



Preparation and Characterization of Ozonated Liposomes Loaded with Curcumin: A Potential Approach for Diabetic Retinopathy Treatment

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Abstract

Background: Limitations in drug penetration are overcome by nanotechnology, particularly liposomes, which enhance targeted administration of ocular medications. Liposomes can augment the therapeutic effects of curcumin, a natural compound with positive impacts on the retina. Treatments for eye diseases can also benefit from the antibacterial properties of ozonated oil liposomes.

Objectives: This study aims to develop and evaluate ozonated liposomes loaded with curcumin for ocular drug delivery applications, specifically targeting diabetic retinopathy (DR).

Methods: Liposomal nanoparticles were prepared using thin-film hydration, incorporating phospholipids, lecithin, cholesterol, ozonated oil, and curcumin. We assessed physicochemical properties including particle size, zeta potential, encapsulation efficiency (EE), and morphology. Drug release profiles and stability studies were also conducted.

Results: The optimized liposomes showed a particle size of 84.77 nm, a zeta potential of -16.8 mV, and an EE of 94.73%. The formulation exhibited sustained curcumin release over 24 hours, reaching 82.09% cumulative release. Stability studies indicated minimal changes in key parameters over three months at room temperature.

Conclusions: The developed ozonated liposomal formulation demonstrates promising characteristics for curcumin delivery, potentially enhancing its therapeutic efficacy in ocular applications, particularly for DR.

Keywords: Curcumin, Liposomes, Ozonated Oil, Ocular Drug Delivery, Diabetic Retinopathy

1. Background

Ocular drug delivery (ODD) presents unique challenges due to the eye's complex anatomy and protective mechanisms. Traditional eye drops often suffer from poor bioavailability and limited retention time on ocular surfaces (1, 2). Nanotechnology-based strategies, particularly liposomes, have shown promise in overcoming these limitations, including poor bioavailability and short retention times (3, 4). Diabetic retinopathy (DR), a common complication of diabetes affecting millions worldwide, often requires invasive treatments with limited efficacy (5, 6). There is growing interest in natural compounds as potential therapeutic

agents for this condition (7, 8). Curcumin, derived from turmeric, has shown promising effects in various ocular disorders, including DR (9-12). However, its clinical application is hindered by poor solubility and low bioavailability (11, 12). Liposomal encapsulation of curcumin can overcome these issues by providing targeted delivery to ocular tissues and reducing the frequency of drug administration due to sustained drug release (13, 14). Additionally, ozonated oils (OO) have demonstrated antimicrobial and wound-healing properties, making them promising candidates for ocular applications (15-18). Combining OO with curcumin in a liposomal formulation offers a novel approach that may synergistically enhance therapeutic

outcomes in DR and other ocular diseases (17, 18). This study aimed to develop and characterize ozonated liposomes loaded with curcumin, evaluating their physicochemical properties, drug release profile, and stability for potential ODD applications, with a specific focus on DR treatment.

2. Objectives

This paper discusses the development of a double-encapsulated liposome formulation containing ozonated oil and curcumin for topical ocular administration, establishing a foundation for a biocompatible ophthalmic drug system for eye diseases.

3. Methods

3.1. Chemicals

We sourced cholesterol, lecithin, curcumin, methanol, and chloroform from Merck (Germany). All other chemicals used were of analytical grade (19).

3.2. Liposome Preparation

We prepared liposomes using the thin film hydration method as described by Zhang (20). Briefly, phospholipids, lecithin, cholesterol, ozone oil (10% and 20% of total lipids), and curcumin were dissolved in a 2:1 chloroform: Methanol mixture. The solvent was evaporated using a rotary evaporator at 60°C and 120 rpm to form a thin lipid film, which was then vacuum-dried overnight. The film was hydrated with phosphate buffer solution at 45°C, followed by vortexing and stirring for 20 minutes.

3.3. Physicochemical Characterization

3.3.1. Particle Size and Zeta Potential

Particle size was measured at 25°C using a particle size analyzer. The mean droplet size of the samples was determined with the SCATTER SCOPE 1 QUIDIX (South Korea) based on photon correlation spectroscopy, with a wide measurable size range of 1 - 7000 nm. Surface charge was assessed using the Zeta Analyzer, and the Zeta Potential of samples was determined with a Malvern Zetasizer Nano-range instrument (Malvern Instruments Ltd., Malvern, UK). Each sample was measured in triplicate (19, 21).

3.4. Morphological Analysis

For SEM imaging, the shape and structure of dried liposome samples were examined at 15 kV using a 6300-

field emission scanning electron microscope (Hitachi, S-4160) (19).

3.5. Encapsulation Efficiency

For this purpose, liposomal formulations were centrifuged at 12,000 rpm for 30 minutes. We then separated the liposomes from the surface of the solution and centrifuged the aqueous solution containing the unloaded drug once more. This time, we isolated the aqueous solution and the liposomes again for an additional 15 minutes. To calculate the amount of unloaded drug, the underlying liquid was isolated, and the drug quantity was measured. The amount of loaded drug was determined by subtracting the amount of unloaded drug from the total drug used. To confirm the test results, the upper liposomal phase was dissolved in methanol to break the liposomes, allowing the measurement of the loaded drug amount. In this way, both loaded and unloaded drug quantities were calculated, and the percentage of loaded drugs was determined using the following equation (19, 21):

$$EE (\%) = \frac{(Total\ drug - Free\ drug)}{Total\ drug} \times 100$$

3.6. In Vitro Drug Release Study

We studied drug release using fabricated diffusion cells. The donor compartment contained 2 mL of liposomal suspension, while the acceptor compartment contained artificial tear fluid (pH 7.4). Samples were collected over 24 hours, and curcumin content was analyzed using HPLC with a UV-Vis detector. Various kinetic models were evaluated to determine the best fit for the release profile (21).

3.7. Stability Studies

We evaluated the stability of the liposomal formulations by storing samples at room temperature for three months and monitoring changes in particle size, zeta potential, and encapsulation efficiency (EE) (19, 21).

3.8. Curcumin Quantification

The amount of drug was determined by HPLC using a C18 column at a wavelength of 425 nm (22).

3.9. pH Measurement

The pH of the samples was measured using a Mettler Toledo pH meter (21).

Table 1. Liposome Formulation Components and Characteristics

Formula	Drug Con(mg/ml)	%OO/LI	Sonicate Time(min)	Factorial State	%EE	PZ	PDI	Zeta P	%D Release 1	%D Release24
Lip-Cur-1	1	20	10	+++	90.81 ± 0.10	130 ± 7.55	0.264 ± .012	-17.57 ± .58	34.15 ± .32	91.32 ± .32
Lip-Cur-2	0.5	20	10	++	88.65 ± 0.43	156 ± 50	0.263 ± .005	-17.47 ± 3.65	30.89 ± .73	96.05 ± .61
Lip-Cur-3	1	20	5	++	96.37 ± 0.26	179 ± 7.21	0.283 ± .018	-17.37 ± .93	30.14 ± .28	96.94 ± .09
Lip-Cur-4	0.5	20	5	+-	92.5 ± 0.37	184 ± 30	0.273 ± .014	-17.37 ± .93	29.51 ± .58	98.49 ± .73
Lip-Cur-5	1	10	10	++	94.47 ± 0.18	84 ± 30	0.267 ± .022	-16.93 ± 1.1	36.45 ± .46	79.88 ± .60
Lip-Cur-6	0.5	10	10	+-	89.52 ± 0.19	91.33 ± 5.03	0.282 ± .016	-16.47 ± 2.81	33.33 ± .24	87.25 ± .11
Lip-Cur-7	1	10	5	+-	97.33 ± 0.12	126 ± 5.57	0.283 ± .022	-17.77 ± 1.01	33.86 ± .46	94.65 ± .27
Lip-Cur-8	0.5	10	5	--	95.27 ± 0.10	147 ± 7.55	0.287 ± .024	-15.6 ± 2.36	32.07 ± .55	94.58 ± .73
Lip-Cur-Optimum	1	10	9.47	++	94.73 ± 0.66	84.77 ± 0.71	0.263 ± .011	-16.8 ± 1.32	36.91 ± 0.41	82.09 ± 0.45

Abbreviations: %OO/LI, %Ozonated Oil/ Lipid; Zeta p, zeta potential; EE, Encapsulation efficiency; PZ, particle size; PDI, polydispersity index.

3.10. Statistical Analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post-hoc test, with $P < 0.05$ considered statistically significant (19).

4. Results and Discussion

4.1. Optimization of Liposomal Formulation

We employed a factorial design to optimize the liposomal formulation, evaluating the effects of curcumin concentration, ozone oil percentage, and sonication time on particle size and EE (Table 1). The optimized formulation (Lip-Cur-Optimum) demonstrated a particle size of 84.77 ± 0.71 nm, a polydispersity index (PDI) of 0.263 ± 0.011 , and an EE of $94.73 \pm 0.66\%$. These results are comparable to those reported by Salimi et al. for naringenin-loaded liposomes (19), indicating the potential of this formulation for ocular drug delivery (ODD) (Figure 1).

4.2. Liposome Physicochemical Characterization

4.2.1. Particle Size and Zeta Potential

Liposome sizes ranged from 84 to 184 nm, with the optimized formulation averaging 84.77 nm. Zeta potential values ranged from -15.6 to -17.8 mV, with the optimized formulation at -16.8 ± 1.32 mV, indicating good colloidal stability (As shown in Table 2). These results

align with previous studies on liposomal formulations for ocular delivery (12, 23). The size of liposomal particles plays a crucial role in their efficacy and behavior in biological systems (24). Smaller particles provide a larger surface area for adsorption, which may enhance system stability and transparency (25). Changes in particle size can result from aggregation and miscibility phenomena (26). Lecithin serves as the main structural component in liposome formulations, while cholesterol is added to increase stability (27). The addition of cholesterol can influence the binding of hydrophobic molecules within bilayer membranes (28). Previous studies have shown varying effects of cholesterol on liposome particle size, suggesting that the preparation method significantly impacts these results (29, 30). Cholesterol's role in liposomal formulations depends on its concentration and the lipid composition, which affects bilayer fluidity and drug encapsulation (31). Zeta potential is a critical indicator of colloidal stability (32, 33). Higher absolute zeta potential values indicate stronger electrostatic repulsion between particles, which can significantly enhance the stability of the liposomal suspension and prevent aggregation (34-36). The negative charge observed in our formulations may be attributed to interactions between phosphatidylcholine and cholesterol (37).

$$Zeta P = \frac{(-13.9 - 2.22 Drug Con - 0.0325 Ozone)}{Lipid\% - 0.102 Sonicate Time}$$

According to regression analysis, increasing the curcumin concentration, ozone-to-lipid ratio, and sonication time enhances the negative charge of the zeta potential (As shown in Figure 2).

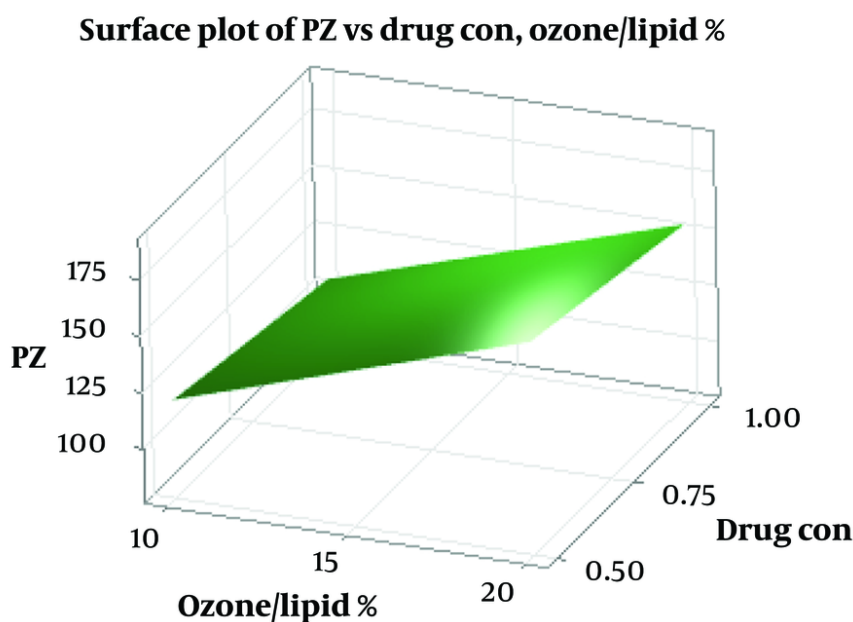


Figure 1. Response surface 3D plot for the effect of concentration of Curcumin (mg/ mL) and % Ozone/lipid on particle size (PZ)

Table 2. Zeta Potential of Liposome Formulations and Optimal Formulation

Zeta potential	1	2	3	Mean
Lip-Cur-1	-16.9	-17.9	-17.9	-17.57 ± 0.58
Lip-Cur-2	-13.3	-19	-20.1	-17.47 ± 3.65
Lip-Cur-3	-17.8	-16.3	-18	-17.37 ± 0.93
Lip-Cur-4	-13.2	-16.5	-17.3	-15.67 ± 2.17
Lip-Cur-5	-15.7	-17.8	-17.3	-16.93 ± 1.1
Lip-Cur-6	-13.5	-16.8	-19.1	-16.47 ± 2.81
Lip-Cur-7	-16.6	-18.3	-18.4	-17.77 ± 1.01
Lip-Cur-8	-12.9	-16.6	-17.3	-15.6 ± 2.36
Lip-Cur-Optimum	-15.3	-17.8	-17.3	-16.8 ± 1.323

4.3. Morphological Analysis

SEM imaging of the optimized formulation revealed spherical particles with smooth surfaces, confirming the formation of liposomes (Figure 3) (19). This morphology is consistent with previous studies on liposomal formulations for ocular delivery (30, 32). The particle size dispersion index, reported in Table 1, indicates a typical range for liposomes.

4.4. Encapsulation Efficiency

Encapsulation efficiency values ranged from 88% to 97%, with the optimized formulation achieving $94.73 \pm 0.66\%$, indicating excellent curcumin loading. According to Figure 4, increasing the percentage of ozone oil to total lipid (% ozone/lipid) shows a decrease in EE%, but increasing the concentration of curcumin shows an increase in EE%. High EE is crucial for the efficacy of liposomal formulations, especially for poorly soluble compounds like curcumin (25). High EE directly impacts drug delivery efficiency and bioavailability in target tissues by improving drug absorption, enhancing

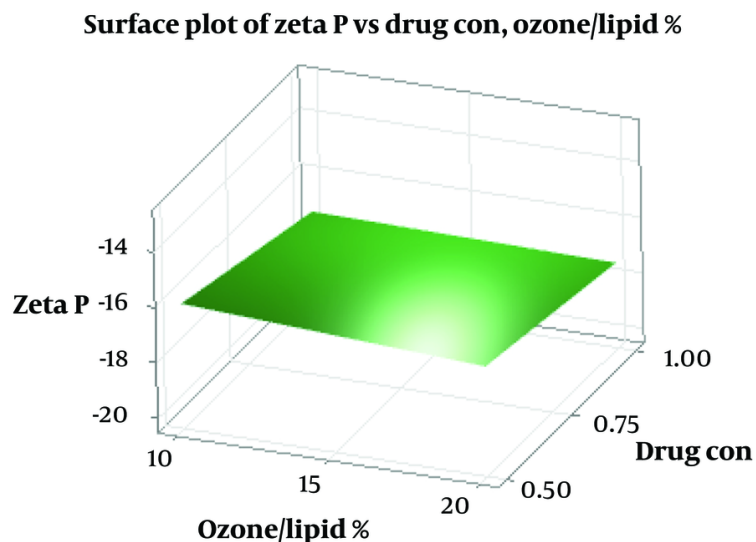


Figure 2. Response surface 3D plot for the effect of concentration of Curcumin (mg/mL) and % Ozone/Lipid on Zeta Potential (Zeta P)

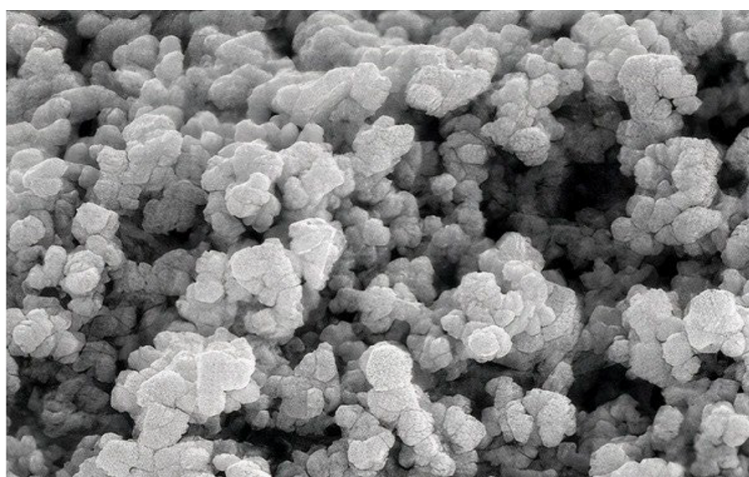


Figure 3. SEM image of optimized liposomal formulation

stability, optimizing distribution within target tissues, and reducing toxicity and side effects (38).

4.5. Drug Release Profile

The optimized formulation demonstrated a biphasic release pattern, with an initial burst release followed by sustained release over 24 hours. Cumulative release at

24 hours was $82.09 \pm 0.45\%$. The release kinetics best fit the Peppas model ($R^2 = 0.985$), suggesting a complex release mechanism involving both diffusion and erosion (As shown in Table 3 and Table 4 and Figure 5) (39, 40). The Peppas model is commonly used to describe drug release kinetics in polymeric systems, indicating a diffusion-controlled release (38). Drug release from nanoliposomes depends on various factors,

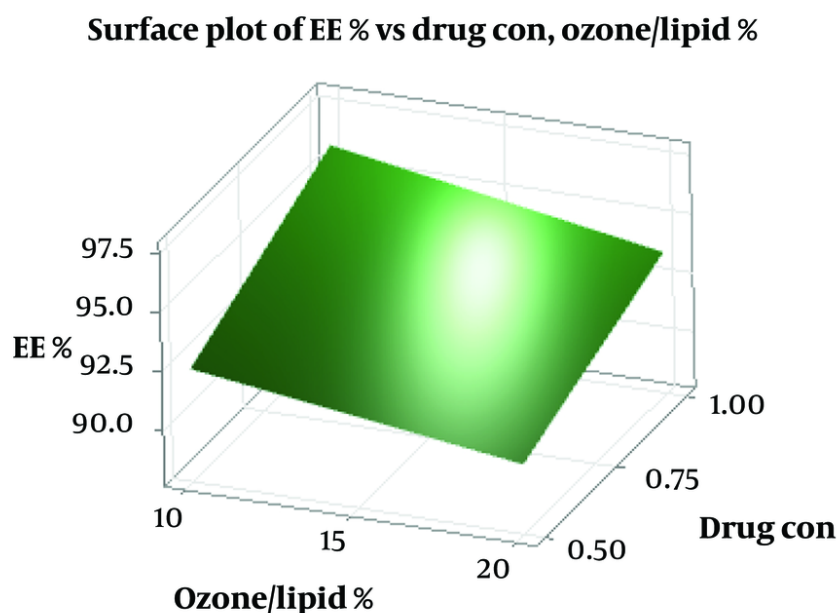


Figure 4. Response surface 3D plot for the effect of concentration of Curcumin (mg/mL) and % Ozone/Lipid on Encapsulation efficiency (EE%)

Table 3. Curcumin Release Percentage from Liposomes at Different Times

Time	Lip-Cur-1	Lip-Cur-2	Lip-Cur-3	Lip-Cur-4	Lip-Cur-5	Lip-Cur-6	Lip-Cur-7	Lip-Cur-8	Lip-Cur-Optimum
0.5	29.37	24.34	23.81	22.45	30.84	28.52	27.81	25.97	31.02
1	34.15	30.89	30.14	29.51	36.45	33.33	33.86	32.07	36.91
2	42.12	40.73	39.93	40.14	42.6	41.88	42.36	40.52	43.27
4	50.59	52.34	53.38	52.95	49.11	53.65	51.88	50.86	49.75
5	58.7	58.85	59.75	60.13	54.38	61.08	58.64	60.23	55.6
6	65.65	66.64	66.73	67.94	61.03	67.39	64.68	69.92	61.48
8	74.94	77.8	76.8	79.84	66.55	74.59	72.51	79.62	66.84
12	83.46	87.09	90.28	90.71	74.05	81.1	82.12	86.77	74.35
24	91.32	96.04	96.94	98.49	79.88	87.25	94.65	94.58	82.09

including composition, synthesis method, drug type, and environmental conditions (such as temperature and pH) (31). The observed release profile is favorable for ODD, potentially providing an initial therapeutic dose followed by sustained release to maintain drug levels (23).

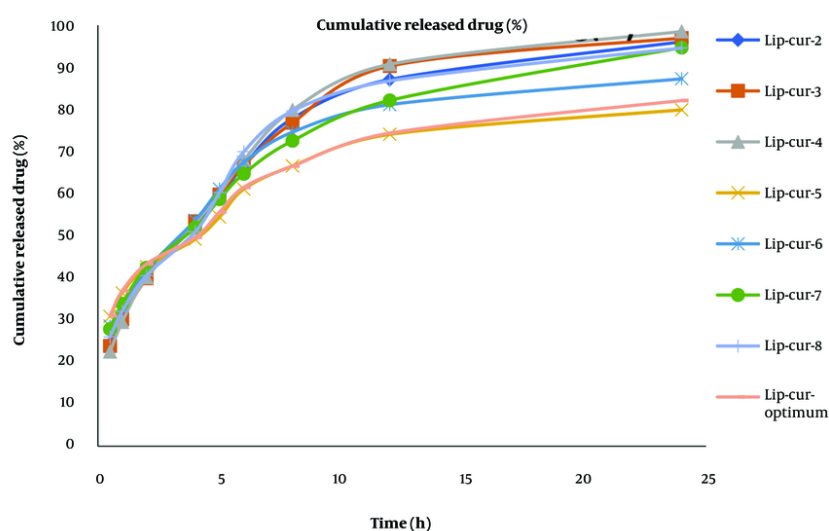
4.6. Stability of Optimized Liposomes

Over three months at room temperature, the optimized formulation showed minimal changes in particle size (As shown in Table 5), zeta potential, and EE,

indicating good physical and chemical stability. This stability is essential for the potential clinical application of the formulation. Results indicated that liposome cake formation did not occur and there was no change in %EE. Changes in particle size for different formulations over 1 day, and at 1, 2, and 3 months, are shown in Table 5. The change in particle size, zeta potential, and PDI for 8 formulations and the optimized formulation over 3 months is displayed in Figure 6, Table 5, and Table 6. Higher zeta potential values indicate greater electrostatic repulsion between particles, which can

Table 4. Comparison of Kinetic Models of Formulations

Formula	Kinetics	RSQ	Slope	Intercept	MPE%	Release% (Q24)	Release% (Q1)
Cu-Li-1	Pepas	0.9761	0.3225	-1.0445	4.2429	91.32 ± 0.32	34.15 ± .32
Cu-Li-2	First	0.9838	-0.1285	-0.2948	6.7184	96.05 ± 0.61	30.89 ± .73
Cu-Li-3	Pepas	0.9828	0.3958	-1.1619	1.9013	96.94 ± 0.09	30.14 ± .28
Cu-Li-4	First	0.9955	-0.1709	-0.1573	3.4024	98.49 ± 0.73	29.51 ± 0.58
Cu-Li-5	Pepas	0.980268	0.263555	-1.0097333	2.719243	79.88 ± 0.60	36.45 ± 0.46
Cu-Li-6	Weibul	0.978542	0.511833	-0.8530214	4.972448	87.25 ± 0.11	33.33 ± 0.24
Cu-Li-7	First	0.995995	-0.11076	-0.3293264	3.453382	94.65 ± 0.27	33.86 ± 0.46
Cu-Li-8	Pepas	0.971251	0.368815	-1.1085016	4.471019	94.58 ± 0.73	32.07 ± 0.55
Cu-Li-Optimum	Pepas	0.985441	0.265525	-1.0000166	2.432798	82.09 ± 0.45	36.91 ± 0.41

**Figure 5.** Comparison of cumulative curves of curcumin release from different formulations at different times

significantly enhance the stability of the liposomal suspension and prevent aggregation (19, 21).

4.7. Curcumin Quantification

Curcumin quantification was performed using HPLC with a reverse-phase column and UV detection at a wavelength of 425 nm, as shown in Table 7. Significant differences in curcumin content were observed among different formulations ($P < 0.0001$).

4.8. pH Measurement

The pH of all formulations ranged from 6.41 to 6.64, with the optimized formulation at 6.52 ± 0.017 , suitable for ophthalmic application and curcumin stability. This

pH range is compatible with the ocular environment (typically 6.5 - 7.5) and is expected to be well tolerated (As shown in Table 8) (21, 24).

4.9. Conclusions

This study successfully developed and characterized an ozonated liposomal formulation loaded with curcumin for ocular drug delivery, specifically targeting DR. The optimized formulation demonstrated favorable physicochemical properties, including a small particle size of 84.77 nm, high EE of 94.73%, and sustained drug release, with 82.09% cumulative release over 24 hours. These characteristics indicate the potential for enhanced curcumin delivery and therapeutic efficacy in

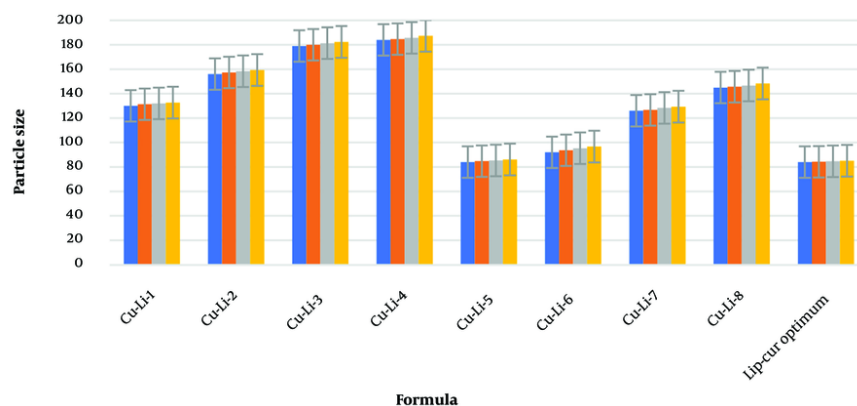


Figure 6. Comparison of the bar graph of the change in the particle size of the formulations in 0, 1, 2, and 3 months

Table 5. Changes in Particle Size of Different Formulations During 1,2 and 3 Months

Formula	P(nm) 1st day	P(nm) 1st month	P(nm) 2st month	P(nm) 3st month
Lip-Cur-1	130 ± 7.55	131.33 ± 7.64	132 ± 7.21	132.67 ± 7.09
Lip-Cur-2	156 ± 5.0	157.33 ± 5.51	158.33 ± 4.51	159.33 ± 4.51
Lip-Cur-3	179 ± 7.21	180 ± 7.21	181.33 ± 7.09	182.33 ± 6.11
Lip-Cur-4	184 ± 3.0	184.67 ± 2.52	185.67 ± 1.53	187.33 ± 2.08
Lip-Cur-5	84 ± 2.65	84.77 ± 2.31	85.33 ± 2.08	86.12 ± 1.53
Lip-Cur-6	92 ± 6.0	93.67 ± 5.51	95.33 ± 3.51	96.67 ± 2.52
Lip-Cur-7	126 ± 5.57	126.67 ± 5.13	128.33 ± 4.04	129.33 ± 4.04
Lip-Cur-8	145 ± 7.55	145.67 ± 8.08	146.67 ± 7.23	148.33 ± 6.66
Lip-Cur-Optimum	84 ± 0.71	84.23 ± 1.31	84.67 ± 1.08	85.07 ± .53

Table 6. Changes in Particle Size, PDI, and Zeta Potential During 3 Months at Room Temperature

Formulations	Condition Time (Months)	Particle Size (nm)	PDI	Zeta Potential
Lip-Cur-1	3	132.67 ± 7.09	0.265 ± .017	-17.59 ± .58
Lip-Cur-2	3	159.33 ± 4.51	0.264 ± .015	-17.51 ± 2.45
Lip-Cur-3	3	182.33 ± 6.11	0.285 ± .018	-17.42 ± .43
Lip-Cur-4	3	187.33 ± 2.08	0.276 ± .024	-17.46 ± .73
Lip-Cur-5	3	86.12 ± 1.53	0.271 ± .022	-16.96 ± 1.13
Lip-Cur-6	3	96.67 ± 2.52	0.286 ± .024	-16.49 ± 1.71
Lip-Cur-7	3	129.33 ± 4.04	0.287 ± .042	-17.81 ± 1.31
Lip-Cur-8	3	148.33 ± 6.66	0.289 ± .034	-15.63 ± 1.27
Lip-Cur-Optimum	3	85.07 ± .53	0.264 ± .023	(-16.83 ± 1.23

ocular applications. Stability studies showed that the formulation remains stable over three months at room temperature, with minimal changes in key parameters. The combination of curcumin's therapeutic benefits with the antimicrobial properties of OO offers a novel

approach for ocular drug delivery (ODD), addressing the challenges posed by curcumin's poor solubility and bioavailability. Future research should focus on in vivo evaluations of the formulation's efficacy in treating DR and on assessing its safety profile. Additionally,

Table 7. The Amount of Curcumin in the Formulations

FORMULA	Amount of Curcumin in Formula (mg/ml)
Lip-Cur-1	0.9081 ± 0.010
Lip-Cur-2	0.443 ± 0.013
Lip-Cur-3	0.964 ± 0.010
Lip-Cur-4	0.463 ± 0.011
Lip-Cur-5	0.945 ± 0.018
Lip-Cur-6	0.448 ± 0.035
Lip-Cur-7	0.973 ± 0.006
Lip-Cur-8	0.476 ± 0.009
Lip-Cur-Optimum	0.9473 ± 0.062

Table 8. pH Results of the Formulations

FORMULA	Lip-Cur-1	Lip-Cur-2	Lip-Cur-3	Lip-Cur-4	Lip-Cur-5	Lip-Cur-6	Lip-Cur-7	Lip-Cur-8	Lip-Cur-Optimum
PH	6.64 ± 0.03	6.41 ± 0.02	6.55 ± 0.026	6.46 ± 0.026	6.57 ± 0.02	6.46 ± 0.08	6.54 ± 0.017	6.43 ± 0.02	6.52 ± 0.017

exploring the potential synergistic effects of curcumin and ozonated oil in treating ocular infections and inflammation could provide new therapeutic options, potentially reducing the need for invasive treatments in ocular disorders.

Footnotes

Authors' Contribution: A. S. conceived and designed the evaluation and drafted the manuscript. A. R. participated in designing the evaluation, performed parts of the statistical analysis, and helped to draft the manuscript. M. F., H. H. K. and F. B. re-analyzed statistical data and revised the manuscript. A. S. and B. S. M. approved the final version to be published.

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