

Original Article

Running Title: Curcumin Nano-micelle and Colon Cancer

Received: August 10, 2020; Accepted: April 10, 2021

Evaluation of Curcumin Nano-micelle on Proliferation and Apoptosis of HT29 and Hct116 Colon Cancer Cell Lines

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Abstract

Background: Curcumin is a natural polyphenolic material with antioxidative, anti-inflammatory, and anticancer effects. In this study, we attempted to assay anti proliferative and apoptotic properties of polymeric micelles of curcumin on two colorectal cancer cell lines and normal human fibroblast cells.

Method: In this experimental study, cancer cells HT29, HCT116 and normal human fibroblast cells (HGF) were subjected to concentrations of Nano- curcumin (1, 50, 100, 250, and 500 µg/ml). After incubation for 48 hours, cell viability was assessed with "MTT"(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Annexin V-FITC and Propidium iodide staining were done with flow cytometry for evaluation of apoptosis. The results were shown as mean ± standard deviation. Statistical significance was assessed utilizing ANOVA and Dunnetts t-test ($P < 0.01$).

Results: According to MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay results, IC₅₀ value of Nano- curcumin in HT29, HCT116, and HGF were 70.63, 123.9, and 168.53 µg/ml, respectively. We also discovered that Nano-curcumin can make indicative apoptosis in cancer cells, which could be compared with cisplatin<0.01.

Conclusion: These results revealed remarkable anti-proliferative and apoptotic effects of polymeric Nano-micelles of curcumin in colorectal cancer cell lines.

Keywords: Colonic neoplasms, Cell proliferation, Apoptosis, Curcumin

Introduction

Colorectal cancer is the second most common reason for cancer death in the world.¹ Over the recent years, remarkable progress has been achieved regarding the treatment of this malignancy and new therapeutics have been distinguished, such as 5-Fluorouracil-leucovorin (thymidylate synthase inhibitor), Irinotecan (topoisomerase inhibitor), Oxaliplatin (DNA replication Inhibitor), Bevacizumab (anti-angiogenic), and Cetuximab (monoclonal antibodies targeting the EGFR).¹ However, chemotherapy is believed to be accompanied with side effects and long-term failure of treatment.

Curcumin, a phenolic compound from the rhizome of the *Curcuma longa* linn (*Zingiberaceae*), has a promising anti-cancer effect owing to its different properties, as anti-inflammatory, antioxidant, and anti-angiogenic.¹ In certain countries particularly, where curcumin is part of the diet, lower incidence of urothelial malignancies and colorectal cancer has been reported.¹ In rodent, it has shown that oral use of curcumin inhibited the formation of polyp and increased cell death in colon cancer lesions.¹ As potential pathways involved in these anti-carcinogenic properties, the decrease in nitric oxide synthase, tyrosine kinase receptor activation, and protein kinase C (PKC) changes in transcriptional factors as c-jun/AP-1 and p53 as well as inhibition of arachidonic acid metabolism, lipoxygenase, and cyclooxygenase activity have been shown. In combination with other anticancer chemicals, such as green tea, curcumin could induce an inhibitory effect in oral cancer and non-small cell lung cancer. Accordingly, curcumin may be considered as a potential anti-cancer agent.¹

The effect of curcumin has been studied in numerous human malignancies, for instance, melanoma, head and neck, pancreatic,

prostate, breast, colon, and ovarian cancers.² It could hinder all the internal properties of cancer cells and diminish tumor progression.² Expression of nuclear factor NF κ B, which controls cell proliferation, metastasis, angiogenesis, apoptosis, and resistance to chemotherapy, could reduce with curcumin.² This phenomenon could be regulated through suppression of I κ B kinase activation. Proliferation signaling as PI3K, AKT, mTOR, AP1 (JUN and FOS), JNK, JAK-STAT, PKC, CMYC, MAPK, ELK, CDKs, iNOS, and Wnt/ β -catenin, which is inhibited with curcumin, is considered as one of the important compounds inhibiting cancer development through targeting multiple cell proliferation signaling as down-regulation of Cyclin D1 expression, a proto-oncogene up-regulated in several cancers; this could also play an important role in the progression of cell cycle and proliferation.^{3,4}

Curcumin also prevents additional TGF β receptor signaling, which makes transport epithelial to mesenchymal tissue through offensive and metastasis.^{5, 6} It can induce cell apoptosis, which is both TP53-dependent or -independent and overexpresses the pro-apoptotic molecules as BAX, BIM, PUMA, and down-regulates anti-apoptotic compounds as BCL2, BCL-XL, and Survivin. Moreover, it can activate Caspases, promote apoptosis⁷ and activate Lysosomal proteases, phosphatases, and lipases; this causes autophagy-mediated cell death.^{8,9}

Curcumin, as a water-insoluble chemical, could be used by polymeric micelles as transmitters, which increases its availability to reach certain cells or organs. Hence, fewer drugs can accumulate in healthy tissues and their toxicities diminish; therefore, higher doses of drug can be delivered.² Accordingly, Liu et al. made curcumin-loaded biodegradable self-assembled polymeric micelles based on the

method of solid dispersion, which was simple and easy to be scaled up and resolved its low water solubility. There is a significant difference concerning release profile between quick release of free curcumin and much slower and sustained release of curcumin-loaded micelles.² Raveendran et al. reported that curcumin loaded micelles with amphiphilic Pluronic/polycaprolactone block copolymer can be efficient for increasing curcumin's aqueous solubility.²

Since no studies have been conducted on the efficacy of Nano-curcumin on colorectal cancer, specifically on cell line model, this study aimed to assay anti-proliferative and apoptotic effects of curcumin Nano-micelle in cell line model of colorectal cancer.

Material and Methods

This research is an experimental study conducted in the lab of pharmacology and toxicology department, Mazandaran University of Medical Sciences, during 2016-2018, under project and ethics code of 2824.

Ethics approval

All the procedures were confirmed by Ethical Committee of Deputy of Research of Mazandaran University of Medical Sciences under the ethics code of: IR.MAZUMS.REC.1398.4972.

5-fluorouracil-lecuvorin, RPMI cell culture medium, fetal bovine serum (FBS), and Cisplatin were purchased from sigma Chemical Co. (Sigma Aldrich, Germany). Curcumin nano-micelle (batch No. 321) was provided by Dr. Jafari's Company, Mashhad University of Medical Sciences (Mashhad, Iran). Penicillin and streptomycin from Roach Co., Germany, HT29, Hct116 colon cancer cell line, and HGF human normal fibroblast cells were provided by Institute Pasteur, cell bank, Tehran Iran.

Cell culture

All the cells were cultured in RPMI medium and supplemented with 10% FBS, penicillin (100 IU/mL), and streptomycin (100 µg/mL); the medium was replaced every 2 days. Cell cultures were maintained at 37 °C in a humidified atmosphere containing 95% air: 5% CO₂.¹⁰

Cell viability assay

Cell viability was done with MTT assay.¹⁰ Accordingly, measuring mitochondrial activity based on the reductive cleavage of yellow tetrazolium salt to a purple formazan compound was performed with the dehydrogenase activity of intact mitochondria. Briefly, the cells were initially washed once with PBS before adding 0.1 ml serum free medium containing MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (1 mg/mL) to each well. After three hours of incubation, the supernatant was removed and the formazan product was dissolved in 1 mL dimethyl sulfoxide (DMSO) being stirred for 15 min on a micro titer plate shaker and the absorbance was read at 550 nm. The viable cells percentage in each treatment group was established by comparing their respective absorbance with the control group.

Drug treatments

Cancer cell lines of HT29 and Hct116 colon were split in 96 well plates at a density of 1.0×10^4 well, and treated differently with concentrations of nano-curcumin (1,50,100,250,500 µg/mL,100µL/well) and cisplatin in triplicate for 24 h of incubation. Cisplatin was considered as the positive control. Following 48 h of incubation, MTT solution was put into the culture as the final concentration of 0.5 mg/ml. After 4 hours of incubation at 37°C, the medium was removed and replaced with the equal volume of DMSO to dissolve the purple formazan crystal. The absorbance of the solution was measured spectrophotometrically with

microplate reader (DynexOpsys MR 24100) at 570 nm and compared to the control. IC₅₀ concentration was calculated for each drug utilizing cell viability graph.¹⁰

Apoptosis analysis (Flow cytometry)

Apoptosis was determined with Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, USA (10). Briefly, after completion of the treatments (24h of incubation of the cells with Nano-micelle curcumin and cisplatin at IC₅₀ concentration in 37⁰ Co2 incubator), the cells were harvested and washed with binding buffer and were counted to obtain a final concentration of 1×10^6 cells/ml. Subsequently, Annexin V and propidium iodide (PI) were added and incubated in the dark for 15 minutes. After washing, cell suspension was fixed with 1% formaldehyde for 10 minutes on Ice.; washing was then performed twice with binding buffer followed by the addition of RNAase enzyme (EMD Biosciences, USA) and incubated for 15 minutes at 37°C. Ultimately, the samples were washed and analyzed with FACS Calibur flow cytometer (BD Biosciences, USA) and the software Cell Pro Quest.¹⁰

Statistical analysis

The data were collected as triplicate from at least three independent experiments and analyzed employing SPSS version 21 Software. The results were shown as mean \pm standard deviation. Statistical significance was assessed with ANOVA and Dunnetts t-test ($P < 0.01$).

Results

MTT proliferation assay

According to MTT assay (Figures 1 and 2), Cisplatin, as the positive control in IC₅₀ concentrations (53.77 for HT29 and 77.40 for hct116 cell lines), significantly decreased cell viability in both colorectal cancer cell lines compared with the negative control ($P < 0.01$, Annona assay). In addition, nano-micelle curcumin in 50 to 500 $\mu\text{g/mL}$

concentrations reduced cell-viability compared with the negative control in both cell lines ($P < 0.01$, Annona assay) (Figures 1 and 2). Furthermore, table 1 represents the comparison between IC₅₀ of nano-curcumin and cisplatin in two colon cancer cells lines. For normal cells, HGF Nano- micelle curcumin in concentrations of 1, 50, 100 $\mu\text{g/mL}$ did not demonstrate any effects on cell viability ($P > 0.05$ Annona assay) and only in concentrations of 250 and 500 ($\mu\text{g/mL}$), it decreased cell viability ($P < 0.01$, Annona assay)(Figure 3). Figure 4 depicts the route of estimation of IC₅₀ determination for cisplatin with cell viability graph of HT29 cells.

Apoptosis analysis

In HT29 cell line, primary apoptosis between Nano-curcumin (%8/06) and the control group and late apoptosis between nano-curcumin (%17/97) and the control group (%7/14) were significant ($P < 0.01$ and $P < 0.001$, Annona assay, respectively). The rate of apoptosis with nano-curcumin was almost similar to that with cisplatin in IC₅₀ concentration (Figure 5).

According to the results of HCT116 cell line, primary apoptosis between Nano-curcumin (%3/46) and the control group (%1/83) in IC₅₀ concentrations did not have any significant differences ($P > 0.01$), yet late apoptosis (%18/26 against %6.80) was significant ($P < 0.001$, Annona assay) (Figure 6).

In the normal cells (HGF), primary apoptosis between Nano-curcumin (%0.75) and the control group (%0.49) ($P > 0.05$, Annona assay) and late apoptosis between Nano-curcumin (%12/99) and the control group (%12/18) were not significant ($P > 0.05$) (Figures 7-13). Accordingly, Nano-curcumin did not show any apoptotic effects on the normal cells whereas it was found to have some effects on the cancer cells.

Discussion

The current study assayed the effect of Nano-micelle curcumin on two colon cancer cell lines (HT29, HCT116). MTT proliferation assay indicated that Nano-micelle curcumin decreased the proliferation of cancer cells significantly and according to IC_{50} , this effect on HT29 was more remarkable than that on HCT116. However, it did not show any significant anti-proliferative or apoptotic effects on HGF (normal human fibroblast cells) in low to medium doses.

The obtained results herein were in line with those of previous studies on Nano-micelle curcumin, in which Nano-curcumin was found to have less toxic effect on normal cells than pure curcumin.¹¹⁻¹³ Furthermore, previous studies have shown that incubation of normal lymphocyte, normal human skin, and rat fibroblasts with Nano-curcumin does not have any cytotoxic effects.¹³ The high capacity anti-oxidant effect of curcumin can explain its protective effect against normal cells and COX-2 and NOS enzymes have been suggested as targets of curcumin in cancer cells.¹⁴

The results of apoptotic assay in our study revealed that Nano-curcumin significantly increased apoptosis compared with the control, which was comparable with cisplatin in the colon cell lines (HT29, HCT116) and on the normal cells (HGF), it did not have apoptotic effects.

Physico-chemical properties of Nano-micelle curcumin makes it more effective in certain types of cancer.¹⁵ This can be owing to its better transmission through cell membranes.¹⁵ Fang XB et al. reported that F68-Cis-Cur micelles have more toxicity against A2780 and SMMC 7721 compared to pure curcumin by reducing mitochondria's potential and making cellular apoptosis more strongly.¹⁶ Moreover, micellar formulation in caveolae-mediated endocytosis in an energy dependent pathway

could increase cellular absorption of curcumin by the cancer cells.¹⁶

In addition, it was revealed that acetal-containing micelles of curcumin has pH-dependent releasing effects in HepG2 (liver cancer) cells with less IC_{50} than the control.¹⁸

In a study by Maling Gou on the identification of anti-cancer activity of Cur / MPEG-PCL micelle, in an in vitro and in vivo model of C-26 colon carcinoma, it was shown that both curcumin and Cur / MPEG-PCL micelle can kill colon cancer cells significantly in a dose dependent manner.¹⁹ Therein, IC_{50} for curcumin was $3.95\mu\text{g/ml}$ and for Cur / MPEG-PCL micelle, it was 5.78 .¹⁹

Mahmoud Khaniki et al. also demonstrated that Nano-curcumin carriers could increase the efficacy of curcumin in the prevention of colon cancer in rat in a colon cancer animal model.²⁰

Our study was in accordance with the above-mentioned studies, particularly with Khaniki and Mailing Gou, who showed numerous properties of Nano-micelle of curcumin in cancer cytotoxicity.^{19,20} Additionally, we exhibited effective apoptotic properties of Nano-curcumin in two colon cancer cell lines for the first time and confirmed the safety of Nano-curcumin in mild to moderate concentrations on normal cells.

The limitation of this study was transferring the samples for flow cytometry from the pharmacy school to another place in Imam Khomeini Hospital, which might have slightly affected the quality of the cells.

Conclusion

According to the results of this study, we could suggest Nano-micelle curcumin as an appropriate drug delivery system specifically in colon cancer chemotherapy regime and as an effective therapeutic with low toxicity on normal cells. Meanwhile,

complementary studies as *in vivo* models or clinical trials are recommended in order to investigate more effects of Nano- curcumin on some other apoptotic or angiogenic proteins and to clarify further aspects of its safeties.

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Table 1. Comparison between IC₅₀ of nano-curcumin and cisplatin in two colon cancer and HGF cell lines

Type cell	IC ₅₀ (Nano-curcumin)	IC ₅₀ (Cisplatin)
HT-29	70.60	53.77
Hct116	141.5(P<0.01)	77.40
HGF	168.53(P<0.01)	12.9

Statistical significance was assessed with ANOVA and Dunnetts t-test ($P < 0.01$).

IC₅₀: half maximal inhibitory concentration is a measure of the potency of a substance in inhibiting a specific biological or biochemical function

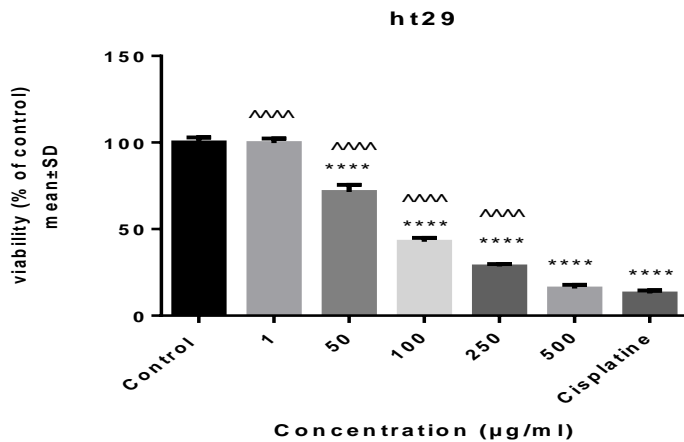


Figure 1. This figure shows the effect of different concentrations of Nano-curcumin on cell viability on HT-29 Cell line.

^^^: Significant compared with cisplatin $P < 0.01$; **: Significant compared with control $P < 0.05$, ***: Significant compared with control $P < 0.001$

HT-29: is a human colorectal adenocarcinoma cell line with epithelial morphology

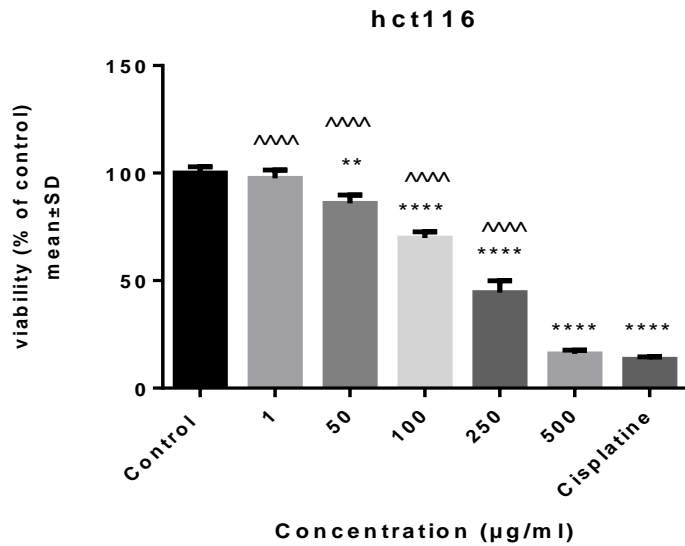


Figure 2. This figure shows the effect of different concentrations of Nano-curcumin on cell viability on Hct116 Cell line.

^^^: Significant compared with cisplatin $P < 0.01$; **: Significant compared with control $P < 0.05$, ***: Significant compared with control $P < 0.001$

hct116: is a human colorectal adenocarcinoma cell line with epithelial morphology

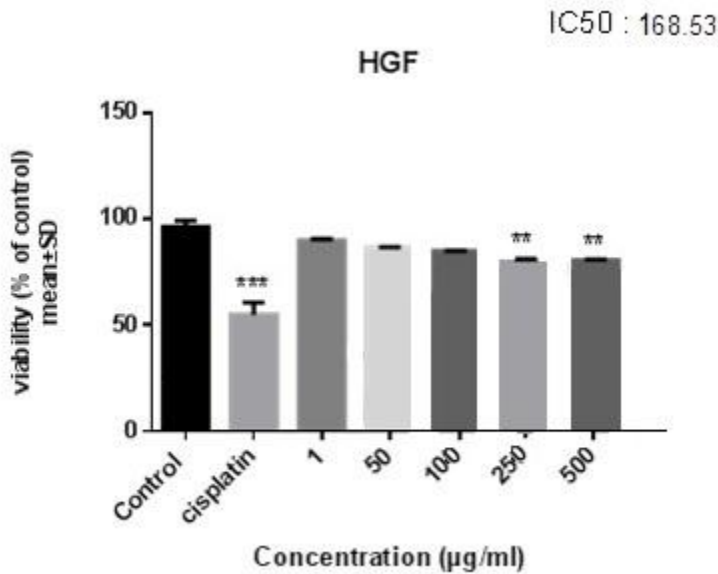


Figure 3. This figure shows the effect of different concentrations of Nano-curcumin on cell viability on HGF Cell line (Normal cells).

** : Significant compared with control $P < 0.05$, ***: Significant compared with control $P < 0.001$

HGF: Primary Gingival Fibroblast; Normal, Human, Adult cell line

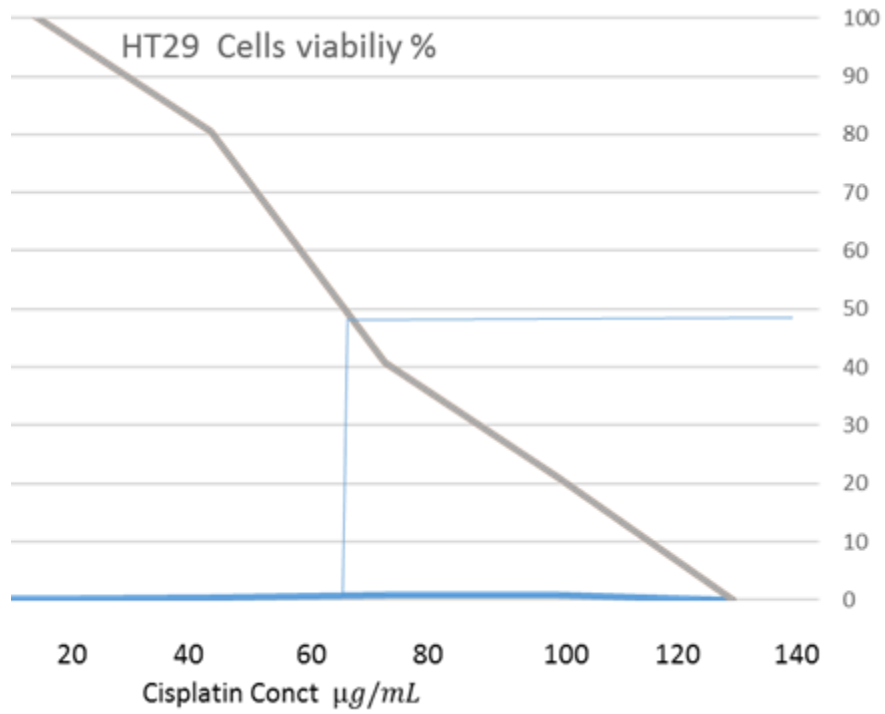


Figure 4. This figure shows the route of estimation of cell viability of cisplatin with cell viability graph of HT29 cells.

HT-29: is a human colorectal adenocarcinoma cell line with epithelial morphology

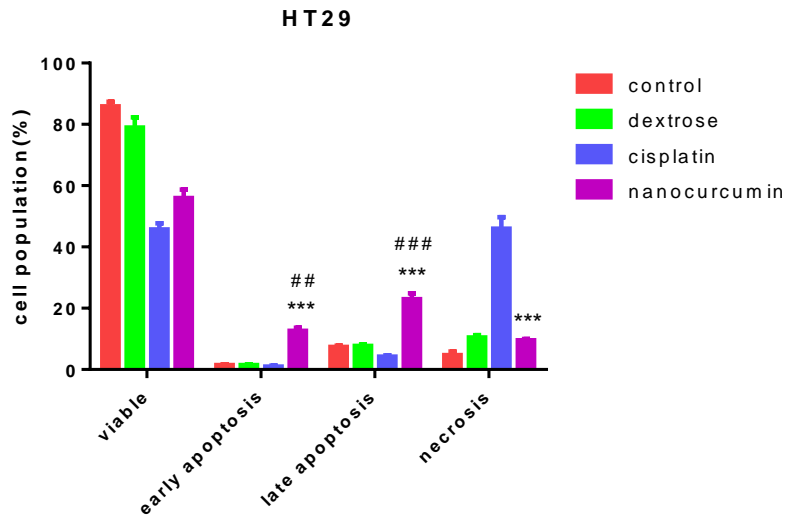


Figure 5. This figure shows the apoptosis assay results with Annexin-V propidium iodide flow cytometry method of Nano-curcumin and cis-platin treatments in IC_{50} concentrations on HT-29 cells following 24 h (incubation time).

***: Significant compared with cisplatin $P < 0.001$. ## : Significant compared with negative control, $P < 0.01$ ###: Significant compared with negative control, $P < 0.001$

Annexin V: Annexin A5 (or annexin V) is a cellular protein in the annexin group. In flow cytometry, annexin V is commonly used to detect apoptotic cells by its ability to bind to phosphatidylserine

IC_{50} : half maximal inhibitory concentration is a measure of the potency of a substance in inhibiting a specific biological or biochemical function

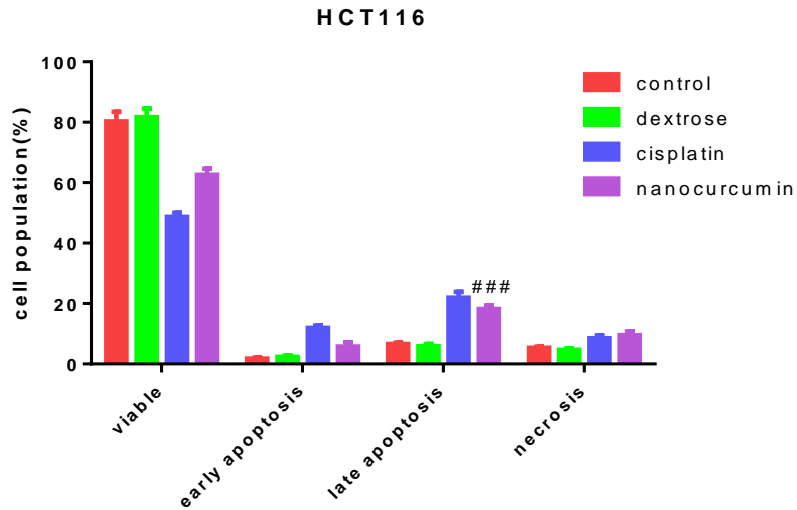


Figure 6. This figure shows the apoptosis assay results with Annexin-V propidium iodide flow cytometry method of Nano-curcumin and cis-platin treatments in IC_{50} concentrations on HCT-116 cells following 24 h (incubation time).

***: Significant compared with cisplatin $P < 0.001$.## : Significant compared with negative control, $P < 0.01$ ###: Significant compared with negative control, $P < 0.001$

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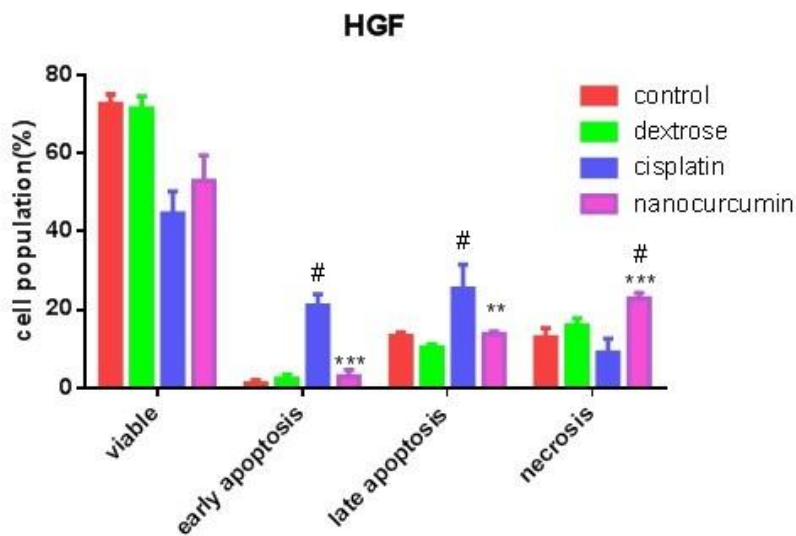


Figure 7. This figure shows the apoptosis assay results with Annexin-V propidium iodide flowcytometry method of Nano-curcumin and cis-platin treatments in IC₅₀ concentrations on HGF normal cells following 24 h (incubation time).

***: Significant compared with cisplatin $P < 0.001$.## : Significant compared with negative control, $P < 0.01$ ###: Significant compared with negative control, $P < 0.001$

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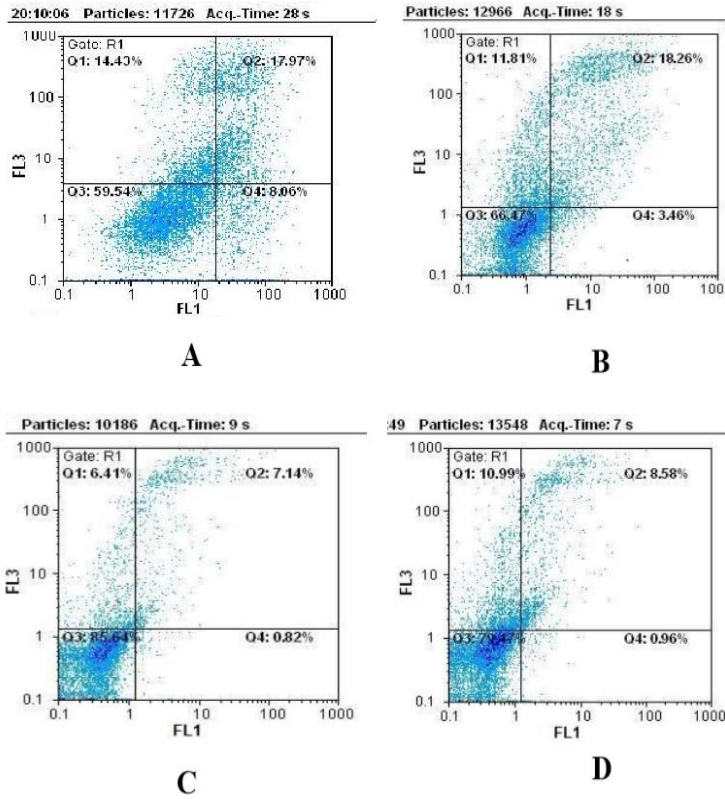


Figure 8. This figure shows the FACS scan analysis of the cells following flow cytometry in HT29 cells; A) treatment with Nano-curcumin; B) treatment with cisplatin; C) negative control; D) treatment with dextrose as drug vehicle.

FACS scan: The FACS (Fluorescence Activated Cell Sorting) is a laboratory technique that allows to examine AND SORT millions of cells, both normal and tumoral cells, in a very short time and receive a lot of information on their biological behavior and scanning

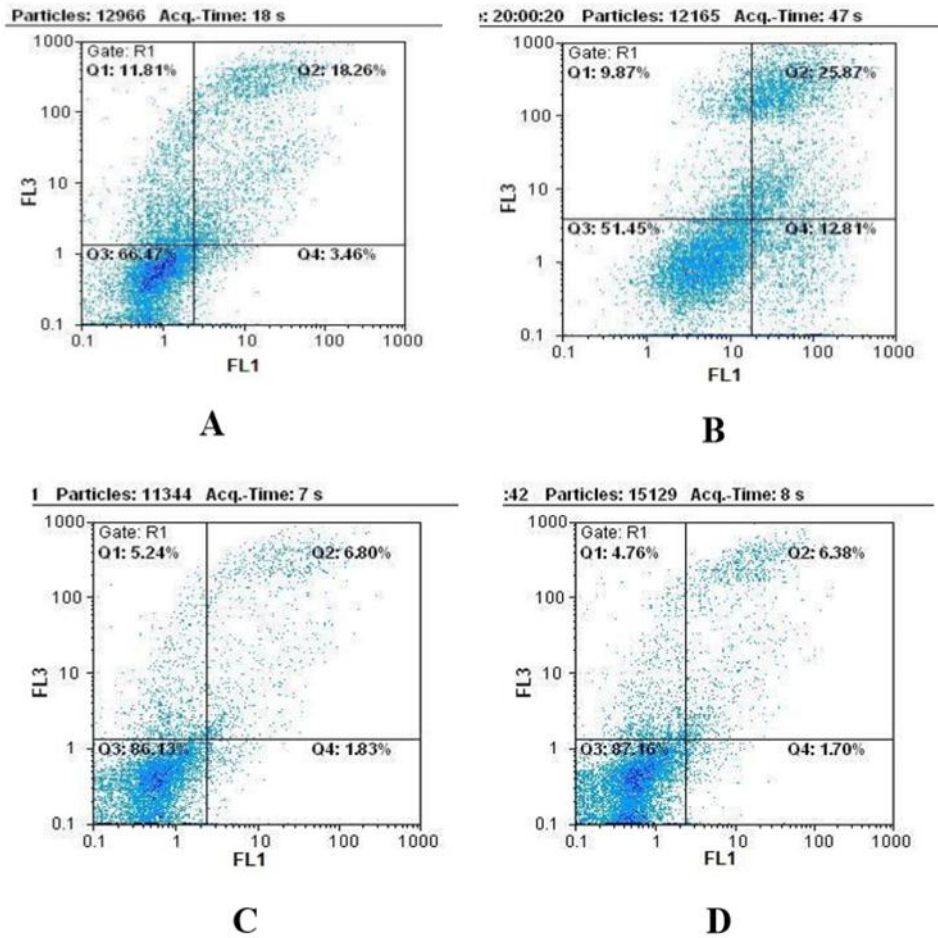


Figure 9. This figure shows the FACS scan analysis of the cells following flowcytometry in HCT116 cells; A) treatment with Nano-curcumin; B) treatment with cisplatin; C) negative control; D) treatment with dextrose as drug vehicle.

FACS scan: The FACS (Fluorescence Activated Cell Sorting) is a laboratory technique that allows to examine AND SORT millions of cells, both normal and tumoral cells, in a very short time and receive a lot of information on their biological behavior and scanning

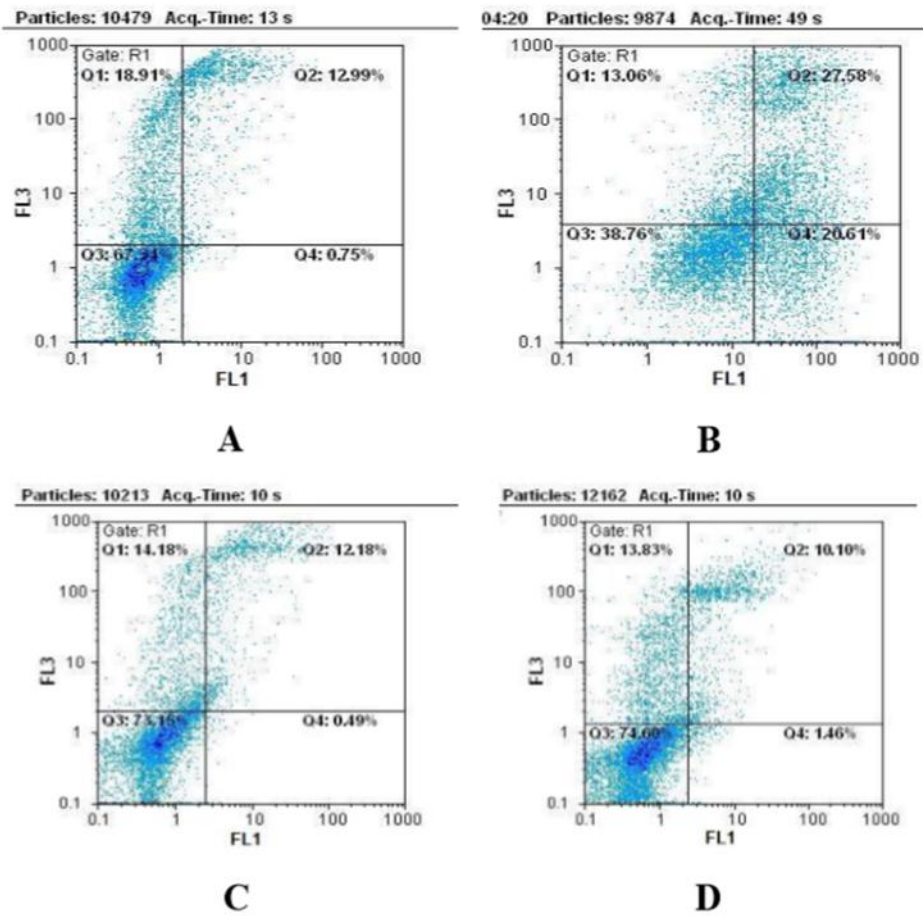


Figure 10. This figure shows the FACS scan analysis of the cells following flow cytometry in HGF normal cells; A) treatment with Nano-curcumin; B) treatment with cisplatin; C) negative control; D) treatment with dextrose as drug vehicle.

FACS scan: The FACS (Fluorescence Activated Cell Sorting) is a laboratory technique that allows to examine AND SORT millions of cells, both normal and tumoral cells, in a very short time and receive a lot of information on their biological behavior and scanning

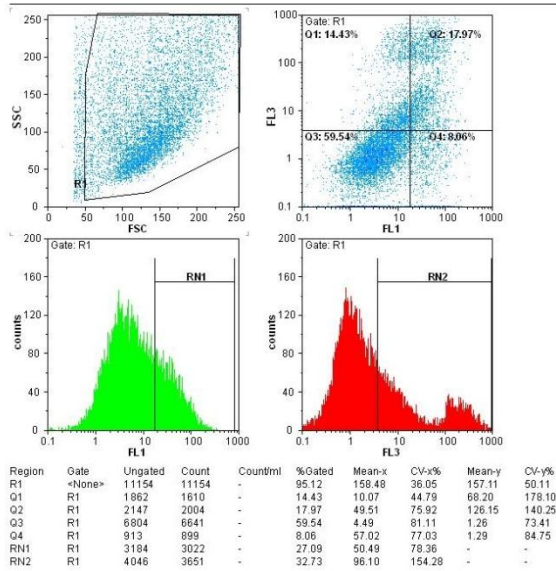


Figure 11. This figure shows the FACS scan and cells analysis following flowcytometry in HT29 cells treated with Nano-curcumin.

FACS scan: The FACS (Fluorescence Activated Cell Sorting) is a laboratory technique that allows to examine AND SORT millions of cells, both normal and tumoral cells, in a very short time and receive a lot of information on their biological behavior and scanning

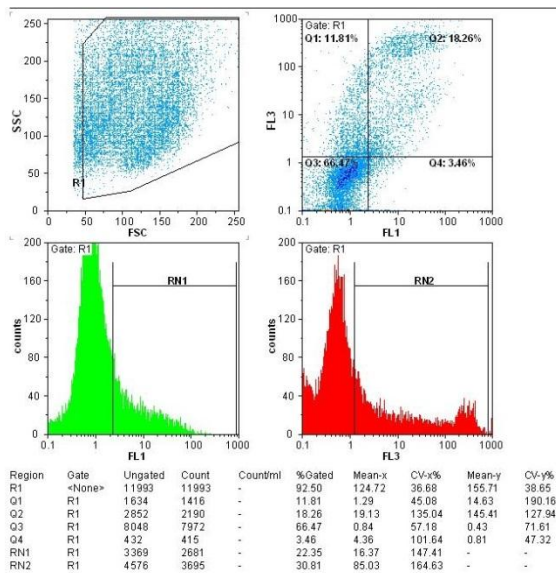


Figure 12. This figure shows the FACS scan and cells analysis following flowcytometry in HCT116 cells treated with Nano-curcumin.

FACS scan: The FACS (Fluorescence Activated Cell Sorting) is a laboratory technique that allows to examine AND SORT millions of cells, both normal and tumoral cells, in a very short time and receive a lot of information on their biological behavior and scanning

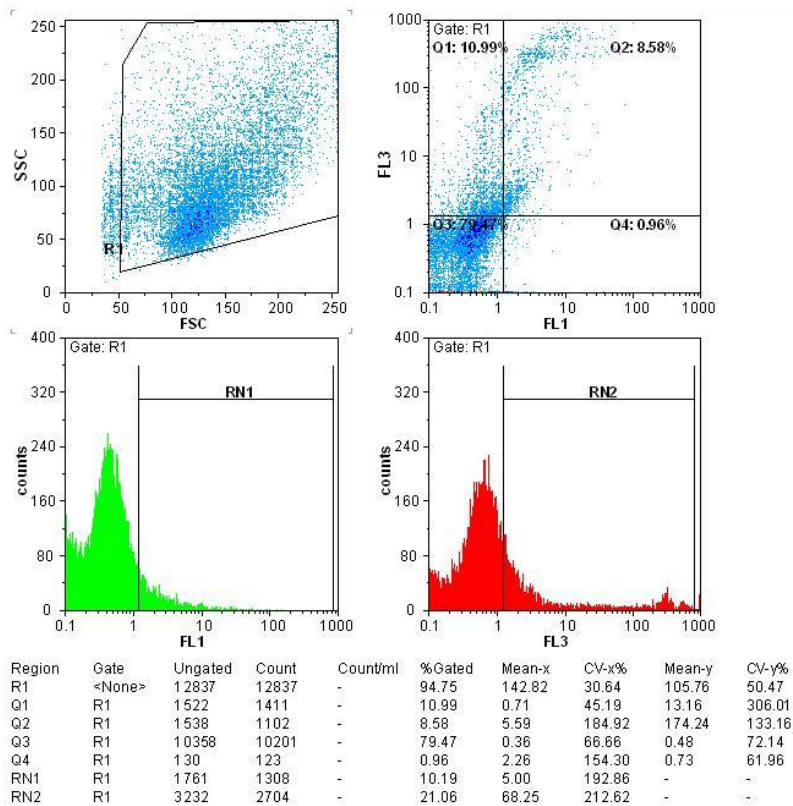


Figure 13. This figure shows the FACS scan and cells analysis following flowcytometry in HGF cells treated with Nano-curcumin.

FACS scan: The FACS (Fluorescence Activated Cell Sorting) is a laboratory technique that allows to examine AND SORT millions of cells, both normal and tumoral cells, in a very short time and receive a lot of information on their biological behavior and scanning