicate) were added. The plate was then incubated at 37 °C for 48 h in CO_2 incubator (WTC Binder, Germany) and MTT (5 mg/ml), 20 µl solution was added to each well [14].

The plate was again incubated for 2 h, DMSO (80 µl) was added to each well. Then the plate was wrapped in aluminum foil to prevent the oxidation of the dye and the plate was placed on a rotary shaker (Remi equipments, Mumbai, India) for 2 h. The absorbance was measured at 470 nm using multiwellplate ELISA reader (BioTek™ Winoosk, USA). The work was done purely under aseptic conditions. The absorbance of the test was compared with that of solvent control to get the percent cytotoxicity [15].

Statistical analysis

The MTT assay data was analyzed using the Graph Pad Prism 6.0 software, one-way ANOVA followed by Bonferroni post hoc test. The statistical significance was assigned at $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ versus control cells.

RESULTS

Characetrization of test nanoparticles

The TNP 20 and ZNP 20 were characterized by TEM, TEM-JEOL-2100F. The primary particle size for the both TNP 20 and ZNP 20 were 18.14±3.09 and 17.39±4.60 nm, shown in fig. 1.

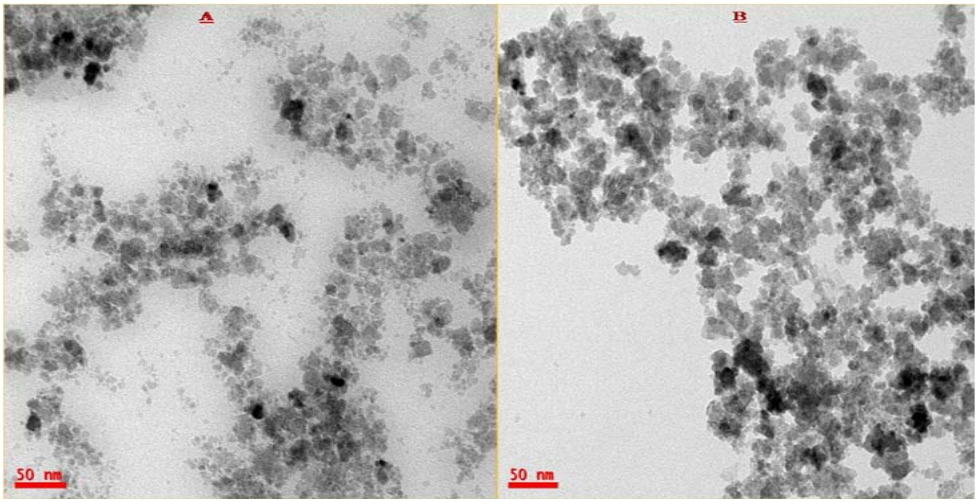

Fig. 1: Characterization of TNP 20 and ZNP 20 particles by transmission electron microscopy (TEM). A-B: A. TNP 20; B. ZNP 20; all dispersions contained nanosized particles; however the particles were mostly dispersed as agglomerates or aggregates

The physicochemical characterization of the test particles size, shape, average hydrodynamic size and surface area were shown in table 1.

Table 1: Physicochemical properties of the titanium and zinc oxide nanoparticles

Particle type	Average TEM diameter of TNP (nm±SD)[A]	Shape[A]	Average hydrodynamic diameter (nm±SD)[B]		Specific surface area (m^2/g)[C]
			MilliQ	2% serum	
TNP 20	18. 14±3.09	Spherical	259.2±28.49	205.7±11.19	46.17
ZNP 20	17.39±4.60	Spherical	298.10±19.60	287.3±21.50	44.85

Data are mean±SD. [A]Average diameter and shape of TNP by Transmission electron microscopy (TEM); [B]Dynamic light scattering (DLS);[C]Specific surface area by Brunauer, Emmett and Teller (BET).

The XRD spectra of test nanoparticles were shown in fig. 2 and 3 and FTIR spectra were shown in fig. 4 and 5 respectively.

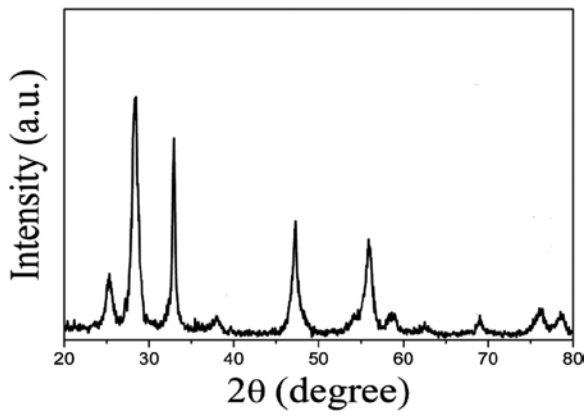

Fig. 2: X-ray diffraction spectrum of TNP 20

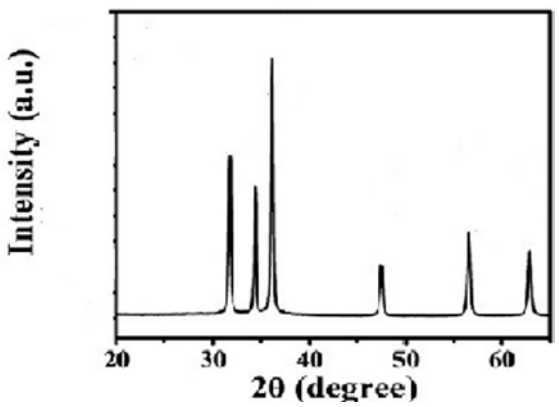

Fig. 3: X-ray diffraction spectrum of ZNP 20

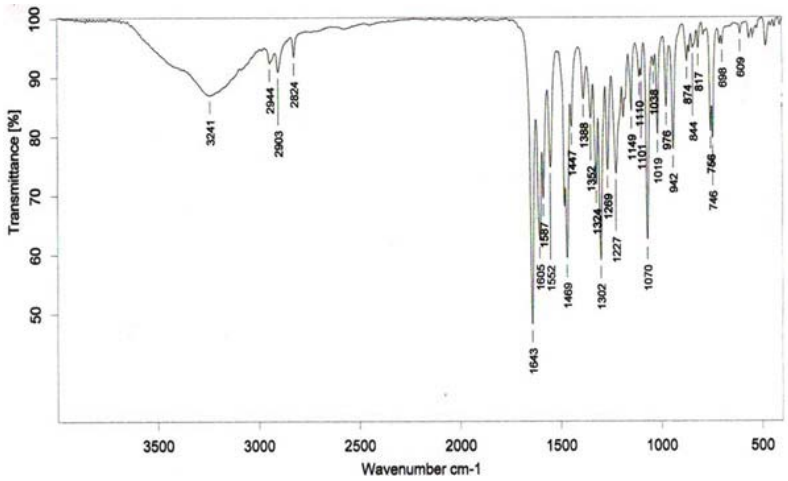

Fig. 4: FTIR spectrum of TNP 20

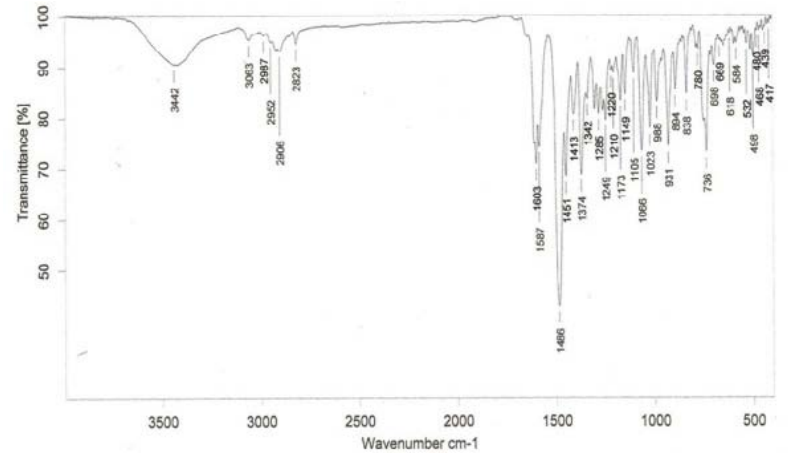

Fig. 5: FTIR spectrum of ZNP 20

Cytotoxicity assessment by MTT assay

TNP 20 and ZNP 20 were exposed to a panel of four different cell lines. The cell lines used for the present study were HaCaT, A549, Hep G2 and Caco-2 cells. According to the results obtained on individual cells were shown in fig. 6, 7, 8, and 9 respectively. The results have shown that reduced cell viability was observed at 100 and 300 µg/ml for the both smaller sized TNP 20 and ZNP 20 in a dose dependent manner significantly on four cell lines. The cytotoxicity produced by TNP 20 in HaCaT cells were shown in fig. 6.

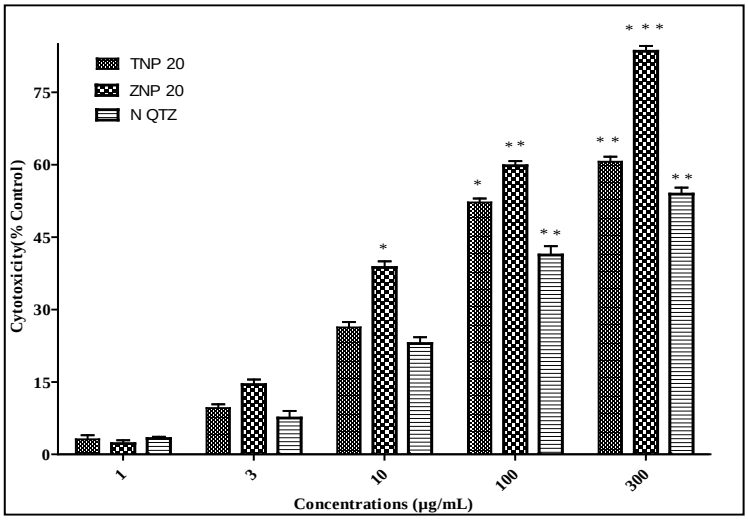

Fig. 6: Percent of cytotoxicity measured by MTT assay on human skin (HaCaT) cells exposure to TNP 20 and ZNP 20 concentrations (1-300 µg/ml) for 48 h. Data are mean±standard deviation (SD) n = 3. Stastical analysis was performed using one-way ANOVA followed by bonferroni post-hoc test. *p<0.05, ***p<0.001, **p<0.01 versus control

TNP 20 reduced cell viability as 26.29 %, 52.18 %, and 60.60 % significantly at the doses 10, 100 (P<0.05) and 300 (P<0.01) µg/ml compared to control (untreated cells) after 48 h post exposure in skin (HaCaT) cells. The cell viability reduction were found to be 38.74%, 59.82%, and 83.50% for the doses 10(p<0.05), 100(p<0.01), 300(p<0.001) µg/ml significantly to the ZNP 20 exposed cells. The TNP 20 has reduced the cell viability as 30.27%, 52.18%, and 62.85% for the doses 10, 100 (p<0.05) and 300 (p<0.05) µg/ml significantly against the A549 cells. The ZNP 20 has reduced the cell viability as 37.40%, 63.40%, and 85.73% at the doses 10 (p<0.05), 100 (p<0.05) and 300 (p<0.001) µg/ml significantly in A549 cells compared to control and NQTZ. The results were shown in fig. 7.

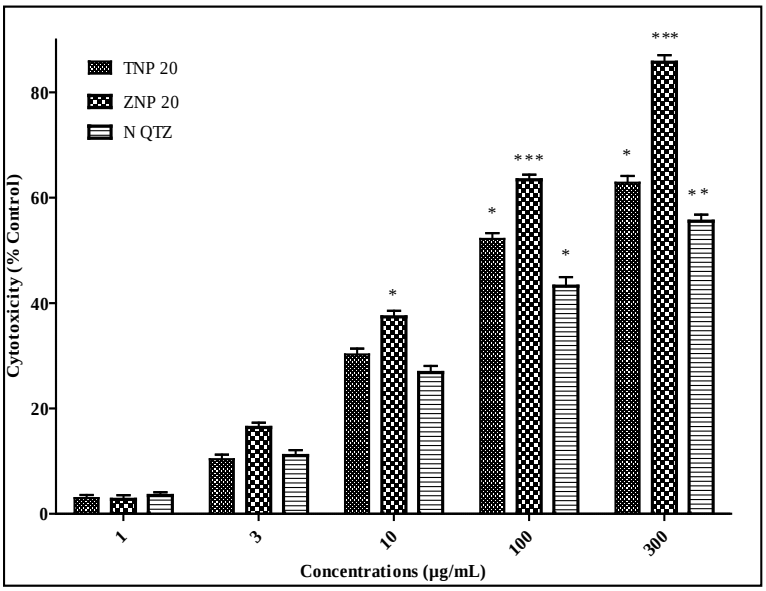

Fig. 7: Percent of cytotoxicity measured by MTT assay on human lung (A549) cells exposure to TNP 20 and ZNP 20 concentrations (1-300 µg/ml) for 48 h. Data are mean±standard deviation (SD) n = 3. Stastical analysis was performed using one-way ANOVA followed by bonferroni post-hoc test. *p<0.05, ***p<0.001, **p<0.01 versus control

The TNP 20 and ZNP 20 cytotoxicity results on Hep G2 cells were shown in fig. 8.

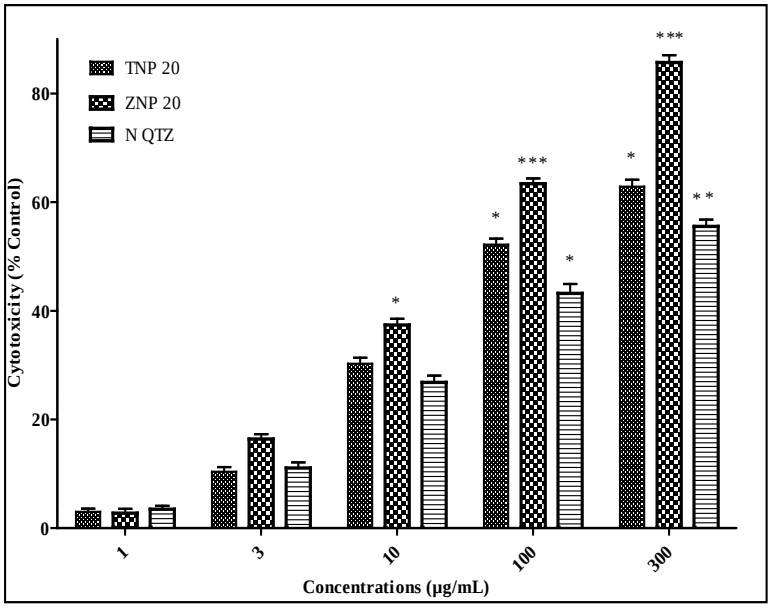

Fig. 8: Percent of cytotoxicity measured by MTT assay on human liver (Hep G2) cells exposure to TNP 20 and ZNP 20 concentrations (1-300 µg/ml) for 48 h. Data are mean±standard deviation (SD) n = 3. Stastical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc test. *p<0.05, ***p<0.001, **p<0.01 versus control

The TNP 20 has 25.39%, 40.82%, and 75.40% at the doses 10, 100 (p<0.05), 300 (p<0.01) µg/ml significantly after 48 h post exposure to Hep G2 cells. ZNP 20 reduced cell viability with increasing dose concentrations as 10 (p<0.05), 100 (p<0.05), 300 (p<0.001) µg/ml were 27.16%, 53.40% and 87.40%. The TNP 20 and ZNP 20 were reduced the cell viability significantly on Caco-2 cells shown in fig. 9.

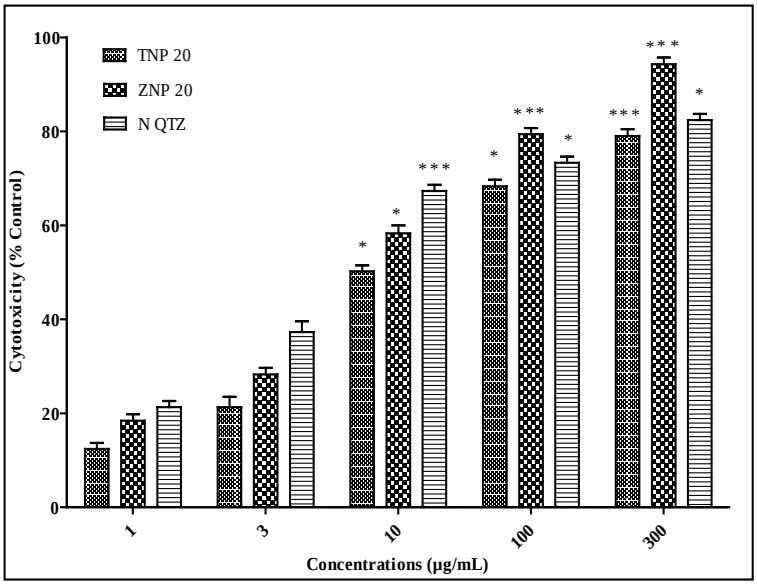

Fig. 9: Percent of cytotoxicity measured by MTT assay on Hhuman colon (Caco-2) cells exposure to TNP 20 and ZNP 20 concentrations (1-300 µg/ml) for 48 h. Data are mean±standard deviation (SD) n = 3. Stastical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc test. *p<0.05, ***p<0.001, **p<0.01 versus control

The cell viability was reduced as 50.20%, 68.30% 79.00% against the doses 10 (p<0.05), 100 (p<0.05) 300 (p<0.01) µg/ml significantly for TNP 20 after exposed to Caco-2 cells for 48 h. ZNP 20 reduced cell viability as 58.30%, 79.38%, and 94.30% at the doses 10 (p<0.05), 100 (p<0.001), 300 (p<0.01) µg/ml compared to control and NQTZ for 48 h post exposure in Caco-2 cells. The non significant cell reduction was found at lower concentrations (1 and 3µg/ml) in all the tested four cell lines.

DISCUSSION

In recent years, small sized particles have been exclusively used in various biological applications including live cell imaging, photodynamic therapy, targeted drug delivery and in cancer imaging [16, 17]. *In vitro* toxicity assays can play an important role for the risk/hazard assessment of NP [18].

TNP 20 and ZNP 20 indicated a range of cytotoxicity responses upon post exposure to human HaCaT, A549, Hep G2 and Caco-2 cells respectively. The TNP 20 and ZNP 20 produced less cytotoxicity on HaCaT cells at lower concentrations (1and 3µg/ml) for 48h. The significant cell viability reduction were observed at higher concentrations 100 (P<0.05) and 300 (P<0.01) for TNP 20 in HaCaT cells after 48 h post exposure. But ZNP 20 has produced cytotoxicity in dose dependent manner at the doses 10 (p<0.05), 100 (p<0.01), 300 (p<0.001) µg/ml significantly when compared to control and NQTZ. The results obtained were similar to the previous work done on three mammalian cells with 11 metal oxides using MTT assay [19]. They concluded that TNP 18.39 nm and ZNP 21.68 nm were produced cytotoxicity, cell viability reduction 50 % and induced membrane damage at concentrations greater than10 µg/ml of the exposure time 48 h [20, 21].

The percent ctotoxicity of TNP 20 and ZNP 20 in A549 cells were shown in fig. 7.

The cells were more sensitive compared to HaCaT cells. The TNP 20, ZNP 20 and NQTZ were produced 50% cell viability reduction at 100 µg/ml significantly. For ZNP 20, the highest cell viability reduction was 85.73% at the 300 µg/ml observed in A549 cells after 48 h post exposure [22]. The percent cytoxtoxicity induced by test TNP 20 and ZNP 20 exposed to Hep G2 cells were shown in fig. 8.

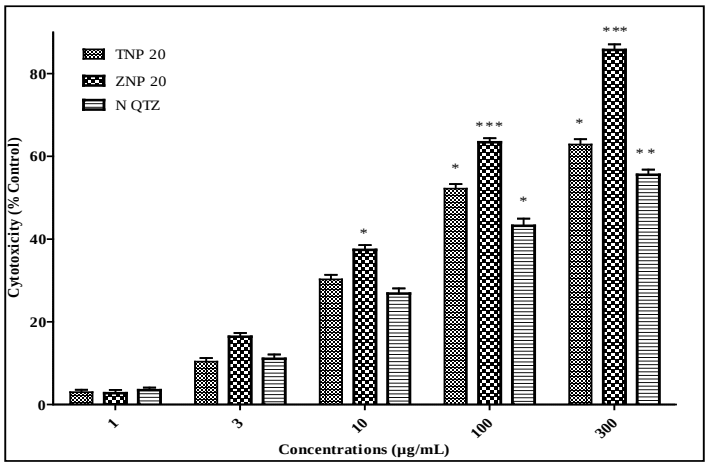

Fig. 7: Percent of cytotoxicity measured by MTT assay on human Llung (A549) cells exposure to TNP 20 and ZNP 20 concentrations (1-300 µg/ml) for 48 h. Data are mean±standard deviation (SD) n = 3. Stastical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc test. *p<0.05, ***p<0.001, **p<0.01 versus control

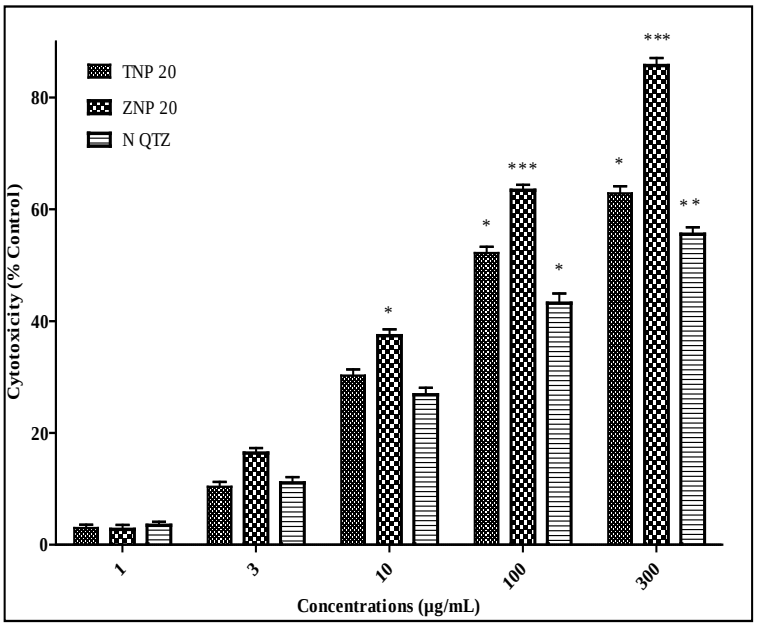

Fig. 8: Percent of cytotoxicity measured by MTT assay on human liver (Hep G2) cells exposure to TNP 20 and ZNP 20 concentrations (1-300 µg/ml) for 48 h. Data are mean±standard deviation (SD) n = 3. Stastical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc test. *p<0.05, ***p<0.001, **p<0.01 versus control

The Hep G2 cells were more sensitive compared to the A549 and HaCaT cells. The highest cell viability reduction was 87.40% at the dose 300 µg/ml for ZNP 20 exposed to Hep G2 cells. The significant cell viability reduction were observed at doses 10 ($p<0.05$), 100 ($p<0.05$), 300 ($p<0.001$) µg/ml for ZNP 20. The above 50% cell viability reduction was observed at the doses 100 and 300 µg/ml for ZNP 20. So results were indicating that the highest cytotoxicity effect was observed at the doses 100 and 300 µg/ml in Hep G2 cells after 48 h post exposure [23].

The percent cytotoxicity in Caco-2 cells after 48 h post exposed TNP 20 and ZNP 20 were shown in fig. 9.

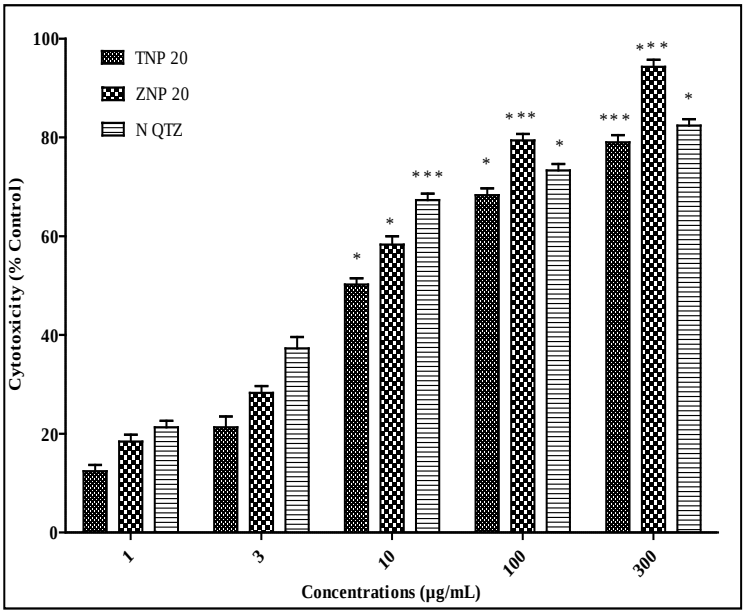

Fig. 9: Percent of cytotoxicity measured by MTT assay on human colon (Caco-2) cells exposure to TNP 20 and ZNP 20 concentrations (1-300 µg/ml) for 48 h. Data are mean±standard deviation (SD) n = 3. Stastical analysis was performed using one-way ANOVA followed by Bonferroni post-hoc test. *p<0.05, ***p<0.001, **p<0.01 versus control

The 50 % cell viability reduction were observed at doses the doses 10 ($p<0.05$), 100 ($p<0.05$) 300 ($p<0.01$) µg/ml significantly for TNP 20. ZNP 20 highest cell viability reduction i.e., 94.30% at the dose 300 ($p<0.01$) µg/ml compared to control and NQTZ for 48 h post exposure in Caco-2 cells. TNP 17.46 nm and ZNP 21.50 nm were potentially damage to upon exposure to these NP. Recent study investigated the potential effects of these food-borne NP on intestinal cells with mechanistic approach. The study report findings

were similar to the results obtained in the present study [24, 25, 26]. In the tested NP, ZNP 20 was the most cytotoxic than TNP 20 on the tested panel of cell lines.

CONCLUSION

The test nanoparticles were induced a significant cell viability reduction on a panel of four cell cultures. The results showed that the cytotoxicity induced by the test TNP 20 and ZNP 20 were concluded as in the order of Caco-2>Hep G2>A549>HaCaT for 48 h post exposed cells.

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AUTHOR CONTRIBUTION

The present work selection, procurement, preparation and screening of cytotoxicity and cell viability reduction study were done Mr. G. Durgaiah under the supervision of Prof. Y. Narsimha Reddy. The technical help was given by Dr. Harikiran Lingabathula for the present work.

CONFLICT OF INTERESTS

The authors declare that there were no conflicts of interest

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