Development and evaluation of optimised nanogel loaded with herbal bioactive antidiabetic agent

Mohammad Rashid[1], Rakesh K Sindhu[1], Farhan J Ahmad[2], Sanjar Alam[3]

[1]School of pharmacy, Sharda University, Greater Noida, U.P.- 201310

[2]Department of Pharmaceutics, School of Pharmaceutical Education & Research, Jamia Hamdard, New Delhi, 110062.

[3]R.V. Northland Institute, G.B. Nagar, Dadri, Greater Noida. U.P.-203207

Corresponding Author :Mohammad Rashid

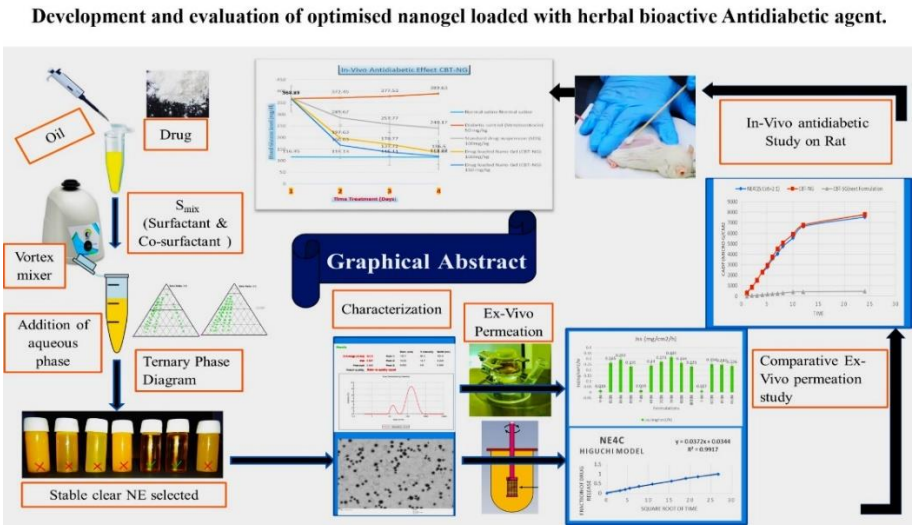
Gmail: mrashid1379@gmail.com

Abstract

This scheme of research was to develop a nanogel loaded with herbal-Bioactive Antidiabetic Cucurbitacin (CBT) from optimised nanoemulsion. Optimisation of nanoemulsion was based on selection of CBT-NE4C on basis of stability, characterisation, profile of in-vitro release kinetics and ex-vivo S.P.S. (skin permeation study). The optimised nanoemulsion was scanned for their Z-average, TEM, ζ-potential, RI, and rheological characteristics. Finally, nanogel (CBT-NG) was developed form nanoemulsion to increase its adherence and application compliance with live-skin. The in-vivo Antidiabetic activity was performed through transdermal route in streptozotocin-induced diabetic rats along with placebo. The data analysis reveals that the CBT in nanogel has better Antidiabetic activity with lower dose as compared to neat formulation. This positive result is attributed towards increased solubility of the drug and enhanced permeability from nanogel.

Methods: The optimized CBT-NE4C was prepared by aqueous titration method with optimised ratios ratio (23.72: 22.08:54.2% v/v) of the oil (Sefsol-218), Smix [surfactants (Tween 80), co-surfactants (plurol oleique)] & aqueous system Milli-Q respectively. Optimised NE was transformed to Nanogel by using carbopol 934.

Conclusion: The Nanogel formulated, was found to exhibit release profile and permeation with correlation coefficient near to one ($r = 0.9088$). Nanogel exhibited significant ($P < 0.01$) Antidiabetic activity as the hypothesis was in scheme of this research work. The nanogel was no-irritating & no-sensitising to live-skin of rat.



Keywords: Nanoemulsion; Permeability; correlation coefficient; Z-average; Nano-gel.

1. INTRODUCTION

Diabetes Mellitus **(D.M.)** is hyperglycemic condition i.e. an increased blood glucose level than normal defined range (Petersmann et al., 2019). Hyperglycemia caused due to quantitative decrease in insulin secretion, decreases concentration available in blood or due to impaired action of insulin.(Care & Suppl, 2018). The threatening phase of DM are its secondary consequences which are Retinopathy, Cardiovascular diseases, Nephropathy, Neuropathy, Polydipsia, Polyphagia, Polyuria, and weight loss etc. (Egan & Dinneen, 2019) (Canadian Journal of Diabetes, 2018).

As per Global report of WHO-2016 on diabetes, D.M. has developed Global burden of diseased population with acceleration of 9.24 million/year from 1980 to 2014 & in adults it's rate of increase was "4.7% in 1980 to 8.5% in 2014" (World Health Organization, 2016). International Diabetes Federation (IDF) has reported that 425 million world population is diabetic and the diabetics in southeast Asia are about 82 million i.e., 19.29% of total diabetics & it can be extrapolated that by 2045 SEAian diabetics will be 51 million. IDF reported that till 2017 there were about 72,946,400 diabetics in India(IDF, 2017). The data is alarming about epidemic scenario of diabetes. If the statistics followed, the diabetics are going to develop huge economic burden globally(Bhatt, Singh, et al.). This will traced into high prevalence of cardiovascular diseases, heart attacks, stroke, kidney failures & blindness etc. (Khursheed et al., 2019).

DM affect body in two-ways, low glucose-fuel to cells of organs which affects its efficiency of working and high glucose concentration fluids of organs which attracts infections and organ

destruction. (Egan & Dinneen, 2014). For D.M., Daytime blood glucose level **(BGL)** range 80-120 mg/dL is diagnosed negative while at night BGL between 100-140 mg/dL in normal & HbA1c up to levels-7% is also no diabetic (Simona et al., 2017).

There are lab synthesised oral-hypoglycaemic drugs in market. For example Metformin HCl, Glimepiride, Glipizide, Glibenclamide, Gliclazide, Pioglitazone, Rosiglitazone, etc. but these show side effects, lean aqueous solubility & side by side have poor bioavailability so effective dose is high (Khursheed et al., 2019). In research history of 10-15 years of medicines for D.M., about 49 out of 100 are over Herbal based (Gothai et al., 2016). The challenges with Phyto-Bioactive antidiabetic agents are there lean efficacy and bioavailability through oral route, and this is due to their solubility-issues, digestive-reactions, Cellular transport and absorption (Al-Snafi et al.). To cope-up with the above challenges, Novel and advanced drug delivery systems can be used (S. Alam et al., 2010) (Ganesan et al., 2017). The Nano-carriers are efficacious to enhance bioavailability of herbal drugs by improving solubility, stability and permeability over traditional drug delivery system. (Harwansh et al., 2015). Nanogel formulations of herbal drugs applied via trans dermal delivery system provides controlled sustained & best permeation rate side by side bypasses the 1st pass-metabolism (Azeem et al., 2012).

In reference to above discussion it can be said that the rationale of engineering, formulation & selection of route of administration of current study as Nano-gel for transdermal application is to enhance the therapeutic-efficacy of drug and to minimize its toxic effects and side effects.

2. MATERIALS AND METHODS:

2.1. Chemicals and excipients:

Cucurbitacin (CBT) [assayed 99%] procured from Sigma-Aldrich Chemicals, Bangalore. Sefsol-218® (propylene glycol caprylate), Stepan D-50 (Isopropyl myristate), Triacetin (Glycerol Triacetate), Peceol® (Glycerol monooleate), Labrafac® were gift samples. Cremophore (polyoxy-35-castor oil), Tween.80, Tween.20, Labrasol, Transcutol, Plurol oleique was procured from R. V. Northland Institute, Greater Noida. The other reagents used during study were of analytical grade.

2.2. Preformulation Studies:

2.2.1. Physical Characterization:

In first step Screening of drug was done by its physiochemical properties. Colour, odor, taste were recorded and compared with reported data.

2.2.2. UV - spectrophotometry:

0.01% w-by-v solution of drugs was prepared in methanol: 0.05M-phosphate buffer. (PB) (pH 7.4) (70:30). The prepared solution was scanned through 200-400 nm wavelength range of UV. (Shimadzu UV-1700 Corporation. Kyoto. Japan.) and the result was recorded.

2.2.3. *Fourier transforms infrared (FTIR):*

KBr + drug powder, in the ratio of (100:1) was pressed in high-pressure hydraulic machine and made pellets. The FTIR spectrometer result was recorded over 1520 (Shimadzu, Japan).

2.2.4. *RP-HPLC analysis of drug (CBT):*

Equipment: A Shimadzu model HPLC used. It was equipped with quaternary LC-10AVP pumps. UV/VIS detector with variable wavelength, SPD-10AVP column oven (Shimadzu), SCL-10AVP system controller (Shimadzu), Rheodyne injector fitted with a 20 µL loop was used. The set was having software Class-VP 5.032.

Chromatographic conditions: The chromatographic conditions used were

Column: 25cm×4.6mm ID SUPELCO 516 C18 DB 5µm RP-HPLC
Mobile phase: Acetonitrile: deionized water (75:25) v/v
Flow rate: 1.2ml. per min.
Run time 10 min
Temperature: 25 ± 0.5°C
Wavelength: 230 nm
Injection Volume: 20 µl

The samples were injected through 20 µL micro-syringe (Hamilton Microliter®; Switzerland). The stock solution of CBT of 1000 µg/ml strength in methanol was prepared. Serial dilution was done to develop Six different aliquots by using same solvent, these were as 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 µg/ml. the dilutions stored in required conditions like temperature at 4°C and protected from light. Mobile phase used was, Acetonitrile: Deionized Water in ratio of 75:25 v/v. in the mobile phase 1% glacial acetic acid was added to maintain pH 2.34. The mixture was sonicated for 12-16 min and degassed prior to use. 0.45 µm (NYL) syringe filter was used to filter all the dilutions before applying in HPLC.

2.3. Screening of Excipients:

For oil phase selection the oils (Sefsol-218®, Stepan D-50, Triacetin, Peceol®, Miglyol 812®) were taken. The Surfactants taken, were - (Labrasol, Tween.80, and Cremophor, Tween.20) and the Co-Surfactants taken, were- (Transcutol.CG and Plurol-Oleique). For solubility assessment, excess amount of drug was added in 2 mL of different oils and distilled water separately in 5 ml capacity vials, and put in vortex mixer (Perfit, India) for proper mixing. The stoppered-vials with solutions were kept for 72 hours at 25±1.0 ^{0}C in a shaker (isothermal) (Nirmal international, Delhi, India) to achieve an equilibrium. After that solutions were centrifuged at 3000 r/m for 15 minutes. (Spinwin.MC-02, from Tarson-India). The supernatant

of centrifuged solution was filtered through a 0.45 µm membrane filter. The concentration of drugs were determined. The soluble-concentration of CBT was individually analyzed in different solvents by RP-HPLC method at λmax 230 nm.(S. Alam et al., 2010)

2.4. Preparation & selection of Nanoemulsions:

The Sefsol 218 is finalised as the oil phase. Tween 80 and Plurol-Oleique were finalised as surfactant and co-surfactant, respectively. Aqueous phase was distilled water. Surfactant and co-surfactant added to develop Smix. The ratios (1:0, 1:2, 1:3, 1:1, 2:1, 3:1, and 4:1) of Surfactant and co-surfactant (w/w) were used for development of Smix (S. Alam et al. 2012).

Table: 1. Surfactant: co-surfactant ratios- taken to make Smix ratios.

S. No	Surfactant used (mL)	Co-surfactant used (mL)	Smix (Ratios)
1	100	0	1:0
2	50	50	1:1
3	33.33	66.66	1:2
4	25	75	1:3
5	66.7	33.3	2:1
6	75	25	3:1
7	80	20	4:1

2.4.1. *Pseudo ternary Phase diagram:*

The Smix (S: CoS) ratios (Table: 1.) are designed for detailed study of the phase diagrams. Now oil: Smix were taken in different ratios by weight from 1:9 to 9:1. These were taken in 16 different glass vials for 16 different ratios of oil: Smix (1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3.5, 1:3, 1:2.3, 1:2, 1:1.5, 1:1, 1:0.7, 1:0.43, 1:0.25, and 1:0.1). These combinations cover maximum ratios possible for the study to delineate the boundaries of phases. Pseudo ternary phase diagrams of (oil: Smix: water) the three phases were traced on the basis of aqueous titration method. Each weight ratio of oil: Smix was titrated slowly with water, and side by side visual observations was done on the following basis:

- ✓ The nanoemulsion visually be transparent and easily flowable, any such o/w Nanoemulsions (NE) marked ok.
- ✓ If any emulsion is milky or cloudy in appearance or any sign of phase separation is visualised, it will marked not ok.

For each and every Smix (S: CoS) ratio the ternary phase diagram was plotted separately. Exactly 2% w/w CBT was dissolved in the oil phase of the nano formulations and was kept constant in all the selected formulations. Thermodynamic stability tests was applied over the above selected formulations.

2.4.2. *Thermodynamic Stability-testing:*

To assess the physical stability of the selected formulations, these were subjected to different thermodynamic stability tests.

Six Heating–cooling cycles, starting from refrigerator temperature (4 ^{0}C) to highest of 45 ^{0}C was applied with storage time not less than 48 hours at each temperature. The formulations visualised for the selection of stable one at these temperatures. The stable formulations were marked and transferred for freeze-thaw cycle test.

Three freeze-thaw cycles were applied over the formulations. The test starts at –21 ^{0}C to highest of +25 ^{0}C. Formulations stored not less than 48 hours at each applied temperatures. Under visual observation the formulations tested for stability, and that which survived in testing of thermodynamic stability were transferred for next study.

2.5. Characterization of Nanoemulsions:

Nanoemulsions may be transparent or semi-transparent/translucent to milky white in appearance. Characterization parameters are:-

2.5.1. *Droplet-Size Analysis:*

The frequency distribution of droplets with respect to to size was determined by P.C.S. (Photon Correlation Spectroscopy). This analyses the fluctuations in light scattering expected due to Brownian motion of the particles. The Zetasizer (Malvern Instruments, Worchestershire, UK) 1000 HS is used for it and the light scattering was monitored at 90- angle.

2.5.2. *Viscosity Determination:*

The viscosity of the formulations determined using a Rheometer (Brookfield DV III ultra V6.0 RV cone and plate) at 25 ^{0}C ± 0.3 ^{0}C.

2.5.3. *Surface Morphology and Structural analysis of droplets by T.E.M.***:**

Transmission electron microscopy (TEM) was done by Topcon 002B operating at 200 kV (Topcon, Paramus, NJ) and capable of point-to-point resolution. The T.E.M. for morphology and structural study of the nanoemulsion was done by dropping of the nanoemulsion directly on the holey film grid. The TEM is applied after drying of the drop on film.

2.5.4. *Rheological studies:*

The selected CBT-NE formulations and the corresponding Neat formulations were evaluated for their Rheology. The Rheometer (Anton Paar, Modular Compact Rheometer-MCR 102) was used with a temperature controller (25 ± 0.5°C) with a 4°/40 mm cone and plate geometry and gap of 0.100 mm. to measure the rheology of NEs. The steady state (After being loaded onto the plate, the NEs were allowed to rest about 10 minutes prior to measurement) rheology of CBT-NEs were recorded as data with controlled rate varying from 0.001 – 100 and 0.0001 – 100 s^{-1} respectively.

2.5.5. *In-Vitro drug release Kinetics analysis for selection of optimised Nanoemulsion*:

Release concentration with time of CBT from nano emulsion was measured using the US Pharmacopeia -XXIV dissolution-apparatus-II (DS 8000, Lab. india, India). This works as dissolving paddle assembly. This is in-vitro dissolution study for release kinetics model setup of stable formulations from each group. Dialysis membrane pouches are inserted in a flask holding buffer after being filled with a nano emulsion carrying a medication dose. 37±0.5° C and 50 rpm of stirring speed are used for this investigation. A 5 mL sample from volume was taken for testing at fixed time intervals & identical amount of fresh dissolving media is always substituted for the sample when it is removed at regular intervals. % Drug release at various time intervals is calculated from data of collected samples. The withdrawn samples were filtered and analysed for CBT content using HPLC method at 230 nm(Sultana et al., 2012).

2.5.6. *Ex-vivo permeation for selection of optimised formulation***:**

Skin permeation study was performed by using hairless abdominal skin. The prepared skin was mounted in the Franz diffusion cell in such a way that its stratum corneum side faced towards the donor compartment and the dermal side towards the receptor compartment. The total effective skin surface area free for diffusion was about 1.767 cm^2. Phosphate-buffer-saline (PBS - pH 7.4) as medium was used at receptor compartment at maintained 37 ± 0.5°C with constant stirring system. CBT-NE1-11, CBT-NG and CBT-CG (1 mL equivalent to 0.1 g of CBT) were individually applied over the skin in donor compartment and studied for their permeation. The assembly was sealed with paraffin film to provide occlusive conditions. the sampling was done from receptor PBS-medium withdrawing 200 µL at fixed time intervals over a period of 24 hours. Each withdrawing was compensated by equivalent volume of fresh PBS to maintain sink conditions. Each study was repeated in triplicated pattern. All samples collected were stored in required conditions after their withdrawal, filtered and suitably diluted. CBT concentration was analysed by RP-HPLC(Tian et al., 2020).

Data collected from this study is total amount of drug permeated through skin after a time period i.e. cumulative concentration of CBT. This data was plotted as a function of time (hrs.). The flux (Jss, µg/ cm^2/h) of permeation was find out by slope of above plot during steady state. Whereas intercept of graph the lag time (t_L). The ER = enhancement ratio (it is calculated according to the equation given bellow)

$$ER = \frac{Jss(Formulation)}{Jss(Control)}$$

2.6. Preparation & characterisation of the Nanogel of optimised nanoemulsion:

2.6.1. *Preparation of Nano Gel:*

The optimised nanoemulsion was transformed to Hydrogel to provide it the needed characteristics for dermal application. The nanoemulsion has low viscosity to be applied over skin and if applied, could be quickly removed, this will alter the dose required. The optimised nanoemulsion CBT-NE4C was transformed to nanogel by using gelling agents, Carbopol-934 or Carbopol-940 polymers. These were used as they are biocompatible, biodegradable, bioadhesive, non-irritating and does not absorbed into body. Among the two polymers mentioned above the carbopol 934 was reported to have more gelling property than carbopol 940 (Aiyalu et al., 2016) so it was preferred. Triethanolamine was added in this mixture to neutralize carbopol.

2.7. In Vivo-Study

2.7.1. *Procuring and rearing of Animals:*

Wistar male albino rats (180-220 g) were selected. Animals were reared in groups of six i.e. (n = 6) at 25 ± 0.5°C and 45-55% R.H. and 12 hrs. Lighting & darkness cycles side by side facilitated w ith free access of feedings with pellet chow (Brook Bond, Lipton India) and fresh-water ad libitum(Arora, Tagde, et al., 2023). The ethical guidelines were strictly followed to perform experiments. The experiment was approved by the Institutional Animal Ethical Committee with approval number: [RVNI/IAEC/2021/01] for the purpose of control and supervision of experiment on animals (CPCSEA), India.

2.7.2. *In-Vivo Efficacy study of the CBT loaded NE-gel as an Antidiabetic:*

It is done in following steps-

Step 1. In-vivo experiments for efficacy studies was approved by I. A. E. Committee of R.V. Northland Institute (Greater Noida, India). The guidelines provided by the committee was strictly followed during the studies.

Step 2. The optimised CBT-NG formulation was tested for anti-diabetic and sustaining action.

Step 3. For diabetes induction in rats- (a). These rats are fasted-16 hours. (b). Streptozotocin (STZ) was selected for diabetes induction as per literatures & solution was prepared in 0.1M Citrate buffer (pH 4.5) (STZ+ CB 4.5). (c). the fresh (STZ+ CB 4.5) was administered i.p. in single dose (Panda et al., 2018).

Dose was calculated as 250 mg/kg according to weight of animal. (e). The Induction of Diabetes is confirmed if-

- ✓ The rat is now showing excessive thirst i.e. Polydipsia.
- ✓ The rat is now showing excessive urination i.e. Polyuria

- ✓ After 72 hrs. of injection of (STZ+CB 4.5) if the rat diagnosed with BGL (Blood Glucose Label) of 250 mg/dl or higher, it was taken as diabetic, is selected for CBT-NG treatment Study.

Step 4. Experimental Grouping was done of diabetic rats on random basis by assigning each group with 6 animals.

Group I. Normal Control Group- having normal rats, i.e. non diabetic, will receive vehicle only.

Group II: Diabetic Control group- also called Toxic group, will receive vehicle only

Group III: Diabetic Reference Group- Receiver of reference standard drug in suspension.

Groups IV - V: Receiver of the optimized CBT-NG.

Step 5. The dose of CBT-NG was calculated as per body weight of the rats(Arora, Gupta, et al., 2023). The gel was to be applied on abdominal region of the rats so hair shaving was done at abdominal region before 12 hrs. o f application, except in the control group and at the shaved abdominal region of all animals CBT-NG applied (except in control group).

Step 6. BGL estimated from the blood of tail vain of rats with Glucometer using an Accutrend GC (Roche) with glucometer strips of Accu-Check. Estimation was done on 1st, 7th, 14th, and 21st, days of application, the estimation was with Non-fasting conditions of rats and before administering the drugs.

2.7.3. *Skin Irritancy Test:*

This is skin safety test. It was performed with optimized nanogel (CBT-NG) to check its safety over skin. Aqil et al Mentioned scoring of live-skin irritancy as 0-1-2-3 on rat and if the value of skin-irritancy score is in range 0-2, it is would be considered as non-irritant and safe for the skin(Aqil et al.) (Azeem, Rizwan, et al., 2009). A single dose of 10 µL of the CBT-NG was applied on the left ear of the rat, while the right ear was a treated as control. The observation was planned for visualisation of erythema and oedema on skin and compared with control. (Van Abbi• et al., 1975)

2.8. Statistical analysis:

Statistically analysis was done by analysis of variance (ANOVA). The analysis followed by Dunnet's Post Hoc Test. The Graph Pad Prism Software-5.0 (San Diego, CA, USA) was used for application of statistical analysis of data.

3. RESULT & DISCUSSION

3.1.1. *Physical Characterization*

The sample of drug was characterized as its Physical form: White Crystalline powder, it is Odorless, Tasteless, have Melting point 188°C (Melting point apparatus)

3.1.2. UV - spectrophotometry:

A 0.01% w/v solution of CRT and CBT was prepared scanned at wavelength mode of 200-400 nm. The λmax of CBT in different solvent was found to be 270 nm.

3.1.3. Fourier transforms infrared (FTIR):

MW= 548.81; $C_{33}H_{56}O_6$; M.p: 190-194°C ; R_f Value: 0.42 (TEF- 5:4:1);

The pellet of CBT was scanned by FT-IR, the obtained absorption spectra was recorded by using KBr pellet. The absorption bands at 3420.24, 1468.82, 1403.45, 1137.04, 824.90, 804.49, 788.83, 764.16, 699.96, 674 and 589.06 were marked and are characteristic of drug (Iqbal et al., 2020).

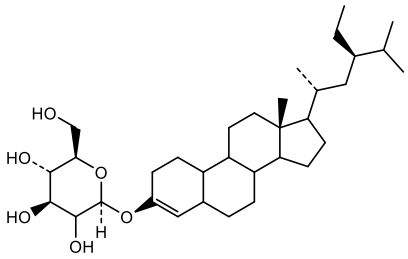


Fig. 1. Cucurbitacin (CBT): Chemical Structure.

3.1.4. HPLC chromatogram of Cucurbitacin:

Representative HPLC chromatogram of Cucurbitacin was developed and shown in Fig. 2**.**

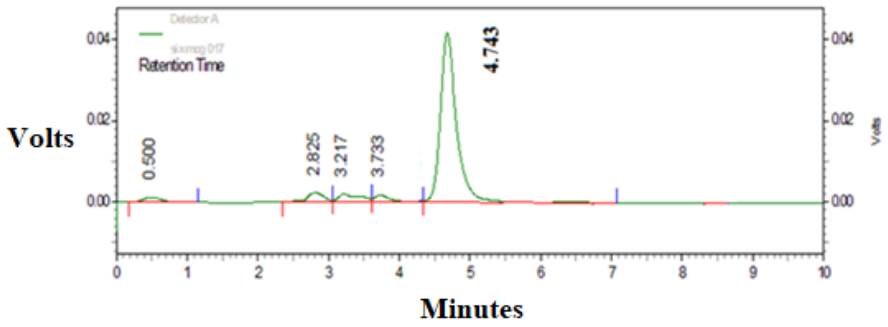


Fig 2: Representative HPLC chromatogram of Cucurbitacin

3.1.5. Calibration Curve- RP-HPLC-PDA:

The optimum separation of CBT was achieved by using the mobile phase at ratio of 75:25 v/v at a flow rate of 1.2 mL/min at 25 ± 0.5°C under the isocratic conditions. The Rt of CBT was found to be 4.75± 0.01 min. A good linear precision relationship between the concentrations (2-20 µg /mL) and peak areas was obtained as the correlation coefficient (r^2) of 0.9992 ± 0.01(Attar & Ghane, 2018).

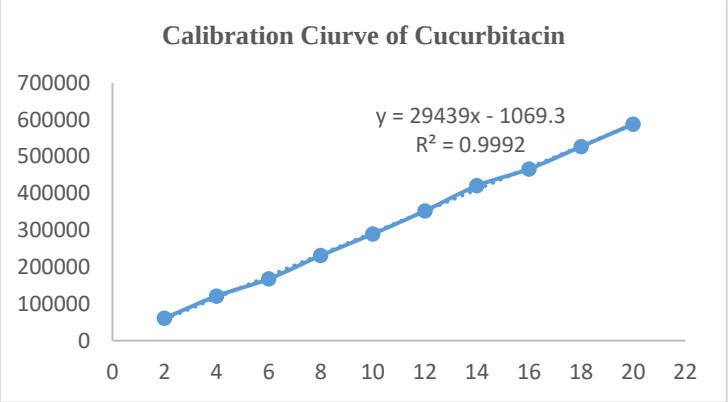


Fig. 3: Calibration curve of Cucurbitacin by RP-HPLC method.

3.2. Screening of Excipients:

The excipients screened through criteria of GRAS (generally regarded as safe) & pharmaceutically acceptable. These are excipients of formulations for transdermal application so must not irritate and sensitizing to the live skin. More will be solubility of CBT in oil phase maximum will be available from NEs for action. After solubility study, the recorded maximum reading was with Sefsol-218 (139.35 ± 3.35 mg/mL), in water it was 0.09 ± 0.14 mg/mL only (Fig. 4). In result, Sefsol-218 was selected as oil phase for the development of the formulations. Values were mean ± SEM (n = 3). *P &**P < 0.05, ***P < 0.01.

Surfactant and co-surfactant was selected on criteria like safety, no-irritancy and no-sensitivity on live-skin. The other important criteria is the Required Hydrophilic Lipophilic Balance (RHLB) > 10 (o/w) (Craig et al., 1995).

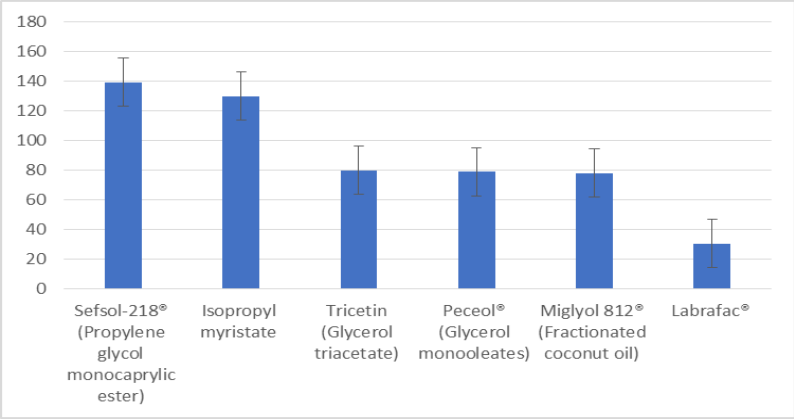


Fig. 4. Solubility studies of CBT in several excipients. Values were mean ± SEM (n = 3). P < 0.05 & *P < 0.01.

This RHLB can be achieved by blending surfactant and co-surfactants. Drug solubility in different surfactant and co surfactant with their various ratios was analysed and on basis of clarity of mixture of Tween 80 & Plurol oleique are selected as emulsifiers (Smix) for placebo formulations (Table: 2) (Pathan et al., 2010).

Table: 2. CBT-Solubility-rating in various SAs & Co-S (surfactants, co-surfactants)

S No.	Surfactants	Observation	Solubility Rating
1.	Labrasol	Turbid	(++)
2.	Cremophore RH	Hazy and thick	(+)
3.	Cremophore-EL	C.Y.S. (clear yellow solution)	(+++++)
4.	Tween.20	C.Y.S.	(++++++)
5.	Tween.80	C.Y.S.	(+++++++++)
Co-surfactants			
1.	Transcutol	Clear	(++++++++)
2.	Plurol oleique	Clear	(+++++++++)
3.	Propylene Glycol	Clear	(++++++++)
4.	Methanol	Clear	(+++++++++)

3.3. Preparation & selection of Nanoemulsions:

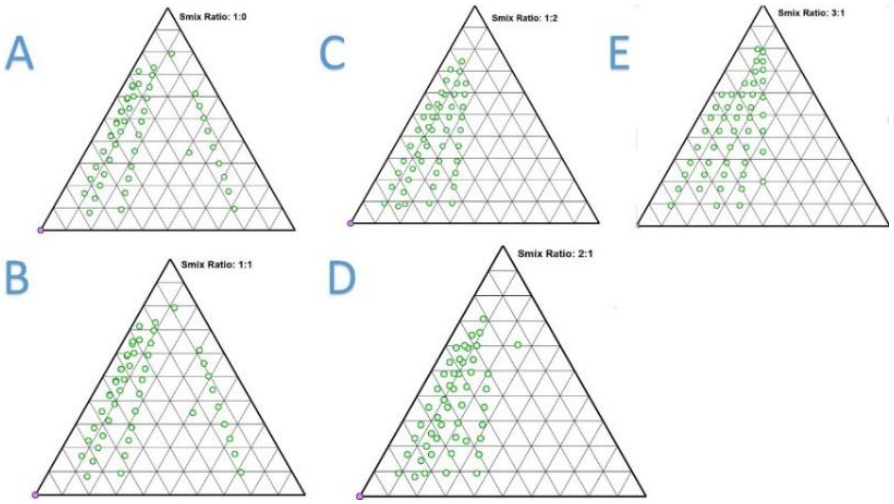


Fig. 5. (A-E). Pseudo-ternary-phase diagrams for o/w nanoemulsion region at different Smix ratios.

Pseudoternary phase diagrams with each Smix ratio and water phase was constructed by using software (Fig. 5.). It concludes all possible three phase combinations for detecting the maximum o/w nanoemulsion available regions **(NEAR).**

In Fig. 5. (A) NEAR shown in diagram of Smix ratio 1:0, seen low NEAR, found at water-rich apex of the PTP diagram. The oil phase that can be dispersed in the above was only 16% w/w using 67% w/w of Smix. Now proceeding for next Smix ratio 1:1 having higher concentration of surfactant, a higher NEAR was observed, because increased concentration results to further reduction of the interfacial tension. In next step of increase of surfactant concentration, i.e. proceeding to Smix 2:1, the NEAR increased as compared with the region in 1:0 and 1:1. That can be dispersed oil was 22% w/w using 52% w/w of Smix. Now proceeded for Smix ratio of 3:1, NEAR was slightly smaller as compared with 2:1, but the disperse ratio of oil with this Smix was 22% w/w with 52% w/w of Smix. In further proceeding at study of Smix ratio of 4:1

NEAR was further de-creased in comparison with 3:1 and 2:1 but was high as compared with 1:0 and 1:1. The oil phase that can be dispersed now was 17% w/w with 67% w/w of Smix. From above data it was concluded that with in conc. Of surfactant as compared with co-surfactant, the NEAR increases till Smix 2:1 ratio, while stats to decrease from Smix 4:1 ratio so further study of Smix ratio of 5:1 was dropped. With increase of CoS, appropriate NEAR was not found as Smix ratios of 1:2 and 1:3 used(Akhter et al., 2008).

Here the conclusion was, the phase diagrams express best NEAR on the basis of the relationship and interaction of three phases. At optimum cases, NE-formation is spontaneous and the resulting dispersions are thermodynamically stable. The ternary phase diagram shows maximum NEAR for optimum proportion of 3 phases (Smix + Oil + Water) to form thermodynamically stable NEs, and will have high potential for the transdermal drug delivery.

3.4.1. *Selection of Nanoemulsion Formulations:*

Excess surfactants cause live-skin sensitivity and irritation. So optimisation of surfactant concentration with respect to safety and effective lowering of surface tension. By using Pseudo Ternary Phase Diagrams, the NEs with optimum three phases (oil +Smix+ Water) were selected which could accommodate required quantity of CBT (Azeem, Ahmad, et al., 2009).

3.4.2. *Thermodynamic Stability Study:*

NEs selected from Phase Diagram were tested for their thermodynamic stability with stressed conditions. The results compiled in Table: 3. for - Heating cooling cycle(H/C), Freeze thaw cycle (F/Th) & Centrifugation(C/f)(S. Alam et al., 2010) (Mostafa et al., 2015).

Table: 3. Stability Study of Selected NEs from Pseudo-ternary Phase diagram study**.**

Smix S:CoS	% Oil (v/v)	% S_{mix} (v/v)	% Water (v/v)	C/f	F/Th	H/C	Codes
1:0 (HLB-15)	27.77	27.77	53.48	X	√	√	NE1A
	25	25	59.18	√	√	X	NE2A
1:1 (HLB-9.6)	14.28	57.14	28.57	√	X	√	NE1B
	12.09	51.61	35.48	X	√	X	NE2B
	22.85	29.3	47.85	√	√	√	NE3B
	25.21	24.61	50.18	√	√	√	NE4B
	25.8	38.7	35.48	√	X	√	NE5B
	29.77	25.75	44.48	√	√	√	NE6B
	41.66	41.66	16.66	X	√	X	NE7B
	40	40	23.07	√	√	√	NE8B

	37.03	37.03	28.57	√	X	√	NE9B
	46.15	30.76	23.07	X	√	√	NE10B
2:1 (HLB- 11.4)	14.28	57.14	28.57	√	√	X	NE1C
	19.35	45.16	35.48	√	X	√	NE2C
	20.51	19.78	59.71	√	√	√	NE3C
	23.72	22.08	54.2	√	√	√	NE4C
	23.07	53.84	23.07	X	√	√	NE5C
	25.85	22.3	51.85	√	√	√	NE6C
	30.3	24.99	44.71	√	√	√	NE7C
	30.76	25.53	43.71	√	√	√	NE8C
3:1 (HLB-12.3)	17.14	40	42.85	X	√	√	NE1D
	18.6	27.9	53.48	√	√	√	NE2D
	19.35	45.16	35.48	√	X	√	NE3D
	20.51	30.78	48.71	√	√	√	NE4D
	22.85	34.3	42.85	√	√	√	NE5D
	25.51	33.78	40.71	√	X	√	NE6D
	32.25	32.25	42.85	√	√	X	NE7D

3.5. Characterization of Nanoemulsions:

3.5.1. *Droplet size (Z-Average), polydispersity index (PDI) and viscosity analysis:*

The droplet size of formulations with Smix ratios (1:1, 2:1, and 3:1) is expressed in Table: 4 (Fig. 6). Formulation NE4C with Smix (2:1) has droplet size (84.03±1.01 nm) that is smallest in the selected all formulations. The smaller droplet size provides larger surface area and consequently the permeation flux is maximum i.e. (Jss= 0.315±0.011). Formulation NE4B with Smix (2:1) has droplet size (90.34±1.19 nm) that is second smallest with permeation flux (Jss=0.293±0.035) and the droplet size of formulation NE2D containing Smix (3:1) has droplet size (103.6±1.17 nm) is third smallest with (Jss=0.254±0.034).

The droplets of all NEs are in the nano range that is from 90.34±1.19 to 148.5±1.18, and their dispersion is uniform which can be concluded on basis of low polydispersity values because the homogenous distribution of droplets is inversely proportional to PDI of NE. The polydispersity of NE4C was lowest (0.587) means the presence of uniformly -dispersed NEs with highest stability (Mustafa et al., 2012).

Table: 4. Droplet Size, PDI, ζ – potential, and Viscosity (η) of the NEs (n=3)

Smix (S:CoS)	Codes	% Oil (v/v)	% S_{mix} (v/v)	% Water (v/v)	Droplet Size Mean±SD (nm)	PDI	ζ - potentia	η- Mean ± SD (Cp)
1:1 (HLB-9.6)	NE3B	22.85	29.3	47.85	138.3±1.17	0.630	-33.9	101.10 ± 1.17
	NE4B	25.21	24.61	50.18	90.34±1.19	0.545	-34.2	119.60 ± 2.21
	NE6B	29.77	25.75	44.48	148.5±1.18	0.624	-32.8	121.10 ± 2.11
2:1 (HLB-11.4)	NE1C	20.51	19.78	59.71	135.6±1.16	0.632	-.34.3	92.12 ± 1.22
	NE3C	23.72	22.08	54.2	138.5±1.21	0.631	-35.3	118.10 ± 1.78
	NE4C	25.85	22.3	51.85	84.03±1.01	0.587	-33.4	110.01 ± 1.11
	NE8C	30.3	24.99	44.71	138.2±11.45	0.624	-33.8	119.10 ± 1.66
	NE10C	30.76	25.53	43.71	134.6±1.81	0.828	-33.9	123.10 ± 1.75
3:1(HLB-12.3)	NE2D	20.21	31.08	48.71	103.6±1.17	0.404	-34.8	91.10 ± 1.21
	NE3D	22.85	34.3	42.85	133.3±11.34	0.625	-35.6	101.10 ± 1.17
	NE4D	25.51	33.78	40.71	148.3±11.34	0.627	-34.4	121.13 1.13

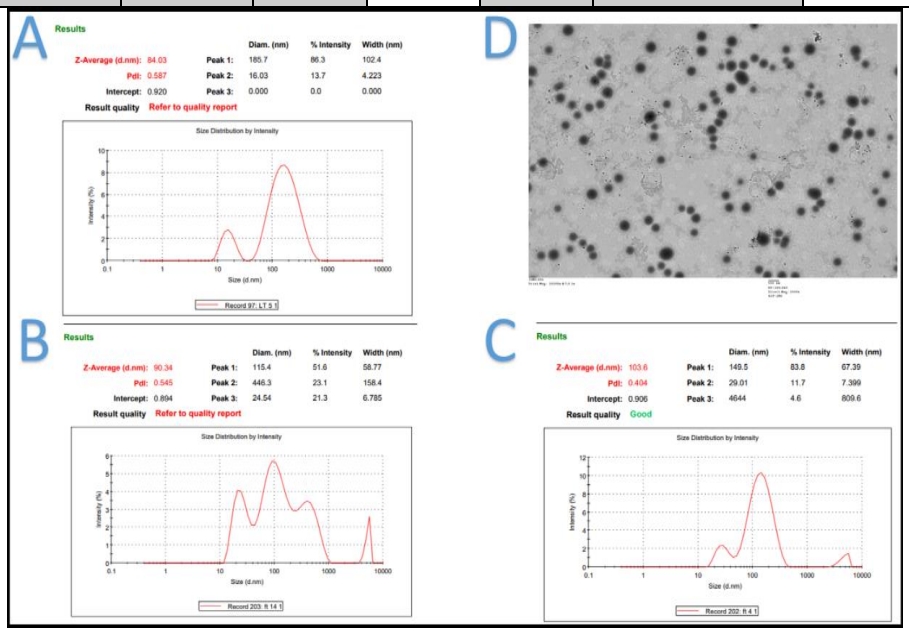


Fig. 6. (A-D). Z-average- droplet size distribution of formulation NE4C is (A), NE4B is (B), NE2D is (C) and TEM image of Nanoemulsion is (D).

The values of Zeta-potential recorded and mentioned in table: 4 & in Fig. 7. The optimum value provides optimum repulsive forces between droplets that are responsible for stability of NEs. The viscosity of the selected NEs were determined Table: 4 (Fig.6.). The viscosity of NE2D (91.10 ± 1.21cP) was lowest, and the difference was significant ($P < .05$). The viscosity of NE10B was highest (123.10 ± 1.75 Cp), NE4C has average viscosity of formulations which was (110.01 ± 1.11). Low Viscosity was expected, because one of the characteristics of NEs is lower viscosity that is why it expresses Newtonian Flow (Azeem, Ahmad, et al., 2009).

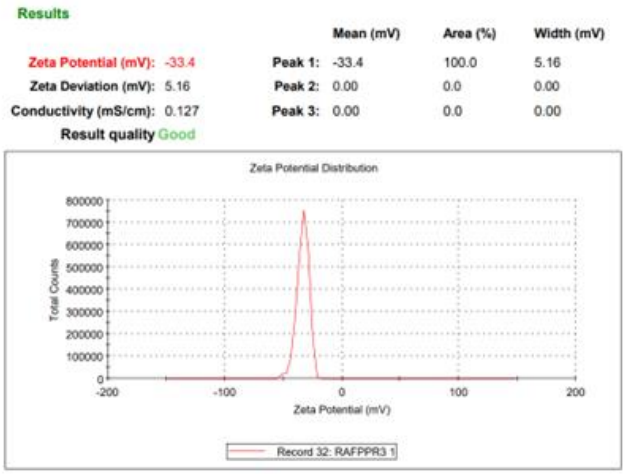


Fig. 7. Zeta (ζ)-Potential of droplet of NE4C Nanoemulsion.

3.5.2. *Refractive Index:*

Table: 5. RI (Refractive Index). Evaluated for Selected NEs (n = 6).

Smix (S:CoS)	**Codes**	**RI ± SD (**Drug loaded**)**	**RI ± SD (**Placebo**)**
1:1 (HLB-9.6)	NE3B	1.403±0.007	1.401±0.007
	NE4B	1.405±0.005	1.404±0.005
	NE6B	1.409±0.006	1.407±0.006
2:1 (HLB- 11.4)	NE1C	1.403±0.004	1.401±0.004
	NE3C	1.405±0.006	1.402±0.006
	NE4C	1.407±0.003	1.405±0.003
	NE8C	1.408±0.003	1.406±0.003
	NE10C	1.411±0.005	1.409±0.005
3:1(HLB-12.3)	NE2D	1.415±0.004	1.411±0.004
	NE3B	1.417±0.007	1.413±0.007
	NE2D	1.421±0.004	1.415±0.004

The mean values (n=6) of the RI of drug-loaded and placebo NEs are in Table: 5. It was found that there was no significant differences between the RI values of drug-loaded and placebo NEs. So it was concluded that the NEs were not only thermodynamically stable but also chemically stable and remained isotropic i.e. no interactions between excipients and drug.

3.5.3. *In-vitro Drug entrapment (EE) & Release Kinetics analysis:*

Table: 6. EE, R^2 values of Selected Nanoemulsions (n = 6), ensuring best fit release Model.

Smix (S:CoS)	**Formulation**	**Entrapment Efficiency (%)**	**Fraction of drug Released at 24 hr.**	**R^2**	**Best Fit Model**
1:1 (HLB-9.6)	NE3B	52.24±5.34	76.5±1.31	0.9394	HIGUCHI
	NE4B	71.12±1.19	96.5±1.23	0.9818	HIGUCHI
	NE6B	63.23±2.25	70.5±0.98	0.9676	HIGUCHI
2:1 (HLB- 11.4)	NE1C	49.92±2.32	71.4±0.99	0.9284	HIGUCHI
	NE3C	64.13±2.12	76.9±0.67	0.9516	HIGUCHI

	NE4C	83.13±1.12	97.9±1.03	0.9917	HIGUCHI
	NE8C	82.12±2.32	76.3±0.93	0.9721	HIGUCHI
	NE10C	82.91±2.11	70.5±0.93	0.9721	HIGUCHI
3:1(HLB-12.3)	NE2D	66.77±2.32	77.3±0.98	0.9656	HIGUCHI
	NE3D	59.22±2.32	73.8±1.05	0.9546	HIGUCHI
	NE5D	50.01±2.13	72.8±1.02	0.9222	HIGUCHI

Selection of optimised NE was done over stable NEs selected. Dissolution studies were performed to confirm release kinetics by R^2 values for individual formulations, having same quantity of CBT (w/w) in each NEs. The best fit model was the Higuchi Model for release of drug from NE (Table: 6) Fig. 8-10.

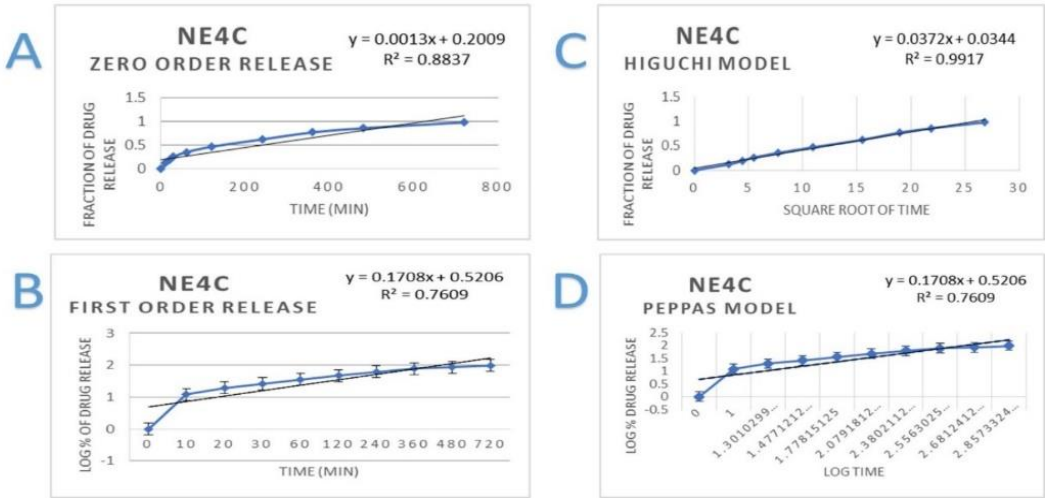


Fig. 8. (A - D). Release kinetics plots: Plot for zero order (A), Plot for 1st order (B), Plot for Higuchi model (C) Plot for Peppas models (D) Plot for NE4C formulation.

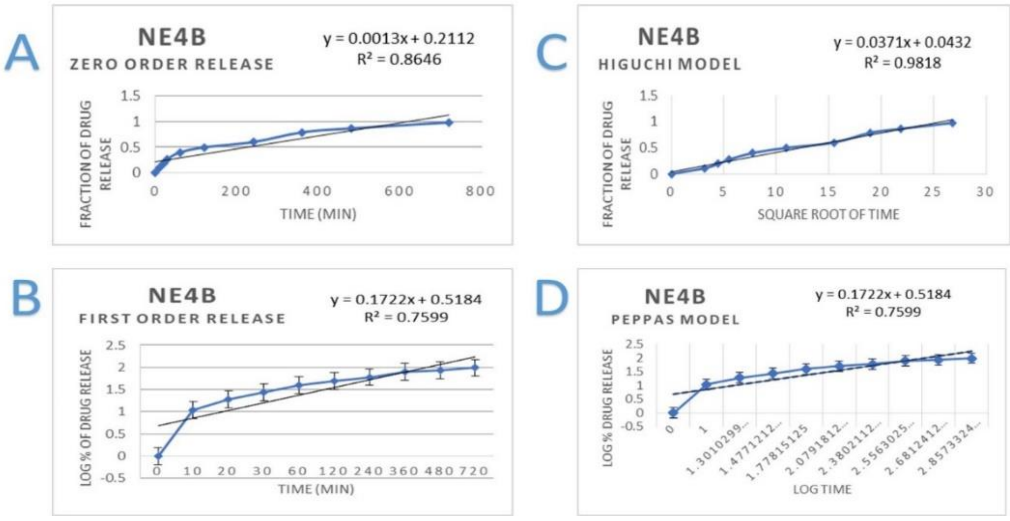


Fig. 9. (A - D). Release kinetics plots: Plot for zero order (A), Plot for 1st order (B), Plot for Higuchi model (C) Plot for Peppas models (D) Plot for NE4B formulation.

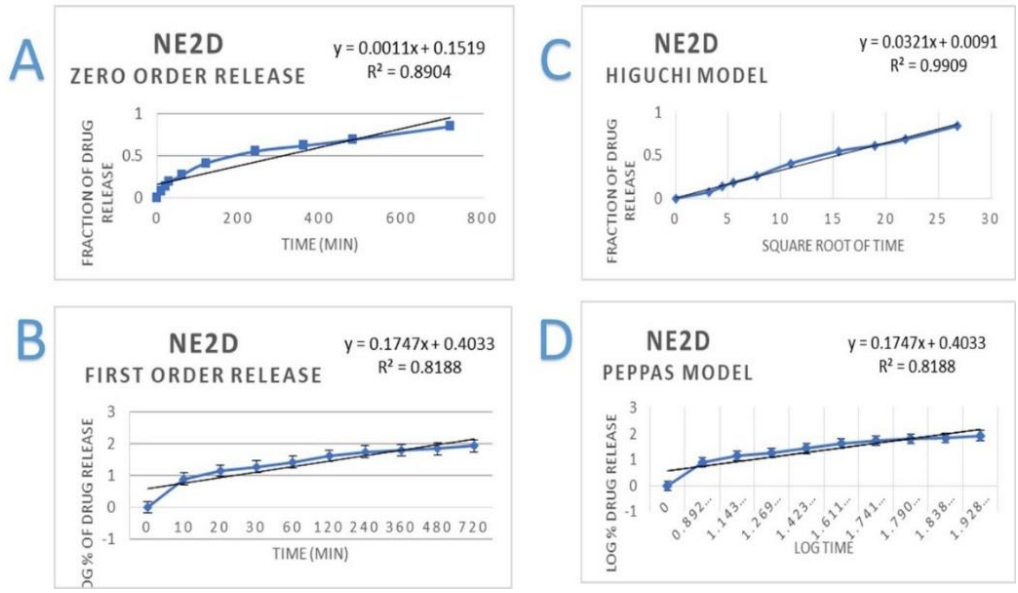


Fig. 10. (A - D). Release kinetics plots: Plot for zero order (A), Plot for 1st order (B), Plot for Higuchi model (C) Plot for Peppas models (D) Plot for NE2D formulation.

3.5.4. *Ex-vivo Skin Permeation Studies:*

Ex-vivo skin permeation studies were performed to obtain permeation profile and comparison parameters of selected formulations. Drug permeation from skin of 11 different NEs and 3 Neat Formulation NF^b (Smix-1:1), NF^c (Smix-2:1) & NF^d (Smix-3:1) (control formulations) was performed and presented in (Table: 7 and Fig. 11.), all having the same quantity of drug w/w. The experimental data analysis concludes that the Permeation Flux (Jss) of all the NEs through rat skin significantly higher ($P<0.01$) in comparison to NF-Control. Permeability parameters like steady-state flux (Jss), and enhancement ratio (Er) were significantly increased in NEs as compared with neat formulations this is because the NEs provide very small size to penetrate maximum, transporting the drug to closest at skin cells and very large contact surface area to cover maximum permeation site on skin. Very high solubility of drug exposes molecular label of particles and activates the faster release and permeation by diffusion. Hydration of skin by external water phase of formulation facilitates faster penetration of drug in skin. Cumulative amount of drug permeation (CAP) (µg/cm^2) of three best Formulations (n = 3)*out of 3 Smix groups are expressed in Table: 8. Fig. 12. NE4C formulation is selected as optimised one.

Table: 7. Permeability Parameter of CBT - Steady-state flux (Jss) and enhancement ratio (Er) w.r.t. (**NF^b**-control), (**NF^c**-control) and (**NF^d**-control). (mean ± SD, n=3) *

Smix (S:CoS)	Formulation Codes	Jss ± SD (mg/cm^2/h)	ER
	NF^b (Neat Formulation)	**0.018±0.002**	**1**
1:1 (HLB-9.6)	NE3B	0.265±0.014	14.7222
	NE4B	0.293±0.035	16.2778

	NE6B	0.231±0.032	12.8333
2:1 (HLB- 11.4)	**NF[c] (**Neat Formulation)	**0.019±0.007**	**1**
	NE1C	0.24±0.024	12.6316
	NE3C	0.273±0.017	14.3684
	NE4C	0.315±0.011	16.5789
	NE8C	0.264±0.013	13.8947
	NE10C	0.231±0.023	12.1579
3:1(HLB-12.3)	**NF[d] (**Neat Formulation)	**0.017±0.005**	**1**
	NE2D	0.254±0.034	14.9412
	NE3D	0.247±0.065	14.5294
	NE5D	0.236±0.067	13.8824

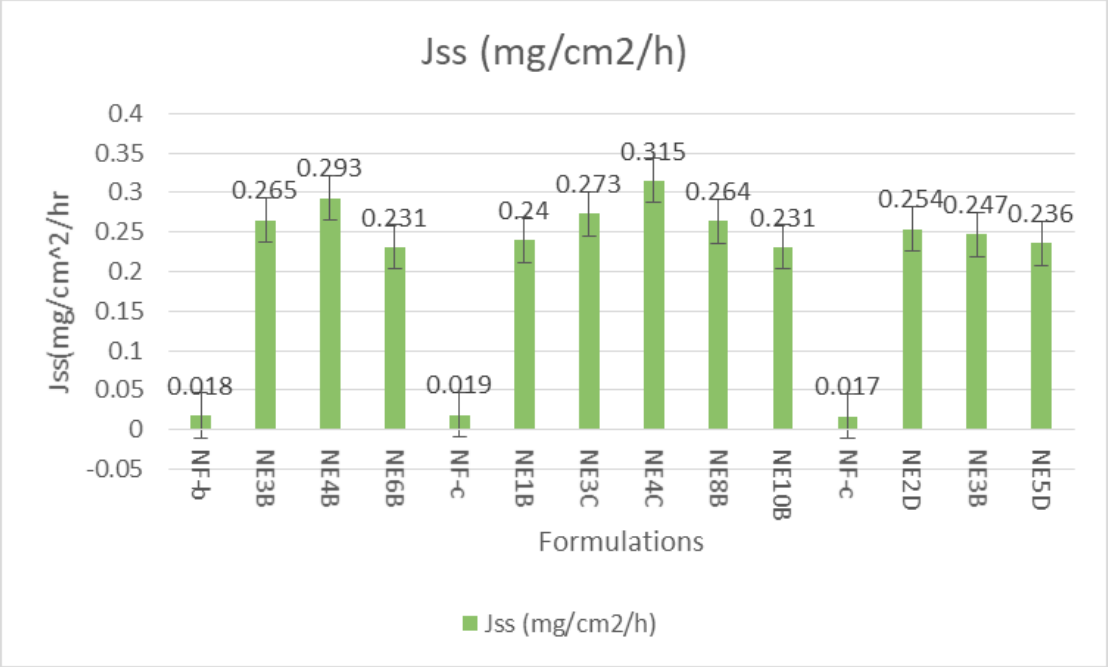


Fig. 11. EX-VIVO skin permeation analysis of drug with Permeation Flux (Jss) of NEs

Table 8. CAP (µg/cm^2) of three best Formulations (n = 3)*out of 3 Smix groups.

CAP (µg/cm^2)			
Time (h)	**NE4B**	**NE4C**	**NE2D**
1	306.67	386.67	240.00
2	626.67	880.00	393.33
3	1190.00	1563.33	630.00
4	1630.00	2263.33	980.00
5	2220.00	2753.33	1286.67
6	2863.33	3563.33	1563.33
7	3276.67	4043.33	1893.33
8	4146.67	4746.67	2276.67
10	4660.00	5560.00	2626.67
12	5306.67	6640.00	4740.00
24	7030.00	7563.33	6090.00

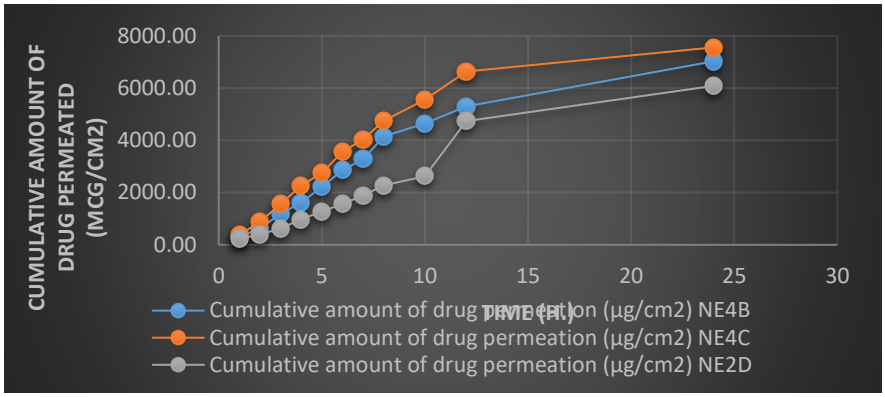


Fig. 12. Comparative EX-VIVO skin permeation profile of drug from three Optimised NEs.

3.5.5. *Effect of surfactant concentration on skin permeation of drug:*

To see the surfactant concentration effect on the of drug permeation from the NE, optimized formulation NE4C was selected and the surfactant concentration was increased 10% (C4S1) and decreased 10% (C4S2) respectively from the optimized surfactant concentration. In vitro skin permeation profile from nanoemulsions C4S1 & C4S2 has been shown in Table: 9. The CAP vs. Time graph was plotted (Fig. 13.). The result shows significant decrease in CAP as compared to optimised formulation P (<0.01). This signifies that any change in the formulation of optimized formulation disturbs the optimum globule size, polydispersity value, viscosity & other characteristics so nano emulsion that decreases the drug permeation (Mudiar & Kelkar-Mane, 2020).

Table: 9. Effect of surfactant concentration on Ex-vivo skin permeation of drug by optimized nanoemulsion CBT-NE4C.

S. No.	Time (h)	NE4BS1 (+10%)	NE4BS2 (-10%)	NE4B (S:CoS=2:1)
1	1	330.00	313.33	386.67
2	2	586.67	603.33	880.00
3	3	1216.67	1323.33	1563.33
4	4	1663.33	1530.00	2263.33
5	5	2153.33	2253.33	2753.33
6	6	2963.33	2530.00	3563.33
7	7	3210.00	3010.00	4043.33
8	8	4080.00	4080.00	4746.67
9	10	4660.00	4460.00	5560.00
10	12	5540.00	5106.67	6640.00
11	24	7073.33	6820.00	7563.33

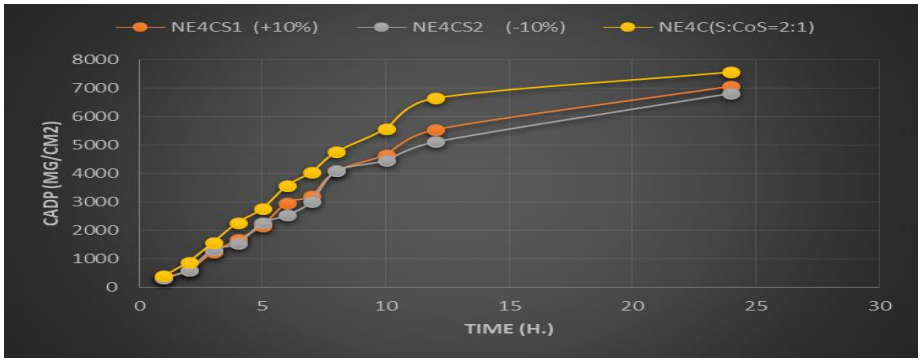


Fig. 13: Effect of surfactant concentration on *in vitro* skin permeation of Optimized nanoemulsion NE4C.

4. TRANSFORMING OPTIMIZED NANOEMULSION(NE4C) IN TO NANO-GEL:

On the basis of the release profile, permeation studies and characterization, the optimized nanoemulsion CBT-NE4C was selected to be formulated in to gel by the use of 1% carbopol 934 which shows better consistency.

4.1. Ex-Vivo Permeation Study of Nano-Gel:

Comparative studies between Drug loaded Nano-gel (CBT-NG), Control **(**CBT-NE4C) and drug loaded Neat Formulation in Conventional gel (NF^{cg}) was performed (mean ± SD, n=3)* (Tab: 10. Fig. 14.). Nano-Gel was better with its permeation profile (Cumulative percentage of Permeation of drug-CPP) (93.56%) as compared with NF^{cg} conventional gel (5.48%) and also with NE NE-4C (90.76%) at 24^{th} hrs. Maximum flux and permeability of CBT was achieved with CBT-NG as compared to other formulations.

Table: 10. Comparative Drug Permeation study through rat skin - Jss of Nanogel (CBT-NG), (CBT- NE4C) & Conventional gel (NF^{cg}). (Mean ± SD, n=3) *

Formulation Code	CAP (µg/cm^2)	CPP (%)	Jss±SD (mg/cm^2/h)
CBT-NE4C	7563.33	90.76	0.315 ± **0**.014
CBT-NG	7796.67	93.56	0.324 ± **0**.086
NF^{cg}	456.67	5.48	0.019 ± **0**.003

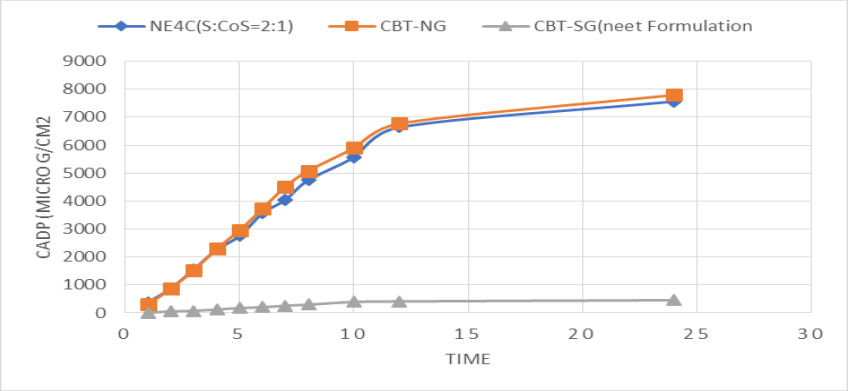


Fig. 14. Plot-In-comparison: Permeation Profile of NE4C, CBT-NG & NF^{cg} through rat skin.

Karl Pearson coefficient of correlation (r = ±1) was applied for the study of relationship between Permeability (**Cumulative %)** and release of drug (**Cumulative %)** from Nanogel of NE4C, it was very near to 1 (r = +0.9088, mean ± SD, n=3) * (Table: 11, Fig. 15).

Table: 11. Correlation study of Permeability and release of drug from Nanogel Formulation.

Time (Hr)	**CPR (%)**	**CPP (%)**
1	23.3	4.64
2	47.9	10.56
4	60.1	27.16
6	76.9	42.76
8	86.5	56.96
12	97.9	79.68
24	99.3	90.76

4.2. Stability Assessment of Nano-Gel:

As per data No change in color, odour, homogeneity, pH, viscosity and net content of the CBT-NG as topical herbal nano-gel formulation was observed after 0,1,2,3 and 6 months. Results are significantly clear stability (Table: 12).

Table: 12. Stability Studies of Optimised nanoemulsion based gel (CBT-NG):

	Optimised Nanogel formulation (CBT-NG) containing 2% w/v of Drug															
							Storage condition									
SN	Parameters	25 °C ± 2 °C/60% RH ± 5% RH					32 °C ± 2 °C/60% RH ± 5% RH					40 °C ± 2 °C/75% RH ± 5% RH				
				Months				Months					Months			
		0	1	2	3	6	0	1	2	3	6	0	1	2	3	6
1	Colour	No change in color					No change in color					No change in color				
2	Odour	No change in odour					No change in odour					No change in odour				
3	Homogeniety			Smooth					Smooth					Smooth		
4	pH	6.45	6.43	6.41	6.37	6.33	6.43	6.42	6.41	6.38	6.36	6.46	6.43	6.41	6.38	6.35
5	Viscosity(Poise)	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.37	0.38	0.38	0.38	0.37	0.36
6	Net Content	99.1	98.3	98.1	98.1	97.4	99.2	99.1	98.2	98.1	97.3	99.1	98.1	97.3	95.2	95.1
7	Microbial load (Bacteria & Fungi)	No microbial growth was observed at 24, 48 and 72 h					No microbial growth was observed at 24, 48 and 72 h					No microbial growth was observed at 24, 48 and 72 h				
8	Sterility test	No microbial growth was observed at 24, 48 and 72 h					No microbial growth was observed at 24, 48 and 72 h					No microbial growth was observed at 24, 48 and 72 h				

5. IN-VIVO STUDIES:

5.1. Antidiabetic study:

The data recorded for 21 days treatment of Nanoemulsion based gel of **drug** CBT-NG on blood glucose level of streptozotocin-induced (STZ) diabetic rats (Table: 15, fig. 16.). The hypoglycemic effect of (CBT-NG) was statistically significant ($P<0.01$, Mean ± SEM); n=6) as compared with Standard Drug Gel (SD^g) and control group-II.

Table: 13. Effect of 21 days treatment of Nano-gel on blood glucose level of streptozotocin-induced (STZ) diabetic rats:

Groups	Treatment		Blood glucose level in mg/dl			
			1^{st} day	8^{st} day	14^{st} day	21^{st} day
I	Normal saline	NA	116.45±3.97*	115.13±1.23*	115.11±1.66*	114.88±1.34*
II	Diabetic control (streptozotocin)	50 mg/kg	367.83±4.15	372.45±2.16	377.53±2.99	389.63±3.36
III	Standard drug (SD^g)	100mg/kg	369.69±4.99	285.67±2.95*	257.77±2.88*	240.17±1.99*
IV	Drug loaded Nano Gel (CBT-NG)	100mg/kg	368.97±4.96	197.63±3.11*	170.77±2.20*	136.5±3.12*
V	Drug loaded Nano Gel (CBT-NG)	150mg/kg	367.27±5.19	166.63±3.15*	137.72±2.67*	119.27±1.98*

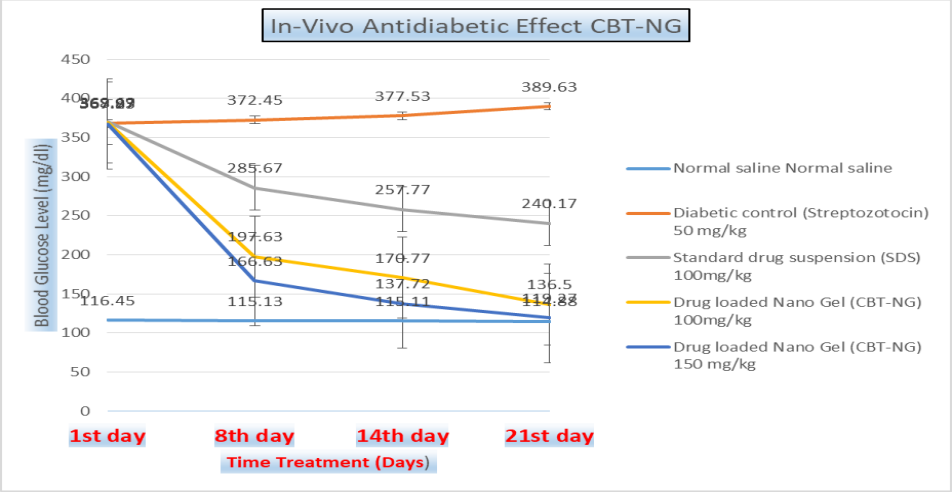


Fig. 16. Plot-In-Vivo Comparative study of Antidiabetic Activity of CBT-NG with SDS & Control.

5.2. Skin-irritancy test:

The mean of live-skin irritancy score for nanogel CBT-NG was found to be 1.25. (Table: 14 & 15). This value indicates that all additives/excipients used in nanoemulsion based gel were safe for transdermal drug delivery.

Table: 14. Skin irritation score, (**A*** Erythema formation score. **B**** Oedema formation score)

Intact skin						Abraded skin				
	24 Hours		48 Hours				24 Hours		48 Hours	
Rats	A*	B**	A*	B**		Rats	A*	B**	A*	B**
1.	1	0	1	1		1.	0	1	1	1
2.	0	0	1	0		2.	1	0	2	0
3.	1	1	0	1		3.	0	2	1	1

Table: 15. Final skin irritation scores of CBT-NG (*= total of A and B from Part A; **= average of all skin reading of 24 and 48 hours)

Intact skin			Abraded skin		
Rats	(I)		(II)		Total Avg.(I + II)
	24 hours	48 hours	24 hours	48 hours	
1.	1	2	1	2	1.5**
2.	0	1	1	2	1**
3.	2	1	2	2	1.75**
Combined Average...					1.25**

CONCLUSION:

The route of administration via transdermal delivery demonstrate & authenticate the concept of drug targeting in the systemic circulation in the safe and effective manner bypassing the first pass metabolism. Encapsulation into nanogel will increase the skin residence time leading to

improved therapeutic efficacy, better dispersibility and good storage stability with increased bioavailability and lower side effects.

Therapeutic dose and Dosing frequency of nanogel system will decrease as compared to available conventional preparation. The optimized nanogel formulation will provide sustained action in the management of diabetes.

Therefore, it was proposed to develop a nanoemulsion based gel system of poorly water soluble antidiabetic drug, with the aim of increasing its solubility and thus its transdermal flux in order to provide action through systemic circulation after absorption in to the blood stream.

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Discloser:

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