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Open Access Research Article

NOVEL TOPICAL LIPOSOMAL GEL OF BENZOYL PEROXIDE AND RESVERATROL FOR TREATMENT OF ACNE

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ABSTRACT

Liposomal formulations have been used in the treatment of a number of dermatological diseases . Benzoyl peroxide is the drug of choiceused for the treatment of acne but suffers from side effects such as skin redness, irritation, itching, oedema and dryness. In this research work benzoyl peroxide was encapsulated into liposomes, alongwith herbal drug resveratrol for treatment of acne . Liposomes were prepared by thin film hydration technique and evaluated for pH , particle size , zeta potential , FT -IR, XRD and TEM etc. which indicated suitable encapsulation of drugs in liposomes. Liposomal gel was prepared using different polymers and evaluated for colour, pH, drug content, homogeneity and grittiness and in vitro release studies .Suitable penetration enhancers were added to the selected preparation . The final selected formulation was evaluated further for viscosity, spreadibility ,drug release, kinetic study and stability studies .Selected liposomal gel showed prolonged release of both drugs and the product was found to be stable .Ex-vivo diffusion studies and drug deposition studies on animals was performed. Skin irritation studies on animals did not show any irritation,dryness or redness.

Key Words: *liposomes,resveratrol,benzoyl peroxide,topical .*

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INTRODUCTION

Liposomes are spherical particles composed of one, several, or multiple concentric membranes [1]. These are potent drug delivery systems for treating hair follicle associated disorders such as acne. [2]. After topical application, liposomes can improve drug deposition within the skin at the site of action, reduces systemic absorption and minimizes the side effects. [3]. They can target the drugs to skin appendages. [4]. They canimprove the therapeutic effect of drugs and decrease the adverse effects.

Benzoyl peroxide (BPO) is a potent antibacterial agent used in treatment of acne vulgaris [5].Benzoyl peroxide is macrolide antibiotic which may be either bacteriostatic or bactericidal depending on the sensitivity of the microorganism and the concentration of the drug.It works against *P.acnes*, helps prevent formation of comedones and anti-inflammatory properties. Topical application of benzoyl peroxide often produces adverse effects like skin redness, irritation, dryness and itching which leads to inconvenience of therapy.

Benzoyl peroxide is an organic compound in the peroxide family. It consists of two benzoyl groups bridged by a peroxide link. Its structural formula is $[C_6H_5(CO)_2]O_2$. The chemical structure of benzoyl peroxide is shown in figure 1.

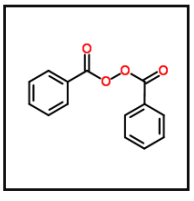

Fig. 1: Structure of benzoyl peroxide

Resveratrol (RES) is an antimicrobial drug and studies has shown its inhibitory effect on *p .acne* It is a type of phenol produced naturally by several plantssuch as red wine, colour berries, and inedible parts of the peanut plant [6] It has variety of other therapeutic properties such as anti inflammatory, antineoplastic ,antioxidant and wound healing activity [7].It is also known to have antiviral, antifungaland antiprotozoal effects [8-10]. It is a powerful antioxidant produced by some plants to protect them against environmental stresses..

Chemically, Resveratrol(RES) is 3,5,4'-trihydroxy-trans-stilbene,a type of natural phenol and a phytoalexin produced naturally by several plants in response to injury or when the plant is under attack by pathogens such as bacteria or fungi. The chemical structure of resveratrol is given in figure 2

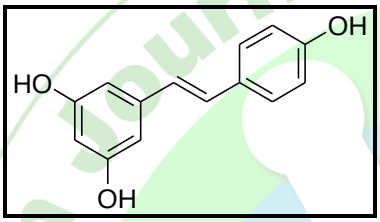

Fig. 2: Chemical structure of Resveratrol

The objective of present work is to formulate and evaluate liposomal gel of benzoyl peroxide and resveratrol for treatment of acne. The present study is based on the hypothesis that encapsulation of benzoyl peroxide and herbal drug resveratrol into liposomes will increase the therapeutic efficacy and reduce the adverse effects. This combination would be beneficial for the treatment of mild to moderate stage of acne vulgaris

MATERIAL AND METHODS

Benzoyl peroxide and resveratrol were obtained as gift samples from JIGS chemicals, Ahmedabad and avanscure life sciences pvt .Ltd, Hydrabad respectively. Phospholipids andcholesterol were purchased from S. D. Fine Chemicals Ltd. (Mumbai, India), chloroform, and methanol were purchased from LobaChemie (Mumbai, India). All the reagents were used without further purifications. Phosphate-buffered saline pH 7.4 (PBS pH 7.4) was prepared as described in the Indian Pharmacopoeia (1996), and necessary chemicals were obtained from the LobaChemie(Mumbai, India). All the chemicals used were of analytical reagent (AR) grade.

Preparation of Benzoyl peroxide and Resveratrol liposomes

The liposomes of benzoyl peroxide and resveratrolalongwith carrier phospholipid and cholesterol were formulated by thin film hydration technique using rotary evaporator. The stabilizer hydroxy propyl beta cyclodextrin(HP- β-CD) was used. Phospholipid , cholesterol andHP- β-CD concentration was optimized by **Box Behnken**Design**(BBD)** .Liposomes using different lipid ,cholesterol and HP- β-CD compositions (as per box behnken design) were prepared.Dissolved lipid, cholesterol and drug benzoyl peroxide in solvents chloroform and methanol (3: l). The solvent was evaporated under vacuum at 45^{o} C while the flask was rotated constantly. A thin film was formed .The aqueous phase is prepared by dissolving resveratrol in methanol while sonicating till it dissolved completely meanwhile hydroxypropyl-β-cyclodextrin was solubilized in water. Mixed both the solutions in ratio 1:1 and allowed to stir for 1 hour. The formed film was then hydrated with aqueous phase of resveratrol complexed with Hydroxy propyl- β-Cyclodextrin(above formed solution). The percent entrapment of all these formulations was determined (Table 1).

Finally the model was further analysed by optimization module in Design Expert Software.5 selected formulations were prepared on the basis of desirability one .Their entrapment efficiency was determined. (table 2) .The final combination S-4 was selected on the basis of entrapment efficiency and minimum standard deviation between theoretical and experimental response value.(table 2).

Evaluation of optimized liposomal suspension (S-4)

Particle size and Polydispersity index

Mean vesicle size of liposomes was determined by using Malvern particle size analyzer model SM 2000, which follows Mie's theory of light scattering. Diluted liposome suspension was added to the sample dispersion unit containing stirrer and stirred at 2000 rpm in order to reduce the interparticle aggregation, and laser obscuration range was maintained between10-20%. The average particle size was measured after performing the experiment in triplicate.

Entrapment efficiency (EE)

Drugs associated with liposomes were separated from unentrapped drug using centrifugation method. Liposomes were centrifuged at 20000 rpm for 1 h at controlled temperature of 4^{0} C .Supernatant containing unentrapped drug was withdrawn and measured UV spectrophotometrically . The amount of drug entrapped in liposomes was determined as follows

EE (%) = [(Cd–Cf)/Cd] 100

Where Cd is concentration detected of total drug and

Cf is concentration of free drug

The entrapment efficiency was obtained by repeating the experiment in triplicate and the values were expressed as mean standard deviation.

Zeta Potential

Surface charge on empty and drug loaded vesicles surface was determined using Zetasizer300HSA (Malvern Instruments, Malvern, UK).Analysis time was kept for 60 s and average zeta potential and charge on the liposome was determined

FT-IR of Liposomes

FT-IR spectrumof liposomes wasdetermined by using FT-IR spectrophotometer by KBr disc method. The KBr and active ingredients separately were mixed thoroughly in a mortar while grinding with the pestle and dried completely. The powdered sample was placed just enough to cover bottom in pellet die and a pressure of 5000-10000 psi was applied to make the pellet. Then the pellet was placed in the sample holder of FT-IR spectrophotometer.

Differential scanning calorimetery (DSC)

DSC scans were recorded for liposomes using differential scanning Calorimeter (DSC) (Perkin Elmer DSC-4000, USA). In brief, 5 mg of sample was sealed in the aluminium pan and heated at the speed of 20°C/min at the temperature range of 300°C. Empty pan was taken as a reference.

XRD analysis (XRD)

The crystalline or amorphous nature of liposome's was determined by XRD measurement which was carried out with an X ray diffractometer (Expert/Pro, Panalytical, Netherlands) . Powder -XRD's (P-XRD) study were performed by exposing the samples to CUK-α (copper) radiation (45 kv, 30 mA) and scanning from 10 degree to 60 degree and step width of 0.05 degree and scan speed 10 degree/minute. The instrument measure interlayer spacing d which is calculated from the scattering angle theta, using bragg's equation

$n\lambda = 2d \sin \theta$.

Where λ is the wavelength of the incident x-ray beam and n is the order interference. Obtained P-XRD patterns were compared with the characteristic drug peak intensity obtained for the pure drug, excipients and physical mixtures.

Lyophilization of liposomal suspension is done by freeze dryer to increase the stability of suspension.

Transmission Electron Microscopy (TEM)

For Transmission Electron Microscopy(TEM) (Philips CM 200),the lyophilized samples were negatively stained with1%aqueous solutions of phosphotungistic acid (PTA). TEM is invaluable tool for providing information on particle size ..

Formulation of liposomal gel [11] [15]

Liposomal gel was prepared using selected formulation S-4 in different gelling agent .LG-1-5 and their drug content was determined .Formulation LG-3 containing 2% carbopol 934 was selected on the basis of pH, drug content .(table 3)This formulation was used to formulate gel with different permeation enhancers .Carbopol 934 was added in minimum amount of distilled water and kept overnight for carbopol to get swelled in water. Added permeation enhancers(DMSO, Propylene glycol, glycerol ,PEG 400, Curcumin) separetely and the pH was then adjusted to neutral using 1N NaOH and stirred slowly till a gel was obtained. Resveratrol and benzoyl peroxide loaded liposomes was added slowly in the above solution with continuous stirring in closed vessel until homogeneous gel were achieved[12]. Composition of permeation enhancers of liposomal gel (LG13.1-3.7)formulation of resveratrol and benzoyl peroxide is given in table 4

Evaluation of gel : These different formulations LG 3.1 to LG 3.8 were evaluated for

Physical examination ,homogeneity and grittiness [13]

The prepared gels were subjected to physical evaluations such as homogeneity and grittiness by physical inspection after the gel have been set in their container.

pH determination: 1.0 gm of gel was taken in test tube and diluted up to 10 ml with distilled water and checked for pH with pH meter (Labindia, Mumbai).

Drug Content determination: 0.5 gm of gel was taken in test tube and diluted up to 5 ml with methanol and centrifuged at 18000 rpm for 30 minutes at 4°C to extract out whole drugs from liposomes incorporated in gel. Supernatant was taken and checked its absorbance at 230 nm and 306 nm .[11]

In-vitro drug release [14]

The diffusion studies were performed using a franz diffusion cell. The cell was locally fabricated and had a 35 ml receptor compartment. The dialysis membrane was mounted between the donor and receptor compartments. The control gel formulation (gel containing both drugs) was applied uniformly on the dialysis membrane and the compartments were clamped together. The receptor compartment was filled with the phosphate buffer (pH 7.4) and the hydrodynamics in the receptor compartment were maintained by stirring with a magnetic bead. 3ml of samples was withdrawn from the receptor compartment at pre-determined time intervals and an equal volume of buffer was replaced.

Spreadibility

Spreadability was determined by apparatus which consists of a wooden block, which was provided by a pulley at one end. By this method, spreadability was measured on the basis of 'Slip' and 'Drag' characteristics of gels. A ground glass slide was fixed on this block. An excess of gel (about 2 gm) was placed on this ground slide. The gel was then sandwiched between this slide and another glass slide having the dimension of fixed ground slide and provided with the hook. A 1 Kg weight was placed on the top of the two slides for 5 minutes to expel air and to provide a uniform film of the gel between the slides. Excess of the gel was scrapped off from the edges.

Spreadability was then calculated using the following formula:

$S = M \times L / T$

Where, S = is the spreadability, M = is the weight in the pan (tied to the upper slide), L = is the length moved by the glass slide and T = represents the time taken to separate the slide completely from each other.

Determination of Viscosity [17]

The viscosity of the formulations was determined using a Brookfield digital viscometer (model DVII, USA) equipped with spindle S63.. The gel sample was placed in the beaker and spindle of the viscometer allowed to settle for 5 min and the viscosity measured at different rotation speed at room temperature.

Drug release kinetic study[18]

The release kinetic was studied by various kinetic models as zero order plot, first order plot, higuchi plot and korsmeyer-peppas.

To study the release kinetics of optimized gel, data obtained from in vitro drug release studies were plotted in various kinetic models: zero order as amount of drug releases Vs time, first order as log percentage of drug

remaining Vs time, Higuchi model as percentage of drug released vs square root of time. The best fit model was confirmed by the value of correlation coefficient near to 1.

Korsmeyer – peppas model

Korsmeyer et al. (1983) derived a simple relationship which described drug release from a polymeric system equation. To find out the mechanism of drug release, first 60% drug release data were fitted in Korsmeyer-peppas model.

$M_t / M_\infty = K_t n$

where Mt / M∞ is a fraction of drug released at time t, k is the release rate constant and n is the release exponent. The n value is used to characterize different release for cylindrical shaped matrices. For the case of cylindrical tablets, $0.45 \leq n$ corresponds to a fickian diffusion mechanism, $0.45 < n < 0.89$ to non-fickian transport, $n = 0.89$ to Case II (relaxational) transport, and $n > 0.89$ to super case II transport. To find out the exponent of n the portion of the release curve, where $Mt / M\infty < 0.6$ should only be used. To study the release kinetics, data obtained from in vitro drug release studies were plotted as log percentage drug release versus log time.

Stability study

The optimized formulation was subjected to stability studies. Optimized liposomal gel was sealed in amber colored bottles with cap covered by aluminium foil and these packed formulation was stored in different temperature viz 1) room temp (RT) 2) 2-8^o C and at 40^{o}Cfor 3 months.The formulation was evaluated before and after periodic interval for change in appearance, pH,viscosity and drug content

Determination of antibacterial activity [19]

Microorganisms: Acne causing microbial cultures *Propionibacterium acnes* MTCC 1951 was procured form microbial type culture collection (MTCC), Institute of microbial technology, Chandigarh, India. The bacterial cultures were preserved at 4°C and sub cultured in every 30 days intervals. The medium used for *P. acnes* is nutrient agar supplemented with 0.1% thioglycollate.

anti acne activity: The prepared liposomal gel formulation, formulation containing 0.5% Benzoyl peroxide and 0.25% Resveratrol in 2% w/w gel and control gel 0.5% Benzoyl peroxide and 0.25% Resveratrol 2% w/w gel containing *P. acnes* for antimicrobial activity for minimum inhibitory concentration calculation. The antimicrobial test was carried out by **micro broth dilution method.** Different concentrations (1g/ml; 0.5g/ml; 0.25g/ml; 0.125g/ml; 0.072g/ml of liposomal gel and same for control gel prepared with dimethyl sulfoxide (DMSO) solvent. The test mixture contained 5 ml of microbiological medium with or without 0.1% thioglycollate for anaerobic culture, 100 µl of test solution /DSMO/nil (as per study design) and 10 µl of microbial suspension (approximately 6X 104 cells per ml) and incubated for 24 hr at 37°C in a bacteriological incubator. After 24hr, the optical density (directly proportional to the microbial growth) was measured at 610 nm by a spectrophotometer for micro-dilution anti microbial assay method.

Skin irritation study

Total 12 healthyWistar albino rat of either sex weighing 120-150 gm were used for this study.The animal's backs were shaven to result in an approximate area of 4cm2 . The rats were treated topically with their respective formulations once daily on day 1 of the 7-day study. Every day visual assessments were made.. The development of erythema was monitored for 6 days.

Ex-vivo diffusion study

Selection of animals: For the present study, adult WistarAlbino rat of either sex (120±10gm) were selected. During all the work, the animals were maintained at a temperature 25±2°C and commercial pellet diet and water ad libitum.

Preparation of skin: The abdominal hair of WistarAlbino rats weighing 120±10g, was removed using depilatory before treatment. After anesthetizing the rats with ether, the abdominal skin was surgically removed from the animal, and adhering subcutaneous fat was cleaned carefully.t. All surgical and experimental procedures were reviewed and approved by the Institutional animal and ethics review committee, Hindu College of Pharmacy Sonepat Reg.. No. ***585/PO/Re/S/02/CPCSEA***:

Ex-vivo permeation studies: A system employing improved Franz diffusion cells with a diffusion area of 3.14cm2 was used for permeation studies. The excised rat skin was set in place with the stratum conium facing the donor compartment and the dermis facing the receptor compartment. Liposomal gel and plane gel was applied to the skin surface in the donor compartment and the receptor compartment was filled with phosphate buffer, pH 7.4 (18 ml). During the experiments, the diffusion cell was maintained at 37±0.5°C and stirred at 200rpm. After application of the test formulation on the donor side, at fixed time intervals, 1 ml of aliquots were withdrawn from receiver compartment through side tube and analysed by UV-Visible spectrophotometer at 230nm.and 306nm**drug deposition studies:[ ]** For determination of drug deposited in skin, cell was dismantled after a period of 24 h and skin was carefully removed from the cell. The formulation applied on skin surface was swabbed first with phosphate buffer pH 7.4 and then with methanol. The procedure was repeated twice to ensure no traces of formulation were left onto skin surface. The skin was then cut into small pieces and drug present in skin was extracted in phosphate buffer pH 7.4 using bath sonicator and determined spectrophotometrically after suitable dilution.

RESULTS

Optimization of lipid, cholestrol and hydroxyl propyl- β – cyclodextrin concentration was done by experimental design Box Behnken design (BBD).**Liposomes were formulated as per box- behnken design matrix** by thin film hydration method. The thin film was hydrated with aqueous phase. containing resveratrol complexed with HP- β-CD. .**The percent drug entrapment** of all prepared formulations was ranged from 46.714 to 96.361 for resveratrol and from 50.325 to 98.048 for benzoyl peroxide. The formulation S-4 was selected on the basis of percent drug content and minimum standard deviation.. The selected drug loaded liposomes

(formulation S-4) were evaluated for physical appearance**, particle size, zeta potential FT-IR,DSC,XRD AND TEM .**Liposome suspension was milky in appearance **Particle size** of optimized formulation of liposomes was found to be with Poly dispersity index value less than 0.3**. Zeta potential graph of Optimized formulation** showed negative value (-15.1). **FT-IR spectrum** of optimized liposomal formulation showed major peaks of both drugs benzoyl peroxide and resveratrol and phospholipid with some minor displacements.**DSC thermogram of final liposomal formulation** showed the endothermic phase transition temperature of the drug loaded liposomes as 267.1° C. A shift in the transition temperature indicated entrapment of drugs into liposome. **XRD of liposomal formulation** revealed the amorphous nature of the encapsulated drug in the formulation due to the absence of the characteristic drug peaks in it. **Lyophilization of liposomal suspension** is done by freeze dryer to increase the stability of suspension. **TEM analysis of lyophilized product of liposomes** revealed the presence of outer coating of bilipid layer entrapping the drugs with an optimum size in the range of 150-350 nm. So the drugs have been successfully encapsulated in liposomes which could be used further for dermatological diseases.

Liposomal gel was formulated by using carbopol 934 in different concentration as 1%, 1.5%, 2%, hydroxypropyl K-100 and sodium carboxymethyl cellulose. Evaluation of liposomal gels was done by pH determination and drug content determination .Percent drugcontent for resveratrol was found to be 75.18 to 89.29 and for benzoyl peroxide was 98.35 to 99.35.. The prepared gels were subjected to homogeneity and grittiness. LG -3 showed maximum homogeneity and no grittiness. In-vitro drug release of different gel formulations was done using phosphate buffer solution pH 7.4. In-vitro drug release of 2.0 % carbopol containing liposomes (LG-3) showed maximum % release for resveratrol and benzoyl peroxide (20.262 %and 19.505 %for 24 hours respectively). Thus this formulation (LG-3) containing 2% carbopol was selected and taken further with permeation enhancers.

Liposomal gel was formulated with permeation enhancers such as. These liposomal gel were further evaluated for percent drug content and in vitro drug release of drug. LG 3.6 showed maximum release for both drugs. pH of LG-3.6 was found to be 7.35. LG-3.6 did not show any grittiness and was highly homogenous .LG 3.6 of optimum spreadibility was selected. Percentage drug release was found to be maximum with combination of 1%DMSO and 10% glycerol (LG-3.6.) as permeation enhancers. Selected formulation LG 3.6was evaluated for viscosity , in vitro drug release, kinetic study and stability study. Viscosity of formulation LG-3.6 was found to be within a limit .Formulation LG-3.6 did not break at different rpm, which represents its mechanical strength. Drug release kinetic study was studied by various kinetic models (zero order plot ,first order plot , higuchi plot and korsmeyer-peppas model .It indicated that formulation LG-3.6 gel has followed Korseymerpeppas model as maximum R^2value closer to 1 was observed in this model. Stability study of LG-3.6 gel was performed by evaluating pH anddrug content of both drugs at different storage conditions. The prepared formulation gel was found to be stable upon storage for 3 months at room temperature, refrigerated temperature and at higher temperature, where no significant changes were observed in the parameters evaluated .Liposomal gel was Propionibacteriunacneees treated to check skin irritation test in rats. There was no redness found on the skin of rat. Anti acne activity was performed on acne causing bacteria Propionibacterium acne. Control gel showed similar antimicrobial effect on p.acne MTCC1951 when compared with selected formulation (LG 3.6) But formulation showed better results than control at the lowest concentration. It was found that antimicrobial effect of liposomal gel more effective in P.acne than the control gel. Ex-vivo permeation studies indicated that maximum %release for resveratrol and benzoyl peroxide as 40.00% and 42.00% respectively. Drug deposition studies showed that %drug retained in skin after 24 hours was found to be 33.52% and with plane gel it was found to be 16.7% .

DISCUSSION

Preparation of Benzoyl peroxide and Resveratrol liposomes

The liposomes of benzoyl peroxide and resveratrolalong with phospholipid cholesterol and Hydroxy propyl beta cyclodextrin were prepared by thin film hydration technique using rotary evaporator. These liposomes were formulated as per Box Behnken Dessign Matrix (Table 1).The percent entrapment of these was also found out and mentioned in table 1

Evaluation of drug loaded liposomes (selected formulation S4)

Visual Appearance: Liposome suspension was milky, in appearance.

Particle Sizeand Polydispersity Index:
Particle size results of optimized formulation of liposomal suspension with PDI value less than 0.3 represents optimum size required to penetrate the skin layer through topical drug delivery system.(fig 1)

Entrapment Efficiency (EE)
Zeta Potential

Negative value of zeta potential (-15.1) of liposomes indicates the brownian motion stability between the particles. (Fig 2)

FT-IR Spectrometry

The FT-IR spectrum of formulation displayed major peaks of both drugs benzoyl peroxide and resveratrol and phospholipid with some minor displacements. (Fig 3)

Differential scanning calorimetry (DSC)

The endothermic phase transition temperature of the drug loaded liposomes was found to be 267.1° C. A shift in the transition temperature indicates a strong hydrophobic interaction between drugs and the phospholipids forming the liposome. DSC thermogram of drug loaded formulation showed entrapment of drug into liposome.(Fig 4)

X ray Diffraction (XRD)

X-Ray diffraction studies of liposomes revealed the amorphous nature of the encapsulated drug in the

formulation due to the absence of the characteristic drug peaks in it. (Fig 5)

TEM analysis of Liposomes

TEM analysis of lyophilized product of liposomes revealed the presence of outer coating of bilipid layer entrapping the drugs with an optimum size in the range of 150-350 nm. (Fig 6)

Preparation of Liposomal gel

Liposomes containing benzoyl peroxide and resveratrol of optimized batch were mixed into carbopol 934 (2%) with a mechanical mixer (25 rpm for 5 min.) and finally the pH was adjusted with triethanolamine and 1N NaOH..Different formulations were prepared.

Evaluation of liposomal gel containing different penetration enhancers

Physical examination and homogeneity

The prepared liposomal gel formulations were inspected visually for their colour ,homogeneity and consistency.All the liposomal gel formulations were found to be homogenouswithout any colour change.The pH value of all the prepared formulation ranged from 6.8-7.0 which is considered to be acceptable for avoiding the risk of irritation upon application to the skin.

pH determination

Drug Content Determination:

0.5 gm of gel was taken in test tube and diluted up to 5 ml with methanol and centrifuged at 18000 rpm for 30 minutes at 4°C to extract out whole drugs from liposomes incorporated in gel. Supernatant was taken and checked its absorbance at 230 nm and 306 nm..(Table 2)

In Vitro drug release (Table3)

Spreadibility (Table 4)

Determination of viscosity

It was found that viscosity of formulation LG-3.6 was found to be within a limit while viscosity of control was found to be more as compare to formulation LG-3.6. formulation LG-3.6 did not break at different rpm, which represent its mechanical strength.(Table 5)

Drug release kinetic

Zero order

First order

Higuchi Model
Korsmeyer –Peppas model

From table 6 it was found that Formulation LG-3.6 gel has followed Korseymerpeppas model (Table 6)

Stability study of LG-3.6 GEL

Stability studies revealed that the physical appearance,rheological properties and drug content remained unchangedupon storage at 2-8^{o} for three months.However changes were observed when the gel was stored at room temperature and 40^{o} C.

Anti microbial studies

It was found that *control gel* showed similar antimicrobial effect on *Propionibacterium acnes MTCC 1951* when compared with formulation containing both drugs in 2% w/w gel at concentration (0.5, 0.25, 0.125 g/ml) but formulation showed better than control at lowest concentration . Overall the antimicrobial effect of *Liposomal gel* was more effective in *Propionibacterium acnesMTCC 1951* infection than control gel and marketed preparation.

Skin irritation study

Liposomal gel was treated to check skin irritation in rats. There was no redness or dryness found on the skin of rats.

Skin permeation studies:

The Ex-Vivo permeation of benzoyl peroxide and resveratrol using Wistar albino rat skin from liposomal gel was compared with that of plane gel containing 2.5% w/w of benzoyl peroxide and resveratrol . The permeation of benzoyl peroxide and resveratrol was calculated at each sampling time points during 24 hours study.

Skin retention study:

Liposomal encapsulation of drugs shows drug reservoir effect in skin so in-vitro skin deposition of benzoyl peroxide and benzoyl peroxide was also calculated. Results of ex-vivo skin deposition are recorded in table . From results shown in table it could be concluded that liposomal encapsulation showed more drug retention compared with plain drug gel. The higher drug skin retention in case of liposomal gel may be due to, creation of reservoir effect for drug in skin due to deposition of other components of liposomes with drug into the skin and thereby increasing the drug retention capacity into the skin

Liposomal gel was Propionibacterium acneees treated to check skin irritation test in rats. There was no redness found on the skin of rat. Anti acne activity was performed on acne causing bacteria Propionibacterium acne. Control gel showed similar antimicrobial effect on p.acne MTCC1951 when compared with selected formulation (LG 3.6) But formulation showed better results than control at the lowest concentration. It was found that antimicrobial effect of liposomal gel more effective in P.acne than the control gel. Ex-vivo permeation studies indicated that maximum %release for resveratrol and benzoylperoxide as 40.00% and 42.00% respectively. Drug deposition studies showed that %drug retained in skin after 24 hours was found to be 33.52% and with plane gel it was found to be 16.7

Table 1 : Box-Behnken Design Matrix

Independent Variables					Dependent Variables	
S.no.	Formulation code	Phospholipid	Cholesterol	HP- β-CD	% Entrapment of Resveratrol	% Entrapment of Benzoyl Peroxide
		(X_1)	(X_2)	(X_3)	(Y_1)	(Y_2)
1	B1	0.1	0.015	0.05	84.608	88.486
2	B2	0.2	0.02	0.1	69.220	90.567
3	B3	0.15	0.015	0.1	75.397	80.021
4	B4	0.15	0.015	0.1	74.680	80.917
5	B5	0.2	0.01	0.1	92.439	93.225
6	B6	0.15	0.02	0.15	96.631	98.048
7	B7	0.15	0.01	0.05	46.714	50.325
8	B8	0.1	0.02	0.1	92.770	93.860
9	B9	0.1	0.01	0.1	86.869	79.925
10	B10	0.15	0.02	0.05	92.715	91.232
11	B11	0.15	0.01	0.15	90.840	90.998
12	B12	0.2	0.015	0.15	93.708	94.070
13	B13	0.1	0.015	0.15	92.991	89.380
14	B14	0.2	0.015	0.05	95.693	96.394
15	B15	0.15	0.015	0.1	73.467	74.691
16	B16	0.15	0.015	0.1	77.162	80.959
17	B17	0.15	0.015	0.1	79.037	80.914

Table 2: Number of Solutions with desirability=1

Number	Formulation code	Phospholipid	Cholesterol	HP-β-CD	% Entrapment of Resveratrol	% Entrapment of Benzoyl peroxide	Desirability	
1	S1	0.15	0.01	0.15	92.4743	90.95641	1.000	
2	S2	0.1	0.02	0.1	93.48979	93.34905	1.000	
3	S3	0.16	0.02	0.11	81.01874	83.17371	1.000	
4	**S4**	**0.16**	**0.01**	**0.14**	**83.81818**	**83.84209**	**1.000**	**Selected**
5	S5	0.15	0.02	0.15	95.18945	96.11434	1.000	

Table 3: Percent Drug content of different gels containing liposomes

S.No.	Formulation Code	% Drug content of Resveratrol (Mean ±S.D)	% Drug content of Benzoyl peroxide) (Mean ±S.D)
1	LG-1	Gel was not formed	
2	LG-2	75.687±1.511	99.383±0.005
3	LG-3	89.290±1.511	98.974±0.009
4	LG-4	77.199±1.511	98.787±0.014
5	LG-5	75.184±0.872	98.350±0.088

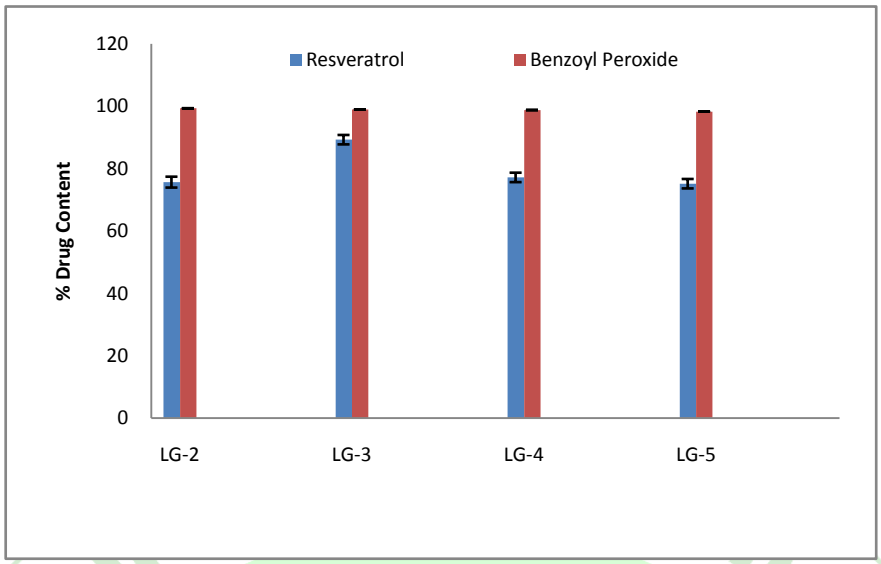

Fig.1: Percentage Drug content of different gels containing liposomes

		Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 411.9	Peak 1:	413.0	100.0	77.93
PdI: 0.263	Peak 2:	0.000	0.0	0.000
Intercept: 0.904	Peak 3:	0.000	0.0	0.000

Result quality : Refer to quality report

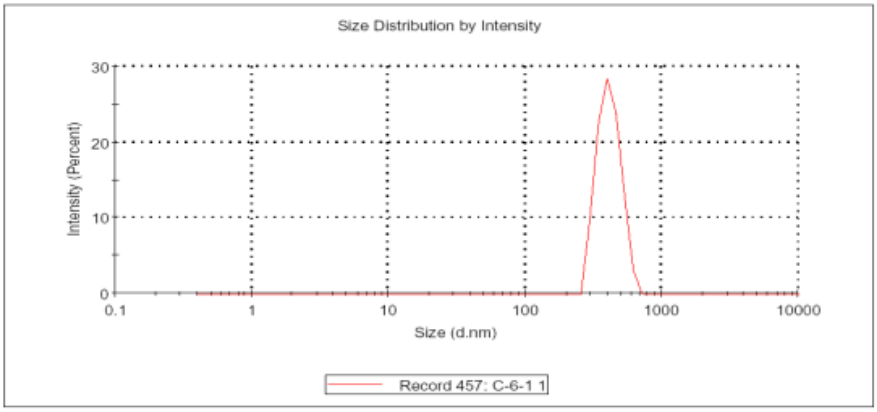

Fig. 2: Particle size peak of Optimized formulation

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -15.1	Peak 1:	-15.1	100.0	3.69
Zeta Deviation (mV): 3.69	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.0411	Peak 3:	0.00	0.0	0.00

Result quality : Good

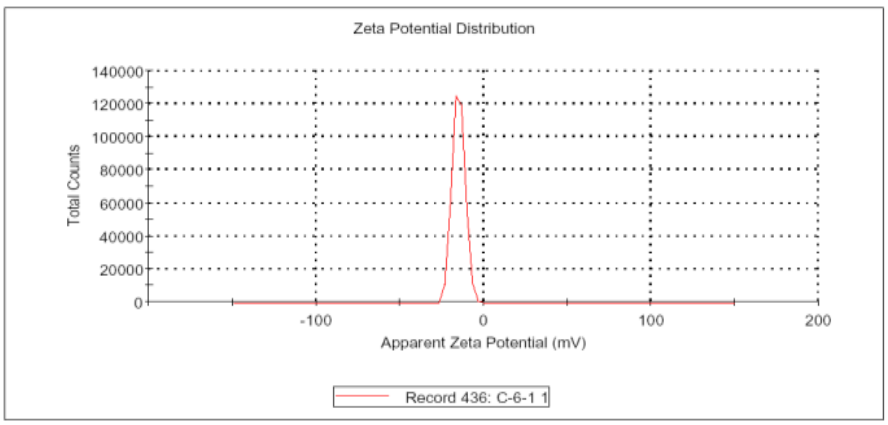

Fig. 2 : Zeta potential graph of Optimized formulation

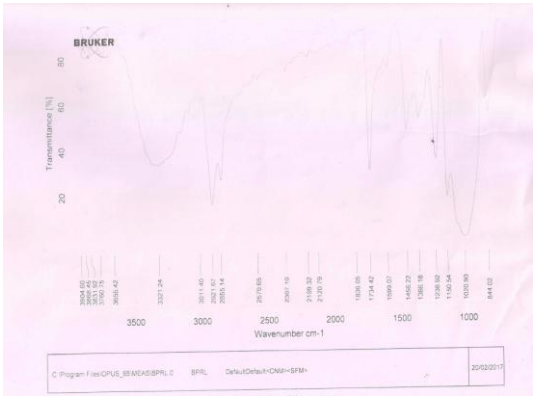

Fig. 4: FTIR spectrum of Liposomal formulation containing Resveratrol and Benzoyl Peroxide

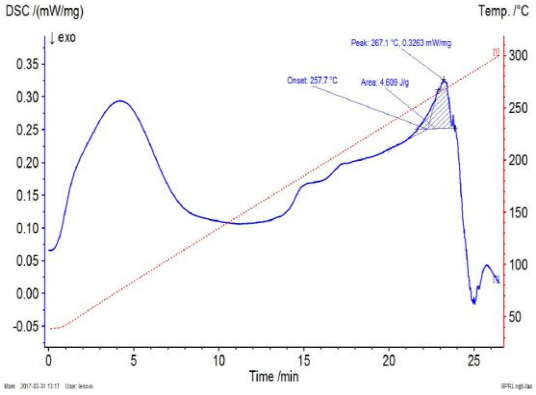

Fig. 5: DSC thermogram of Liposomes containing Resveratrol and Benzoyl Peroxide

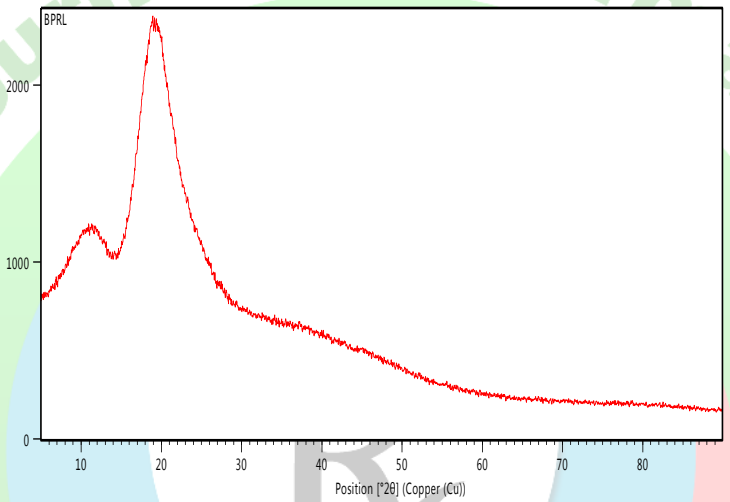

Fig. 6: XRD spectra of Liposomes containing Resveratrol and Benzoyl peroxide

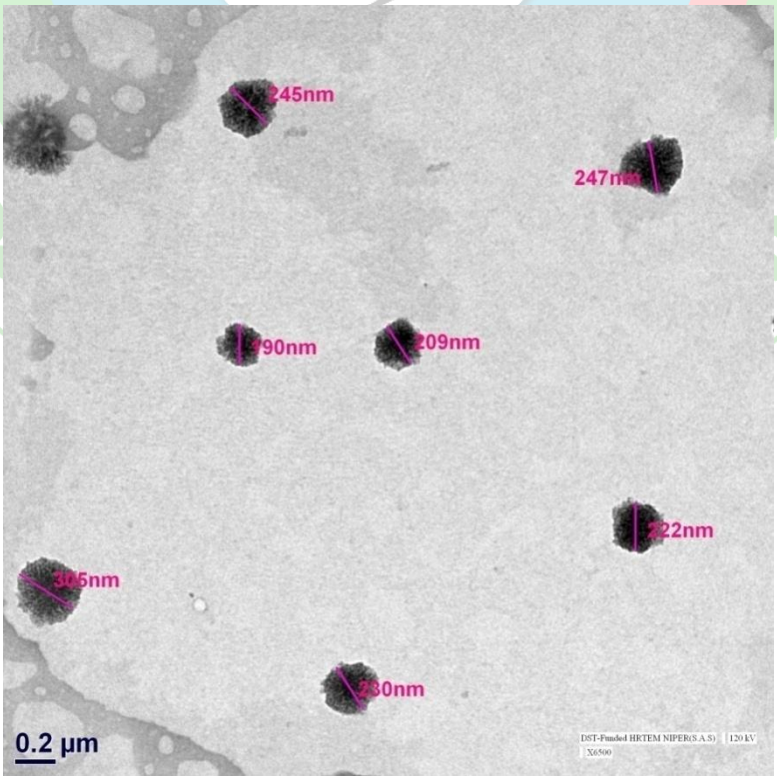

Fig 7: TEM analysis of lyophilized product

Table 3: In vitro Drug Release of LG-3 in Phosphate buffer solution pH 7.4

S.No	Time (hr)	% Release of Resveratrol (Mean± Std.dev)	% Release of Benzoyl Peroxide (Mean ± Std.dev)
1	0.25	0.48±0.052	2.028±0.049
2	0.5	1.555±0.061	5.419±0.022
3	1	2.314±0.08	5.583±0.035
4	2	3.336±0.052	6.857±0.019
5	3	5.117±0.133	7.529±0.061
6	4	7.373±0.052	10.464±0.041
7	5	9.843±0.139	13.72±0.052
8	6	14.427±0.11	17.782±0.059
9	7	18.924±0.061	19.765±0.027
10	8	20.581±0.061	20.027±0.027
11	24	20.262±0.052	19.505±0.138

Table 6: Percent Drug Content of Liposomal gel containing different permeation enhancers

S. No.	Formulation Code	Drug Content of Resveratrol (%)	Drug Content of Benzoyl Peroxide (%)
1	LG-3.1	52.507 ± 0.349	54.393 ± 0.355
2	LG-3.2	70.650 ± 0.872	66.473 ± 0.945
3	LG-3.3	54.024 ± 0.872	52.188 ± 0.997
4	LG-3.4	42.985 ± 0.174	38.962 ± 0.070
5	LG-3.5	28.274 ± 0.302	11.260 ± 0.356
6	LG-3.6	96.847 ± 0.872	87.757 ± 1.010
7	LG-3.7	82.237 ± 0.872	81.909 ± 1.010

Table 5 : pH of Formulations

S. No.	Formulation Code	pH
1	LG-3.1	7.26±0.026
2	LG-3.2	7.11±0.01
3	LG-3.3	7.08±0.02
4	LG-3.4	7.12±0.02
5	LG-3.5	7.07±0.02
6	LG-3.6	7.35±0.03
7	LG-3.7	7.45±0.01

Table 6: Homogeneity and Grittiness of formulation

S. No.	Formulation Code	Grittiness	Homogeneity
1	LG-3.1	-	++
2	LG-3.2	-	++
3	LG-3.3	-	++
4	LG-3.4	-	+++
5	LG-3.5	*	+
6	LG-3.6	-	+++
7	LG-3.7	-	+++

Where, + Satisfactory, ++ Good, +++ Very Good, - No Grittiness, * Grittiness.

Table 7: Spreadbility of different concentration carbapol liposomal gel

S. No.	Formulation Code	Spreadibility (g.cm/s)
1	LG-3.1	18.5±0.48
2	LG-3.2	16.14±1.27
3	LG-3.3	15.6±0.95
4	LG-3.4	14.21±0.80
5	LG-3.5	12.23±1.01
6	LG-3.6	11.5±0.92
7	LG-3.7	14.4±0.55

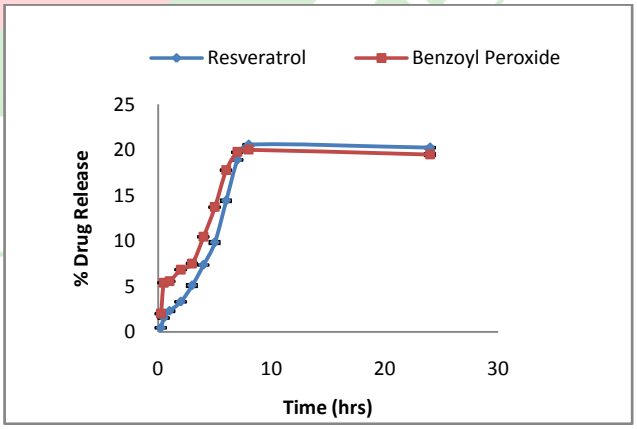

Figure 8:*in-vitro* drug release of **LG-3 in Phosphate buffe solution pH 7.4**

Table 8(a): Percent drug release profile of liposomal gel containing different penetration enhancers

Time (hr)	10% Glycerol (LG 3.3)		1% DMSO (LG 3.1)		3% Propylene glycol (LG 3.2)		30% PEG-400 (LG 3.4)	
	Resveratrol	Benzoyl Peroxide	Resveratrol	Benzoyl Peroxide	Resveratrol	Benzoyl Peroxide	Resveratrol	Benzoyl Peroxide
0.25	1.52±0.061	1.94±0.042	0.251±0.11	0.314±0.095	0.339±0.061	0.347±0.183	2.402±0.03	2.031±0.12
0.5	2.455±0.133	3.828±0.127	0.286±0.03	1.761±0.122	0.586±0.091	0.863±0.101	2.349±0.03	3.983±0.5
1	2.623±0.13	4.435±0.13	1.23±0.23	2.73±0.03	0.69±0.102	1.02±0.16	3.14±0.12	4.43±0.11
2	4.588±0.161	5.18±0.033	2.472±0.122	3.79±0.132	0.921±0.133	2.298±0.058	4.729±0.08	10.29±0.63
3	5.1±0.11	7.552±0.122	3.072±0.274	4.746±0.205	1.926±0.161	3.63±0.218	4.994±0.12	12.14±0.55
4	7.621±0.052	8.496±0.081	4.958±0.08	8.53±0.06	4.042±0.061	4.363±0.142	5.593±0.24	12.741±0.12
5	9.508±0.061	9.457±0.079	8.126±0.199	14.554±0.16	6.052±0.352	6.66±0.118	5.258±0.08	15.917±0.19
6	17.901±0.06	10.344±0.04	13.601±0.04	25.926±0.15	7.885±0.139	7.386±0.027	6.034±0.052	16.133±0.12
7	29.062±0.22	11.894±0.401	21.747±0.08	36.169±0.14	8.661±0.08	9.857±0.138	6.21±0.133	16.005±0.13
8	34.035±0.32	13.921±0.08	22.911±0.12	36.986±0.91	8.679±0.052	9.819±0.105	6.316±0.11	17.09±0.05
24	33.152±0.11	13.441±0.21	22.831±0.09	35.499±0.56	8.926±0.091	8.255±0.048	7.286±0.15	17.274±0.14

Table 8(b): Percent drug release profile of liposomal gel containing different penetration enhancers

Time (hr)	0.1% Curcumin (LG 3.5)		10% glycerol and 1% DMSO (LG 3.6)		5% DMSO and 10% Glycerol (LG 3.7)	
	Resveratrol	Benzoyl Peroxide	Resveratrol	Benzoyl Peroxide	Resveratrol	Benzoyl Peroxide
0.25	0.427±0.052	1.232±0.100	0.444±0.11	1.492±0.128	0.268±0.139	1.0381±0.834
0.5	0.956±0.105	2.206±0.052	1.203±0.03	2.986±0.082	0.921±0.133	2.298±0.155
1	1.10±0.12	2.44±0.04	2.38±0.3	3.64±0.11	1.34±0.08	3.68±0.03
2	3.460±0.030	2.965±0.103	12.241±0.11	8.269±0.578	8.838±0.091	7.428±0.612
3	4.923±0.052	4.364±0.131	22.115±0.091	17.972±0.075	12.84±0.03	13.155±0.102
4	5.117±0.030	4.855±0.082	26.402±0.165	24.916±0.447	19.047±0.052	18.720±0.069
5	5.435±0.061	5.542±0.137	34.923±0.349	36.11±0.209	25.974±0.152	30.742±0.738
6	5.646±0.052	5.89±0.124	40.257±0.275	46.248±0.257	32.278±0.132	35.866±0.519
7	5.822±0.052	6.284±0.099	51.277±0.381	56.388±0.419	37.435±0.132	43.848±0.302
8	6.034±0.091	6.214±0.09	53.393±0.076	62.595±0.144	42.328±0.132	50.684±0.252
24	5.964±0.061	6.038±0.096	52.644±0.349	62.389±0.151	43.298±0.275	50.375±0.243

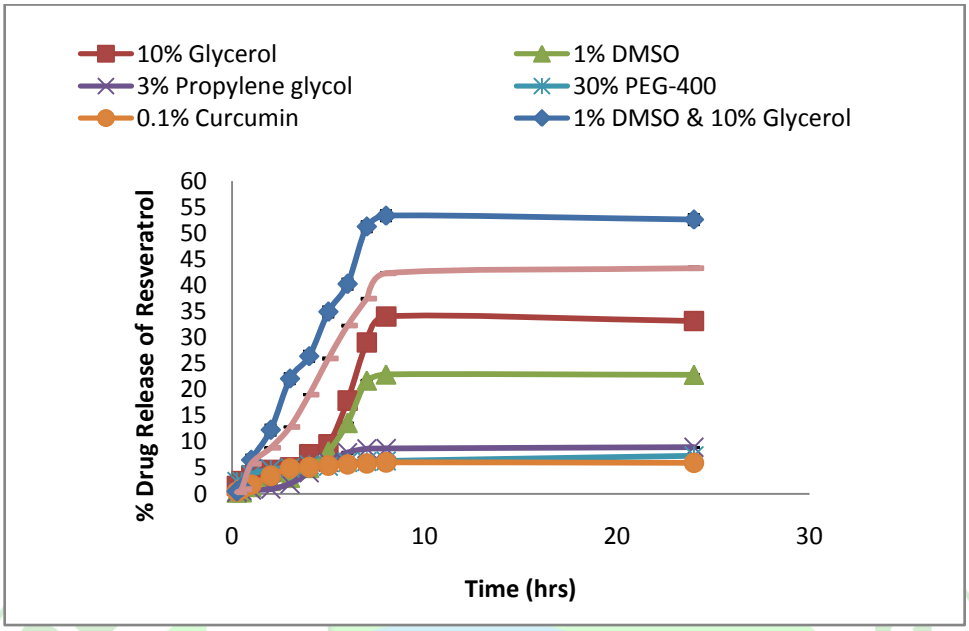

Fig. 6: Percentage drug Release Profile of Resveratrol in Formulation with different permeation enhancer

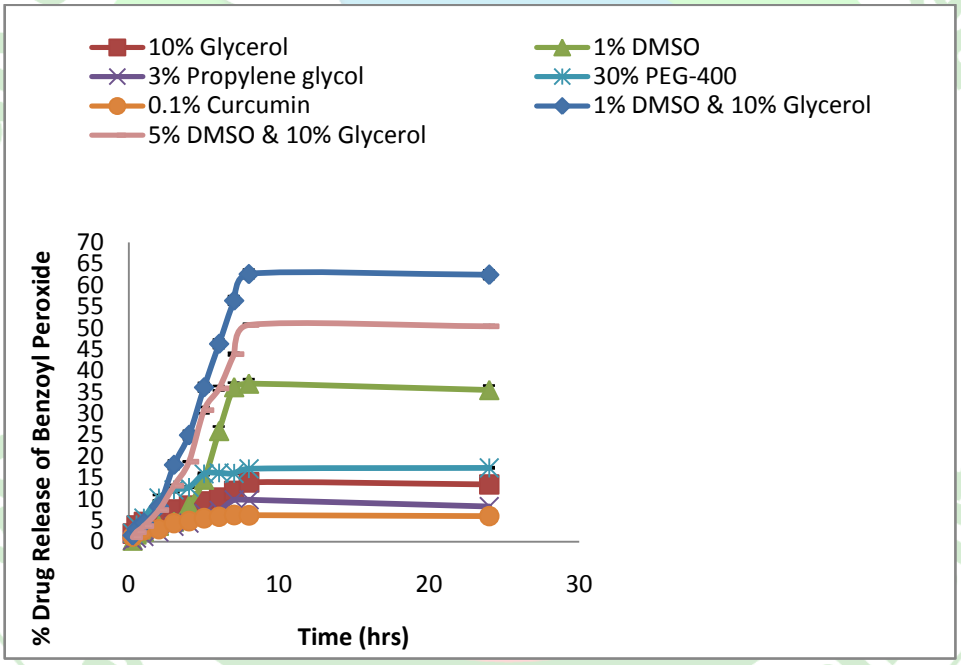

Figure 7: Percentage drug Release Profile of Benzoyl Peroxide in Formulation with different permeation enhancer

Table 9: Viscosity of control gel and formulation LG-3.6 in centipoises

.S.No.	RPM	Viscosity of control gel (cp)	Viscosity of formulation LG-3.6 formulation (cp)
1	10	40389±0.38	25689±1.01
2	20	32576±1.38	19579±0.49
3	50	20457±1.11	11568±0.99
4	100	15241±0.66	4583±1.57

Drug release kinetic

Zero order

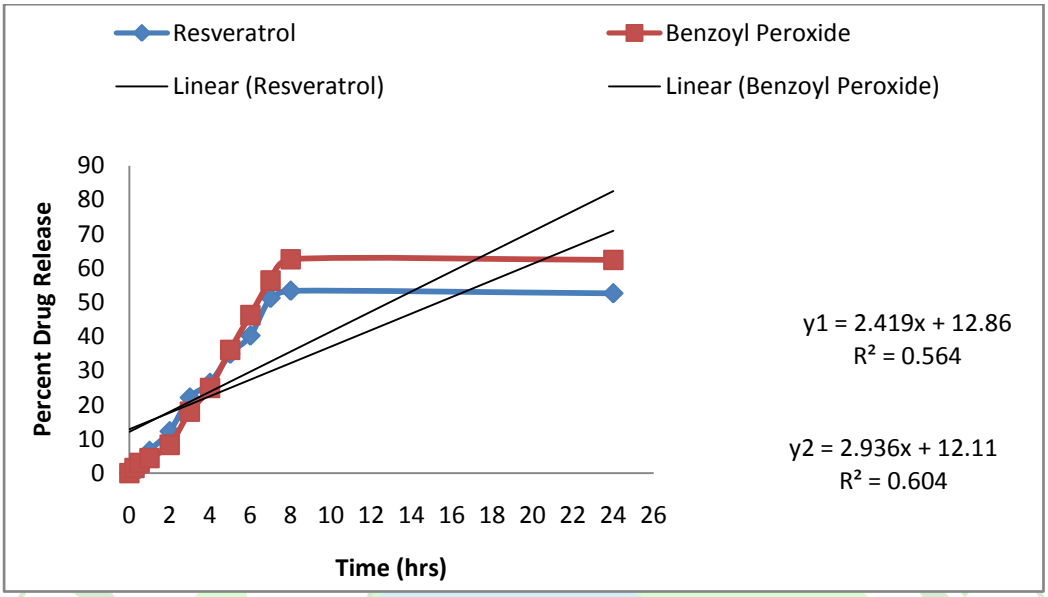

Fig. 9 : Zero order graph for LG-3.6 formulation (y1 corresponds to Resveratrol and y2 corresponds to Benzoyl Peroxide)

First order

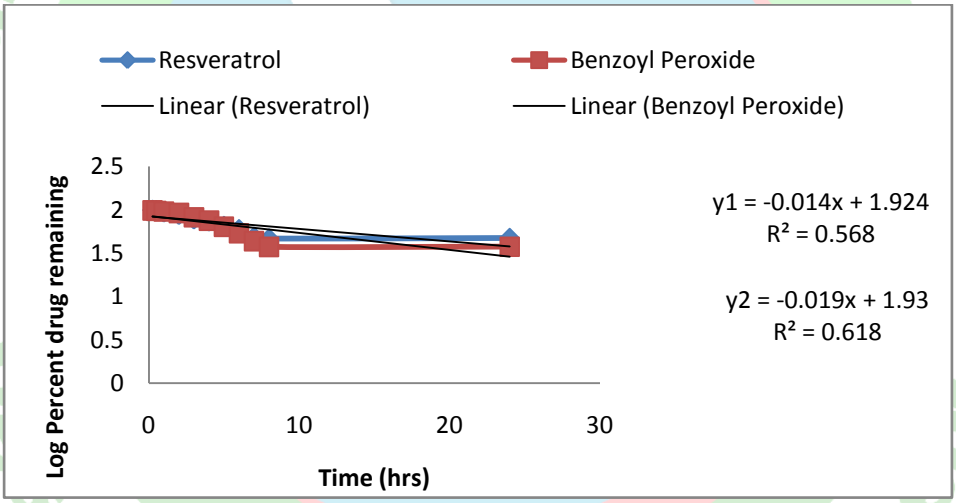

Fig. 10 : First order graph for LG-3.6 formulation (y1 corresponds to Resveratrol and y2 corresponds to Benzoyl Peroxide)

Higuchi Model

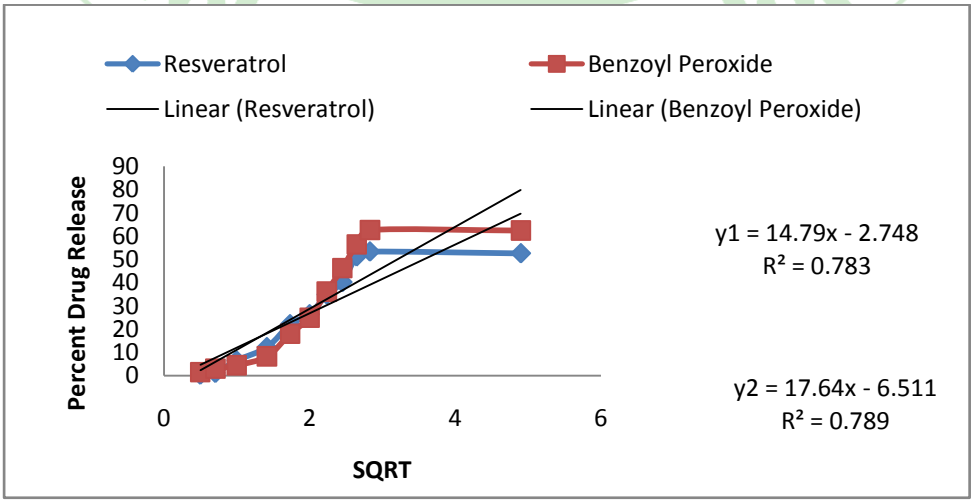

Fig. 11 : Higuchi order graph for LG-3.6 formulation

Korsmeyer –Peppas model

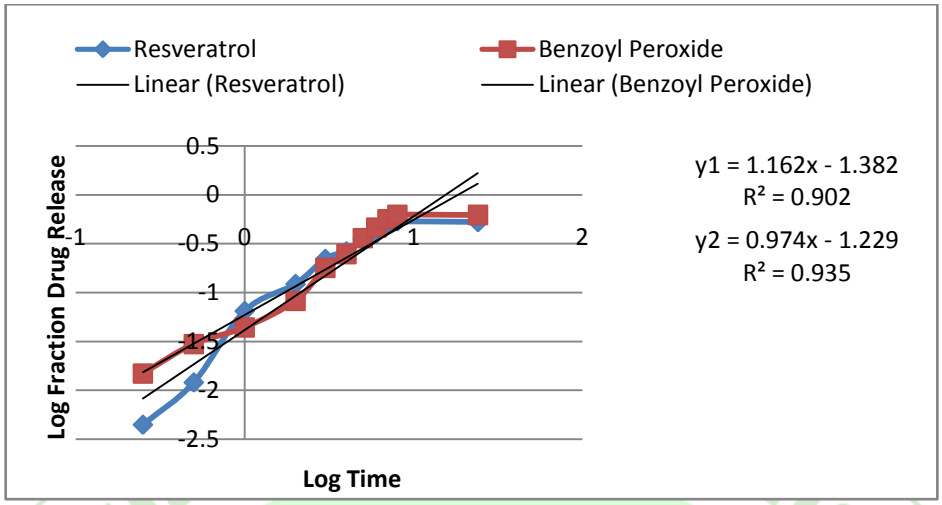

Figure 12: Korsmeyer –Peppas order graph for LG-3.6 formulation

Table 10: Kinetic equation parameter of formulation LG-3.6 formulation

DrugName	Zero order		First order		Higuchi		Korsmeyer-Peppas	
	R^2	K_0	R^2	K_0	R^2	K_0	R^2	N
Resveratrol	0.564	2.419	0.568	-0.032	0.783	14.79	0.902	2.676
Benzoyl peroxide	0.604	2.936	0.618	-0.043	0.789	17.64	0.935	2.243

Table No.11: Drug content of prepared Liposomal gel formulation in different temperatures.

S.No.	Formulation	Initial drug content		% Drug Content after 30 days	
		Resveratrol	Benzoyl Peroxide	Resveratrol	Benzoyl Peroxide
1	LG-3.6 at refrigerator temp.	96.847 ± 0.872	87.957 ± 1.010	94.520 ± 0.755	85.065 ± 0.702
2	LG-3.6 at room temp. (25±2°C)	96.847 ± 0.872	87.957 ± 1.010	92.097 ± 0.577	82.295 ± 0.053
3	LG-3.6 at (40±2°C)	96.847 ± 0.872	87.957 ± 1.010	90.838 ± 0.218	81.244 ± 0.064

Table 12: pH study of LG-3.6 gel at different temperatures

S.No.	Formulation	Initial pH	pH after 30 days
1	LG-3.6 at refrigerator temp (5±3°C)	7.35 ± 0.03	7.42±0.037
2	LG-3.6 at room temp	7.35 ± 0.03	7.33±0.016
3	LG-3.6 at (40±2°C)	7.35 ± 0.03	7.20±0.018

Table 13: Antimicrobial activity of Liposomol gel formulation in comparison to control gel

S.No.	Concentration (g/ml)	Control	Sample (LG-3.6)
1	1	0	0.2
2	0.5	0.2	0.25
3	0.25	0.3	0.31
4	0.125	0.275	0.326
5	0.072	0.398	0.450

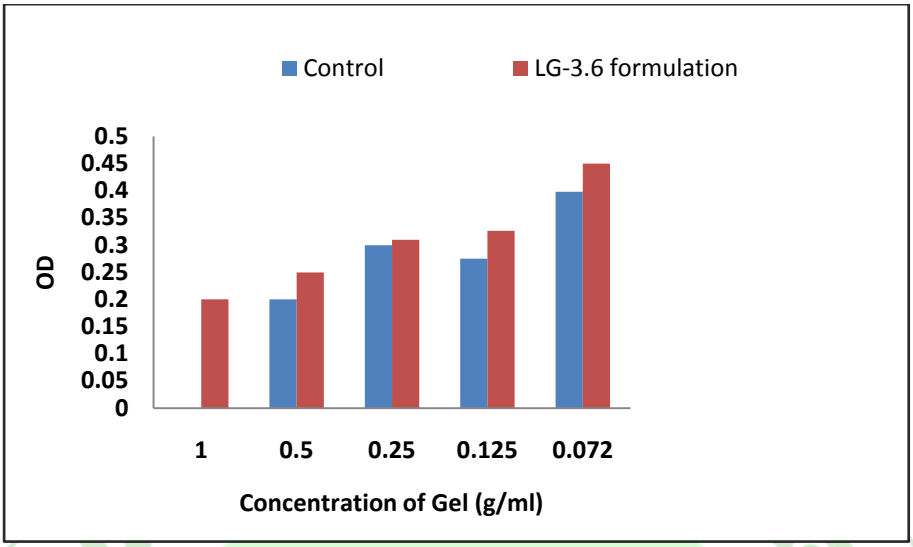

Fig.13: Optical density (OD) of the P. acnes cell growth treated with Control gel, Liposomal gel formulation (LG-3.6)

Table 8 : In vitro Drug Release of LG-3 in Phosphate buffer solution pH 7.4

S.No	Time (hr)	% Release of Resveratrol (Mean± Std.dev)	% Release of Benzoyl Peroxide (Mean ± Std.dev)
1	0.25	0.48±0.052	2.028±0.049
2	0.5	1.555±0.061	5.419±0.022
3	1	2.314±0.08	5.583±0.035
4	2	3.336±0.052	6.857±0.019
5	3	5.117±0.133	7.529±0.061
6	4	7.373±0.052	10.464±0.041
7	5	9.843±0.139	13.72±0.052
8	6	14.427±0.11	17.782±0.059
9	7	18.924±0.061	19.765±0.027
10	8	20.581±0.061	20.027±0.027
11	24	20.262±0.052	19.505±0.138

TABLE 12 : Percentage of drug retained in skin after 24 hours

Test Formulation	% Drug Retained
Plane Gel	16.7%
Liposomal Gel	33.52%

CONCLUSION

The liposomal product of benzoyl peroxide and resveratrol was prepared with a view to improve therapeutic response and reduce the side effects. Here liposomes of these drugs were prepared by thin film hydration technique and evaluated. Maximum entrapment efficiency was found to be96.631 for resveratrol and 98.048 for benzoyl peroxide. The prepared liposomes were found to have good morphological properties and size distribution. From DSC thermo grams of liposomes it could be concluded that significant interaction occur between drug and lipid components of the vesicles that lead to higher entrapment efficiency. The drug entrapment efficiency can be attributed to phospholipids ability to vesiculate independently because they carry two

bulky non polar chains and polar head groups which help them spontaneously form into closed bilayer. Liposomal gels of the optimized liposomes were prepared to increase the rate of permeation into the skin and also to decrease the adverse side effects. Carbopol 934, HPMC was used for gel preparation. The gel was evaluated for colour, clarity, pH, drug content. Viscosity, spreadibility and homogeneity In vitro drug diffusion and skin retention for liposomal gel was found to be respectively. Stability studies indicated that the formulation is stable over a period of three months when stored at 2-8^{o}C .

The present study has been a satisfactory attempt to formulate and evaluate liposome of benzoyl peroxide and resveratrol and liposomal gel .Hence, finally from skin permeation study and *in vivo* study it was concluded that the prepared liposome of benzoylperoxide and resveratrol may prove to be potential candidate for safe and effective topical drug delivery .

CONFLICTS OF INTEREST

Authors declare that there is no conflict of interest.

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