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Preparation of novel quercetin-loaded solid lipid nanoparticles: Formulation and evaluation of their anticancer potential on SK-MEL-30 melanoma cell line

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ABSTRACT: Quercetin, a hydrophobic compound with well-documented anticancer activity, presents challenges for therapeutic application due to its water insolubility, thus representing a promising candidate for encapsulation within solid lipid nanoparticles (SLNs) to enhance its therapeutic application. In this study, SLNs were developed using an optimized formulation of Precirol ATO 5, Tween 80, Span 80, and ethanol, using the modified hot microemulsion technique. Quercetin was encapsulated within these nanoparticles, and the particle size, zeta potential, and polydispersity index (PDI) were analyzed to assess the formulation's characteristics and stability. Final formulations were further characterized by electron microscopy. The anticancer efficacy of quercetin-loaded SLNs was evaluated in SK-MEL-30 (melanoma) and HaCaT (keratinocyte) cell lines. Additionally, an in vitro release assay was performed to monitor quercetin release dynamics. The encapsulation efficiency and release profile of quercetin were quantified using high-performance liquid chromatography (HPLC).

KEYWORDS: Drug delivery systems; solid lipid nanoparticles; quercetin; melanoma.

1. INTRODUCTION

Solid lipid nanoparticles (SLNs) are colloidal carrier systems with particle sizes ranging from 50 to 1000 nanometers. They are made up of lipids that remain solid at room and body temperature, surfactants that lower interfacial tension, and water [1-3]. SLNs offer several benefits, such as excellent physical stability, protection of encapsulated drugs from degradation due to external factors, controlled drug release, targeted delivery, high drug transport efficiency, scalability in production, and the absence of toxicity and sterilization issues [1,2]. These characteristics make SLNs suitable for various routes of administration, including parenteral, pulmonary, dermal, and topical applications, and they can be designed to carry both hydrophilic and hydrophobic drugs [4-7].

Depending on the production method of SLNs, three models can be discussed in terms of drug integration [4,5]. These models are as follows: SLN Type I, where the drug is evenly distributed within the lipid core; SLN Type II, with a drug-free lipid core surrounded by a shell containing both lipid and drug; and SLN Type III, where the drug concentration near saturation causes it to precipitate in the core, forming a lipid coating [8].

The hot microemulsion technique was selected for its superior ability to encapsulate hydrophobic compounds like quercetin, offering high drug loading capacity and scalability compared to conventional methods such as cold homogenization or ultrasonication. This method also ensures uniform particle size distribution, which is critical for the stability and bioavailability of the nanoparticles. Furthermore, the scalability of the hot microemulsion method makes it suitable for industrial-scale production [9].

Cancer, a condition marked by the unchecked growth and replication of cells caused by damage to their genetic material from various factors, often evades detection by the immune system. Research has shown that natural products may help prevent cancer development without producing undesirable side effects [10,11].

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Quercetin (QC), one of these natural products, a 3,5,7,3',4'-pentahydroxyflavone and 7-hydroxyflavonol with the chemical formula $C_{15}H_{10}O_7$, is the main representative of the flavonol subclass. Quercetin is a yellow, bitter-tasting crystalline solid that does not dissolve easily. While it is generally insoluble, it shows slight solubility in alcohol and aqueous alkaline solutions [12,13].

It has the potential to aid in the prevention of chronic diseases, while also offering a range of benefits such as anti-inflammatory, antioxidant, antiviral, antidiabetic, antiplatelet, anti-tumor, antihypertensive, cardiovascular protective, immune-modulating, antimicrobial, neuroprotective, antiallergic, and anti-asthmatic properties [14].

One of QC's most significant effects is its ability to inhibit the proliferation of various cancer types [15]. An excess of reactive oxygen species leads to oxidative stress, which damages DNA and encourages mutation formation. QC works to neutralize these reactive species, thereby preventing the DNA damage they would otherwise cause. Moreover, at elevated concentrations, QC induces oxidative stress and cytotoxic effects, harming tumor cells and initiating apoptotic pathways [16].

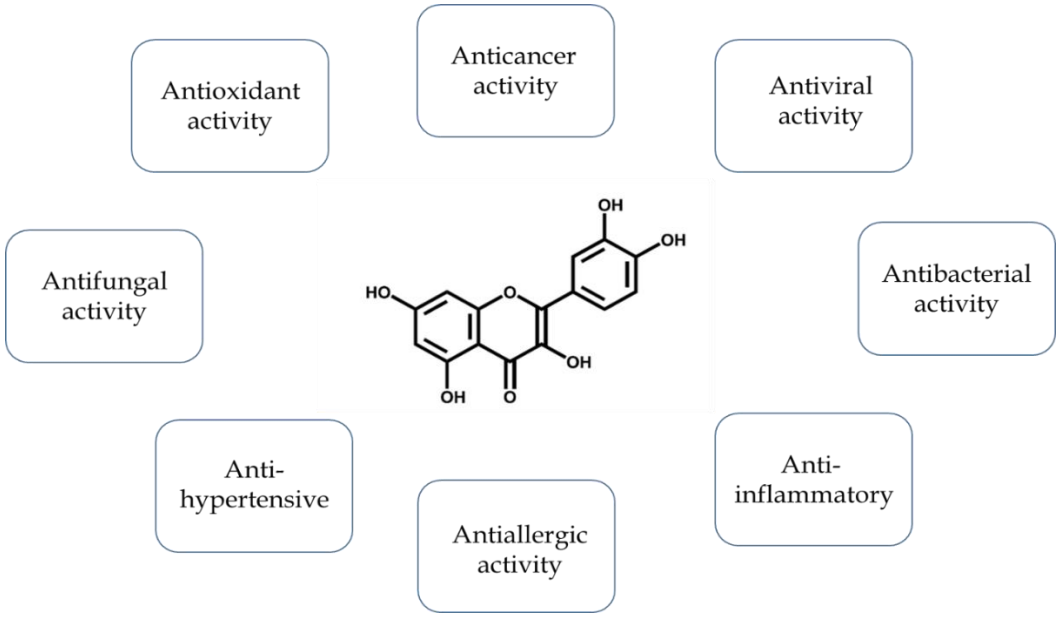

Figure 1. Structure and potential applications of quercetin

QC's low water solubility, rapid excretion from the body, low intrinsic bioactivity, and hydrophobic nature limit its clinical application [14]. To address these limitations, QC-loaded nanoformulations have recently been explored [17]. Among various nanoformulations, solid lipid nanoparticles (SLNs) offer superior biocompatibility, minimal toxicity, efficient transport of lipophilic drugs, cost-effectiveness, and the ability to form stable nanosuspensions over prolonged periods. SLNs also provide protection against external degradation and enable controlled drug release [2,4,14].

Based on this background, the present study investigates the anticancer effect of QC-loaded SLNs prepared via the hot microemulsion method.

2. RESULTS

2.1. Formulation

In this study, Precirol ATO 5 was selected as the solid lipid, with Tween 80 and Span 80 as the surfactants and ethanol as the co-surfactant. The surfactant ratio was optimized at 3:1:2 for Tween 80, Span 80, and ethanol, respectively. Following titration, a point within the green stable microemulsion zone of the triangular phase diagram was selected for the solid lipid nanoparticle (SLN) formation. This region represented a stable formulation zone, resulting in the desired transparent microemulsion, as depicted in Figure 2.

The final formulation, chosen from this phase diagram, consisted of 10% solid lipid, 50% surfactant mixture, and 40% ultrapure water. Quercetin was incorporated by reducing the solid lipid content by 1% and substituting it with quercetin. The physical stability of both the original and quercetin-loaded

formulations was assessed, with stability data shown in Figure 3, indicating robust formulation integrity after one month.

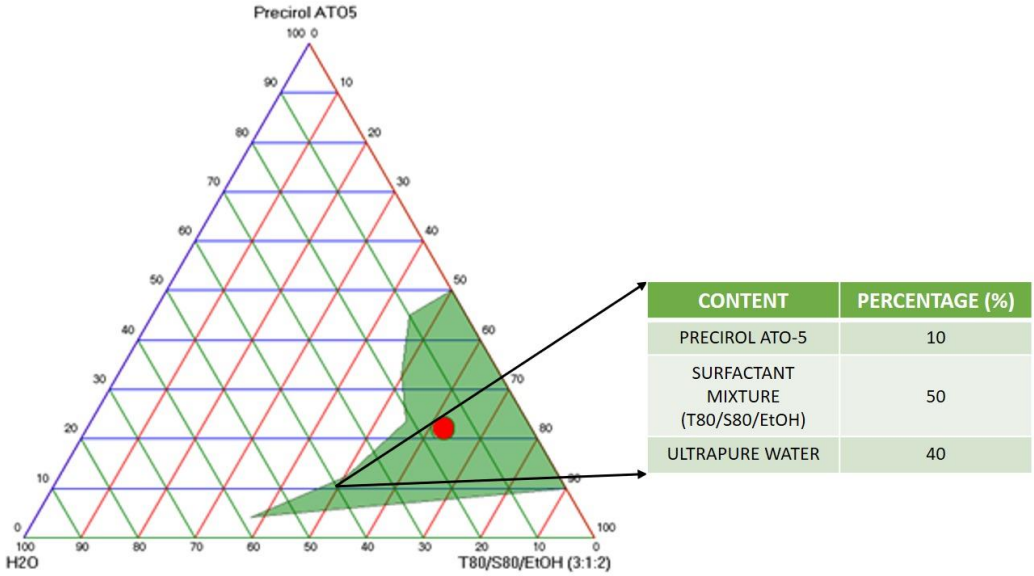

Figure 2. Ternary phase diagram (T80: Tween 80, S80: Span 80, EtOH: Ethanol, H_2O: Water)

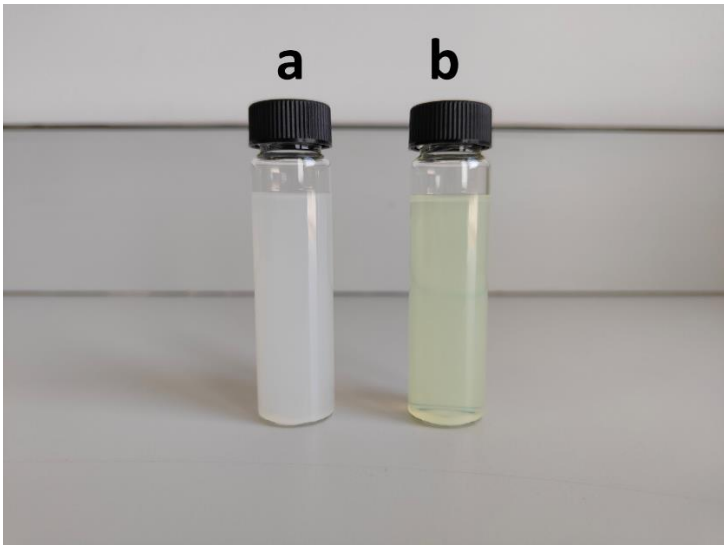

Figure 3. The physical appearance of the formulations
Empty formulation (a) and quercetin loaded formulation (b)

2.2. Characterization

2.2.1. Viscosity and pH measurements

The viscosity measurements revealed that the empty formulation (F) had a viscosity of 0.38 cP, while the quercetin-loaded formulation (Fq) exhibited a slightly higher viscosity of 0.43 cP. This increase in viscosity can be attributed to the incorporation of quercetin, which may influence the internal structure and flow properties of the formulation. Both formulations demonstrated low viscosity values, indicating their suitability for various administration routes, including parenteral and topical applications. The blank formulation exhibited a pH of 6.45 ± 0.04, while quercetin-loaded SLNs showed a slightly acidic shift (6.22 ± 0.02), consistent with quercetin's phenolic proton release.

2.2.2. DLS (Dynamic Light Scattering) measurements

The particle size, polydispersity index (PDI), and zeta potential (ZP) of both the quercetin free formulation (F) and the quercetin-loaded formulation (Fq) were measured using the Malvern Zetasizer

Advanced PRO (Malvern instruments, UK). The DLS measurements taken immediately after the preparation of the formulations and after 6 months of storage at +4 °C are presented in Table 1. The initial measurements show that the quercetin-loaded formulation (Fq) has a smaller particle size (114.9 ± 1.33 nm) and lower polydispersity index (PDI = 0.23) compared to the empty formulation (F), suggesting improved uniformity and stability due to the incorporation of quercetin. After 6 months of storage, both formulations maintained consistent particle sizes and PDIs, with Fq showing a slight increase in size (127.2 ± 3.13 nm) but remaining within a stable range. The zeta potential values for both formulations shifted to around -15 mV after 6 months, indicating a stable colloidal system with sufficient electrostatic repulsion to prevent aggregation. Overall, the results demonstrate that the formulations, particularly Fq, exhibit good physical stability over time under refrigerated conditions.

Table 1. DLS measurement results (PS: Particle Size, PDI: Polydispersity Index, ZP: Zeta Potential)

SAMPLE	PS (nm)	PDI	ZP (mV)	Conductivity (mS/cm)
F	124.4 ± 0.83	0.30	-11.79 ± 0.79	0.005353
Fq	114.9 ± 1.33	0.23	-15.15 ± 0.48	0.005067
F (6 month)	99.5 ±0.44	0.28	-15.2 ± 0,4	0.005242
Fq (6 month)	127.2 ± 3.13	0.30	-14.7 ± 0.3	0.006374

2.2.3. Transmission Electron Microscopy

Transmission electron micrographs were obtained using FEI Tecnai G2 high-contrast transmission electron microscope (FEI Co., USA). Final quercetin loaded formulation photographed and showed in Figure 4. As observed in the TEM image, the particle size corroborates the DLS measurements. Particles have spherical shape also, as can be seen in Figure 4.

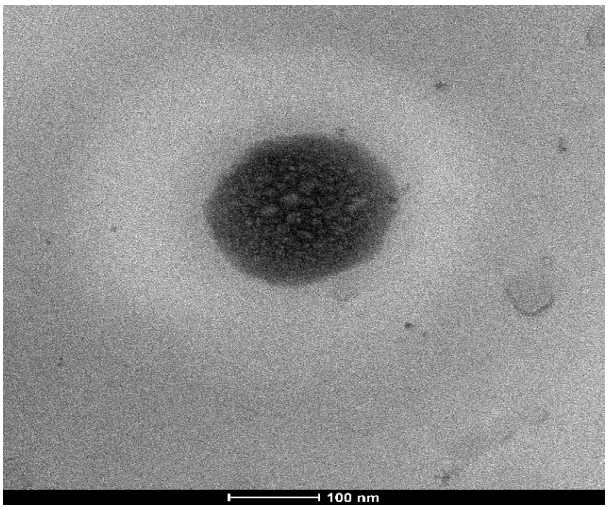

Figure 4. TEM photograph of a Fq particle

2.3. Cytotoxicity

The MTT cytotoxicity assay was performed on SK-MEL-30 melanoma and HaCaT keratinocyte cell lines, with 48-hour results illustrated in Figures 5 and 6. HaCaT cells were used as a non-cancerous control for melanoma cell response comparison. In these figures, doses are presented in µl per well. HaCaT cells exhibited no significant difference between the two groups (p=0.1046). Figure 5 presents the 48-hour MTT cytotoxicity results for SK-MEL-30 melanoma cells treated with the blank formulation (F) and the quercetin-loaded formulation (Fq) at various doses (1, 2, 3, 5, and 8 µL/well). The quercetin-loaded formulation (Fq) consistently exhibited lower cell viability percentages across all doses compared to the blank formulation (F), indicating enhanced cytotoxicity. At the 1 µL/well dose, Fq reduced cell viability to approximately 71%, compared to 103% for F, marking the most notable difference. Similar trends were observed at higher doses, with Fq showing cell viability reductions to 75%, 59%, 71%, and 53.5% for doses of 2, 3, 5, and 8 µL/well, respectively, in contrast to F values of 86.32%, 71.18%, 74.38%, and 65.5%.

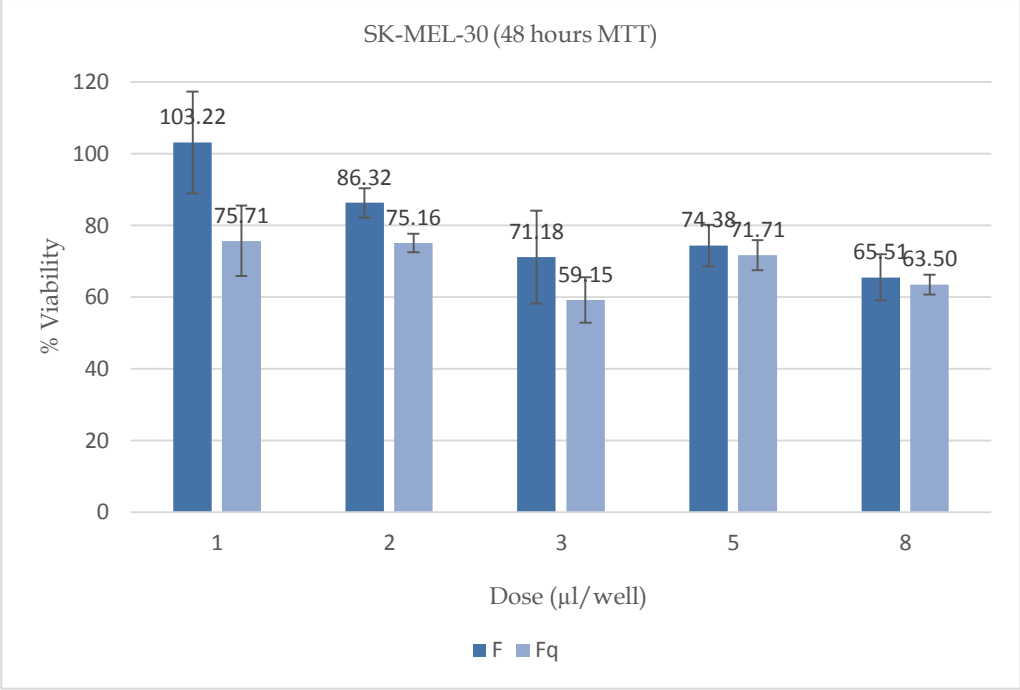

Figure 5. SK-MEL-30 cell line MTT assay

2.4. Encapsulation Efficiency of Quercetin

The encapsulation efficiency of quercetin in SLNs was determined by centrifuging the nanoparticle suspension at 4,000 rpm for 10 min to pellet unencapsulated quercetin. The supernatant containing SLNs was carefully separated, while the pellet was dissolved in ethanol. Encapsulation efficiency was calculated as 90,8%.

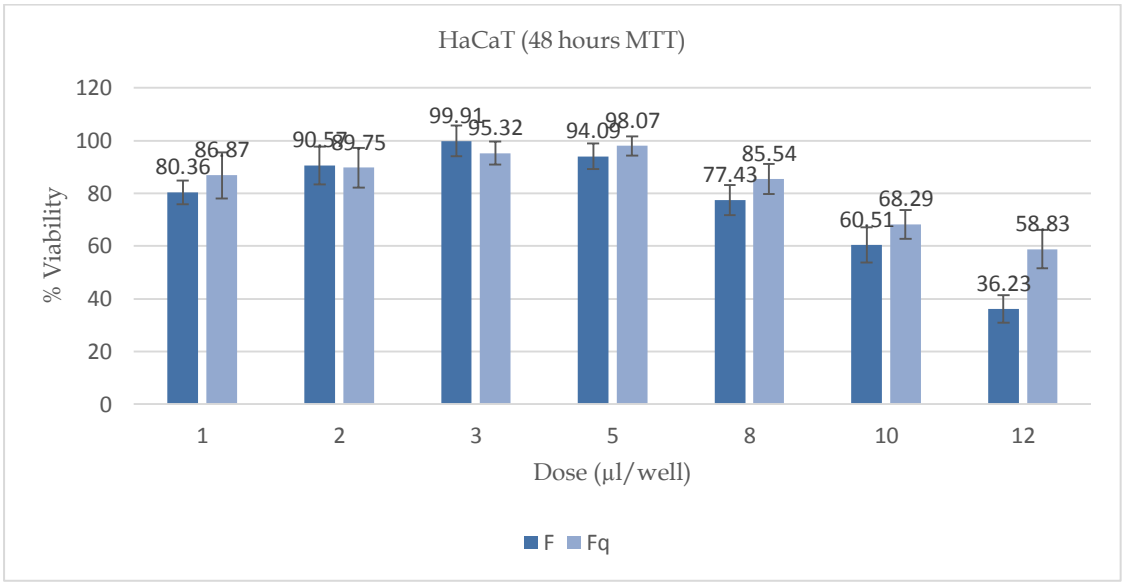

Figure 6. HaCaT cell line MTT assay

2.5. *In vitro* release study

The *in vitro* release study results, presented in Figure 7, demonstrate a sustained and controlled release profile of quercetin from the solid lipid nanoparticles. Release percentages were calculated relative to the total encapsulated quercetin. The in vitro release profile of quercetin from SLNs demonstrated sustained release characteristics (Figure 7). Notably, no detectable release occurred at 0.5 h, likely due to the extremely

low concentration below the HPLC detection limit, confirming effective initial retention of quercetin within the lipid matrix.

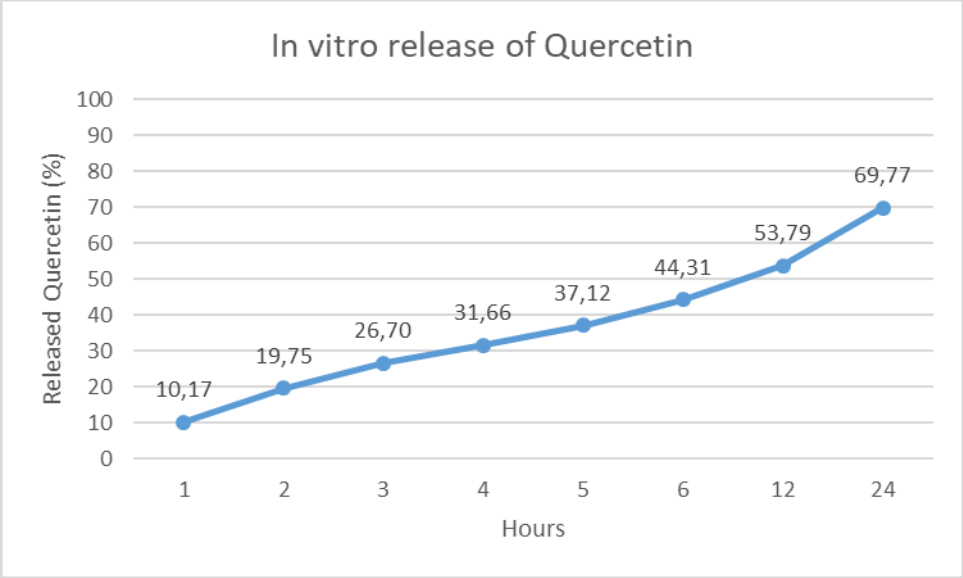

Figure 7. *in vitro* release profile of quercetin from SLNs

3. DISCUSSION

In developing an effective quercetin-loaded solid lipid nanoparticle (SLN) system, careful consideration of lipid and surfactant components was essential to ensure stability, compatibility, and optimal release characteristics. The selection of Precirol ATO 5 as the solid lipid and the combination of Tween 80, Span 80, and ethanol as surfactants and co-surfactant was based on their recognized compatibility and efficiency in forming stable solid lipid nanoparticle (SLN) systems. Precirol ATO 5 is known for its suitable melting point and hydrophobic nature, which aligns well with quercetin's properties, providing an efficient encapsulation matrix. The surfactant mixture was optimized at a 3:1:2 ratio of Tween 80, Span 80, and ethanol, based on empirical studies effectively balancing surface tension reduction and particle stabilization. The final selection of formulation parameters from the green stability region of the phase diagram (Figure 2) suggests that this approach ensured the formation of a stable microemulsion, leading to SLNs with desirable size and stability characteristics [1]. The hot microemulsion method proved to be highly effective in producing uniform and stable nanoparticles, as evidenced by the DLS and TEM results.

Quercetin incorporation, achieved by a slight reduction of the solid lipid content, did not compromise formulation stability, as evidenced by the stability assessment over one month (Figure 3). This outcome confirms that the selected lipid and surfactant ratios effectively stabilize the SLN system, even with the hydrophobic quercetin incorporated. These findings align with prior studies that highlight the role of lipid composition and surfactant ratio in maintaining nanoparticle stability and promoting sustained drug release. The encapsulation of quercetin within SLNs has been shown to significantly improve its bioavailability and stability, as reported in previous studies [14]. The optimized formulation thus presents a promising platform for further evaluation in targeted therapeutic applications.

The viscosity values of 0.38 cP for the empty formulation (F) and 0.43 cP for the quercetin-loaded formulation (Fq) suggest that the incorporation of quercetin slightly alters the rheological properties of the formulation. This increase in viscosity is consistent with previous studies, where the addition of hydrophobic compounds like quercetin has been shown to affect the internal structure of lipid-based nanoparticles [18]. Despite this slight increase, both formulations maintained low viscosity, which is advantageous for ease of administration and ensures uniform distribution upon application. These results further support the potential of the developed formulations for clinical use, particularly in scenarios requiring controlled drug delivery. The observed pH reduction in quercetin-loaded SLNs suggests superficial localization of some quercetin molecules, where phenolic group dissociation may influence surface charge. This aligns with the zeta potential trend and warrants further stability studies under physiological pH gradients.

Dynamic Light Scattering (DLS) analysis was conducted to assess key parameters—average particle size, polydispersity index (PDI), and zeta potential (ZP)—all of which are critical in evaluating the stability and performance of nanoparticle-based drug delivery systems size is particularly significant in colloidal carrier systems, as it influences not only the stability of the suspension but also its interaction with biological barriers and uptake efficiency [19]. In this initial particle size of the solid lipid nanoparticles (SLNs) was measured at 124.4 nm. Upon incorporating quercetin, particle size increased to 114.9 nm, a change of approximately 9.5 nm, indicating that quercetin addition impacted the particle matrix. This size increase, however, remains within a favorable range for SLN stability, demonstrating that the formulation maintained structural integrity and is suitable for sustained delivery applications. Measurements of zeta potential for SLNs offer insights into the storage stability of colloidal dispersions [20]. The zeta potential measurements provided further insights into the formulation's colloidal stability. Generally, ZP values between -30 mV and -60 mV indicate moderate electrostatic stabilization, while values exceeding -60 mV provide stronger stabilization by preventing particle aggregation. In our results, zeta potential for formulation was recorded as -11.79 mV. After quercetin loading, this value decreased to -15.15 mV, reflecting a shift of approximately 3.36 mV toward the negative range. While this change suggests a better electrostatic stability, the particle system remained stable within the experimental conditions, indicating that the formulation is capable of maintaining a stable dispersion over time [21]. The 6-month stability measurements reveal that both the empty (F) and quercetin-loaded (Fq) formulations maintained their particle size (PS) and polydispersity index (PDI) within acceptable ranges, indicating good physical stability over time (Table 1).

Polydispersity index (PDI), also known as the heterogeneity index, represents the degree of non-uniformity in particle size distribution within a sample, providing insights into the range of particle sizes within lipid-based nanocarrier systems. In general, a PDI below 0.05 indicates a highly monodisperse population, while values above 0.7 suggest a broad particle size distribution that may be unsuitable for dynamic light scattering (DLS) analysis due to excessive heterogeneity. In our study, the PDI of the formulation was initially 0.379 ± 0.039, and this decreased slightly to 0.369 ± 0.018 following quercetin loading, reflecting a minor reduction of 0.01. This decrease in PDI indicates a slightly improved homogeneity in particle size distribution, which is within an acceptable range for colloidal stability [22].

Transmission electron microscopy (TEM) provided detailed insights into the morphology and surface characteristics of the quercetin-loaded solid lipid nanoparticles (SLNs). The TEM images confirmed that the particles maintained a spherical shape with a smooth surface, indicative of successful encapsulation and lipid stability. The uniform particle shape observed in the TEM analysis supports the findings from the DLS measurements, suggesting consistency in particle size and distribution. These morphological features are essential for ensuring the SLNs' stability and performance in drug delivery applications, as they facilitate effective interaction with biological membranes.

The MTT assay results demonstrate the enhanced cytotoxic potential of quercetin-loaded solid lipid nanoparticles (Fq) against SK-MEL-30 melanoma cells in comparison to the blank formulation (F). The significant reduction ($p<0.05$) in cell viability observed with Fq, especially at the 1 µL/well dose, suggests that quercetin incorporation effectively boosts the formulation's anticancer properties. This can likely be attributed to quercetin's known pro-apoptotic and anti-proliferative effects on melanoma cells. Statistical analysis using Student's t-test revealed significant differences at various doses: 1 µL/well ($p < 0.001$), 2 µL/well ($p < 0.001$), 3 µL/well ($p < 0.05$), and 8 µL/well ($p < 0.001$). No significant difference was observed at the 5 µL/well dose ($p > 0.05$). These results indicate that the quercetin-loaded formulation exhibits dose-dependent cytotoxicity against SK-MEL-30 melanoma cells. Overall, these findings support the potential of quercetin-loaded SLNs as an effective strategy for targeted melanoma therapy, highlighting the formulation's promise for clinical applications.

The MTT cytotoxicity assay, which evaluates mitochondrial activity as an indicator of cell viability, provided clear evidence of the selective anticancer efficacy of quercetin-loaded solid lipid nanoparticles (SLNs) on SK-MEL-30 melanoma cells. In our study, a dose of 1 µL per well of the blank formulation (F) did not impact cell viability, while the quercetin-loaded formulation (Fq) at this same dose reduced viability to 75%, representing the most pronounced difference between F and Fq. This decrease in cell viability was consistent across all doses for Fq, highlighting the potent anticancer effect of quercetin within the SLN formulation. Conversely, no cytotoxic effect was observed on HaCaT cells, our non-cancerous control, demonstrating the formulation's selective cytotoxicity. These results support the potential of quercetin-loaded SLNs as a targeted therapeutic strategy for melanoma, emphasizing their capability to differentiate between cancerous and non-cancerous cells [23].

The high encapsulation efficiency of quercetin into solid lipid nanoparticles (SLNs), achieving 90.8%, suggests a successful loading process. This outcome indicates the potential of SLNs as an effective delivery

system for quercetin, which could enhance its bioavailability and therapeutic efficacy. The *in vitro* release profile of quercetin-loaded SLNs exhibited a characteristic controlled release behavior (Figure 7). No release was detected in the initial half-hour, indicating a very low concentration release below the limits of analysis. This observation confirms that the formulation effectively retained quercetin initially. The release profile indicates that the lipid matrix maintained its integrity during the initial hours, modulating quercetin release in a controlled manner. These findings support the potential use of the developed SLN formulation as a suitable controlled release system for quercetin. Especially in dynamic environments like the gastrointestinal system, such biphasic release profiles can provide advantages in terms of both rapid onset action and sustained therapeutic concentrations.

4. CONCLUSION

In this study, we successfully formulated quercetin-loaded solid lipid nanoparticles (SLNs) with an optimized combination of Precirol ATO 5, Tween 80, Span 80, and ethanol using the hot microemulsion technique. The SLNs demonstrated a suitable particle size, stable zeta potential, and acceptable polydispersity index, indicating high stability and uniformity of the formulations. Electron microscopy confirmed the spherical morphology and well-defined structure of the nanoparticles, supporting their potential for drug delivery applications.

In this study, quercetin-loaded solid lipid nanoparticles exhibited significant anticancer activity in SK-MEL-30 melanoma cells, while showing no significant cytotoxic effects in HaCaT cells, which served as our non-cancer control. This selectivity proves that the potential of quercetin-loaded SLNs for safe and targeted anticancer therapy. The in vitro release assay demonstrated a sustained release profile, making these formulations a suitable option for controlled quercetin delivery.

However, further in vivo studies and long-term stability assessments are needed to confirm the efficacy and safety of quercetin-loaded SLNs in clinical settings. Future research should also explore optimizing formulation parameters to maximize encapsulation efficiency and therapeutic outcomes. Overall, this work supports the use of SLNs as a promising platform for the delivery of hydrophobic anticancer agents like quercetin. Future studies should focus on in vivo evaluation of the developed formulation, as suggested by Pérez-Herrero and Fernández-Medarde (2015), to confirm its efficacy and safety in clinical settings [11].

5. MATERIALS AND METHODS

5.1. Materials

The materials used in the formulation of solid lipid nanoparticles included Precirol ATO 5 (Gattefossé), Tween 80 (Sigma-Aldrich), and Span 80 (Fluka). Absolute ethanol (Isolab) was employed as a co-surfactant. Quercetin (Sigma-Aldrich) was incorporated as the active ingredient. PBS tablets purchased from bioshop. MTT was provided from Carl Roth. HPLC grade methanol and phosphoric acid (%85 v/v) were provided from Sigma-Aldrich.

5.2. Methods

The hot microemulsion method was used to obtain solid lipid nanoparticles [24]. 10 mg quercetin was dispersed into the solid lipid nanoparticles while the formulation process.

5.2.1. Preparation of Solid Lipid Nanoparticles Using the Hot Microemulsion Method

Solid lipid nanoparticles were prepared using the hot microemulsion technique, with Precirol ATO 5 serving as the solid lipid component. The formulation mixture, comprising surfactants and lipids, was heated to a temperature at least 10°C above the melting point of the solid lipid (70°C for Precirol ATO 5) and maintained in a constant-temperature water jacket, as depicted in Figure 8. Following heating, the mixture was titrated with ultrapure water to construct a ternary phase diagram (Figure 2).

In this diagram, the green region represents the stable microemulsion zone. A specific point within this W/O (water in oil) microemulsion region was selected, and the hot microemulsion mixture was promptly added to 20 mL of cold water (4°C), resulting in the formation of solid lipid nanoparticles.

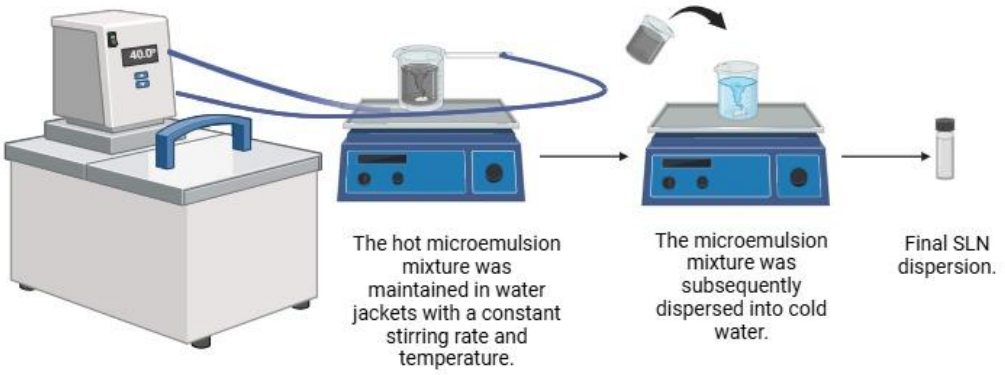

Figure 8. Schematic representation of hot microemulsion method

5.2.2. Characterization of formulations

Viscosity and pH measurements

The viscosity of the formulations was determined using Vibro Viscometer SV-10 (A&D Company, Japan). The pH of blank and quercetin-loaded SLN dispersions was measured in triplicate using a calibrated Mettler Toledo SevenCompact pH meter at 25°C. Measurements were conducted at 25 °C to ensure consistency with standard experimental conditions. Each formulation was analyzed in triplicate, and the average viscosity and pH values were calculated to ensure reproducibility and accuracy.

DLS (Dynamic Light Scattering) measurements

Dynamic Light Scattering (DLS) measurements were performed using a Malvern Zetasizer Advanced PRO (Malvern instruments, UK). The scattering angle was set to 173° for particle size measurements. Zeta cuvettes were employed for zeta potential measurements, and the zeta potential was calculated using the Smoluchowski equation through the instrument's software. Three independent measurements were conducted for each sample.

TEM (Transmission Electron Microscopy) imaging

The morphological characterization of the formulations was conducted using a FEI Tecnai G2 high-contrast transmission electron microscope (FEI Co., USA). Approximately 10 µL of each sample was deposited onto carbon-coated 200-mesh copper grids and allowed to air-dry overnight at ambient temperature. Imaging was performed at an accelerating voltage of 120 kV.

5.2.3. Cell Culture

Cytotoxicity was assessed using the MTT cell viability assay [25]. The MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) relies on the ability of viable cells to reduce MTT to purple formazan crystals, indicative of mitochondrial activity. Briefly, cells were seeded in 96-well plates at a density of 5,000 cells per well. After 24 hours, when the cells reached approximately 70% confluency, the formulations were applied. Plates were then incubated at 37°C in a humidified 5% CO_2 atmosphere. Following the incubation period, the MTT reagent was added to each well at a final concentration of 0.5 mg/mL. The plate was incubated with MTT for an additional 4 hours at 37°C. After the MTT incubation, the media were carefully discarded, and the formazan crystals were dissolved by adding 100 µL DMSO per well. Absorbance was measured spectrophotometrically at a wavelength of 570 nm. Treatments were performed in triplicate, and untreated wells served as controls, with control cell viability defined as 100%. Cell viability was calculated using the following formula:

$$\% \text{ Viability} = \text{Abs(sample)} \times 100) / \text{Abs(control)}$$

5.2.4. Encapsulation Efficiency of Quercetin

The encapsulation efficiency was determined by separating free quercetin via centrifugation (4,000 rpm, 10 min) [14]. The pellet was resuspended in ethanol, and quercetin content was measured with HPLC

[26]. Total quercetin was derived from the initial formulation input. The encapsulation efficiency was calculated as:

EE (%) = (total quercetin added – free quercetin)/total quercetin*100

5.2.5. In vitro Release Study

In vitro release study was conducted using a modified version of the protocol by Talarico and colleagues [14].

The release profile of quercetin from SLNs was evaluated using a dialysis membrane method (MWCO 8 kDa). Briefly, 4 mL of purified nanoparticle suspension was placed in the dialysis bag and immersed in 80 mL of release medium (PBS:EtOH, 75:25 v/v) maintained at 37°C with gentle stirring. Aliquots (1 mL) were withdrawn at predetermined intervals (0.5-24 h) and replaced with fresh medium to maintain sink conditions. Quercetin concentration was quantified by HPLC [26].

5.2.6. Chromatographic conditions

Chromatographic analysis of quercetin was performed with an Agilent 1260 Prime II HPLC connected to a diode array detector and a ZORBAX Eclipse C18 column (4.6 × 100 mm, 3.5 µm) was used (Agilent Technologies, USA). HPLC method employed the isocratic mobile phase 2% phosphoric acid: methanol (20:80, v:v) at a flow rate of 0.6 mL/min. The separation was carried out at temperature of 30°C, 25 µL injection volume with 372 nm detecting wavelength. The method was validated according to the International Conference on Harmonization guidelines. Calibration equation was y=0.685x-43.410 (R^2=0.9996) within the concentration range 1-20 µg/mL. Limit of detection and limit of quantification values were 0.54 and 0.89 µg/mL, respectively. Average RSD% values of intraday and inter day studies were 1.32 and 1.71%, respectively. Accuracy results for different concentrations of quercetin (5, 10 and 15 µg/mL) were 101.4, 98.7 and 99.1%.

900 µL methanol was added on 100 µL sample which taken from acceptor compartment, mixed with vortex stirrer for 1 minute and kept in ultrasonic bath for 5 minutes at ambient temperature. All samples were filtered through 0.45 µm nylon filter before injecting to column.

5.2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0. The data were presented as mean ± standard deviation. A significance level of $p < 0.05$ was considered for all tests.

Author contributions: Concept – U.K., K.Ş.; Design – U.K.; Supervision – U.K.; Resources – U.K.; Materials – U.K., Ç.D.; Data Collection and/or Processing – U.K.; Analysis and/or Interpretation – U.K., K.Ş., Ç.D.; Literature Search – U.K., K.Ş.; Writing – U.K., K.Ş; Critical Reviews – U.K.
Conflict of interest statement: The authors declared no conflict of interest.

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