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IN VITRO CYTOTOXICITY EVALUATION OF ESCITALOPRAM LOADED NANOPARTICLES AFTER EXPOSURE TO NEUROBLASTOMA CELL LINES

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ABSTRACT: The aim of the present studies is to evaluate the cytotoxic effects of newly formulated polymeric nanoparticles (ETP NP's) of Escitalopram oxalate (ETP) on NB41A3 cell lines. They were made by ionic gelation method with particle size range between 60 - 115nm, showing spherical morphology. Moreover, zeta potential of the same was recorded as -1.89 ± 0.052 mV and its permeability release showed 98.45 ± 1.82 % in 24 hour. The cytotoxicity evaluation of the optimized formulation (A3) included qualitative method of cell morphology (microscopic and DAPI staining evaluation) and quantitative colorimetric assays like - neutral red (NRU), MTT and LDH. The results suggested that, in presence of ETP, there was a remarkable decrease in cell count whereas; ETP NP's showed lesser cytotoxicity. performed. Further, in NRU membrane integrity was assessed and it showed 95.2 ± 0.83 % and 72.2 ± 0.56% of cell viability in ETP NP's and ETP pure respectively. However, MTT assay summed up the cell viability up to 93.6 ± 0.68 % in ETP NP's in comparison to 88.2 ± 0.35 % of ETP pure and LDH assay results for both , ETP NP's (5.105 unit/ml) and ETP pure (5.779 units/ml) suggested that the cell viability is more dose and incubation time dependent. These results confirms the efficacy and non toxicity of formulated ETP NP's and thus, it can be further applied to *in vivo* model to study its various pharmacokinetic and pharmacodynamic parameters to establish its therapeutic potentials completely.

INTRODUCTION: Nanoparticle based formulations are proving to be a potential targeted, drug delivery system as they have substantial advantages over the conventional dosage forms like – prolonged stability, protects drug against chemical and enzymatic degradation, sustained drug release at targeted site and easy penetration into small capillaries [1]. Drug deliveries through polymeric nanoparticles have shown effective and increased bioavailability of drugs across the biological barriers [2] especially in case of central nervous system disorders (CNS).

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In CNS disorders, the influx mechanisms that regulate the concentration of drug via cardiovascular system to the target site depend on the high selectivity by BBB (blood brain barrier), BCB (blood cerebrospinal fluid barrier), BTB (blood tumor barrier). Therefore, reducing down the bioavailability and therapeutic index of the drug compound. Escitalopram (ETP) is one such example of drug which undergoes for extensive hepatic metabolism forming secondary metabolites like S-demethylcitalopram (S-DCT) and S-didemethylcitalopram (S-DDCT) and has less bioavailability (80%) with its oral dosage forms which is 5 - 20mg/ml/day [3, 4].

It is an antidepressant, approved by FDA for treatment of general anxiety disorder (GAD), major depressive disorder (MDD) etc. [5, 6] Now, this drug although being a potential one for the treatment of

mentioned medical disorders has less therapeutic index due to its excessive hepatic metabolism. So, to increase its pharmacological effects, there arises a need to formulate a novel drug delivery system for the same [7, 8]. Therefore, a chitosan based ETP loaded nanoparticles were prepared by ionic gelation method, which was further characterized and evaluated for *in vitro* drug release kinetics. The results showed that the optimized ETP loaded polymeric nanoparticles exhibited particle size between 60-115nm with PDI (polydispersity index) and zeta potential score of 0.117 ± 0.06 and -1.89 ± 0.27 mV respectively, indicating the homogenous nature of nanoparticle solution.

The TEM and SEM analysis further confirmed nanometric size of the formulation with spherical morphology [9-11]. Also, the FT – IR results showed that there was no bond formation between the drug molecule and polymer compound. So, after confirming and validating the nanoformulation it was further required to test the cytotoxicity for the same [9]. Hence, the present study is focused on evaluating the *in vitro* cytotoxicity of pre formulated Escitalopram (ETP) loaded nanoparticles on NB41A3 cell lines [12]. These are the neuroblastoma cell line of mouse brain which has the peculiar enzyme secretory characteristics for neurotransmitter synthesis.

MATERIALS AND METHODS: NB41A3 cell lines were procured from NCCS, Pune. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serums (FBS), Trypsin EDTA were supplied by Himedia. Phosphate buffered saline solution (PBS), MTT, Coomassie blue, Neutral Red, Sodium pyruvate, NADH and Tris-HCl buffer was from Sigma-Aldrich Chemicals (Bornem, Belgium).

Preparation of Escitalopram loaded polymeric nanoparticles: Escitalopram (ETP) loaded nanoparticles were formulated by ionic gelation method using chitosan and TPP polymers in ratio (1:1.5 mg/ml) with ETP drug concentration of 2.5mg/ml. Characterization of ETP loaded nanoparticles validated the particle size range between 60-115nm. Further, after preparation process nanoparticles were washed and centrifuged thrice at 12750g for 40 minutes and were lyophilized for further *in vitro* assessment. The stock nanoparticles (1mg/ml) were further dispersed in Milli Q water [13].

Cell culturing and experimental model: For cytotoxicity analysis of ETP nanoparticles, NB41A3 cell line was taken as a model system to assess neurotoxicity. Their morphology is elongated and is loosely adherent with multicell aggregates. It was propagated in DMEM media with cell concentration of 2.5 $X10^5$ cell/ml with 5% FBS (Fetal bovine serum) at 37^0C temperature and 5% CO_2. The cells got adhered within 24 hours and after getting the optimal growth of cells in the flask, cells were trypsinized. After removing the media, cell layer is briefly rinsed with 0.25% (w/v) Trypsin-0.53mM EDTA solution (1.5ml of 1X). Cells were then seeded at the concentration of 2.5 $X10^5$ cell/ml in 6-well, 24-well and 96-well plates, as per the requirement in experimental setup and incubated for 24 hours in same experimental conditions. After that, cells were treated with test samples (ETP pure, only NP's and ETP NP's solutions) at different time intervals for further qualitative and quantitative evaluations [14].

Qualitative evaluation: Microscopic Evaluation: Microscopic evaluation was conducted to observe and analyze the morphology of cells (at 40x) treated with test samples on inverted microscope (Olympus CKX-31). After seeding of cells in 6 well plates, test samples were added in each well and were observed at different time intervals (*i.e.* 4, 8 and 24 hours). Then bright field micrographs were acquired for all the samples [15, 16].

Fluorescence Microscopy: In parallel, with the microscopic evaluation of test samples, they were further studied by the imaging and micrographs acquired by fluorescence microscope (Olympus IX71). The cell viability of the same area, selected for bright field micrograph was treated with DAPI (4', 6 – diamidino – 2 - phenylindole) stain for fluorescence imaging (at 100x). Cells were seeded onto the cover slip and were treated with test samples for 4 hours. After that, media was completely removed from the wells and PBS (Phosphate buffer saline, pH – 7.2) wash was given to the cells and fixed with 1% formaldehyde.

Thereafter, cells were stained with DAPI dye and washed with PBS to remove excess dye. Lastly, a small drop of glycerol was placed on glass slide and cover slip was turned upside down gently and observed under fluorescence microscope for cell viability.

Quantitative evaluation: MTT Assay: MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5 - diphenyl-2H-tetrazolium bromide) assay was performed to check the cell viability by monitoring the mitochondrial activity of viable cells. After seeding, NB41A3 cells were treated with 100µl of test samples (optimized escitalopram drug solution of 2.5mg/ml concentration, only NP's solution (Chitosan-1mg/ml and tri-polyphosphate- 1.5mg/ml) and escitalopram loaded nanoparticles (ETP NP's)) were added to wells in triplicates and incubated for 24 hours.

Subsequently, MTT reagent (20µl) was added in each well and kept for incubation (4 hours). Meanwhile, formazan crystals were formed due to the reaction between MTT and dehydrogenase enzymes, which were further dissolved with Dimethyl sulfoxide (DMSO) solution and absorbance was taken on multidetection micro plate reader at 570nm wavelength [17-19].

Neutral Red Assay: To ensure the cell membrane integrity, neutral red uptake assay (NRU) was performed. Here, cell viability assessment is based upon the ability of viable cells to attach to neutral red within lysosomes and usually done on adherent cells. Neutral red is basically a weak cationic dye which permeates through the cell membrane and aggregate inside the lysosomes, further attaching to lysosomal mould (anionic).

Hence, making it possible to count upon the dead, viable or damaged cells as the absorbance recorded at 540nm is directly related to the count of undamaged cells. After incubating the cells with test samples (ETP pure, ETP NP's and only NP's for 24 hrs), cell layer was washed with DPBS buffer and 100µl of 0.4% (w/v) neutral dye solution (diluted in 1:80 ratio with culture medium) was added in each well, then after 3hrs. Incubation extraction of dye was done by adding 100µl of 1% acetic acid solution prepared in 50% (v/v) ethanol and absorbance was taken on multidetection micro plate reader at 540nm wavelength [15, 17, 20, 21].

Lactate Dehydrogenase (LDh) Assay: Lactate Dehydrogenase assay was performed to estimate the release of enzyme (LDh) by the cells (NB41A3). This enzyme act as a self indicator for presence of stress condition and cytotoxicity in cells treated with different test samples. If test compound is toxic, it causes damage to cell membrane, release of lactate dehydrogenase enzyme in culture media and then cell death. After seeding of cell in 24-well plate for 24 hours the same was incubated with test samples for 24 hrs. After that, culture media were collected, centrifuged at 3187g for 5 minutes and supernatants were collected. Then, reaction mixture was prepared for each test sample with the composition of 500µl of sodium pyruvate (30mM) prepared in Tris-HCl buffer (0.2M, pH 7.3), 20µl of NADH (6.6mM) and 250µl of Tris-HCl buffer and incubated at 25 ^{0}C for 5 minutes. After that, 20µl of each of supernatants were added one at a time to this reaction mixture, incubated for 30 minutes and absorbance were recorded at 340nm wavelength [22-26].

Statistical Analysis: The experiments were performed in the sets of triplicate and statistical data were expressed as mean value of ± standard deviation. All the experimental data were assessed by one way analysis of variance (ANOVA) and were considered significant at $p < 0.01$ [27].

RESULT AND DISCUSSION:

Microscopic Evaluation: At different time intervals (4, 8 and 24 hours), effects of different test samples (ETP pure, ETP NP's and only NP's) were tested on NB41A3 cell lines shown in **Fig. 1**. It was observed that there was a slight change in the morphology and count of the cells. The ETP pure, was showing decrease in the cell count whereas cells treated with ETP NP's and only NP's were not affected much in 4 hours and almost similar results were obtained after 8 hours. However, after 24 hours only NP's were showing remarkable cell growth, which could be due to the presence of chitosan nanoparticles, providing increased surface area. Also, ETP NP's were showing less cytotoxicity as compared to ETP pure.

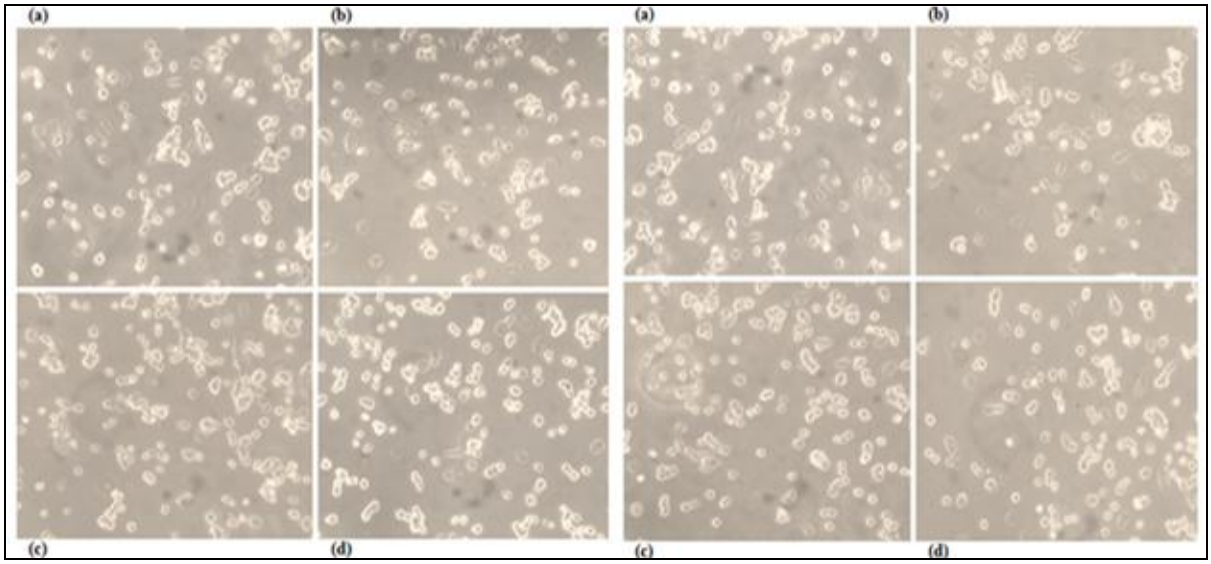


IMAGE I **IMAGE II**

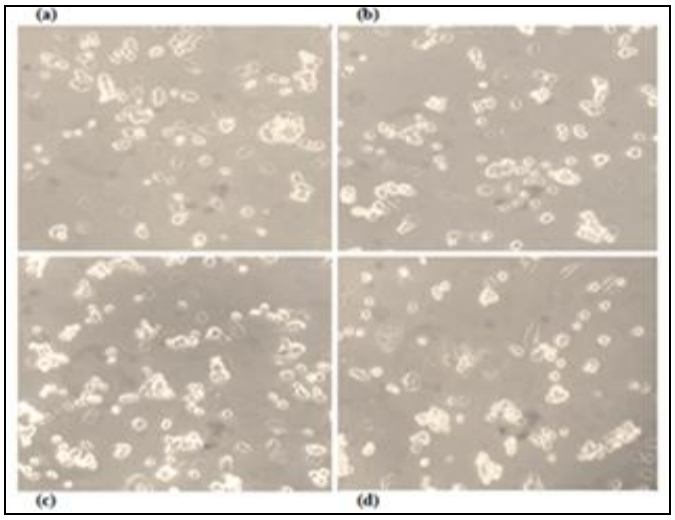


IMAGE III

FIG. 1: IMAGES SHOWING MORPHOLOGY AND CELL CHARACTERISTICS OF NB41A3 CELL LINE AFTER 4 (IMAGE I), 8 (IMAGE II) AND 24 (IMAGE III) HOURS OF TREATMENT ((a) CONTROL SET WITHOUT ANY TEST SAMPLES, (b) ETP PURE DRUG, (c) ONLY NP'S AND (d) ETP NP'S TREATED CELLS)

Fluorescence Microscopy: Since, not much of the change was observed in the cell morphology and viability at 4 and 8 hour, therefore fluorescence tagging of cells with DAPI dye was performed at 24 hours to confirm the microscopic evaluation done earlier.

In this process, blue fluorescent zone of viable cells was observed under microscope. The results suggested that in comparison with ETP pure drug, ETP NP's showed more cell viable zones and only NP's reflected viable zones equivalent to control suggesting that ETP NP's are less toxic than ETP pure drug (**Fig. 2**).

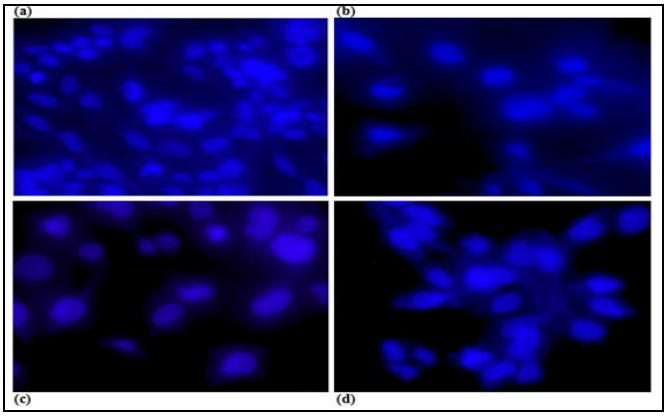
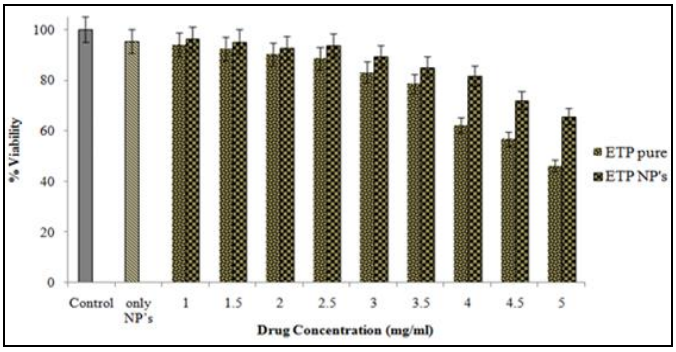


FIG. 2: MICROSCOPIC IMAGES OF DAPI STAINED NB41A3 CELL LINES AFTER 24 HOURS OF TREATMENT WITH (A) CONTROL SET, WITHOUT ANY TEST SAMPLES, (B) ETP PURE DRUG, (C) ONLY NP'S AND (D) ETP NP'S

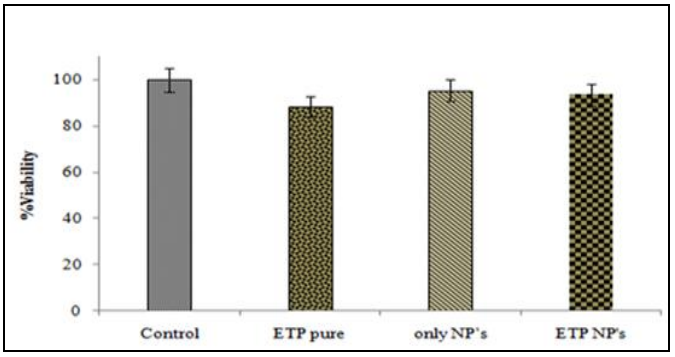
Quantitative Analysis:

MTT Assay*:* The results obtained after the completion of MTT assay, suggests that there is direct relationship between the ETP pure concentration and cell viability, as with the increase in drug concentration (ETP pure) there was a significant increase in cell toxicity, reducing the cell count from 94.1 % (1 mg/ml) to 46.2 % (5 mg/ml). However, in ETP NP's and only NP's there was not much noticeable drop in cell count as it decreased from 96.3 ±0.27% (1 mg/ml) to 65.7 ±0.46% (5 mg/ml) in case of ETP NP's and it remained 95.4 ±0.62% for only NP's, which is lesser than the dose dependent toxicity shown by ETP NP's (**Fig. 3(A)).**

After this dose dependent cytotoxicity analysis, the drug dose concentration of 2.5 mg/ml of ETP in all test samples (ETP pure and ETP NP's) was again tested by MTT assay and results showed in **Fig. 3(B)** exhibited that ETP NP's had maximum cell viability (93.6 ± 0.68%) in comparison to ETP pure (88.2 ± 0.35%).



A



B

FIG. 3: *IN VITRO* CYTOTOXICITY ANALYSIS OF ((A) THE DIFFERENT CONCENTRATION OF TEST SAMPLES; (B) TEST SAMPLES WITH 2.5mg/ml OF ETP CONCENTRATION) ON NB41A3 CELL LINES AFTER 24 HOURS OF TREATMENT. Abbreviations: only NP's = nanoparticles without ETP drug loading, ETP pure = Escitalopram drug only, ETP NP's = Escitalopram loaded nanoparticle formulation

Neutral Red (NR) Assay: The results of NRU assay showed that during the test period there was no significant toxicity enforced by the ETP NP's and only NP's showing cell viability of 95.2± 0.83 % and 91.5±0.17% respectively but escitalopram drug solution (ETP pure) of 2.5mg/ml concentration was showing significantly decreased cell viability of 72.2±0.56% (**Fig. 4**). Therefore it was finally concluded that escitalopram loaded nanoparticles have higher cell viability than ETP pure so it can be effectively used further for the targeted drug delivery.

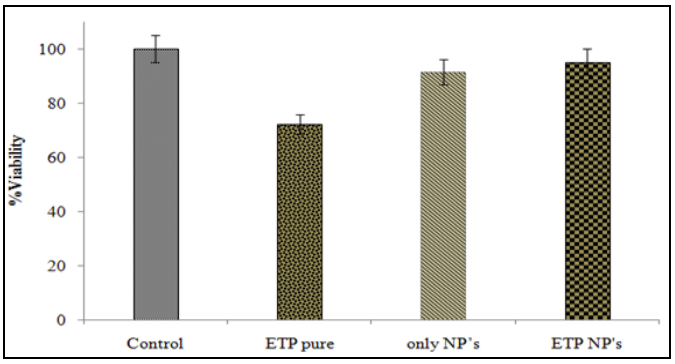


FIG. 4: *IN VITRO* CYTOTOXICITY ANALYSIS OF TEST SAMPLES (WITH 2.5mg/ml OF ETP) ON NB41A3 CELL LINES AFTER 24 HOURS OF TREATMENT BY NRU ASSAY

Lactate Dehydrogenase (LDh) Assay: This assay was performed for NB41A3 cell line to analyze the cytotoxic effect of different test samples (pure ETP, only NP's and ETP NP's) (**Fig. 5**). The results shows that during the test period there was decrease in LDH activity of cells treated with ETP nanoparticles as compared to the cells treated with pure ETP. It was also observed that LDH activity of ETP nanoparticles treated cells was slightly higher than untreated cell.

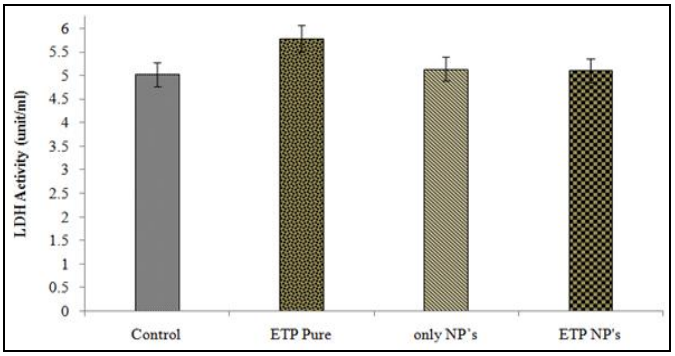


FIG. 5: *IN VITRO* CYTOTOXICITY STUDIES OF OPTIMIZED NANOPARTICLES ON NB41A3 CELL LINE

CONCLUSION: Nanoparticle has been used to protect the drug in the systemic circulation, to deliver the drug at a controlled and sustained rate to the site of action. Since, Escitalopram oxalate had high hepatic metabolism, so to increase its efficacy, sustained delivery through nanoparticles based formulation was opted. Hence, Escitalopram loaded nanoparticles were prepared to decrease the loss of drug entering systemic circulation due to high hepatic metabolism of the drug. The drug after getting entrapped in nanoparticles becomes more stable and shielded from enzymatic degradation. The nanoparticles were prepared after optimization of various process parameters and were successfully made with 1mg/ml chitosan, 1.5mg/ml tripolyphosphate polymers and 2.5mg/ml drug concentrations.

Further confirmation from characterization showed their particle size range between 60 - 115nm with spherical morphology without any encapsulated impurities. FT-IR results revealed that there is no bond formation between polymer and drug and *in vitro* permeability release kinetics also showed sustain release.

Further, *in vitro* cytotoxicity effect of ETP NP's was evaluated on NB41A3 cell line. In qualitative evaluation, ETP pure was showing more morphological and viability changes on cell line as compared to ETP NP's . MTT assay results showed that ETP NP's were not toxic to the cell lines during the test period (93.6 ± 0.68%). Similarly, in NRU and LDH assay, percentage viability and LDH activity of cells treated with ETP pure and ETP NP's were 72.2±0.56% (NRU), 5.779 units/ml (LDH) and 95.2 ± 0.83 % (NRU) 5.105 unit/ml units (LDH) respectively that shows ETP nanoparticles were less toxic than pure ETP. Therefore, it can be proposed from the present study that the optimized ETP loaded nanoparticles (ETP NP's) can be further evaluated on *in vivo* model system to study its various pharmacokinetic and dynamic parameters.

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