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Comparision of anticancer activity and drug internalization ability of rutin,rutin nanoparticles and hyaluronic acid surface modified rutin nanoparticles.

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Abstract

This study explores rutin (RT), rutin nanoparticles (RTN), and hyaluronic acid-coated rutin nanoparticles (HA RTN) effects on essential cellular processes in MCF-7 breast cancer cells. Analyzing cell cycle dynamics, apoptosis induction, intracellular reactive oxygen species (ROS) levels, and cellular uptake aimed to understand their influence on cancer cell behaviour. Treatment induced changes in cell cycle distribution, reducing cells in the G2 phase, suggesting a possible G2 arrest and associated cytotoxicity aligned with apoptosis pathways. Assessment of apoptosis revealed a significant increase across all treatments. Notably, HA RTN showed the most substantial increase in apoptotic cells, implying its potential for programmed cell death induction. Concurrently, ROS level evaluation highlighted notable increases, especially in HARTN-treated cells, indicating enhanced rutin accumulation and ROS production. Cellular uptake analysis demonstrated robust drug internalization, particularly in HA RTN-treated cells, indicating its efficient cellular penetration compared to RT and RTN formulations. In conclusion, HA RTN exhibited superior cytotoxicity, prominently induced apoptosis, elevated intracellular ROS levels significantly, and notably enhanced drug internalization in MCF-7 cells. These findings position HA RTN as a promising avenue for targeted cancer therapy and suggest its potential role in innovative treatment strategies.

Key words:Nanoparticles, Cancer cell, Rutin, RTN and HA RTN.

Introduction

The growing global interest in utilizing herbal treatments for tumors has emphasized the significance of flavonoids, ubiquitous compounds found in various herbs. Among these, rutin

has garnered attention due to its diverse advantages, including its ability to inhibit platelet aggregation, anti-inflammatory properties, antioxidant effects, and its potential in reducing blood fat and cholesterol levels [1-3]. Researchers have also observed promising outcomes indicating rutin's capability in potentially reducing precancerous complications and inducing apoptosis cancers[4]. To optimize the therapeutic potential of rutin, we have explored the conversion of rutin into rutin nanoparticles and coating them with hyaluronic acid [5-7]. These modifications serve multiple purposes, enhancing the drug's efficiency and targeting ability. Rutin nanoparticles, being on a nanoscale, possess a larger surface area, facilitating improved cellular uptake and increasing the drug's bioavailability. Moreover, the coating of these nanoparticles with hyaluronic acid offers specific advantages. Hyaluronic acid is known for its biocompatibility and ability to specifically bind to receptors overexpressed on cancer cells, allowing targeted drug delivery [8-10]. This study focuses on evaluating the prepared rutin nanoparticles to ascertain their efficacy in enhancing cellular delivery. By converting rutin into nanoparticles and encapsulating them with hyaluronic acid, the aim is to potentially increase drug uptake by target cancer cells. The strategy seeks to exploit the advantages of nanoparticles, such as enhanced cellular penetration and hyaluronic acid's specificity for targeting cancer cells, with the ultimate goal of improving the therapeutic effectiveness of rutin in cancer treatment.

Materials and methods

Cell cycle analysis

Proliferation inhibition was further assessed by analyzing the distribution of cells across the cell cycle phases. The cell culture process commenced by seeding cells into 25cm^2 culture flasks at 10^6 cells/ml, facilitating overnight adhesion. Subsequently, cells were treated with rutin, RTN, and HA RTN at their respective IC_{50} concentrations for 24 hours. Post-treatment, the cells were collected, and did centrifugation at 1,000 rpm for 10 min. The resultant pellet was washed with cold PBS. The cells were then re-suspended in 500µl PBS. Followed by fixation in cold ethanol (70%) at -20°C for a 2 h1[11,12].

Following PBS washing and centrifugation, the cells underwent incubation with a PI/RNase mixture (propidium iodide - 400µl and RNase A - 100µl). The stained cells were then incubated in darkness at room temperature for 30 minutes before subsequent analysis. The flow cytometer was utilized to determine the cell cycle profile without any identifiable differences from the original protocol [13].

Cellular Apoptosis

Apoptosis analysis was conducted through flow cytometry utilizing an annexin V-FITC apoptosis detection kit in strict accordance with the manufacturer's provided guidelines. MCF-7 cell lines, obtained from NCCS Pune, were cultured and exposed to the IC50 dose of rutin, RTN, and HA RTN samples for a duration of 24 hours. Following treatment, cells underwent two cold PBS washes, followed by suspension in 1X binding buffer at $1x10^6$ cells/ml concentration. The cells were meticulously categorized in tounstained, control, annexin, PI, and treatment groups. Subsequently, annexin V FITC and PI each at 5 µL were added to their respective labelled tubes. Following overtaxing and a 15-minute incubation at room temperature, 1X Binding buffer was introduced to each tube. Analysis was performed using a Flow Cytometer (BD FACS LyricTM) within 1 hour [14-16].

Reactive oxygen species

MCF-7 cells were initially seeded in 6-well plates, with density 5000 to 8000 cells/well, using 1ml DMEM medium. The medium was supplemented with 1% antibiotic solution and 10% FBS. The cells were left to incubate for a period of 24 hours at 37°C in an environment containing 5% CO2 to facilitate adhesion. Following the incubation period, the old medium was discarded, and fresh culture medium was added prior to the commencement of treatment. Subsequently, the cells were treated with the respective IC_{50} concentrations of RT, RTN, and HA RTN, after which they were further incubated for an additional 24 hours. Upon completion of the incubation period, the medium was carefully removed. The cell detachment was encouraged with trypsin EDTA. The detached cells were efficiently collected in 1.5 ml tubes and washed once with 500µl chilled PBS. Finally, the cell pellets were suspended in 100 µl PBS containing 2µM DCFDA before the acquisition of samples using a Flow Cytometer (BD FACS Calibur, USA) within a timeframe of 1 hour. The data obtained from the Flow Cytometer were analyzed using Flowing Software version 2.5.1 to discern and interpret the experimental outcomes [17-19].

Cellular uptake analysis

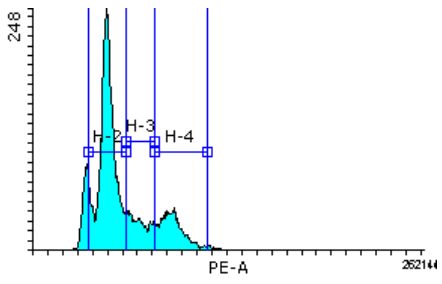
To assess and compare the internalization of RT, RTN, and HA RTN compounds, their concentrations within MCF-7 cell lines were evaluated using flow cytometry. MCF-7 cells (1.2 × 105) were initially cultured in 12-well plates and allowed to incubate for 24 hours. Subsequently, RT, RTN, and HA RTN at their respective IC50 concentrations were introduced into corresponding wells, and cells were incubated for 1, 2, 4, and 12 hours. At specific time intervals, the cells underwent harvesting, followed by two washes with PBS to eliminate any unbound drug and subjected to flow cytometry analysis to measure the fluorescence using the FITC channel. Additionally, to visualize the uptake profiles through fluorescence microscopy,

MCF-7 breast cancer cells (1.5×10^5) were seeded in a 96-well plate and incubated until treated with RT, RTN, and HA RTN for 12 hours. Post-treatment, cells were washed with PBS, fixed with PFA for 10 minutes, and immersed in PBS. The cellular uptake of nanoparticles was observed and documented using imaging techniques via the In Cell 2000 Analyzer, allowing for the visualization and analysis of the extent and patterns of uptake within the cells treated with RT, RTN, and HA RTN. FITC was used as a fluorescent probe for the study [20,21]

Result and discussion

Cell cycle analysis

Analysis revealed distinct distributions in various samples. The control sample exhibited 48.4% of cells in G1, 11.27% in S, and 14.56% in G2 phases. Rutin treatment showed alterations with 46.84% in G1, 12.06% in S, and 10.81% in G2 phases. Rutin nanoparticles exhibited a rise with 51.92% in G1, 12.13% in S, and 11.09% in G2 phases. Hyaluronic acid treatment displayed 47.06% in G1, 13.06% in S, and 10.32% in G2 phases (figure 1-5). Each treatment demonstrated unique shifts in cell cycle phases compared to the control, indicating specific effects on cell proliferation dynamics. The decline in the G2 phase cell population, observed across the treated groups compared to the control, suggests a G2 arrest phenomenon induced by the treatments. This decrease indicates a halt in cell cycle progression at the G2 phase. Simultaneously, the increase in proliferation denotes an absence of cell cycle arrest at G0 and S phases. This contrasting pattern in cell cycle phases signifies a specific interference with G2 progression due to the treatments. Moreover, the observed induction of cytotoxicity is linked to mechanisms closely associated with apoptosis[22,23].



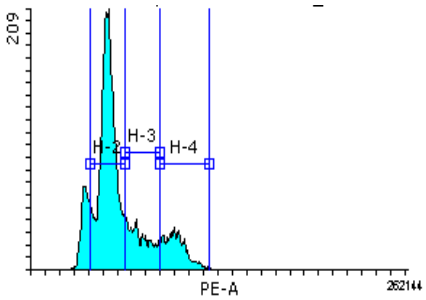
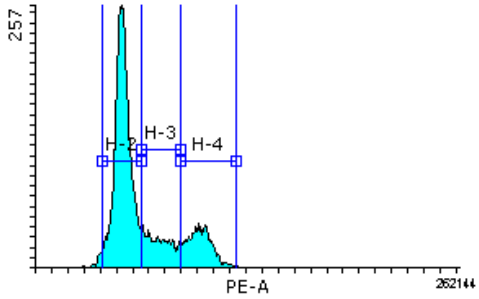


Figure 1: Control

Figure 2: Rutin



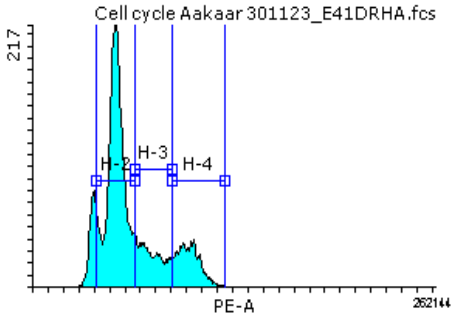


Figure 3: RTN

Figure 4: HA RTN

Table 1: Cell Cycle analysis

	G1 Phase(%)	S Phase (%	
Control	48.4	11.27	14.56
Rutin	46.84	12.06	10.81
RTN	51.92	12.13	11.09
HA RTN	47.06	13.06	10.32

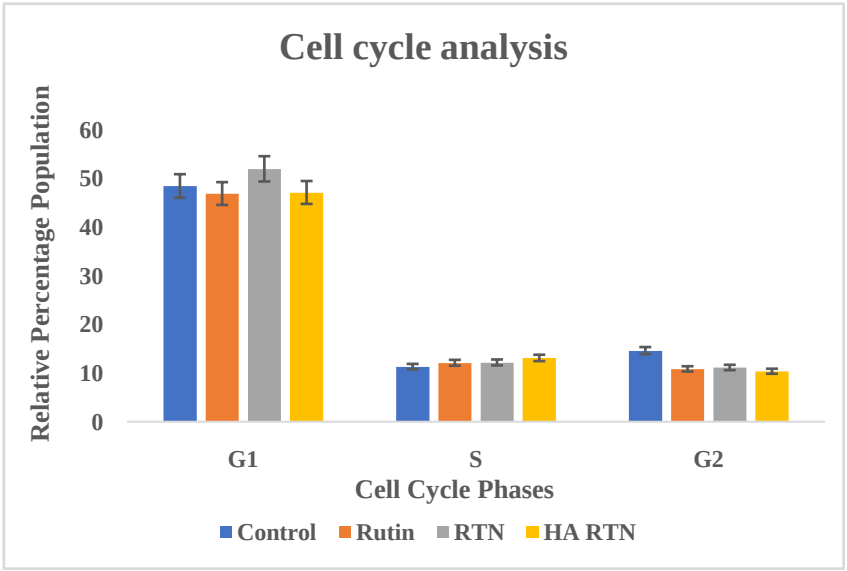


Figure 5: Cell cycle analysis

Cell apoptosis

The Annexin V apoptosis assay relies on a crucial characteristic of apoptotic cells, specifically the migration of phosphatidylserine from the inner layers of the plasma membrane to the outer surface of the cell. The identification of this translocation can be accomplished by tagging it with a fluorescent compound of annexin V, a calcium-dependent protein that binds to phospholipids with a strong affinity for phosphatidylserine. The assay's ability to differentiate between apoptosis and necrosis is facilitated by simultaneous staining with annexin V-FITC and propidium iodide (PI). In this context, PI is utilized to recognize cells undergoing necrosis, characterized by permeabilized plasma membranes [24,25].

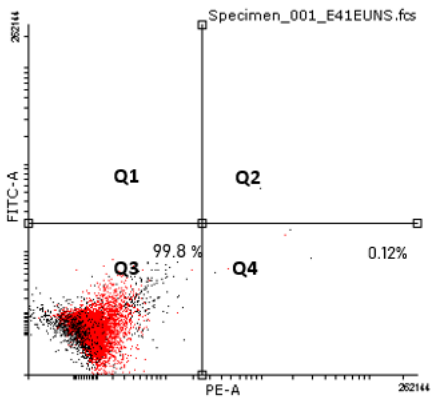


Figure 6: Control

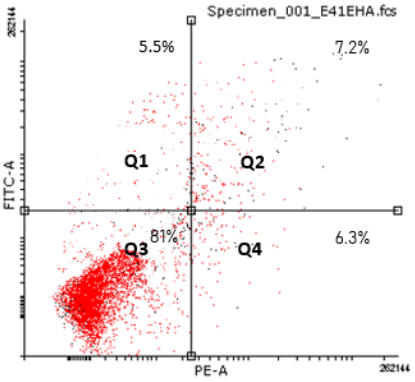


Figure 7: Rutin

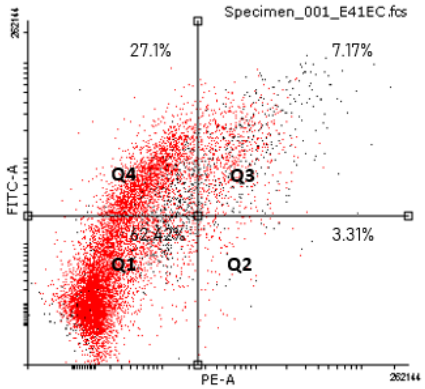


Figure 8: RTN

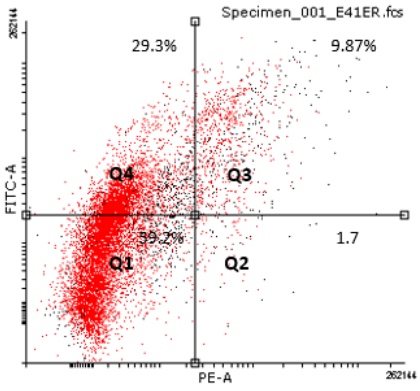


Figure 9: HA RTN

Table 2: Cell apotosis

Category	Early Apoptosis	Late Apoptosis	Necrosis
Rutin	6.3	7.2	5.5
RTN	3.3	7.2	21.7
HA RTN	1.7	9.9	29.3

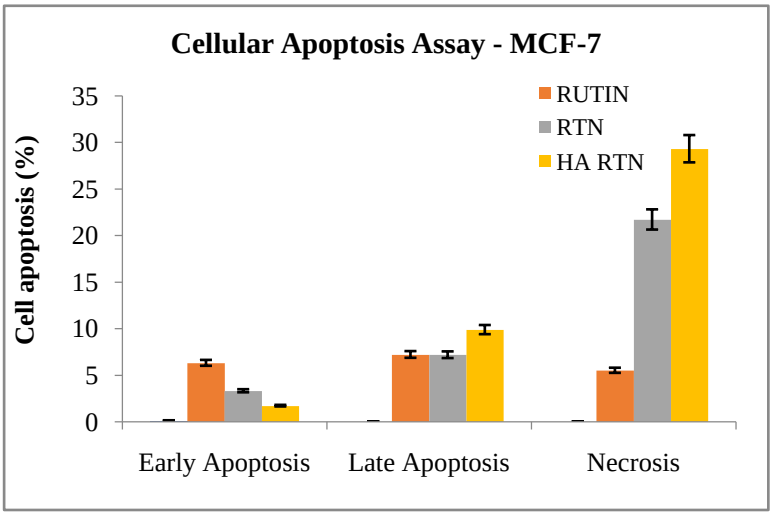


Figure 10: Cell apoptosis

The assessment of cellular apoptosis was performed on MCF-7 cells using flow cytometry analysis with Annexin V-FITC staining. This methodology was employed to ascertain the percentage of apoptotic cells within populations treated with RT, RTN, and HA RTN. In all treated cell groups, a substantial increase in apoptotic cell count was observed compared to the control.

Treatment with RT displayed 6.3% early phase apoptotic cells, 7.2% late phase apoptotic cells, and 5.5% necrotic cells, while 81% of the cells remained viable. RTN treatment resulted in 3.3% early phase apoptotic cells, 7.2% late phase apoptotic cells, and 21.7% necrotic cells, with 67.8% cell viability. Meanwhile, HA RTN treatment demonstrated 1.7% of cells in the early phase of apoptosis, 9.9% in the late phase of apoptosis, and 29.3% exhibiting necrosis. The viable cell count for this group was notably lower, with only 59.1% of cells remaining viable (Figure 6-10).

The comparative analysis of apoptosis induction between RT, RTN, and HA RTN treatments demonstrates a consistent escalation in apoptotic effects from RT to RTN and further to HA RTN. This progression is evidenced by the increase in both early and late phase apoptotic cells, along with a rise in necrotic cells, reflecting a heightened cellular response towards programmed cell death. Notably, HA RTN, when compared to RT and RTN, exhibited the most pronounced rise in apoptotic cells, underscoring its superior potential in promoting cellular apoptosis among the investigated treatments. This increase may be due to the increased accumulation of the drug in the cells [26,27].

Reactive oxygen species

The assessment of intracellular ROS levels in MCF-7 cells via flow cytometry, utilizing the redox-sensitive fluorescent probe DCFH-DA, revealed distinctive findings. The Mean Fluorescence Intensity (MFI) of positive cells indicated significant differences among the treatments. Specifically, Sample Rutin exhibited 10.65% MFI, RTN showed 18.78%, and HA RTN demonstrated 8.27%, contrasted with the Control's 5.04% (figure 12).

These results underscore the notable capability of RT, RTN, and HA RTN in significantly elevating intracellular ROS accumulation within MCF-7 cells. Particularly, HA RTN displayed the highest proportion of cells exhibiting ROS levels compared to RTN and rutin. This heightened activity potentially suggests the enhanced accumulation of rutin within the cells, contributing to the escalated ROS production induced by HA RTN.

Table 3: Reactive oxygen species

Category	MFI Positive Cells (%)
Control	5.04
Rutin	10.65
RTN	18.78
HA RTN	25.62

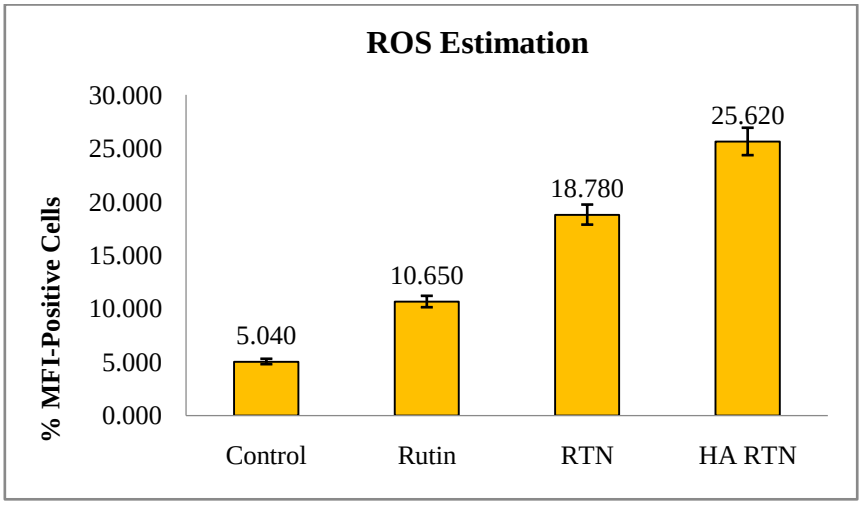


Figure 11: Reactive oxygen

Cellular uptake analysis

Flow cytometry was employed to investigate the cellular uptake of DAPI-labelled RT, RTN, and HA RTN. The recorded fluorescence intensity of the control group stood at 133.02. Contrastingly, RT-treated cells exhibited a notable increase to 142.63 fluorescence intensity.

Remarkably, the fluorescence intensity significantly surged in the RTN and HA RTN treated groups, reaching 158.042 and 165.60, respectively. This substantial surge in drug internalization, particularly in the HA RTN treated group, suggests a pronounced enhancement in the internalization of rutin, contributing to the heightened drug uptake observed in these treatments[28-31].

Table 4: Cellular uptake analysis

Sample Code	Mean Fluorescence
Control	133
Rutin	143
RTN	158
HA RTN	166

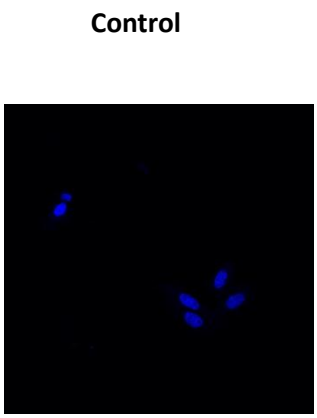


Figure 12. Control

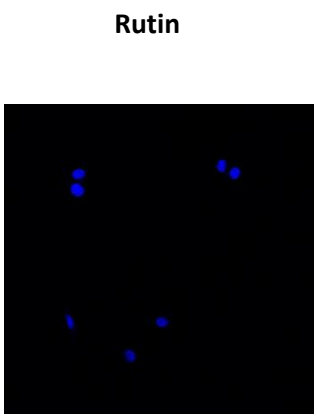


Figure 13. Rutin

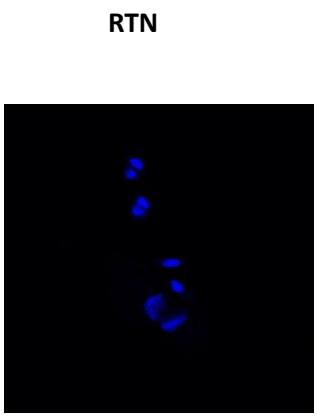


Figure 14. RTN

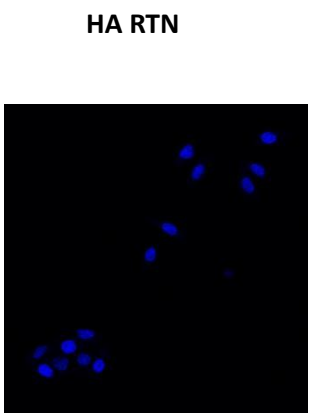


Figure 15. HA RTN

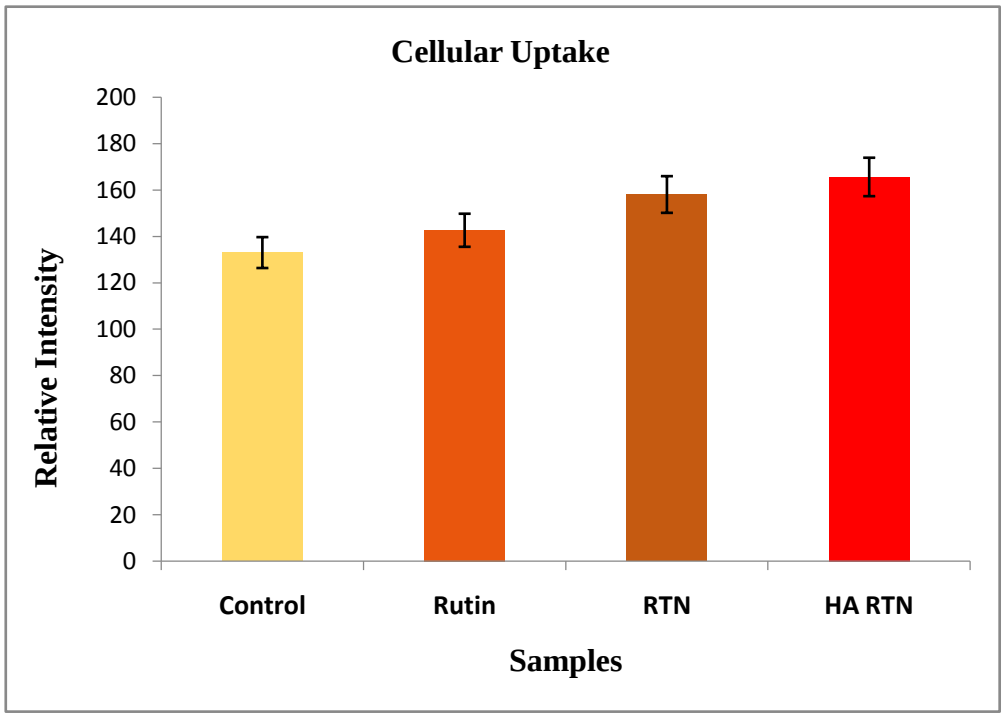


Figure 16: Cellular uptake

Conclusion

The investigation into rutin (RT), rutin nanoparticles (RTN), and hyaluronic acid-coated rutin nanoparticles (HA RTN) on MCF-7 breast cancer cells provided comprehensive insights across multiple cellular dynamics. Treatment with these compounds induced distinctive alterations in cell cycle progression, prominently reducing the population of cells in the G2 phase, indicative of a G2 arrest phenomenon. This interference in cell cycle dynamics potentially contributes to the observed cytotoxic effects linked to apoptosis.

Assessment of cellular apoptosis via Annexin V-FITC staining revealed a significant increase in apoptotic cells across all treatments, with a clear progression in apoptotic effects from RT to RTN and notably to HA RTN. Particularly, HA RTN demonstrated the most substantial rise in apoptotic cells, highlighting its superior potential in promoting cellular apoptosis among the tested compounds. The evaluation of intracellular ROS levels exhibited a notable increase in ROS accumulation following treatment with RT, RTN, and HA RTN. HA RTN notably displayed the highest proportion of cells with elevated ROS levels, indicating enhanced rutin accumulation and heightened ROS production associated with this treatment.Cellular uptake analysis demonstrated a remarkable increase in drug internalization, particularly evident in the HA RTN treated group. This significant enhancement in drug compound uptake underscores the superior ability of HA RTN to penetrate cells effectively. In summary, HA RTN showcased superior cytotoxicity, induced apoptosis most prominently, elevated intracellular ROS levels significantly, and notably increased the internalization of drug compounds within MCF-7 breast cancer cells. These collective findings underscore the potential of HA RTN as an advanced and promising strategy for targeted cancer therapy. Its unique capability to enhance cellular uptake and exert potent anti-cancer effects suggests HA RTN as a promising candidate for further exploration and development in innovative cancer treatment modalities.

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