

Indian gooseberry (*Phyllanthus emblica* L.) based liposomes: Formulation, characterization, *in vitro* and *ex vivo* antioxidant activity evaluation

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ABSTRACT: The research was focused on incorporating the polyphenols from Indian gooseberries (*Phyllanthus emblica* L.) in phospholipid nanocarriers, aiming to enhance skin penetration, bioavailability and stability. Design-Expert® aided in achieving the optimal liposomal formulation, characterized by particle size, zeta potential, and a polydispersity index. Liposomes were prepared by applying the film hydration method. The liposomes were further loaded into a hydrogel (Carbopol 934) for its controlled release and stabilizing effects on liposomes. Multiple antioxidant assessment methods DPPH, ABTS, FRAP, CUPRAC were performed. Additional evaluations encompassed FTIR, SEM, rheological studies, and *in vitro/ex vivo* diffusion comparisons between liposomal-loaded gel (GEL-LE) and pure extract with gel (GEL-E). Depending on the formulation and extract amount, the total antioxidant content per sample varies between 59.3-486.75 mg. When the optimal formulation (LE) contained 1.8 % soybean and 0.07 % cholesterol the mean particle size was 74.66 nm, zeta potential – -50.35 and polydispersity index– 0.3. *In vitro* results exhibited 42.28 % cumulative release for GEL-LE and GEL-E by 23.44 %. *Ex vivo* findings showed a 6% discrepancy in cumulative release (21% for GEL-LE). These outcomes emphasize liposomes' potential for enhanced antioxidant delivery and release, contributing to potential advancements in cosmetic and skincare applications.

KEYWORDS: Loaded liposomes; gel; free radicals; antioxidant; *P. emblica* L.

1. INTRODUCTION

Free radicals are unstable molecules or atoms with an odd electron number, which have gained scientific interest [1]. Reactive oxygen species and reactive nitrogen species (ROS and RNS) are subsets of free radicals [2]. Free radicals can originate internally (metabolism, inflammation) and obtained externally through (radiation, smoking). High levels of ROS and RNS cause oxidative or nitrosative stress, linked to various diseases [3-5].

Antioxidants combat aging and oxidative damage resulting from free radicals [6]. A diet rich in different antioxidants, including vitamins E and C, is crucial [7]. Antioxidants vary in function; they can be radical scavengers, donors, and enzyme inhibitors [8]. The cosmetic industry is embracing natural ingredients, including plant extracts rich in vitamins, antioxidants, and essential oils. These extracts offer effective skincare benefits and appeal to environmentally conscious consumers [9]. Common plant extracts used in cosmetics include green tea, rosemary, grape seed, and blueberry, known for their natural antioxidants [10-11]. Antioxidants are categorized as primary (minerals, vitamins, and phyto-antioxidants) and secondary (propyl gallate, metal chelating agents tertiary butylhydroquinone) (synthetic antioxidants) and play a role in preventing oxidative degradation each with different mechanisms [12-18]. Indian gooseberry, also known as *Phyllanthus emblica* L. (synonym of *Emblica officinalis* Gaertn) or Amla, is valued for its potent antioxidant, immune-modulating, and anticancer properties. It contains high levels of vitamin C, tannins, flavonoids, and phenolic compounds. Studies suggest its benefits in various conditions, including diabetes, inflammation, tumor growth, gastric ulcers, and retroviruses like HIV [19-20] Phenolic compounds

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in *P. emblica* contribute to its strong antioxidant activities [21]. Liposomes show promise in stabilizing these compounds and enhancing their bioavailability for potential cosmetic treatments [22].

Drug delivery systems offer alternative methods for administering pharmaceutical substances, including peptide and protein therapies [23]. Liposomes, spherical vesicles with lipid membranes, can improve drug distribution in the skin by incorporating lipophilic drugs into lipid bilayers and hydrophilic drugs into the aqueous compartment [24]. In the context of UV radiation, liposomes have been explored for their UV-blocking effects, with particle size playing a role in the effectiveness of sunscreen action [25]. The stratum corneum acts as a primary barrier in the skin, and since liposomes have similar lipid composition, it can enhance drug delivery while limiting systemic absorption [26].

Studies have demonstrated the effectiveness of liposomes as a superior delivery system compared to conventional formulations. Octyl methoxycinnamate, a UV absorber, exhibited better SPF quality when delivered through liposomes, with reduced penetration into deeper skin layers and limited systemic absorption [27]. Antioxidant delivery is another area where liposomes have shown promise. Sodium ascorbyl phosphate, a potent antioxidant, exhibited enhanced stability and penetration through the stratum corneum when loaded into liposomes [28]. Similarly, dispersed ascorbyl palmitate, aided by cathodal electric assistance, demonstrated effective skin penetration when incorporated into liposomes [29]. Liposomes offer numerous advantages as a drug delivery system. They improve drug solubility, provide sustained release, enhance efficacy, enable site-specific targeting, improve molecule transport, and enhance drug stability. Additionally, liposomes are non-toxic, flexible, biocompatible, and non-immunogenic. Overall, liposomes are a versatile and effective approach for delivering drugs and molecules [30]. Gels are semisolid systems composed of a liquid phase within a three-dimensional polymeric matrix [31]. They offer several advantages, such as avoiding first-pass metabolism easy preparation and application, and targeted drug delivery [32]. Gels provide continuous drug input, reduce fluctuations in drug levels, and allow for easy termination of medications [33]. They have a larger area of application compared to other dosage forms and can selectively deliver drugs to specific sites [34]. Gels also improve physiological and pharmacological response, enhance patient compliance, and are suitable for self-medication [35]. Our aim is to enhance the penetration and stability of antioxidants and phenols from *P. emblica* fruit. While some studies have shown satisfactory results with liposomal delivery combined with the fruit in cream formulations, there are challenges in maintaining the stability of ascorbic acid in such formulations. Factors like emulsion choice, oil phase selection, consistency, pH level, and emulsifying agents are critical for stability.

2. RESULTS

2.1. Total phenolic, flavonoid, and antioxidant content

Four different methods were employed to assess the antioxidant activity of the formulations. These four antioxidant experiments (CUPRAC, FRAP, DPPH and FRAP) are the most used tests in the literature. Six formulations were evaluated, with three containing hydrogel-loaded liposomes and three containing only loaded liposomes. The inclusion of different formulations aimed to determine if the hydrogel had any interaction, if none, with the antioxidant activities. The samples had varying amounts of extract, which were deliberately varied to showcase the relative increase in antioxidant power with concentration. Among the four methods used to evaluate antioxidant activity, DPPH proved to be the most accurate. Formulations containing higher amounts of the extract exhibited greater antioxidant power. In terms of the hydrogel formulation, it demonstrated minimal to no interaction with antioxidant activity (Table 1).

2.2. Liposomal characteristics

To determine the 4 various ingredient compositions, D-optimal experimental design using Design-Expert® (version 13.0.4.01, Stat-Ease Inc., Minneapolis, MN, USA) was used. Two independent variables' effects include: amounts of SPC and cholesterol on four response variables: zeta potential, mean particle size and PDI were evaluated as shown in Table 2. The thin film hydration approach was used to create unloaded liposomes, which were further characterized. Table 3 lists the fitting models, equations, and statistical parameters. To determine the significance level $p < 0.05$ was utilized. Design Expert® software produced equation-based three-dimensional response surface graphs. The optimal parameters for the formulations were generated using the desirability approach [36-37]. The experimental design resulted in 11 combinations, encompassed 3 replications at the central point. The analysis of variance (ANOVA) tables were generated to assess the effect and regression coefficients of individual linear models and to determine

the relationships between the variables. The statistical significance of all terms within the polynomial was evaluated by

Table 1. Total phenolic, flavonoid and antioxidant content of samples. (Results are means $\pm$ standard deviations of triplicate analyses. Different letters in the same column indicate significantly different values at $p < 0.05$.)

Sample	Total Phenolic Content ¹	Total Flavonoid Content ²	Total Antioxidant Content ^{3,4}			
			DPPH	FRAP	CUPRAC	ABTS
G1	29.7 $\pm$ 0.8 ^d	0.52 $\pm$ 0.01 ^e	103.7 $\pm$ 4.4 ^c	59.3 $\pm$ 4.2 ^b	90 $\pm$ 5.9 ^b	96.1 $\pm$ 6.4 ^d
G2	67.1 $\pm$ 0.46 ^c	0.94 $\pm$ 0.02 ^c	211.3 $\pm$ 26.4 ^b	142.2 $\pm$ 6.4 ^d	208.4 $\pm$ 4.06 ^d	244.49 $\pm$ 14.6 ^b
G3	119.7 $\pm$ 2.52 ^a	1.7 $\pm$ 0.06 ^a	370.1 $\pm$ 25.05 ^a	217.12 $\pm$ 13.05 ^a	358.4 $\pm$ 16.8 ^b	384.4 $\pm$ 22.7 ^a
L1	33.8 $\pm$ 2.3 ^d	0.4 $\pm$ 0.002 ^b	102.57 $\pm$ 6.7 ^c	80.5 $\pm$ 6.5 ^b	120 $\pm$ 9.1 ^b	92.5 $\pm$ 2.4 ^d
L2	70.1 $\pm$ 5.1 ^c	0.74 $\pm$ 0.001 ^d	209.14 $\pm$ 11.8 ^b	181.6 $\pm$ 9.4 ^c	263.2 $\pm$ 20.2 ^c	140 $\pm$ 1.96 ^c
L3	91.2 $\pm$ 5.2 ^b	1.15 $\pm$ 0.005 ^b	360.1 $\pm$ 30.6 ^a	262.4 $\pm$ 19.7 ^a	486.75 $\pm$ 14.96 ^a	221.9 $\pm$ 18.1 ^b

¹ mg gallic acid equivalent total phenolic content per sample

² mg quercetin equivalent total flavonoids content per sample

³ mg trolox equivalent total antioxidant content per sample

⁴ DPPH: 2,2-Diphenyl-1-picrylhydrazyl radical scavenging assay, FRAP: Ferric reducing antioxidant power assay, CUPRAC: Cupric reducing antioxidant capacity assay, ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay

Table 2. Optimal design displaying component and response variables for the unloaded liposome formulations

Run No.	Independent Variables	Independent Variables	Response Variables	Response Variables	Response Variables
	Soybean (%)	Cholesterol (%)	Zeta potential (mV)	Mean particle size (nm)	PDI
1	1.8	0.6	-45.0	77.1	0.27
2	1.9	0.6	-48.8	80.0	0.28
3	1.9	0.5	-49.0	103.2	0.23
4	1.9	0.6	-48.0	80.0	0.28
5	1.9	0.6	-48.2	81.2	0.29
6	2.0	0.5	-50.2	75.0	0.26
7	1.8	0.7	-50.7	74.6	0.30
8	1.9	0.5	-50.8	108.3	0.23
9	1.8	0.7	-50.0	74.7	0.3
10	2.0	0.5	-50.4	75.2	0.26
11	1.8	0.6	-45.0	78.7	0.28

calculating the F value with a significance level set at $p < 0.001$. In order to get an acceptable model, there must be an insignificant lack of fit. The statistical values of PDI response are presented in Table 3. The 0.8980 value of adjusted R^2 is close to the predicted R^2 0.7240. The design area can be navigated using this model. The PDI values varied between 0.23 and 0.3, indicating monomodal size distributions [38]. The statistical values of mean particle size response are presented in Table 3. The predicted R^2 of 0.9726 agrees with the adjusted R^2 of 0.9332 rationally. Also, the particle size of the unloaded liposomes was relatively small, the mean particle size of all formulations was smaller than 110 nm, ranging between 74.6 and 108.3 nm meaning the particles are all well made to conveniently penetrate the all show significant penetration through the skin [39]. Moreover, the zeta potential response statistical values are presented in Table 3. The 0.9122 value of adjusted R^2 is close to the predicted R^2 0.7940. Furthermore, in this research, zeta potential values were all shown to be negative for all of our unloaded formulations and results varied between -50.8 to -45.0 mV. The negative values are preferable when it comes to penetration and release on the nanoparticles. A study showed that negatively charged particles yield better release than positively charged particles [40]. The best results were achieved when 1.85 % of SPC and 0.65 % of cholesterol were used for preparation of liposomes.

Numerical optimization and the application of a desirability function were employed for the optimization of the fitted polynomials. The determined optimal conditions were subsequently validated through experimental testing. Responses were monitored and the results were compared with model prediction. To facilitate visualization of the data the Origin Pro 9.5 program OriginLab Corporation, Northampton, MA, USA, was utilized. The procedure was optimized for each of the three responses after creating the simplified model polynomial equations that link the dependent and independent variables

(Table 4). Based on the criteria of achieving the smallest value of particle size, zeta potential and PDI the best formulation was chosen. Utilizing the extensive grid search and feasibility search features offered by the Design Expert program, the final ideal experimental parameters were determined. Numerical optimization of the liposomal matrix using the desirability function has been performed. The results showed that experimental values did not significantly differ from the predicted values ($p > 0.05$). The optimal composition of unloaded liposomes has been determined as follows: 1.8 % of SPC, 0.7 % of cholesterol (Table 4).

Table 3. Fitting Models, equations, and statistical parameters of the experimental design

Response	Model	R ²	R ² Adjusted	R ² Predicted	Final equation
Zeta potential (mV)	Quartic	0.9473	0.9122	0.7940	$= +1.34343 \times 10^5 A$ $+7.64661 \times 10^6 B$ $+51.66782 C$ $-81205.78985 A * B$
Mean particle size (nm)	Quartic	0.9836	0.9726	0.9332	$1/(\text{mean particle size}) =$ $-43028.00352 A$ $-5.14307 \times 10^7 B$ $+7.58012 \times 10^5 A * B$ $-2432.57808 A * B * (A-B)$
PDI	Quartic	0.9388	0.8980	0.7240	$1/(PDI) =$ $+3.96465 \times 10^7 A$ $+3.60533 \times 10^{10} B$ $-360.25365 C$ $-5.37382 \times 10^8 A * B$ $+1.76484 \times 10^6 A * B * (A-B)$

Table 4. Optimization of components amount using desirability function

Independent variables	Amount value	Predicted amount value
SPC	1.85-2	1.8
Cholesterol	0.6-0.7	0.7
Response variables		
Mean particle size	76.62	74.66
Zeta potential	-49.8	-50.35
PDI	0.29	0.3

Lastly, the optimal liposomal formulation was used to load *P. emblica* extract and the characterization of the liposome with *P. emblica* extract (LE) was performed. The particle size is one of the most important measurements that can influence the active ingredient penetration across the membrane and the stability of the formulation [41]. The size of the liposome formulation was found to be around 96.63 ± 3 nm. In comparison with unloaded formulation the mean particle size increased in LE formulation but there was no significant difference ($p < 0.05$). Similar results were found by Pham et al. have prepared caffeine loaded niosome and liposome vesicular formulations. While they have obtained blank formulation of niosome and

liposomes' size 89 ± 7 nm and 112 ± 8 nm, respectively; they have found loaded formulations 95 ± 5 and 117 ± 7 nm as well [42]. Muhammadi et al. stated similar results around 100 nm, while using the same lipid carriers for the preparation of doxorubicin-loaded liposomes [43]. It was observed that the LE liposome formulation shows a homogeneous particle size distribution and it was found out that PDI around 0.27 ± 0.001 . The PDI is a valuable parameter to make assumption of the stability of dispersed systems. The dispersions are physically stable when PDI values are less than 0.3 [40]. Furthermore, zeta potential value was negative of the formulation and it was about -49.34 mV, as well-known the nanocarriers' negative surface charges may have an impact on the processes that lead to their transport from the stratum corneum [40]. Moreover, the encapsulation efficiency (EE) is another important parameter of nanoparticles due to the amount of drug per weight of a given formulation composition. Hence, it can be found from the composition of formulation when the active compound is fully encapsulated into nanoparticles. The %EE values of the total amount of polyphenols in the LE formulation has been found to be above 95.35 ± 3.6 %.

2.3 Liposomal morphology

SEM is the most convenient visual technique to probe the mean size and the surface morphology of prepared Nano formulations [44]. The surface morphology of the optimal liposome formulation was evaluated using SEM analysis. SEM picture was taken to obtain more information about the morphology of the prepared UL and LE formulations (Figure 1). In Figure 1 it could be seen that the particles were almost spherical, uniform in size with smooth surfaces and the SEM images taken are supportive of the nanosized measurement results.

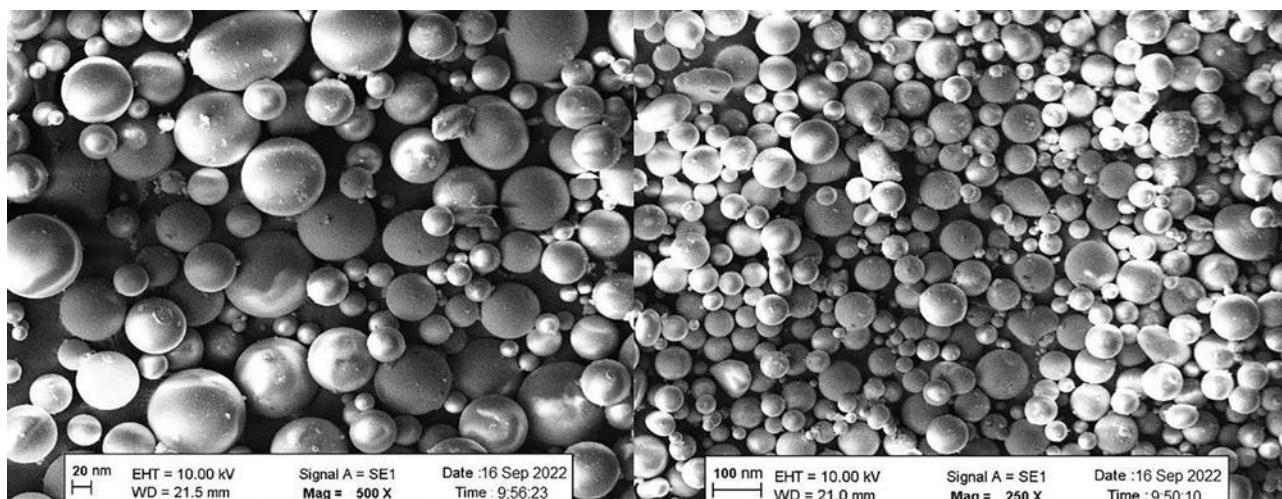


Figure 1. Liposomal morphology imaged by scanning electron microscope at magnifications 250 X and 500 X

2.4 Rheological studies

The evaluation of rheological properties for gels is one of the most important parameters for predicting *in vivo* behavior. The rheological properties especially affect both ease of application and retention within the application area. The measurements were done for GEL, GEL-E, GEL-UL, GEL-LE formulations. Measurement conditions were chosen to be conducted under the following ambient conditions of ($25 \pm 0.5^\circ\text{C}$) and ($37 \pm 0.5^\circ\text{C}$). Shear rate, which is the difference of velocity of two layers within the bulk of material divided by a distance, was applied to the formulation from 10 rpm to 100 rpm while viscosity was monitored and noted. The formulations showed proper textbook non-newtonian pseudoplastic properties. Viscosity was reported to be around +800 cps which is the reference range for carbopol hydrogel formulations in cosmetics [45]. A change in viscosity was observed after a certain shear rate allowing better flowability an obvious increase in viscosity relative to the increase in temperature was noted similar to another paper that was studying rheological behaviors or Carbopol [46]. The increase of viscosity with the increase of temperature can be interpreted in many ways but one article explained that with temperature the solvent-polymer interaction grows stronger thus explaining the increase of viscosity results were expressed in cPs and Pa s [47]. Obtained results showed that in continuous shear rheometer, all liposomal and without liposomal formulations showed a non-Newtonian pseudo-plastic flow, showing decreasing viscosity with progressive increases in the shear rate both at 25°C and 37°C (Figure 2).

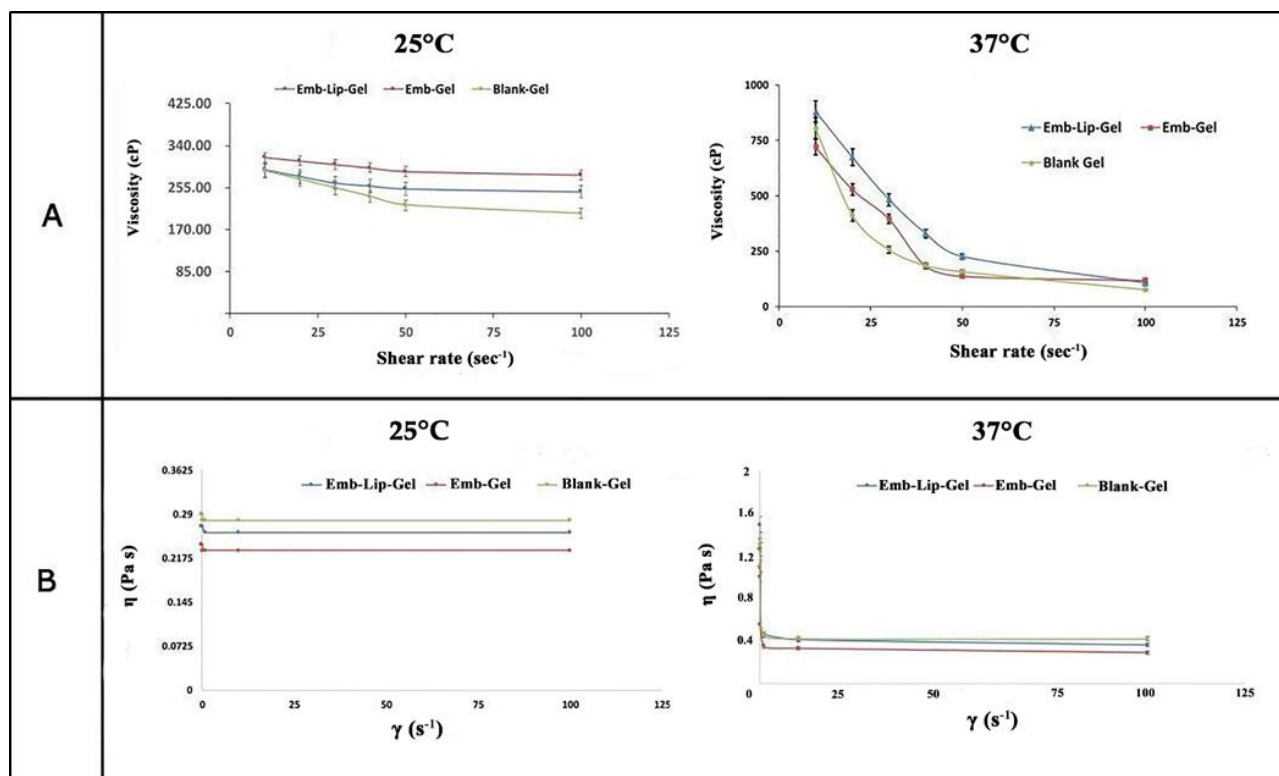


Figure 2. Viscosity versus shear rate graphs of the formulations (A) GEL, GEL-E, GEL-UL, GEL-LE formulations, when shear rate 10 rpm was used and ambient conditions of $(25 \pm 0.5^\circ\text{C})$ (B) GEL, GEL-E, GEL-UL, GEL-LE formulations when shear rate 100 rpm was used and ambient conditions of $(37 \pm 0.5^\circ\text{C})$.

2.5. Fourier Transform Infrared Spectroscopy (FTIR-ATR) Analysis

FTIR test was conducted for UL, LE, GEL, GEL-E, GEL-UL, GEL-LE (Figure 3). The samples underwent proper lyophilization overnight and had their wavelength scanned respectfully between 400 - 4000 cm^{-1} wavelength. The presence of a hydroxyl group was found in the *P. emblica* extract with the wavenumber of 3348 cm^{-1} which is due to OH stretching vibration [48]. Wave numbers recorded between 1712 cm^{-1} , 1613 cm^{-1} and 1553 cm^{-1} all indicated the presence of double bonds (C=O, C=C and C=N), Aryl-substituted C=C and Conjugated C=C C=C-C Aromatic ring stretch [48]. Wavenumbers were also noted around 1448 cm^{-1} , 1344 cm^{-1} and 1212 cm^{-1} which translates as Aromatic primary amine, CN stretch Primary or secondary, OH in-plane bend Phenol, and C-O stretch [48]. 872 cm^{-1} , 764 cm^{-1} and 628 cm^{-1} wavenumbers were absorbed meaning a possibility of Vinylidene, C-H out-of-plane bend Peroxides, C-O-O- stretch Alkyne. C-H bend 680-610 cm^{-1} . The 1652 cm^{-1} band could indicate the existence of flavonoid stretching vibration of C=O and of C=C, asymmetric bending vibration of N-H. 1448 cm^{-1} could be related to CH₃, CH₂, flavonoids and aromatic rings [49].

2.6. In vitro release studies

The assessment of *P. emblica* extract release and transport through an acetate cellular membrane is a valuable indicator for gauging the bioavailability of the extract. The release of *P. emblica* extract from the

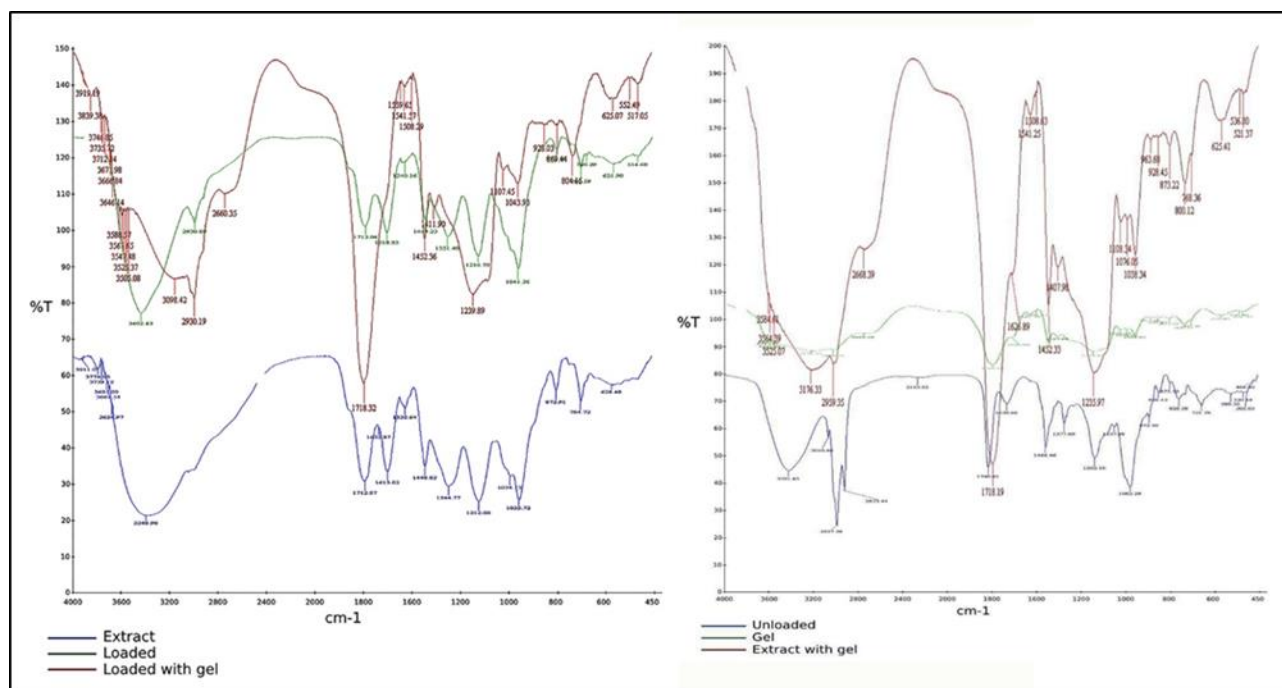


Figure 3. FT-IR spectra of UL, LE, GEL, GEL-E, GEL-UL, GEL-LE formulations.

formulation (GEL-LE and GEL-E) is notably influenced by the partition coefficient of the extract in GEL-LE and GEL-E formulations and the solubility of the phosphate buffer system at pH 6.4. A higher drug solubility in the external phase enhances its partition from the formulation, consequently increasing drug release [50]. To evaluate the antioxidant activity of the produced formulations the release studies by *in vitro* assay toward stable radical DPPH was done. The pharmacological effect of the extract primarily stem from the polyphenol compounds, which exhibit amphiphilic properties facilitating their antioxidant activity in both the aqueous and lipid phases [51]. The presence of these compounds capable of scavenging the stable radical DPPH in the receptor solutions signifies high release of the extract from the formulations. Therefore, DPPH was useful as a marker to detect the release of the extract and assess it in terms of antioxidant activity in the receptor solutions. The GEL-E and GEL-LE formulations loaded with 0.25 % *P. emblica* extract inhibited 18.84 ± 4.24 % and 42.28 ± 2.42 %, respectively, of DPPH radical activity (Figure 4). The GEL-LE formulation, which contained liposomal extract, demonstrated higher DPPH radical scavenging activity, deeper penetration, and a significant difference compared to the GEL-E formulation. The *in vitro* results of the scavenging activity of the formulations released through the artificial membrane towards DPPH confirm the sufficient release of polyphenolic compounds. Therefore, when applied to the skin, this product could effectively penetrate and provide beneficial effects.

2.7. Ex vivo permeation studies

The permeation studies for GEL-LE and GEL-E formulations were done through pig skin using Franz-diffusion cells. The *P. emblica* extract amount permeated versus time profiles of formulations was shown in Figure 5. The GEL-LE formulation showed better penetration through the pig skin thus yielding a relatively higher antioxidant amount. The amount was reached after 240 minutes with a total of $1540 \text{ ug} \pm 12.8$ per sample which is 21.5 ± 0.78 % of total cumulative drug release while on the other hand, the GEL-E formulation had an antioxidant amount of $1131 \pm 35 \text{ ug}$ after 300 minutes, that is 15 ± 3.09 % of total cumulative drug release. It was found that cumulative amount of *P. emblica* extract from GEL-LE formulation was significantly higher than the amount permeated by GEL-E formulation ($p < 0.05$). The improved permeation of *P. emblica* extract from GEL-LE formulation may be attributed to the high flexibility of liposomes, so they can penetrate the skin easily and overcome the barrier function by squeezing through the intracellular lipid of the stratum corneum [52].

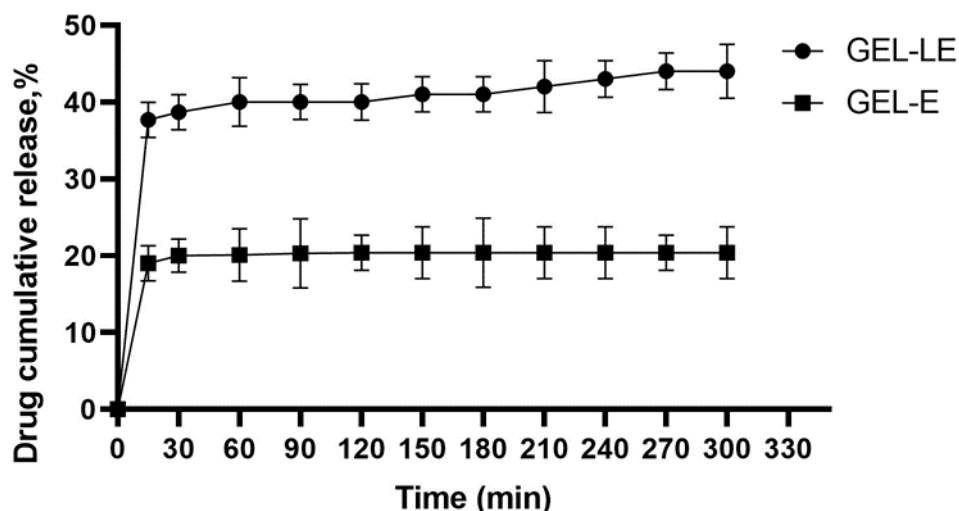


Figure 4. *In vitro* drug release comparison between GEL-LE formulation and GEL-E formulation. Graph expressed as total cumulative drug release% $\pm$ SD over time.

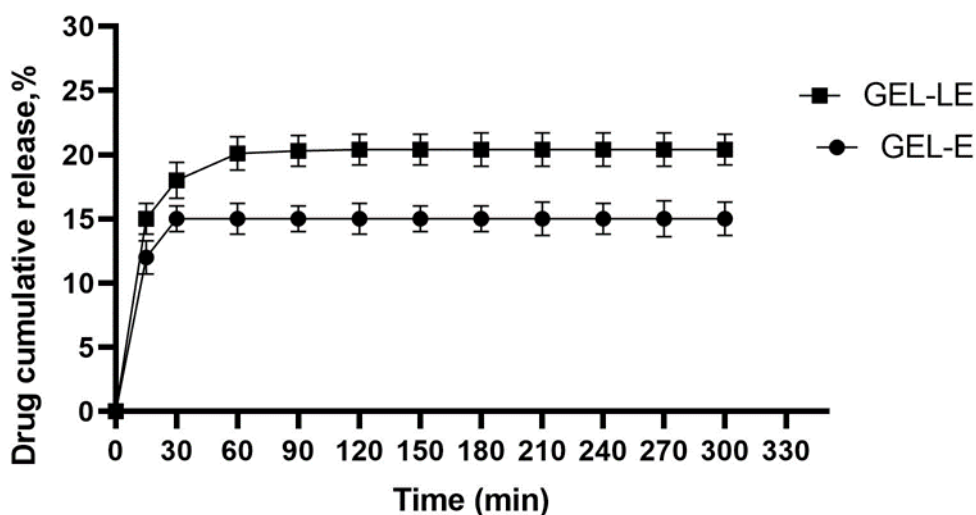


Figure 5. *Ex vivo* release study comparison between GEL-LE formulation and GEL-E formulation. Graph expressed as total cumulative drug release % $\pm$ SD over time.

3. CONCLUSION

This study is done to point to the potential of an innovative composite formulation based on liposomal *P. emblica* extract included in gels in terms of ease of administration and improved drug (antioxidants) bioavailability. Antioxidants are required to be underneath the stratum corneum inside the cells where they can function and perform their duty in combating the free radicals and creating an anti-aging effect. At the end of this research, we are provided with a functional anti-aging gel that is rich in antioxidants and encapsulated with nano lipid carriers that proved to have higher bioavailability than formulas that didn't.

4. MATERIALS AND METHODS

4.1. Materials

As a plant material, dried fruits of *P. emblica* were bought from a local market in Pakistan Punjab region. Soybean phosphatidylcholine (SPC), chloroform, methanol, ethanol, sodium hydroxide and sodium chloride were purchased from Sigma Aldrich (Munich, Germany). Cholesterol, copper (II) chloride, neocuproine, ammonium acetate, trolox, potassium bromide, quercetin, gallic acid, aluminum chloride, sodium hydroxide, DPPH (2,2-Diphenyl-1-picrylhydrazyl), iron trichloride, TPTZ (2,3,5-Triphenyltetrazolium chloride), sodium acetate were purchased from Merck-millipore (Munich, Germany). Potassium dihydrogen phosphate was purchased from Supelco, Darmstadt, Germany. Carbopol 934 was purchased from Serva (Heidelberg, Germany).

4.2. Extraction method

35 g of dried *P. emblica* fruit was ground using a laboratory mill (K3104, Arcelik, Istanbul, Türkiye). Powdered plant material was extracted with 500 mL of 50 % ethanol through an orbital shaker (IKA KS50, Staufen, Germany) set to 150 rpm for about 14 hours in a dark environment by the maceration method. After the maceration period, the ethanolic solvent was filtered by filter paper with 0.2 mm thickness and the ethanol portion was evaporated via a rotary evaporator (Heidolph, Schwabach, Germany) at 45 °C and 80 mbar pressure. The remaining water portion was frozen at -20 °C in the refrigerator, then it was lyophilized by freeze-drier (Christ, Germany) for 2 days at -50 °C and 0.3 mbar vacuum pressure conditions. Yield of the crude extract was weighed as 13 g (34 % of total weight), then poured inside an amber bottle for further light protection and stored at +4 °C throughout the study.

4.3. Design optimal experimental design

Using D-optimal mixture experimental design, the optimal liposomal formulation was found. D-optimal design was used to assess the effects of four independent variables on response variables, including mean particle size (MPS), zeta potential (ZP) and polydispersity index (PDI). The independent variables were the amount of SPC (A), cholesterol (B), chloroform (C). Previous studies were used to identify the parameter range for the current inquiry. In order to pick the best fitting model and improve the process, Design-Expert® (version 13, Stat-Ease Inc., Minneapolis, MN, USA) was utilized. Table 5 displays the variables and their levels that were employed in the design. The analysis of variance tables was performed, the effect and regression coefficients of the various linear models, as well as the correlations between the variables, were calculated. The experimental design resulted in 11 combinations (Table 5). After evaluating various statistical characteristics such as the coefficient of variation, the multiple correlation coefficient (R^2), and adjusted multiple correlation coefficient (adjusted R^2), the most suitable mathematical model was selected. The statistical significance of all terms in the polynomial was determined by calculating the F value with a significance set at $p < 0.05$. The numerical optimization was employed to optimize the fitted polynomials. By conducting experiments under the circumstances listed in Table 5, the ideal conditions were confirmed.

Table 5. Optimal design displaying component and response variables for the preparation of unloaded liposomes.

Sample No.	Independent Variables	
	A: Soybean phosphatidylcholine (%)	B: Cholesterol (%)
1	1.8	0.6
2	1.9	0.6
3	1.9	0.5
4	1.9	0.6
5	1.9	0.6
6	2.0	0.5
7	1.8	0.7
8	1.9	0.6
9	1.8	0.7
10	2.0	0.5
11	1.9	0.6

4.4. Liposomes preparation

Unloaded liposomes were produced by the Bangham method; the composition of the samples is presented in Table 5 [36]. Cholesterol (0.5-0.7 %) and soybean phosphatidylcholine (SPC) (1.8-2 % w/w) were dissolved in 10 mL of chloroform and methanol mixture (2:1) in the flask and then, the rotary evaporator (Heidolph, Germany) was set at a constant rotation speed and temperature of 40 °C to form a thin film. Once the organic phase was evaporated, the formulation was passed through nitrogen gas to prevent the oxidation risk of phospholipids. Then the thin film was dissolved in 10 mL of distilled water and the samples were all put into an ultrasonic probe sonicator device for 8 minutes, 70 % amplitude, x7 cycle [37]. Finally, the dispersed liposomal formulation was allowed to cool at room temperature (23 ± 0.5 °C) and stored at 4 °C. Loaded liposomes with *P. Emblica* extract preparation procedure. Loaded liposomes were prepared by the Bangham method. The optimal unloaded liposome formulation was used. The desired amount of *P. Emblica* extract was dissolved in organic solvent phase and the procedure was repeated as described in the unloaded liposome preparation part.

4.5. Characterization studies of liposomes

Mean particle size and polydispersity index (PDI) were determined using a Nano ZS 3600 instrument (USA) equipped with differential light scattering (DLS) technology. The DLS instrument was operated in zeta mode to obtain measurements of zeta potential. Zeta potential was measured using electro cuvettes.

4.6. Determination encapsulation efficiency

Encapsulation efficiency of *Phyllanthus emblica* extract active compounds' in liposome suspensions was determined using an indirect method. The calculation involved subtracting the amount of non-entrapped *Phyllanthus emblica* extract remaining in the supernatant from the total amount added to the loading solution. To obtain the supernatant, the liposome suspension was centrifuged (Spectra Por, Germany) at 10,000 rpm for 20 minutes. The number of polyphenols in the supernatant was then determined using the spectrophotometer method. Encapsulation efficiency was calculated according to the following formula [53]:

$$EE(\%) = \frac{qt - qu}{qt} \times 100 \quad (\text{Eq.1})$$

Where qu is the number of unloaded polyphenols (mg/mL) and qt is the total polyphenols quantity of taken (mg/mL)

4.7. Scanning electron microscopy (SEM) analysis

The morphology of the liposomes was analyzed using a scanning electron microscope (SEM) model Quattro S (Thermo Scientific, USA). Before the analysis, the samples were lyophilized and then coated with a 20 nm thickness of Au-18k using an Ion sputter mc1000 (Hitachi High Tech, USA). Carbon tape was employed to securely hold the lyophilized liposome powders in place on the SEM stub. Prior to capturing the final images, any excess powder was gently removed by tapping the stub. The SEM was operated at an accelerating voltage of 15.00 kV, and the surfaces of the prepared samples were coated with a thin layer of gold and palladium using a sputter instrument (LEICA EM ACE200, Leica Microsystems, Germany) at 3 kV for 60 seconds. SEM images were captured at various magnifications under high vacuum conditions [53].

4.8. Sample preparation for total antioxidants, phenols, and flavonoids

6 samples were prepared, 3 of them containing liposomes loaded with extract and the other 3 were liposomes loaded with extract mixed with gel formulation. All samples consisted of soybean phosphatidylcholine, cholesterol, and extract powder. The liposome formulations were prepared by Bangham method and followed the above mention procedure. Gel contained 2.6 g of carbopol and mixed with 100 mL of purified water and stirred with a magnetic stirrer. The required amount of gel was used to prepare the G1, G2 and G3 formulations. The composition of the formulations is shown in Table 6. Lastly, 8 mL of each sample were taken and mixed with 8 mL of methanol (1:1) poured inside eppendorf tubes centrifuged then filtered and stored inside vials and was used for further studies.

Table 6. Samples measurement that were used in total phenolic, flavonoid and antioxidant tests. (G: loaded liposomes with *P. emblica* extract and carbopol 934, L: loaded liposomes with *P. emblica* extract)

Sample	Cholesterol (mg)	Soybean (mg)	Purified water (mL)	Extract (mg)	Gel (Carbopol, 2.5 %)(mL)	Total size (mL)
G1	70 mg	180 mg	10 mL	250 mg	20 mL	30 mL
G2	140 mg	320 mg	10 mL	500 mg	20 mL	30 mL
G3	140 mg	320 mg	10 mL	750 mg	20 mL	30 mL
L1	70 mg	180 mg	10 mL	250 mg		10 mL
L2	140 mg	320 mg	10 mL	500 mg		10 mL
L3	140 mg	320 mg	25 mL	750 mg		25 mL

4.9. Total phenolic content

Total phenolic content of samples was evaluated according to the rapid assessment of Folin-Ciocalteu reducing capacity method of Magalhaes et al [54]. 50 μ L of water, 50 μ L of 10 % Folin-Ciocalteu reagent and 100 μ L of 0.35 M sodium hydroxide were added to microplates containing 50 μ L of either sample solution, 50 μ L of standard (gallic acid, 4-125 μ g/mL, $y=54.683x-0.0319$, $R^2=0.9969$) or blank (50 % ethanol). After 3 minutes, absorbance of the blue color formed was measured at 760 nm wavelength by a microplate reader (Thermo Fisher, Waltham, USA).

4.10. Total flavonoid content

50 μ L of either sample solution, standard (quercetin, 16-250 μ g/mL, $y=0.0421x-0.0442$, $R^2=0.9995$) or blank (50 % ethanol) were added inside each cell of the microplate then topped with 30 μ L of aluminum chloride, 30 μ L of 1 M sodium acetate and 130 μ L of water. Reaction was kept at room temperature for 30 minutes furthermore absorbance was measured at a 415 nm wavelength [55].

4.11. Total antioxidant content

Ferric Reducing Antioxidant Power Assay (FRAP): 20 μ L of sample solution, standard (Trolox, 4-128 μ g/mL, $y=0.1238x+0.0068$, $R^2 = 0.9993$) or blank (50 % ethanol) were mixed with 280 μ L FRAP reagent (2×10^{-2} M FeCl_3 , 1×10^{-2} M TPTZ and pH 3.6 sodium acetate buffer were mixed with a ratio of 1:1:10, respectively). After a 6 minutes incubation period, the absorbance of the blue color formed in the reaction was detected at a wavelength of 595 nm [56].

DPPH Radical Scavenging Assay: 280 μ L of 0.1 mM ethanolic DPPH solution (2,2- Diphenyl-1-picrylhydrazyl, abs. ~ 0.7) was added onto 20 μ L of sample solution, standard (Trolox, 6-250 μ g/mL, $y=0.0583x+0.003$, $R^2=0.996$), or blank (50 % ethanol). Reactions were kept for half an hour in a dark environment then the change in purple color at the beginning of the reaction was determined at a wavelength of 520 nm [57].

ABTS Radical Scavenging Assay: Initially 20 μ L of sample solution, standard (Trolox, 4-125 μ g/mL, $y=0.0741x+0.0179$, $R^2=0.9949$), or blank (50 % ethanol) were added to the microplate wells. The volume was completed to 300 μ L by adding 280 μ L of ABTS reagent consisting of 7×10^{-3} M of ABTS and 2.45×10^{-3} M of potassium persulfate. The lightening of the blue color in the reactions was found at a wavelength of 734 nm. [58].

CUPRAC: Cupric Reducing Antioxidant Capacity assay was conducted with some modifications of the study of Apak et al [59]. 280 μ L reagent consisting of 1×10^{-2} M copper (II) chloride, 7.5×10^{-3} M neocuproine and 1 M ammonium acetate (pH 7) was mixed with 20 μ L of sample solution, standard (Trolox, 8-128 μ g/mL, $y=0.0545x+0.0098$, $R^2=0.9994$) or blank (water). The solutions were incubated for half an hour in the dark and the intensity of the yellow color formed in the reactions was evaluated at 450 nm wavelength.

4.12. Preparation of loaded liposome-based gel

The polymer (Carbopol 934) at a concentration of 2.6 % was added to the distilled water and placed on a magnetic stirrer to ensure thorough mixing. To ensure proper hydration of the polymer and minimize the presence of air bubbles, the formulation was left overnight. Subsequently, the pH was adjusted to a range of 5.5-6.5 by using triethanolamine. The composition of the formulations is presented in Table 7.

Table 7. Formulation ingredients of gels (g, w/v)

Formulation codes	<i>Phyllanthus emblica</i> extract (mg/30 mL)	SPC (mg/30 mL)	Cholesterol (mg/30 mL)	Carbopol 934 (g/100 mL)
UL	-	180	70	-
LE	250	180	70	-
GEL	-	-	-	2.6
GEL-E	250	-	-	2.6
GEL-UL	-	180	70	2.6
GEL-LE	250	180	70	2.6

UL: unloaded liposome; LE: loaded liposomes with *P. emblica* L. extract; GEL: Carbopol 934 gel; GEL-E: *P. emblica* extract in Carbopol 934 gel; GEL-UL: unloaded liposome in Carbopol 934 gel; GEL-LE: loaded liposomes with *P. emblica* extract in Carbopol 934 gel.

4.13. Fourier transform infrared (FTIR) analysis

The interactions between the formulation components were evaluated by FT-IR (Nicolet iS50 FT-IR, Thermo-Fischer, USA). All 6 samples were prepared and freeze-dried before conducting the analysis samples were as follows (UL, LE, GEL, GEL-E, GEL-UL, GEL-LE). Using Fourier transform infrared at a wavenumber between 400 and 4000 cm⁻¹ the spectrum was measured. Samples were cut using a scalpel blade and were added to the pellet mixed and ground with potassium bromide. It was then added to the crystal where the absorbance was measured [60].

4.14. Rheological behavior estimation of the formulation

Rheometer was used to further study to determine the rheological properties and behaviors of the formulations (GEL-E, GEL-UL, GEL-LE). The rheometer of the Couette type with C18 concentric cylinders was used. The sample total volume was around 2 mL. Formulations were added to the surface of the sample to prevent any further evaporation of the solvent. Extended shear analysis of each formulation was conducted. In shear rate ramp mode and using parallel steel plate (CP4/40) (0.8 mm of the gap). Flow curves were measured ranging from 0.1 s⁻¹ to 100 s⁻¹. The measurements were done at 25.0 ± 0.5 °C and 37 ± 0.5 °C. To ascertain the maximum strain amplitude for the gel, also strain-controlled measurements were conducted on each sample. The three-dimensional network of the gel was destroyed over a specific strain amplitude. As a result, measurements above this point do not capture the physical characteristics of a gel. The greatest strain was used for all subsequent rheological property measurements.

4.15. *In vitro* release studies

In vitro release of *P. emblica* extract from the GEL-E and GEL-LE gel formulations was evaluated using a Franz diffusion cell system. Franz cell diffusion method was conducted for this test, 6 cells were used 3 for each sample, the samples of choice were 2. The release membrane used in this experiment was artificial cellulose acetate (Spectra / Por Regenerated Cellulose, Molecular weight cut off 8-10 kDa). The diffusion area was 5.29 cm² and the volume of the receiving phase was 20 mL of 0.9 % isotonic solution of NaCl pH (5.8) with a temperature of (37 ± 0.5°C) using a circulating water bath. 2 g of each sample each containing 0.25 mg of *P. emblica* extract (0.05 % w/w) was placed on the membrane respectfully covered with parafilm, injectors were used to draw 1 mL from the receiving phase in these following time intervals (5 min, 15 min, 30 min, 60 min, 120 min, 180 min, 240 min and 300 min). Subsequently, the solutions within the receptor compartments were subjected to assessment for their antioxidant activity using DPPH method. The experiment was carried out in triplicate and the results are expressed as the mean ± standard deviation [61].

In vitro radical scavenging activity test was done by above mention method in materials and methods in total antioxidant content section.

4.16. *Ex vivo* skin permeation studies

The pig ears were sourced from Acibadem Experimental Research Center (Acibadem University, Istanbul, Turkey) within a few hours after the animals were euthanized. The full-thickness skin of the dorsal side was carefully separated from the underlying cartilage using a scalpel. All skin samples were disinfected with ethanol and washed carefully with distilled water then wrapped in aluminum foil and stored at - 80 °C for further use [62].

In vitro release of *P. emblica* extract from the GEL-E and GEL-LE gel formulations was evaluated using a Franz diffusion cell system as mentioned in the *in vitro* release studies section.

4.17. Statistical analysis

The software program Minitab 17 was used to evaluate the results and statistical significance of multiple outcomes and findings in this research. The results were determined by the use one-way analysis of variance and the order of significance was determined using the Tukey pot-hoc test. Most methods were done in this research in form of triplicates and results were given as mean $\pm$ SD. Concerning significant levels it was defined as $p \leq 0.05$ with CI of 95%.

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