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Curcumin and quercetin loaded nanocochleates gel formulation for localized application in breast cancer therapy

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ABSTRACT

After surgical excision of breast cancer, chemotherapy is recommended to eradicate any undiagnosed cancer cells and lower the likelihood of the cancer recurring. Curcumin and quercetin are two old flavonoid medicines used to treat breast cancer. Besides ambient popularity, they possess poor water solubility and poor bioavailability, limiting their usefulness. Hence to overcome these limitations, the present research aims to formulate curcumin and quercetin-loaded nanocochleates and convert them into a gel for localized application to enhance the breast cancer treatment. In this research article, we have developed curcumin and quercetin-loaded nanocochleates gel for breast cancer adjuvant therapy. The particle size, zeta potential encapsulation efficiency, and drug release of quercetin nanocochleates were 327 nm, −16.8 mV, 83.28 %, and 80.23 %, respectively, and that of curcumin nanocochleates were 328.6 nm, −15.0 mV, 82.30 %, and 77.19 %, respectively. The quercetin and curcumin-loaded nanocochleates gel was further characterized for pH, spreadability, and viscosity. The in vitro drug release behaviour of gel is controlled compared to plain quercetin and quercetin nanocochleates. The release of quercetin and curcumin from nanocochleates gel was 78.19 %, and 77.19 %, respectively. The MTT assay results showed quercetin and curcumin-loaded nanocochleates have maximum inhibition compared to control, quercetin alone, quercetin liposomes, and quercetin nanocochleates. Thus the quercetin and curcumin combination nanocochleates gel formulation can be a better option for the localized application in the breast cancer treatment.

1. Introduction

Curcumin and quercetin are ancient popular drugs employed for various activities ranging from dietary supplements to anticancer moiety. Besides great therapeutic applications, they have poor water solubility, bioavailability, and absorption, decreasing utility. Different novel drug delivery systems have been formulated to improve their pharmacokinetic properties. Out of these, lipid-based systems have proven their use as efficient drug delivery system. In recent years nanocochleates have been one of the lipid-based carriers studied extensively for various ailments and demonstrated their efficacy in therapy [1–3].

Quercetin is one of the flavonoids used widely for its therapeutic utility. It is abundant in onions, apples, broccoli, berries, tea, and

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red wine. Despite wide therapeutic applications, it has drawbacks such as rapid clearance, fast metabolism, and enzymatic degradation, decreasing therapeutic action potential. Various drug delivery systems have been tried out to overcome these problems [4]. The basic mechanisms of quercetin as an anticancer agent have different pathways in different types of cancers. In breast cancer, it involves an increase in cell apoptosis and cell cycle progression. In colon cancer, it stimulates apoptosis by mitogen activating protein kinases. In pancreatic cancer, it reduces tumor growth and drug resistance. In liver cancer, it stimulates apoptosis. In lung cancer, it reduces phosphorylation histone 3, an epigenetic factor in tumor growth. Quercetin in prostate cancer has efficiency by decreasing tumor improvement and down-regulation of Ki67, thereby enhancing caspase 7 and downregulating growth factors such as VEGF and EGF [5].

Curcumin is the most active component of curcuma longa plant. Curcumin is a yellow crystalline powder. The possible mechanisms of curcumin as an anticancer agent are in several ways, out of which few are described here. One of the mechanisms is acting on the STAT pathway, specifically STAT3 regulating oncogenes. Apart from it modulating cytokines and growth factors. Curcumin also suppresses the Nf Kβ receptor, which is involved in the expression of different proteins [6].

Nanocochleates is a novel rod-shaped drug delivery system invented in 1975. In earlier days, it was applied only for gene delivery but later explored many therapeutic applications. Nanocochleates as delivery have many potential applications as better stability over liposomes. It also has better encapsulation efficiency as being rod shape. It also holds the drug in more controlled fashion because of its bilayered structure. Apart from many advantages they possess drawbacks as they require specific storage conditions. They may aggregate; hence addition of aggregation inhibitor is required [7]. Numerous studies have been carried out on the formulation and evaluation of nanocochleates of various anticancer agents like fisetin [8], paclitaxel [9,10], raloxifene [11], quercetin [12], erlotinib, and dexketoprofen [13], doxorubicin with folic acid [14], docetaxel [15], curcumin [16], hydrocamptothecin [17], cytarabine [18, 19], and imatinib with dexketoprofen [20] and demonstrated to improve the efficacy for the treatment of various types of cancers.

Quercetin has a number of disadvantages, including greater dose, reduced oral bioavailability, and chemical instability, which reduces its therapeutic usefulness. Several lipid-based drug delivery methods have been shown to improve therapeutic efficiency. In recent years, many researchers investigated nanocochleates, which is one of the lipid-based drug delivery systems used in cancer therapy [21–33]. Encapsulating quercetin in nanocochleates, which are known to have high tissue penetration, allows for controlled drug release and improved efficacy. According to a literature search, curcumin and quercetin synergistically to prevent cancer cell proliferation by apoptosis [34,35]. Hence this research aimed to formulate quercetin and curcumin nanocochleates to enhance the localized concentration of both the drugs in tumor tissue and enhance the efficacy of quercetin by the synergistic effect of curcumin. In this study, we prepared curcumin and quercetin liposomes by ethanol injection approach and then treated with calcium chloride to form nanocochleates. Nanocochleates were incorporated into the gel for ease of application.

2. Materials and methods

2.1. Materials

Curcumin (95.78 %) was obtained as a gift sample from Sami Labs, Bangalore, India. Quercetin (95 %) was purchased from Sigma Aldrich, (Merck) Bangalore, India. Phosphatidylcholine (90G) was received as gift sample from Lipoid GmBH Ludwigshafen, Germany. Ethanol was purchased from central drug house Ltd India. Cholesterol and Carbopol 940 were purchased from Molychem Mumbai, India. All other solvents and chemicals are of analytical grade.

2.2. Methods

2.2.1. Preparation of curcumin and quercetin loaded liposomes

Curcumin and quercetin liposomes were prepared by the ethanol injection method separately as described by Nadaf and Kiledar [15]. In 2 mL of ethanol, 50 mg of phosphatidylcholine, 15 mg of cholesterol, and 10 mg of curcumin were dissolved and heated to 40 °C. The warm solution was then immediately injected into 10 mL of 40 °C distilled water and swirled at a particular rpm on a magnetic stirrer (Remi, India) for 30 min at a speed of 560 rpm. The same process was used to create quercetin liposomes with 10 mg of quercetin. To obtain purified liposomes, curcumin and quercetin liposomes were filtered via a 0.45 m membrane filter. The liposomes were then sonicated for 2 min in an ice bath using a probe sonicator (Ultrasonic probe sonicator VCX-130) [36–38].

2.2.2. Formulation of curcumin and quercetin-loaded nanocochleates

Curcumin and quercetin-loaded nanocochleates were formed by vortex mixing curcumin liposomes and quercetin liposomes for 2 min with 50 μL of calcium chloride (0.1 mM) as a bridging agent. As calcium ions come into contact with liposomes, the liposomal solution becomes murky, and aggregates of nanocochleates can be observed, confirming the development of curcumin and quercetin-loaded nanocochleates. The formed nanocochleates were then sonicated for 2 min on an ice water bath with a probe sonicator (40 amplitude) (Ultrasonic probe sonicator VCX-130) [8].

2.2.3. Formulation of nanocochleates gel

About 0.2 gm of carbopol 940 was weighed and added to previously formulated 10 mL of quercetin and curcumin loaded nanocochleates suspension with constant stirring for 30 min and kept aside for 2 h to allow for complete swelling of a polymer. The resulting mixture was neutralized with triethanolamine to produce a clear gel. The final concentration of carbopol gel is 1 %.

2.2.4. Determination of particle size

The particle size of curcumin liposomes, nanocochleates, quercetin liposomes, and nanocochleates was determined using a zeta sizer (Malvern, USA). Before measurement, each sample was diluted with distilled water.

2.2.5. Zeta potential determination

The zeta potential of the optimized formulation of quercetin and curcumin liposomes as well as nanocochleates has been recorded by using Malvern zeta sizer using the dynamic light-scattering technique at (25 ± 2 °C).

2.2.6. Surface morphology

The surface morphology of optimized curcumin nanocochleates was studied using scanning electron microscopy (Zeiss, Germany). Using a dual ion beam sputtering system (DIBS), a few drops of an aqueous dispersion of nanocochleates were coated with gold. This is set to a voltage of 5.0 kV with magnifications of 10–20 kX and a temperature of 252 °C for SEM.

2.2.7. Determination of entrapment efficiency (EE)

1 mL of the formulation was placed in a centrifuge tube to for determination of entrapment efficiency. The mixture was then centrifuged at 15,000 rpm for 30 min to separate the non-encapsulated curcumin and quercetin. Supernatant and sediment were separated by centrifugation, and ethanol was added to extract curcumin and quercetin. After diluting with methanol, and absorbance was measured at 426 nm and 378 nm by a UV spectrophotometer. The calibration curve has been plotted, and the regression equation is noted from the calibration curve.

Entrapment efficiency is calculated by using the formula

$$\frac{\%\ EE = amount\ of\ total\ drug - \ the\ amount\ of\ free\ drug}{Amount\ of\ total\ drug} \times 100 \tag{1}$$

2.2.8. Drug loading efficiency

The drug loading content of curcumin and quercetin was determined by dissolving 20 mg of nanocochleates in 1 mL methanol. The UV absorbance for curcumin was measured at 426 nm and quercetin was measured at 378 nm by using UV spectrophotometer. Theoretical drug loading content, actual drug loading content and % drug loading efficiency were calculated by using following formula (eqs. (2)–(4))

$$\mathrm{DLC}_{\mathrm{Theoretical}}\% = \frac{Weight\ of\ free\ drug}{Weight\ of\ free\ drug, phosphatidylcholine\ and\ cholesterol}\ \mathrm{x}\ 100 \tag{2}$$

$$\mathrm{DLC}_{\mathrm{actual}}\ \% = \frac{weight\ of\ drug\ in\ blend\ nanocochleates}{weight\ of\ blend\ nanocochleates}\mathrm{x}\ 100 \tag{3}$$

$$\mathrm{DLE}\% = \frac{DLC_{actual}}{DLC_{Theoretical}} \times 100 \tag{4}$$

2.2.9. pH measurement

The pH of the gel formulation has been analysed by using a pH meter (LABINDIA Pico+). All readings were taken in triplicate, and the average of them was recorded.

2.2.10. Spreadability

Spreadability has been determined using the laboratory apparatus suggested by Multimer [39]. A 1 gm sample of Nanocochleates gel was put between two slides, and a 100 gm weight was positioned on the slides for 5 min to compress the sample into a homogeneous layer. The time necessary to separate the two glass slides was used to calculate spreadability.

The formula for calculating spreadability is,

$$\mathrm{S} = \mathrm{mL/t} \tag{5}$$

where S = spreadability in gm. cm/sec,

L = length of a glass slide,

m = weight tied to upper slide,

t = time in seconds.

Glass slide of length 8.3 was used and weight to upper slide 105 g used throughout the experiment.

2.2.11. Viscosity of gel

The viscosity of gel was determined using a Brookfield viscometer (DV II Pro+, Brookfield Engineering Lab., Inc., Middleboro, USA). The graph of shear rate versus viscosity was obtained.

2. 2.12. In vitro drug release studies

In vitro drug release experiments of drug-loaded nanocochleates and drug-loaded nanocochleates gel were carried out in phosphate buffer pH 5.3, which is the pH of cancer cells, and the results were compared to that of free drugs. The 1 gm sample was kept in a donor compartment of the Franz diffusion cell. Buffer was filled into a receptor compartment. This assembly was placed on a magnetic stirrer at 100 rpm at 37 ± 0.5 °C. The 1 mL aliquots were withdrawn at specific time intervals (30, 60, 90, 120, 180, 210, 240, 300, 360, 480, 600 and up to 1440 min), each time replacing with the equal volume of phosphate buffer. Cellophane membrane is used for dialysis. Aliquots were withdrawn at specific time intervals and analysed by HPLC method developed and validated in our laboratory (unpublished data). The limit of detection for curcumin and quercetin was found to be 0.052 μg/mL and 0.087 μg/mL respectively. Reverse phase C18 column was used to develop HPLC method. A mobile phase containing acetonitrile: methanol: citric acid (0.1 %) (5:4:1) with flow rate of 1 mL/min was used for simultaneous estimation. The analysis was carried out at 360 nm and at ambient temperature. The concentration of the drug was calculated using a calibration curve [40].

2.2.13. In-vitro anticancer studies

The anticancer activity was evaluated at Deshpande Laboratories in Bhopal, employing MCF7 cell lines and using the MTT assay method. When MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)] is cleaved by live cells, it transforms into a dark blue formazan product. Active mitochondria are required for the conversion of blue formazan. As a result, the number of live cells is directly proportional to the amount of MTT cleaved by active mitochondria. Colorimetric methods are used to examine the amount of cleaved MTT. All materials were dissolved in DMSO, and further dilutions were produced to achieve a variety of test concentrations.

The DMSO content in all samples was 0.1 %. MCF7 was stored under appropriate conditions and seeded in 96 well plates. The plates were treated with various concentrations of test samples. These plates were then incubated for 96 h at 37 °C with 5 % CO2. After that, MTT reagent was applied to the wells and the timer was set for 4 h. The produced dark blue formazan product was dissolved in DMSO and analysed at 550 nm using a safety cabinet. The percentage inhibition by samples was also calculated. For the computation of IC50 values, a graph between % inhibition and concentration was also plotted [40–44].

2.2.14. Ex-vivo skin permeation studies

Ex-vivo skin permeation investigations were conducted on goat skin [38,45,42]. Because it is a byproduct of the slaughterhouse, the ear pinna skin was employed. The hairs on the ear pinna skin were removed using an electric shaver, and the skin was first washed with water and then with a normal saline solution. The skin was cut into the circular pieces and mounted on the diffusion cell, stratum layer facing towards the donor cell. The effective surface area for diffusion was 5.652 cm^2. The receptor cell was made up of a phosphate buffer (8 mL), pH 5.5, and was kept at 37 ± 0.5 °C. The *ex–vivo* skin permeation was performed on the formulated gel formulation containing equivalent of 10 mg drug, for 24 h and compared with marketed gel formulation. 2 mL of aliquots were removed at specific time intervals and promptly replenished with the same amount of new diffusion medium and analysed by HPLC method described in section 2.2.11. On completion of permeation studies, the media was analysed to estimate the amount of drug retained in skin.

The receptor cell was made up of a phosphate buffer (8 mL), pH 5.5, and was kept at 37 0.5 °C. Ex-vivo skin permeation was done for 24 h on the produced gel formulation containing the equivalent of 10 mg medication and compared to the marketed gel formulation. At certain time intervals, 2 mL of aliquots were extracted and promptly replenished with the same amount of new diffusion medium before being analysed using the HPLC method described in section 2.2.11. Following the completion of the permeation studies, the media was analysed for the amount of medication retained in the skin.

The slope of the linear component of the graph representing the cumulative amount of quercetin permeated through skin plotted as a function of time was used to compute the permeation rate/flux values through the ear pinna skin. Kp (cm^2/h) was estimated using Equation (2): $Kp = J/C_o$ (2) where Kp is the permeability coefficient, J is the steady state flux and C_o represents the drug concentration that stayed constant in the vehicle [40,43].

2.2.15. Stability studies

Stability studies help predict the change in the efficacy of a drug or formulation over a period of time. Nanocochleates gel formulation was stored in a sealed glass container at 25 °C and 4 °C for a period of 3 months for stability studies as per ICHQ1R2 guidelines. The samples were withdrawn at the specified interval and analysed for drug content, spreadability, and pH.

3. Results and discussion

The ethanol injection method was selected for the preparation of curcumin and quercetin nanoliposomes. This method was used since it gives smaller-sized liposomes and reproducibility. These formulated liposomes were further sonicated by using a probe sonicator (Ultrasonic probe sonicator VCX-130) for 2 min for uniformity in liposome shape and size. These liposomes were taken further as a precursor for converting into nanocochleates.

The formulated liposomes of curcumin and quercetin were further converted to nanocochleates by using 0.1 M $CaCl_2$ as a bridging agent. It was added dropwise to curcumin and quercetin under the vortex mixing. The turbidity indicates the formulation of nanocochleates. The nanocochleates suspension was further sonicated by using a probe sonicator (Ultrasonic probe sonicator VCX-130) for 2 min to have uniformity in the size of nanocochleates. The transparent yellow, uniformly textured gel was obtained by incorporating nanocochleates into carbopol 940 base. The viscosity of the gel increases with increasing pH. The pH of the gel was set to 6.5–6.7, which is good for the stability of a carbopol gel. The viscosity of the gel was observed in the range of 750–947 pa. s (gm/sec).

3.1. Particle size, zeta potential, and entrapment efficiency

Particle size, zeta potential, and entrapment efficiency of curcumin liposomes and nanocochleates, quercetin liposomes and nanocochleates are shown in Table 1.

Particle size and encapsulation efficiency were found to be increased in the case of nanocochleates. The reason for increased particle size may be a shape change from spherical to rod shaped. The increase in encapsulation efficiency of nanocochleates may be due to the further encapsulation of the free drug remaining unentrapped in liposomal suspension during the conversion from liposomes to nanocochleates. Hence, nanocochleates offer better encapsulation efficiency over liposomes. The decrease in zeta potential may occur because of interaction between calcium ions with the phospholipid [46].

3.2. Drug loading efficiency

The drug loading efficiency were calculated as per the formulas mentioned in literature [47]. The drug loading content (theoretical) for curcumin and quercetin were found to be 13.33 % Actual drug loading content for curcumin and quercetin was found to be 10 %. The drug loading efficiency for curcumin and quercetin was found to be 75.01 %.

3.3. Surface morphology

Surface properties of curcumin nanocochleates were studied using scanning electron microscopy. The nanocochleate structure (Fig. 1) was revealed to be rod-shaped. Aggregates of nanocochleates were also visible in the image, which is a typical feature of the nanocochleate trapping method. The size of nanocochleates is also within the range necessary for nanoparticles.

3.4. Viscosity of gel

The viscosity of gel was determined using a Brookfield viscometer (DV II Pro+, Brookfield Engineering Lab.,Inc., Middleboro, USA). A graph was also generated between shear rate and viscosity. The gel's viscosity was found to be between 750 and 947 gm/sec. It can be seen from the graph that the system is shear-thinning. Fig. 2 depicts a graph of shear rate vs viscosity.

3.5. In vitro drug release studies

In vitro drug release experiments were carried out in order to figure out how a drug would behave *in vivo*. The Franz diffusion cell was used for in vitro release. The release profiles of pure drugs, quercetin and curcumin-loaded nanocochleates, and quercetin and curcumin-loaded nanocochleates gel were compared in this work, as shown in Fig. 3.

The first purple line represented the 24-h release of pure drug. According to the graph, about 90 % of the drug was released up to 5 h. In the case of quercetin nanocochleates, the drug release was further delayed and observed to be in a controlled form. The graph clearly showed that the release profile of quercetin and curcumin nanocochleates exhibited a biphasic pattern: an initial burst release within the first few hours, followed by a slower and more controlled release over a 24-h period. This could be due to the instantaneous diffusion of the drug entrapped in the surface layer of the nanocochleates in the first stage, whereas the drug inside the deep layers slowly diffused into the media when the lipid layers eroded in the second stage. Up to 24 h, around 80 % of the drug was released. The release of both drugs was further delayed in the case of nanocochleates gel. The release of 78.19 % quercetin and 77.19 % curcumin from nanocochleates gel was found. The release pattern was examined further for several models, and it was discovered that the Korsmeyer Peppas model best matched the drug release pattern, indicating a diffusion process in drug release. The values of n for quercetin release from nanocochleates and nanocochleates gel were found to be 0.21 and 0.30; in case of curcumin release from nanocochleates and nanocochleates gel, the value of n was found to be 0.25 and 0.35, indicating a diffusion mechanism as all the values were less than 0.5. Thus, by entrapping drugs in nanocochleates and nanocochleates gel, controlled release was achieved.

3.6. In vitro anticancer studies

The MTT assay was performed on quercetin, quercetin liposomes, quercetin nanocochleates, and quercetin and curcumin-loaded nanocochleates. The IC50 values for each sample have been calculated (Table 2).

According to Table 2, quercetin has a 53.64 ± 1.68 % inhibition on cancer cells at a dose of 10 μg/mL, while quercetin liposomes have a 56.62 ± 2.56 % inhibition at this concentration. Quercetin nanocochleates kill cancer cells more effectively than quercetin

Table 1
Particle size, zeta potential, and entrapment efficiency of curcumin liposomes and nanocochleates, quercetin liposomes and nanocochleates.

Formulation	Particle size (nm)	Polydispersity Index	Zeta potential (mV)	Entrapment Efficiency (%)
Curcumin liposomes	235.6 ± 4.78	0.328 ± 0.0026	−25.6 ± 1.35	75.83 ± 3.211
Curcumin nanocochleates	328.6 ± 5.84	0.312 ± 0.002	−15.0 ± 1.70	82.30 ± 2.056
Quercetin liposomes	160.5 ± 1.970	0.493 ± 0.0020	−27.4 ± 1.85	76.69 ± 0.944
Quercetin nanocochleates	327 ± 3.681	0.396 ± 0.0045	−16.8 ± 2.01	83.28 ± 1.745

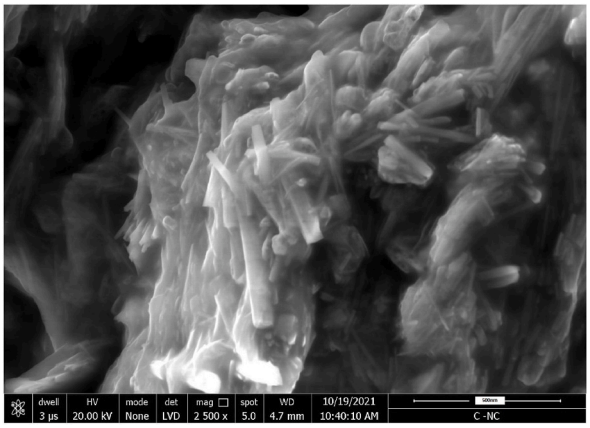

Fig. 1. SEM image of curcumin nanocochleates.

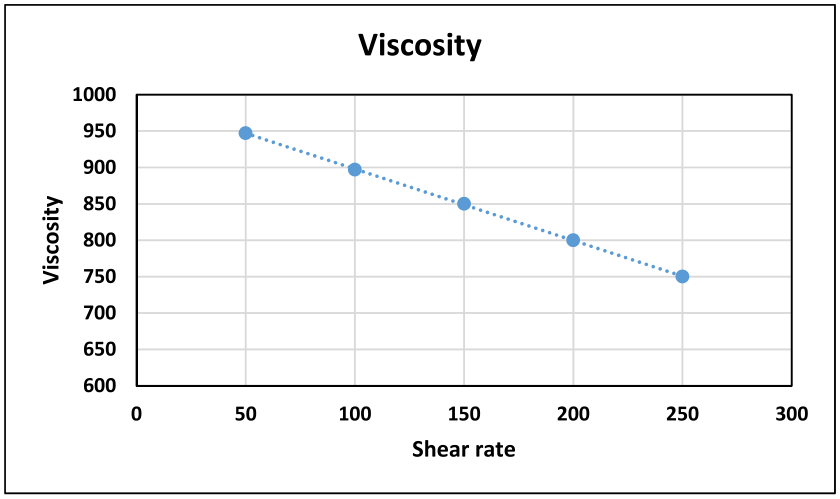

Fig. 2. Plot of shear rate vs viscosity of gel.

liposomes, with a 61.25 ± 0.91 % inhibition rate. When curcumin and quercetin encapsulated in nanocochleates, a synergistic effect was observed on inhibiting cancer cells, and hence there is improvement in anticancer action, i.e., resulting in 69.85 ± 0.71 % inhibition. The IC 50 values were determined to be 3, 2, 1, and 0.7 g/mL for quercetin, quercetin liposomes, quercetin nanocochleates, curcumin, and quercetin loaded nanocochleates, respectively. As a result, it is clear that curcumin and quercetin-loaded nanocochleates require the lowest dose for inhibiting cancer cells by 50 %.

From Fig. 4, it can be observed that cancerous cells remain intact in control (Fig. 4 A), whereas when treated with quercetin (Fig. 4 B) and quercetin liposomes (Fig. 4C) (slightly better), the viability of cancer cells decreases. When compared to quercetin liposomes, quercetin nanocochleates (Fig. 4 D) increased activity even more. As a result, it has been established that nanocochleates are more effective than liposomes for cancer therapy. When cancer cells were treated with a combination of quercetin and curcumin nanocochleates (Fig. 4 E), the anticancer activity was excellent, demonstrating the synergistic effect of both drugs. The obtained synergistic results are consistent with studies described in the literature [35,48,49,50]. As a result, nanocochleates are a more effective choice for delivering curcumin and quercetin together for cancer therapy. Mansourizadeh et al. developed nanoparticles loaded with curcumin and quercetin with horse spleen apoferritin for breast cancer therapy. MTT assay was performed on curcumin and quercetin in combination and alone on MCF cell lines. It has been observed that as compared to individual drugs quercetin and curcumin in combination have synergistic effect for anticancer therapy. The half-effective maximal concentration (EC50) was calculated for individual drugs and combination. The EC50 value for quercetin, curcumin and quercetin-curcumin with horse spleen apoferritin nanoparticles were found to be > 100 µM, 58.5 µM, 11 µM. Hence it was found that curcumin and quercetin show synergistic effect when combined with human serum apoferritin and loaded in nanoparticles. Srivastava et al. studied the effect of curcumin and quercetin individually and in combination on MCF-7 cancer cell lines. They have performed MTT assay for analysing percentage inhibition. The IC50 values for quercetin, curcumin and for curcumin and quercetin were found to be 45 µM, 18 µM, and 3.1 µM respectively, showing synergistic effect of curcumin and quercetin. The researcher also performed an isobologram approach to verify

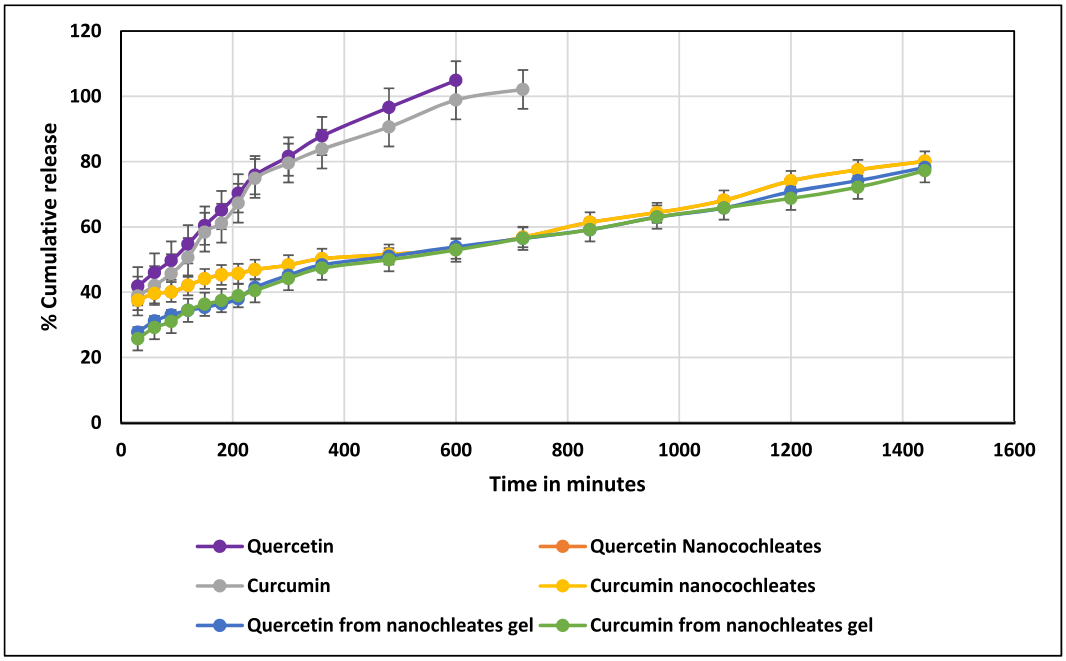

Fig. 3. *In vitro* release behaviour of pure drugs, drug loaded nanocochleates, and drug loaded nanocochleates gel.

Table 2
% Growth inhibition in MCF7cell line at different concentrations of drug in liposomes and nanocochleates.

Concentration (μg/mL)	Quercetin	Quercetin liposomes	Quercetin nanocochleates	Quercetin and curcumin nanocochleates
10	53.64 ± 1.68	56.62 ± 2.56	61.25 ± 0.91	69.85 ± 0.71
1	41.28 ± 0.73	45.55 ± 1.13	47.85 ± 0.81	51.26 ± 0.52
0.1	13.26C0.15	21.65 ± 0.37	14.51 ± 0.30	38.64 ± 0.63
0.01	8.75 ± 0.60	4.58 ± 0.30	6.98 ± 0.21	18.51 ± 0.26
0.001	2.59 ± 0.29	3.15 ± 0.13	2.57 ± 0.34	14.57 ± 0.56
IC50	3	2	1	0.7

synergism [48,49].

3.7. Ex vivo skin permeation studies

Goat ear pinna skin was used for ex vivo skin permeation studies. Ear pinna skin is used for this purpose since it is a waste product of the slaughterhouse [42,43]. Skin permeability and flux were determined using a formula from the literature [43,46,51,52]. The following is the formula:

$$Kp = J/\ C_O \tag{6}$$

where p = permeability coefficient
J = flux
C_O= Initial concentration

The permeation profiles of quercetin through ear pinna skin from formulated gel and marketed gel was shown in Fig. 5. Over a 24-h period, the amount of drug permeated from nanocochleates gel was found to be 72.86 %, while the marketed gel formulation was found to be 46.19 %. In case of the nanocochleates gel significant increase in skin permeation observed. The reason might be the lipid delivery has eased penetration and avoided barriers to skin permeation. Quercetin is available in free form in marketed formulation hence it might not permeated in skin as compared to nanocochleates gel where it was entrapped in lipid bilayer that made eased permeation. (Fig. 5).

The flux was determined to be 20.125 μg/cm^2 h for nanocochleates gel and 8.61 μg/cm^2 h for the commercialized gel formulation. It was discovered that the permeability coefficients for the commercial gel and the nanocochleates gel were 0.004 and 0.1, respectively (Table 3).

Also, from microscopic images (Fig. 6), it has been clear that the formulated gel formulation has permeated through skin. The skin's yellow colouring is visible in the photograph obtained following the study (Fig. 6 b), but there is no such colour in the image taken

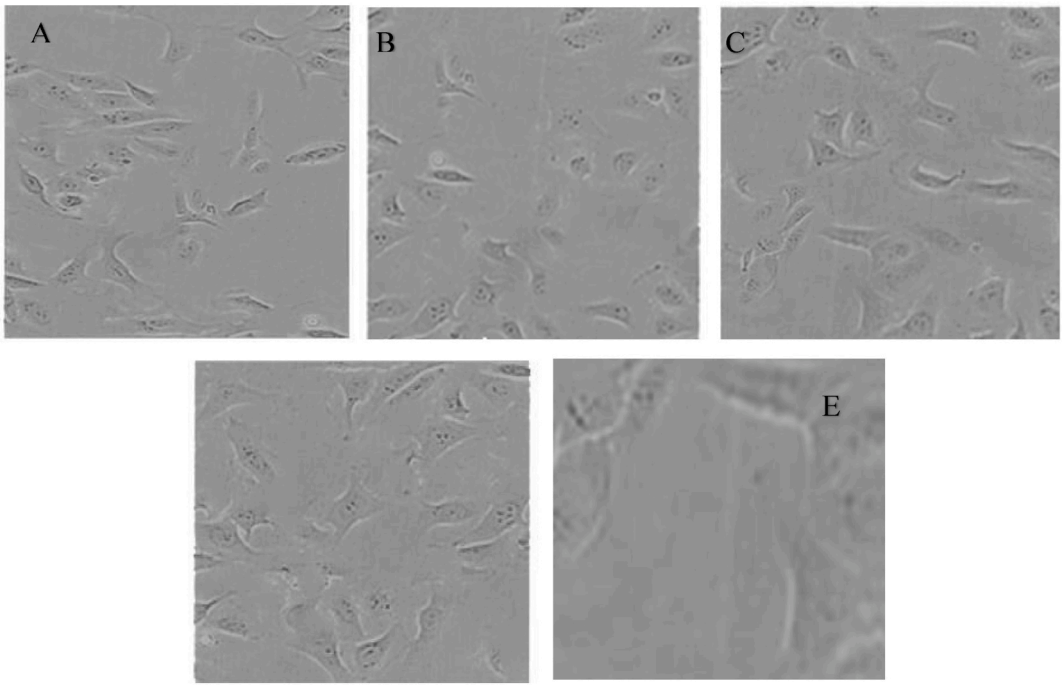

Fig. 4. Image showing effects of control (A), quercetin (B), quercetin liposomes (C), quercetin nanocochleates (D), curcumin and quercetin loaded nanocochleates (E) on cancer cells.

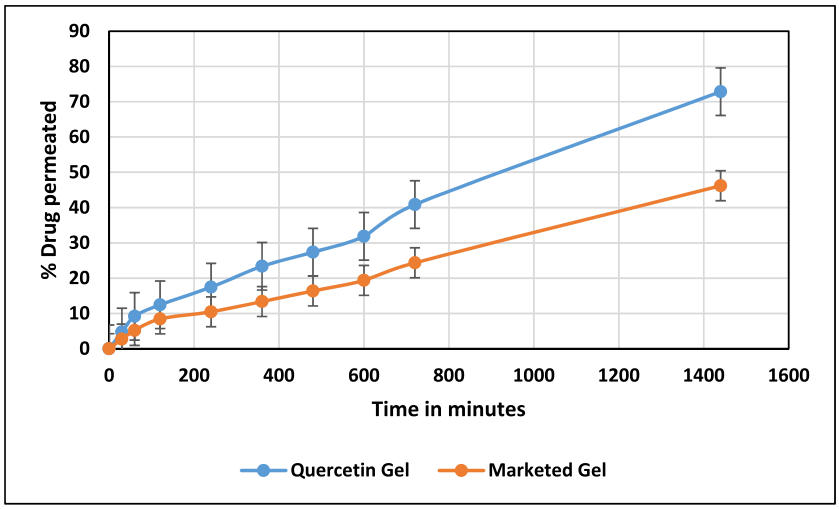

Fig. 5. Ex vivo skin permeation of quercetin from nanocochleates gel and marketed quercetin gel formulation.

Table 3
Permeation parameters for quercetin release for nanocochleates gel and quercetin marketed gel.

Formulation	Flux (μg/cm^2 h)	Permeability coefficient (cm^2/sec)
Nanocochleates gel 8.2 mg/gm	20.125 ± 0.94	0.1
Marketed gel 50 mg gm	8.61 ± 0.13	0.004

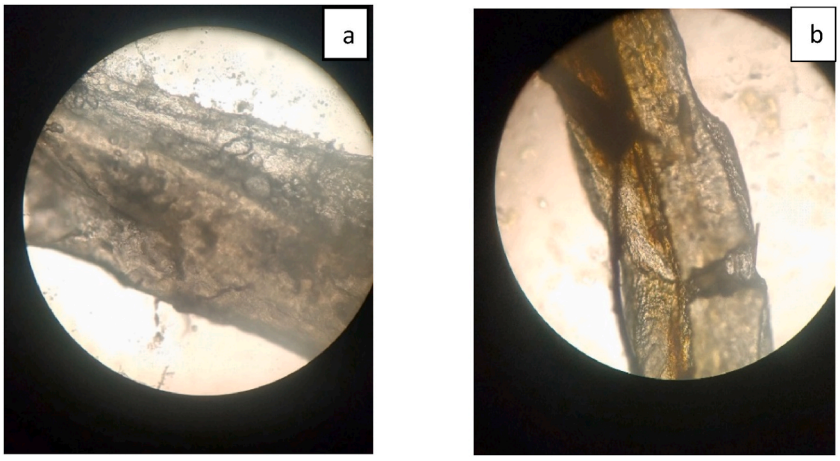

Fig. 6. Ear pinna skin a) before gel application and b) after gel application.

prior to the examination (Fig. 6 a).

. any colouration wheras (Fig. 6 a) also proven the permeation of gel through skin.

3.8. Stability studies

Stability studies are carried out to ascertain the product's shelf life, guaranteeing that the formulation stays within its specified limits for the duration of the required period. According to ICH recommendations, stability investigations of gel were conducted for three months under a range of temperature and relative humidity settings [53]. The formulation was taken out first at 15-day intervals, then once a month, and its pH, spreadability, and percentage of drug content were assessed. No significant differences were observed in the initial and final stability data, indicating a stable formulation.

4. Conclusion

Quercetin and curcumin-loaded nanocochleates gel was successfully prepared in this study. The developed formulation was characterized by particle size, zeta potential encapsulation efficiency, spreadability, drug release, and ex vivo drug permeation. The in vitro drug release behaviour of gel was exhibited as a controlled release compared to plain quercetin and quercetin nanocochleates. The stability studies for three months indicate a stable formulation. The formulated novel nanocochleates gel was found to be promising in treating breast cancer based on in vitro anticancer testing. The MTT assay results showed quercetin and curcumin-loaded nanocochleates have maximum inhibition compared to control, quercetin, quercetin liposomes, and quercetin nanocochleates. Thus, it can be considered a suitable delivery option in breast cancer adjuvant therapy.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Meena Tilawat: Writing – original draft, Validation, Methodology, Investigation, Data curation, Conceptualization. **Smita Bonde:** Writing – review & editing, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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