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DESIGN OF AN ENCAPSULATED TOPICAL FORMULATION FOR CHEMOPREVENTION OF SKIN CANCER

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ABSTRACT: This study aims to design resveratrol loaded microemulsion (ME) topical formulation for use in skin cancer. Encapsulation of resveratrol can improve its intrinsic solubility and enhance penetration into the skin for topical use as an alternative to injectables. Preformulation studies by DSC and FTIR confirmed the compatibility of chosen excipients with resveratrol. Resveratrol loaded microemulsion was developed using phase diagrams based on oleic acid, labrasol and transcutol P as the oil phase, surfactant and co-surfactant respectively. Optimization was based on the particle size and % drug diffused. Optimized resveratrol loaded microemulsion was transparent and stable without phase separation. Optimized ME had an average particle size of 243 nm, zeta potential -32.9 ± 2.5 mV and % drug content of 92.216 ± 1.056. Optimized resveratrol ME was incorporated into a gel base to form a microemulgel that exhibited a spreadability of 34.3 ± 0.34 gcm/s, the viscosity of 6000 ± 1.67 cps and sustained i*n-vitro* and *ex-vivo* drug release. Amount of drug diffused (*in-vitro*) at the end of 24 h was 98.21 ± 0.03% from the conventional gel. In comparison, 71.11 ± 0.47 and 68.15 ± 0.12 was the % drug diffused from the ME gel. Results of drug retention for both *in-vitro* as well as *ex-vivo* permeation study was considerably higher for resveratrol microemulgel than control. Data of antioxidant activity, tyrosinase inhibition, and cytotoxicity in B16F10 melanoma cell line prove that resveratrol loaded ME gel is a promising treatment option for skin cancer chemoprevention.

INTRODUCTION: The three skin cancer types are cutaneous malignant melanoma, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCC and SCC are classified as nonmelanoma skin cancers, whereas melanoma skin cancer is an aggressive type that can metastasize and cause death.

Actinic keratosis (AK), also known as solar keratoses, is the most frequent dermatological premalignant presentation exhibiting a proliferation of atypical keratinocytes confined to the deeper epidermis. This effect is dependent on the cumulative effect of UV radiation from sun exposure on the skin [1].

Studies for chemoprevention of melanoma by agents that prevent, inhibit or reverse its development are being investigated [2]. Novel topical formulations are non-invasive drug delivery systems in comparison to parenteral and can provide sustained therapy with a single application, avoid first-pass hepatic metabolism, gastric

degradation, frequent dosing, and inconvenience of parenterals. As a result, enhanced topical delivery of drug to the target site can be achieved. Marketed topical creams and solutions of 5 fluorouracil (5-FU) and imiquimod used in skin cancers are beset with severe side effects like skin irritation, burning, redness, dryness, pain, swelling, tenderness or changes in skin color at the site of application. There is a growing need for newer topical formulations which can increase penetration and retention of the drug on the skin and provide benefits like extended drug release, improved drug stability, and lower skin irritation by avoiding direct contact of the drug with the skin's surface [3]. Use of novel drug delivery systems like polymeric and lipid nanoparticles, nanoemulsions, dendrimers and liposomes to enhance skin penetration of drugs are reported [4, 5].

Microemulsions are transparent, colloidal, isotropic and thermodynamically stable liquid dispersions of oil and water prepared using surfactants and co-surfactants. Surfactants of choice are non-ionic surfactants because of their cutaneous tolerance and balanced hydrophilic-lipophilic nature. ME's offer several advantages for topical delivery including controlled droplet size, ability to efficiently dissolve lipophilic drugs, enhanced skin permeation and an extended release of lipophilic and hydrophilic drugs. Reports suggest that oleic acid microemulsions demonstrate a higher solubilizing ability and a higher concentration of drug retention within the skin [6]. Microemulsions have high drug loading capacity, and due to high drug solubilizing capacity, they can overcome stratum corneum barrier and partition the drug into the skin [7]. Non-ionic surfactants used in topical formulations include polyoxyl castor oil derivatives like cremophor EL and cremophor RH 40, polysorbates sorbitan monooleate and various polyglycolyzed glycerides like labrafils and labrasol [8, 9]. It is generally accepted that surfactants with low HLB of 3-6 are used for the formulation of w/o ME, whereas surfactants with high HLB values of 8 to 18 are preferred for the formation of o/w ME. Surfactants having HLB greater than 20 require the presence of co-surfactants for the formation of microemulsions [10].

Resveratrol, a natural polyphenolic compound, has high antioxidant activity but published data shows that penetration of polyphenols into the skin is limited due to its poor solubility [11]. Hence, innovative methods are essential to improve penetration of resveratrol into the skin. Published reports suggest that the formulation of MEs achieve improved penetration of resveratrol into the skin, especially into the dermis [12]. Resveratrol has low bioavailability which is attributed to poor water solubility. It also shows photodegradation and low stability against environmental stress. Encapsulation of resveratrol can be promising to overcome these limitations and as a result enhance the solubility, thereby improving its bioavailability and also provide stability against trans-to-cis isomerization, and overcome susceptibility to photodegradation. Due to the insoluble nature of resveratrol, conventional topical formulations like solutions, creams, and gels remain ineffective [13]. As MEs have low viscosity, incorporation of optimized drug loaded microemulsion in a gel base can be beneficial for ease of application and better absorption through the skin.

The objective of the proposed work is directed towards encapsulation of resveratrol to overcome its limitations and provide efficacious treatment with enhanced skin delivery and improved patient compliance for use in premalignant skin cancer as an alternative to injectables. The overall goals of this study were (i) To develop and characterize microemulsion of resveratrol (ii) improve intrinsic solubility issue of resveratrol (iii) evaluate subsequent effects on *in-vitro* and *ex-vivo* release and retention of drug on the skin and iv) estimate antioxidant, antityrosinase and melanoma cell line cytotoxicity activities of the optimised formulation.

MATERIALS AND METHODS:
Chemicals and Reagents: Resveratrol was a gift sample from Sami Labs Pvt. Ltd., India; oleic acid, ethanol, disodium hydrogen orthophosphate, potassium dihydrogen phosphate, and propylene-glycol were procured from S.D. Fine Chemicals Ltd. (India); isopropyl myristate, polysorbates, PEG-400 and PEG-600, were gifted by Mohini Organics Pvt. Ltd., India, Capryol PGMC, Labrafac Lipophile WL1349, Labrasol, and Transcutol-P were received as gift samples from Gattefosse Pvt. Ltd., India; Carbopol Ultrez10NF was a gift from Lubrizol Pvt. Ltd., India.

DPPH and mushroom tyrosinase were procured from Sigma Aldrich Pvt. Ltd., India. All other chemicals used were of laboratory reagent grade.

Formulation of O/W Microemulsion:

Screening of Oils, Surfactants and Co-Surfactants for Microemulsion Formulation using Saturation Solubility Method: The saturation solubility of Resveratrol in various oils such as oleic acid, capryol PGMC, iso propyl myristate, Labrafac lipophilic WL 1349, [oleic acid + Labrafac lipophilic WL 1349 (1:1)]; Surfactants like Labrasol®, Tweens® and Co-surfactants/ Co-solvents like Transcutol® P, PEG-400, PEG-600 and propylene glycol was determined. Briefly, an excess amount of Resveratrol, *i.e.* 100 mg was added in 2 ml of either oil, surfactant and cosurfactant in a 5 ml stoppered vial separately and this mixture was mixed in a vortex mixer (Citizen CY 120). The vials were then shaken on an orbital shaker for 24 h followed by centrifugation at 6000 rpm for 30 min. The concentration of Resveratrol in the filtrate was determined by UV spectrophotometer (UV 1800 Shimadzu version 2.33) after appropriate dilution with ethanol at λmax of 235 nm. The excipients that showed the highest solubility of Resveratrol were shortlisted for further studies.

Emulsification Ability of Surfactants and Co-Surfactants: Various surfactants were screened for their emulsification ability in the selected oil phase. 300 mg of surfactant was mixed with 300 mg of the selected oil phase and the mixture gently heated at 45 ℃ to 60 ℃ for homogenizing the components. 50 mg of this isotropic mixture was accurately weighed and diluted with double distilled water to 50 ml to yield a fine emulsion. The ease of formation of emulsions was monitored by noting the number of volumetric flask inversions required to obtain a uniform emulsion. The resulting emulsions were observed visually for relative turbidity. The emulsions were allowed to stand for 2 h, and the percent transmittance was measured at 638.2 nm by UV-spectrophotometer (UV 1800 Shimadzu version 2.33) using double distilled water as a blank. Similarly, emulsification ability of co-surfactant was evaluated.

Construction of Pseudo Ternary Phase Diagram: Pseudo-ternary phase diagrams containing oil, surfactant, co-surfactant, and water were developed using water titration method [14]. Briefly, mixtures of (Oleic acid) and (Labrasol + Transcutol P) were prepared as the oil phase and Smix (surfactant and co-surfactant). The concentration of surfactant to cosurfactant (Smix) was varied from 1:1, 1:2 and 2:1. Selected Smix were mixed with the oil phase in ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9. Distilled water was titrated drop by drop to the oil and Smix mixture with constant stirring using a magnetic stirrer at ambient temperature. After each addition, the mixture was checked for appearance. The endpoint of the titration was the point where the solution turned turbid or cloudy. The quantity of distilled water required to make the mixture turbid was noted. The percentages of the components were calculated, and the same procedure was followed for the other Smix ratios. Pseudo-ternary phase diagrams were plotted for the prediction of clear and stable microemulsion zones using Chemixschool 3.6 software.

Loading of Resveratrol in the Optimised ME: From the obtained pseudo-ternary phase diagrams, a formulation containing maximum microemulsion region was selected. Four points were selected from this region of the phase diagram, and micro-emulsions were prepared. An accurately weighed quantity of drug (1% w/w) was added to the oil and stirred for 15 min, at 30 - 40 ℃ until the drug was solubilized. To this mixture, surfactant and co-surfactant were added and stirred until a homogeneous mixture was obtained.

Characterization of Resveratrol Loaded ME:

Macroscopic Appearance: Visual observation for color, homogeneity, presence of precipitate or phase separation were evaluated after 48 h of ME preparation.

Mean Droplet Size, Polydispersity Index and Zeta Potential: Optimised resveratrol loaded microemulsion was subjected to droplet size analysis, polydispersity index and zeta potential measurement using Zeta sizer Nano-ZS, UK. Samples were loaded into 1 cm^2 cylindrical cuvettes and placed in a thermostat controlled scattering chamber. The aperture of the photomultiplier tube was set at 50 nm.

Percent Transmittance: Percent transmittance of each batch was checked at 638.2 nm, using doubled distilled water as a blank.

Morphology by Transmission Electron Microscopy: A drop of resveratrol loaded optimized batch sample was placed on the copper grid, and phosphotungstic acid was applied as the negative stain. The grid was examined under a Transmission electron microscope.

Differential Scanning Colorimetry (DSC): Differential scanning calorimetry of the optimized resveratrol loaded microemulsion was carried out using DSC (EXTAR6000 SERIES: DSC6220).

Formulation and Optimization of Resveratrol ME-based Gel: As microemulsions possess low viscosity and have poor retention on the skin, incorporation in a gel base helps increase viscosity. Carbopols 974 P NF, 980 NF, 971 P NF, Ultrez 10 NF, and HPMC were used in a concentration of 2 to 3% w/w as gelling agents. The optimized microemulsion was incorporated into these gel bases to form Microemulsion based gel. Carbopol Ultrez 10 NF (3% w/w) was chosen for incorporating the drug-loaded microemulsion as it formed an appealing, transparent and stable gel. A weighed amount of Carbopol Ultrez 10 NF (3% w/v) was soaked in distilled water for 30 min. In a separate beaker, oil and Smix were weighed and homogeneously mixed. To this, accurately weighed Resveratrol (1% w/v) was added and sonicated for 5 min. Methyl paraben, propyl paraben, and butylated hydroxyl anisole were then added in concentrations of 0.18., 0.02 and 0.1% w/w respectively. The calculated quantity of distilled water was then added to this mixture and stirred for 10 min to ensure the formation of the microemulsion and then added to the pre-soaked carbopol. pH was adjusted to 6 using triethanolamine to obtain resveratrol microemulsion based gel.

Characterization and Evaluation of Resveratrol Microemulsion Based Gel:
Appearance: The optimized Resveratrol ME-based gel was checked for color, homogeneity, and consistency.

Determination of pH: The pH of the optimized Resveratrol ME-based gel was determined using pH meter (Eutech instrument) which was previously calibrated with pH 4 and 7 standard buffer solutions. pH of the optimized resveratrol ME-based gel formulation was measured in triplicate.

Drug content / Assay: The optimized ME-based gel (0.25 g) was taken in 10 ml of volumetric flask and dissolved in ethanol. Resveratrol was quantified using the developed UV spectroscopic method for analysis.

Spreadability: 1 g of the gel was placed between two glass slides and a 500 g weight was placed on the upper side of the slide for few minutes to compress and uniformly spread the gel between the slides. A specific weight was placed on the pan, and it was subsequently increased till the glass slide started to slide. The time required for separation of the two slides and the distance traveled by the glass slide was taken as a measure of spreadability. It was calculated using the formula: $S = M.L/T$, where, S = spreadability (gm.cm/sec), L = length of glass slide (cm) and T = time in seconds.

Thermodynamic Stability Studies: To assess stability, optimised formulation was subjected to six heating-cooling cycles at 4 °C and 45 °C for 48h; centrifugation at 10,000 rpm for 30 min using Eltek TC 4815D centrifuge and freeze-thaw cycles between -4 °C and room temperature for not less than 48 h at each temperature and examined for phase separation.

Viscosity: Viscosity of the formulation was checked using Brook-field viscometer (DV-III Ultra programmable rheometer D220).

***In-vitro* Diffusion:** Franz diffusion cell with an effective diffusion area of 2 cm^2 was used for performing the i*n-vitro* diffusion studies. Dialysis membrane having a molecular weight of 10,000 Da was held between the donor and the receptor compartment of the Franz diffusion cell (DBK instrument). Resveratrol loaded microemulsion based gel was placed on the membrane and the amount of drug release estimated. The receptor medium was filled with 22 ml of diffusion medium (Phosphate buffer 5.5: ethanol 1:1). The receptor medium was maintained at 37 ± 2 °C and was stirred magnetically at 100 rpm. Aliquots were withdrawn at predetermined time intervals and

analyzed by UV spectrophotometer. After each sampling, the fresh buffer solution was replenished into the receptor chamber. Percent cumulative release of the drug was then plotted as a function of time.

***Ex-vivo* Diffusion Studies:** The same procedure as used for *in-vitro* drug release was used by replacing the dialysis membrane with pig ear skin.

***In-vitro* Drug Release Kinetics:** To determine the mechanism of release of resveratrol from the selected microemulsion formulation, the data obtained from *in-vitro* and *ex-vivo* release studies were fitted into the kinetic models of zero-order, first-order, Higuchi and Korsmeyer pepper [15].

Occlusivity: 25 ml of water was added to two beakers and covered with cellulose filter paper and sealed. 100 mg of sample was spread evenly with a spatula on the filter surface of one beaker. The other beaker without applied sample served as control. These beakers were then placed at 30 ± 2 °C/ for a period of 72 h. The samples were weighed after every 24 h to determine the water loss due to evaporation (water flux through the filter paper [16]. Occlusivity factor (F) was calculated about the control using the following formula

$$F = 100(A-B) / A$$

Where A = water loss without sample (control) and B = water loss with the sample.

Antioxidant Activity by Radical Scavenging by DPPH Free Radical Method: DPPH(1,1-diphenyl-2-picrylhydrazyl), (0.010g) was dissolved in 100 ml of 80% ethanol solution. Resveratrol was dissolved in 20 ml of 80% ethanol, and solutions at concentrations varying from 1 to 10 mg/ml in 80% ethanol were prepared. 2 ml of prepared DPPH solution was added to 2 ml of each antioxidant solution in vials. The solutions were shaken well and incubated for 30 minutes at room temperature. Two ml of 80% ethanol with 2 ml DPPH mixture was used as the control. The absorbance of control and sample solutions were measured at 517 nm by using UV-Vis spectrophotometer [17].

Tyrosinase Inhibition Activity: 20 µl of mushroom tyrosinase (1000 U/ml), 20 µl of 0.1 M phosphate buffer (pH 6.8) and 100 µl of the test sample solution (20%) containing 20 µl of the sample were mixed (sample solution with enzyme). Sample solutions without enzyme were also prepared by repeating all previous steps but without the sample. Blank solutions with and without enzyme were also prepared without test sample solution. Positive controls of 0.5 mg/ml kojic acid solutions (with water), with and without enzyme were used. Twenty 20 µl of 0.85 mM L-DOPA solution as the substrate was added to every sample and blank. These assay mixtures were incubated at 25 °C for 10 min. The amount of dopachrome produced in the reaction mixture was measured at 475 nm ($e475 = 3600$ $M^{-1}cm^{-1}$). Percent inhibition of tyrosinase activity was calculated using the following equation

$$\% \text{ tyrosinase inhibition} = \frac{(A - B) - (C - D)}{(A - B)} \times 100$$

Wherein A = absorbance of the blank solution with an enzyme, B = absorbance of blank solution without enzyme, C = absorbance of the sample solution with enzyme and D = absorbance of sample solution without enzyme [18].

B16F10 Melanoma Cell Line Cytotoxicity Study: Resveratrol loaded ME formulations containing 0.5% w/w and 1% w/w drug was tested for their efficacy to inhibit B16F10 melanoma cells by the *in-vitro* MTT Assay.

MTT [(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide)] is a pale-yellow substrate that is cleaved by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even freshly dead cells do not leave a significant amount of MTT. Thus the amount of MTT cleaved is directly proportional to the number of viable cells present, which is quantified by colorimetric methods. Briefly, the compounds were dissolved in DMSO and serially diluted with complete medium to get the concentrations a range of test concentration. DMSO concentration was kept < 0.1% in all the samples. B16 F10 melanoma cells maintained in appropriate conditions were seeded in 96 well plates and treated with different concentrations of the test samples and incubated at 37 °C, 5% CO_2 for 96 h. MTT reagent was added to the wells and incubated for 4 h; the dark blue formazan product formed by the cells was dissolved in DMSO under a safety cabinet and read at 550 nm. Percentage

inhibitions were calculated and plotted with the concentrations used to calculate the IC_{50} values.

Stability Studies: The optimized microemulsion based gel was subjected to stability as per ICH guidelines (ICH Q1 R2). The optimized microemulsion based gel formulation was kept in glass vials (10 ml capacity) covered with aluminium foils and stored at different temperatures and humidity conditions, *viz.* 4 ℃, 25 ℃ / 60 ± 5% RH and accelerated temperature 40 ℃ / 75 ± 5% RH for three months. Microemulsion-based gel was evaluated periodically for physical appearance, particle size, drug content, and drug release.

RESULTS AND DISCUSSION:
Preformulation Studies:
Characterization of API Melting Point: Done by the capillary method wherein melting point was found to be 261 °C and confirmed by DSC.

Drug-Excipient Chemical Compatibility:
Drug Excipient Compatibility was Evaluated by FTIR by using IR Affinity, Shimadzu:

Following is the FT-IR data of drug and excipients blend after three months of storage instability chamber Osworld, India. From **Fig. 1** and **2**, the IR spectrum was observed to have frequencies similar to that of resveratrol, thus confirming that there was no interaction of the drug with excipients blend and no complexes were formed.
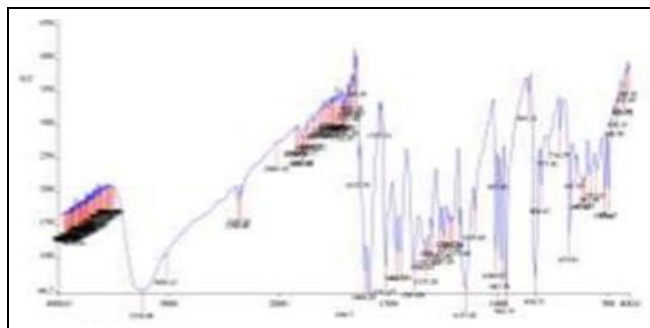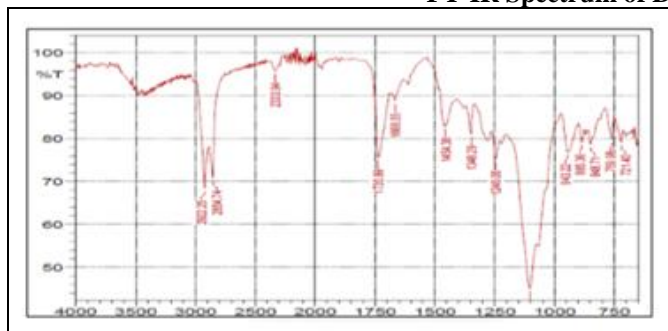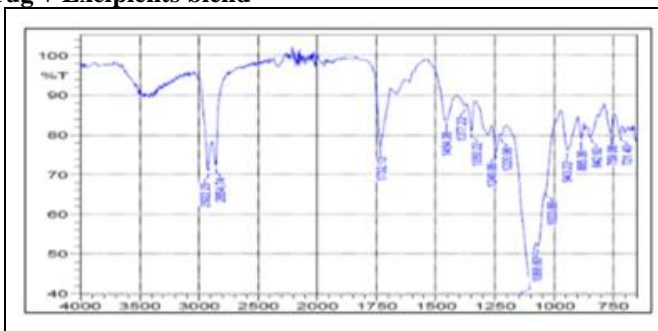

FIG. 1: FTIR SPECTRUM OF RESVERATROL

Functional group	Observed wave number (cm^{-1})	Reported range (cm^{-1})
-OH (Alcohol)	3505.65, 3569.99	3300-3600
C-O (Alcohol)	1105.84, 117.02	1050-1200
C= (Aromatic)	1512.47, 1535.54	1450-1600
C=C (Alkene)	1632.39, 1648.18	1630-1670

FT-IR Spectrum of Drug + Excipients blend

FT-IR Spectrum of Drug + Excipients blend at 25 ℃ ± 2 ℃ / 60% RH ± 5% RH

FT-IR Spectrum of Drugs + Excipients blend at 40 ℃ ± 2 ℃ / 75% RH ± 5% RH

FIG. 2: FTIR SPECTRA OF RESVERATROL AND EXCIPIENTS

Analytical Method Development:
UV Method: The maximum absorption wavelength of resveratrol in methanol was found to be at 306 nm and the standard equation of calibration curve was y = 0.1527x - 0.0501 with regression value *i.e.* R² = 0.9987 The maximum absorption wavelength of resveratrol in Ethanol: buffer pH 5.5 (1:1) was found to be 306 nm and the standard equation of the calibration curve was y = 0.1527x - 0.0501 with regression value R² = 0.9987. The developed method was validated as per the ICH Q2B guidelines validation of analytical

procedures, and following validation, parameters were obtained

HPLC Method: Samples were chromatographed on Inertsil ODS-2, 5μ. 1.6 × 150 mm column (Temperature 25 ℃). The mobile phase was methanol: phosphate buffer (pH 6.8 adjusted with 0.5% (v/v) orthophosphoric acid solution in Milli-Q water) (63:37%, v/v) and was delivered at a flow rate of 0.8 ml/min. The injection volume was 50 μl. The eluate was monitored by an ultraviolet detector set at 307 nm.

Formulation Design of Microemulsion:
Screening of Oils, Surfactants, and Co-Surfactants for Microemulsion Formulation by Saturation Solubility Method: Solubility of resveratrol was estimated in various oils, surfactants and co-surfactants / cosolvents.

The solubility of resveratrol was highest in oleic acid among oil components tried. Resveratrol showed maximum solubility in labrasol which was chosen as the surfactant. Among the four co-surfactants / co-solvents tried, resveratrol showed maximum solubility in Transcutol P. Hence, Oleic acid, Labrasol, and Transcutol P were used as oil, surfactant and co-surfactant for further studies. **Fig. 3** is a compilation of resveratrol solubility data.

Construction of Pseudo-Ternary Phase Diagrams: To obtain the appropriate concentration ranges of oil, surfactant, and co-surfactant in the microemulsion formulation, pseudo-ternary phase diagrams were constructed for three ratios *viz.* 1:1, 1:2 and 2:1 ratios of Smix. From the three plotted phase diagrams, the largest microemulsion region was observed in the ME containing 2:1 ratio of Smix. **Fig. 4, 5** and **6** represent the pseudo-ternary phase diagrams of 1:1, 1:2 and 2:1 ratios of Smix to oil respectively. It is observed from the three pseudo-ternary phase diagrams obtained, that an increase in the surfactant concentration and decrease in co-surfactant concentration (2:1 ratio), the obtained ME region is larger in comparison to **Fig. 4** and **5**.
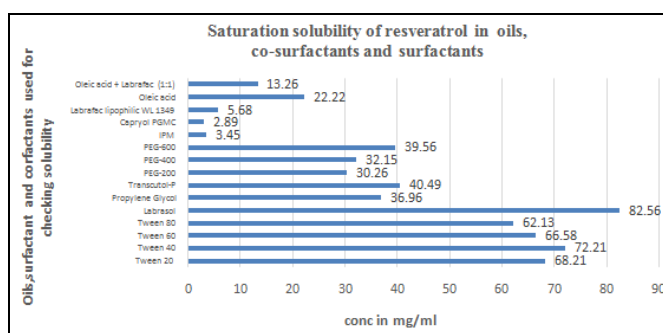

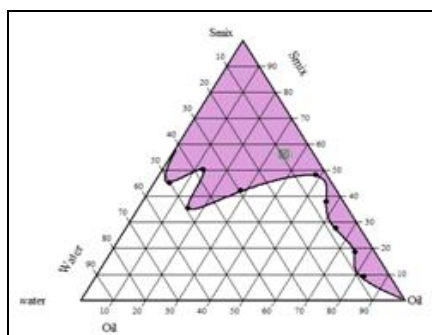**FIG. 3: SATURATION SOLUBILITY OF RESVERATROL IN OILS, CO-SURFACTANTS AND SURFACTANTS**

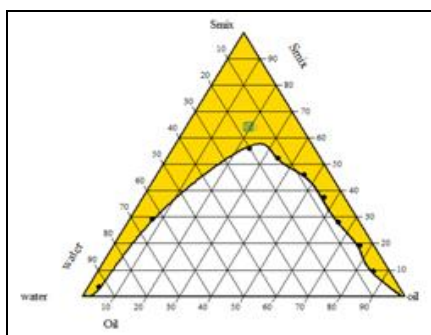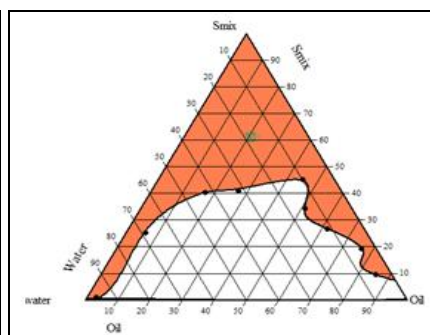| FIG. 4: TERNARY PLOT OF ME WITH Smix 1:1 | FIG. 5: TERNARY PLOT OF ME WITH Smix 1:2 | FIG. 6: TERNARY PLOT OF ME WITH Smix 2:1 |

Chosen compositions of resveratrol loaded O/W microemulsion from plot exhibited in **Fig. 6** [Smix-Labrasol: Transcutol (2:1)] which shows the maximum ME region are tabulated in **Table 2**. The chosen batch was coded B.

TABLE 1: COMPOSITION OF BATCHES SELECTED FROM PSEUDOTERNARY PHASE DIAGRAM

Batch code	Oil % (Oleic acid)	Smix (2:1)% (Labrasol: Transcutol)	Water %
A	5	48	47
B	5	50	45
C	5	52	43
D	5	58	37

Characterization of Resveratrol Loaded Microemulsion: Macroscopic Appearance and Visual Observation: The optimized batches of micro-emulsions were found to be transparent, optically clear and homogeneous with no phase separation.

ME Ultrastructure by TEM: The microstructure transitions of the optimized ME can be visualized when investigated by transmission electron microscopy. Resveratrol loaded ME showed spherical shaped non-aggregated droplets as observed in **Fig. 7**.
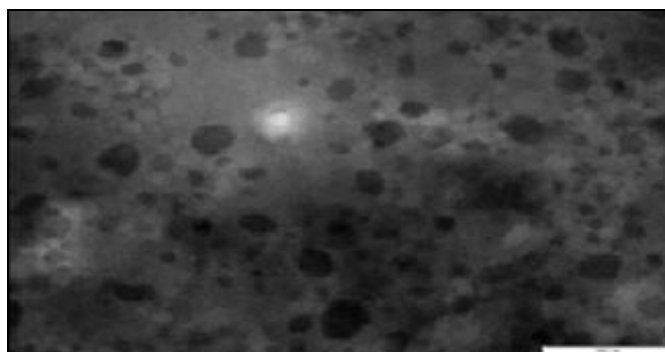
FIG. 7: TEM OF OPTIMIZED BATCH

Following **Fig. 8** and **9** depict the average droplet size (247.8 ± 0.9601 nm) with a polydispersity index of 0.213 and a zeta potential of -32.9 obtained for the optimized resveratrol ME.
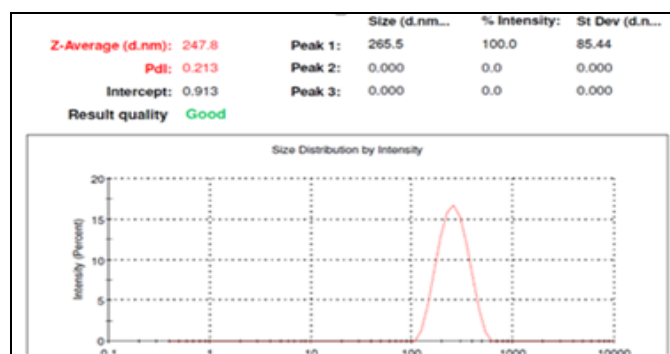

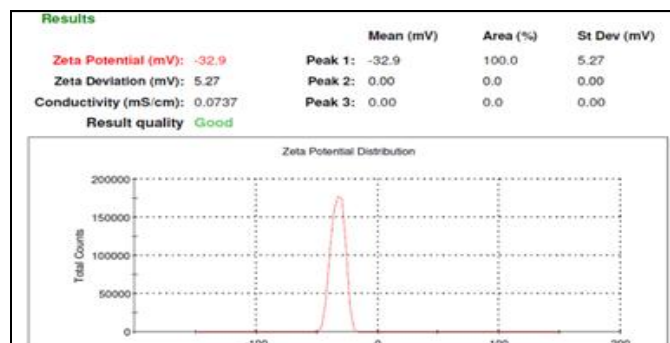**FIG. 8: AVERAGE PARTICLE SIZE AND PDI OF RESVERATROL LOADED ME**

FIG. 9: AVERAGE ZETA POTENTIAL OF RESVERATROL LOADED ME

Percent Transmittance: The four batches of developed resveratrol loaded microemulsions were evaluated for percent transmittance at 638.2nm. Results are tabulated in **Table 2**.

TABLE 2: PERCENTAGE TRANSMITTANCE OF OPTIMIZED MICROEMULSIONS

Batch code	Percent transmittance
A	95.14 ± 0.158
B	95.87 ± 0.257
C	95.93 ± 0.586
D	98.15 ± 0.620

Percentage transmittance was used as one of the responses to optimize the amount of Smix and oil to get the desired microemulsion. All four batches showed good transmittance proving transparency of the ME.

Thermodynamic Stability Studies: On exposure of the four batches to heating-cooling cycles, freeze-thaw cycles and centrifugation studies, none of the batches showed phase separation.

Differential Scanning Colorimetry (DSC): Fig. 10 illustrates an overlay of DSC of the oil phase used in the formulation and resveratrol loaded ME which proves the compatibility of drug and excipients.
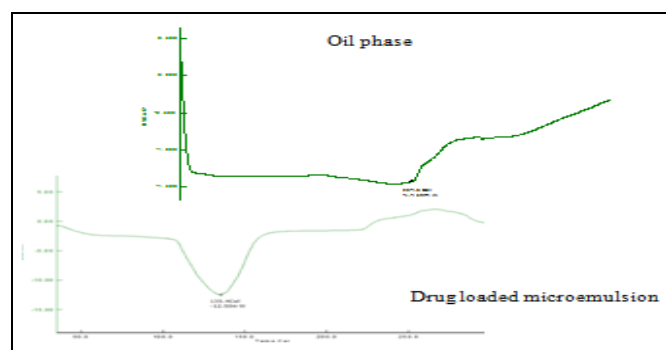

FIG. 10: DIFFERENTIAL SCANNING COLORIMETRY OF OIL PHASE AND RESVERATROL LOADED ME

In-vitro **Drug Release and Resveratrol ME Release Kinetics:** To evaluate the ability of the ME vehicle to allow the release of resveratrol and the rate at which it occurred, *in-vitro* drug diffusion study was carried out for the four prepared batches in comparison with a conventional gel formulation of resveratrol**.**

From the results shown in **Fig. 11**, batch B was shortlisted since it exhibited the desired drug diffusion pattern. Diffusion of resveratrol from this ME batch was continuous and increasing upto 24 h without burst release or plateau formation indicating a sustained release. This may be due to the drug's confinement in droplets coated with a surfactant layer or due to the partitioning of the drug between oil and aqueous phases. **Fig. 12** shows the percent of drug diffused (*in-vitro*) at the end of 24 h as 98.21 ± 0.03 from the conventional gel. In comparison, 71.11 ± 0.47 and 68.15 ± 0.12 was the percent drug diffused from the ME gel *in-vitro* and *ex-vivo* respectively.
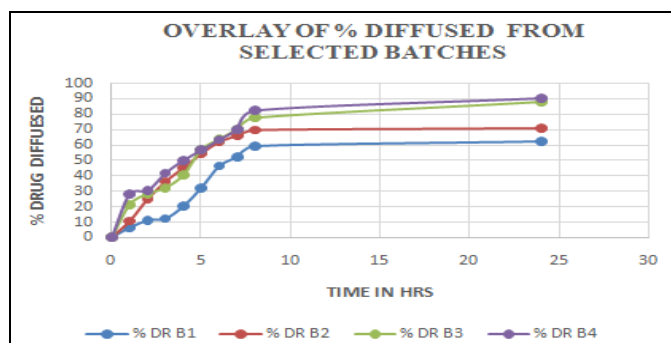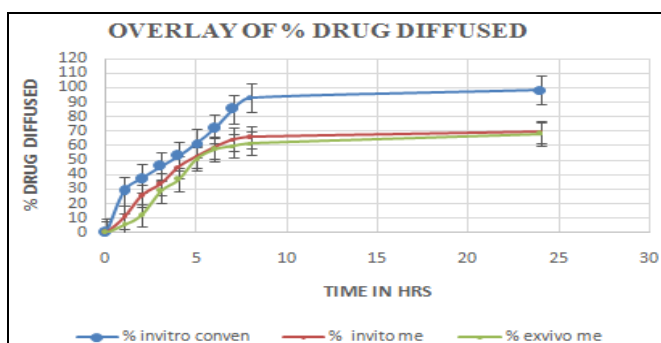
FIG. 11: OVERLAY OF DRUG RELEASE FROM SELECTED BATCHES

FIG. 12: OVERLAY OF DRUG RELEASE FROM OPTIMIZED BATCH

Drug Release Kinetics: Drug release data of optimized formulations were fitted into different mathematical models namely zero order, first order, Higuchi and Korsmeyer peppers. The theory states that zero order kinetic model describes systems where the drug release rate is independent of its concentration, whereas the first order kinetic model proves that drug release is a concentration-dependent process. Higuchi's model describes the release of drugs from the formulation as the square root of time-dependent process, based on Fickian diffusion. The model that best fitted the release data of resveratrol loaded ME gel formulation was evaluated by the highest regression coefficient (R^2). The final ME gel batch of resveratrol showed R^2 values of 0.9969 for *in-vitro* and 0.9753 for *ex-vivo* drug release for the Higuchi model as represented in **Fig. 13**.
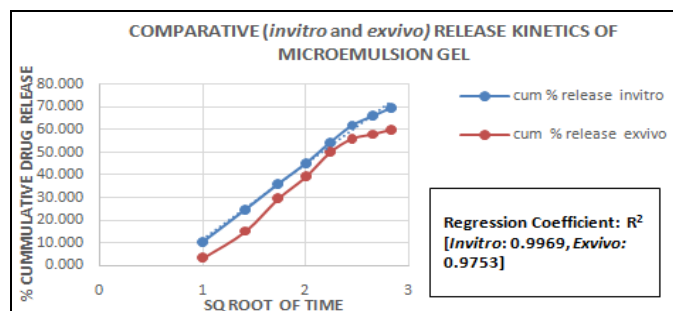

FIG. 13: COMPARATIVE *IN-VITRO* AND *EX-VIVO* RELEASE KINETICS

This suggests that the drug release from microemulgel followed Higuchi model indicating controlled release of drug explained by the drug diffusion from the surface in contact with the skin. Progressively, more drug moves out from the microemulgel matrix in a sustained manner.

Drug Content: The average amount of drug in final optimized batch Bas determined in triplicate was 90.97 ± 1.92 %.

Spreadability: The values of spreadability indicate that the gel is easily spreadable on the application of a small amount of shear and it was found to be 35.42 ± 0.81gm.cm.sec$^{-1.}$

Viscosity: Viscosity of the optimized Resveratrol microemulsion based gel was found to be between 6500 cps and 7800 cps.

Skin Retention: The quantity of *ex-vivo* resveratrol skin retention of the optimized batch at the end of 24 h was 30.49 ± 1.5549% which shows that the developed formulation can remain on the skin and release the drug in a sustained manner.

Occlusivity: Occlusivity of the optimized batch was tested gravimetrically. The occlusivity provided by formulations enables prevention of water loss from the skin surface which helps increase the hydration level in stratum corneum. This in turn facilitates is helpful in drug transport. A result of 71.24 ± 0.96% occlusivity indicates that the formulation can have acceptable permeable properties.

Assay for Tyrosinase Inhibition: Results of tyrosinase inhibition of resveratrol ME gel depict 98.58 ± 0.022% inhibition as against 98.67 ± 0.058 observed for the pure drug. This proves that the developed formulation is very promising as a tyrosinase inhibitor which can prove beneficial in certain skin cancers wherein the enzyme tyrosinase is present in abnormal quantities.

Antioxidant Activity: DPPH (1, 1-diphenyl-2-picrylhydrazyl) is considered a stable radical and gives a strong absorption band at 520 nm. A substance has antioxidant activity if there is a color change from deep violet to pale violet color at 520 nm. DPPH % inhibition of resveratrol ME gel was

98.12 ± 0.0559, and that of the standard resveratrol was 95.28 ± 0.047 thereby indicating the excellent antioxidant potential of the developed formulation.

B16F10 Melanoma Cell Line Cytotoxicity: ME formulations containing 0.5% w/w and 1% w/w were checked for their cytotoxic potential against melanoma cells *in-vitro.* **Table 3** illustrates the % inhibition values at specified concentrations.

Fig. 14 and **15** are plots of concentration in ul/ml (X-axis) of the ME formulations versus percentage inhibition (Y-axis) using B16F10 Melanoma cell line. IC_{50} values of 1% resveratrol ME and 0.5% resveratrol ME are 0.2 and 5 respectively.

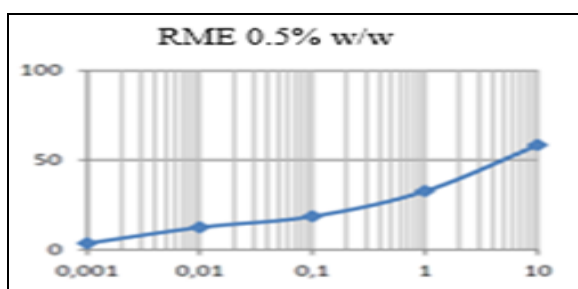Results prove that both formulations are active and the activity is dose-dependent.
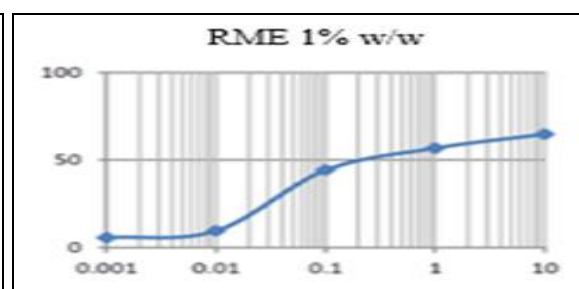

FIG. 14: IC_{50} OF 0.5% RESVERATROL LOADED ME

FIG. 15: IC_{50} OF 1% RESVERATROL LOADED ME

TABLE 3: IC_{50} DATA OF RESVERATROL LOADED ME

Concentration	RME 0.5% w/w	RME 1% w/w
10	58.41	64.81
1	32.75	56.83
0.1	18.62	44.27
0.01	12.48	9.64
0.001	3.63	5.66
IC_{50} value (µl/ml)	5	0.2

Stability Studies: Microemulsion based gel was evaluated periodically for physical appearance, particle size, drug content, and drug release.

Table 4 depicts the stability data of optimized resveratrol ME gel batch.

TABLE 4: STABILITY DATA OF OPTIMISED BATCH B

Parameters	24 hours	1 week	1 month	3 months
Size (µm)	0.247 ± 0.0126	0.391 ± 0.9601	0.396 ± 0.9965	0.411 ± 0.23691
Visual appearance	Homogeneous	Homogeneous	Homogeneous	Homogeneous
Drug content (%)	92.216 ± 1.056	91.016 ± 1.009	90.118 ± 1.589	90.015 ± 0.236
% Drug release	71.11 ± 0.47	69.81 ± 0.698	70.433 ± 1.25	69.254 ± 0.953

CONCLUSION: The main focus of this study was to develop an encapsulated topical drug delivery system for poorly soluble resveratrol with enhanced therapeutic efficacy by improving the solubility and skin permeability of the drug through the skin. A microemulsion formulation containing 1% resveratrol was developed using water titration method with the help of pseudo-ternary phase diagrams for optimization. The optimized final formulation was incorporated in a gel base to increase its an appeal as a product which exhibited the desired characteristics of thermodynamic stability. From *in-vitro* and *ex-vivo* drug release data, it can be concluded that the developed resveratrol loaded ME gel follows the Higuchi model of drug release kinetics which depicts controlled release of the medicament. Efficacy of the developed formulation was tested using antioxidant, tyrosinase inhibition and melanoma cell line cytotoxicity and very promising results were obtained for the respective activities. Hence, we can conclude that a user-friendly topical formulation having the potential for use in chemoprevention of skin cancer was successfully developed.

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CONFLICT OF INTEREST: Authors declare no conflict of interest.

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