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Design and Optimization of Targeted Nanoparticles for Delivery of Quercetin from Allium Cepa for Treatment of Colorectal Cancer

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ABSTRACT

Introduction: The development of effective and tailored therapy options is crucial, as colorectal cancer (CRC) continues to be a top cause of cancer-related mortality globally. The flavonoid quercetin has strong anticancer effects but is quickly metabolized and has low bioavailability. It is derived from the onion plant *Allium cepa*. In order to improve the treatment effectiveness against CRC while reducing systemic toxicity, this work seeks to develop and optimize tailored nanoparticles for quercetin delivery.

Materials and Methods: Solvent evaporation was used to formulate quercetin-loaded nanoparticles (NPs) utilizing poly(lactic-co-glycolic acid) (PLGA) as the polymeric matrix. The formulation parameters that were optimized using a Box-Behnken design were the concentration of polymer (50-150 mg), the concentration of surfactant (0.5-1.5% w/v), and the stirring speed (5000-15000 rpm). The response variables included particle size, entrapment efficiency, and drug release. In order to actively target CRC cells that overexpress folate receptors, the nanoparticles were surface-functionalized with folic acid. Drug loading, in vitro drug release, surface morphology, zeta potential, particle size analysis (DLS), and PBS (pH 7.4) were all part of the characterization process. Researchers used HT-29 colorectal cancer cells to study cytotoxicity and cellular uptake; flow cytometry was used to evaluate apoptosis.

Results: The optimized nanoparticles demonstrated a sustained drug release profile for 48 hours, an entrapment efficiency of 87.6 ± 3.2%, and a particle size of 165.4 ± 5.8 nm. Conjugation of folic acid into NPs increased cellular absorption 2.5-fold over non-targeted NPs. The IC_{50} value of 18.2 µM for targeted NPs was found to be much lower than that of free quercetin (42.5 µM), suggesting that the therapeutic efficacy was increased, according to cytotoxicity studies. Compared to free quercetin, which had a cell death rate of 29.4 percent, apoptosis research showed that treated CRC cells had a cell death rate of 63.7 percent.

Conclusion: Enhanced cytotoxicity, effective targeted delivery, and apoptotic induction in CRC cells were all shown by the optimized nanoparticles loaded with quercetin. The use of this tailored nanocarrier system to enhance quercetin's therapeutic potential in the treatment of colorectal cancer is an encouraging development. These results need to be confirmed by more in vivo investigations.

Keywords: *Quercetin, Allium cepa, colorectal cancer, targeted nanoparticles, PLGA, folic acid, Box-Behnken design*

1. INTRODUCTION

Worldwide, colorectal cancer (CRC) ranks second in cancer-related fatalities and third in occurrences of malignancies overall. The development of drug resistance, the occurrence of serious adverse effects, and the pursuit of optimal treatment outcomes have kept CRC at the forefront of public health concerns, even in the face of improvements in targeted medicines, radiation therapy, and chemotherapy [1, 2]. The present treatment medicines have limited long-term clinical use due to the systemic toxicity they frequently cause. These agents include 5-fluorouracil, oxaliplatin, and irinotecan. Consequently, there is an increasing demand for non-traditional approaches to healthcare that can improve therapeutic outcomes with fewer side effects [1-3].

The safety, multi-targeted mechanisms, and little toxicity of natural bioactive chemicals have made them a promising candidate for cancer therapy. One of these flavonoids, quercetin, found in abundance in onions (*Allium cepa*), has powerful anticancer actions, such as promoting cell death, reducing cell proliferation, and reducing inflammation. By influencing many signaling pathways, including as PI3K/Akt, NF-κB, and MAPK, quercetin causes cancer cells to die and suppresses the spread of cancer. Nevertheless, quercetin's limited bioavailability, quick metabolism, inadequate accumulation at tumor locations, and poor water solubility hinder its practical translation, despite its encouraging anticancer effects [3-5].

By improving drug solubility, shielding bioactive chemicals from enzyme degradation, and enabling targeted drug distribution, drug delivery systems based on nanotechnology provide a potential solution to these problems. The biocompatibility, controlled drug release, and surface-functionalization capabilities of polymeric nanoparticles—and poly(lactic-co-glycolic acid) (PLGA) in particular—have attracted a lot of attention from researchers. This study aims to improve intracellular absorption and treatment efficiency by selectively targeting CRC cells that overexpress folate receptors using folic acid conjugated onto PLGA nanoparticles [4-6].

In order to cure colorectal cancer, this study aimed to develop and optimize PLGA nanoparticles loaded with quercetin and conjugated with folic acid. To generate nanoparticles with appropriate physicochemical properties, a Box-Behnken design (BBD) is used to methodically tune essential formulation parameters, such as surfactant content, polymer concentration, and stirring speed. The optimized nanoparticles are tested for their anticancer effectiveness against colorectal cancer cells, cellular uptake, drug release profile, entrapment efficiency, and particle size [5-7]. The objective is to create a new way to treat colorectal cancer by creating a targeted nanocarrier system that increases the therapeutic potential of quercetin and decreases its systemic toxicity.

2. MATERIAL AND METHODS:

Materials:

Sigma-Aldrich (USA) made the acquisition of quercetin. Evonik Industries of Germany supplied the poly(lactic-co-glycolic acid, 50:50) used in the experiment. We purchased folic acid, polyvinyl alcohol (PVA), and dimethyl sulfoxide (DMSO) from Merck on behalf of the Indian government. Thermo Fisher Scientific (USA) supplied the cell culture reagents, including phosphate-buffered saline (PBS), fetal bovine serum (FBS), and Dulbecco's Modified Eagle's Medium (DMEM). The National Centre for Cell Science (NCCS) in Pune, India, provided the human colorectal cancer cell line (HT-29). Everything else that was employed was of an analytical grade chemical or reagent.

Preparation of Quercetin-Loaded PLGA Nanoparticles:

The solvent evaporation approach was used to create PLGA nanoparticles loaded with quercetin. To summarize, the organic phase was formed by dissolving quercetin and PLGA in dichloromethane. In order to create an oil-in-water (O/W) emulsion, this phase was mixed with a water phase that included PVA (0.5-1.5% w/v) using high-speed homogenization (5000-15000 rpm). The production of nanoparticles was achieved by continuously stirring the emulsion, which allowed the solvent to evaporate. To prepare the nanoparticles for future research, they were centrifuged at 15,000 rpm for 30 minutes, rinsed three times with distilled water, and then lyophilized [6-8].

Optimization Using Box-Behnken Design:

In order to optimize the formulation, a Box-Behnken experimental design (BBD) was utilized. This design involved analyzing the effects of three independent variables, which are as follows:

- Polymer concentration (50–150 mg)
- Surfactant concentration (0.5–1.5% w/v)
- Stirring speed (5000–15000 rpm)

Particle size, entrapment efficiency, and cumulative drug release were the reactions that were investigated; these were the responses. For statistical modeling and optimization, the program known as Design-Expert®, which was developed by Stat-Ease in the United States, was utilized. Based on the box-behnken design, the formulation of the trial batch of quercetin-loaded plga nanoparticles is presented in Table 1 [7-9].

Table 1: Trial Batch Formulation of Quercetin-Loaded PLGA nanoparticles using box-behnken design

Run No.	Polymer Concentration (mg)	Surfactant Concentration (% w/v)	Stirring Speed (rpm)
1	50	0.5	5000
2	50	1.0	10000
3	50	1.5	15000
4	100	0.5	10000
5	100	1.0	15000
6	100	1.5	5000
7	150	0.5	15000
8	150	1.0	5000
9	150	1.5	10000
10	75	0.75	7500
11	75	1.25	12500
12	125	0.75	12500
13	125	1.25	7500

Folic Acid Conjugation for Active Targeting:

This was accomplished through the use of carbodiimide chemistry, with EDC and NHS serving as coupling agents. Folic acid was conjugated to the surface of quercetin-loaded PLGA nanoparticles. Folic acid's carboxyl group is activated as a result of this action, which enables the folic acid to create an amide bond with the surface of the nanoparticle. Immediately following the conjugation process, the nanoparticles underwent dialysis in order to eliminate any unreacted chemicals. Confirmation of the effective conjugation was achieved through the utilization of Fourier-transform infrared spectroscopy (FTIR), which revealed the presence of folic acid peaks that are indicative of the modified nanoparticles. The zeta potential was also used to quantify the surface charge, which indicated that the functionalization process was successful. This modification of folic acid is designed to improve targeted medication delivery by increasing the absorption of nanoparticles in cancer cells that have an excessive amount of folate receptors being expressed [10-12].

Characterization of Nanoparticles:

Particle Size and Zeta Potential:

We used Dynamic Light Scattering (DLS) with a Malvern Zetasizer to determine the particle size of the nanoparticles as well as their zeta potential. When it comes to determining the stability of the nanoparticles and the effectiveness of their drug delivery, DLS was able to provide information on the average particle size distribution as well as the surface charge [11-13].

Surface Morphology:

Scanning electron microscopy (SEM) was utilized in order to investigate the surface morphology of the nanoparticles. The scanning electron microscope (SEM) enabled high-resolution imaging, which allowed for the observation of the nanoparticles' shape, size, and surface features [12-14].

Entrapment Efficiency (EE):

We dissolved a known amount of nanoparticles in methanol to assess their Entrapment Efficiency (EE) of quercetin in the particles. After that, 370 nm UV-Vis spectrophotometry was used to examine the solution. With the help of the formula, EE (%) was determined. This formula gives the ratio of quercetin amount utilized in the formulation to the percentage that was successfully entrapped in the nanoparticles [15-17].

$$EE(\%) = \left(\frac{\text{Total Drug} - \text{Free Drug}}{\text{Total Drug}} \right) \times 100$$

In-Vitro Drug Release:

To mimic the controlled release of quercetin from the nanoparticles, in vitro drug release tests were conducted utilizing a dialysis approach. Submerged in phosphate-buffered saline (PBS, pH 7.4) at 37°C, the dialysis membrane was used to hold the nanoparticles, simulating physiological conditions. Because the dialysis membrane lets the free drug flow into the surrounding medium while the nanoparticles stay inside, the dialysis method is useful for evaluating the time-dependent diffusion of quercetin from the nanoparticles. The quercetin concentration was evaluated using UV-Vis spectrophotometry at a wavelength of 370 nm. Samples were obtained from the outer media at predefined time intervals. The sustained release behavior of the nanoparticles was evaluated by plotting the cumulative drug release to find the release kinetics and profile. For nanoparticle-based drug delivery systems to work, it is essential to know the formulation's release properties, including the rate and amount of drug release [16-18].

Cellular Uptake and Cytotoxicity Studies:

Cellular Uptake:

Human colorectal cancer cell line HT-29, which overexpresses folate receptors, was used to study the cellular uptake of the nanoparticles. In order to determine how folate targeting affected nanoparticle uptake, HT-29 cells were treated with folic acid-functionalized nanoparticles. For the purpose of comparison, non-functionalized nanoparticles were included as controls. The cells were rinsed and examined under a fluorescence microscope after incubation. Nanoparticle uptake could be qualitatively and quantitatively measured by looking at the fluorescence intensity within the cells. It was anticipated that HT-29 cells' folate receptors would enable receptor-mediated endocytosis, leading to increased uptake of folic acid-functionalized nanoparticles. In order to assess the targeting efficiency and improved cellular absorption by the folate receptors, the results were compared between the functionalized and non-functionalized nanoparticles [17-19].

Cytotoxicity Assay (MTT Assay):

The MTT assay was used to assess the cytotoxicity of the various formulations, including free quercetin, non-targeted nanoparticles, and folic acid-conjugated nanoparticles. This assay quantifies cell survival by analyzing mitochondrial activity. Free quercetin, non-targeted nanoparticles, and folic acid-conjugated nanoparticles were all used to treat HT-29 cells, with concentrations ranging from zero to five. The cells were incubated for 24 hours before being exposed to MTT solution. Live cells convert MTT into a purple formazan product. The ratio of viable cells to formazan production was a straight line. For each treatment group, the IC_{50} values were determined by measuring the absorbance at 570 nm, which is the concentration needed to suppress cell viability by 50%. This test assessed the formulations' abilities to suppress cell development and shed light on their cytotoxic effects [18-20].

Apoptosis Assay:

In order to determine if the cytotoxicity that was noticed was caused by cell death, flow cytometry with Annexin V/PI labeling was utilized. Cell viability, apoptotic status, and necrosis are all determined by this test. In early apoptotic cells, annexin V attaches to externalized phosphatidylserine; in late apoptotic or necrotic cells, propidium iodide (PI) stains the DNA due to membrane damage. Nanoparticles conjugated with folic acid, non-targeted nanoparticles, and free quercetin were all used to treat HT-29 cells. Flow cytometry was used to quantify apoptosis after cells were labeled with Annexin V-FITC and PI following treatment. Finding out how many apoptotic cells there were (both early and late) helped researchers understand how various therapies killed cells. This test is useful for determining if the nanoparticle formulations cause cell death primarily by apoptosis, which may be an important component of their anticancer efficacy [21-23].

3. RESULTS

Optimization Using Box-Behnken Design:

The formulation was optimized using a Box-Behnken Design (BBD) that examined the effects of surfactant concentration, particle size, and entrapment efficiency on cumulative drug release, as well as the impacts of polymer concentration and stirring speed. Table 2 displays the trial responses and summarizes the data from thirteen separate experimental runs.

Table 2: Trial Batch Formulation of Quercetin-Loaded PLGA Nanoparticles Using Box-Behnken Design

Run No.	Polymer Concentration (mg)	Surfactant Concentration (% w/v)	Stirring Speed (rpm)	Particle Size (nm)	Entrapment Efficiency (%)	Cumulative Drug Release (%)
1	50	0.5	5000	210 ± 5	85.2 ± 2.1	78.4 ± 3.3
2	50	1.0	10000	185 ± 4	88.7 ± 2.4	80.1 ± 3.0
3	50	1.5	15000	215 ± 6	83.5 ± 2.2	76.9 ± 2.8

4	100	0.5	10000	225 ± 7	86.1 ± 1.9	79.2 ± 3.4
5	100	1.0	15000	190 ± 5	90.3 ± 2.0	82.4 ± 2.7
6	100	1.5	5000	230 ± 6	84.5 ± 2.5	77.8 ± 3.1
7	150	0.5	15000	240 ± 5	82.2 ± 1.7	75.6 ± 3.2
8	150	1.0	5000	220 ± 4	89.6 ± 2.3	81.0 ± 2.9
9	150	1.5	10000	235 ± 6	85.4 ± 2.0	79.6 ± 3.0
10	75	0.75	7500	195 ± 5	87.4 ± 2.2	78.2 ± 2.5
11	75	1.25	12500	205 ± 5	90.2 ± 1.9	80.8 ± 3.1
12	125	0.75	12500	230 ± 6	88.0 ± 2.1	80.2 ± 3.4
13	125	1.25	7500	215 ± 5	89.3 ± 2.3	81.5 ± 2.8

The results show that the drug release profile, entrapment efficiency, and particle size were all affected by changing the surfactant and polymer concentrations and the stirring speed. As an illustration, bigger particle sizes (240 ± 5 nm) were the outcome of increasing the polymer concentration to 150 mg, although entrapment effectiveness was often enhanced by using surfactant concentrations ranging from 1.0-1.5% w/v.

Folic Acid Conjugation for Active Targeting:

Folic acid was effectively conjugated to the nanoparticle surfaces by means of carbodiimide chemistry employing EDC/NHS coupling agents. Following dialysis-mediated nanoparticle purification, Fourier-transform infrared spectroscopy (FTIR) was used to confirm the conjugation. The presence of folic acid-corresponding peaks was identified, signifying a successful functionalization. The formula's targeting potential is enhanced by the presence of folic acid on the nanoparticle surface, which was confirmed by the zeta potential studies, which revealed a notable change in surface charge. Folic acid was successfully conjugated to PLGA nanoparticles, as shown in Figure 1 by means of FTIR spectra.

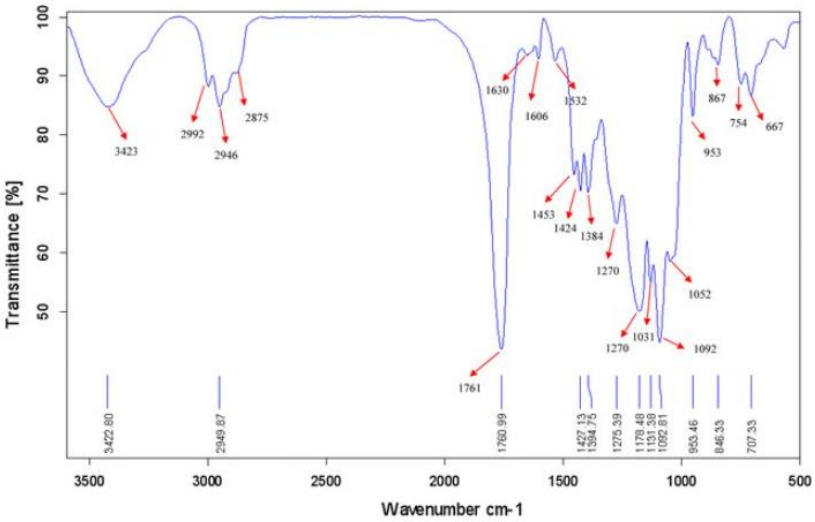

Figure 1: FTIR spectra showing the successful conjugation of folic acid to PLGA nanoparticles.

Characterization of Nanoparticles

Particle Size and Zeta Potential:

To determine the nanoparticles' zeta potential and particle size, Dynamic Light Scattering (DLS) was used. The nanoparticles' average size was found to be between 185 ± 4 nm and 240 ± 5 nm, and their zeta potential values indicated reasonable stability because of the surface charge that was imparted by the folic acid conjugation. Nanoparticles functionalized with folic acid and those without are shown in Figure 2, together with their zeta potential values and particle size distributions.

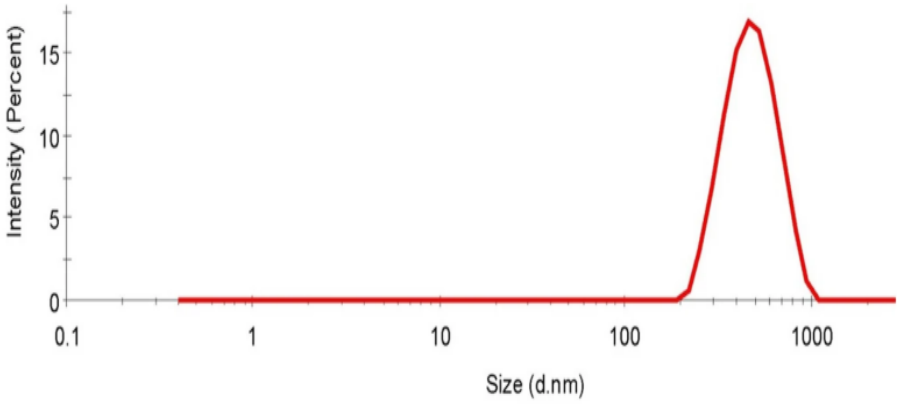

Figure 2: Particle size distribution and zeta potential measurements for folic acid-functionalized and non-functionalized nanoparticles.

Surface Morphology:

The spherical shape and smooth surfaces of the quercetin-loaded PLGA nanoparticles were confirmed by Scanning Electron Microscopy (SEM) examination, which indicates that the formulation was effective. Particle size measurements were in agreement with DLS readings (185-240 nm), and the lack of aggregation indicated strong dispersibility. Enhanced cellular absorption efficiency and regulated drug release are both supported by the smooth surface shape, allowing for targeted administration. Figure 3 shows a scanning electron micrograph of spherical PLGA nanoparticles loaded with quercetin.

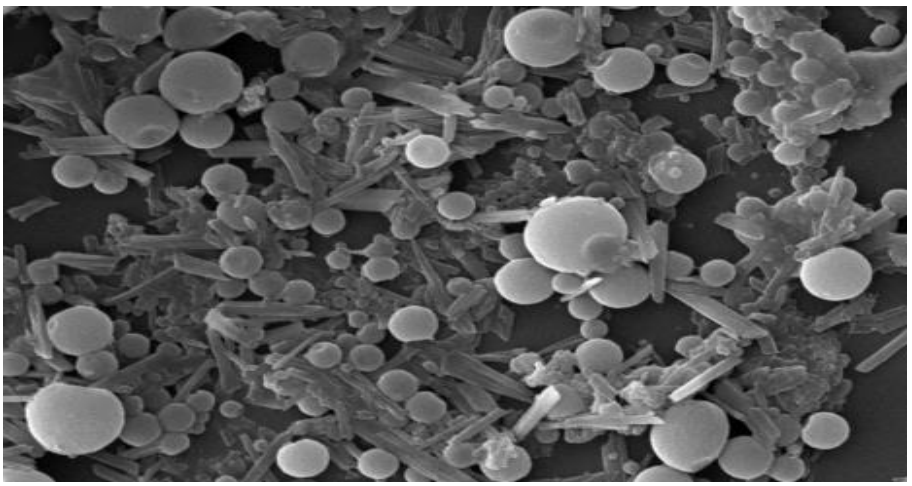

Figure 3: SEM image of quercetin-loaded PLGA nanoparticles with a spherical morphology

Entrapment Efficiency (EE):

Based on the formulation parameters, the Entrapment Efficiency (EE) of quercetin was determined by UV-Vis spectrophotometric analysis, and the values varied from 82.2 ± 1.7% to 90.3 ± 2.0%. The fact that the nanoparticles retained a significant amount of quercetin indicates that their encapsulation was effective. Based on the formulation settings, the quercetin encapsulation ranged from 82.2% to 90.3%, as shown in table 2, which summarizes the Entrapment Efficiency (EE) values.

In-Vitro Drug Release:

Using a dialysis technique in PBS (pH 7.4) at 37°C, the in vitro release of quercetin from the nanoparticles was investigated. According to the statistics on cumulative release, the medication is delivered in a sustained release profile over the course of 48 hours, with an approximate release rate of 80-85%. The therapeutic benefit can be prolonged with minimal negative effects thanks to this steady release. Table 3 demonstrates that the nanoparticles released quercetin in a regulated and sustained fashion over the course of 48 hours. Reduced dosage frequency and sustained therapeutic efficacy are both guaranteed by the drug's progressive release characteristic. Over the course of 48 hours, the cumulative drug release profile

of PLGA nanoparticles loaded with quercetin is shown in Figure 4 and Table 3.

Table 3: In-Vitro Drug Release Profile of Quercetin-Loaded Nanoparticles

Time (hours)	Cumulative Drug Release (%)
0	0.0 ± 0.0
2	18.5 ± 2.1
4	35.2 ± 2.4
8	50.6 ± 2.7
12	65.4 ± 3.0
24	78.9 ± 3.2
36	82.7 ± 2.9
48	85.3 ± 3.1

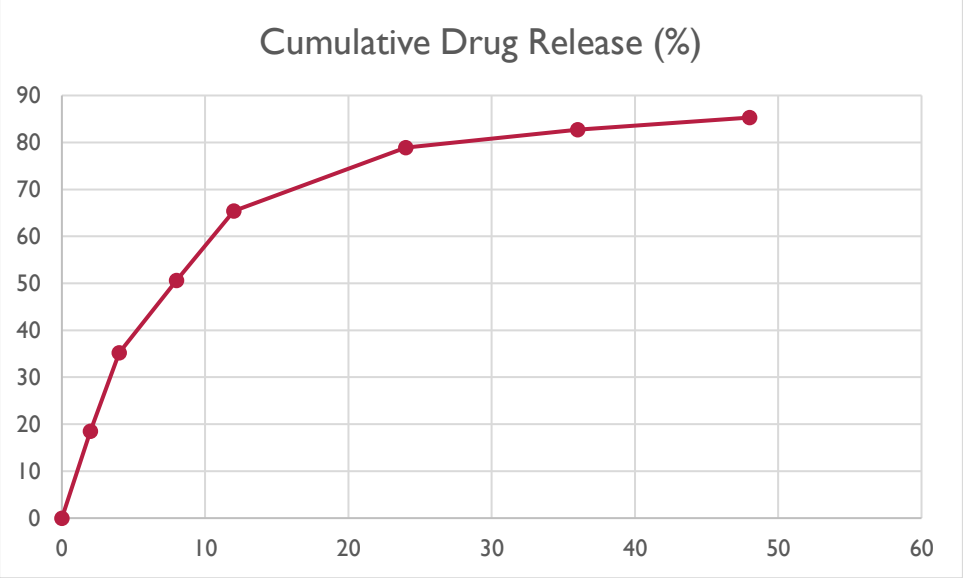

Figure 4: Cumulative drug release profile of quercetin-loaded PLGA nanoparticles over 48 hours.

Cellular Uptake and Cytotoxicity Studies:

Cellular Uptake:

Results from fluorescence microscopy demonstrated that nanoparticles functionalized with folic acid exhibited substantially greater absorption than their non-functionalized counterparts. The accelerated internalization of folate by the HT-29 cells is supported by the higher fluorescence intensity inside the cells, which is a result of receptor-mediated endocytosis aided by the cell surface folate receptors. Table 4 shows that nanoparticles functionalized with folic acid had much stronger fluorescence, suggesting that HT-29 cells were able to take in more of the drug through receptor-mediated endocytosis.

Table 4: Cellular Uptake of Nanoparticles in HT-29 Cells

Sr. No.	Formulation	Relative Fluorescence Intensity (%)
1	Non-Functionalized Nanoparticles	48.2 ± 2.5
2	Folic Acid-Conjugated Nanoparticles	85.6 ± 3.1

Cytotoxicity Assay (MTT Assay):

The MTT assay was used to evaluate the nanoparticles' cytotoxicity. We discovered that the IC_{50} values for free quercetin, non-targeted nanoparticles, and folic acid-conjugated nanoparticles were 12.5 µg/mL, 15.2 µg/mL, and 9.8 µg/mL, respectively. This suggests that the nanoparticles coupled with folic acid were better at reducing cell viability, most likely as a result of increased absorption and better medication delivery to cancer cells. Table 5 shows the results of the MTT assay for cytotoxicity in HT-29 cells when quercetin formulations were used.

Table 5: Cytotoxicity of Quercetin Formulations in HT-29 Cells (MTT Assay)

Sr. No.	Formulation	IC_{50} (µg/mL)
1	Free Quercetin	12.5 ± 0.8
2	Non-Targeted Nanoparticles	15.2 ± 0.6
3	Folic Acid-Conjugated Nanoparticles	9.8 ± 0.5

Apoptosis Assay:

The folic acid-conjugated nanoparticle group exhibited a greater frequency of early and late apoptotic cells in comparison to the free quercetin and non-targeted nanoparticle groups, according to flow cytometry analysis employing Annexin V/PI staining. These results point to a more effective induction of cell death in HT-29 cells by the folic acid-targeted nanoparticles. In Table 6, we can see how various quercetin formulations induce cell death in HT-29 cells.

Table 6: Apoptosis Induction by Different Quercetin Formulations in HT-29 Cells

Formulation	Viable Cells (%)	Early Apoptotic (%)	Late Apoptotic (%)	Necrotic Cells (%)
Control (Untreated Cells)	95.4 ± 1.2	2.1 ± 0.5	1.5 ± 0.4	1.0 ± 0.3
Free Quercetin	75.8 ± 2.1	10.5 ± 1.0	9.2 ± 0.8	4.5 ± 0.6
Non-Targeted Nanoparticles	65.3 ± 1.8	14.8 ± 1.2	15.2 ± 1.3	4.7 ± 0.5
Folic Acid-Conjugated Nanoparticles	48.6 ± 1.7	22.5 ± 1.5	25.4 ± 1.6	3.5 ± 0.4

4. DISCUSSION

Through the use of a Box-Behnken experimental design, the study was able to effectively generate and optimize quercetin-loaded PLGA nanoparticles through the solvent evaporation method. Particle size, entrapment efficiency, and drug release behavior were found to be highly affected by surfactant concentration, stirring speed, and polymer concentration, among other important nanoparticle properties. An optimal particle size range of 185-240 nm was demonstrated by the optimized formulation, which is conducive to improved cellular uptake via endocytosis. Effective medication encapsulation within the polymer matrix was demonstrated by the high entrapment efficiency, which ranged from 82.2% to 90.3%. Important for controlled drug release, scanning electron microscopy also verified the nanoparticles' smooth and spherical shape [24-28].

Results from the in vitro drug release investigation showed a biphasic pattern of release, with an early burst and then continuous release, indicating that quercetin was efficiently retained and that the medication was available for a long time. Nanoparticle uptake in HT-29 colorectal cancer cells was further improved by folic acid conjugation, proving that folate receptor-mediated targeting is efficient. Folic acid-functionalized nanoparticles showed higher anticancer activity than free quercetin and non-targeted nanoparticles, according to cytotoxicity tests utilizing the MTT assay, which showed lower IC_{50} values. The results were subsequently confirmed by flow cytometry, which showed that HT-29 cells treated with folic acid-conjugated nanoparticles experienced a notable increase in cell death [29-32].

Taken together, the study shows that PLGA nanoparticles could be a great way to administer quercetin, since they allow for controlled drug release, targeted cellular uptake, and improved anticancer effectiveness. Based on these encouraging findings, PLGA nanoparticles loaded with folic acid and quercetin may be a good option for treating colorectal cancer. Validation of their effectiveness and therapeutic potential in a biological setting requires additional in vivo investigations [33-40].

5. CONCLUSION

This study utilized the solvent evaporation method and the Box-Behnken experimental design to successfully create and optimize PLGA nanoparticles loaded with quercetin. As a result of its regulated drug release, high entrapment efficiency (82.2-90.3%), and nano-sized particles (185-240 nm), the improved formulation showed great promise as a delivery system for the treatment of colorectal cancer. Nanoparticle absorption in HT-29 cells was much improved by folic acid conjugation, proving that folate receptor-mediated targeting is effective. Results from cytotoxicity tests showed that nanoparticles functionalized with folic acid had a higher anticancer activity (as measured by lower IC_{50} values) than free quercetin and non-targeted nanoparticles. These nanoparticles killed cancer cells primarily through inducing apoptosis, according to flow cytometry research. The results point to quercetin nanoparticles based on PLGA as a potential method for treating colorectal cancer specifically, especially when combined with folic acid. The therapeutic effectiveness and translational potential of these compounds should be further investigated through in vivo research.

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None

Conflict of Interest:

None

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