ORIGINAL ARTICLE

In Vitro-In Vivo Characterization of Oleuropein loaded Nanostructured Lipid Carriers in the Treatment of *Streptococcus pneumoniae induced Meningitis*

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Abstract

Background: Olive leaf, a versatile plant obtained from Olea europaea which was enriched with various secondary metabolites likes polyphenols, secoirodiods eg. Oleuropein, Hydroxytyrosol etc. Current treatment modalities for meningitis were ended up with MDR. Hence, a new strategy is mandate to alleviate bacterial meningitis. **Objective:** Current research structured to identify the potential of Oleuropein loaded NLC in the inhibition of Bacterial Meningitis. Glycerol is used as bio-solvent in hot blanching assisted microwave extraction & extracts were analyzed by using UV, FTIR, HPTLC and HPLC to quantify the Oleuropein. **Methodology:** NLC was formulated by melt dispersion ultrasonication method using Box-behnken design. Formulated NLC was analyzed for particle size, zeta potential and in-vitro drug release studies. Toxicity and Histo-pathological examination was done in albino Wistar rats using cerebellar cistern puncture method to estimate In-vivo potential of Oleuropein in bacterial meningitis. **Results and Discussion:** From the data, it was observed that hot blanching process potentiates the extraction and the data depicts that aqueous glycerol extract contain huge amount of Oleuropein. Optimized NLC resides with 165nm of size, ZP of -40mv and PDI of 0.3 with a sustainable release of drug for 12hrs. By Histo-pathological examination, regeneration of brain tissue was initiated in the hippocampus region in treated group as compared to control group. **Conclusion:** Combination of hot blanching with microwave extraction for Oleuropein and subsequent loading of drug into NLC shown significant positive effect on treatment of Streptococcal meningitis.

Key words: Meningitis, Nanostructured lipid carrier, Neural damage index, Vascular lesion index

INTRODUCTION

Olive leaf, a widespread versatile plant obtained from *Olea europaea* enriched with various secondary metabolites such as polyphenols and secoiridoids, for example, oleuropein and hydroxytyrosol.[1] Oleuropein content was rich in olive leaves compared to olive fruit; henceforth, leaves were chosen for further studies.[2] Extraction of oleuropein from olive leaves was done using solvents such as ethanol or hydroalcoholic which were ended up with a major drawback of lack in extraction efficiency and extensive solvent consumption.[3] Taking these cons into consideration, there is a need to chalk out a new strategy which can encapsulate all the demands such as reduced cost, feasibility of technology transfer from laboratory scale to pilot scale, and increasing the productivity of the extract.[4,5] In the current research, pre-treatment, i.e., hot blanching assisted microwave extraction technique using glycerol as biosolvent was chosen for extraction process to reduce matrix effects and enhance leaching of oleuropein.[6-9] Meningitis, a

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chronic disease characterized by inflammation in meninges of brain. At present, the second-generation cephalosporins are preferred choice to alleviate the disease which offers a serious disadvantage like multidrug resistance (MDR).[10,11] Hence, it is a prerequisite to develop a new herbal drug-loaded lipid carrier, i.e., nanostructured lipid carriers (NLCs) to combat the symptoms of meningitis and eradicate bacteria without resulting any side effects and maximize the efficacy toward *Streptococcus meningitides*. The objective of the current research was to design the oleuropein loaded NLCs using response surface methodology to predict the pharmacological efficacy of oleuropein toward meningitis.

MATERIALS AND METHODS

Olive leaves were collected from Rajasthan Olive Cultivation Limited, India. Solvents such as ethanol, methanol and glycerol, hydrochloric acid, sulfuric acid, and chloroform were purchased from Merck, India. Solid lipid Precirol ATO 5 was kindly gifted from Gattefosse; liquid lipid Capmul MCM was kindly gifted by ABITCEH Corporation; surfactant lecithin and stabilizer poloxamer 188 was purchased from Merck, India. All other materials and solvents were of analytical reagent grade.

Methods

Preliminary investigation of solvent efficacy in extraction of phenolics

Before commencement of extraction process and to predict the efficacy of solvent in extracting oleuropein, all the processed and dried olive leaves were grouped and micronized using a mixer. The powder (1 g) was extracted (before hot blanching) with various solvents such as methanol, ethanol, water, water:glycerol (1:3) in a volume of 20 ml and further stirred with the help of vortex mixture for 5 min. The extraction was employed by cold maceration and Soxhlet technique. The extracts were screened and analyzed for phenolic content by an aforementioned method called Folin-Catechu and percentage yield was estimated.[12]

Hot blanching assisted microwave extraction of oleuropein from olive leaf

Olive leaves were subjected to the hot blanching at optimum conditions (60–65°C temperature and 20–25 min duration of blanching), and concomitantly, the leaves were shade dried to remove moisture content, i.e., up to 10%.

Procedure for microwave-assisted extraction

Extraction process was done using domestic microwave oven (Samsung MW718). A measured weight of 3 g of powdered sample having PS of 250 µm was mixed with 70% (v/v) aqueous glycerol at solvent ratio of 50 ml/g in a closed Duran bottle. The mixture was irradiated at 150 W, 300 W, and 450 W for about 2–10 min. The extract is not allowed to super boil. On irradiation, the extract was screened using fine cloth and filter using 0.45 µ syringe filter and analyzed the drug concentration by high-performance thin-layer chromatography (HPTLC). Culmination of extraction was singling out using TLC plate and absence of colored spot on plate indicates shattering of extraction process. Extracts were screened using a paper filter of pore size 0.45 µm and concentrate by deploying Rotary flash evaporator; terminally, the extracts are stored at a temperature of −20°C until further usage.[13-15]

Qualitative identification of oleuropein by UV- Visible spectrophotometer

About 10 mg of olive leaf extract was dissolved in 10 ml of water, sonicated well to get a homogenous solution of concentration (1 mg/ml). Filter the solution by 0.45 µ filter paper, collect the filtrate, and dilute to get a concentration of 20 µg/ml. Analyze the sample by UV-visible spectrophotometer at a wavelength of 200–400 nm.[16]

Spectral interpretation of functional groups in extract by fourier transform infrared (FTIR)

FTIR study was performed by direct sampling technique using Agilent technologies FTIR spectrophotometer. When electromagnetic radiation passes through sample ranging from 4000/cm to 400/cm bond stretching and bending mechanisms will take place, which reflects the structural arrangements of atoms in the extract.

Chromatographic identification of oleuropein in extract by TLC

Development of TLC plate

TLC method development was done according to Trease and Evans, 2002.[17-24]

Precoated silica gel plates of size 20 cm × 10 cm were selected and drop 10 µL of extract on plate with capillary. Dip the spotted silica plate into the saturated developing chamber and allow the solvent front to raise up to a level of 3/4th height of the plate. Measure the solvent front without disturbing the plate and then, the plate was dried, marked, and detected using ferric chloride spraying reagent.

Quantitative estimation of oleuropein by HPTLC technique

The objective of HPTLC is to develop a method [Table 1] which promotes the separation of oleuropein from the mixture of metabolites.

Preparation of sample and standard stock solution of oleuropein

A stock solution of oleuropein standard was prepared by dissolving 10 mg of oleuropein in 10 ml of methanol. From this solution, 1 ml was pipetted out and dilute to 10 ml with methanol to get final concentration of 100 µg/ml and same procedure was deployed to prepare sample olive extracts.

Development of HPTLC chromatogram

Samples of volume 250 µl were spotted in the way of a band of width 80 mm with a Camag Microlitre Syringe on precoated silica gel aluminum plate using a Camag Linomat V sample applicator with an application rate of 1 µl/s. The split bandwidth was set at 20 nm and each track was scanned at 5 nm using a solvent composition of chloroform:methanol (4:1 V/V). Linear ascending method development was carried in 20 × 10 cm twin trough saturated glass chamber (Camag). After the development, the chromatograms were dried and scanned by Camag TLC scanner using WIN CATS Software 1.46. Concentration of the compound oleuropein in chromatogram was determined by the intensity of diffusely reflected light. The analysis and interpretation of chromatogram was done by comparing the peak areas of standard and extract and peak purity was analyzed by comparing Peak start, Peak apex, and peak end. Extracts rich in oleuropein was selected and subjected to gravity chromatography.[26-28]

Separation of oleuropein by gravity chromatography

Methodology

Select a column of desired size and packed the column by placing the porous cotton, followed by bed of sand of 3 cm length. 200 g of column silica gel of 60–120 mesh was made into the slurry with a suitable liquid (n-Hexane:dichloromethane [DCM]) of volume 400 ml and poured into the column through the funnel. Allow the adsorbent to settle and pack the column up to the 3/4th length. load it on the top of the column up to the level of mobile phase. Specific quantity of sample was dissolved in solvent (ethanol) pass the mobile phase through the sample, collect fractionates in a volume of 20 ml concentrate it and then analyze by spectral studies.

Table 1: Summary of validation parameters for HPTLC quantification of oleuropein

Validation parameters	Method property value
Linearity for oleuropein (ng/spot)	100–600
Correlation coefficient for gallic acid (r)	0.9994
Intraday precision (RSD % *n*=6) on different days for oleuropein	1.08
Interday precision (RSD % *n*=6) on same day oleuropein	1.13
Limit of quantization of gallic acid (ng/µl)	0.0667
Limit of detection of gallic acid (ng/µl)	0.045
Specificity	Specific

HPTLC: High-performance thin-layer chromatography, RSD: Relative standard deviation

Qualitative and quantitative estimation of oleuropein by High-performance liquid chromatography (HPLC)

An existed and validated method which was chalked out by Fuad-Al-Rimawi, in 2013, has been deployed to carry out the reverse-phase (RP) HPLC analysis for the isolate.

Standard stock solution of oleuropein with 1000 ppm concentration was prepared by dissolving 100 mg of drug in 100 ml of mobile phase. From the above stock solution, a series of six serial dilutions were prepared in a range of 50 ppm, 100 ppm, 150 ppm, 200 ppm, and 250 ppm, respectively, using a mobile phase as dilution media. Linearity curve was constructed using these aforementioned concentrations. The chromatographic evaluation of the isolate was functionalized on an Agilent 1200 HPLC with Diode-array detector, RP-18 end capped column having dimensions of 5 µm, 150 mm × 4.6 mm ID which was followed by UV detection at 280 nm. The optimized mobile phase was screened using 0.22 µm filter and degassed by sonication prior use which was then delivered by isocratic mode of elution at a flow rate of 1 ml/min and injection volume of 20 µl. Analysis was accomplished at room temperature 25°C after baseline stabilization for at least 30 min.[29-33]

Formulation of oleuropein NLCs by melt dispersion ultrasonication method

NLCs were prepared by melt dispersion ultrasonication method. Formulation additives were selected based on compatibility studies by FTIR. The solid lipid (Precirol) and liquid lipid (Capmul) in a ratio of 7:3%w/v were blended around 55°C Melting point, followed by homogenous dispersion of oleuropein (0.5%w/v) to form a uniform and clear lipid phase. Meanwhile, the aqueous phase containing surfactant poloxamer (2% w/v) in double distilled water was maintained at 55°C. The oil phase was added to the aqueous phase, at a drop flow rate of 1 drop/sec and both phases were mixed at 55°C by the aid of agitation at 600 rpm for 10 min with the help of magnetic stirrer to form a microemulsion. This warm microemulsion was diluted in cold water (2–3°C) under mechanical stirring to form NLCs dispersion such that the concentration of oleuropein in the final dispersion remains 2% w/v. The obtained emulsion was ultrasonicated for 5–15 min with amplitude of 80% and 5 s pulse rate using

Probe Ultrasonicator. The formulated NLCs were subjected to particulate characterization.[33-36]

Box–behnken experimental design for formulation of oleuropein loaded NLCS

The selected variables fixed in 3^3 Box–Behnken experimental design are shown in Tables 2 and 3. Protocol for this study was designed with the help of design expert 11 Software, Stat-ease, Inc., USA. 13 formulations with four centric points and nine factorial runs were generated and each variable was analyzed at three levels. The dependent variables shown for this study were PS nm, zeta potential (ZP) mV, and entrapment efficiency and the independent variables selected are conc. of solid lipid in mg, surfactant in % weight base, and ultrasonication time in min. In this design, response surface model was implemented to correlate and justify the effect of independent variable on dependent variables (responses). Selected independent variables are notified as A for solid lipid, i.e., Precirol; B for liquid lipid, i.e., lecithin; and C for ultrasonication time at three different levels, coded as low (−1), medium (0), and high (+1). Anova was statistically used to compare the percentage variation between the runs and it helps to figure out whether the variables are showing significant effect or not. It was helpful in deciding how the model is good enough to bring a relationship between variables and responses.[33-36]

Validation of experimental design

The numerical and graphical analysis was done using statistica 10 to obtain optimum values for the variables based on criteria of desirability. The obtained optimum values were used to prepare a checkpoint NLCs formulation which was then compared with predicted values to calculate with the predicted error, to validate the experimental domain and polynomial equation. ANOVA was conducted to confirm the fitted mathematical model.

Evaluation of oleuropein NLC to predict particulate characters

PS and PS distribution measurements

The PS distribution, mean PS (PS-Z average in nm), and PI of NLCs were measured using a Horiba nanoparticle size analyzer (SZ-100 nanopartica series). Dilute the samples with distilled water and analyse. Analyze by filtering the above solution using 0.45 µ membrane filter. The dynamic light scattering intensity was fixed automatically by the instrument based on the viscosity of medium, i.e., 90° light scattering for low viscous samples and 170° light scattering for high viscous sample. The PS for NLCs should be in the range of 10–500 nm and PI should be <0.3, which indicates a unimodal monodisperse system with uniform size distribution. All measurements were carried out in triplicate (n = 3).[37]

ZP (ζ)

The ZP or surface charge potential [Table 2] was determined using the Horiba nanoparticle size analyzer (SZ-100 Nanopartica Series). NLCs were diluted with saline to get a desirable particulate intensity and the dispersions were injected into the probe in an electrophoretic cell through which an 80 mV electric field was supplied. All measurements were carried out in triplicate at 25°C. The ZP was then directly determined using the Smoluchowski equation as follows.[37,38]

$$\zeta = \mathcal{E}\mu/\eta$$

Where, ζ-ZP, μ-Electrophoretic mobility; $\mathcal{E}$-electric permittivity of the liquid; and η is the viscosity of the liquid

Surface morphology studies-scanning electron microscope (SEM) studies

The surface morphology of optimized formulation N1 (Oleuropein NLCs) was observed by SEM (Hitachi S-3000N). Lyophilized NLCs powder samples were coated with platinum of 600 Å using a sputter coater and examined through SEM lyophilized NLCs were then mounted on a sample holder and scanned through an electron beam. The electron beam strikes NLC particles and emits secondary electrons based on the nature of the surface, which gives the surface morphology of NLCs. compare the mean PS of NLCs obtained by SEM with NLCs size obtained by Horiba Nano PS analyzer.[39,40]

Entrapment efficiency (%EE)

EE was determined by centrifugation method. In this study, 5 ml of NLC dispersion was taken in centrifuge tube priorly filled with 50 ml of pH 7.4 phosphate buffer and centrifuged at 15,000 rpm for 1 h in REMI centrifuge, to extract the free drug from NLCs carrier. After 1 h, 5 ml of sample was withdrawn from phosphate buffer saline. the drug concentration was determined by UV spectrophotometer at 280 nm using phosphate buffer as blank solution. Same protocol was followed for blank NLCs preparation and used as blank for UV analysis. The analysis was done in triplicate (n = 3). Percentage entrapment efficiency was calculated by the following equation.[41]

$$\%EE = \frac{Xs - Xt \times 100}{Xs}$$

Where, Xs – total amount of drug used for formulation;

Xt – amount of drug in 5 ml sample.

In vitro drug release studies

In vitro drug release for NLCs formulation was performed over 12 h using the dialysis membrane method. 1 ml of NLCs formulation was taken in dialysis membrane and both the open ends of dialysis membrane were tied. The

Table 2: Evaluation of the effect of independent variables (solid lipid concentration, surfactant, and ultrasonication time) on responses (particle size, ZP, entrapment efficiency) by Box–Behnken design at three levels (−1, 0, +1)

Run	Independent variable						Dependent variable		
	Solid Lipid-Precirol (mg)	Surfactant-Lecithin (%)	Ultrasonication time (min)	Actual value of factors			R1 particle size (nm)	R2 ZP (mV)	R3 entrapment efficiency (%)
	Factor A	Factor B	Factor C	Factor A	Factor B	Factor C			
N1	−1	1	1	5	3	20	168.0±2.54	−42.1±3.54	89.40±3.64
N2	1	−1	1	10	1	20	544.9±2.36	−11.1±2.54	58.84±3.60
N3	1	0	1	10	2	20	472.8±2.68	−13.4±3.42	54.50±3.98
N4	0	0	0	7.5	2	10	544.9±3.54	−11.1±3.66	56.28±3.84
N5	−1	−1	1	5	1	20	239.5±2.42	−16.1±2.54	77.24±3.76
N6	−1	0	−1	5	2	5	204.7±3.54	−33.5±2.68	76.84±3.66
N7	−1	1	−1	5	3	5	208.8±2.54	−26.7±2.72	75.42±2.86
N8	0	−1	−1	7.5	1	5	280.6±2.40	−12.9±2.44	62.54±3.42
N9	1	1	0	10	3	10	594.4±2.54	−22.2±2.68	54.34±4.24
N10	1	1	−1	10	3	5	357.8±3.56	−31.3±2.74	56.44±3.42
N11	−1	−1	0	5	1	10	239.5±3.54	−16.1±2.54	76.62±3.62
N12	1	−1	−1	10	1	5	540.6±2.68	−12.3±2.46	53.42±3.76
N13	0	1	1	7.5	3	20	336.6±3.24	−30.0±2.62	64.46±3.26

*All values expressed as mean±SD, *n*=3, −1: Low level, 0: Moderate level, +1: High level

Table 3: Summary on results of regression analysis for responses of R^1 and R^2

Parameter	Intercept	A	B	C	D	AB	AC	AD	BC	BD	A^2
Particle size R1	544.9	145.24	−18.19	−11.26	16.69	−100.8	−149.5	−120.35	−58.65	−177.65	−235.3
P values		0.0358	0.5857	0.0228	0.6146	0.3543	0.2957	0.2493	0.3612	0.2777	0.1695
Zeta potential R2	−11.1	4.42	−8.38	−3.07	0.4	−1.7	−3.75	−2.65	−3.35	−7	−13.45
P values		0.1159	0.0367	0.02042	0.8312	0.7630	0.6084	0.6065	0.3700	0.4245	0.1754
Entrapment efficiency R3	88.83	−3.0564	0.6556	0.3524	8.2353	0.3571	−1.8323	−3.14	−1.26	−3.64	−1.335
P values		0.0510	0.6513	0.8140	0.0093	0.8833	0.4246	0.5432	0.3692	0.4324	0.3804

membrane was allowed to immerse in a 100 ml phosphate buffer solution with 6.8 pH. The system was stirred continuously with magnetic stirrer at 50 rpm. The samples were collected at an interval of 0, 1, 2, 4, 6, 8, 10, and 12 h, and oleuropein content in the receptor compartment was determined by UV spectrophotometer at 280 nm. Sink conditions were maintained in the receptor compartment during *in vitro* release studies. The experiment was carried out in triplicate (n−3).[42]

Toxicity and tolerability studies

Acute toxicity study of water:glycerol fractionate was functionalized in Wistar strain albino rats using varying doses of 100, 200, 400, 800, and 1000 mg/kg body weight. These doses were administered orally to male albino Wistar rats by excluding control group and observed for morbidity and mortality after day 1, day 7, and day 14. Oleuropein was administered orally once daily at 24 h intervals for 28 days and the vehicle (physiological saline) was administered to the control group.[43]

In vivo Method for inducing bacterial meningitis

The animal use protocol has been reviewed and approved by the Institutional Animal Ethical Committee (SVCOP/IAEC/005/2016-17) of Sri Venkateswara college of Pharmacy. Albino Wistar rats aged 21 days and weighted 180–240 g were randomly selected to the experimental group (n = 6) and the control group (n = 6). Animals were anesthetized by intraperitoneal injection of pentobarbital sodium (40 mg/kg) and meningitis was induced to experimental group using cerebellar cistern puncture method. About 20–40 µL of cerebrospinal fluid (CSF) was removed, and 50 µL of *Streptococcus pneumoniae* ATCC strain 6303 bacterial suspension was injected into CSF. Rats in the control group were injected with 50 µL saline. after 24 h of bacterial inoculation, rats of each test group were administered with oleuropein NLC dispersion (100 mg/kg, qd, intraperitoneal injection) for 7 days.[43,44]

Histological examination

At the end of the predetermined experimental time, rats were anesthetized, and left ventricular perfusion was performed using 4% paraformaldehyde to fix body tissues. Brains were fixed overnight in 4% paraformaldehyde and embedded in paraffin the following day. The brains were sectioned along the coronal place into 5-µm slices and stained by Hematoxylin and eosin. Inflammation of the meninges and brain tissue was observed; vascular disease index and a neuronal damage index were estimated. Six slices were used from each specimen and 10 random images from each slice were used for observation.[2,44]

RESULTS AND DISCUSSION

The UV spectrum of extract is depicted in Figure 1.

From the study, it was revealed that in all the extracts, maximum absorbance was laid in the range of 275–280 nm. This spectral analysis supports the presence of oleuropein in olive leaf, which is a prerequisite for further studies.

The results of IR spectrum are shown in Figure 2. The IR spectrum screens out the presence of aromatic OH group at 3278 cm^{-1} and 3321 cm^{-1}, respectively, which confirms the presence of phenolic functional groups in the extracts. The wavenumber's at 2931.6c m^{-1}, 2927 cm^{-1}, and 2975.8 cm^{-1}indicates the presence of alkanes which is due to C-H Strech and the presence of Ar C=C Strech followed by Ar-CH=CH-R group reflects the presence of aromatic ring with conjugated diene confirms the presence of oleuropein in the extracts.

From the TLC study, it was observed that solvents chloroform:methanol, ethyl acetate:formic acid:acetic acid:water, and chloroform:methanol:acetic acid show clear separation of oleuropein from the extract. The solvents used for separation are tabulated in Table 4. R_f values of the separated compounds are depicted in Table 4 and it was found to be 0.85, 0.83, and 0.75 for three solvents, respectively.

Linearity tracks of HPTLC are depicted in Table 5 and Figure 3. The linearity range for oleuropein was 100–600 ng/spot with correlation coefficient (R^2 value) of 0.9994 and 0.9997, respectively. Comparative interpretation of HPTLC chromatograms is shown in Table 5. The identity of band oleuropein in the olive leaf extract was confirmed by overlay in UV absorption spectra with those of standard oleuropein using CAMAG TLC scanner. The purity of bands was confirmed by overlaying the absorption spectra at the start, middle, and end position of band. From the results, it was observed that, in extract 1, the chromatogram shows five peaks (0.08, 0.12, 0.44, 0.67, and 0.83), whereas, in extract 2, it shows three peaks and the R_f values are 0.40, 0.75, and 0.87, in extract 3, it shows major one peak and the value is 0.88, and in extract 4, it shows three peaks at 0.82, 1.12, and 1.19. In all the extracts, the major peaks eluted at an R_f of 0.87, 0.82, 0.88, and 0.83. The observed results are mapping with the R_f of standard oleuropein. The amount of oleuropein was found to be 25 µg/ml, 34 µg/ml, 36 µg/ml, and 29 µg/ml in various extracts, respectively. Using n-Hexane:ethyl acetate (90:10) as eluent, the separation of the extract was very digester and compounds are separated effectively. In case of solvents such as ethanol and acetone, the intermolecular forces are predominating which results in the retention of the compound in column itself. Hence, those are not preferred for the isolation of oleuropein from olive leaf extract. The isolates are crystallized to get pure compound for further analysis.

The IR spectrum for isolates is shown in Figure 4 which screens out the presence of aromatic OH group at 3208 cm^{-1} and 3263 cm^{-1}, respectively, in spectrum, which confirms the presence of functional groups present in the oleuropein. The wavenumber at 1653.1 cm^{-1} indicates the presence of alkenes and 1407.1 cm^{-1} indicates the presence of aromatic compounds with conjugated diene, and confirms the aromatic compounds to phenolic OH olive leaf extracts. The wavenumber at 1686.6 cm^{-1} indicates the presence of carboxylic acid will also add some information regarding the presence of oleuropein in the extract.

HPLC calibration curve is shown in Figure 5 and HPLC chromatograms for isolates I and II are shown in Figure 5 which depicts a clear, sharp, and characteristic peak at 280 nm, indicate the presence of oleuropein in both the isolates. The opted method shows a good separation of oleuropein with good resolution. Quantitative interpretation of the chromatogram clearly gives information about the

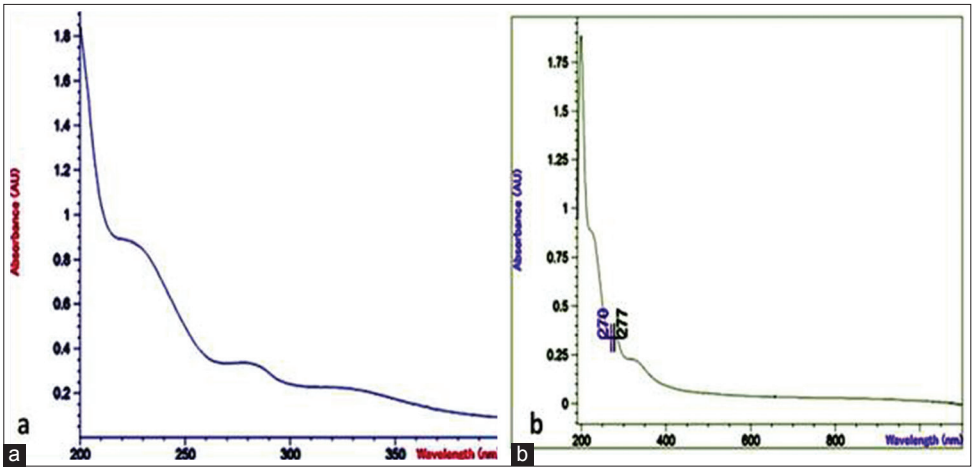

Figure 1: Overlaid ultraviolet spectrum of (a) oleuropein (b) olive leaf extract (aqueous glycerol)

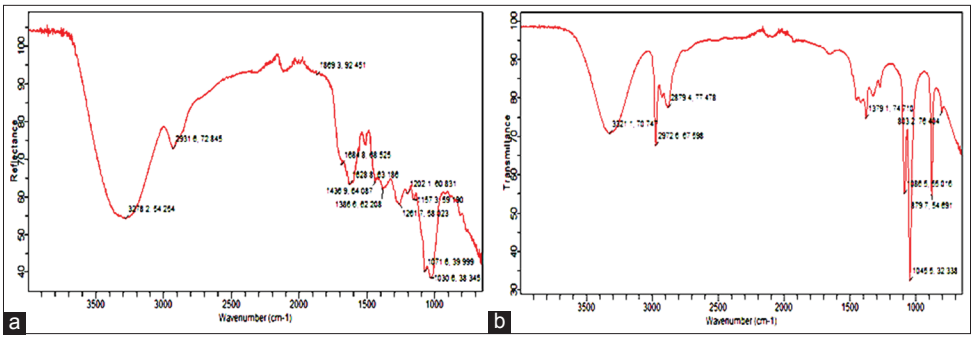

Figure 2: Fourier transform infrared spectrum; (a) oleuropein standard; (b) olive leaf extract

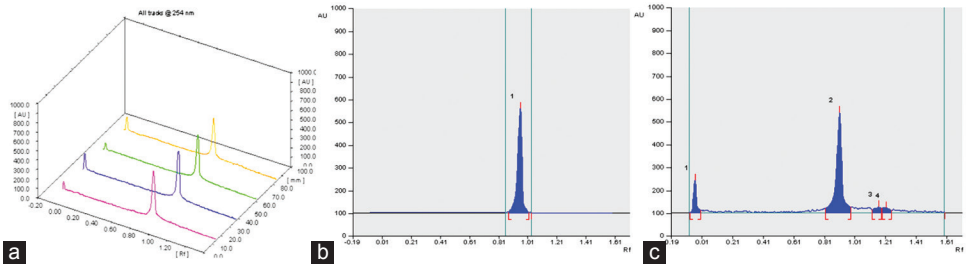

Figure 3: High-performance thin-layer chromatography chromatogram; (a) linearity of oleuropein; (b) oleuropein pure drug;(c) aqueous glycerol oleuropein extract

Table 4 : Detection of phenolic compounds in the oleuropein extract using R_f value

S. No.	Mobile phase	R_f	Number of compounds
1.	Ethyl acetate: methanol: water	-	-
2.	Chloroform: ethyl acetate: formic acid	0.78	1
3.	Chloroform: ethyl acetate: water	0.72, 0.75	2
4.	Chloroform: ethyl acetate: formic acid	0.82, 0.89	2
5.	Chloroform: methanol	0.85	1
6.	Butanol: acetic acid: water	-	
7.	Ethyl acetate: formic acid: acetic acid: water	0.83	1
8.	Chloroform: methanol: acetic acid	0.75	1
9.	N-Hexane: acetic acid	0.89, 0.95	2
10.	Ethyl acetate: acetic acid: water	-	-

approximate concentrations of the oleuropein in both isolates.

From the data, the water:glycerol extract (80:20% v/v) (n-Hexane:DCM isolate) possesses rich concentration of oleuropein when compared to other extracts.

Results of experimental design

The designing of NLC using experimental design was tabulated in Table 2 and Figure 6. Response surface analysis was plotted based on model polynomial equation depicts that three independent factors (solid lipid, liquid lipid, and surfactant concentration) selected shown a significant impact ($P < 0.05$) on the dependent variables (PS, ZP, and entrapment efficiency) are shown in Table 3.

Effect of dependent factors on ZP

The independent factors ascertain a significant effect ($P < 0.05$) on ZP. The polynomial equation was derived from the Box-Behnken design, produced by the changes in the ZP based on the independent variables is as follows:

$$ZP = -11.1 - 8.38\ B - 3.07C$$

The positive sign indicates synergistic effect on the examined dependent factors, whereas negative sign indicates antagonistic effect. Solid lipid and liquid lipid concentration shows a direct relation with ZP and the concentration of factor A and factor B at low levels shows lower values. This relation is depicted in Figure 6. The ratio of A:B and factor C had shown an indirect proportionate relation with ZP.

Lower values of ZP were obtained at a higher concentration of surfactant which is due to reduction of interfacial tension between drug and lipid phase. The response surface showed a minimum value at solid lipid:liquid lipid ratio of 7:3, ZP was −42.1 + 3.54 mV

Effect of dependent factors on PS

The three dependent factors together affect significantly on particle size of drug-loaded NLC ($P < 0.05$). The response surfaces of this effect are shown in the Figure 6. The mathematical model derives that a polynomial equation was found to be

$$PS=544.9+145.24\ A-11.26C$$

Where, a=Lipid concentration; b=Surfactant concentration, and C = Ultrasonication time

In Figure 6, the response surfaces exhibit a minimum value at solid-liquid lipid ratio of 7:3; the particle size was found to be 168 + 2.54. The particle size obtained was in the range of 168 + 2.54 (Sample 1)–544.9 + 2.56 (Sample 4) within the selected levels of variables, while the mean particle size was found to be 357.8 nm, which is the intercept of the model. The ratio of solid lipid-to-liquid lipid shows synergistic effect on PS, which is due to increasing the rigid structure of lipid matrix. Surfactant concentration lowers the particle

Table 5: Comparative interpretation of HPTLC chromatograms to quantify oleuropein in olive leaf

Track	Peak	Start position	Start height	Max. position	Max. height	Max.%	End position	End height	Area	Area %
1	1	0.08 R_f	3.9 AU	0.09 R_f	17.0 AU	3.03	0.12 R_f	0.7AU	216AU	2.07
1	2	0.12 R_f	1.1 AU	0.14R_f	11.0 AU	1.97	0.17 R_f	0.1AU	89.8AU	0.86
1	3	0.44 R_f	1.2 AU	0.48 R_f	12.4 AU	2.21	0.47 R_f	2.4AU	98.8AU	0.95
1	4	0.67 R_f	4.5 AU	0.70 R_f	19.0 AU	3.37	0.71 R_f	8.8AU	200.2AU	1.92
1	5	0.83 R_f	20.0 AU	0.91 R_f	502.3AU	89.4	0.99 R_f	35.1AU	9837.8AU	94.21
2	1	−0.07 R_f	7.2 AU	−0.05 R_f	167.7 AU	23.54	−0.02 R_f	7.2AU	1499.7AU	12.67
2	2	0.40 R_f	2.8 AU	0.41R_f	11.5 AU	1.61	0.43R_f	0.6AU	90.4AU	0.76
2	3	0.75 R_f	4.4 AU	0.76 R_f	14.9 AU	2.09	0.78 R_f	5.3AU	104.2AU	0.88
2	4	0.87 R_f	23.3 AU	0.97 R_f	518.3 AU	72.76	1.04 R_f	33.6AU	10142.8AU	85.69
3	1	−0.06 R_f	0.2 AU	−0.05 R_f	71.8 AU	12.80	−0.01 R_f	2.7AU	643.7AU	6.58
3	2	0.88 Rf	27.7 AU	0. 96R_f	489.2 AU	87.20	1.05 R_f	26.0AU	9137.8AU	93.42
4	1	−0.07 R_f	0.1 AU	−0.03R_f	144.1 AU	22.72	0.00 R_f	11.6AU	1433.3AU	13.68
4	2	0.82 R_f	19.5 AU	0.91Rf	439.1 AU	69.33	0.98 R_f	25.6AU	8001.1AU	76.37
4	3	1.12 R_f	13.2 AU	0.16R_f	27.1 AU	4.27	1.18 R_f	18.4AU	537.9AU	5.13
4	4	1.19 Rf	18.8 AU	1.21R_f	23.4 AU	3.69	1.25 R_f	10.3AU	504.5AU	4.82

HPTLC: High-performance thin-layer chromatography

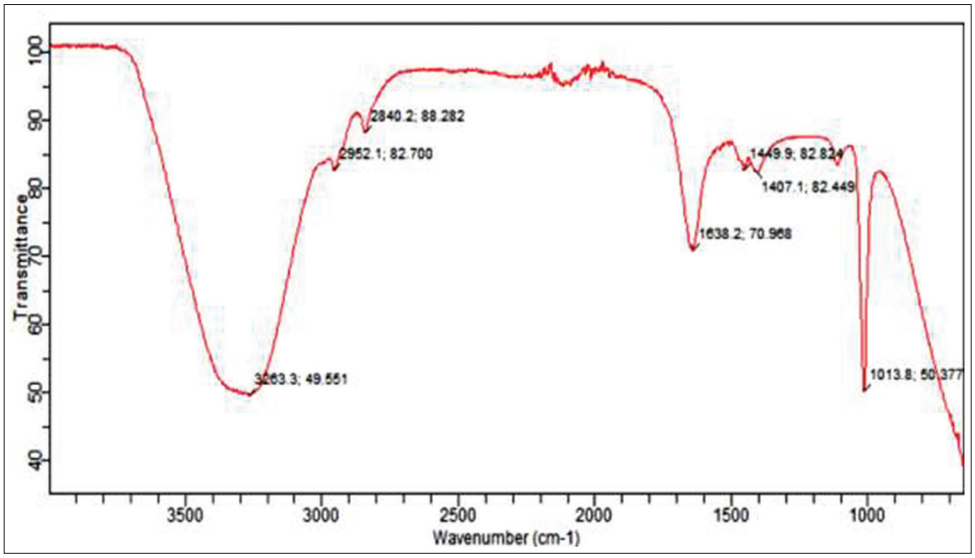

Figure 4: Fourier transform infrared spectrum of n-Hexane:dichloromethane isolate I

size with increased concentration which is due to micellar solubilization of drug in lipid matrix. Interaction effects have non-significant effect on PS.

Effect of Dependent Factors on Entrapment Efficiency (%EE)

The EE was varied from 53.42 + 3.76% (Sample 11) to 89.4 + 3.64% (Sample 1) within the selected levels of variables. The independent factors had shown a significant effect on %EE which reveals that a solid, liquid lipid concentration in combination with surfactant does not influence entrainment of drug oleuropein. Factor C shows a linear relationship with %EE. Interaction effects have non-significant effect on EE.

$$EE = 88.83 - 3.05A + 0.0093B$$

Where, A=Lipid concentration; B=Surfactant concentration

Selection of Optimized Formulation

NLC formulations were randomly picked from the factorial design space. By estimating ZP, PS, and polydispersity index (PDI), a simpler function could be defined to choose the optimum formulation. In general, factorial design requires a

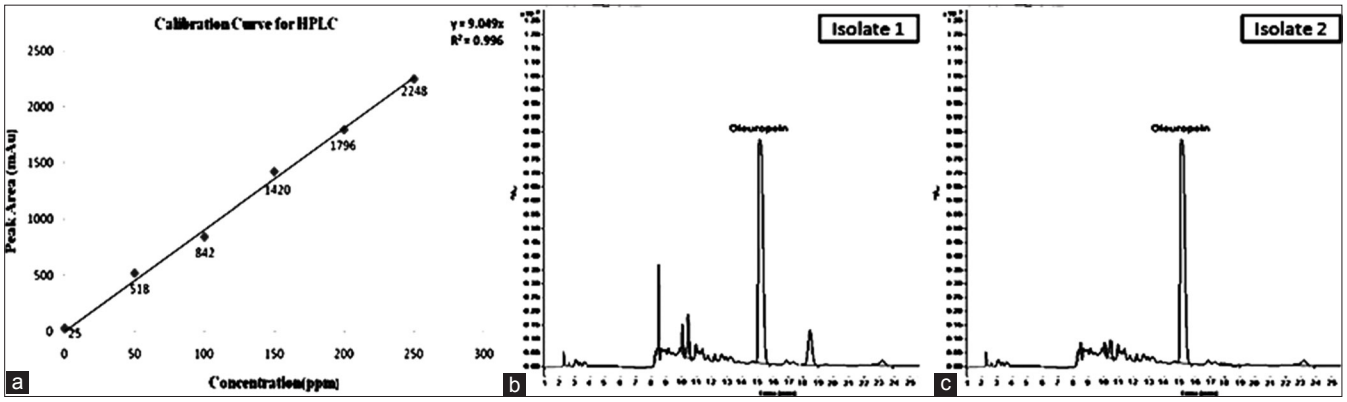

Figure 5: (a) Oleuropein standard graph; high-performance liquid chromatography chromatograms n-Hexane:dichloromethane fractionates (b) isolate I and (c) isolate II

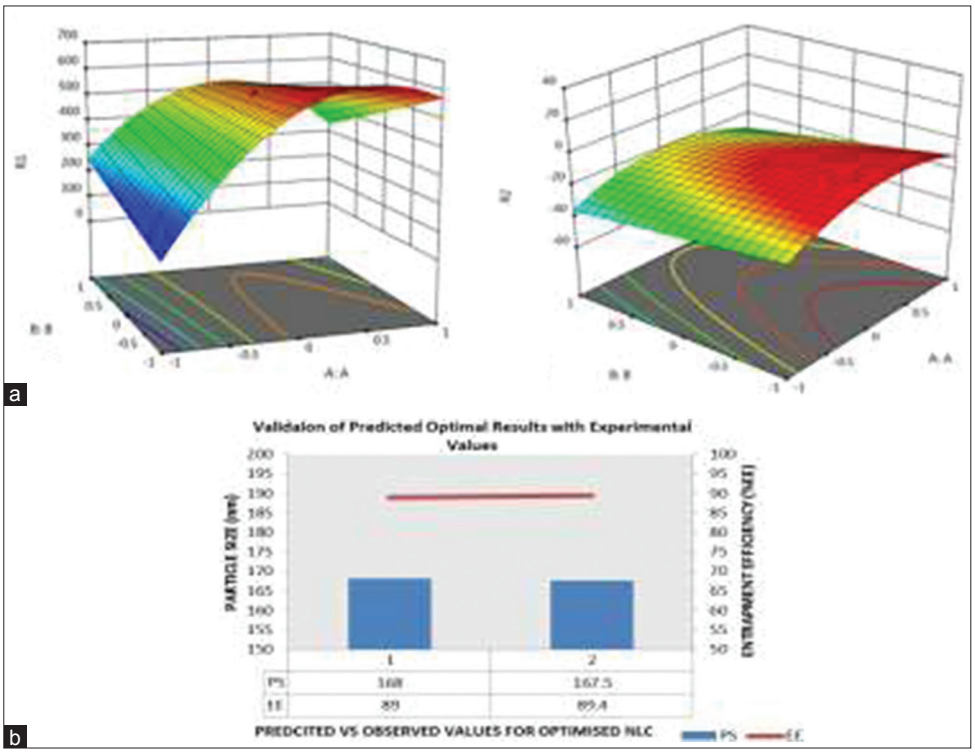

Figure 6: Response surface contour plot represents the mathematic relationship between factor A and factor B on R1, R2. (a) Effect of solid lipid concentration and surfactant on particle size nm; (b) effect of solid lipid concentration and surfactant on particle size nm on zeta potential

large number of samples so to reduce this following equation is used at its minimum value. The equation is as follows:

$$\text{Optimum} = \text{Min}(\text{ZP} + \text{PS} + \text{PDI} + \text{Span Value} + \text{SL/LL} + \text{SL: LL/S})$$

From the equation, the optimum values were obtained for the formulation of NLC which will result in the formation of stable formulation. Stable NLC was obtained at a concentration of 5 mg of solid lipid, 3%w/v of surfactant, and 20 min of ultrasonication time (−1, 1, and 1 levels). Particulate characteristics of optimized formulation was shown in Figure 7a and 7b.

Optimization and Validation

Validation of a model and to predict how well the model is useful in deriving a constructive conclusion between dependent and independent variables was identified by checkpoint analysis. Predicted and observed values of the dependent factors were plotted and the R^2 values were estimated using linear regression model.

Particulate Morphology by SEM

The SEM images were shown in Figure 7c and 7d studies are shown in Figure 7c and d, in which the NLCs were found to be oval in shape with groups and linked like a chain together, which infers that the NLCs will enhance the penetration of drug through the blood–brain barrier and deliver oleuropein in an effective manner in CSF.

Release studies of drug from NLC

In vitro drug release studies for N1 formulation show better control of drug release as 81.15 ± 2.42% in 12 h than oleuropein extract, which shows immediate drug release pattern, i.e., 98.11 ± 2.10% in 1 h as shown in Figure 8. From the data, it was concluded that oleuropein NLC follows sustained release kinetics.

In vivo Studies

Acute toxicity study was done at doses ranging from 100 to 1000 mg/kg in 180 to 250 g/kg body weight rats. Animals were observed for any toxic sign and symptoms periodically. After completion of the study, animals were sacrificed; their hematological, biochemical parameters were analyzed and histopathology of vital organs was done. No mortality and any clinical signs of toxicity were found in oleuropein administered group of animals. There were no significant alterations in hematological and biochemical parameters

Histopathological studies

Two parameters, i.e., neuron damage index and vascular lesion index were chosen to estimate the effect of oleuropein loaded NLC on bacterial meningitis. The values obtained for positive, negative control and treated group are tabulated in Table 6.

Histopathological images derived at a magnification of ×100 are shown in Figure 9, After 24 h duration of bacterial inoculation, visual observation of brains in the experimental group revealed edema, and the brain parenchyma had minor bleeding. The brains of rats inoculated with the bacterial suspension had more obvious changes. Histopathological studies show normal cytoarchitecture of hippocampus region of brain tissue in control group [Figure 9a]. Subarachnoid blood vessels and brain parenchyma showed edema, vasodilatation in bacterial-induced group [Figure 9b]. Regeneration of brain tissue initiated in hippocampus region of brain tissue in treated group [Figure 9c]. No meningeal inflammation or no neuronal damage was observed in the control group. Bacterial inoculum shows significant difference with the pathological vascular index ($P <$

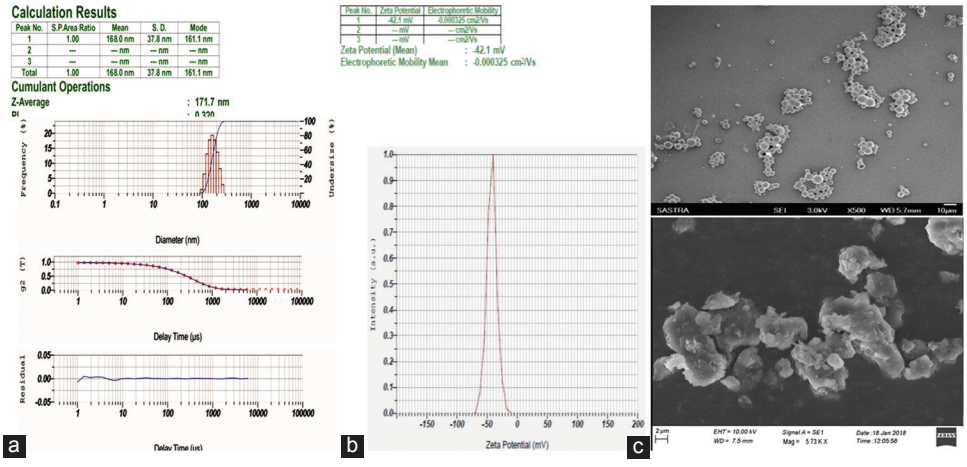

Figure 7: Particulate characteristics of optimized oleuropein nanostructured lipid carriers (NLC) (N1) (a) minimal particle size of –168 nm; (b) Maximum zeta potential of –42.1 mV; (c and d) scanning electron microscope report of Oleuropein NLC in different magnitude

Table 6: *In vivo* studies of oleuropein on *Streptococcus pneumoniae*-induced animals and determination of its efficiency in meningitis by estimating clinical parameters

Compared item	Control	Negative control	Positive control	*P*
Vascular lesion index	1.0±1.1	2.8±1.2[a]	1.3±1.0[a,b]	<0.05
Neuron damage index	0.8±1.2	2.5±0.5[a]	1.2±0.5[a,b]	<0.05

Compared with control group, [a]*P*<0.05, compared with control group and negative control [b]*P*<0.05, *All values expressed as mean±SD, *n*=3

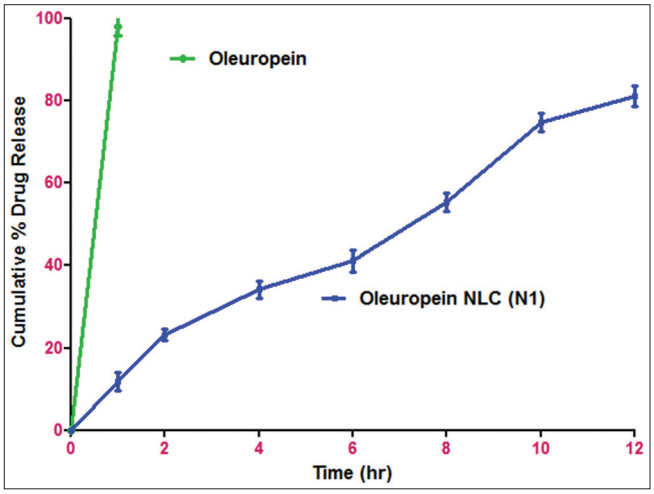

Figure 8: Cumulative percentage drug release profile of oleuropein versus oleuropein nanostructured lipid carriers

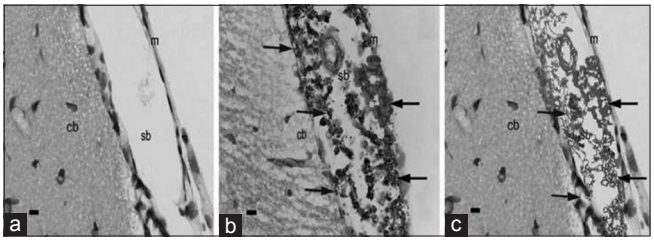

Figure 9: Effect of oleuropein nanostructured lipid carriers on histopathological studies of brain observed at a magnification of ×100 (a) normal cytoarchitecture of hippocampus region of brain tissue; (b) degenerative changes in hippocampus region of brain tissue; (c) regeneration of brain tissue initiated in hippocampus region of brain tissue

0.01) [Table 6]. The neuronal damage index shows significant difference with the concentration of bacterial inoculums ($P < 0.01$) [Table 6]. The area of neuronal loss was smaller in oleuropein treatment group when compared to bacterial inoculums treatment group [Figure 9c]; larger necrotic lesions were visible within the brain parenchyma when compared to treatment group [Figure 9c]. After oleuropein treatment, 7 days after inoculation, the density of some cortical neurons was increased. The arrangement of neurons was haphazard, and there was evidence of glial cell proliferation.

SUMMARY AND CONCLUSION

The collected olive leaf was processed, examined for the preliminary investigation of secondary metabolites, and percentage yield of olive leaves was estimated using two different methods, i.e., cold maceration and Soxhlet extraction. From the observation, it was depicting that the olive leaves were predicted as perfect house for phenolic compounds and the percentage yield was a step high in cold maceration aqueous glycerol extract, which is due to hydrolysis of few phenolic compounds during Soxhelation. A biosolvent, glycerol was functionalized to establish a green effective cumbersome method for the extraction of secoiridoid from olive leaves. By the observation, it showcases that aqueous glycerol solvent possesses an ability to potentiate the leaching of phenolic compounds which justifies the acceptability of glycerol as biosolvent. TLC findings resulted that the solvent chloroform:methanol, ethyl acetate:formic acid:acetic acid:water, and chloroform:methanol:acetic acid projects the better separation of compounds. The Rf values of the extract in the specified solvent gives an idea about the presence of phenolic compounds in olive leaves.

HPTLC, a versatile technique, used to quantify and quantity the secoiridoid present in the olive leaves. Chromatograms are developed using solvent chloroform:methanol (3:1% v/v). The densitograms resulted were mapped with standard and the amount of oleuropein was speculated to be 25 µg/ml, 34 µg/ml, 36 µg/ml, and 29 µg/ml in various extracts, respectively. This shows that the aqueous glycerin extract resides huge amount of oleuropein compared to all other solvents. By this research, the effectiveness of biosolvents in the extraction of phenolics was estimated successfully.

Spectral studies by UV and FTIR confirm the maximum absorption of the compounds at a wavelength of 280 nm and FTIR spectral studies, reflect the presence of the major phenolic O-H group at 3208 cm^{-1} and 3263 cm^{-1}, respectively, and the other main bond which was predicted to be responsible for biological activity is conjugated diene, Ar C = C stretch, and C-O stretch, also confirms the presence of oleuropein in olive leaves. Results depicted that the expected functional groups were present in all the extract. Hence to channelize the research, there is a surge to develop further. Thus, a column chromatography was chalked out to separate and isolates the compounds. Using n-Hexane:ethyl acetate (90:10) as eluent, three fractionates were eluted and with n-Hexane:DCM (90:10), two fractionates were eluted. The collected fractionates were analyzed by FTIR and HPLC to confirm and quantify the oleuropein. By the FTIR analysis, it was confirmed that major functional groups such as Ar–OH, conjugated diene, and aromatic acid were identified in the spectra of isolates

I and III. The concentration of oleuropein was quantified as 40 µg/ml (isolate III), which was very high when compared to other extracts. Oleuropein loaded NLCs were formulated by melt-dispersion ultrasonication method using Precirol. Histopathological examination was done using Oleuropein NLCs. Regeneration of brain tissue was initiated in the hippocampus region of treated group as compared to control group.

In this Research, it was concluded that n-Hexane:ethyl acetate is suitable to extract high amount of oleuropein from olive leaves, in turn, it proves that oleuropein NLCs show a better *in vivo* meningitis activity in bacterial-induced animal model.

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REFERENCES

1. Persia FA, Mariani ML, Fogal TH, Penissi AB. Hydroxytyrosol and oleuropein of olive oil inhibit mast cell degranulation induced by immune and non-immune pathways. Phytomedicine 2014;21:1400-5.
2. Liu Y, McKeever LC, Malik NS. Assessment of the antimicrobial activity of olive leaf extract against foodborne bacterial pathogens. Front Microbiol 2017;8:113.
3. Rigacci S, Guidoi V, Monica B, Parri M, Nediani C, Cerbai E, *et al*. Oleuropein aglycon prevents cytotoxic amyloid aggregation of human amylin. J Nutr Biochem 2010;21:726-35.
4. Yateem H, Afaneh I, Al-Rimawi F. Optimum conditions for oleuropein extraction from olive leaves. Int J Appl Sci Technol 2014;4:153-9.
5. Ahmad-Qasem MH, Barrajón-Catalán E, Micol V. Influence of freezing and dehydration of olive leaves (var. Serrana) on extract composition and antioxidant potential. Food Res Int 2013;50:189-96.
6. Rabiei Z, Bigdeli MR, Rasoulian B, Hashemi P, Pour MR, Mohagheghi F, *et al*. The neuroprotective effect of olive leaf extract is related to improved blood-brain barrier permeability and brain edema in rat with experimental focal cerebral ischemia. Phytomedicine 2011;18:170-5.
7. Kamran M, Hamlin AS, Scott CJ, Obied HK. Drying at high temperatures for a short time maximizes the recovery of olive leaf biophenols. Ind Crops Prod 2015;78:29-38.
8. Cifa D, Skrt M, Pittia P, Di Mattia C, Ulrih NP. Enhanced yield of oleuropein from olive leaves using ultrasound-assisted extraction. Food Sci Nutr 2018;6:1128-37.
9. Romero JM, Lama-Munoz A, Rodriguez G. Obtaining sugars and natural antioxidants from olive leaves by steam-explosion. Food Chem 2016;210:457-65.
10. Sudjana AN, Orazio CD, Ryan V. Antimicrobial activity of commercial *Olea europaea* (Olive) leaf extract. Int J Antimicrob Agents 2009;33:461-3.
11. Woehrl B, Klein M, Grandgirard D, Koedel U, Leib S. Bacterial meningitis: Current therapy and possible future treatment options. Expert Rev Anti Infect Ther 2011;9:1053-65.
12. Erbay Z, Icier F. Optimization of hot air drying of olive leaves using response surface methodology. J Food Eng 2009;91:533-41.
13. Rios JJ, Gutiérrez-Rosales F. Comparison of methods extracting phenolic compounds from lyophilised and fresh olive pulp. Food Sci Technol 2010;43:1285-8.
14. Yu H, Liu P, Tang H, Jing J, Lv X, Chen L, *et al*. Oleuropein-a natural extract from plants, offers neuroprotection in focal cerebral ischemia/reperfusion injury in mice. Eur J Pharmacol 2016;775:113-9.
15. Japan-Luja R, de Castro MD. Superheated liquid extraction of oleuropein and related biophenols from olive leaves. J Chromatogr A 2006;1136:185-91.
16. Guinda A, Castellano JM, Santos JM. Determination of major bioactive compounds from olive leaf. Food Sci Technol 2015;64:431-8.
17. Wagner H, Bauer R, Melchart D, Xiao PG, Staudinger A. Chromatographic Fingerprint Analysis of Herbal Medicines. Thin-layer and High Performance Liquid Chromatography of Chinese Drugs. Vienna: Springer-Verlag; 2011. p. 225-30.
18. Nassima T, Amani T, Gómez-Caravaca AM, Fernández-Gutiérrez A, Segura-Carretero A. Phenolic compounds in olive leaves; Analytical determination, biotic and abiotic influence and health benefits. Food Res Int 2015;77:92-108.
19. Trease GE, Evans WC. Pharmacognosy. 15th ed. Philadelphia: W.B. Saunders Company Ltd.; 2002.
20. Kurth MJ. Thin-layer chromatographic separation of phenols. J Chem Educ 1986;63:360-3.
21. Crabtree AN, McGill AE. Thin-layer chromatography of phenolic compounds. Microchimica Acta 1967;55:85-7.
22. Aly OM. Separation of phenols in waters by thin layer chromatography. Water Res 1968;2:587-92.
23. Medi-ari M, Jasprica I, Bubalo AS, Mornar A. Optimization of chromatographic conditions in thin layer chromatography of flavonoids and phenolic acids. Croat Chem Acta 2004;77:361.
24. Harborne JB. Phenolic compounds. J Chromatogr Libr 1983;22:B407-33.
25. Nair CV, Ahamad S, Khan W, Anjumand V, Mathur R. Development and validation of high-performance thin-layer chromatography method for simultaneous determination of polyphenolic compounds in medicinal

plants. Pharmacognosy Res 2017;9 Suppl 1:S67-73.
26. Heyden YV. Extracting information from chromatographic herbal fingerprints. LCGC Europe 2008;21:438-43.
27. Attimarad M, Ahmed KK, Aldhubaib BE, Harsha S. High-performance thin layer chromatography: A powerful analytical technique in pharmaceutical drug discovery. Pharm Methods 2011;2:71-5.
28. Yuan JJ, Wang CZ, Ye JZ, Tao R, Zhang YS. Enzymatic hydrolysis of oleuropein from *Olea europaea* leaf extract and antioxidant activities. Molecules 2015;20:2015-221.
29. Al-Rimawi F. Development and validation of a simple reversed-phase HPLC-UV method for determination of oleuropein in olive leaves. J Food Drug Anal 2014;22:285-9.
30. Aouidi F, Dupuy N, Artaud J, Roussos S, Msallemd M, Gaime IP, *et al*. Rapid quantitative determination of oleuropein in olive leaves (*Olea europaea*) using mid-infrared spectroscopy combined with chemometric analyses. Ind Crops Prod 2012;37:292-7.
31. Ranelli A, Contento S, Lucera L, Di Febo M, Marchegiani D, Di Fonzo V, *et al*. Factors affecting the contents of iridoid oleuropein in olive leaves (*Olea europaea*) L. J Agric Food Chem 2006;54:434-40.
32. Salah MB, Abdelmelek H, Abderraba M. Study of phenolic composition and biological activities assessment of olive leaves from different varieties grown in Tunisia. Med Chem 2012;2:107-11.
33. Al-Rimacoi F, Yaleem H Afanesh I. Optimum conditions for oleuropein extraction from olive leaves. Int J Appl Sci Technol 2014;4:153-7.
34. Gambhire MS, Bhalekar MR, Gambhire VM. Statistical optimization of dithranol-loaded solid lipid nanoparticles using factorial design. Braz J Pharm Sci 2011;47:503-11.
35. Gupta VK, Karar PK. Optimization of process variables for the preparation of chitosan alginate nanoparticles. Int J Pharm Pharm Sci 2011;3:78-80.
36. Yassin AE, Anwer MK, Mowafy HA, El-Bagory IM, Bayomi MA, Alsarra IA. Optimization of 5-fluorouracil solid-lipid nanoparticles: A preliminary study to treat colon cancer. Int J Med Sci 2010;7:398-408.
37. Limón D, Amirthalingam E, Rodrigues M, Halbaut L, Andrade B, Garduño-Ramírez ML. Novel nanostructured supramolecular hydrogels for the topical delivery of anionic drugs. Eur J Pharm Biopharm 2015;96:421-36.
38. Parida P, Mishra SC, Sahoo SS, Behera A, Nayak BP. Development and characterization of ethyl cellulose based microsphere for sustained release of nifedipine. J Pharm Anal 2016;6:341-4.
39. Mathiowitz E. Encyclopedia of Controlled Drug Delivery. Vol. 1, 2. New York: John Wiley and Sons; 1999. p. 236-48.
40. Gaba B, Fazil M, Khan S, Ali A, Baboota S, Ali J. Nanostructured lipid carrier system for topical delivery of terbinafine hydrochloride. Bull Fac Pharm Cairo Univ 2015;53:147-59.
41. Esposito E, Ravani L, Mariani P, Huang N. Effect of nanostructured lipid vehicles on percutaneous absorption of curcumin. Eur J Pharm Biopharm 2014;86:121-32.
42. Vrignaud S, Anton N, Gayet P, Benoit JP, Saulnier P. Reverse micelle-loaded lipid nanocarriers: A novel drug delivery system for the sustained release of doxorubicin hydrochloride. Eur J Pharm Biopharm 2011;79:197-204.
43. Liu XJ, Zhang XL, Han QZ. Establishment of rat pneumococcal meningitis models: A histopathological analysis. Int J Clin Exp Pathol 2015;8:2242-8.
44. Krishnappa LG, Marie MA, John J, Dabwan KH, Shashidhar PC. Serological and molecular capsular typing, antibiotic susceptibility of *Streptococcus pneumoniae* isolates from invasive and non-invasive infections. Acta Microbiol Immunol Hung 2014;61:173-9.

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