

RESEARCH PAPER

Preparation and Evaluation of a Liposome Drug Delivery System in Cancer Treatment in vitro

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ABSTRACT

Cancer is a fatal disease and relatively widespread in the world; Breast cancer is the most prevalent cancer among women. Hydroxyurea (HU) is a chemotherapy drug for the cure of cancer different types in patients, for example breast cancer, but has several defects, for to remove these problems in this study a nanoliposome (NL) suspension for Hydroxyurea (HU) delivery in breast cancer cell therapy was developed. HU was encapsulated into NLs. Size was measured by nanosizer. The release of the liposomal formulation was assessed during 36 h. FTIR analysis for liposomal Hydroxyurea and free Hydroxyurea was carried out. The uptake capacity of the formulation was determined by transfection of nanoliposomal hydroxyurea (NL-HU) in MDA-MB231 cells via flow cytometer and fluorescence microscopy studies, the cytotoxicity of NL-HU and free HU was evaluated in cells. Size of NL-HU was 174nm, HU encapsulation efficiencies in NLs was 81%. FTIR analysis showed the stability of HU in the liposome and no improper interaction between liposome and HU, release after 36h depicted sustained release behavior. NL-HU had suitable uptake in MDA-MB231 cells. Cytotoxicity of NL-HU on cells was considerable. We confirmed these nanoliposomes are potentially useful for delivery of Hydroxyurea in breast cancer cells treatment.

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INTRODUCTION

Cancer is a common disease in the world and breast cancer for women has the highest occurrence and death rate. Current methods for breast cancer therapy are chemotherapy, radiotherapy, and surgery [1, 2]. Between the chemotherapy agents, Hydroxyurea (HU) is well-known, safe, low cost and effective that is applied extensively in the treatment

of the cancer. In addition to these useful properties, rapid clearance from blood circulation, short tumor exposure time and several side effects such as bone marrow and skin disorders have been reported in patients, also this drug show the best response to treatment with high dose that increases side effects in organs [3-6], some methods were developed to dissolve these problems. Various pharmaceutical

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carriers are used in the clinic that among them, the liposome is the one of most successful in cancer therapy. Liposomes are spherical structures composed of double layers lipids surrounding an aqueous part that can to pass through biological barriers, delivery, and release of the drug to the target tissue and protection of drugs against demolition [7-8]. Several studies in this field done, Alavi et al. prepared liposomal HU and evaluated the cytotoxicity on MCF7 cells [9]. Tabrizi et al. incorporated luckily HU into nanotransferosomes to the delivery of HU in breast cancer cells [10]. We in this work constructed first nanoliposomal Hydroxyurea with these components and drug to lipid ratio with a size lower than 200 nm and high encapsulation efficacy to upgrade the HU therapeutic properties and decrease its adverse effects in MDAMB-231 breast cancer cells in the world.

MATERIALS AND METHODS

Materials and cell line

The HU, lecithin, cholesterol, DSPE-mPEG 2000 and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and Chloroform were prepared by Merck (Darmstadt, Germany). Polycarbonate membranes were bought from Northern Lipids (Vancouver, BC). Penicillin-glutamine-streptomycin (P/G/S), Sephadex G50 and FBS were obtained from Invitrogen (Burlington, ON). The MDAMB231 cell line was taken from the ATCC.

Preparation of liposomes and drug loading

Thin film hydration technique was applied for the preparation of nanoliposomes. Briefly, lecithin: chol:DSPE-mPEG2000 at 7:4:0.18 molar ratios were dissolved in chloroform: methanol (2:1), After drying, the film was suspended in 50 ml of PBS (pH=7.4) containing sucrose (1% w/v) and HU (molar ratio drug/lipid=0.2/1) or without drug (blank), Stirred and heated to 50 °C in water bath. The resultant suspension was homogenized by a homogenizer (T18 Ultra Turrax, IKA, US) in 16,000 rpm for 4 min and extruded through filters (100nm) for ten cycles (LiposoFast, Avestin, Ca) afterward freeze-dried (Alpha1-2LD Christ, UK) at -40 °C for 48 h.

Characterization of formulations

Size, the zeta potential of NL-HU

The size and zeta potential of the nanoliposomes

were determined by laser light scattering (DLS, Malvern Zeta sizer, UK), samples were filtered and placed in a quartz cuvette, the result was manifested as mean with standard deviation.

Morphology of NLs

The morphology of the nanoliposomes was investigated by scanning electron microscope (SEM, KYKY-EM3200, China) at an acceleration voltage of 26 kV. Briefly one drop of liposome was spread on a stub and dried at environment, next was coated with a layer of gold and was assessed with SEM.

Entrapment efficiency in NL

Entrapment efficiency (EE) study was done in such a way that, 1 ml of the nanoliposomal HU suspension was centrifuged at 13000 rpm for 2 h. Then using a spectrophotometer (1601PC, Shimadzu), liposomal HU supernatant absorbance was monitored at 214 nm, after that by using plot Standard Curve and below Formula [11-13], the encapsulation efficiency was computed.

$$\text{Encapsulation Efficacy\%} = (\text{Total weight of encapsulated HU in liposomes}) / (\text{Weight of total HU}) \times 100$$

In Vitro Release Study

In order to determine the released HU from nanoliposomes, one ml from NL-HU and 10 ml PBS were poured to the dialysis sacks and settled on a shaker for 36 h at 37°C. The amount of obtained HU in PBS was determined with Spectrophotometer at 214 nm and the standard curve.

FTIR Analysis

Interactions among HU and nanoliposomes were evaluated using FTIR (Tensor 27 FTIR spectrophotometer, Bruker, Germany) operating in the range of 800–4000 cm⁻¹.

Uptake of NL-HU to cells

Qualitative uptake of formulations on MDA-MB231 cells was investigated. The cells (70×10³/well) were cultured on 6-well culture plates (Costar, US). Next day, the cells were incubated with FITC-labeled NL-HU and PBS as control, at 37°C for 4 hours. The cells were washed with cold PBS and were fixed with paraformaldehyde in PBS for 10 minutes, the cells were then rinsed with cold PBS and were imaged using a fluorescence

microscope (Olympus IX 71, Tokyo, Ja). Also, quantitative uptake of FITC-NL-HU and FITC-HU into MDA-MB231 cells was evaluated using flow cytometry, 300×10^3 Cells per well were placed into 6-well plates then were incubated with FITC-NL-HU and free HU at 37°C for 4 h. The cells were then rinsed with PBS. Flow cytometry was performed using FACS (Becton Dickinson, Canada).

Cytotoxicity assays on cells

MDA-MB231 Cell line in RPMI with (P/S/G) at incubator with 5% CO_2 and 37°C maintained. After growth subcultured. fifty thousand cells / well in $200\ \mu\text{L}$ of cell culture media at 37°C with NL-HU, and free HU at different concentrations was incubated for 48 h. $20\ \mu\text{L}$ of MTT in PBS was added to wells. The formazan crystals were solubilized in $100\ \mu\text{L}$ of DMSO, and the absorbance was monitored using microplate reader (Bio-TEK, Winooski, Vermont, U.S.A.) at 570 nm and triplicate. Cell viability percent was calculated. Also, the IC_{50} (Half-maximal inhibitory concentration, mg concentration that produced a 50% reduction in control absorbance)[14], was determined, using graph pad prism software (version 6.0). Result were depicted as mean \pm SD.

Statistical analysis

All of the tests carried out triplicate. The data analyzed by ANOVA with SPSS software version 20 (IBM, Armonk, NY) and $p < 0.05$.

RESULTS AND DISCUSSION

Characterization of the liposomes

As is seen in Table 1, the particle size of the NL-HU was 174nm. Size is a significant factor for the development of suitable drug delivery system that affects on the toxicity, release, to evade the uptake by reticuloendothelial systems and the ability of penetration into the tumor cells [15-18]. The optimum size should be between 10 and 200 nm for reach to these goals, that 174nm was in this range and lower than Alavi et al. report [9]. In comparison with blank liposomes, there was no significant increase in particle size for NL-HU; this was because HU is a hydrophilic drug and loaded in the aquatic core of the liposomes and did not influence on the lipidic layer [19]. Liposomes showed zeta potential of $-29 \pm 0.21\text{mv}$, this parameter of the nanoliposome is one of the most important factors in stability determination. The negative zeta potential on the surface of

the liposomes was related to charge of lipids in liposomes and ratio drug to lipid that creates the stability of the liposomes via electronic repulsion [20], this zeta potential was enough to keep the stability of liposomal formulations. The shape of the nanoliposomes was examined by SEM. According to SEM images in Fig.1, the nanoliposomes containing Hydroxyurea were spherical with a size close to that attained by the DLS. The dimensions of nanoliposomes observed using SEM was slightly lesser than that of the DLS technique. DLS confirmed the hydrodynamic diameter of nanoliposomes, but SEM depicted the dried form of the nanoliposomes[21]. The EE% of the liposomes was 81% that is more than Alavi et al. work [9], the high EE% of HU was attributed to appropriate types and amounts of materials have been used for the preparation of nanoliposomes [22]. As is seen in Table 1 these results had no distinct difference with blank NL declared drug loading gave no significant effect on the physical properties of nanoliposomes.

Release study

Drug release graph of the nanoliposomal HU at the $\text{pH}=7.4$ is presented in Fig. 2. The HU release behavior of the NL-HU showed that during 5 hours of release at 37°C in the buffer, $19.23 \pm 1.64\%$ of HU released, it continued for up to 36 hours that produced $22.36 \pm 2.06\%$ sustained HU release that was in accordance with Alavi et al. result [9]. Rapid

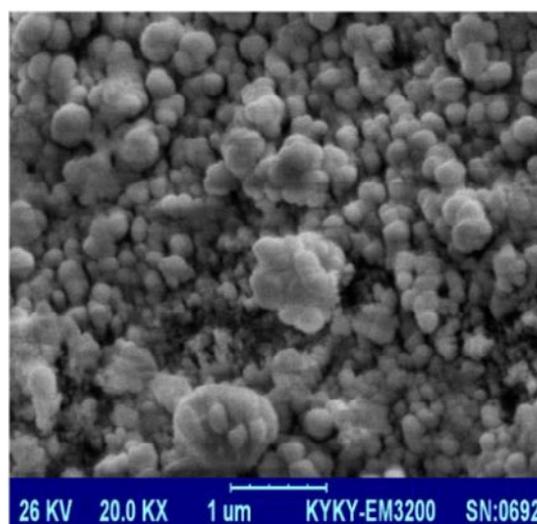


Fig. 1. SEM of pegylated nanoliposomes containing hydroxyurea with 20 \times magnification

Table 1. Physicochemical characteristics of nanoliposomes (mean± Standard Deviation, n=3)

Sample	Particle size(nm)	Zeta Potential(mV)
NL-HU	174± 3.5	-29±0.21
Blank NL	173±2.1	-29±0.18

Abbreviations: NLs, nanoliposomes; PDI, polydispersity index; HU, Hydroxyurea; SD, standard deviation.

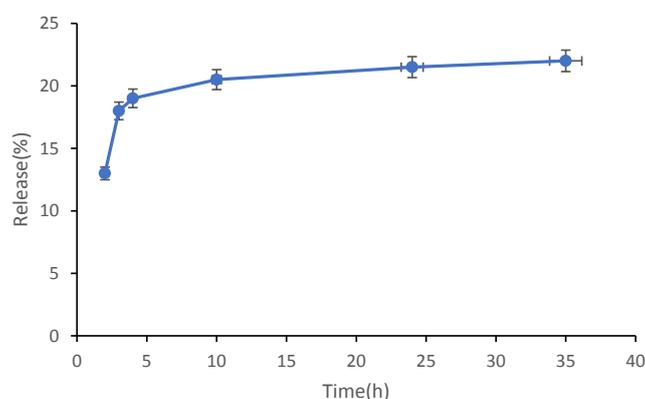


Fig. 2. Release studies of HU encapsulated liposomes in PBS buffer at pH=7.4 and 37°C. (mean± SD ,n=3)

release of HU over about 5h that were probably related to adsorbed HU on the nanoliposome surface, followed by a sustained release depend on liposome erosion and HU diffusion mechanisms [23,24].

FTIR analysis

Fig.3A showed the FTIR spectra of nanoliposomal HU. The graph displayed the bands of the O-H or N-H stretching vibrations (3420.20 cm^{-1}), the -CH stretching bonds (2924.51 cm^{-1}), the C=O stretching group (1635.98 cm^{-1}), the N-O stretching vibrations (1384.17 cm^{-1}) and the C-N,P=O or C-O stretching vibrations (1111.49 cm^{-1}). The FTIR spectrum of HU in Fig.3B exhibited O-H or N-H stretching vibration ($3412.38, 3302.17, 2802.65\text{ cm}^{-1}$), C=O band (1629.99 cm^{-1}), N-O group ($1585.07, 1482.24, 1407.23\text{ cm}^{-1}$) and stretching vibration of C-N group (1104.01 cm^{-1}). Findings confirmed that chemical bonds of HU in nanoliposomes remain intact and there were no chemical interactions between the loaded HU, cholesterol, lecithin and DSPE-mPEG2000 in liposomes [25,26].

Cellular Uptake Study

Qualitative cellular internalization of FITC-NL-HU was assessed by fluorescence microscopy. As is observed in Fig .4, the FITC-NL-HU showed the high uptake in comparison with PBS as control, thereupon, the superior internalization of FITC-

NL-HU in cells representing that the liposomes had a high affinity to the cells. Also, quantitative cellular internalization of FITC-NL-HU and FITC-HU in MDA-MB231 cells assessed after 4h incubation at 37 °C by flow cytometry. As is seen in Fig.5, the percentage of uptake in cells was $72.13\pm 5.21\%$ for the FITC-NL-HU and $45.94\pm 2.26\%$ for FITC-HU, the higher cellular uptake of FITC-NL-HU demonstrating the great affinity between the liposomes and cell and the increase of HU accumulation in the cells because by entrapping of HU into nanoliposomes can easily diffuse into the cells [27].

Cytotoxicity studies

Cytotoxicity of nanoliposomal HU was investigated in contrast with free HU with the same drug concentration on MDAMB231 cells after 48 incubation at 37 °C. Results in Figs .6&7 showed NL-HU, and free HU had no significant difference in cell viability and IC50 against MDAMB231 cells, therefore no remarkable distinction in toxicity, that can be related to slow release behavior of the HU from the nanoliposomes, cell type, exposure time and liposome charge in these cells for 48 h [28].

CONCLUSION

In this study, nanoliposomes were employed as a carrier to prepare pharmaceutical formulation. This nanoliposomal hydroxyurea had the suitable release, uptake and good cytotoxicity in cellular studies that will significantly improve the efficacy

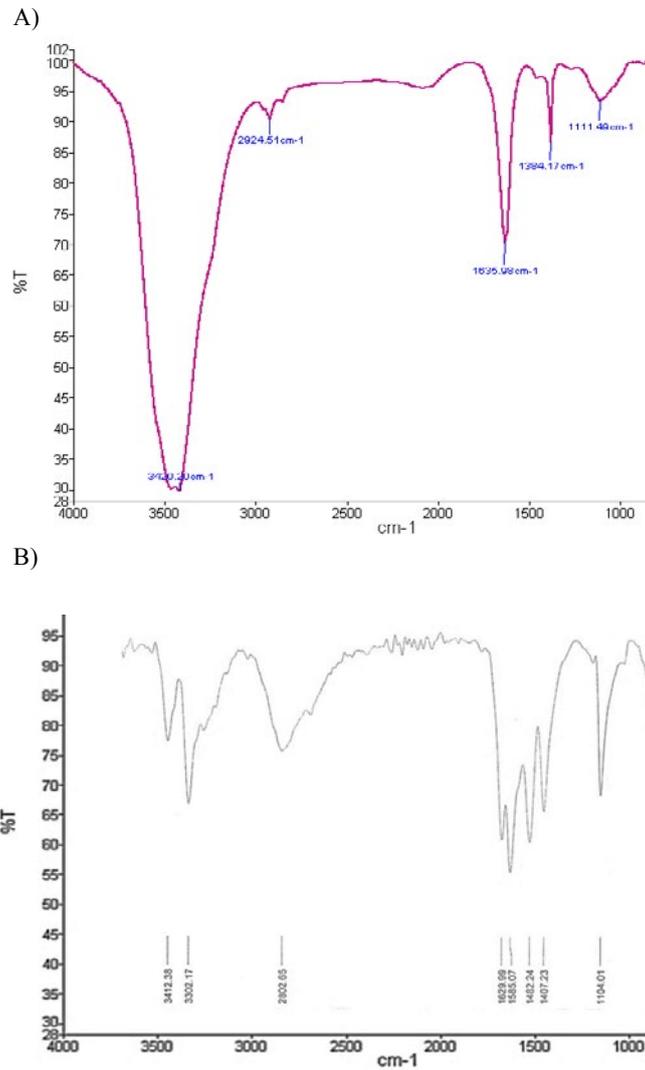


Fig. 3. FTIR spectra of NL-HU(A) and free HU(B)

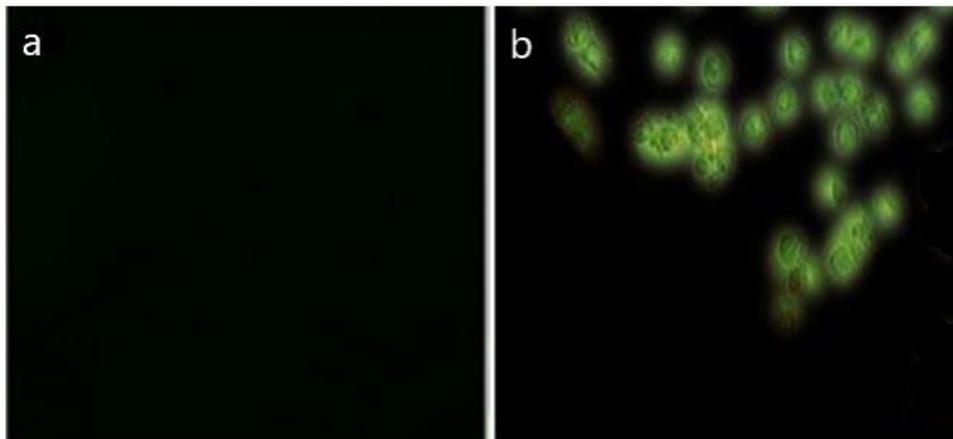


Fig. 4. Uptake of PBS control and FITC-NL-HU into MDA-MB231 cells (a, b) by fluorescence microscopy after 4hours

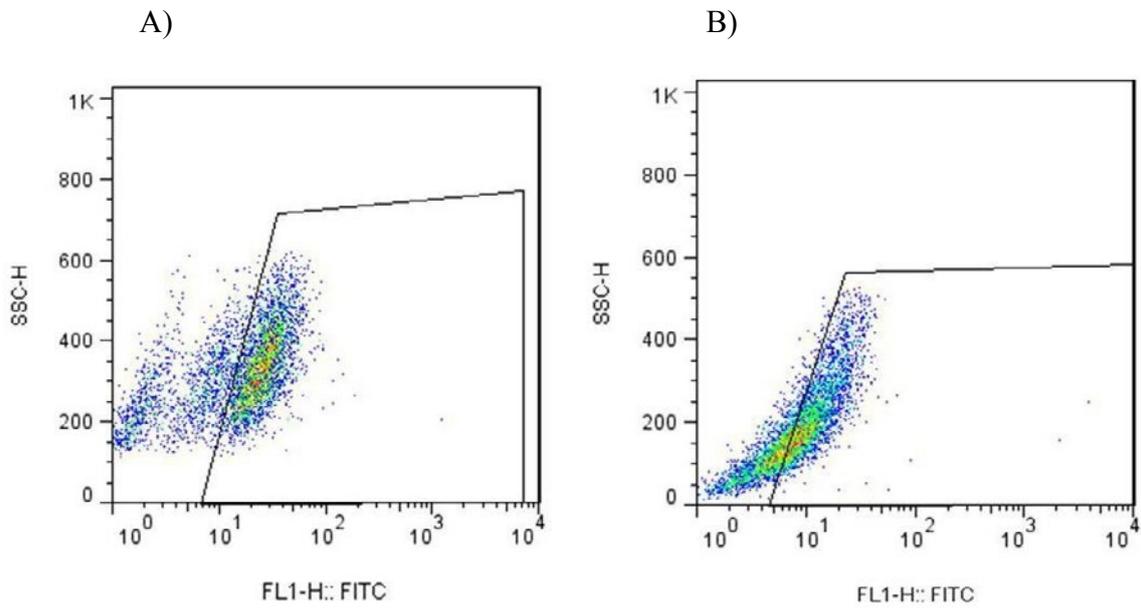


Fig. 5. Uptake of FITC-HU (A) and FITC-NL-HU(B) on MDA-MB231 cells by flow cytometry after 4hours

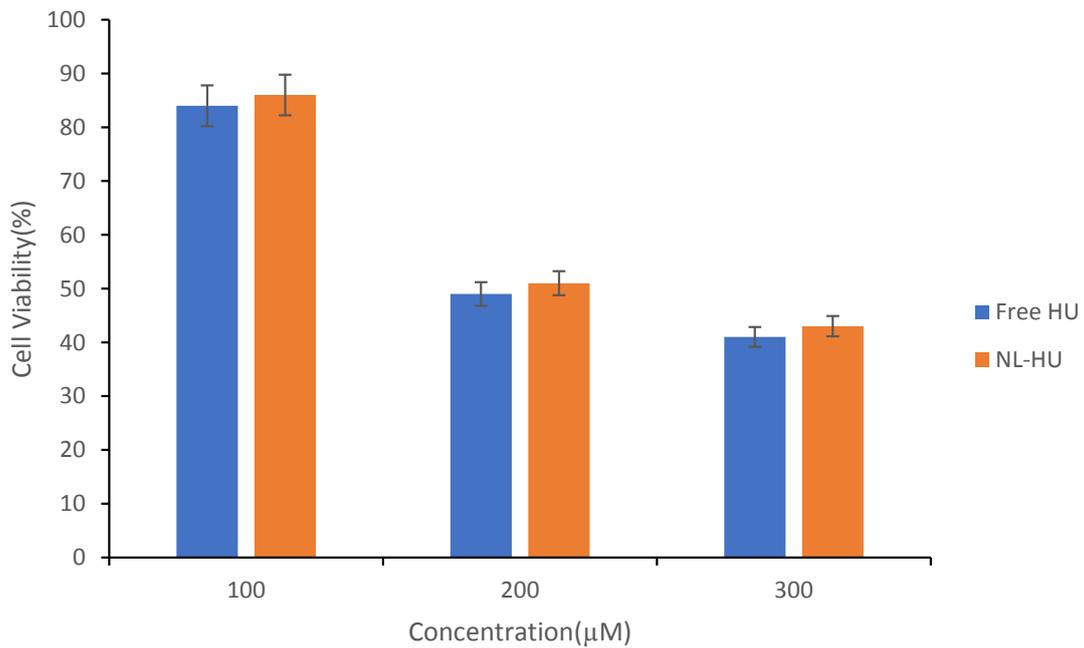


Fig. 6. Viability of MDA-MB231 cells at Different concentrations with HU formulations after 48 hours

of HU in the clinic and decrease side effects and drug dosage. These results demonstrate this liposomal drug system can potentially useful for delivery of hydrophilic anticancer drugs.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.



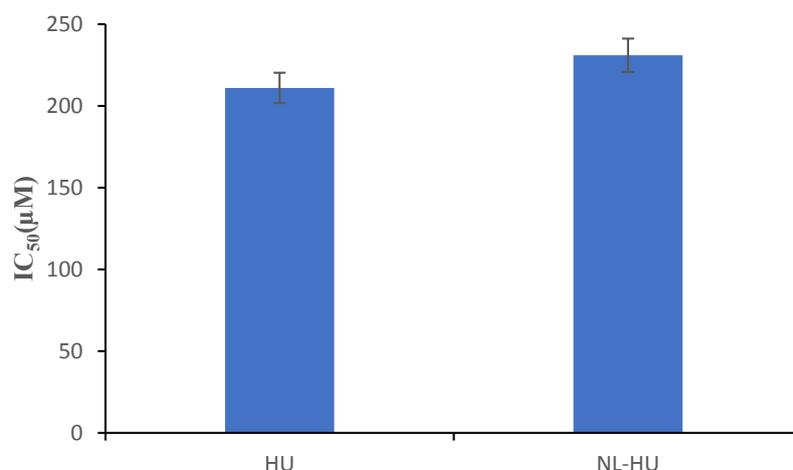


Fig. 7. IC₅₀ of free HU and NL-HU on MDA-MB231 cells

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