

Enhanced antitumor efficacy of resveratrol-loaded nanocapsules in colon cancer cells: physicochemical and biological characterization

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Abstract. – OBJECTIVE: The aim of present work was to prepare resveratrol-loaded lipid-core-nanocapsule (RSV-LNC) and to characterize its ability to target the colon cancer cells.

MATERIALS AND METHODS: The lipid-core nanocapsule was prepared by precipitation method. The nanoparticle was prepared and evaluated regarding physical, chemical and biological parameters.

RESULTS: The average size of optimized nanocapsule was ~159 nm with a uniform size distribution index of 0.15 (PDI). The RSV-LNC showed a controlled and sustained release pattern with maximum release up to ~70% by the end of 48h study period. LNC showed an excellent cellular uptake potential. LNC showed a typical endocytosis-mediated cellular internalization process and located in the cell cytoplasm.

DISCUSSION: Importantly, RSV encapsulated in a nanocapsule showed a superior anticancer effect in HT29 cancer cells than compared to that of free RSV. Consistently, RSV-LNC showed a remarkable ~36% of cell apoptosis indicating its superior anticancer effect.

CONCLUSIONS: Based on the *in vitro* studies, RSV encapsulated in a nanocapsule showed promising potential to increase the therapeutic efficacy in colon cancer cells; however, further studies on animal models are warranted to confirm the improved effects of RSV nanoformulations.

Key Words:

Nanocapsules, Colon cancers, Resveratrol, Anticancer effect.

Introduction

Colon carcinoma is one of the most common forms of cancer among Western people with more than 1 million new cases every year¹. Colon carcinoma is specifically a neoplastic disease which occurs in the large intestine. Colorectal carcinoma

causes approximately 50,000 deaths in the United States and 655,000 deaths throughout the world per year^{2,3}. Despite the advances in technology and biological knowledge, colon cancer is still third most leading cause of mortality among cancer patients (lung, female breast, colorectal and stomach cancers)⁴. For the treatment, chemotherapy showed lots of potential in cancer patients; however, chemotherapeutic drugs suffer from high adverse effects to normal tissues. Therefore, the full benefits could not be reaped until now^{5,6}. In this regard, specific strategies have to be designed to increase the chemotherapeutic efficacy as well as to decrease the associated side effects.

In this perspective, resveratrol (RSV) is a natural polyphenol that has attracted a significant attention of researchers across the globe owing to its benefits including, antioxidant, anti-inflammatory, neuroprotective, chemopreventive and chemotherapeutic effects⁷⁻¹⁰. The most important pharmacological effects of this dietary phytochemical are cardioprotective activity and antitumor effect. RSV could effectively induce the apoptosis pathway to inhibit cancerous cell proliferation, as well as to enhance the sensitivity of drug-resistant cancer cell^{11,12}. This property of RSV allows it to combine with either other anticancer drug or as a single drug itself and, thereby, expected to reduce the side effects in chemotherapeutic treatments¹³. Unfortunately, RSV has a very short half-life of 8-15 min making it one of the most instable drugs in the systemic circulation. In addition to that poor solubility of RSV and inefficient systemic delivery limits its further clinical applications¹⁴. Therefore, efforts have to be made to overcome the physicochemical and pharmacokinetic limitations of RSV and to increase its systemic performance.

Various nanoparticle-based formulations have reported to improve its property; however, none resulted in satisfactory results. In this regard, li-

lipid-core nanocapsules (LNC) are a class of nanocapsules that has an oil core which is formed by a dispersion of a liquid lipid and a solid lipid (sorbitan monostearate) and then surrounded by a polymeric wall, stabilized by surfactants¹⁵. Specifically, oil core was covered or immersed with a mixture of polymers and particle water interface was stabilized by Tween 80 (surfactant). LNC has been reported to stabilize the incorporated drugs, control its release pattern, improve its therapeutic performances and ultimately increased the activity of the drug in the body¹⁶. LNC has shown to increase the therapeutic efficacy of RSV and mitigated its deleterious effects in glioma cells. However, there is no such study has been performed in colon cancer cells¹⁷.

The aim of present study was to investigate the potential of RSV-loaded lipid-core-nanocapsules (LNC) in inhibiting the proliferation of colon cancer cells (Figure 1). For this purpose, RSV was loaded in LNC carrier and its physicochemical and biological characteristics have been evaluated. The anticancer effect of RSV was tested in HT-29 cells by MTT assay. Furthermore, apoptosis assay was carried out to observe the apoptosis potential of RSV in colon cancer cells.

Materials and Methods

Materials

Resveratrol, sorbitan monostearate, vitamin E-TPGS, and polycaprolactone (PCL) were purchased from Sigma-Aldrich (Shanghai, China). All other chemicals were of reagent grade and used without further purification.

Preparation of Resveratrol (RSV)-Loaded Lipid-Core Nanocapsule

The lipid-core nanocapsule was prepared by precipitation method. In brief, PCL (0.1 g), sorbitan monostearate (40 mg), TPGS (20 mg), and RSV (15 mg) was added to acetone at 38°C. The organic phase was then added dropwise into an aqueous solution (40 ml) containing Tween-80 (1%) and stirred for 30 min. The organic phase was the removed under reduced pressure. The resulting drug-loaded nanocapsule was stored at refrigerated conditions.

Drug Loading

The amount of drug loaded in the nanocapsule was determined from HPLC method. For this purpose, the drug loaded nanocapsule dispersion was centrifuged at high speed and the supernatant was removed to evaluate the amount of drug untrapped. Following equations were applied:

$$EE (\%) = \frac{\text{Amount of drug in supernatant-total amount of drug added}}{\text{Total amount of drug added}} \times 100$$

$$DL (\%) = \frac{\text{Amount of drug in supernatant-total amount of drug added}}{\text{Total mass of nanoparticles}} \times 100$$

The HPLC conditions were; high-performance liquid chromatograph from Shimadzu (Kyoto, Japan), HPLC Class VP series with two LC-10AT-VP pumps, SPD-10AVP variable wavelength programmable UV-vis detector, and SCL-10AVP system controller was used. Phenomenex C18 col-

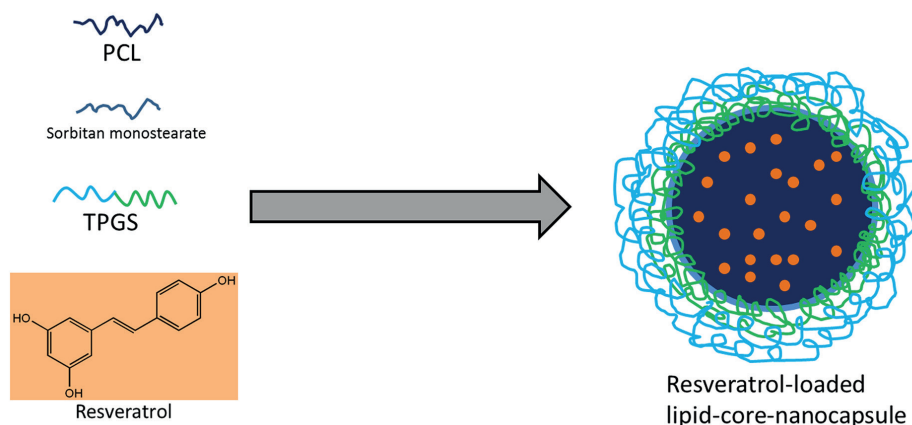


Figure 1. Schematic illustration of preparation of resveratrol-loaded lipid-core-nanocapsule.

umn (250 mm × 4.6 mm, 5 μm) equipped with a guard column (4 mm × 3 mm × 5 μm) (Torrance, CA, USA) was used. The mobile phase used was methanol: phosphate buffer (pH 6.8 adjusted with 0.5% (v/v) orthophosphoric acid solution in Milli-Q water) (63:37%, v/v) set at a flow rate of 1 ml/min and wavelength was set at 306 nm.

Particle Size Analysis

The average particle size distribution was analyzed using Zetasizer[®] Nano series (Malvern, UK). The samples were suitably diluted in ultrapure water at 25°C. The experiments were performed 3 times.

Morphology Analysis

The morphology of nanocapsule was observed using transmission electron microscope (TEM; H 7500, Hitachi, Tokyo, Japan). The diluted samples were placed on a copper grid and the excess was drawn off with a filter paper. Samples were subsequently stained with 1% of uranyl acetate solution for 1 min.

Release Study

The *in vitro* release profile of RSV from RSV-LNC was carried out using dialysis method as per the reported protocols. In brief, release study was carried out in phosphate buffered solution (pH 7.4) and acetate buffered solution (pH 5.0) at 37°C under constant shaking in an incubator. The drug loaded-nanoparticle pellet was redispersed in 1 ml water, mixed homogeneously and transferred to dialysis tubes. The dialysis tube containing the nanocapsule dispersion was placed in respective release medium (25 ml) and then incubated in a shaker bath. At predetermined time intervals, 1 ml of release media was taken from the beaker and replenished with fresh PBS and ABS buffers. The released drug in both the release medium was measured using a HPLC as mentioned above. HPLC Class VP series with two LC-10ATVP pumps was used and methanol: phosphate buffer (pH 6.8 adjusted with 0.5% (v/v) orthophosphoric acid solution in Milli-Q water) (63:37%, v/v) was used a mobile phase.

Cellular Uptake

HT29 colon cancer cell was used to observe the cellular uptake. The cells at a density of 5×10^5 cells/well were seed in a 6-well plate containing a cover slip. The cells were incubated for 1 day and then treated with rhodamine-b-loaded nanocapsule and incubated for 1h. After which cells were washed with PBS and fixed with 4% paraformaldehyde

solution for 15 min. The cells were then washed again with PBS and exposed with DAPI for 10 additional min. The cover slip containing the cells was mounted and cellular uptake was observed using a confocal microscope (Leica SP 5 II).

Cell Viability

The cell viability of RSV and RSV-LNC was evaluated using of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. In brief, HT29 cells at a seeding ratio of 1×10^4 per well in 100 μL of complete culture medium was seeded in a 96-well plate. Next day, cells were treated with RSV and RSV-LNC at different concentrations and incubated for additional 24h. After incubation for the specified time at 37°C in a humidified incubator, cell viability was determined by MTT. MTT solution was added to each well and incubated for 4h and 0.1 mL of buffered dimethyl sulfoxide (DMSO) was added to each well. After 30 min, the absorbance was recorded on a microplate reader at the wavelength of 540 nm.

Apoptosis Study

The apoptosis study was evaluated utilizing of FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA). In brief, HT29 cells at a seeding ratio of 2×10^5 per well in 100 μL of complete culture medium was seeded in a 6-well plate. Next day, cells were treated with RSV and RSV-LNC at different concentrations and incubated for additional 24h. Cells were then harvested in annexin V binding buffer provided in the FITC Annexin V Apoptosis Detection Kit I. Cells were stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's protocol. The cells were finally analyzed by flow cytometer.

Statistical Analysis

All data are presented as mean ± SD. Student's *t*-tests were used to compare measurements between groups, and $p < 0.05$ was considered statistically significant.

Results

Physicochemical Characteristics of RSV-loaded Nanocapsule

The particle size and size distributions of RSV-LNC were determined using laser diffraction method (Figure 2a). The Dynamic Light Scattering (DLS) analysis showed that average particle

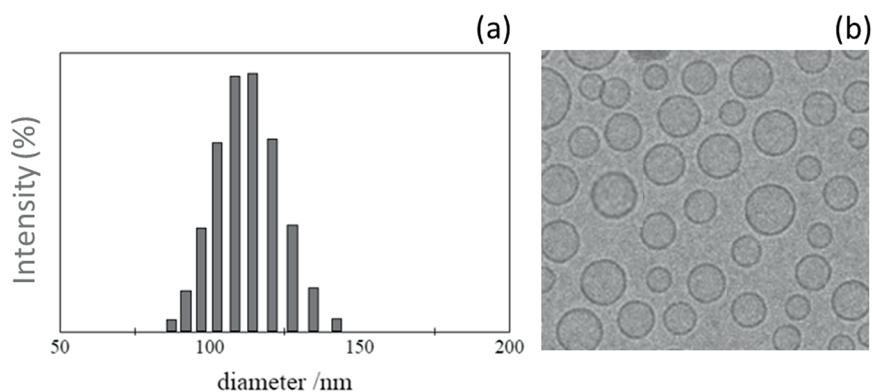


Figure 2. (a) Particle size distribution of RSV-LNC determined using laser diffraction methods; (b) TEM image of RSV-LNC

size of RSV-LNC was around ~ 150 nm with an excellent polydispersity index (PDI ~ 0.15). Moreover, particles exhibited an entrapment efficiency of more than 95% with an active drug loading of $\sim 13.5\%$.

Morphological Analysis

The particle size and morphology was further confirmed by Transmission Electron Microscopy (TEM) imaging (Figure 2b). Consistent with the DLS analysis, the particle was nanosized and uniformly dispersed on the TEM grid. The parti-

cles were spherically shaped with a clear boundary with each other. As one can see a clear outer membrane was seen indicating the interface. The spherical shaped particles further confirmed its suitability for cancer targeting.

In vitro Drug Release

The *in vitro* drug release was carried out by dialysis method. The study was carried out up to 48 h. As seen (Figure 3), throughout the release period, LNC exhibited a controlled and sustained release profile of RSV. Notably, LNC exhibited a pH-sensi-

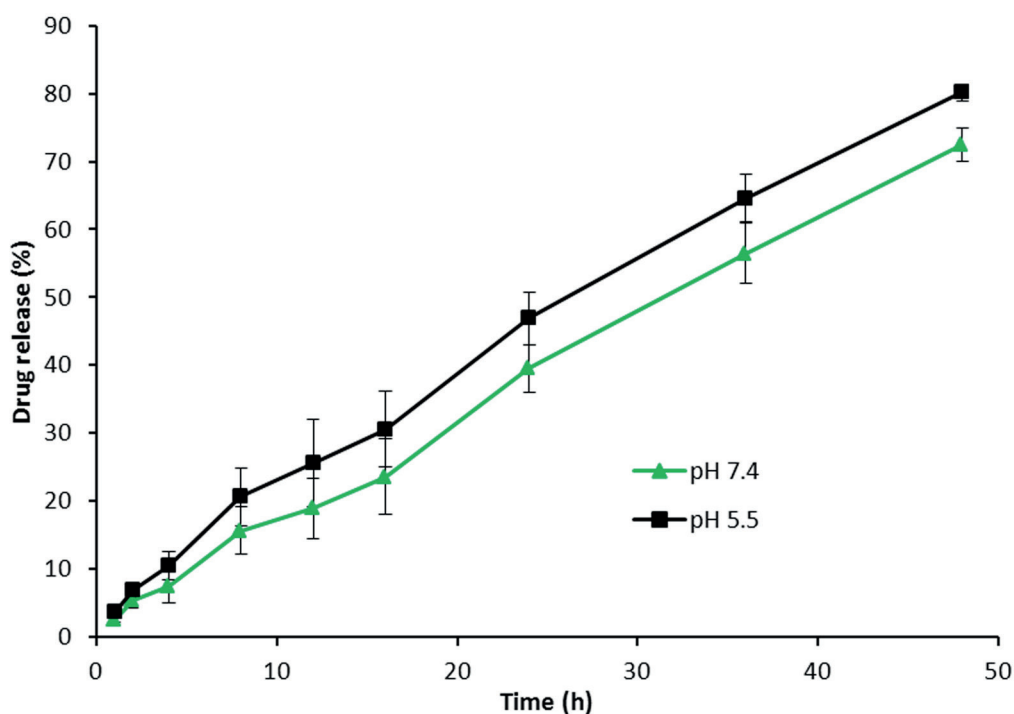


Figure 3. *In vitro* release profile of RSV from RSV-LNC. The release study was carried out by dialysis method and carried out up to 48h in PBS and ABS media.

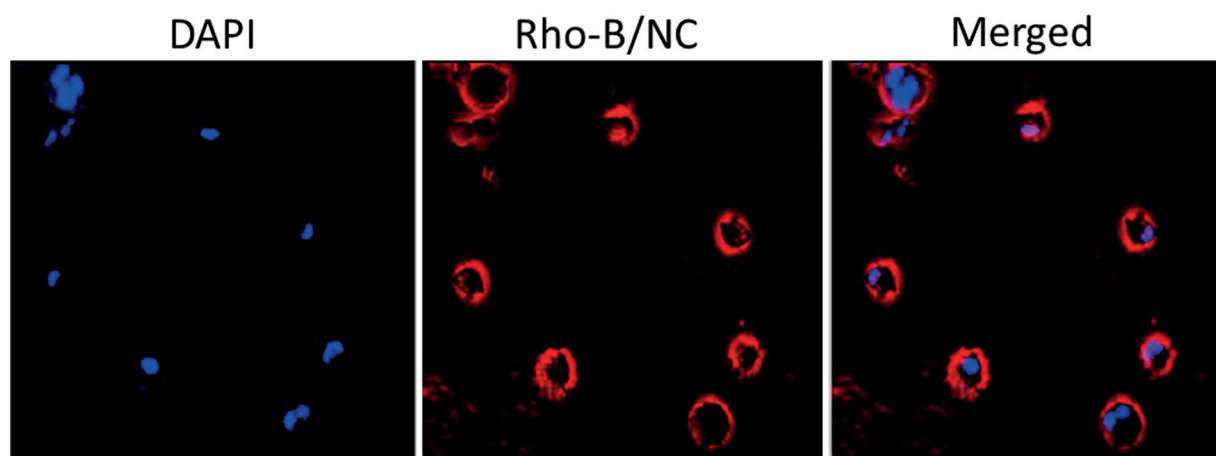


Figure 4. *In vitro* cellular uptake of LNC carrier in HT29 colon cancer cells. The Rhodamine-B was used as a fluorescent dye and DAPI was used to stain the nucleus. The images were captured using confocal microscopy.

tive release pattern with slight higher release at tumor pH. Originally, LNC doesn't have any pH-responsive components, yet it showed pH-dependent release pattern indicating that some of the components may be unstable in lower pH.

In vitro Cellular Uptake

Cellular uptake potential of RSV-LNC was observed in HT 29 colon cancer cells using a confocal microscope. As can be clearly seen (Figure 4), red fluorescence is present in the cytoplasmic region but not in the nucleus indicating that the particles were internalized via endocytosis process and located in the cell circumference.

Cytotoxicity Assay

The antiproliferative effects of free RSV and RSV-LNC were evaluated in HT 29 colon cancer cells. The study was performed for 24 and 48 h incubation. The results clearly showed a time and dose-dependent cytotoxicity (Figure 5). IC₅₀ value was calculated to determine the concentration required to kill 50% of cancer cells. The IC₅₀ of RSV was around ~50 µg/ml while it was ~40 µg/ml for RSV-LNC after 24 h incubation.

Apoptosis Assay

The remarkable tumor suppressive effect of RSV and RSV nanoformulations prompted us to determine the underlying mechanisms. Therefore, we have carried out the apoptosis assay via Annexin-V/PI staining protocol. It has been reported that RSV induces the cancer cell death via apoptosis pathway. In the present work, we have observed a marked apoptosis effect of the RSV

(Figure 6). As seen, free RSV induced nearly ~15% cell apoptosis whereas RSV-LNC induced a remarkable ~36% of cell apoptosis indicating its superior anticancer effect in the case of HT 29 colon cancer cells.

Discussion

RSV could effectively induce the apoptosis pathway to inhibit cancerous cell proliferation, as well as to enhance the sensitivity of drug-resistant cancer cell. Especially, RSV is now regarded as a chemopreventive as well as a proapoptotic agent which is very effective in inhibiting the cancer progression. However, beneficial advantages of RSV are hampered due to its high hydrophobicity and rapid systemic clearance resulting in a low therapeutic effect. In the present study, therefore, we have used a unique delivery system to counter its drawbacks¹⁸. To this front, we have encapsulated RSV in a lipid-core-nanocapsule because of its excellent *in vitro* and *in vivo* stability. It has been reported that LNC possesses remarkable advantages over other carriers such as liposomes, nanoemulsions, and solid lipid nanoparticles.

The thorough characterization of RSV-LNC is an important step towards the development of nanoparticles with ideal properties for cancer targeting and to ensure its physicochemical stability. The DLS analysis showed that average particle size of RSV-LNC was around ~150 nm with an excellent polydispersity index (PDI ~0.15). Such nanosized particles will be suitable for cancer targeting. Moreover, zeta potential was

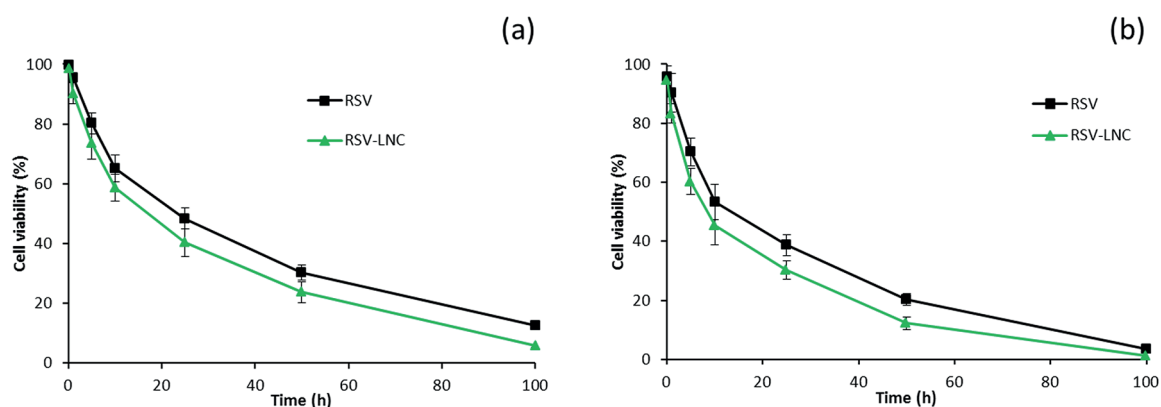


Figure 5. *In vitro* cytotoxicity assay of free RSV and RSV-LNC in HT29 colon cancer cells. The cytotoxicity potential of formulations was determined by MTT assay and the study was carried out at 24h and 48h incubation.

evaluated to further confirm the storage stability. It was observed that average surface charge of RSV-LNC was approximately ~ 3.5 mV indicating the values towards neutral. Although neutral charges on the surface are regarded as unstable formulations; however, in the present case, it should be considered that the particles developed here have steric stabilization, a consequence of the presence of a non-ionic polymer on the interface particle/water.

LNC exhibited a controlled and sustained release profile of RSV. A controlled release of RSV was followed throughout the release study. For example, $\sim 38\%$ and $\sim 45\%$ of RSV was released in pH 7.4 and pH 5.0 release conditions and a similar trend was followed at the end of 48h with the overall release was close to $\sim 70\%$. No burst release pattern was observed in either pH conditions indi-

cating that the drug was stably incorporated into the lipid core. Such sustained release of the drug might be beneficial for the cancer targeting.

Cellular uptake potential of RSV-LNC was observed in HT29 colon cancer cells using a confocal microscope. For this purpose, Rhodamine-B was replaced with RSV in order to track the fluorescence within the cellular cytoplasm or nucleus. The nucleus was stained with DAPI (blue color) where as nanocapsule has been tracked with red fluorescence from rhodamine B. It can be seen that red fluorescence did not migrate towards the nucleus suggesting the stability of particles in the acidic compartment. We have incubated the cells with 1h and within this stipulated time, we did not observe any drug release in the cellular environment.

Cytotoxicity assay clearly demonstrated that RSV loaded in a nanocapsule was more effective

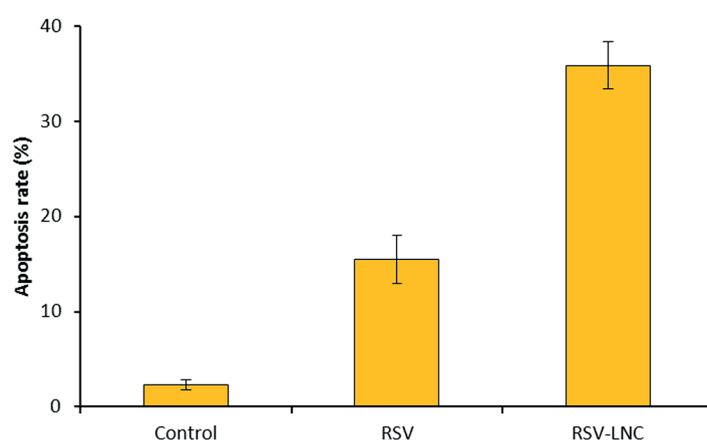


Figure 6. Apoptosis analysis of free RSV and RSV-LNC in HT29 cancer cells. The apoptosis potential of formulations was determined by staining with Annexin-V/PI double staining assay kit.

in exhibiting the anticancer effect in the cancer cells in a concentration and time-dependent manner. Based on the *in vitro* anticancer effect, it can be expected that nanocapsule would be more effective at the *in vivo* level¹⁹. Further, apoptosis assay was evaluated using Annexin-V/PI staining protocol. As seen, free RSV induced nearly ~15% cell apoptosis whereas RSV-LNC induced a remarkable ~36% of cell apoptosis indicating its superior anticancer effect in the case of HT29 colon cancer cells. Earlier, it has been reported that RSV kills tumor cells in part through the generation of the high amount of ROS intracellularly. The resulting high ROS levels could induce DNA damage and activates p53 related apoptotic cascade. Such RSV interacts with the H₂O₂-oxidase system and generates ROS in HT 29 cancer cells and leads to cancer cell apoptosis²⁰.

Conclusions

RSV-loaded lipid-core-nanocapsule was successfully prepared and characterized for its ability to target the colon cancer cells. The average size of optimized nanocapsule was ~159 nm with a uniform size distribution index of 0.15 (PDI). The RSV-LNC showed a controlled and sustained release pattern with maximum release up to ~70% by the end of 48h study period. LNC showed an excellent cellular uptake potential. LNC showed a typical endocytosis-mediated cellular internalization process and located in the cell cytoplasm. Notably, RSV encapsulated in a nanocapsule showed a superior anticancer effect in HT29 cancer cells than compared to that of free RSV. Consistently, RSV-LNC showed a remarkable ~36% of cell apoptosis indicating its superior anticancer effect. Based on the *in vitro* investigations, RSV encapsulated in a nanocapsule showed promising potential to increase the therapeutic efficacy in colon cancer cells. However, further studies on animal models are warranted to confirm the improved effects of RSV nanoformulations.

Acknowledgement

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Conflict of interest

The authors declare no conflicts of interest.

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