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Original Article

Quercetin-loaded Liposomes Effectively Induced Apoptosis and Decreased the Epidermal Growth Factor Receptor Expression in Colorectal Cancer Cells: An *In Vitro* Study

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What's Known

- Quercetin has good pharmaceutical properties; however, its bio-medical applications are constrained by factors such as insufficient bioavailability, poor solubility, and low permeability.
- The epidermal growth factor receptor, which is involved in the cell signaling pathway of metastasis, is increasingly expressed in colorectal cancer cells.

What's New

- Loading more quercetin on nanoliposomes could enhance its toxicity, mostly by inducing apoptosis against colon cancer cells.
- Quercetin-loaded nanoliposomes significantly decreased the epidermal growth factor receptor gene expression, indicating the effectiveness of this compound in inhibiting gene expression and cancer development.

Abstract

Background: Quercetin is a flavonoid having anti-cancer properties; however, it has low stability, insufficient bioavailability, and poor solubility. This study aimed to load quercetin on nanoliposomes to enhance its efficiency against SW48 colorectal cancer cells. The cytotoxicity of free-quercetin and quercetin-loaded nanoliposomes on the expression of the epidermal growth factor receptor (EGER) gene was investigated.

Methods: This present *in vitro* study was conducted at Yasuj University of Medical Sciences (Yasuj, Iran) in 2021. In this *in vitro* study, the lipid thin-film hydration method was used to synthesize quercetin-loaded liposomes. Additionally, high-performance liquid chromatography (HPLC) analyses, dynamic light scattering (DLS), and transmission electron microscopy (TEM) investigations were used to characterize nanomaterials. Following that, MTT, flow cytometry, and real-time PCR were used to investigate the cytotoxicity of quercetin-loaded liposomes on the colorectal cancer cells SW48 cell line, the incidence of apoptosis, and the expression of the EGFR gene in these cells. Statistical analysis was performed using the SPSS (version 26.0), and the graphs were created with the GraphPad Prism version 8.4.3. $P<0.05$ was considered statistically significant.

Results: The nanoparticles were spherical, homogenous, and 150±10 nm in size. According to HPLC, Quercetin had a 98% loading capacity. Although both free quercetin and quercetin-loaded liposomes indicated significant cytotoxicity against cancer cells ($P<0.001$), the combined form was significantly more active ($P=0.008$). 50 µg/mL of this compound reduced the viability of SW48 cells by more than 80% (IC_{50}: 10.65 µg/mL), while the viability of cells treated with free quercetin was only 66% (IC_{50}: 18.74 µg/mL). The apoptosis was nearly doubled in the cells treated with quercetin-loaded nanoliposomes compared to free quercetin (54.8% versus 27.6%). EGFR gene expression, on the other hand, was significantly lower in cells treated with quercetin-loaded liposomes than the quercetin alone ($P=0.006$).

Conclusion: When combined with nanoliposomes, quercetin had greater anti-proliferative, apoptotic, and anti-EGFR expression than free quercetin.

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Keywords ● Neoplasms ● Quercetin ● Colorectal neoplasms ● ErbB receptors ● Lipids ● Nanoparticles

Introduction

Colorectal cancer (CRC) is the second deadliest cancer and the third most common malignant tumor worldwide, accounting for approximately 10% of new cancer and death cases.[1] The number of new CRC cases in developing countries is expected to reach 2.5 million by 2035.[2] According to reports, in about 25% of patients, cancer had metastases at the time of diagnosis. Moreover, about 50% of patients will face developing metastases during their lifetime. Furthermore, the five-year survival rate in individuals with metastatic disease was about 14%.[3] Despite significant advances in cancer treatment methods, there is still no certain treatment for CRC, especially in patients with liver metastasis, a common type of distant metastasis in CRC and considered a major cause of death in these patients.[4]

The epidermal growth factor receptor (EGFR), which is involved in cell proliferation, apoptosis, and angiogenesis, is crucial to the metastasis cell signaling pathway. EGFR belongs to the ErbB family and receptor tyrosine kinases (RTKs), which regulate the growth and homeostasis of epithelial tissues. Most malignancies have point mutations or activity intensification in the genomic locus of this receptor.[5, 6] EGFR-inducing mutations and methods promote pro-oncogenic signaling pathways such as RAS-RAF-MEK-ERK MAPK and AKT-PI3K-mTOR. These pathways, subsequently, stimulate cancer cell proliferation.[7] Previous studies showed that this growth receptor is overexpressed in a variety of malignancies, including glioblastoma, cervical cancer, and breast cancer.[8, 9] In addition, anti-EGFR monoclonal antibodies are used in the clinical phase for various cancers, including CRC. Although antibodies had a good therapeutic effect, drug resistance was simultaneously established.[10]

Given the ever-increasing emergence of various drug resistance, natural substances for treating diseases have recently received a lot of attention. In this regard, phytocompounds were indicated to be more accessible and effective.[11, 12] Flavonoids, a type of polyphenolic secondary metabolites, that are prevalent in fruits, soy, and vegetables showed significant anti-inflammatory and antimicrobial activities.[13] These compounds inhibit a variety of signaling pathways involved in apoptosis, cell cycle arrest, mitogen-activated protein kinase (MAPK), (PI3K/AKT) kinase, adhesion, migration, and metastasis.[14] Quercetin is a well-known flavonoid that was found to have anti-tumor properties in both *in vivo* and *in vitro* research studies.[15]

The scientific communities have recently welcomed nanotechnology as an emerging field in the diagnosis and treatment of cancer. In this regard, controlled drug release and encapsulation are just two great applications of nanoliposome technology for cancer therapy.[16, 17] Liposomes were approved as suitable drug carriers due to properties such as biocompatibility, biodegradability, and their unique capacity to enclose the drug in both hydrophilic (within the aqueous nucleus) and hydrophobic (within the layers) sections.[18]

This study aimed to enhance the cytotoxicity of quercetin against SW48 colorectal cancer cells by loading it on nanoliposomes and evaluating the effect of free quercetin and quercetin-loaded nanoliposomes on the EGFR gene expression. Accordingly, quercetin-loaded nanoliposomes were synthesized and characterized. Then, the activity of these compounds was tested against the colorectal cancer-derived SW48 cell line using the MTT assay. Additionally, real-time PCR and flow cytometry were used to assess the incidence of apoptosis and the expression of the EGFR gene in cells exposed to nanoliposomes and free quercetin.

Materials and Methods

This *in vitro* study was conducted at Yasuj University of Medical Sciences (Yasuj, Iran) in 2019. This study was approved by the Ethics Committee of Yasuj University of Medical Sciences (IR.YUMS.REC.1400.020).

Cell Culture

SW48 cell lines were purchased from Pasteur Institute of Iran (Tehran, Iran). All cell lines were cultured in RPMI-1640 medium with 10% FBS and 1% antibiotic (Penicillin-Streptomycin; 5,000 U/mL). The cells were incubated at 37 °C in 5% CO_2 and 90-95% humidity.

Nanoliposomes Synthesis

Quercetin-loaded nanoliposomes were prepared using the lipid thin-film hydration method, as described previously.[19] First, 5 mL of chloroform was used to dissolve the lyophilized phospholipids (cholesterol and Distearoylphosphatidylcholine), and quercetin (molar ratio: 3, 7, and 2, respectively). The obtained mixture was then transferred to a round bottom flask, and the organic solvent was extracted under vacuum conditions using a rotary evaporator at 40 °C. The lipid film formed at the bottom of the flask was then gradually hydrated using phosphate buffer (PBS). Following liposome synthesis, fat chains were inserted in the lipid layer and hydrophilic part on the surface of the liposome.

Characterizations

A High-performance liquid chromatography (HPLC; KNAUER, Germany) system was used to assess the loading capacity of quercetin on liposomes. Furthermore, the size of nanoparticles was determined using the dynamic light scattering (DLS) technique (HORIBA Ltd., Japan).[20] The morphology and size of the Quercetin-loaded nanoliposomes were investigated using a transmission electron microscope (TEM; CM30, Philips).

Biological Activities Assessments

Cytotoxicity: As previously mentioned, the MTT assay was performed to determine the cytotoxicity of the nano-compound against SW48 cells.[21]

In a 96-well plate containing 100 µL of RPMI medium, 5000 cells per well were cultured and incubated for 24 hours. Following that, different concentrations of quercetin-loaded nanoliposomes and free quercetin (3–50 µg/mL) were mixed with 100 µL of culture medium, added to each well, and incubated for 24 hours. Then, 20 µL of MTT solution (5 mg/L; Sigma, Germany) was added to each well and incubated for four hours. Afterward, the wells' medium was discarded, 100 µL of dimethyl sulfoxide (DMSO) was replaced, and then the cell culture plate was incubated in the dark for 40 minutes. Finally, a microplate reader was used to measure the absorbance of each well at 570 nm (Bio-Tek Instruments Inc., Vermont, USA). Each assay was performed with three repeats. The untreated cells were also used as a control.

Flow Cytometry: To compare the incidence of apoptosis in cells treated with quercetin-loaded nanoliposomes to untreated cells, cells were stained with fluorochrome dye of Annexin and propidium iodide (PI), according to the instructions of the FITC Annexin V Apoptosis Detection Kit (BD biosciences, USA), and studied using a flow cytometry device. In this regard, the cells were cultured on a six-well culture plate and treated for 24 hours with IC_{50} concentration of quercetin-loaded nanoliposomes and free quercetin. The control group received no treatment. Cells were then collected and transferred to the falcon tube containing fresh medium and centrifuged at 300 ×g for five minutes. The precipitate was re-suspended in 1 mL PBS on ice. The cells were once again suspended in 5 mL of binding buffer. The mixture was then incubated in the dark for five minutes at room temperature with 5 mL of Annexin V-FITC and 5 mL of Propidium Iodide. The stained cells were then analyzed using a Becton Dickinson FACS Calibur flow cytometer (BD Company, USA). Besides, the FITC signal detectors (BD biosciences, USA) were used to detect Annexin V-FITC binding, and the Phycoerythrin emission signal detector was used to quantify PI binding.

EGFR Gene Expression: The real-time PCR technique was used to assess EGFR gene expression in the cells treated with free quercetin and quercetin-loaded nanoliposomes.[22] Briefly, the treated cells were detached from the culture plates, and after rinsing with PBS, RNAs were isolated from the cells using an RNA extraction kit (Behgene, Iran). Then, using a DNA synthesis kit (Takara, Japan), cDNA was prepared from mRNA. The EGFR gene expression was measured using the SYBR® Green method in real-time PCR technique with specific primers.[23] The following real-time PCR program was used: initial denaturation at 95 ˚C for 15 minutes, followed by 40 cycles, including the denaturation phase (20 second, 95 ˚C), annealing phase (30 seconds, 60 ˚C), and elongation (30 seconds, 72 ˚C). The HPRT1 gene was used as a housekeeping gene. The sequence of primers as well as additional information are presented in table 1.

Statistical Analysis

All experiments were carried out with three replicates, and results were shown as mean±SD. All statistical analyses were performed with the statistical package for social sciences, version 26.0 (IBM Software Group's Business Analytics Portfolio) and GraphPad Prism version 8.4.3 (GraphPad Software Inc., California, USA). The one-way ANOVA and unpaired *t* test were used to analyze the relationship. $P<0.05$ was considered statistically significant.

Results

Physicochemical Characteristics of Quercetin-loaded Nanoliposomes

Based on the TEM analysis results, the nanoliposomes were successfully synthesized

Table 1: Sequence of Primers used for analyzing the EGFR and HPRT1 genes expression

Primers	Sequences
HPRT1	Forward: 5′-GCCCTGGCGTCGTGATTAG-3′ Reverse: 5′-TCGAGCAAGACGTTCAGTCC-3′
EGFR	Forward: 5´-CATGAGAAGTATGACAACAGCCT-3' Reverse: 5´-AGTCCTTCCACGATACCAAAGT-3'

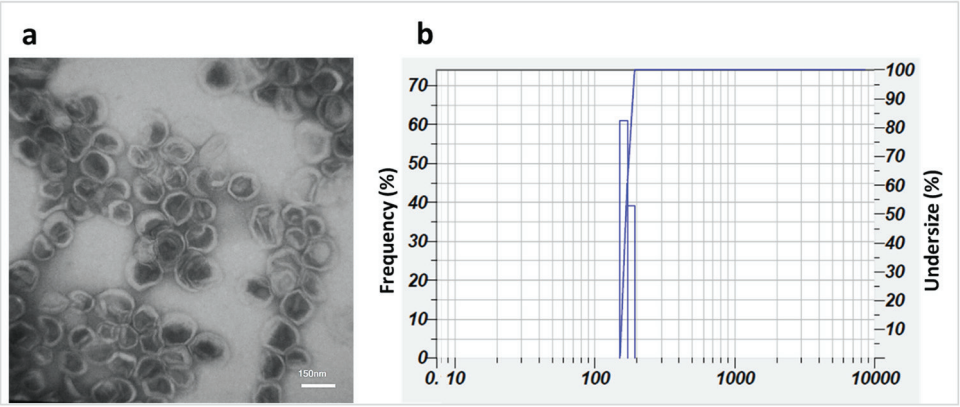

Figure 1: This figure represents the TEM (a) and DLS (b) analysis of quercetin-loaded nanoliposomes. Both analyses show a uniform size of 150±10 nm for these compounds.

and had a spherical and uniform shape measuring 150±10 nm in diameter (figure 1a). These results were compatible with DLS analysis, which indicated that these particles had an average size of 167 nm (figure 1b). Concerning the quercetin loading capacity, the HPLC technique indicated that the drug loading rate on nanoliposomes exceeded 98%.

Cytotoxicity Assessment

As seen in figure 2, there is a significant difference in cytotoxicity between free quercetin and its nanoliposomes-loaded form. At a concentration of 50 µg/mL, the nano-compound inhibited the proliferation of SW48 cells by more than 80%. Moreover, an IC_{50} of 10.65 µg/mL was determined for this compound. Free quercetin significantly reduced cytotoxic activity. With an IC_{50} of 18.74 µg/mL, this had the potential to impair the cell viability by 65%.

Apoptotic Cells Detection

The flow cytometry analysis revealed that both quercetin and quercetin-loaded on nanoliposomes significantly induced apoptosis in SW48 cells (figures 3 and 4). In the cells treated with quercetin-loaded on nanoliposomes, the percentage of early apoptosis increased from 16% in cells exposed to quercetin at equal concentrations to 44%. The proportion of late apoptosis in both treatments was about 11%. For these compounds, the sum of primary and late apoptosis was 27.6% and 54.8%, respectively. These results were in agreement with the MTT results, indicating that the nano compounds could enhance quercetin cytotoxicity. Notably, there was no evidence of necrosis in the cells.

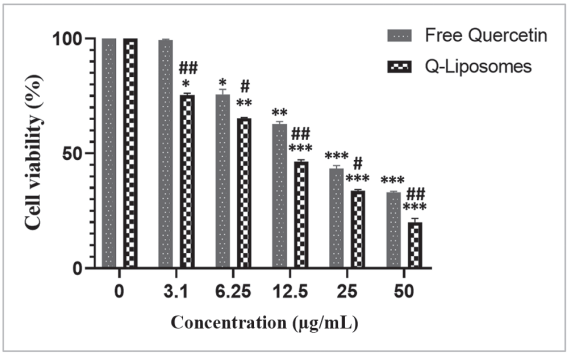

Figure 2: This chart compares the viability of SW48 cells treated with free quercetin and quercetin-loaded on nano-liposomes after 24 hours. *, **, and ***Indicate significant differences between the treatment groups and the control (0 µg/mL) with P=0.035, P=0.006, and P<0.001, respectively. # and ##Show the significant difference between quercetin-loaded nanoliposomes and free quercetin (P=0.041 and P=0.008, respectively).

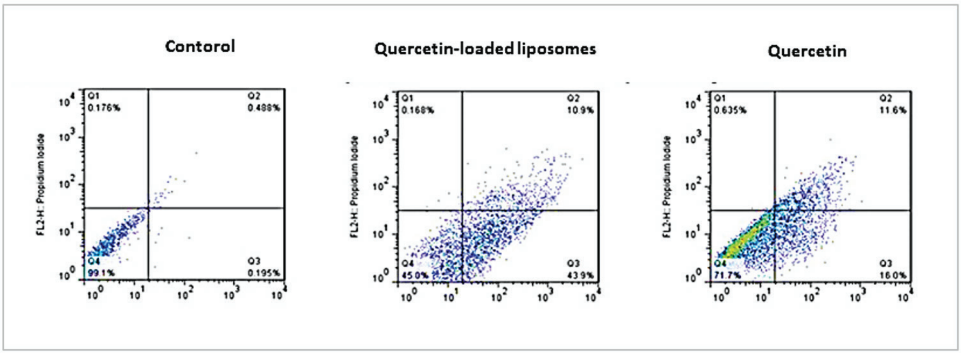

Figure 3: The flow cytometry analysis reveals the percentage of apoptosis induced in SW48 cells exposed to the quercetin and quercetin-loaded nanoliposomes. Q4 represents the percentage of viable cells, Q3 shows the percentage of early apoptosis, Q2 indicates the percentage of cells in the late stage of apoptosis/dead, and Q1 shows the necrotic cells.

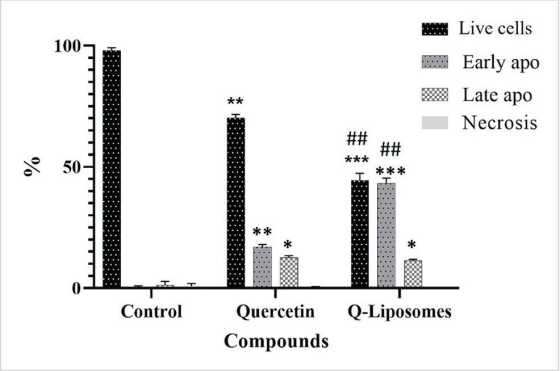

Figure 4: The chart compares the percentage of live and apoptotic SW48 cells treated with quercetin and quercetin-loaded liposomes. *, **, and *** Represent significant differences between the treatment groups and the control group (P=0.027, P=0.004, and P<0.001, respectively). ##Shows the significant difference between quercetin-loaded nanoliposomes and free quercetin (P=0.005).

EGFR Gene Expression

The real-time PCR analysis of the results revealed that quercetin and quercetin-loaded on nanoliposomes reduced the expression of the EGFR gene in SW48 cells (figure 5). There was also a significant difference between the activity of the free drug and the drug-loaded nanoliposomes. When compared to the control, the quercetin-loaded on nanoliposomes reduced the expression of the gene by about 60%, while the free drug reduced this feature about 44%.

Discussion

The purpose of this study was to use a nanoliposome-based drug delivery system to boost the quercetin efficiency against SW48 colorectal cancer cells. The findings revealed that loading the quercetin on nanoliposomes significantly increased the toxicity of this drug against CRC cells. Besides, we found that the quercetin-loaded on nanoliposomes significantly induced apoptosis in cancer cells and decreased the expression of the EGFR gene, which plays a crucial role in tumor development.

Various molecular mechanisms are involved in the development and progression of CRC, one of the most common causes of cancer death. Although metastatic cancers, particularly CRC, frequently overexpress the epidermal growth factor receptor (EGFR), metastatic cancers gradually become resistant to anti-EGFR therapies,[10] indicating the need for more effective approaches. According to the findings of the present research, quercetin significantly reduced the EGFR gene expression and induced apoptosis in CRC cancer cells (SW48). It is noteworthy that the activity of this drug was significantly increased by loading it onto liposomes.

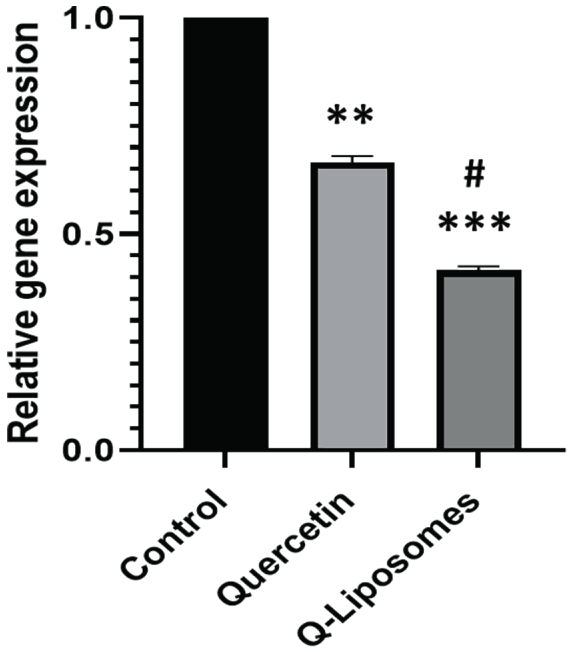

Figure 5: This figure represents the relative EGFR gene expression in the SW48 cell line under free quercetin and quercetin-loaded nanoliposomes (Q-Liposomes). The expression of the EGFR gene in the presence of these compounds is considerably reduced, causing inhibition of metastasis in these cells. ** and *** Represent significant differences between the treatment groups and the control (P=0.005 and P<0.001, respectively). #Shows the significant difference between quercetin-loaded nanoliposomes and free quercetin (P=0.006).

In addition to activating the signaling pathways, such as MAPK cascades downstream of EGFR, Notch, PI3K/AKT, Wnt, and transforming growth factor β (TGF-β), tumor cells can benefit from the increase in PD-L1 levels to escape and inhibit $CD8^+$ T cells. Cheng and others showed that EGF potentiates PD-L1 by increasing signal transducer and activator of transcription1 (STAT1) protein levels to amplify the IFNr-JAK1/2-mediated signaling axis in $EGFR^+$ cancers. Inhibiting EGFR significantly reduced Programmed death-ligand 1 (PD-L1) expression and might be a potential strategy for increasing therapeutic efficacy.[24]

The primary flavonoid, quercetin, was shown to have anti-cancer and anti-inflammatory properties by regulating the pathways involved in the proliferation and apoptosis of cancer cells.[25, 26] Quercetin was indicated to inhibit cell growth and induce apoptosis in human lung cancer cell lines by targeting receptor tyrosine kinases (RTKs), such as EGFR.[27] Another study on nasopharyngeal cancer (NPC) found that quercetin treatment significantly reduced the survival rate of NPC039 cells. Quercetin significantly reduced VEGF and NF-κB genes expression.[28] A recent study also suggested that quercetin inhibited the invasion and migration

of H-Ras-induced MCF10A human epithelial cells by targeting the protein kinase PI3K.[29] The anticancer activity of quercetin might be related to its antioxidant properties.

Despite its good pharmaceutical properties, bio-medical applications of quercetin face some limitations, including low stability, insufficient bioavailability, poor solubility, and poor permeability.[30] Recently, some nanotechnology-based techniques, such as using drug delivery systems, have been proposed to increase the efficiency, solubility, and bioavailability of compounds.

In this study, we investigated quercetin activity against CRC cells in both its free form and when loaded on nanoliposomes. In general, it was found that adding liposomes to this anticancer agent enhanced its cytotoxicity, doubled its ability for apoptosis induction, and increased its activity in decreasing the EGFR gene expression in cancer cells. These improvements can be attributed to liposome functions. Liposomes can contain both hydrophilic and lipophilic molecules, owing to their aqueous core and lipid bilayer. In this way, they can increase the solubility of the compounds. The advantages of using liposomes as drug carriers are as follows:[31] i) inhibition of the drug's chemical and biological degradation during storage and administration; ii) improvement of drug efficacy and therapeutic index, subsequently reducing side-effects induced by nonspecific toxicity; iii) facilitation of targeted drug delivery by chemical modifications with specific surface ligands, and iv) biodegradability and eco-friendly synthesis process.

In line with the findings of this study, some recent studies reported similar results. Quercetin and resveratrol were combined to develop a dual drug-loaded nanostructured lipid carrier (NLC) gel, which increased their absorption in the dermal and epidermal layers. According to the findings of this study, NLC gel could be used as a potential carrier to transport quercetin and resveratrol to deeper layers of the skin, making it a promising formula for treating skin cancer.[32] Liu and colleagues demonstrated that the solubility and bioavailability of quercetin in liver tissue were enhanced by liposome nanoparticles. They also showed that the nanoliposome-quercetin compound effectively protected mice from acute liver injury and could be a new therapeutic and protective agent for patients with liver disease.[33] Another study, which aimed to develop new liposomes for the simultaneous delivery of two polyphenols (quercetin and gallic acid) for the treatment of Vulvovaginal candidiasis (caused mostly by *Candida albicans*), found that quercetin and gallic acid worked synergistically and exhibited increased antioxidant and anti-inflammatory effects. Furthermore, while polyphenol-liposomes did not show cytotoxicity, the liposome-quercetin-gallic acid composite significantly inhibited the growth of *C. albicans*.[34] One of the limitations of this study was the lack of facilities and financial resources to measure the release rate of the drug-loaded with nanoliposomes in cells.

Conclusion

The findings showed that combining quercetin with nanoliposomes significantly increased quercetin toxicity against CRC cells. Moreover, quercetin loaded on nanoliposomes significantly induced apoptosis in cancer cells and significantly decreased EGFR gene expression, both of which play an important role in tumor development. It is suggested to perform this method in different cell lines as well as animal models to get more precise results.

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Authors' Contribution

F.K: Substantial contributions to the conception, conducting experiments, analyzing data and article submission, drafting and revising the manuscript; M.D: Conducting experiments, performing real-time PCR, drafting and revising the manuscript; H.B: Statistical analysis and interpretation of experiments and Construction of nanoliposomes, drafting the manuscript; F.Kh and P.N: Performing flow cytometry and analyzing the results, Collaboration in the process of cell culture, and drafting the manuscript; Gh.Gh: The principal designer of the study, analyzing data and drafting and final editing of the manuscript. All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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