Olive Leaves (*Olea europaea* L) Extract Loaded Lipid Nanoparticles: Optimization of Processing Parameters by Box-Behnken Statistical Design, *in-vitro* Characterization, and Evaluation of Anti-oxidant and Anti-microbial Activity

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Abstract: The present study was aimed to prepare and evaluated solid lipid nanoparticles (SLNs) of olive leaves extract powder (OLP) which contained many anti-oxidant and antimicrobial agents like oleuropein, a natural polyphenol. The major issue concern OLP was the instability due to environmental conditions and hence compromised bioactivity. To overcome this problem, SLNs were designed by hot homogenous followed by sonication technique to protect the drug and improve its antioxidant and antimicrobial activity. Lipids like compritol 888ATO and surfactant like tween 80 were used for the development and stabilization of SLNS and optimization was done by Box-Behnken statistical design (3x3). The optimized batch (F9) showed particle size, entrapment efficiency, PDI, and zeta potential 277.46 nm, 80.48%, 0.275, and −23.18 mV respectively. Optimized formulation (F9) exhibited a sustained release pattern up to 24 h with first-order release kinetic ($R^2 = 0.9984$) and the mechanism of drug release was found to be Fickian diffusion type ($n = 0.441$). Upon the stability study, it could be found that SLNs formulation was stable. Anti-oxidation and anti-microbial studies were conducted on optimized formulation and findings suggested that SLNs showed an improved radical scavenging activity and anti-microbial activity against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria. Finally, it was concluded that developed SLNs were able to protect and suitable for the delivery of OLP.

Key words: *Olea europaea*, oleuropein, solid lipid nanoparticles, olive leaves extract, anti-oxidant activity, anti-microbial activity

1 Introduction

The Dicotyledons Oleaceae family consists of about 30 genera of deciduous plants including olive trees and it has related around 600 species. Oleaceae members are well grown in topical and sub-temperate reasons of Asia. Olive tree (*Olea europaea*) oil obtained from different parts like fruit, leaves, etc. has many health benefits upon regular consumption. Traditionally it was used as a folk medicine for remedying various types of fevers and other problems associated with body.

Olive leaves extract exhibited several therapeutics activities like anti-hypertensive, hypoglycemic, antimicrobial, hypouricemic, etc. Anti-viral activity against HIV-1 infection has been demonstrated and reported. In addition, oleuropein (a typical secoiridoid) obtained from the olive plant exhibited hypocholesterolic and hypoglycemic activity with potent anti-oxidant and as well as anti-inflammatory activity. Degradation product of oleuropein i.e. hydroxytyrosol also exhibited all above mention activity along with potential free radical scavenging action. Association all these therapeutic activity concerned to olive plant makes it an important contributor in the field of health.

In this project, the authors are concerned with the anti-microbial and anti-oxidant properties of extract of Olive leaves. The anti-microbial activity of the olive extract is
due to p-hydroxybenzoic acid, cyclotrisiloxane hexamethyl, cycloitetrasiloxane octamethyl, and cyclopentasiloxane decamethyl, vanillic, caffeic, protocatechue, syringic, gallic acid, etc. Other ingredients like oleuropein, quercetin, tyrosol, and elenolic acid are also responsible for anti-microbial activity. The antimicrobial potential of all these substances has been reported previously by several researchers against bacteria, yeast, fungi, viruses, retroviruses, and other parasites.

Dietary intake of natural anti-oxidant sources like fruits, leaves have been gained a lot of attention and showing a positive effect on the health of the human being. Results of epidemiological studies explored that the intake of polyphenol-rich food reduces the incidence of coronary artery disease. The CHD like MI (myocardial infarction), IS (ischemic stroke), etc are associated with atherosclerosis. The recent studies explored that oxidative damage is an important etiological factor for the progress of atherosclerosis. Especially, according to the oxidative stress theory, oxidative modification of low-density lipoprotein is thought to play a key role in the development of atherosclerosis. Therefore, the inhibition of such a process is considered to be an important therapeutic approach.

Various important constituents like Oleuropein (hydroxytyrosol, tyrosol), verbascoside (also known as acteoside or kusaginin), ligstroside, apigenin-7-glucoside, diosmetin-7-glucoside, luteolin, catechin, were obtained for the leaves of olive and exhibiting anti-oxidant activity as reported previously. Hydroxytyrosol is the principal olive leaves oleuropein that has patent anti-oxidant property.

Besides this activity, olive leaves extract exhibited other pharmacological activities like anti-inflammatory, anti-cancer, anti-viral, hypoglycemic, hypolipidemic, anti-platelet aggregation, etc. An agency like EFSA (European Food Safety Agency) and EMA (European Medicine Agency) gave their assessment about the therapeutic applicability of olive leaves extractants. Along with the major applicability, the extraction products of olive leaves are suffered from instability problems in atmospheric conditions (temperature, light and oxygen) as well as biological conditions and hence it exhibited poor bioactivity. To improve the stability, various nano based formulation have been developed to explore the various activity of olive leaves extract. These formulations include nano-emulsion, spray dry product, electrostatic extrusion, bipolar complexes, nano-liposomes, inclusion complexes, micro-encapsulation, PLA nanoparticles, and NLCs. Various problems are associated with these formulations like low yield value, time-consuming procedure, compromised entrapment efficiency, residual solvent contamination, high particle size. So, to regulate these disadvantages, lipid nanoparticles came into existence. Lipid nanoparticles especially solid lipid nanoparticles offer many advantages like better entrapment efficiency, controlled particle size, biocompatibility, biodegradability, and ease of preparation. Moreover, lipids used for the preparation of SLNs have some synergistic action on anti-inflammatory activity along with the entrapped drug. To date, no formulation based on solid lipid nanoparticles for exploring its anti-microbial and anti-oxidant properties as well to improve the stability of olive leaf extract product has been developed.

Therefore, the research work was aimed at the development of solid lipid nanoparticles (SLNs) for Olive leaves (Olea europaea L.) extract powder (OLP). The active principals were separated by the extraction of olive leaves using ethanol. The solvent was removed by drying to get the dry powder product. Olive leaves extract powder was incorporated into SLNs and optimization was done by expert design software using Box-Beanken design. Three factors like a drug to lipid ratio, surfactant concentration and homogenization speed were taken as independent parameters and factors like particle size, entrapment efficiency, and polydispersity index (PDI) were selected as dependent parameters. Optimized formulation was evaluated for morphological study, DSC study, in-vitro release study, and stability study. Finally, antimicrobial and anti-oxidation activities were evaluated to check the anti-microbial and anti-oxidant potential of developed SLNs formulation.

2 Materials and Methods

Olive leaves were obtained from the local garden. Fresh leaves were dried and powdered for extraction. Various lipids like precirol ATO 5, compritol 888 ATO were procured from Gattefosse (Germany). Glyceryl monostearate (GMS), palmitic acid, stearic acid were purchased from central drug house ltd (New Delhi, India). 2, 2-Diphenyl-1-Picryl hydrazyl-hydrate free radicals were procured from Merck (Darmstadt, Germany). Ascorbic acid, potassium dihydrogen phosphate, ammonium acetate, methanol, sodium hydroxide, poloxamer 188, ethanol, nutrient agar, dialysis bag (M. Wt cut off 12,000 kD) were procured from Sigma Aldrich (St Louis, USA). All other chemicals used for the study are analytical grade.

2.1 Extraction of phenolic components

Fresh leaves of olive were dried and powdered for extraction. The active principals were extracted from the power with the help of solvent (ethanol:water, 4:1 v/v). A sufficient quantity of powder (250 mg) was taken in the extraction flask and the solvent was added (1000 mL) ethanol: water, 4:1 v/v) and the mixture was continuously agitated for the duration of 24 h. After this step, the physical
mixture was filtered and the solvent was evaporated to dryness at 40°C to get the powder.

2.2 Preparation and optimization of SLNs: Preliminary screening study and pre-optimization of formulation variables

For the selection of suitable lipid, surfactant, homogenization speed & time, sonication time, etc., preliminary studies were conducted. The selection of suitable lipid depends upon the drug solubility in lipid and selection of surfactant is depending upon the solubility of lipid in surfactant.

The concerned lipid (100 mg) was melted above its melting points (around 10°C above) in the vial followed by the incorporation of the drug (OLP) with continuous shaking. The appearance of light pale color indicates the endpoint. Similar experiments were performed for the selection of suitable surfactants.

2.3 Experimental design

In the present study, BBD of expert design software with three factors and three levels was applied for optimization purposes. In the study, three factors like a drug to lipid ratio (A, 1:3-1:6), surfactant concentration (B, %, 1.5-4.5%), and homogenization speed (C, rpm, 3000-6000 rpm, for two h) were taken as independent parameters and particle size (nm, Y1), entrapment efficiency (% Y2) and PDI (Y3) were taken as dependent parameters. The aim of applying this design was to optimize the above said three independent parameters and to achieve the optimum particle size, maximum entrapment efficiency, and least possible PDI. The data were fitted in the expert design software for BBD. Total 17 batches were generated with 5 batches having a similar composition (five center points). The second-order polynomial equation was used to demonstrate the influence of various independent parameters on particle size, entrapment efficiency as well on PDI.

2.4 Development of SLNs

OLP-loaded SLNs were prepared by hot homogenization followed by sonication method. The composition of different OLP-loaded SLNs batches is given in Table 1. First of all, the lipid (compritol 888 ATO) was melted around 10°C of its melting point and the desired quantity of OLP was added (1:3-1:6 drug to lipid ratio). Meanwhile, the required quantity (1.5-4.5%) of surfactant was dissolved in deionized water and the temperature of this phase was maintained the same as that of the lipid phase. The aqueous phase was then incorporated in the lipid phase gradually and subjected to hot homogenization at variable speed ring (rpm, 3000-6000 rpm, two h) to get the course SLNs dispersion. The obtained emulsion was allowed to

| FC  | D:L | SC (%) | HS (rpm) | PS (nm) Actual | PS (nm) Predicted | EE (%) Actual | EE (%) Predicted | PDI Actual | PDI Predicted |
|-----|-----|--------|----------|---------------|------------------|--------------|-----------------|------------|---------------|
| F1  | 1:3 | 1.5    | 4500     | 237.05        | 237.17           | 60.55        | 60.73           | 0.307      | 0.305         |
| F2  | 1:6 | 1.5    | 4500     | 259.57        | 260.10           | 86.46        | 86.49           | 0.379      | 0.363         |
| F3  | 1:3 | 4.5    | 4500     | 112.35        | 111.82           | 50.17        | 50.13           | 0.315      | 0.310         |
| F4  | 1:6 | 4.5    | 4500     | 222.07        | 221.95           | 69.35        | 69.17           | 0.402      | 0.418         |
| F5  | 1:3 | 3.0    | 3000     | 187.46        | 187.56           | 63.65        | 63.66           | 0.258      | 0.247         |
| F6  | 1:6 | 3.0    | 3000     | 251.37        | 251.06           | 83.51        | 83.16           | 0.339      | 0.332         |
| F7  | 1:3 | 3.0    | 6000     | 134.27        | 134.58           | 51.57        | 51.41           | 0.348      | 0.346         |
| F8  | 1:6 | 3.0    | 6000     | 204.24        | 204.15           | 76.73        | 76.72           | 0.429      | 0.413         |
| F9  | 1:4.5 | 1.5 | 3000     | 277.46        | 277.25           | 80.48        | 80.29           | 0.275      | 0.282         |
| F10 | 1:4.5 | 4.5 | 3000     | 185.12        | 185.55           | 63.25        | 63.27           | 0.352      | 0.366         |
| F11 | 1:4.5 | 1.5 | 6000     | 217.79        | 217.36           | 67.91        | 67.89           | 0.432      | 0.423         |
| F12 | 1:4.5 | 4.5 | 6000     | 145.35        | 145.56           | 56.79        | 56.98           | 0.388      | 0.379         |
| F13 | 1:4.5 | 3.0 | 4500     | 193.67        | 193.68           | 70.01        | 69.79           | 0.328      | 0.317         |
| F14 | 1:4.5 | 3.0 | 4500     | 192.68        | 193.68           | 69.48        | 69.79           | 0.328      | 0.317         |
| F15 | 1:4.5 | 3.0 | 4500     | 197.35        | 193.68           | 71.23        | 69.79           | 0.328      | 0.317         |
| F16 | 1:4.5 | 3.0 | 4500     | 192.79        | 193.68           | 70.18        | 69.79           | 0.328      | 0.317         |
| F17 | 1:4.5 | 3.0 | 4500     | 191.89        | 193.68           | 70.00        | 69.79           | 0.328      | 0.317         |

FC = Formulation code, D:L = drug to lipid ratio, SC = surfactant concentration, HS = homogenization speed, PS = particle size, EE = entrapment Efficiency, PDI = polydispersity index
cool and then sonicated for 10 min at 100% amplitude with the aid of a sonicator (Probe type, Vibra-Cell® VCX 130; Sonics, CT, USA) to obtain the final SLNs dispersion. The dispersion was collected in a glass vial and stored in a refrigerator for further study. Similarly, blank SLNs were developed without API (drug).

2.5 Characterization of OLP-SLNs

2.5.1 Evaluation of particle size, PDI, and zeta potential

Photon Correlation Spectroscopy (PCS) using a zeta sizer machine (Malvern, nano ZS 90, Malvern Instruments, UK) was used for the measurement of the average particle size and polydispersity index (PDI) of various batches. The temperature was kept at 25°C and the scattering angle was set at 90°. Deionized water was used for the dilution of original OLP-SLNs dispersion. Zeta potential which indicates the surface charge was determined with the help of the same instrument with electric field strength around 20 V/cm.

2.5.2 Morphological study

Transmission electron microscope (TEM, FEI electron optics, Japan) using copper grid coated carbon was used to examine the morphology and shape of prepared OLP loaded SLNs (optimized batch F9). Negative staining with phosphotungstic acid stain (2% w/w, duration 20-30 seconds) was performed to stain the OLP-SLNs sample followed by drying at room temperature. Finally, the sample was detected by TEM.

2.5.3 Evaluation of entrapment efficiency

Entrapment efficiency (EE) was determined by examining the un-entrapped amount of drug present in the OLP-SLNs dispersion. The centrifugation technique was used for this purpose. A measured quantity (10 mL) of OLP-SLNs was taken in a centrifuge tube and allowed to sediment with the help of a cooling centrifuge at 12000 rpm for 15 minutes. The presence of an un-entrapped drug was analyzed by a UV-visible spectrophotometer (Model 1800, Shimadzu Japan) at 230 nm. The percent drug entrapment was determined by the following formula (equation 1):

\[
EE(\%) = \frac{\text{Entrapped drug (OLP)}}{\text{Total amount of OLP (Entrapped + unentrapped drug)}} \times 100
\]  

(1)

2.5.4 Differential scanning calorimetry (DSC) study

The purpose of this study was to evaluate the thermal behavior of various formulations viz pure OLP, compriot 888 ATO, and optimized OLP-SLNs formulation (F9) using differential scanning calorimetry (Mettler, Toledo, USA). Each sample was packed in an aluminum pan and scanned at the temperature range of 20-350°C (rate 10°C/min) using an empty sealed pan as a reference in an inert atmosphere (nitrogen). DSC curves were obtained and interpreted.

2.5.5 Drug release study (%)

The drug release study of optimized batch (F9) was done by Franz diffusion cell using dialysis membrane. Before application of dialysis membrane, it was developed by treating 0.35% w/v sodium sulfite solution at 80°C for 1-2 min followed by acidification with H₂SO₄ (0.2%, v/v) and then stored for 12 h in distilled water. Saline phosphate buffer (pH 7.4) was filled in the receptor compartment and SLN dispersion (9, 1 mL) was taken in the donor compartment. The experiment was performed at 37 ± 0.5°C with continuous stirring (50 rpm). Aliquots (1 mL) were taken from the receptor compartment at predetermined time intervals and the amount of drug present in each sample was detected by the UV-visible spectrophotometer at 230 nm.

The suitable release kinetic from optimized SLNs was detected by fitting the release data into zero, first, Higuchi model and Korsmeyer-Peppas model as given the Table 2 and value of R² (Correlation coefficient) was determined. The model with the highest R² value was considered as an optimized model.

2.5.6 Stability evaluation

The stability of optimized formulation (F9) was examined as per the International Council for harmonization (ICH) guidelines. SLN dispersion was taken in three separate glass vials (10 mL each). Among the three vials, one was stored in the refrigerator (4°C ± 2°C), the second at room temperature (25 ± 2°C/60 ± 5% RH), and the last one was stored in a stability chamber (Thermo Scientific, Sweden) at 40 ± 2°C/75 ± 5% RH. At a specific time point viz 1, 3, 6 months, the samples were taken out and examined for certain parameters like particle size, entrapment efficiency, zeta potential & PDI, etc, were determined and compared statistically with zero time data.

2.5.7 Antioxidant activity

One of the most useful methods for the detection of antioxidant potential is 2,2-Diphenyl-1-Picryl-Hydrazyl-Hydrate free radicals (DPPH) method. Radical scavenging activity (i.e., analyzing their ability of scavenging DPPH free radicals) of OLP-SLNs was evaluated spectrophotometrically at 517 nm. Antioxidant activity of OLP, as well as OPL-SLNs, was determined with the help of (DPPH) method. Solution of DPPH was prepared (0.1 mM) in ethanol. OLP-SLNs (equivalent to 0.2 mg/mL of OLP) were dispersed in phosphate buffer and left for 24 h to release the drug. An equivalent concentration of OLP (0.2 mg/mL) and blank SLNs was also prepared in the same medium. In this study, ascorbic acid of concentration 0.2 mg/mL was taken as a standard and while DPPH solution was used as a control. 3300 µL DPPH was mixed in each solution of OLP-SLNs (500 µL), blank SLNs, and OLP solution (500 µL). Each reaction mixture was kept on a shaker water bath at 37 ± 0.5°C for 30 min under protected conditions from the light. Finally, the absorbance of each sample was determined by a UV-visible spectrophotometer (Shimadzu 1800, Japan) at 517 nm using ethanol was used as a blank. Each experiment was evaluated three times and values are presented as mean ± SD. The value of anti-oxidant activity in the form of % DPPH scavenging activity was determined by the following formula (equation 2):

\[
\text{Antioxidant activity (%) = } \frac{A_0 - A_t}{A_0} \times 100
\]  

(2)
% DPPH scavenging activity =
\[
\frac{\text{Absorbance of DPPH solution} - \text{absorbance of test solution}}{\text{Absorbance of DPPH solution}} \times 100 \ (2)
\]

2.5.8 Anti-microbial study

The antimicrobial study of OLP-loaded SLNs (optimized batch F9) was conducted by utilizing the agar well diffusion method. Bacterial strains like *Staphylococcus aureus* (Gram’s positive) and *Pseudomonas aeruginosa* (Gram’s negative) were used for the evaluation \(^9\). Nutrient agar plates were prepared under sterile conditions and bacterial suspension (100 µL, concentration around \(10^6\) CFU/mL) of each bacterium was placed individually on each sterile nutrient agar plate with the help of sterile swabs incubated at 37 ± 0.5°C for 1 h. Four wells around 8 mm diameters each were produced on each plate with the help of a sterile cork borer. Here, blank SLNs, positive control (pure extract), physical mixture (OLP extract + blank), and SLNs and optimized OLP-SLNs preparation (100 µL) were used for the antimicrobial study and placed in each well. Plates were incubated at a temperature of 37 ± 0.5°C. At predetermined time points (6, 12, 24 h), the plates were withdrawn and the zones of inhibition around the walls were measured in mm with the help of a caliper.

### 3 Results and Discussion

#### 3.1 Preliminary screening and pre-optimization of formulation variables

The highest solubility of OLP was found to be in compritol 888 ATO as depicted in Fig. 1. The descending order of OLP solubility/100 gm in different lipid was compritol 888 ATO (36.93 ± 4.92 mg) >Precirol ATO 5 (29.37 ± 3.01 mg) >GMS (23.84 ± 2.73 mg) >palmitic acid (21.41 ± 2.81 mg) >stearic acid (16.25 ± 1.65 mg). Compritol 888 ATO had been used as a lipid for the preparation of oral-based SLNs for the preparation SLNs \(^2\).

Based on the results of the preliminary screening study, tween 80 and compritol 888 ATO were selected as a surfactant and lipid, respectively. Other parameters like a drug to lipid ratio (1:3-1:6), surfactant concentration (%), 1.5-4.5%), and homogenization speed (rpm, 3000-6000 rpm), homogenization time (2 h), sonication time (10 min) were decided by conducting the preliminary experiments. Factors like a drug to lipid ratio (A), surfactant concentration (B), and homogenization (C) were again optimized by using response surface methodology (RSM) in conjunction with 3-factors and 3-levels BBD, and their influence was detected on dependent factors viz particle size (Y1), entrapment efficiency (Y2) and PDI (Y3).

#### 3.2 Preparation of SLNs and optimization of OLP loaded SLNs

The values of pre-optimized parameters as described above were fitted to BBD of expert design. The composition of the total of seventeen formulations was explored by BBD with 5 center points. All formulations were developed and values of dependent parameters like particle size, entrapment efficiency, and PDI were examined and further fitted to BBD to get the final result. Polynomial equations and 3D graphs were generated showing the influence of independent factors on dependent factors. The positive sign of the polynomial equation explored a positive effect and the negative sign indicated a negative effect on dependent variables. Value of actual and predicted dependent variables regression analysis and Analysis of variance data of models is presented in Tables 1, 2, and 3 respectively. The quadratic model was considered as the best-fitted model for all responses as in this case the highest value of regression coefficient was observed.

#### 3.3 Effect of certain parameters on particle size (Y1)

Following polynomial equation was obtained from the BBD indicating the effect of different independent factors on particle size (Y1):

\[
Y_1 = +193.68 + 32.26A - 40.87B - 24.97C + 21.80AB + 1.49AC + 4.97BC + 0.99A^2 + 13.09 B^2 - 0.34C^2 \ (3)
\]

The above-given equation exhibited that compritol 888 ATO showed a positive effect on the particle size while tween 80 and homogenization exhibited a negative influence on the particle size.

Here, variables A, B, C, AB, BC, and \(B^2\) had a significant effect on the particle size. At 95% confidence interval, the lack of fit was insignificant (\(p > 0.05\)) while the remaining parameters were found to be significant (\(p < 0.0001\)) with adequate precision (\(>4\)) (Table 3). Based on the \(R^2\)
Table 2  Mathematical model used for detection of release kinetic.

| S. No. | Kinetic model       | Equation                              | $R^2$ |
|--------|---------------------|---------------------------------------|-------|
| 1      | Zero order          | $M_0 - M = kt$                       | 0.8522|
| 2      | First order         | $\ln m = kt$                         | 0.9984|
| 3      | Higuchi’s model     | $M_0 - M = kt^{1/2}$                 | 0.9704|
| 4      | Korsmeyer-Peppas model | $\log (M_0 - M) = \log k + n \log t$ | 0.9869, n=0.441 |

$M_0 =$ initial drug content, $M =$ drug content at time $t$, $n$ is the release exponent.

Table 3  ANOVA of quadratic model for responses of developed SLNs.

| ANOVA results | Particle size (nm) (Y1) | Entrapment efficiency (%) (Y2) | PDI (Y3) |
|---------------|-------------------------|-------------------------------|----------|
| Regression    |                         |                               |          |
| Some of square | 29948.34             | 1657.03                       | 0.038    |
| Degree of freedom | 9                  | 9                              | 9        |
| Mean square   | 3327.59                | 184.11                        | $4.247 \times 10^{-3}$ |
| F-value       | 1180.59                | 842.92                        | 1189.13  |
| $P$           | $< 0.0001$            | $< 0.0001$                    | $< 0.0001$ |
| Influence     | Significant            | Significant                    | Significant |
| Lack of fit test |                     |                               |          |
| Some of square | 1.27                   | 1.14                          | $2.500 \times 10^4$ |
| Degree of freedom | 3                   | 3                              | 3        |
| Mean square   | 0.42                   | 0.38                          | $8.333 \times 10^{-4}$ |
| F-value       | 0.091                  | 3.94                          | 4.672    |
| $P$           | 0.9610                 | 0.1093                        | 0.011    |
| Influence     | Non-significant        | Non-significant                | Non-significant |
| Residual      |                         |                               |          |
| Some of square | 19.73                  | 0.78                          | $2.500 \times 10^4$ |
| Degree of freedom | 7                   | 7                              | 7        |
| Mean square   | 2.82                   | 0.11                          | $3.571 \times 10^{-5}$ |

Table 4  Statistics model summary of regression analysis results for response Y1, Y2 and Y3.

| Model             | R$^2$ | Adjusted R$^2$ | Predicted R$^2$ | SD  | % CV | Remark |
|-------------------|-------|----------------|-----------------|-----|------|--------|
| Particle size (Y1)|       |                |                 |     |      |        |
| Linear            | 0.9078| 0.8865         | 0.8143          | 14.58|      |        |
| 2F1               | 0.9748| 0.9597         | 0.9012          | 8.68 |      |        |
| Quadratic         | 0.9996| 0.9985         | 0.9984          | 1.68 | 0.84 | Suggested |
| Entrapment efficiency (Y2)|       |                |                 |     |      |        |
| Linear            | 0.9625| 0.9538         | 0.9291          | 2.12 |      |        |
| 2F1               | 0.9824| 0.9719         | 0.9411          | 1.71 |      |        |
| Quadratic         | 0.9991| 0.9979         | 0.9886          | 0.47 | 0.68 | Suggested |
| PDI (Y3)          |       |                |                 |     |      |        |
| Linear            | 0.7981| 0.7515         | 0.6193          | 0.024|      |        |
| 2F1               | 0.9112| 0.8579         | 0.7179          | 0.018|      |        |
| Quadratic         | 0.9993| 0.9985         | 0.9995          | 0.002| 0.55 | Suggested |
Olive Leaves (Olea europaea L) Extract Loaded Lipid Nanoparticles

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(0.9996) value, the quadratic model was considered as the best-fitted model with adequate signal (Table 4).

The particle size of different batches was found to be in the range of 112.35-277.46 nm. If other variables were maintained constant, the particle size was increased on enhancing the drug to lipid ratio (Table 1, batch F1 237.05 nm and F2 259.57 nm). This might be due to the aggregation of the particles due to the insufficient surfactant to disperse the particles. On the other hand, surfactant (1.5-4.5%) exhibited a negative effect on the particle size (Table 1, batch F1 237.05 nm and F3 112.35 nm). This might be due to a decrease in interfacial tension between the aqueous and lipid phase which prevents particle aggregation. Homogenization speed exhibited a negative impact (Table 1, batch F5 187.46 nm and F7 134.27 nm) on the particle size due to the generation of high force which breaks the particles and hence reduces the particle size. Equation 3 indicated that the effect of surfactant (coefficient value -7.23) had a more prominent effect on particle size as compared to homogenization speed (coefficient value -5.76). Figure 2 explored the influence of various independent parameters on particle size.

3.4 Effect of certain parameters on entrapment efficiency (Y2)

Following polymer equation was obtained from BBD showing the influence of different independent parameters on the entrapment efficiency:

\[
Y_2 = +69.99 + 12.02A -7.23B - 5.76C - 1.93AB + 1.70AC + 1.28BC - 0.74A^2 - 2.37B^2 - 0.26C^2
\] (4)

Here, variables A, B, C, AB, AC, BC, A², and B² had significant influence on the entrapment efficiency. At 95% confidence interval, the lack of fit was insignificant (p < 0.05) while remaining parameters were found to be significant (p<0.0001) with adequate precision (>|4|) (Table 3). The quadratic model (R² = 0.9991) was considered as the best-fitted model with adequate signal (Table 4).

From the above equation 4, it is observed that lipid (compritol 888 ATO) exhibited a positive effect while the remaining two parameters exhibited a negative influence on entrapment efficiency. The surfactant (coefficient value -7.23) had a more prominent effect as compared to homogenization speed (coefficient value -5.76). The positive effect of lipid on entrapment efficiency (Table 1, batch F1 and F2) was due to the availability of more lipid for the accommodation of available drugs. A variable effect was observed on entrapment efficiency. At the initial stage, on increasing the surfactant, the value of entrapment efficiency was increased but on further increasing the surfactant, it was decreased due to leakage of the drug in the external environment (Table 1, batch F1 and F3). Homogenization speed exhibited a negative effect on entrapment efficiency due to more shear force which might responsible for the expulsion of the drug (Table 1, batch F5 and F7). Figure 3 explored the effect of various independent parameters on entrapment efficiency.
3.5 Influence of independent parameters on PDI \( Y_3 \)

Following polynomial equation showing the influence of various parameters on the PDI:

\[
Y_3 = +0.33 + 0.046A + 0.014B + 0.048C + 0.010AB + 7.500E - 0.03AC - 0.037BC + 5.000E - 0.03A^2 + 0.025B^2 - 0.017C^2
\] (5)

Here, variables like A, B, C, AB, AC, BC, \( A^2 \), and \( C^2 \) had a significant effect on the PDI. At 95% confidence interval, the lack of fit was insignificant \( (p > 0.05) \) while the remaining parameters were found to be significant \( (p < 0.0001) \) with adequate precision \( (>4) \) (Table 3). The quadratic model \( (R^2 = 0.9991) \) was considered as the best-fitted model with adequate signal (Table 4).

From equation 5, it is stated that all the three independent parameters like a drug to lipid ratio, surfactant, and homogenization speed had a positive effect on the PDI. The dominant effect was due to homogenization speed (coefficient value 0.048) followed by lipid (coefficient value 0.046) and the least was surfactant (coefficient value 0.014). Due to homogenization, the kinetic energy of the system became somewhat high which cause the collision and aggregation of lipid nanoparticles (Table 1, Batch F9 and F11). A high concentration of surfactant produced more small particles that form a bridge with big particles and hence produce non-uniformity which enhances PDI. Increasing the lipid concentration at a constant surfactant level caused the coagulation of particles resulting in a non-uniform particle size distribution. Figure 4 explored the influence of various independent variables on the PDI \( Y_3 \).

Based on three characters i.e. particle size, entrapment efficacy, and PDI the batch F9 was considered as an optimized formulation with the value of particle size, entrapment efficiency, and PDI 277.46 nm, 80.48% and 0.275 respectively.

3.6 Characterization of OLP-SLNs

3.6.1 Evaluation of particle size, PDI, and zeta potential

The value of particle size, PDI, and zeta potential of the optimized batch (F9) was found to be 277.46 nm, 0.282, \( \text{Fig. 5A} \) and \(-23.18 \text{ mV} \) respectively. The small value of PDI \( (<0.5) \) indicates the uniform or mono distribution of particles without any aggregation in the developed SLNs dispersion. A similar finding was observed by Yasir et al. during the production of Buspirone-loaded SLNs for the nose to brain delivery \( (32) \). Both particle size and surface charge are important in the case of nanoparticles drug delivery. The particle size less than 500 nm is thought to be escaping from the phagocytosis mechanism induced by macrophages \( (41) \). Zeta potential in the form of a negative surface charge (around \(-20 \text{ mV}\)) is desirable for the proper stability of nano-formulation. The observed value of zeta potential was \(-23.18 \text{ mV} \) indicating good physical stability.
Olive Leaves (Olea europaea L) Extract Loaded Lipid Nanoparticles

3.6.2 Morphological study
The optimized batch of developed OLP-SLNs (F9) indicated the roughly spherical shape as observed by the TEM study (Fig. 5B).

3.6.3 Entrapment efficiency (%) The entrapment efficiency of developed batches of OLP-SLNs was found to be in the range of 50.17-86.46% with 80.48% of optimized formulation (F9) indicating a good drug entrapment ability of developed SLNs.

3.6.4 Differential scanning calorimetry (DSC) study Properties like crystallinity and thermal behavior of the developed OLP-SLNs are important properties that ensure their application in drug delivery. The DSC thermogram of OLP (drug), lipid (compritol 888 ATO), and optimized formulation (F9) is represented in Fig. 6. The thermal spectrum of OPL showing a short and broad endothermic peak at 65.23°C but it went up to 100.96°C. The compritol 88ATO showed an endothermic peak at 70.5°C which resembling with its melting point. The characteristics peak of OLP was absent in the thermogram of optimized formulation (F9). Here, only one comparatively broad peak around 67.81°C was observed signifying the entrapment of OLP in the lipid matrix leading to the formation of OLP-SLNs. 44

3.6.5 In-vitro drug release
The release of drug from optimized OLP-SLNs formulation (F9) was found to be 95.29 ± 8.13% as shown in Fig. 7. The release from the optimized formulation exhibited a bi-
phasic pattern i.e. initial fast release ($23.83 \pm 4.51$ in first 1 h) due to the release of surface absorbed drug and later sustained ($95.29 \pm 8.13\%$ in 24 h) due to drug release from SLNs matrix. The release kinetic of OLP form optimized formulation was detected by putting the obtained drug release data into the different kinetic models as represented graphically in Fig. 8. The maximum $R^2(0.9984)$ value was found to be for the first-order kinetic model. Therefore, first-order kinetic was considered as the best-fitted model. The release mechanism was found to be the Fickian diffusion type with the value of release exponent ($n$)$0.441$.

3.6.6 Stability evaluation

Under the proper storage conditions, any developed dosage form is expected to be stable up to its duration of usage (expiry date). Here, developed optimized OLP-SLNs

**Fig. 6** DSC curve of pure OLP (A), Compritol 888ATO (B), and OLP-SLNs optimized formulation (F9) (C).

**Fig. 7** *In-vitro* drug release for optimized OLP-SLNs.

**Fig. 8** Fitting of difference kinetic model.
Olive Leaves (Olea europaea L) Extract Loaded Lipid Nanoparticles

J. Oleo Sci. 70, (10) 1403-1416 (2021)

formulation (F9) was stored at certain storage conditions as per the specification provided by ICH guidelines. The formulation stored at 4 ± 2°C (refrigerator) and room conditions (25 ± 2°C /60 ± 5% RH) were not significantly (p < 0.05) differing from the initial data (zero time) in respect of particle size, PDI, surface charge (zeta potential) and entrapment efficiency. A significant reduction (p < 0.05) in zeta potential (-19.27 mv) and entrapment efficiency (73.29%) and a significant increment (p < 0.05) in particle size (388.37 nm) was observed in the formulation stored at 40 ± 2°C /75 ± 5% RH. It might be due to the partial loss of surfactant covering (hence zeta potential reduced) which leads to aggregation of particles (hence particle size increased) and leakage of a drug in the external environment (hence entrapment efficiency reduced). 3.6.7 Antioxidant activity

The main action of OLP is to prevent peroxidation by cleaning free radicals and contributes to the amelioration of injuries caused by oxidative stress. As shown in Fig. 10, the maximum zone of inhibition of OLP for Staphylococcus aureus (Gram’s positive) and Pseudomonas aeruginosa (Gram’s negative) was 7.5 ± 1.25 mm and 8 ± 1.30 mm respectively. Similarly, the value of zone of inhibition for the physical mixture (OLP extract + blank SLNs) against Pseudomonas aeruginosa and Staphylococcus aureus was found to be 8.25 ± 1.9 mm and 8.60 ± 2.1 mm respectively. On the other hand, OLP-loaded SLNs exhibited the antimicrobial effect for a longer time (up to 24 h) as compared to free OLP and physical mixture. This is due to the sustained release of OLP from SLNs formulation. The antimicrobial effect of OLP-loaded SLNs against both gram-posi-

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The maximum zone of inhibition for positive control and physical mixture (OLP extract + blank SLNs) was observed within the first 6 h. After this duration (first 6 h), there was no change in the zone of inhibition of positive control and physical mixture. The value of zone of inhibition for positive control against Pseudomonas aeruginosa and Staphylococcus aureus was 7.5 ± 1.25 mm and 8 ± 1.30 mm respectively. Similarly, the value of zone of inhibition for the physical mixture (OLP extract + blank SLNs) against Pseudomonas aeruginosa and Staphylococcus aureus was found to be 8.25 ± 1.9 mm and 8.60 ± 2.1 mm respectively.

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The study exhibited the anti-microbial potential of developed SLNs formulation of OLP (F9) against the bacteria like Staphylococcus aureus (Gram’s positive) and Pseudomonas aeruginosa (Gram’s negative) as shown in Fig. 10. The result exhibited that there was no zone of inhibition for blank formulation as it was free from the OLP extract. The study exhibited the anti-microbial potential of developed SLNs formulation of OLP against both the bacteria as shown in Fig. 10. The result exhibited that there was no zone of inhibition for blank formulation as it was free from the OLP extract. The study exhibited the anti-microbial potential of developed SLNs formulation of OLP against both the bacteria as shown in Fig. 10. The result exhibited that there was no zone of inhibition for blank formulation as it was free from the OLP extract.

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tive and gram-negative bacteria was observed up to 24 h. The antimicrobial effect of OLP-SLNs was significantly ($p < 0.001$) more than that of OLP extract and physical mixture against Pseudomonas aeruginosa and Staphylococcus aureus. The value of the zone of inhibition of OPL-SLNs against Pseudomonas aeruginosa and Staphylococcus aureus was observed $14.75 \pm 2.25$ mm and $16.30 \pm 2.1$ mm in 24 h respectively. Moreover, study findings indicated that all formulation containing OLP extract exhibited better anti-microbial efficiency towards Gram-positive Staphylococcus aureus as compared to Gram-negative Pseudomonas aeruginosa 46,47. To the best of our knowledge, and the previous report showed that the major constituents for antimicrobial activity present in the olive extract are Cyclotrisiloxane hexamethyl($36.98\%$), Cyclotetrasiloxane octamethyl($15.18\%$), and Cyclopentasiloxane decamethyl($14.59\%$) 39.

4 Conclusion

In this study, olive leaves extract powder which was less stable in normal environmental conditions was successfully converted into stable SLNs formulation. Optimized formulation exhibited a promising particle size, entrapment efficiency as well as surface charges. The optimized formulation exhibited a sustained drug release pattern up to 24 h following the first order drug release kinetic and Fickian diffusion type of drug release mechanism. The stability study was conducted and optimized formulation(F9) was stable under the stated storage conditions. OLP formulation exhibited a promising anti-oxidant property as justified by the DPPH assay method. The antimicrobial activity against Gram’s positive (Staphylococcus aureus) and Gram’s negative (Pseudomonas aeruginosa) bacteria. Finally, it was concluded SLNs could be the promising carriers for the delivery of olive leaves extract powder.

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Conflict of Interest

Authors have no conflict of interest.

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