

Demonstration of Multivariate Data Analysis for the Development of nanoemulsions containing Active Herbal Principle of *Boswellia serrata* for Topical Application

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Abstract

The chemometric techniques have been used to demonstrate the role of nanoemulsion components on release of active herbal principle present in the spray dried *Boswellia serrata* extract. Isopropyl myristate, Tween 80 and Transcutol P^R were selected after determining the solubility of boswellic acids (BAs) and used to draw phase diagrams. Simplex lattice mixture design was applied to optimize the percentual composition of nanoemulsions. The Permeability coefficient and droplet size were modelled with set of variables by partial least squares (PLS). Partial least square regression analysis was done through Excel STAT to influence mixture composition on permeation behavior of drug from the nanoemulsion as calculated models revealed good predictive abilities. Ex vivo skin permeation and in vivo anti-inflammatory study were conducted to evaluate the potential of optimized nanoformulations. About 3.25fold increase in flux was seen in case of nanoemulsion, nanogel showed 1.45 fold increase in flux as compared to carbopol gel with highest enhancement ratio 4.57 and 1.59 respectively. Physicochemically stable and non-irritant hydrogel showed significant percentage inhibition in rat paw edema. Our study illustrated scientific and statistical evidence for the potential of developed nanoemulsion as possible alternative to traditional topical formulations.

Keywords: *Boswellia serrata* extract, nanoemulsion, Pseudo ternary phase diagram, Nanoemulsion based hydrogel, Simplex lattice design, Partial least square analysis.

Introduction

Globally there is renewed interest in drugs derived from herbal sources due to its promising beneficial therapeutic effects and better tolerance with no side effects. Currently, pharmacological and clinical studies indicate that gum resin of *Boswellia serrata* [*B. serrata*] contains six types of boswellic acids (BAs) as active herbal principle which play significant role in treatment of inflammatory disorders by inhibiting 5-lipoxygenase (5-LO)[1]. Herbal drugs have therapeutic potential which shall be explored through some value added new drug delivery system. Recent advancement in formulation of micro/nanoemulsion has attracted much attention in pharmaceutical Industry. Nanoemulsions are optically isotropic, transparent, thermodynamically stable systems of oil, surfactants and water with narrow droplet size usually in the range of 10-100 nm having high solubilization capacity for lipophilic drugs[2], ease of preparation, and size of emulsion droplets influence its target distribution [3] thereby improve the penetration of drug into skin[4].

Chemometric methodologies proved to be an effective tool during the formulation optimization of pharmaceutical dosage forms [5]. Since nanoemulsion can be developed only for some set of the

components [6]. Simplex lattice design initially was used to investigate the optimum ratio of components in order to determine existence of nanoemulsion. Then Partial least square regression analysis was applied to emphasize the role of nanoemulsion components. In this perspective, permeation rate of drug and droplet size were modelled as function of the mixture percentual composition.

Nanoemulsions as the topical carrier offer significant advantages including low skin irritation, greater permeation ability due to nano droplet size and high drug-loading capacity [7], but low viscosity restricts its topical application. As hydrogel thickened agent change the viscous properties of nanoemulsions to make suitable for topical delivery[8, 9]. Very few work has been reported on nanoemulsion based hydrogel delivery for herbals. Many active herbal principles have been incorporated in nanoemulsion for transdermal delivery, but boswellic acids have not been evaluated into nanoemulsion vehicle. However novel Boswellic acids nanoparticle have been developed for prostate cancer cell death[10] and 3 Acetyl-11 keto-B boswellic acid loaded polymeric nanomicelles significantly enhanced topical anti-inflammatory and anti-arthritis activity [11].

Thus, the present research aims to optimize the formula of nanoemulsions using Multivariate data level techniques and to



evaluate the effects of boswellic acids formulated in novel nanoemulsion based hydrogel which accomplish significant anti-inflammatory effect on rats using carrageenan-induced rat paw edema model.

Material and Method

Material

The dry extract of *Boswellia serrata* (*B. Serrata*) was gifted by Pharmeda Herbal Pvt. Ltd., Gujarat, India. Coconut oil and clove oil were purchased from S.D Fine chemicals, Mumbai, India. Caprylic triglyceride polyethyleneglycol-4 complex (Labrafac®), Diethylene glycol monoethyl ether (Transcutol P®), Oleoyl macroglycerides EP (Labrafil), Polyglyceryl-6-dioleate (Plurol-Oleique®), were received as gift samples from Gattefossé (Cedex, France). Sasol Imwitor, Acrysol (Hydrogenated castor oil) were provided as gratis samples from Abitec Corporation, Mumbai, India. Oleic acid, Triacetin, and Isopropyl myristate, (IPM) were procured from SD Fine chemicals, (Mumbai, India) Span 20, Span 60, Tween 80, and Carbopol 940 were purchased from Sigma Aldrich (St. Louis, MO). HPLC grade methanol, and phosphoric acid, Acetonitrile were purchased from SD Fine chemicals (Mumbai, India). All other chemicals, buffer solution components and solvents were of analytical grade. Water was obtained from Milli Q water purification system (Millipore, MA).

Excipient screening method

The equilibrium solubility study was performed by adding an excess amount of *B. serrata* extract in 2ml of various oils (isopropyl myristate, Labrafac, oleic acid, Labrafil, coconut oil, clove oil, almond oil and Sasol Imwitor), surfactants (Tween 80, Span 60, Span 20, and Acrysol), and cosurfactants (Plurol Oleique and Transcutol P) in 5-mL capacity stoppered vials separately vortexed using a Cyclo mixer [CM 101, REMI (INDIA)]. The vials were then kept at 25±0.5 C in an orbital shaker (CSI-24 BL, Remi Laboratories, and Ahmadabad, India) for 72 h to reach equilibrium. Following attainment of equilibrium, the supersaturated samples were centrifuged at 2,000 rpm for 15 min to separate undissolved amount. The obtained supernatants were quantified followed by filtered through a 0.45-µm membrane filter (Membrane Technologies, (Mumbai, India) using a HPLC (Shimadzu, Tokyo, Japan).

Construction of phase diagrams and formulation of BAs-loaded NE

Construction of pseudo ternary phase diagrams

On the basis of solubility studies, selected as oil (IPM), surfactant (Tween 80), co-surfactant (Transcutol P) and distilled water were used to construct phase diagram. Surfactant and co-surfactant (Smix) were mixed in different weight ratios (Km 1:1, 2:1, 3:1, 4:1, and 1:2, 1:3). For drawing phase diagrams, ten different combinations of oil and Smix (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2,

9:1, and 1:0) were combined in different glass tubes to cover entire boundaries of three phases precisely formed in the phase diagrams. Water titration method was adopted and samples were examined for transparency and flowability by visual observations. The existence of nanoemulsion zone can be illustrated by constructing pseudo ternary phase diagram using ProSim ternary software.

Formulation of *B. Serrata* loaded nanoemulsion system

To prepare *B. Serrata* extract-loaded NE, appropriate amount of oil surfactant and cosurfactant were mixed together in accordance with obtained microemulsion region in the phase diagrams shown in fig. 2, and equilibrated with gently vortexing to get the initial concentrate. Then appropriate amount of *B. serrata* extract (133.2mg) was dissolved in the oil phase followed by addition Smix to prepare initial preconcentrate. The blend was mixed using high speed homogenizer at 6000RPM upto 10min. [IKA Pvt. Ltd, Germany] as the extract was resinous in nature. The drop wise addition of water was done to resulting mixture till nanoemulsion was formed.

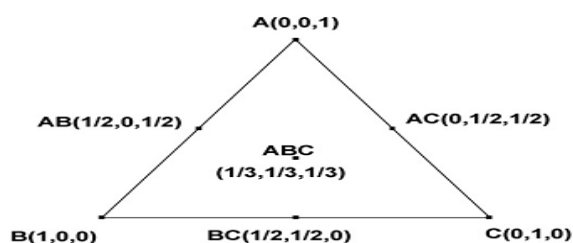
Preparation of BAs loaded NE based hydrogel

In general, most nanoemulsion possess a very low viscosity, which may restrict their topical application [12]. To overcome this, Carbopol 940 was selected as the hydrogel matrix for obtaining nanoemulsion based hydrogel. An amount of drug representing 2%w/w of the formulation consisting of the chosen oil and Smix was vortexed until the drug completely dissolved. An aqueous phase of Carbopol 940 was prepared by dispersing an amount of the gelling agent, equivalent to 1% w/w of the formulation in water. After complete swelling of the Carbopol in water, the pH of the aqueous phase was adjusted by adding triethanolamine and the nanoemulsion based hydrogel obtained by adding the oily phase to the water-swelled carbopol gel.

Application of Multivariate data techniques for optimization of BAs loaded NE.

Application of Multivariate data techniques, called design of experiments (DOE) and partial least square regression (PLSR) involve the concept of 'mixture designs' for changing mixture composition and explaining how such changes will affect the properties of the mixture[13]. Percentual composition of nanoemulsion mixtures were optimized using Simplex lattice mixture design represented as three-components by an equilateral triangle in two-dimensional space. Seven formulations were selected from each vertex (A, B and C), at the halfway point between vertices (AB, BC and AC), and last one at the center point (ABC)[14].





Equilateral triangle representing simplex lattice design for three components.

The percentual composition of oil, S_{mix} and water were selected as independent variables. The mean droplet size and cumulative

permeation of drug from nanoemulsions were chosen responses. For the formulation design, selected responses for seven formulations were used to fit polynomial equations which can help to predict the properties of all possible formulations within the design space. Polynomial equations were generated and model graphs in the form of contour plots were constructed through Design Expert 7.0.1 Software (Stat-Ease, Inc., Minneapolis, MN). Comparison of the observed and predicted responses were critically made and percentage prediction error was also calculated with respect to the observed responses. From pseudo-ternary diagrams, appropriate ranges of the components were chosen, and the actual concentrations of independent variable were transformed and droplet size and permeation rate of BAs from developed nanoemulsions were measured and the results of observed and predicted responses were shown in Table 1.

Table: 1 Actual and transformed values, mean droplet size and cumulative permeation of seven different formulations as per simplex lattice design.

Mixtures	Formulation Components/ transformed components value			Response: R_1 Droplet size [nm]		Response: R_2 Qn permeation [$\mu\text{g}/\text{cm}^2$]	
	A	B	C	Experimental result	Predicted result	Experimental result	Predicted result
NE-1	8/1	8/0	5/0	23.38±1.98	22.01±2.83	20.95±2.54	22.01±2.83
NE-2	2/0	36/1	5/0	21.8±2.35	22.51±2.32	20.44±1.28	22.51±2.32
NE-3	2/0	8/0	20/1	29.31±3.45	21.59±3.02	19.48±1.54	21.59±3.02
NE-4	4/0.5	18/0.5	5/0	12.89±1.25	23.54±3.80	19.17±2.45	23.54±3.80
NE-5	4/0.5	8/0	10/0.5	18.48±2.84	22.91±2.43	23.24±4.01	22.91±2.43
NE-6	2/0	18/0.5	10/0.5	15.98±3.21	23.32±2.74	25.15±1.47	23.32±2.74
NE-7	3/0.33	12/0.33	7/0.33	25.33±2.24	21.99±2.50	20.48±2.78	21.99±2.50

Where, A=%oil, B = % S_{mix} , C = %Water as independent variables.

Data of mean droplet size (R_1) and cumulative permeation (R_2) were shown as mean± SD (n= 3).

Partial least square regression analysis

PLS regression analyses a set of components that performs a simultaneous decomposition of variable (X) and response (Y) with the constraint explaining possible covariance between them. The partial least squares regression approach was selected. [15-17], where new set of orthogonal variables obtained as linear combinations of the original ones. These variables are calculated in such a way to avoid collinearity present in the independent block and correlating them with the chosen responses. [18]. The three components oil, S_{mix} and water were selected as independent

variables and permeability coefficient and droplet size were chosen as dependent variables. The correlation matrix with independent and dependent variable with its labels is shown in Table 2. The selection of the best set of independent variables was performed using a step-wise algorithm [19]. A model that shows lowest predicted sum of squares (PRESS) is used for prediction purposes. The PRESS was evaluated through a leave-one-out cross validation procedure [6], an option available in most of the softwares that can handle chemometric analysis. EXCEL stat software was used to carry out PLSR in this investigation.

Table: 2 Mixtures of the experimental design and corresponding observed responses as per PLS regression

A	B	C	AB	BC	AC	A ²	B ²	C ²	Kp (10-2)	Mean Droplet size
8	36	5	288	180	40	64	1296	25	3.13	23.38
2	36	5	72	180	10	4	1296	25	7.06	21.8
2	8	20	16	160	40	4	64	400	6.72	29.31
4	18	5	72	90	20	16	324	25	4.74	12.89
4	8	10	32	80	40	16	64	100	3.58	18.48
2	18	10	36	180	20	4	324	100	3.5	15.98
3	12	7	36	84	21	9	144	49	5.02	25.33

Where, A = % oil, B = % S_{mix} , C = % Water as independent variables, and Kp = Permeability coefficient and Mean droplet size were dependent variables for PLSR modelling

Characterization of nanoemulsion

The morphology and structure of the droplet present in nanoemulsion was analyzed using electron microscope (Tecnai G2 20 TEM, Phillips Holland) operating at 70 kV capable of point-to-point resolution. The differential scanning calorimeter (DSC-6 Perkin Elmer, USA) was used for detecting changes in specific heat capacities of sample in an aluminum crimped pan.

The mean droplet size and zeta potential of the various nanoemulsions was determined by photon correlation spectroscopy using a Zetasizer 1000 HS (Malvern Instruments, Worcestershire, UK). Sample was extemporaneously diluted in Milli-Q water (Millipore Corp., USA) and injected in the apparatus. Each sample was analyzed twice, each analysis consisting of five replicates. The viscosity of the nanoemulsion was determined using Brookfield viscometer LV model (DV-III Ultra Programmable Rheometer) using spindle no. S-61 in triplicate at 25°C. The refractive index of the prepared nanoemulsion was measured by an Abbe refractometer (Nirma International, Mumbai) to reveal isotropic character of NE by placing one drop of the formulation on the slide in triplicate at 25°C. In order to verify the isotropic nature of nanoemulsion, optimized sample was examined using cross-polarized light microscopy (Leica microsystem, Germany) by putting drop of sample between a coverslip and glass slide and then observed under cross-polarized light. The equilibrium surface tension of samples was measured using Surface tensiometer (Force Tensiometer K100, Kruss, Germany). The instrument room was maintained at 25°C.

The pH values of nanoemulsions were determined using Digital pH meter (Weltronix PM100, EIE, INDIA) in triplicate at 25°C. Electrical conductivity (κ) of the sample was measured using a conductivity meter CDM230 in duplicate to ensure reproducibility. To determine the solubility of BAs in NE, excess B. Serrata was added into 2.5 ml initial preconcentrate, then 5 ml water was added and the resulting dispersion was shaken at 25°C for 72 h using an orbital shaker and centrifuged at 10,000 rpm. The supernatant was diluted with methanol appropriately and the content of BAs was determined by HPLC. The formulations were exposed to centrifugation (10,000RPM for 20 min), and heating-cooling cycle (-4°C and 40°C for 48hrs.) to assess thermodynamic stability.

Evaluation of NE based Hydrogel [NBH]

The viscosity of nanoemulsion based hydrogel [NBH] was measured at 25°C using Brookfield viscometer employing spindle S96 rotated at 10 rpm. The pH of prepared NBH formulation was measured using digital pH meter at 25°C in triplicate. The spreadability of nanoemulsion based hydrogel was determined by placing 0.5 g of NBH within 1 cm diameter circle pre-marked on a glass plate, over which second plate is placed. A weight of 500 g is allowed to rest on the upper glass plate for 5 min. The increase in diameter was due to hydrogel was noted.

In-vitro permeation experiments

In-vitro drug diffusion study

In vitro diffusion studies of nanoemulsions were performed using a modified Franz diffusion cell attached with thermostatic water bath at 25°C±1°C. A dialysis membrane (MWCO: 12,000 Da, Himedia), with a pore size of 0.22µm was used. Each one gm of drug loaded nanoemulsion was placed in the donor compartment. The receiver compartment was filled with phosphate buffer (pH 7.4) as dialysis medium at 25°C±1°C and 400rpm on magnetic stirrer. Aliquots were periodically withdrawn at suitable time interval from the receiver compartment through a side tube and analyzed using HPLC.

Ex-vivo skin permeation study

The ex vivo skin permeation study was performed using on a vertical Franz diffusion cell having effective diffusional area 7.03 cm² and 15 ml of receiver chamber capacity. Skin samples were prepared by removing subcutaneous fat and connective tissues, washed and examined for integrity. Skin was soaked in the receptor solution for 1 h before the permeation experiment. Skin sample was then clamped between the donor and the receptor chamber of vertical diffusion cells. Samples were gently placed in the donor chamber and the receptor chamber was filled with PBS (pH 7.4) and thermo stated at 25°C±1°C. The solution in the receptor chamber was stirred using magnetic stirrer at 100 rpm throughout the experiment. Samples were withdrawn at 0.5, 1, 2, 4, 6, and 8 hr., filtered through a 0.22µm membrane filter, and analyzed for BAs permeant content using HPLC method. Calculation of permeation data was done by plotting the graph of the cumulative amount of drug permeated through the skin [mg/cm²] as a function of time [t, h] for formulation. The flux at steady state [J_{ss}] was calculated by dividing the slope of the linear portion of the graph by the area of diffusion cell. The permeability coefficient [K_p] and Enhancement ratio was calculated using following equation 3 and 4 respectively [20]:

Calculation Permeation Data using following equations 1 and 2
..... Eq. 1

$$E_r = \frac{J_{ss} \text{ of formulation}}{J_{ss} \text{ of control}}$$

..... Eq. 2

Skin irritation Study

The skin irritation test was carried out on Male Sprague Dawley rats weighing 250-300 g. Animals were housed in cages with free access to standard diet and water ad libitum. A single dose of 10mg of the nanoemulsion hydrogel was applied to the left ear of the rat, with the right ear as a control. The development of erythema was monitored for 6 days.

In-vivo pharmacodynamics study using carrageenan-induced rat paw edema



Male Sprague Dawley rats weighing (250-300gm, 12-14 weeks age) were housed in cages with free access to standard diet and water ad libitum, acclimatized to surrounding for one week prior to experiment. The experimental protocol was approved by Institutional Animal Ethics Committee of Faculty of Pharmacy, Dharmsinh Desai University, Nadiad, India [Reg. no/date: No. 1338/c/CPCSEA, 07/04/2010] as per the guidance of CPCSEA, Ministry of Social Justice and Experiment, Government of India with approved Protocol No: DDU/FOP/14/04, dated 19th March 2014. The committee guidelines were followed for the skin permeation, skin irritation and anti-inflammatory studies. The carrageenan-induced hind paw edema method was used to examine anti-inflammatory effect of the optimized nanoemulsion based hydrogel formulation. Rats were fasted for 12 hour prior to the treatment although the water was in access during the fasting period. Male Sprague Dawley rats were randomly allocated to three groups, each containing six animals. Group I (n=6): served as Normal control; received plain carbopol gel without drug. Group II (n=6): served as standard group; received Standard Piroxicam gel. Group III (n=6): served as test group; received nanoemulsion based hydrogel formulation. A total of 0.1 ml 1% (v/v) suspension of carrageenan in normal saline was injected intradermally into subplantar region of right hind paws of the rats. Each test compound was applied topically 1 h prior to the injection of carrageenan. The digital vernier caliper [RSK, Mumbai] was used to measure paw width and thickness before and at 1st, 2nd, 3rd, 4th and 5th hour after injection of carrageenan. The paw volume was then calculated from width (a) and thickness (b) measurement using the following Eq. (3) [21]:

$$\text{Volume} = \pi \times a^2 \times b$$

..... Eq. 3

The percentage inhibition of paw edema was calculated for each group with respect to its vehicle-treated control group by using the formula:

$$\text{Percentage inhibition of edema} = (1 - V_t/V_c) \times 100$$

Where, V_t is the inflammatory increase in paw volume in test groups, and V_c is the inflammatory increase in paw volume in normal control group of rats. Percentage inhibition of edema is proportional to anti-inflammatory activity.

Statistical analysis

The statistical significance difference of mean values was assessed using one way ANOVA followed by Dunnet's multiple comparison Test using GraphPad prism 6 software. Statistical probability (p) values less than 0.05 were considered significantly different.

Stability study on the selected nanoemulsion and nanoemulsion based Gel

Liquid and gel formulations of NE-5, NBH were stored in well-stoppered glass containers for 6 months at 40 ± 2 C and $75 \pm 2\%$ RH. The stored samples were checked for optical clarity and phase separation by visual inspection. The pH, refractive index, mean droplet size, and Boswellic acids content were measured at specific time interval during storage.

Results and Discussion

Screening of oil for NE

The solubility of boswellic acids was determined in different oils (Figure: 1-A). It was evident that BAs exhibited highest solubility in isopropyl myristate (192.71 ± 0.89 mg/2ml) as polarity of the poorly soluble drugs favors their solubilization in small/medium molar volume oils. Edible oils cannot depicts large microemulsion region due to their rancid nature. IPM was selected for the preparation of nanoemulsions due to its well-known permeation-enhancing property and biocompatibility.

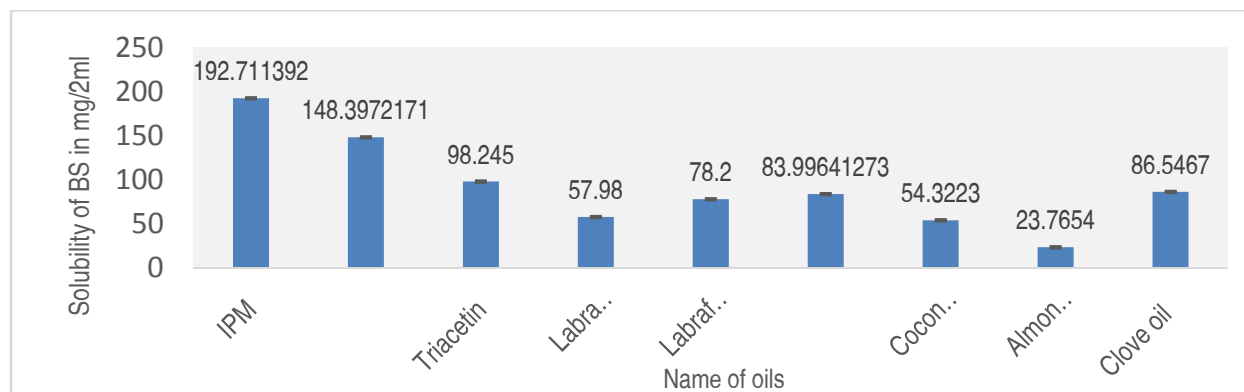


Figure: 1-a Solubility of Boswellic acids in various oils. (n=3)

Selection of surfactant co-surfactant system

It is necessary to determine the type and concentration of surfactant as nonionic surfactants known to be less affected by



changes in pH and ionic strength (Azeem *et al.*, 2009). Amongst various surfactants screened, Tween 80 exhibited (93.8 ± 1.23 mg/2ml) highest solubilization capacity of BAs. Whereas various cosurfactants were screened for solubility as well miscibility with surfactant, Transcutol P was revealed greater solubilization capacity (101.98 ± 1.02 mg/2ml) as well as it forms transparent system ($99.87\%T$) more than Plurol Oleique ($96.78\%T$) with

selected surfactant at different Km values. Transcutol P also increased permeant partitioning into and solubility within the stratum corneum depicts as permeation enhancer. The result of solubility of BAs in various surfactant and cosurfactants are shown in fig. 1-B. In conclusion, IPM, Tween 80 and Transcutol P were subsequently chosen as the oil phase, surfactant and cosurfactant for the construction of phase diagrams.

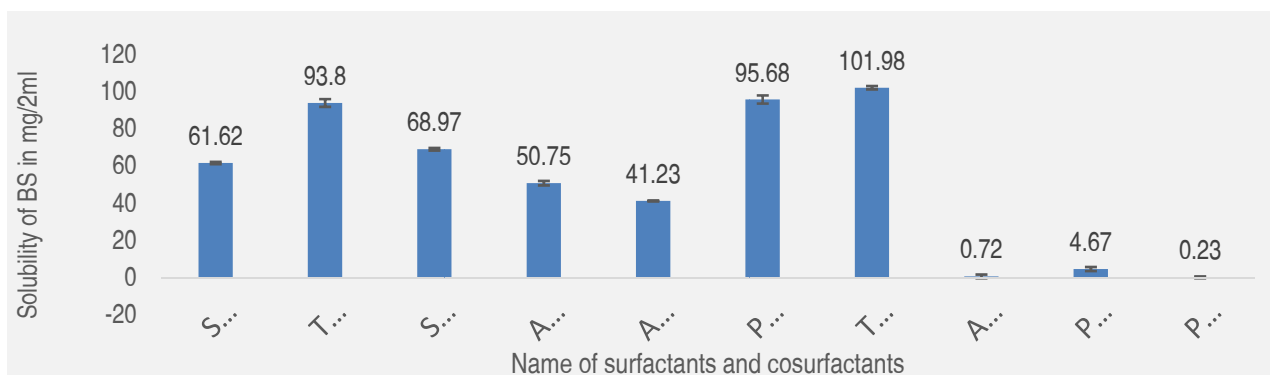


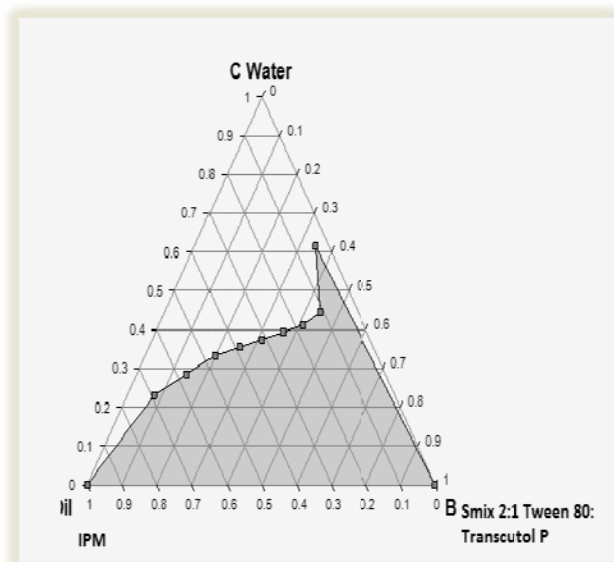
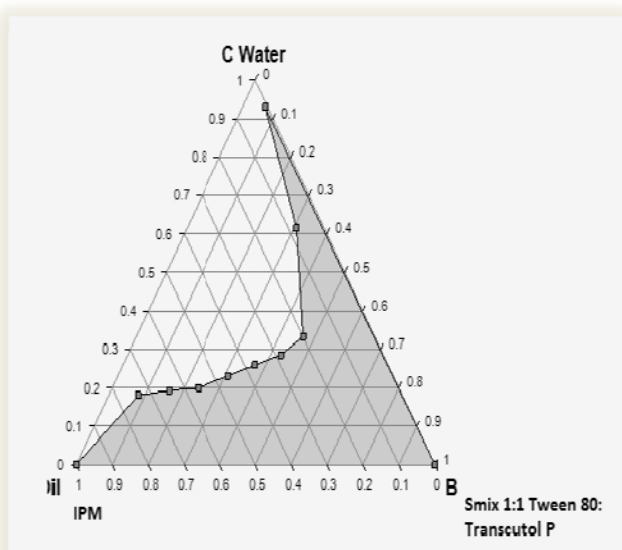
Figure: 1-b Solubility of Boswellic acids in various surfactants and co surfactant (n=3).

Construction of Pseudo ternary phase diagram

Pseudo ternary phase diagrams were constructed in the absence of BSE to identify nanoemulsion region depicts an important tool to study the phase behavior of nanoemulsion. It can be represented in a triangular format (triangle) which has three coordinates. (1) Oil phase, (2) Surfactant/co-surfactant phase and (3) aqueous phase at fixed weight ratios (Smix ratios 1:1, 2:1, 3:1, 4:1, 1:2, 1:3). The different blend of surfactant and cosurfactants (Km) were chosen in increasing concentration of surfactant with respect to co-surfactant and increasing concentration of co-surfactant with respect to surfactant for detailed study of the phase diagrams [2]. The concentrations of components were recorded in order to complete the pseudoternary phase diagrams, and then the contents of oil, Smix and water at appropriate weight ratios were selected based on these results.

An o/w nanoemulsion region was found towards the water-rich apex of the phase diagram. As the surfactant concentration was increased in the Smix ratio 1:1 (Figure. 2(a)), a higher

nanoemulsion region was observed. The probable reasons are reduction of the interfacial tension, increased the fluidity of the interface. Kawakami K and coworkers reported that greater penetration of the oil phase in the hydrophobic region of the surfactant monomers [22]. From the phase diagrams in fig.2 (e) & (f), formulation with Km 1:2, 1:3 showed very narrow area of microemulsion formation compared with others. So, further study in direction of increasing concentration of cosurfactant was not carried out. Phase diagram in Figure. 2 (a) & (c) with Km 1:1 and 3:1 has largest area of microemulsion formation followed by Km 4:1 (Figure. 2 (d)). Beyond this point, there is decrease in the area of NE formation. Various blends were selected from the microemulsion region to obtain *B. serrata* loaded nanoemulsions. From pseudo ternary phase diagrams, the formulations in which the amount of oil phase completely solubilized the drug and which could accommodate the optimum quantity of Smix and distilled water were selected for the optimization study.



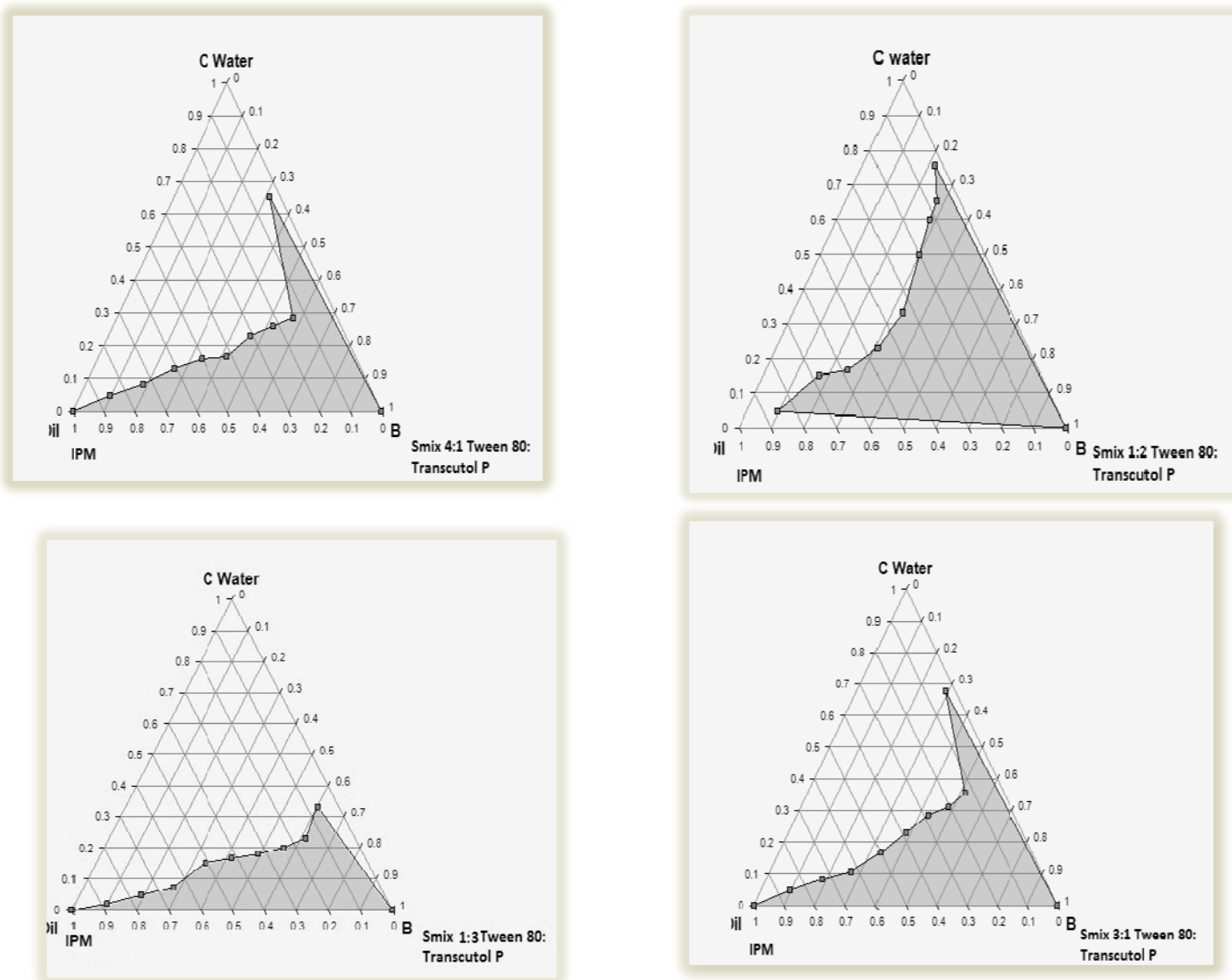


Figure 2 Pseudo ternary diagrams with Km (a) 1:1, (b) 2:1, (c) 3:1 and (d) 4:1, (e) 1:2 (f) 1:3

Demonstration of Multivariate analysis techniques

Simplex lattice mixture design was used to optimize the composition of nanoemulsions. The mixture components and response variables were related using polynomial equation with statistical analysis through Design-Expert® software. The approximations of response values (R_1 , R_2) based on the special cubic model was most suitable with lowest PRESS value. The value of the coefficients exhibit the effect of these variables on the response. The polynomial equations comprise the coefficients for intercept, first-order main effects, interaction term, a positive sign of coefficient indicate a synergistic effect while negative term indicates an antagonistic effect upon the response. After generating the polynomial equations through MLRA (Multiple linear regression analysis) relating the dependent and independent

variables, mixture components were optimized for the responses R_1 and R_2 . When the ratio of Smix was close to 1:1 (w/w), droplet size of nanoemulsion was lowest, also the permeation of Boswellic acids from nanoemulsion was high, because the small droplet size of nanoemulsion facilitates penetration of the drug for topical absorption. Topical absorption is influenced by nano droplet sized in vehicle and the partition coefficient. The solubility of drug in vehicle is also increased because of IPM and Transcutol P are present in nanoemulsion which contributes as penetration enhancer.

The result of polynomial equation for selected responses were obtained through Design expert software as shown in Eq. 4 and Eq. 5.



Droplet Size R1 = +34.66 *X₁ +16.95 *X₂ +11.81 *X₃ +0.61* X₁ X₂ +15.63* X₁X₃ + 68.35 *X₂ X₃- 400.6 *X₁X₂X₃ . (r²= 0.998)
 Eq. 4

Cumulative Permeation [mg/cm²] R2 = +20.19 * X₁+23.35* X₂ +23.60*X₃ -2.55*X₁X₂ -5.15 *X₁X₃ -10.81 *X₂X₃+28.2*X₁X₂X₃. (r²=0.998)..... Eq. 5

Eqs.(4) and (5) may be used to calculate the predicted values for other formulations in the design space. Table 1 contains the predicted values of droplet size and cumulative permeation with % prediction error of seven formulation with respect to observed

responses. The equation has good prediction ability. Model graphs of the droplet size and the cumulative amount of Boswellic acids permeated were constructed in the form of contour plots (Figure. 3-A.1,3-A.2) and the optimized formulation was chosen by superimposing the contour plots of the two responses, shown in overlay plot.(Fig. 3-A.3). The result of predicted data of the two responses for the optimized formulation demonstrate close agreement between the predicted and experimental results. In order to obtain nano droplet size and high permeation ability, the appropriate amount of components were chosen as oil (2%), Smix (18%), and water (10%).

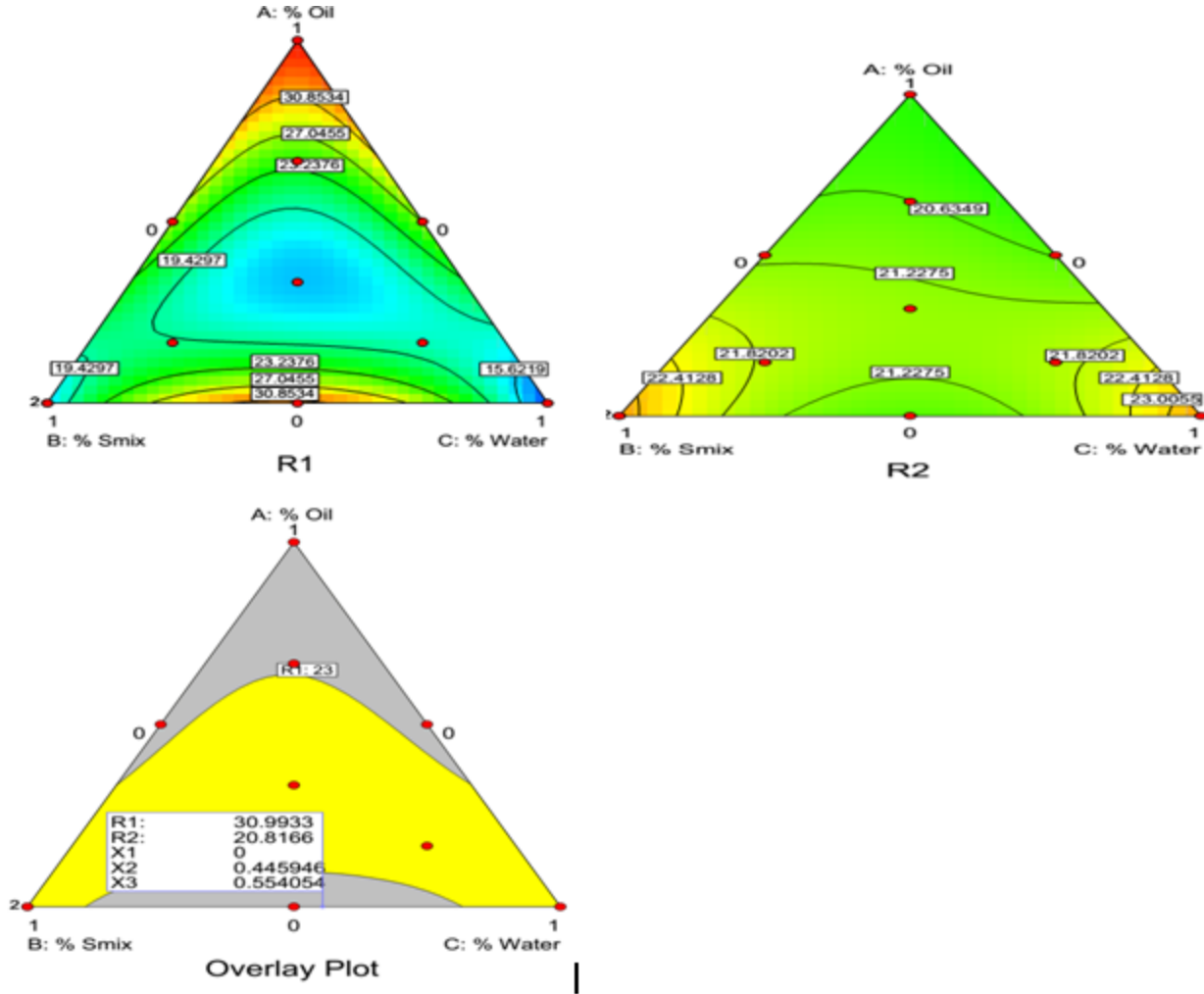


Figure 3 shows contour plots for R1-Droplet size, R2-percutaneous absorption and overlay plot of superimposing the contour plots of the two responses.

Partial least square regression analysis

The equations obtained through partial least square regression for selected responses shown in Eq. 6 and Eq. 7 obtained through XL State software.

Permeability coefficient: K_p (10⁻²)= 4.30-0.10*A 1.09E-03*B+4.72E-02*C -1.55E-03*AB+3.78E-03*AC-1.95E-03*BC-9.08E-03*A²+4.64E 05*B²+2.22E-03*C²
 Eq. 6



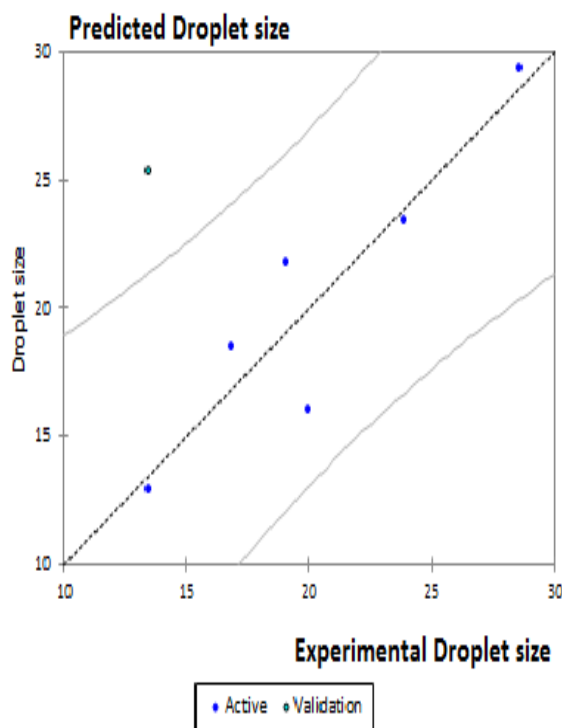
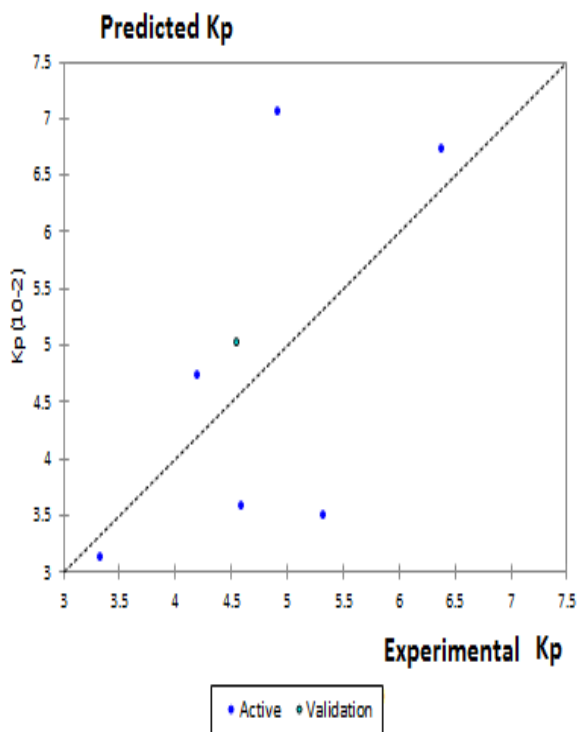
$$\text{Droplet size} = 3.42 - 5.79E-02 * A + 5.37E-02 * B + 0.34 * C + 5.73E-03 * AB + 4.16E-02 * AC + 0.10 * BC + 1.27E-02 * A^2 + 2.08E-03 * B^2 + 1.70E-02 * C^2 \dots \dots \dots \text{Eq. 7}$$

The resultant output from PLSR models for calculated responses were shown in Table 3. The results of microemulsion are expressed as pseudo-ternary phase diagram. A limited area shows the combinations of the variables that will result into formation of microemulsion. The classical multiple regression analysis is unsuitable to evolve a model which incorporates interaction and polynomial terms since the number of trials are few in the microemulsion region. PLSR can be used to handle this issue. The PLS models often used for prediction purpose rather than

interpretation. The independent and the dependent variables with original data matrix was expanded to incorporate interaction and polynomial terms before performing PLSR which shown in Table 2. The graph of the experimental and predicted responses for both dependent variables are shown in (Figure. 3-B.1, 3-B.2). Biplot chart generated through modelling of data illustrated in fig. 3-B.3 indicates partial least square analysis. PRESS value from PLSR was nearer to zero as compared to simplex lattice model. Fairly linear relationship was observed and hence it is concluded that PLSR proved to be an effective tool to predict responses. The use of PLS and its active cross-validation makes the results more stable and reliable.

Table: 3 Experimental, predicted and calculated permeability coefficients and droplet size by means of the best PLS model

Mixtures	Kp exp.	Kp pred.	Kp calc.	DLS exp.	DLS pred.	DLS calc.
NE-1	3.13	4.29	4.68	23.38	21.03	19.93
NE-2	7.06	6.58	5.49	21.8	22.88	20.36
NE-3	6.72	5.59	5.71	29.31	26.48	24.58
NE-4	4.74	3.88	4.04	12.89	18.45	19.54
NE-5	3.58	2.66	4.95	18.48	19.63	20.31
NE-6	3.5	3.80	3.71	15.98	13.31	13.03
NE-7	5.02	5.42	5.70	25.33	21.28	22.28



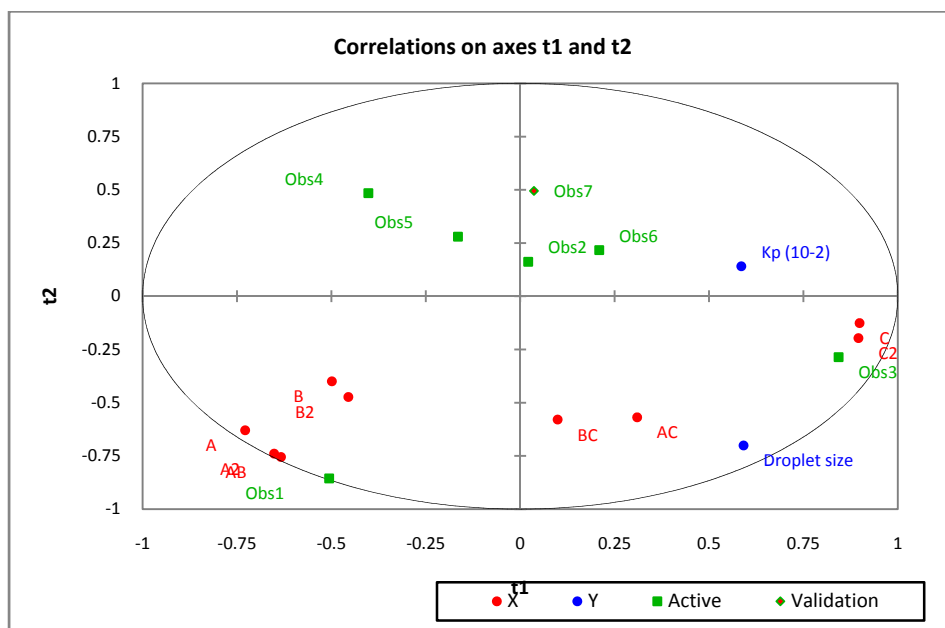


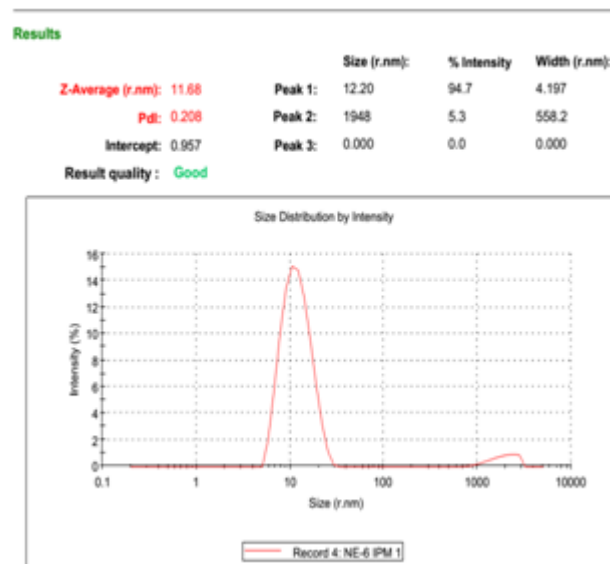
Figure : 4 shows Plot of predicted vs experimental Kp (Figure. 4-a) and droplet size (Figure 4-b) , where Fig. 4-c shows Biplot chart obtained from final PLS model

Characterization of nanoemulsion

TEM positive image of optimized NE showed spherical droplets without aggregation confirmed the findings of dynamic laser scattering technique (fig. 5-A.1, 5-A.2). DSC was applied to confirm the alteration in physical property of the drug. As specific endothermic peak of untreated drug was found at 104.92°C with heat capacity of -777.75 mJ, while in case of drug loaded microemulsion, the specific endothermic peak was found at 78.47°C with heat capacity of -46.16 mJ. The droplet size of prepared formulations were in the range of 11.2 ± 0.25 nm to 34.78 ± 1.25 nm. The low polydispersity values observed for all the formulations, Zeta potential determination of the prepared formulations were in range of 0.226 ± 0.01 mV to -0.846 ± 0.40 mV. The nanoemulsions consisted of non-ionic components, which show relatively neutral charge. This means that absorption is not influenced by charge of membrane in body. The viscosity of nanoemulsions were found in range of 22.3 ± 1.89 to 29.1 ± 2.56 m.Pa.s. As the concentration of oil globules was increased, viscosity of the formulation was increased. The pH of prepared formulation were found in range of 6.23 ± 0.02 to 6.67 ± 0.08. The refractive index of prepared formulations were found in the range of 1.28 ± 0.01 to 1.99 ± 0.02. However little increase in the refractive index was seen in case of formulation having more oil content and less water content, as refractive index of water, i.e. 1.334.

Examination under cross polarizing microscope showed dark field indicating no change in isotropic character. No crystals of the drug were detected that confirmed optical isotropic property of formulation and drug was not in liquid crystalline phase. Percentage transmittance of prepared formulation were found in range of 96.24 ± 0.087 to 99.87 ± 0.036 indicating transparency and

optical isotropic nature of the formulations. The conductance of the formulation increased as water level was increased indicates O/W type of nanoemulsion. The 5.5 fold increasing in solubility of BAs found by incorporating in nanoemulsion system. The viscosity of NE based hydrogel were found in range of 92.3 ± 2.89 mPa. S with 6.61 ± 0.05 pH having Spredability 2.35 cm.gm/sec.



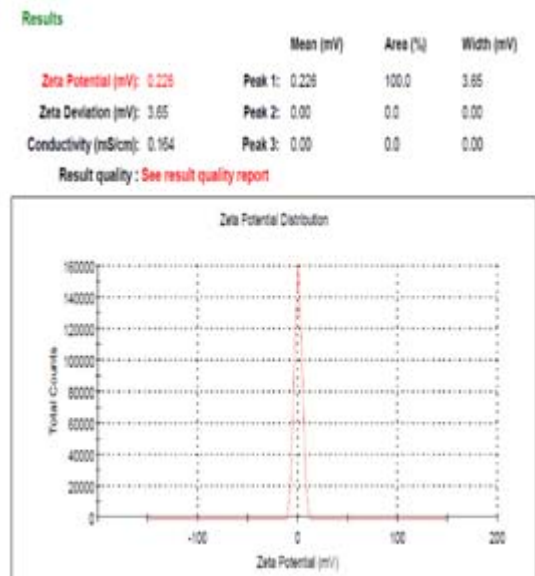


Figure: 5-A.1 Droplet size distribution and Zeta potential analysis of Nanoemulsion.

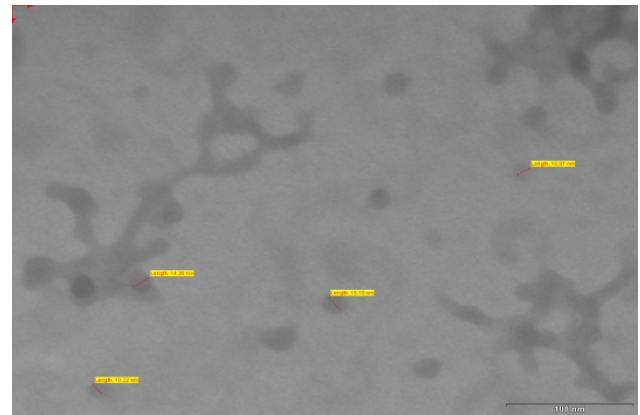


Figure: 5.A.2 TEM image of optimized Nanoemulsion formulation at 100nm scale.

In vitro Drug Diffusion study

In vitro diffusion studies were performed to ensure drug release prior to ex vivoskin permeation studies. The release profiles of boswellic acids from the different nanoemulsion formulations are illustrated in Figure. 6-A.1. The maximum drug diffusion in NE-5 could be due to lowest droplet size, and lowest viscosity. NE-7 batch was excluded for further study as having poor thermodynamic stability.

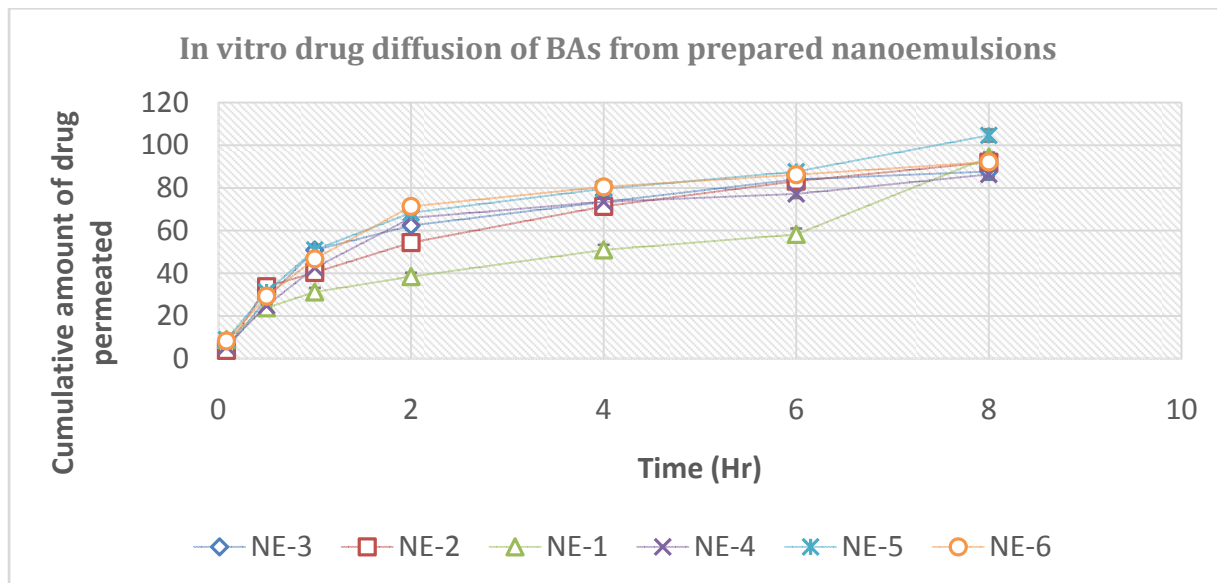


Figure: 6-A.1 In vitro diffusion profile of prepared nanoemulsion formulations. (n=3)

Ex-vivo permeation study

The formulation NBH showed a significant skin permeation profile. The skin permeation profile of NE-5 was significantly different when compared with that of carbopol gel (CG) and nanoemulsion based hydrogel (Figure: 6-B.2). The significant difference in BAs

permeation between liquid nanoemulsion formulations, NE based hydrogel, and CG was probably due to the mean size of internal phase droplets, which were significantly smaller in nanoemulsions.

Permeation data analysis



Permeability parameters like steady-state flux (Jss), permeability coefficient (Kp), and enhancement ratio (Er) were higher in nanoemulsions and nanoemulsion based hydrogel [NBH] formulation as compared with CG. This is because nanoemulsions and NBH excipients contain permeation enhancers like IPM and Transcutol P. The result depicts optimized nanoemulsion

formulation and NE based hydrogel showed enhanced flux (Jss) and permeability coefficient (Kp) as compared to the plain carbopol gel. Optimized NE-5 showed highest flux of $0.306 \pm 0.011 \mu\text{g}/\text{cm}^2/\text{h}$, in case of NE based hydrogel showed $0.137 \pm 0.058 \mu\text{g}/\text{cm}^2/\text{h}$ while carbopol gel showed $0.0944 \pm 0.019 \mu\text{g}/\text{cm}^2/\text{h}$.

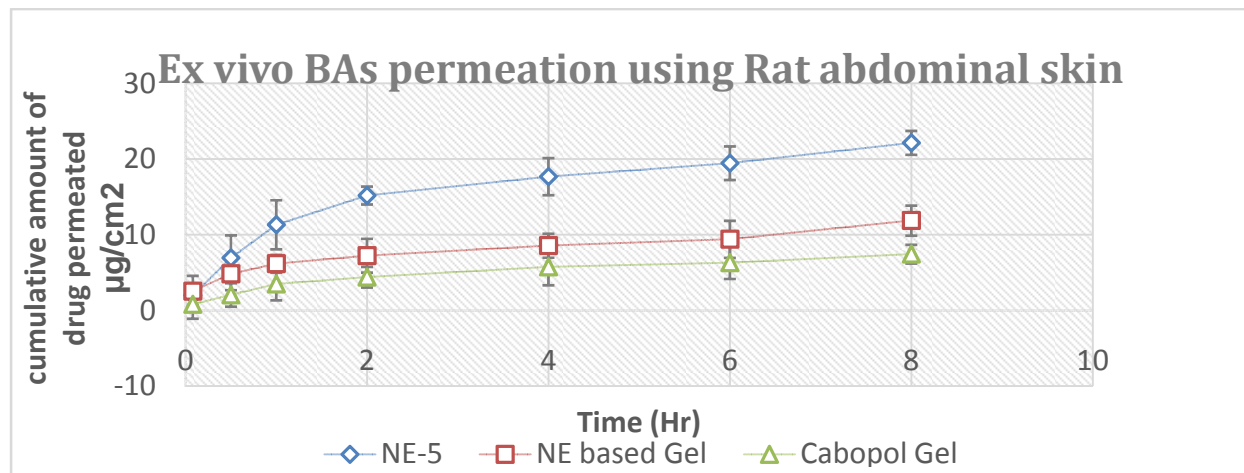


Figure: 6-B.2 Ex-vivo percutaneous skin permeation profile of optimized nanoemulsion, NE based hydrogel, and Carbopol gel. (n=3)

As compare to carbopol gel, 3.25-fold increase in flux was seen in case of liquid nanoemulsion as well 1.45-fold increase in flux was seen for NE based Hydrogel with highest enhancement ratio (Er) 4.574 and 1.59 respectively. As the viscosity of nanoemulsion is much lower than NE based hydrogel, the mobility of drugs in nanoemulsion is more facile. Moreover the nanoemulsions affect the stratum corneum structure and reduce the diffusional barrier by acting as a permeation enhancer[23-25].

Skin irritation study

The skin irritation test was performed to confirm the safety of the optimized nanoemulsion formulation. Van-Abbe and coworkers mentioned that a value between 0 and 9 indicates that the applied formulation is generally not an irritant to human skin [26]. The mean skin irritation score for NBH was 2.42 ± 0.76 . From this results, it was concluded that the nanoemulsion based hydrogel formulation was safe to be used for topical drug delivery.

In-vivo anti-inflammatory using carrageenan induced paw edema

The Intraplantar injection of carrageenan to hind paw in rats induced an increasing in the paw thickness. These edema had a rapid onset, and reached a peak at 5 hour after the challenge. Pretreatment with NE based hydrogel containing BAs, standard Piroxicam gel resulted in 31% and 29% inhibition in paw edema respectively at 5 hour as compared to plain carbopol gel show

significantly suppressed the increase in paw edema after carrageenan injection. Hence as shown in fig. 5, it is evident that NE based Hydrogel on pretreatment showed significant ($p < 0.05$) percentage inhibition of edema as compared to plain carbopol gel which could be due to enhanced permeation of Boswellic acids through skin. Hence prepared NE based hydrogel containing Boswellic acids produced significant anti-inflammatory effect.

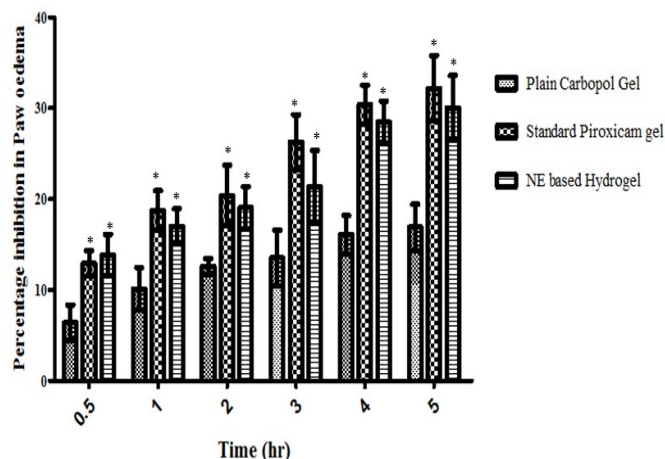


Figure: 7 Time-dependent inhibitory effect of the topical application of NE based Hydrogel of Boswellic acids against carrageenan-induced paw edema in rats.

Three groups of animals were pre-treated with the tested formulations 1 h before subplantar injection of 0.1 ml carrageenan (1%, w/v). Data are expressed as mean \pm S.E.M. (n = 6/group). Asterisks denote significance levels when compared with control values. *p < 0.05

Three groups of animals were pre-treated with the tested formulations 1 h before subplantar injection of 0.1 ml carrageenan (1%, w/v). Data are expressed as mean \pm S.E.M. (n = 6/group). Asterisks denote significance levels when compared with control values. *p < 0.05

Accelerated Stability studies

Developed formulations were found to be stable over period of six month. There is no phase separation, aggregation revealed physically stable formulation, no significant change in droplet size, zeta potential and drug content was seen during consequent analysis upon exposure to stress conditions of temperature and humidity.

Conclusion

This present research concluded the therapeutic potential of boswellic acids nanoemulsion for topical delivery through multivariate data techniques. The statistical models from PLS regression showed good predictive abilities of the boswellic acids permeability from nanoemulsion with its narrow droplet size of components. The results suggested that the nanoemulsion components played key role in permeation enhancing effect. Compared with Carbopol gel, the skin permeation ability of boswellic acids was significantly increased by nanoemulsion. The dose of BSE used to treat inflammation could be decreased due to the high solubility of drug in nanoemulsion and greater permeation ability of BAs nanogel inculcates significant anti-inflammatory effect. Present research focused on formulation development of a novel nanoemulsion based drug delivery system containing *Boswellia serrata* extract, which may provide important initiative and facet by enhancing bioavailability. The use of chemometric tools is also favored by FDA in this era of quality by design and process analytical technology.

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