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PREPARATION, CHARACTERIZATION AND *IN-VITRO* EVALUATION OF BERBERINE LOADED NANOEMULSION FOR ENHANCED ANTIMICROBIAL EFFICACY

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Keywords:

Berberine hydrochloride, Herbal nanoemulsion, Water titration method, Antimicrobial activity and *in-vitro* release

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ABSTRACT: Aim: The current research aimed to formulate and evaluate herbal nanoemulsion of poorly soluble Berberine hydrochloride, which can provide a realistic alternative for treating microbial infections. **Materials and Methods:** The drug was characterized using various analytical techniques such as Ultraviolet Spectroscopy, Fourier transform infrared spectroscopy, and Differential Scanning Calorimetry study. The herbal nanoemulsion was prepared using the water titration method by constructing pseudo-ternary phase diagrams. Formulations were evaluated by thermodynamic stability studies, droplet size analysis, viscosity, drug content, refractive index, polydispersity, and *in-vitro* release study. **Results:** On the basis of the analytical study, no interaction between the drug and excipients was found. Based on visual observation nanoemulsion was optimized by using thermodynamic stability study and freeze-thaw cycle. The optimized nanoemulsion (NE-2) shows droplet size of 203.7 nm, Viscosity-94 centipoises, Zeta potential -19mv, polydispersity index- 0.366. The optimized formulation NE-2 shows 91.7% drug release in 24 hours and was further evaluated for antimicrobial activity using Bacillus subtilis and *Candida albicans*. The zone of inhibition was found to be 56.26± 1.78 mm for antibacterial activity and 52.62±1.78 mm for anti-fungal activity with respect to control, *i.e*. 7.59±0.62 mm at 500 ppm. This indicates that the prepared formulation showed the most significant antimicrobial activity. **Conclusion:** The developed nanoemulsion systems can be a suitable carrier for transdermal delivery of berberine and can be used to treat bacterial and fungal infections such as blastomycosis, histoplasmosis, onychomycosis, *etc*.

INTRODUCTION: Nano-emulsions (NEs) are colloidal dispersions of two immiscible liquid phases, such as oil and water, in which one step is dispersed in other phases using a surfactant system, resulting in oil-in-water (o/w) or water-in-oil (w/o) nano-droplet systems [1]. Nanoemulsions are colloidal particulate systems with sub-micron particle sizes that respond as drug carrier particles. Their sizes vary from approximately 1,000nm.

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Nanoemulsion is a rapidly expanding technology, particularly in the food and pharmaceutical application, new dosage forms for drugs, and lipid-soluble substances like flavorings, color combinations, essential fats, *etc*. [2]. Optical clarity, good stability to separation, flocculation, and coalescence are some of the advantages of nanoemulsions [3].

It also enhances active compound absorption and bioavailability [4]. In recent years, there has been a growing interest in topical vehicle systems to alter drug penetration through the skin. To achieve these aims, several dermal carriers incorporate chemical enhancers and solvents. However, because many of these pharmaceutical enhancers are irritants, their

usage may be hazardous, especially in long-term use [5]. Transdermal drug delivery devices provide a simple, dependable technique for delivering drugs when a fast onset is not required [6].

Transdermal medication administration avoids enterohepatic circulation, producing a more consistent clinical effect [7]. With many medicines, transdermal administration has the same effectiveness as a continuous IV infusion but in a noninvasive method [8].

A microorganism, also called a microbe, is a pathogen and is a single-cell organism. The study of microorganisms is known as microbiology [9]. Microorganisms include bacteria, fungi, archaea, and protists. Microbial infection may result from the invasion of pathogens into the microbe, their proliferation, and the sponsor tissue's response to such agents.

Examples are bacteria, viruses, parasites, fungi, and other microbial pathogens [10]. These microbial infections are used to treat and manage many agents, also called antimicrobial agents [11]. Types of microbial infection: two types of microbial infection first are external microbial infection and another is internal microbial infection [12]. Berberine hydrochloride (BBH) is an iso-quinoline alkaloid found in medicinal plants. Berberine hydrochloride has multiple pharmacological actions [13].

These pharmacological actions, like antimicrobial, anti-tumor, or anti-cancer [14] and anti-inflammation, also show irritable bowel syndrome properties such as diarrhea, constipation, and abdominal disorders [15]. These have been considered effective antimicrobial agents because they inhibit bacterial cell replication by interacting with bacterial DNA [16].

On the other hand, Berberine hydrochloride is safe and is not mutagenic to human cells [17]. The nanoemulsion technology is one of the most promising approaches for improving transdermal medication penetration [18].

Nanoemulsion carriers are the best delivery techniques for poorly water-soluble drugs since they enhance drug solubility penetration [19] and, eventually, bioavailability *via* transdermal treatment systems [20]. The nanoemulsion for less aqueous soluble drugs is commendable and has multiple benefits over other drug formulations [21].

MATERIALS AND METHODS:

Materials Required:

TABLE 1: LIST OF MATERIALS

Sr. no.	Material	Sources
1	Berberine Hydrochloride	PRS infotech & engineers Sanjay Enclave, Faridabad, New Delhi, India.
2	Tween 20	Chemical storehouse in R.V. Northland Institute
3	Tween 80	Chemical storehouse in R.V. Northland Institute
4	Span 20	Chemical storehouse in R.V. Northland Institute
5	span 80	Chemical storehouse in R.V. Northland Institute
6	Ethanol	Chemical storehouse in R.V. Northland Institute
7	Glycerol	Chemical storehouse in R.V. Northland Institute
8	Olive oil	Chemical storehouse in R.V. Northland Institute
9	Castor oil	Chemical storehouse in R.V. Northland Institute
10	Isopropyl myristate	Chemical storehouse in R.V. Northland Institute
11	Methanol	Chemical storehouse in R.V. Northland Institute
12	Salicylic acid	Chemical storehouse in R.V. Northland Institute
13	Zinc oxide	Chemical storehouse in R.V. Northland Institute
14	Butylated hydroxyl anisole	Chemical storehouse in R.V. Northland Institute
15	Semipermeable membrane	Pharmacology Research Lab. in R V Northland Institute
16	Skin	Animal House Facility in R V Northland Institute
All of the chemicals and reagents were of analytical quality		

Pre-formulation Study:

Melting Point and Color: The melting point (VEEGO Model-Vmp-0) instrument was used to determine the melting point of berberine hydrochloride [22].

Ultraviolet Spectroscopy: Berberine hydrochloride was serially diluted. Initially, a 1000 mcg/ml solution was prepared by weighing 10 mg of the drug with an electronic balance (Shimadzu, AU X 220) and dissolving in 10 ml of methanol. From this stock solution, various dilutes ranging 1 to 20 ml were prepared, and the absorbance of the sample's solution was measured on a PC with methanol solution as a blank. For the detection of Berberine Hydrochloride, the detection wavelength was set at 348 nm. The concentration (X-axis) and absorbance (Y-axis) were shown on a graph [23-24].

DSC (Differential Scanning Calorimeter): DSC of the standard drug was determined using a differential scanning calorimeter calibrated with Indium (Perkin Elmer DSC-7) and every sample was tested in triplicate [25]. The following settings were done on the instrument shows in **Table 2.**

TABLE 2: DSC SETTING PARAMETERS SET ON INSTRUMENTS

Sr. no.	Parameter	Set
1.	Atmosphere	Nitrogen free
2.	Gas flow order	20 ml/min
3.	Heating order	10ºC/min
4.	Temperature:	-30 - 200ºC
5.	Size of Sample	0.5 mg.

FTIR (Fourier-Transform Infra-Red Spectroscopy): The FT-IR study was carried out using the Shimadzu FT-IR spectrophotometer model IR affinity-1CE. Using the potassium bromide pellet method, the infrared spectrums of the drug sample (BBH powder) were acquired in the region of 800-4000 cm^{-1} and evaluated for additional interaction [26].

Screening of Solubility: The solubility of BBH in different oils (Olive oil, Castor oil, Oleic acid, Isopropyl myristate), surfactants (tween 20, tween80, Span 20, pan 80), and cosurfactants (PEG-400, Glycerol, and ethanol) was determined by dissolving an extreme amount of BBH in 5 ml of each of the identified oils, surfactants, and co-surfactants in 10 ml 27.

In an isothermal mixer (Nirmal International Delhi, India), the mixture vials were kept at 37 0C for 72 hours to attain equilibrium. The shaker samples were equilibrated and centrifuged at 3000 rpm for 10 minutes. Using a 0.45 mm membrane filter, the tubes were centrifuged and filtered.

Formulation Development:

Construction of Pseudo-ternary Phase Diagram: Based on the solubility analysis, olive oil was used as the oil phase and Tween-20 and glycerol was used as the surfactant and co-surfactant. To assess overall concentrations of constituents for such natural range of nanoemulsion, a pseudo ternary phase diagram is constructed at room temperature (24°C) using the water titration technique. The surfactant system (Surfactant and Co-surfactant) was combined in various ratios (1:0, 1:1, 1:2, 2:1, 3:1, and 3:2). Oils and surfactant system mixtures (O-S/Co) was mixed gently in a selection of volume ratios (1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, and 9:1). Taken oil, surfactant, and co-surfactant combinations in various concentration ratios were used to titrate using drop by drop of distilled water. Physical clarity of such a resulting mixture was observed until the titration endpoint (turbid was observed). At this stage, the amounts of water, oil, surfactant, and cosurfactant applied were recorded and utilized to create a pseudo-ternary graph. Mono-phasic, transparent, less viscosity and stable thermodynamic systems are known as nanoemulsions (NE) and shown for the NE area [28].

Drug-Loaded Nanoemulsion: The drug-loaded nanoemulsion was made by dissolving a specified amount of berberine hydrochloride in designated oil, then S/Cos-mix was added to it in water titration tube and the mixture was placed upon a vortexer until the mixture was miscible. The resulting mixture was titrated with definite amount of water to produce drug loaded nanoemulsion.

Thermodynamic Stability Studies: This experiment aimed to observe how phase separation and temperature affect NE Drug delivery formulations. These preparations are subjected to the following thermodynamic stability study in order to assess their physical stability:

Heating Cooling Cycle: Formulation was kept between 4°C (refrigerator temperature) and 45°C for 48 h. Centrifugation testing was needed for the stable formulae.

Centrifugation Test: After dilution with water, the preparations that showed stability in the heating-cooling testing were centrifuged at 15,000 rpm for

15 minutes. The freeze-thaw stress test was done on the formulations that completed this test.

Freeze-Thaw Period: Three freeze-thaw processes were conducted between temperatures of (-4°C) and (+40°C), with the formulation being deposited for at least 24 h at each temperature.

Dispersibility Tests: This test is often used to calculate emulsification performance and duration. After pre-concentrate dilution, the emulsification period is the amount of time it takes to produce a homogeneous mixture. In a nutshell, 0.5 ml of the formula was combined with 250 ml of deionized water at 100 rpm, and the temperature was set to 37 ± 0.5 C.

TABLE 3: GRADE DISPERSIBILITY

S. no.	Grade Dispersibility	Appearance Time
1.	Clear or slightly bluish emulsion	<1 min
2.	Slightly less clear or bluish white emulsion	<2 min
3.	White emulsion	<3 min
4.	Grayish white Emulsion	>3 min
5.	Poor or minimal emulsification	>3 min

Droplet Size Analysis: Based on the laser light scattering phenomenon, the droplet size of Nano emulsion diluted with purified water using a photon correlation spectrometer (Zeta-sizer) was analyzed.

***In-vitro* Release Study:** A dialysis bag diffusion analysis was conducted to determine in vitro drug release study. The Semi-permeable membranes had a penetration depth of 2.0 nm as well as a molecular weight cut off of 10,000–12,000 Da (Dialysis membrane-70, Hi Media, Mumbai, India).

Stability / Activation of Semi-permeable Membrane for Permeation Studies: For 12 to 24 hours, the membrane was immersed in phosphate buffer pH 7.2. Finally, the semi-permeable membrane was cleaned in distilled water and examined for physical damage. Wrapping the semi-permeable membrane with aluminum foil and storing it at room temperature overnight worked well.

The bags were soaked in 7.2 pH Phosphate buffer for 24 hours prior to usage. The prepared nano emulsion (10 ml) was precisely weighed and deposit sealed dialysis bag in the basket of USP. The basket was submerged in 500 ml of dissolving medium, a 0.1 percent w/v tween 20solution kept at 37±50 ^{o}C and 60 rpm. For sample time, 1, 2, 4, 6, 8, 12 and 24 hours were chosen. To keep a stable volume, the same volume of new dissolving media was replenished. UV spectroscopy at 350 nm wavelength was used to examine the samples.

***Ex-vivo* Permeation Studies:** Static Franz diffusion cells were utilized in this research. These cells are donor and receptor chambers separated by a cell membrane. *Ex-vivo* studies were conducted using the skin of albino rats (body weight of 250 ± 20 g and 6–8 weeks old male) issued from the Animal house of R V Northland institute, Dadri, UP, India (Approved Ethical No: 1149 /PO /Ere /S/07/ CPCSEA). The study was carried out following the guideline for animal care and the use of laboratory animals.

A Franz diffusion cell assessed the transdermal permeation of the optimized formulation NE-2 and BBR-S. Rat skin was obtained from the Institute animal house, and hairs were shaved using an electric shaver. The skin was made free from fatty debris and unwanted adhered tissues. The skin was placed between both chambers (donor and receptor) so that the dermal side faced the Phosphate buffer solution medium (pH 7.0), and the epidermis faced the loaded sample. The release medium was stirred using beads (1250 rpm) at 37 ± 2 ◦C. Furthermore, sampling was performed at varied time points in hrs.(1, 2, 4, 6, 8, 10, 12, and 24), and the drug concentration was estimated using the validated HPLC method at 350nm.

Calculation of Permeation Parameters: The cumulative amount of berberine hydrochloride permeated per unit of ratskin surface area, mg = cm^2 was plotted as a function of time (t, h).

Cumulative Amount of Drug Permeated = concentration (µg/ml) x dilution factor / Area of the permeation cell

The Steady-State Drug Flux (Permeation Tate): was estimated by dividing the linear component of the curve by the diffusion cell area (mg cm2 h^1). The permeability coefficient (Kp) was determined by dividing Jss by the drug's initial concentration in the donor cell (cm h^1).

The enhancement ratio (ER) = flux from formulations / Flux from saturated solution of drug

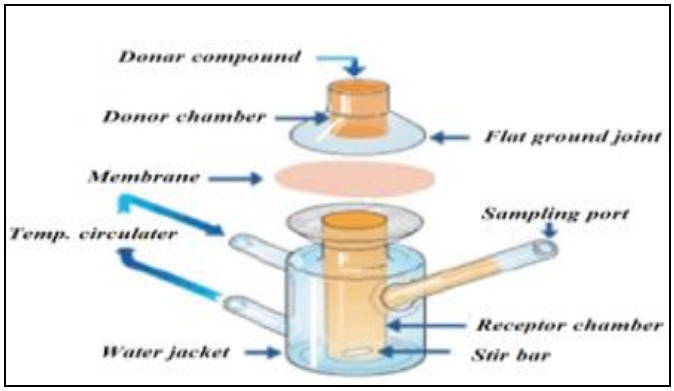

FIG. 1: FRANZ DIFFUSION CELL

Viscosity Determination: A concentric cylinder Brookfield viscometer (Brookfield Engineering Laboratories) was used to evaluate the viscosity of nano emulsion.

Drug Content: The sonication approach was used to extract berberine hydrochloride from an optimizednano emulsion formula based on the pseudo ternary phase diagram results. After appropriate dilution, the extract was spectro-photometrically examined for berberine hydrochloride concentration at 260 nm.

pH: The pH of the berberine hydrochloride nanoemulsion was determined using a digital pH meter

Refractive Index: The Refractive index of the optimized nanoemulsion formulation NE-2 was determined using an Abbe's refractometer. As a control, distilled water was used. Typically, the Refractive index of a nanoemulsion must be comparable to that of pure water (1.33). As a consequence, it is feasible to claim that all of the formulae were designed straightforwardly.

Zeta Potential: The zeta potential is a measurement of the quantity of particle repulsion or attraction, it defines the surface charge characteristic, and its value reflects system behaviour. For 60 seconds, prepared nanoemulsion formulations were diluted in a 1: 100 ratio with distilled water. The zeta potential value was then calculated using Zetasizer (Malvern Instruments, UK), and on molecules of sufficient size, a strong zeta potential gives stability and resistance to aggregation.

Dilution test: This analysis was extremely important for formulation and development. The proper emulsifier combination is required to create a stable nanoemulsion formulation. Berberine hydrochloride was diluted with 10 parts purified water 7.2 pH phosphate buffer for 1 part medication for nanoemulsion preparation.

Polydispersity Index: The Zetasizer nano S90 (Malvern Instruments, Malvern, UK) was used to estimate the Polydispersity Index.

Surface Morphology: The morphology and structure of the nanoemulsion were studied using transmission electron microscopy (TEM; Morgagni 268D SEI, USA) operating at 200 KV and capable of a 0.18 nm point-to-point resolution. A combination of bright field imaging at increasing magnification and diffraction modes was used to reveal the form and size of nanoemulsion droplets. To perform the TEM observations, a drop of the nanoemulsion was directly deposited on the holey film grid and observed after drying.

Antimicrobial Activity: The disc diffusion method was chosen to investigate the antimicrobial activities of berberine hydrochloride. Bacillus subtilis (bacteria) and Candida albicans (fungi) cultures were grown on a Sabouraud-dextrose agar medium to carry out these experiments. (Amin, Subbaiah *et al.* 1969) Each Petri dish (10-cm diameter) received 15 ml of media containing 24-hour subcultures of Bacillus subtilis and *Candida albicans*, which were then allowed to harden and dry. The microbial suspension was put on the surface of the medium using a sterile cotton swab after it had solidified. (Sahibzada, Sadiq *et al.* 2018). The filter paper disc was created using Whatman's filter paper, which was then treated with a berberine hydrochloride formulation before being streaked across the surface of agar plates containing a microbe culture. The diameter of the inhibitory zone (in mm) was then determined. The experiment was carried out in an aseptic room [29].

Homogeneity and Skin Irritation test: The homogeneity of the formulations was evaluated visually and by touching. The irritancy test of the optimized formulations was evaluated in albino Wistar rats obtained from the R.V. Northland institute animal house laboratory (Reg. No. 1149/PO/Ere/S/07/ CPCSEA). 1ml nanoemulsion

was administered to the albino Wistar rat's left ear, whereas the right was used as a reference. The reported approach was used to monitor the development of erythema and oedema for three days.

Stability Study: The nanoemulsion formulation was kept at various and different temperatures for 60 days. Following that, the consistency and concentration of berberine hydrochloride were determined or studied and three packs of produced nanoemulsion-based gel were submitted to 40±5 °C and 75±5% relative humidity for this investigation (RH). After 0, 15, 30, 45, and 60 days, the samples were extracted, diluted with methanol and analyzed by HPLC [30].

RESULTS AND DISCUSSION:

Pre-formulation Studies

Melting point and Color: The results of the melting point and color was shows in **Table 4.**

TABLE 4: MELTING POINT AND COLOUR

Melting Point and Colour			
S. no.	**Test**	**Specification**	**Result**
1.	Colour	Yellow powder	Yellow powder
2.	Melting point (M P)	193-196° C	194° C

UV-Spectroscopy: The U.V Spectroscopy (Shimadzu) UV spectrum of BBH solutions in methanol was studied at 400 to 200 nm. The wavelength of maximum absorption (max) was determined, and maximal absorption wavelength (lambda max) was calculated in **Table 5.**

Calibration Graph of Berberine Hydrochloride: Berberine hydrochloride was observed to satisfy Beer Lambert's law at 348 nm in the range of concentrations of 1-20 ug/ml, with a correlation value of 0.999 **Table 5 Fig. 2**. The linearity curve's regression coefficient was y = 0.0899x**.**

TABLE 5: UV ABSORBANCE OF BERBERINE HYDROCHLORIDE

UV Spectroscopic Data			
S. no.	**Concentration**	**Absorbance**	**Parameter**
1.	1	0.107	Maximal Absorption (λ-max) 348 nm
2.	2	0.178	Dilutions 1-20ug/ml
3.	4	0.355	Regression equation (y = bx + a) y = 0.0899x
4.	5	0.452	
5.	8	0.719	
6.	10	0.899	Correlation coefficients (r^2) r^2 = 0.9999
7.	20	1.797	

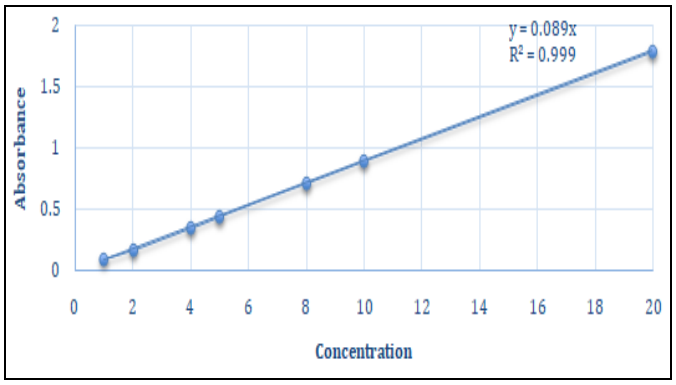

FIG. 2: CALIBRATION GRAPH OF BERBERINE HYDROCHLORIDE

Differential Scanning Calorimetry (DSC): The melting point of the powder was determined to be 198.165°C, which is consistent with the standard monograph (195°C) of berberine hydrochloride, confirming the existence of berberine hydrochloride in powder form.

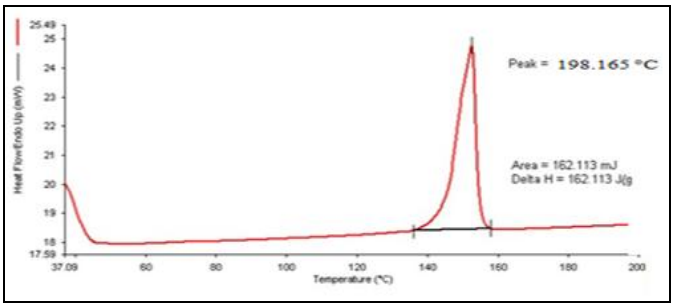

FIG. 3: DSC ANALYSIS OF BERBERINE HYDROCHLORIDE POWDER

Fourier Transform Infra-Red Spectroscopy (FTIR): The sample's FT-IR interpretation peaks for Aromatic C-H stretch, Aromatic C-H, C=C stretch, C=N stretch, and C-O stretch were almost identical to those of the standard range, as shown in **Table 6**. As a result of the interactions, the medication samples appear to be quite stable and inert to the standards.

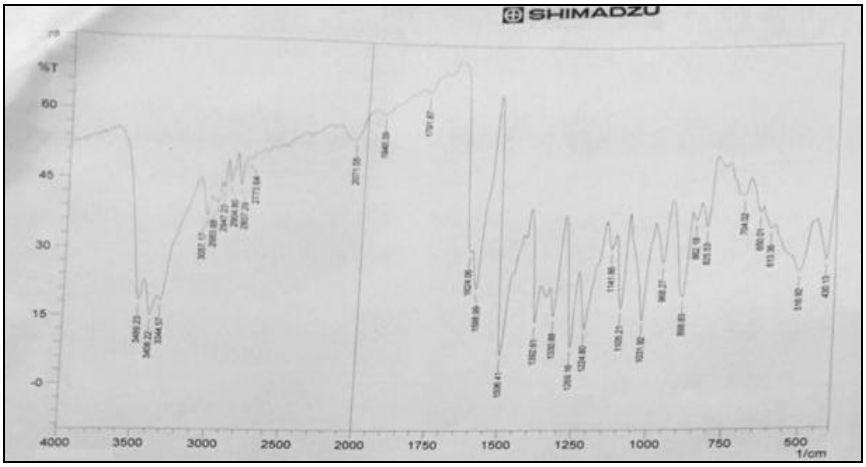

FIG. 4: FTIR ANALYSIS OF BERBERINE HYDROCHLORIDE POWDER

TABLE 6: COMPARATIVE FT-IR SPECTRA OF BBH POWDER WITH STANDARD BBH

Comparative FT-IR Spectra Data		
Wave number (cm^{-1}) and Compound type	**Functional group**	**Sample detected At (cm^{-1})**
2850-2500 cm^{-1} AROMATIC	Ar C-H stretch	2845.87
900-600 cm^{-1} AROMATIC	Ar C↓H	859.27
1650-1535 cm^{-1} Cyclo-alkene/s	C=C stretch	1641.65
1690-1520 cm^{-1} Heterocyclic amines	C=N stretch	1673.98
1330-1050 cm^{-1} Lactone	C-O stretch	1058.27

Solubility Screening: The content of berberine hydrochloride in each oil, surfactant and co-surfactant was measured using UV Spectroscopy at 348nm. **Table 7** shows the solubility of berberine hydrochloride in many pharmaceutical additives. In the oil phase, olive oil solubilizes the most berberine hydrochloride, but in the surfactant or co-surfactant phase, Tween-20 and glycerol demonstrated great berberine hydrochloride solubility.

TABLE 7: SOLUBILITY OF BERBERINE HYDROCHLORIDE WITH DIFFERENT COMPONENTS

Solubility Studies Data		
Sr. no.	**Components**	**Solubility (mg/ml) (mean ± SD; n = 3)**
1.	Olive oil	96.22±1.22
2.	Oleic acid	79.12± 0.83
3.	Isopropyl myristate	48.22±1.01
4.	Castor oil	29.23± 0.52
5.	Tween 20	113.12± 1.02
6.	Span 20	27.33± 0.82
7.	Tween 80	39.41± 0.75
8.	Span 80	66.22± 2.01
9.	Ethanol	60.51± 1.23
10.	Glycerol	104.25± 1.18
11.	PEG 400	63.51± 1.23

Formulation Development:

Development and Construction of Pseudo-ternary Phase Diagrams: Based on the physical appearance was selected many formulations for the construction of Pseudo Ternary Phase Diagram [PTPD], Surfactant Systems like surfactant and co-surfactant (S-Mix) ratio 1:0, 1:1, 2:1, 1:2, 3:2 with the help of PCB Triangle Software for the created formulations and shown **Fig. 5.**

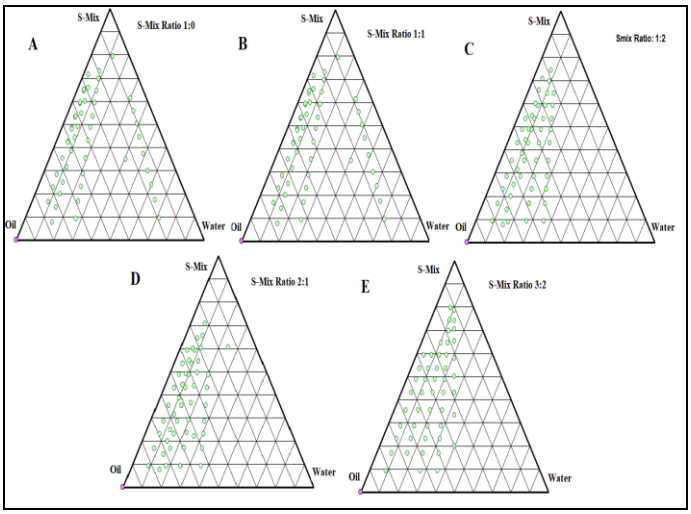

FIG. 5: PSEUDO-TERNARY PHASE DIAGRAMS OF THE O/W NANO EMULSION AREA FOR THE S-MIX DIFFERENT RATIOS

Drug-loaded Nanoemulsion: Berberine hydrochloride was mixed in the oily phase to create a drug-loaded nanoemulsion. As with drug-loaded nanoemulsion with, the appropriate quantity of surfactant was added, and water was added drop by

drop until a clear and transparent liquid was produced. The Nano-emulsion s were securely sealed and kept at room temperature. Result of drug loaded nano emulsion was shows in **Table 8.**

TABLE 8: DRUG LOADED NANOEMULSION FORMULATIONS

Formulations Code	Drug (%)	Ingredients (%)				S-Mix Ratio
	Berberine Hydrochloride	Surfactant -Tween 80	Cosurfactant - Glycerol	Oil - Olive oil	Water	
NE- A	1.00	34.55	34.55	9.90	21.00	1:1
NE 1	1.00	14.65	29.30	29.70	26.35	1:2
NE- B	2.00	32.10	16.10	19.80	20.00	2:1
NE- 2	2.00	19.20	19.20	9.90	49.30	1:1
NE- C	1.00	14.65	29.30	29.70	26.35	1:2
NE – 3	2.00	39.60	13.20	19.20	28.00	3:1

Thermodynamic Stability: The effect of increasing the dilution factor from (1:10, 1:50, and 1:100) was studied and they mostly swiftly created transparent nanoemulsion. Throughout all cases, higher dilution resulted in the nanoemulsion remaining clear. During the stability investigations, no phase separation or drug precipitation and thermodynamic stability result show in **Table 9.**

TABLE 9: THERMODYNAMIC STABILITY

Thermodynamic Stability			
Formulations code	Heating-cooling cycle	Centrifugation test	Freeze-thaw period
NE- A	P	P	F
NE-1	P	P	P
NE-B	P	F	F
NE-2	P	P	P
NE-C	P	F	F
NE-3	P	P	P

P- pass; F- fail.

Dispersibility Test: The result of the dispersibility study of berberine hydrochloride loaded nanoemulsion are shown in **Table 10.**

TABLE 10: DISPERSIBILITY STUDY

Dispersibility study		
S. no.	Formulations code	Dispersibility study result
1.	NE-A	@@@
2.	NE-1	***
3.	NE-B	@@@
4.	NE-2	***
5.	NE-C	@@@
6.	NE–3	***

*** pass and @@@ fail

Droplet Size Analysis: Based on thermodynamic stability and dispersibility test, formulation NE-1, NE-2, and NE-3for droplet size analysis. Droplet size analysis of the selected formulation of nanoemulsion NE-1, NE-2 and NE-3 was determined and Results shows in **Table 11** and **Fig. 6, 7,** and **8.**

TABLE 11: DROPLET SIZE ANALYSIS

Droplet Size Analysis		
Sr. no.	Selected Formulations	Result
1.	NE-1	274.1
2.	NE-2	203.7
3.	NE-3	467.5

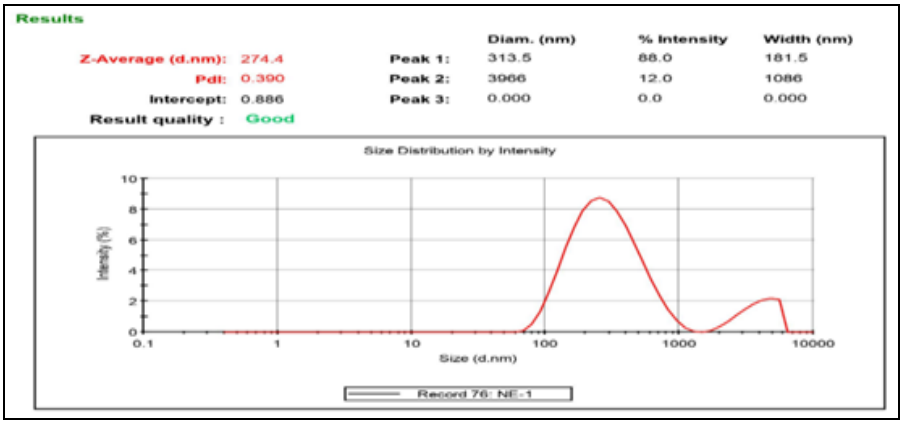

FIG. 6: DROPLET SIZE ANALYSIS NE-1 FORMULATION

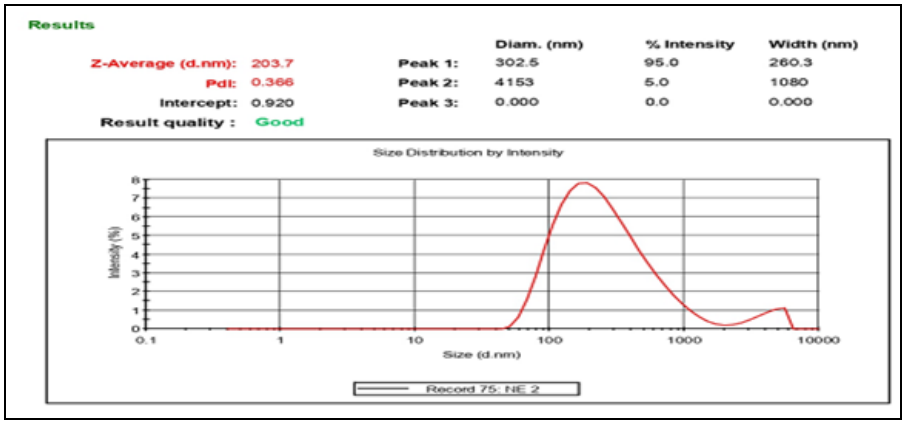

FIG. 7: DROPLET SIZE ANALYSIS NE-2 FORMULATION

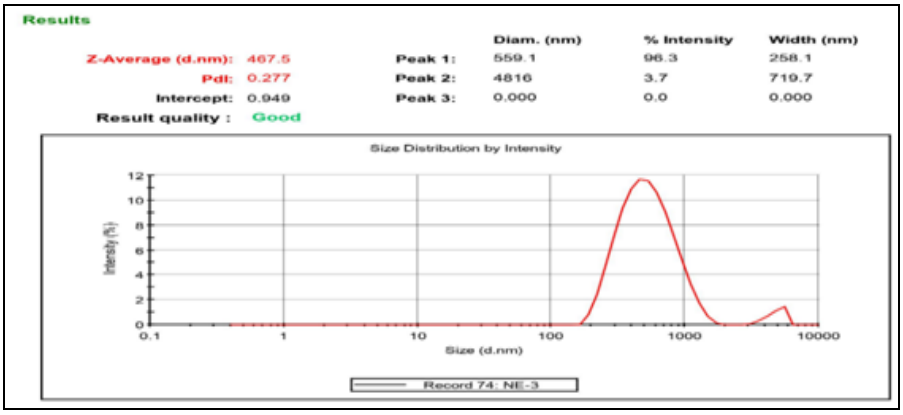

FIG. 8: DROPLET SIZE ANALYSIS NE-3 FORMULATION

***In-vitro* Release Study:** Results of the *in-vitro* release study of the formulations NE-1, NE-2 and NE-3 indicates that the optimized nanoemulsion NE-2 formulation has the highest drug release, 91.7% at 24 hours, compared to the NE-1 and NE-3 formulations, which shows drug releases of 65.1% and 55.9% respectively.

TABLE 12: % CPDR STUDY OF NANOEMULSION FORMULATIONS

Time (hours)	% Cumulative percent drug release		
	NE-1	NE-2	NE-3
1	32.5%	41.6%	31.1%
2	44.5%	49.7%	35.5%
4	52.99%	58.9%	44.5%
6	56.4%	69.4%	47.5%
8	59.2%	73.7%	50.5%
12	60.5%	79.2%	53.5%
24	65.1%	91.7 %	55.9%

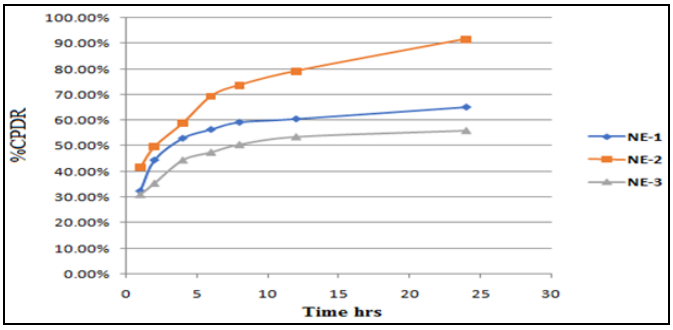

FIG. 9: %CPDR STUDY

***In-vitro* Skin Permeation Study:** Franz diffusion cell was used to carry out the permeation study. Based on particle size analysis are selected optimized formulation of NE-2 nanoemulsion was studied for in vitro skin penetration. The impacts of oil and surfactant combination concentration on berberine hydrochloride skin penetration were analyzed. Permeability tests revealed that formulation NE-2, which included 9.90% oil phase (olive oil), 19.20% Tween 20, 19.20% Glycerol, and 49.30% distilled water (from its phase diagram of surfactant system ratio 1:1), having a greatest 207.48µg/cm^2/ hpermeation flux. As a result, nanoemulsion NE-2 was considered the most suitable nanoemulsion for further research activity.

TABLE 13: *IN-VITRO* SKIN PERMEATION OF OPTIMIZED NANOEMULSION (NE-2) USING FRANZ DIFFUSION CELL

			***In-vitro* permeation Study**					
Sr. no.	**Time (h)**	**Mean area (n=3)***	**Concentration (µg/ml)**	**Cumulative amount of drug permeation. (µg)**	**Cumulative percentage of drug permeation.**	**Cumulative amount of drug permeation. (µg/cm^2)**	**Flux (µg/cm^2/h)**	**Pbx10^2**
1.	0	0	0	0	0	0		
2.	1	22889	5.94	594	2.97	198		
3.	2	82209	9.78	978	4.89	326		
4.	4	120293	23.52	2352	11.76	784		
5.	6	131992	43.22	4322	21.61	1440.66	207.48	1.0374
6.	8	143818	61.55	155	30.77	2051.66		
7.	10	150049	78.90	7890	39.45	2630		
8.	12	160222	87.77	8777	43.88	2925.66		
9.	24	172938	130.98	13098	65.49	4366		

TABLE 14: *IN-VITRO* SKIN PERMEATION OF CONTROL (BERBERINE SUSPENSION)

			***In-vitro* permeation Study**				
Sr. no.	**Time(h)**	**Mean area (n=3)***	**Concentration (µg/ml)**	**Cumulative amount of drug permeated (µg)**	**Cumulative amount of drug permeated (µg/cm^2)**	**Flux(µg/cm^2 /h)**	**Pbx10^2**
1.	0	0	0	0	0		
2.	1	89481	2.56	256	85.33		
3..	2	135532	3.826	382.6	127.53		
4.	4	241827	6.94	694	231.33		
5.	6	323102	9.44	944	314.66		
6.	8	459176	13.10	1310	436.66		
7.	10	550924	16.80	1680	560.00	58.983	0.2949
8.	12	761697	22.38	2238	746		
9.	24	1468133	42.88	4288	1429.33		

TABLE 15: RESULTS SHOWS COMPARATIVE *IN-VITRO* PERMEATION STUDY

Sr. no.	**Formulations**	**Flux(µg/cm^2/h)**	**Pbx10^2**	**ER**
1.	Optimized Nano emulsion	207.48	1.0374	
2.	Berberine Suspension	58.983	0.2949	3.5176

Comparative Study of Optimized Nanoemulsion Formulations and Marketed Berberine Suspension: The *in-vitro* permeation study of Berberine hydrochloride loaded nanoemulsion, and Berberine hydrochloride Suspension (Control) is depicted in the table above. The result shows that the flux of optimized nanoemulsion (NE-2) is the highest value compared to the control.

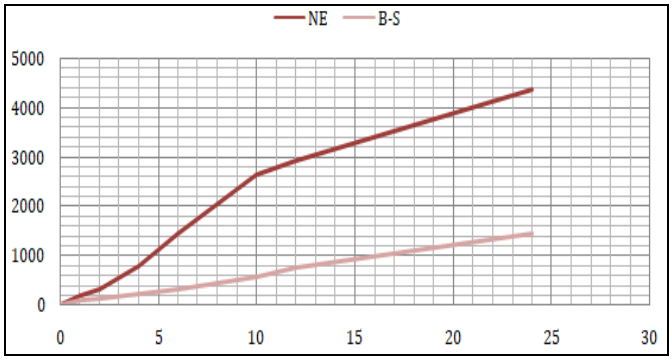

FIG. 9: COMPARISON OF *IN-VITRO* PERMEATION STUDY. NE: Nanoemulsion, B S-Berberine hydrochloride Suspension (Control)

Viscosity Determination: The optimized nanoemulsion formulation NE-2 viscosity shows to be 94 centipoises.

Drug Content: The optimized nanoemulsion formulation NE-2 drug content shows to be 98.20 ±2.25 %.

pH: The optimized nanoemulsion formulation NE-2 pH was reported to be 5.9, indicating it to be sufficient and non-irritant for skin application.

Refractive Index: The optimized nanoemulsion formulation NE-2 refractive index was found to be 1.321.

Zeta Potential: The optimized nanoemulsion formulations' NE-2zeta potential was found to be -19.0 mv, suggesting that they are stable.

Dilution test: The Optimized nanoemulsion formulations NE-2 found to be stable with diluted in a water and 7.2 pH phosphate buffer.

Polydispersity Index: The optimized nanoemulsion formulation NE-2 PDI was found to be 0.366.

TABLE 16: CHARACTERIZATION OF OPTIMIZED NANO EMULSION

Characterization of optimized Nano emulsion		
Sr. no.	**Evaluation Parameter**	**Result**
1.	Viscosity	94 Centipoises
2.	Drug Contant	98.20±2.25%
3.	pH	5.9
4.	Refractive Index	1.321
5.	Zeta potential	19.0 mv
6.	Dilution Test	Stable with water and 7.2 pH phosphate buffer.
7.	Polydispersity index	0.366

Surface Morphology: A TEM was used to analyze the nano emulsion's morphology. With the light surroundings, the droplet on the nanoemulsion appeared darkish. The globules are spherical in shape, according to TEM images **Fig. 10.**

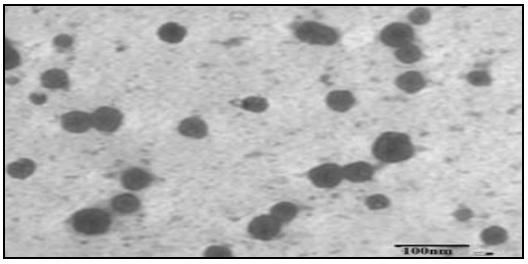

FIG. 10: SURFACE MORPHOLOGY (TEM)

Antimicrobial Activity: By comparing the zone of inhibition values of the test (BBRNE) with the control, it is discovered that the optimized formulation (NE2) has a larger zone of inhibition, that is, 44.66 ± 1.24 and 40.45 ± 1.36 for bacteria and fungi at 250 ppm/disc and 56.26 ± 1.93 and 52.62 ± 1.78 for bacteria and fungi at 250 ppm/disc and 500 ppm/disc, in both; as a result, it is possible to infer that its modified formulation NE-2 has improved antimicrobial efficacy.

TABLE 17: ZONE OF INHIBITION OF THE OPTIMIZED FORMULATION (NE-2)

Sr. no.	Category	Test organism	Diameter of zone of inhibition (in mm)			
			Formulation			
			250ppm/disc		500ppm/disc	
1.	Bacteria	*Bacillus subtilis*	BBR NE	BBR-S	BBR NE	BBR-S
			44.66 ± 1.24	7.96 ± 0.62	56.26 ± 1.93	7.59 ± 0.49
2.	Fungi	Candida albicans	40.45 ± 1.36	5.34 ± 0.66	52.62 ± 1.78	6.55 ± 0.71

*BBH-NE= Berberine Hydrochloride nano emulsion *BBH-S =Berberine Hydrochloride Suspension.

Homogeneity: The optimized formulation NE-2 applied a small amount of nanoemulsion gel is rubbed here between thumb and index finger and the consistency of the gel (whether homogenous or not, and whether any coarse particles emerge or are detached on the fingers) is noted.

Skin Irritation Test: To ensure that the herbal mixture was acceptable and effective, the skin irritation test was applied. A skin irritancy score of 0 to 2 indicates that the formulation is non-irritant and safe for human skin. The mean skin irritancy score for the formulation was found to be 1.16 **Table 18**. This result indicates that all of the excipients included in the formulation were safe to use topically.

TABLE 18: SKIN IRRITATION TEST OF OPTIMIZED FORMULATIONS (NE-2)

a. Skin Irritation Scores of Formulations (A=Erythemaformations core; B = Oedema formation score)

Rats	Intact skin				Abraded skin			
	24 hrs		72 hrs		24 hrs		72 hrs	
	A1	A2	A1	A2	A1	A2	A1	A2
1.	0	1	1	0	1	1	0	1
2.	1	1	0	1	1	0	1	0
3.	0	0	1	0	0	2	1	1

b. Final skin irritation scores of formulations (* =total of a and b from parta.; **=average of all skin reading of 24 and 72 hours)

Rats	Intact skin		Abraded skin		Total Average (A)+(B)
	24 h and 72 h		24 h and 72 h		
	(A)		(B)		
1.	1	1	2	1	1.25
2.	2	1	1	1	1.25
3.	0	1	2	2	1.25
					Combined av g.=1.25

Stability Study: The nanoemulsion formulation was kept at various and different temperatures for 60 days. Following that, the consistency and concentration of berberine hydrochloride were determined or studied, and three packs of produced nano emulsion-based gel were submitted to 40±5 °C and 75±5% relative humidity for this investigation (RH). After 0, 15, 30, 45 and 60 days, the samples were extracted, diluted with methanol, and analyzed by HPLC. (Ryu, McClements, *et al.* 2018) During stability studies, no changes in physical characteristics such as clarity were detected. The drug content and *in-vitro* dissolution profile did not vary significantly. Since there was no phase separation the end of the 60-day stability tests, suggesting that berberine hydrochloride were chemically stable in nanoemulsion.

TABLE 19: STABILITY STUDY

Stability Study				
Sr. no.	**Sampling Time**	**Drug Content**	**Drug Release**	**Clarity**
1.	0 days	98.20 ± 2.25.	91.7%	Clear
2.	15 days	99.10 ± 1.15	90.3%	Clear
3.	30 days	99.40 ± 2.45	88.1%	Clear
4.	45 days	99.70 ± 2.90	87.4%	Clear
5.	60 days	101.80 ± 2.95	85.9%	Clear

CONCLUSIONS: An o/w nano emulsion containing berberine hydrochloride was developed for transdermal use. The components for the formation of nanoemulsion and their concentration ranges were determined using a pseudo-ternary phase diagram. Various formulation parameters were examined in arrive at an optimal formulation with acceptable ex-vivo effectiveness. When compared to control or drug-loaded suspension, the optimum formulation of the nanoemulsion consisted of 39.60 percent mixture of oil (Olive oil) and ratio (1:1), 19.20 percent surfactant (Tween-20) and Co-surfactant (Glycerol), and 19.20 percent water shows an increase (P 0.01) in the steady-state flux (Jss) and permeability coefficient (Kp)-302.1 of berberine hydrochloride. An antibacterial activity analysis was also undertaken for the improved formulation. The results revealed that the antibacterial activity of produced formula was higher. Based on physicochemical parameters and ex vivo data, it can be concluded that the developed nanoemulsion system can be a promising carrier for the transdermal delivery of berberine hydrochloride for a long time with minimal side effects and can

provide a novel treatment option for bacterial and fungal infections such as blastomycosis, histoplasmosis and onychomycosis.

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CONFLICTS OF INTEREST: Nil

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