Comparative Anti-inflammatory Potential of Crystalline and Amorphous Nano Curcumin in Topical Drug Delivery

Abdulmohsen H. Al-Rohaimi*

Abstract: The problem of poor bioavailability and clinical efficacy of curcumin can be sorted out after converting crystalline Curcumin (CrysCur) into amorphous NanoCurcumin (NanoCur). Amorphous NanoCur was prepared by converting into nanoemulsion (o/w) using water titration method. The formulation were pre-screen by different physical stress tests, followed by in vitro release study, zeta potential, viscosity, transmittance, globule size distribution and ex vivo studies. The morphology of the NanoCur was determined using transmission electron microscopy (TEM) which revealed fairly spherical shape and good correlation with droplet size distribution study. The NanoCur was converted to gel using Cabopol 934. The composition of optimized NanoCur was curcumin (0.154% w/w), Carbopol 934 (0.702% w/w), ethanolic oil phase [ethanol (0.013% w/w); Capryol 90 (0.015%w/w), Tween 20 (0.076%w/w) as surfactant, PEG 200 (0.038%w/w) as a co-surfactant and distilled water (q.s) as hydration phase. The steady state flux (Jss), permeability coefficient (Kp) and enhancement ratio (Er) of NanoCur gel was determined and compared with CrysCur gel. Anti-inflammatory effects of the formulations were evaluated in carrageenan-induced paw edema method in rats using Diclofenac as a reference. These anti-inflammatory effects of NanoCur was highly significant (p<0.001) compared to CrysCur and significantly (p<0.05) comparable with standard Diclofenac. The histology of the formulation treated skin showed insignificant changes in the integrity except in the group treated with NanoCur. The slight disruption in the integrity of skin may be because of surfactant present in the nano formulations. Short term storage stability showed insignificant changes in the droplet size and zeta potential, proving its high shelf-life. Finally, it was concluded that NanoCur could be a promising tool in the management of topical inflammation.

Key words: curcumin, anti-inflammatory, nanoemulsion, phase diagram, thermodynamic stability, permeation flux

1 Introduction

Curcumin (Curcuma longa L.) has been used from centuries in traditional medicine and reported to possess multiple bioactivities, such as antioxidant, anticancer, and anti-inflammatory properties. In addition, clinical evidences have shown that, it is as much effective in various neurological disorders. Despite the favorable biological properties of curcumin, there are some limitations in the development of curcumin as a potential therapy, which include its low solubility and instability\(^1\),\(^2\). The level of curcumin after its administration has been evaluated by many studies and has been reported to be either negligible or only low in serum or tissue after oral administration\(^1\),\(^2\). In addition to poor bioavailability, curcumin is unstable in aqueous solution at physiological pH and undergoes rapid degradation into molecular fragments\(^6\). Because of these, the clinical applicability of curcumin has been significantly hampered.

The development of transdermal drug delivery systems (TDDS) designed to have systemic effects appears to be very advisable and beneficial. Several study showed tremendous advancement in terms of bioavailability and tolerability that TDDS offered over conventional routes of drug administration\(^7\)

-\(^9\). But, TDDS is restricted by skin, a natural barrier. A range of molecules that can achieve therapeutic concentration at their target site following TDDS is restricted by stratum corneum.

Despite of this limiting factor, numerous advantages are associated with TDDS. The most important is, it circumvents the hepatic first pass metabolism and therefore an alternative route for drugs with a low oral bioavailability.
TDDS also controlled, drug input; decrease fluctuation in drug plasma levels; inter and intra patient valuations and most importantly patient compliance. Therefore transdermal route for administering curcumin warrants investigation especially for the treatment of ailments like rheumatoid arthritis and its related disorders, which need prolonged therapy with desirable local application. Since TDDS offers the greatest opportunity for drug delivery and to overcoming the problem of low skin permeability represents a crucial challenges to prove its therapeutic benefit. The use of penetration enhancers offers a cheap, simple and convenient method of improving transdermal bioavailability\textsuperscript{19}. But it was observed that, penetration enhancers sometime leading to irreversible changes in the integrity of skin. Several formulation approaches have been adopted to overcome permeation problem associated with penetration enhancers. One possibility for increasing drug permeation across the skin is the use of vesicular carrier system\textsuperscript{11,12}. Now a day, nanocarrier system emerging as a best alternative for TDDS. Nanoemulsion, a most stable nano-carrier system combined the advantages of other innovative carrier systems with highest physical stability, excellent tolerability and minimal inter-subject variability\textsuperscript{13}. Nanoemulsion because of its excellent flexibility can permeate the intact stratum corneum through intracellular route or Trans-cellular route without much affecting the integrity of the skin\textsuperscript{14}. Therefore present study was design to prepare and investigate a nanoemulsion system for enhanced permeation of curcumin in the management of topical inflammation.

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2.1 Materials
Curcumin (E,E)-1,7-bis(4-Hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) was purchased from Sigma-Aldrich Chemicals Co. Pvt. Ltd., Bangalore, India. Polyoxymethylene (20) sorbitan mono oleic acid (Tween 20\textsuperscript{b}), Polyethylene glycol (PEG 200) and ethanol were purchased from SDFCL (Mumbai, India). Capryol 90 was a gift samples from Gattefosse (Saint Priest, Cedex, France). Water was obtained from a Milli-Q water purification system (Millipore, Billerica, MA).

2.2 Analytical methodology
The concentration of curcumin was estimated by in-house developed HPLC analysis using a Shimadzu LC 2010 system (Kyoto, Japan) consisting of quaternary LC-10A VP pump, SPD-10AVP column oven, variable wavelength programmable UV/VIS detector. The analysis was carried out on a LiChrospher\textsuperscript{c18} (150 mm \times 4.6 mm i.d., 5 μm particle size; Merck, India) column. The mobile phase consisted of acetonitrile: Acetic acid solution (3%) in ration of 80:20 respectively. The flow rate was maintained at 1 mL min\textsuperscript{-1} and the retention time was 3.2 min. The detection was performed by UV/VIS detector at 425 nm. All the assays were performed at ambient temperature (25 ± 0.5°C) and Class VP 5.03 version software was used to process the chromatograms.

2.3 Solubility study and preparation of oil phase
Solubility of curcumin in various oils was determined using isothermal water shaker maintained at 37 ± 0.5°C. For determining the solubility of cur in different oil phase, an excess quantity of cur was added to a stopper vial (5 ml capacity) containing 2 ml of different oils. The vials were kept into the isothermal shaker for 72 h to equilibrium. After 72 h, the samples were taken out and passed through membrane filter and assayed the oil sample using validated HPLC method. Parallel miscibility studies of different oils were also conducted using ethanol as an internal phase to get alternative oil phase. Primarily, cur was solubilized in ethanol and then mixed with different ratios (1:1, 1:2 and 1:3) of oil phase in eppendorf tube. After standing for 72 h, each eppendorf tube was centrifuge followed by heat-shock treatment. The samples were visualized against the bright light to see any phase separation or boundary layers and precipitation\textsuperscript{14}.

2.4 Map construction for existence of nanophas
In order to find out the concentration range of various components for the existence range of nanolipid carrier, pseudoternary phase diagrams were constructed by aqueous titration method\textsuperscript{15}. Pseudoternary phase diagrams of oil phase, aqueous phase, and cosurfactant (CoS)/surfactants (S)/mixtures were constructed at fixed CoS/S weight ratios. Here, ethanolate capryol 90 was used as an internal phase (oil) where as mixture of tween20 and PEG 200 was used as Smix. Distilled water was used as an external aqueous media for titration. Surfactant and cosurfactant (Smix) were pre-mixed in different volume ratios (1:0, 1:1, 1:2, 1:3, 2:1, 3:1, and 4:1). For each phase diagram, oil phase and specific Smix ratio was mixed thoroughly in different volume ratios (1:9 to 9:1). Slow titration with aqueous phase was done for each combination of oil and Smix separately\textsuperscript{18}. The physical state of the nanoemulsions was marked on a pentameric pseudo ternary phase diagram with one axis representing the aqueous phase, oil phase (ethanolic capryol 90) and the third representing a mixture of surfactant and co-surfactant. After the pseudoternary phase diagrams were plotted and compared, optimal surfactant, co-surfactant and lipid combinations were selected.

2.5 Formulation development
Different formulations were selected from the phase diagram showing nanoemulsion region on the following
basis.
1. The oil phase should sufficient to dissolves the required dose of curcumin.
2. External phase concentration (water) must be always greater than the internal phase (oil phase).
3. For each percentage of oil selected, the emphasis was given to those compositions which contained minimum concentration of Smix to give nanoemulsion.

2.6 Characterization

2.6.1 Physical stress tests

In the search of a robust formulation and to overcome the problem of metastable formulation, physical stress tests were performed as already discussed in our previous study. Selected formulations were centrifuged (REMI, India) at 5000 rpm for 30 min and observed for phase separation, creaming or cracking, turbidity and drug precipitation. The formulations which showed none of the mentioned problems were taken for heating and cooling cycle.

Six heating-cooling cycles between the refrigerator (0 ± 0.5°C) and heating temperature (45 ± 0.5°C) temperature were performed with storage at each temperature for not less than 48 h. Those formulations, which were stable at this temperature cycle with no phase separation, creaming or cracking, turbidity and drug precipitation, were subjected to freeze and thaw cycle.

In freeze and thaw cycle, selected formulations were exposed to three freeze-thaw cycles between −21 ± 0.5°C and +25 ± 0.5°C with storage at each temperature for not less than 48 hours.

2.6.2 In Vitro Drug Release

In vitro drug release study was performed as a pre-screened parameter for those formulation which survived physical stress tests. In vitro release study was performed using acetate buffer pH 5.4 with Tween 80 (1.5% w/v) as a dissolution medium, in USP XXIV method (dissolution apparatus USP-II, at 50 rpm and 37 ± 0.5°C). One ml of optimized formulation (Table 1) was placed in treated dialysis bag (MWCO 12,000 g mole−1; Sigma, St. Louis, MO, USA) and 0.5 mL samples was withdrawn at regular time intervals (0, 0.25, 0.5, 0.75, 1, 2, 5, 10 and 12 h) and replenish with same amount of acetate buffer pH. The samples were diluted with methanol and analyzed for the drug content by using developed validated RP-HPLC at 445 nm.

2.6.3 Percentage Transmittance

Percentage transmittance of the system was measured at 630 nm (visible range) using UV spectrophotometer (UV 1601, Shimadzu, Japan) keeping distilled water as a reference solution.

2.6.4 Refractive Index

Refractive index (n) of the optimized formulations was determined using an Abbe type refractometer. Standardization was performed using cedarwood oil. n was measured after placing 1 drop of optimized formulations on the slide and viewed against the bright light.

2.6.5 Droplet size and surface charge

Droplet size diameter was determined using a photon correlation spectrometer (PCS; Zetasizer-1000 HAS, Malvern Instruments, UK) based on the laser light scattering phenomenon, which analyzes the fluctuations in light scattering. Light scattering was monitored at 90° angle (25°C). Properly diluted samples of the optimized formulation (0.1 mL) were used for particle size analysis to minimize any possibility of droplet aggregation. Average droplet diameter (Δdm) and polydispersity index (pi) were recorded.

Zeta potential (ζ) measurements were carried out with the same diluted sample using the same equipment and operating conditions. Zeta potential was measured by applying an electric field across the dispersion medium. Particles within the dispersion with a zeta potential migrated towards the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential.

2.6.6 Rheological measures

Brookfield DV III ultra V6.0 RV cone and plate rheometer (Brookfield Engineering Laboratories, Inc, Middleboro, MA) with spindle # CPE40 at 25 ± 0.5°C was used for the determination of viscosity of the formulations. All measurements were carried out at a temperature of 25 ± 0.5°C. The software used for the calculations was Rheocalc V2.6.

2.6.7 Transmission electron microscopy

Surface morphology of the optimized formulation was studied with the help of transmission electron microscope (TEM) (TOPCON 002B) operating at 200 KV, capable of point to point resolution. Properly diluted samples (dilution factor, 1:1000) of nanoemulsion system were used for TEM observations. A drop of diluted NanoCur was allowed to deposit directly on the circular copper film grid of 400 size mesh and stained with 2% (w/v) phosphotungstic acid for 30 s and placed for observation after air drying. Combination of bright field imaging at increasing magnification and diffraction modes were used to determine the form and size of the nanoemulsion.

2.7 Formulation of NanoCur gel, CrysCur gel and Diclofenac gel

On the basis of in vitro characterization and optimization of nanoemulsions, formulations were converted into gel. Amorphous NanoCur gel, CrysCur gel and Diclofenac gel was prepared after dispersing Carbopol-934 in water followed by admixing different optimized formulations. In brief, the carbopol gels (0.5% w/v) were prepared by dispersing in distilled water. After complete dispersion, the Carbopol-940 dispersion was kept in dark for 24 h for the complete swelling. The mixture was then neutralized by drop-wise addition of triethylamine (25-50% w/w), until a transparent gel appeared. The optimized NanoCur, Simple
Table 1  Different physical stress tests of nano-formulations selected from phase diagrams.

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Surfactant-Co-surfactant ratio (Smix), Heating-cooling cycle (H/C), Centrifugation study (Cent), Freeze-thaw cycle (Freez-Thaw).
Cur and Diclofenac was added slowly into the gel with continuous stirring. The obtained gel was left for 24 h to get homogeneous dispersion of gel.

2.8 Permeation study
2.8.1 Preparation of skin

The preparation of rat’s skin, Wister rats was used. The rat abdominal surface hairs were removed by a clipper and skin (full thickness) was surgically removed. The subcutaneous layer was removed manually and dermis side was wiped with isopropyl alcohol to further remove residual adhering of fat. Thus full thickness of skin was washed with buffered (phosphate saline), wrapped in foil (aluminum) and stored at \(-20 \pm 0.5^\circ C\) till further use.

2.8.2 Ex vivo skin permeation

Ex vivo skin permeation studies were performed using the Franz diffusion cell with an effective diffusion surface area of 0.636 sq. cm and 4.8 mL of receiver chamber capacity using excised rat abdominal skin. The cleaned skin was washed with distilled water and stored in the deep freezer at \(-21 \pm 0.5^\circ C\) till further use. Before ex vivo permeation, the skin was brought to room temperature and mounted between donor and receiver compartments of the Franz diffusion cell facing stratum corneum side toward the donor side and dermal side facing the receiver compartment. Multiple cycles of skin stabilization was done with continuous stirring. The obtained gel was left for 24 h to get homogeneous dispersion of gel.

The enhancement ratio (Er) was also calculated by dividing the Jss of respective formulation with Jss of control formulation by using the following equation:

\[
Er = \frac{J_{ss_{NanoCur}}}{J_{ss_{Control}}}
\]

2.9 Short term storage stability study

Short term storage stability (90 days) was performed to observe droplet growth and changes in surface charge after storing formulations at regulated temperature and humidity. Initially, droplet size and zeta potential measurements were carried out to optimize the NanoCur formulation. Properly sealed samples were stored at regulated temperature and humidity (40 \(\pm 2{^\circ C})\), (65 \(\pm 5\% RH\)). Samples were withdrawn after 90 days for particle size and zeta potential measurements.

2.10 In vivo study

2.10.1 Anti-inflammatory effects of NanoCur

NanoCur formulations showing excellent ex vivo permeation and short term storage stability profile were selected for in vivo anti-inflammatory study. The anti-inflammatory and sustaining action of the optimized NanoCur formulation was performed by carrageenan-induced hind paw edema method in Wistar rats. Young male Wistar rats (180–220 g), were randomly divided into four groups having six rats in each group. The animals were kept under standard laboratory conditions at temperature of 25 \(\pm 0.5^\circ C\) and relative humidity of 55 \(\pm 2.5\% RH\). The animals were housed in polypropylene cages (6 per cage), with free access to standard laboratory diet (Lipton Feed, Mumbai, India) and water ad libitum. The drug dose for the rats was calculated on weight basis. Curcumin acts as anti-inflammatory agents by inhibiting TNF-\(\alpha\) and NO release in a dose dependent manner. The effective range curcumin in the treatment of rheumatoid arthritis varied between 30-100 mg kg\(^{-1}\) in wistar rats. In the present study, 30 mg kg\(^{-1}\) was selected as an effective curcumin dose. The abdominal region of the rats was shaved 24 h before starting the experiments. Formulation, NanoCur and CrysCur gel were applied on the shaved abdominal region just half an hour before subplantar injection of carrageenan in right paws. The paw edema was induced by injecting 0.1 mL of 1% w/v suspension of carrageenan in normal saline. The volume of paw was measured at 0.5, 1, 2, 3, 6 and 12 h after injection using digital plethysmometer (Basile, Ugo, Italy). Percent inhibition of edema produced by gels treated group was calculated against the respective control group and marketed diclofenac (DICLOMAX\(^{R}\), Torrent Pharmaceuticals limited, Ahmedabad, INDIA) topical gel. The results of anti-inflammatory effects were compared using Dunnett test of one-way analysis of variance.

2.10.2 Histology of formulation treated skin

After anti-inflammatory study, the abdominal skin was removed surgically to evaluate the histological changes. The obtained skin was fixed with 10% (v/v) formalin solution for at least 72 h before routine processing. The Coronal sections of 3-mm thickness were made, and its blocks were embedded in paraffin. Sections of 5 \(\mu m\) thickness were cut in the coronal plane and stained with eosin.

pseudoternary phase diagrams were constructed using aqueous phase titration technique at room temperature (5000 rpm for 5 min) and two cycle of heat shock (0 ± 0.5°C and 45 ± 0.5°C) treatment. Therefore, combination of ethanol (50% v/v) and Capryol 90 (50% v/v) was selected to make the internal phase.

Based on nanophasic area, primary screening of different Smix combinations were done using selected oil phase (ethanol:capryol 90::1:1). The Smix combination (Tween 20 and PEG 200) showed significantly high (p < 0.01) nanophasic area compared to Smix of Cremophor EL with Transcutol-P, Tween 20 with Transcutol-P, Plurol oleique with Transcutol-P and Cremophor EL with PEG 200. Although, the nanophasic area with Smix of Plurol oleique and PEG 200 was also significantly comparable (p < 0.005) to Smix of Tween 20 and PEG 200, but the consistency of the former was highly viscous leading to large metastable nanogel area. Tween 20 and PEG 200 were selected as a Smix for developing NanoCur formulations.

3.2 Map construction for existence of nanophase

In order to find out the concentration range of various components for the existence range of nanophasic area, pseudoternary phase diagrams were constructed using aqeous phase titration technique at room temperature (27 ± 0.5°C). The care was taken in order to ensure that observations should be avoided on metastable systems. Surfactant (Tween 20) and co-surfactant (PEG 200) were pre-mixed in different volume ratios (1:0, 1:1, 1:2, 1:3, 2:1, 3:1, 4:1). These Smix ratios were chosen in increasing concentration of co-surfactant with respect to surfactant and vice versa for comprehensive study. Pseudoternary phase diagrams were constructed separately for each Smix ratio selected previously, so that O/W nanoregions could be defined. The common phasic behavior obtained during phase titration is shown in Fig. 1. It was clear that, the compositions consisting of low oil phase and high surfactant phase give metastable nanogel region. Although, the obtained nanogel region spontaneously converted into nanoemulsion region, but sometime it may lead to failure of the system. Therefore, we tried to discard the formulation coming in this region (Fig. 1). Although, we selected some metastable formulation to check the in vitro performance (Table 1). The phase diagram consisting of Smix 1:1 to 1:3, a decreasing trend of nanophasic region was observed. On the other hand, in the phase diagram consisting of Smix 2:1 to 4:1, an increasing trend of nanophasic area was obtained. O/W nanoemulsion region appeared towards the aqueous rich apex of the phase diagram with higher concentration of Smix, showing that Tween-20 could be used alone without co-surfactant, but liquid crystalline form (Smix: 2:1, 3:1, 4:1) limits its implication and therefore it was advised to use a co-surfactant to minimize the possibility of the rigid crystalline interface. The phase diagram of Smix 1:0 gave large liquid crystalline (LC) region that was unable of breaking the interfacial tension between oil-water interfaces which is required to get the spontaneous nanoemulsification. Therefore nanophasic area obtained was less. But after adding co-surfactant (Smix 1:1, 1:2 and 1:3), an increased nanophasic area was obtained. The co-surfactant helped in making more flexible interfacial film leading to decreased in LC area appeared. The maximum concentration of oil that was solubilized by Smix 1:3 was nearly 15 ± 2.62% v/v by incorporating Smix around 43 ± 2.36% v/v. It was observed that increasing the concentration in surfactant (Smix 1:1 and 1:2), a marked decline in liquid crystals appeared and more solubilization of an oil (15-20% v/v) phase with justified Smix contribution was...
obtained. However on increasing the surfactant concentration in Smix ratios (Smix 2:1 - 4:1); a significant increase in metastable nanogel followed by increased spontaneous nanoemulsion region were observed. This could be formation of flexible interfacial film that helped in reducing interfacial tension and increase in fluidity at interface. A decrease in interfacial tension further increases the entropy of the system leading to spontaneous emulsification\(^26\). The increased in nanophase area was insignificant when surfactant concentration in Smix was increase from 2:1. This could be the metastable gel region generated by Tween-20 and it was continuously decreased after adding the PEG 200. These results conclude that free energy of nanoemulsion formation is somehow dependent on the extent to which the surfactant and co-surfactants lower the interfacial tension of the oil-water interface\(^26,27\). The increase in free energy and dispersion entropy leading to the formation of spontaneous and thermodynamically stable nanoemulsion\(^29\). Therefore, while selecting the formulations composition from each phase diagrams, the care was taken to select those which could accommodate optimum quantity of oil phase by using lowest possible Smix to further avoid the possibility of liquid crystalline or metastable gel region.

### 3.3 Formulation development

Considering pseudoternary phase diagrams, maximum regions were shown by Smix ratios of 1:1, and 2:1 devoid of LC region where as Smix 1:3 and 4:1 also gave large nanoemulsion region which was associated with the LC region. Although, Smix 1:2 and 1:3 showed very high kinetically stable nanoemulsion region but the area of thermodynamically stable nanoemulsion was insignificant. Different formulations were selected at different points from the pseudo ternary phase diagram which justified the drug dose considering the drug solubility in the oils phase\(^1\). Therefore, from each phase diagram different concentrations of oil, at which nanoemulsions formed, were selected at a difference of 2% (10, 12, 14, 16 and 20%) so that maximum formulations could be selected covering the nanoemulsion area of the phase diagram. Almost in all cases, Smix concentration was up to 40% of total formulation was selected.

### 3.4 Characterization

#### 3.4.1 Thermodynamic stability test

In the search of a robust formulation and to overcome the problem of metastable formulation, thermodynamic stability/ physical stability tests (centrifugation, heating-cooling cycle and freeze-thaw cycle) were performed as already discussed in our previous study\(^12,15\). In physical stability testing some formulations became turbid and some leading to immediate phase separation. One reason of this instability may be due to Ostwald ripening in which molecules move as a monomer and coalesce of smaller dropet take place, resulting in the formation of large droplets by diffusion process driven by the gain in surface free energy. The other reason may be that when temperature quench occur during stress stability study, instability of nanoemulsion occurs due to separation of oil phase and droplet distribution of smaller size is favoured by the change in curvature free energy\(^29\). Only those formulations, which showed no phase separation, creaming, cracking, coalescence and phase inversion during stress stability tests, were selected for further studies. Those formulations, which survived \textit{in vitro} physical stability tests, were taken for further studies. The compositions of these selected formulations are shown in \textit{Table 1}.

#### 3.4.2 In Vitro Drug Release

Studies were performed to compare the release of drug from different NanoCur formulations that survived different physical stress tests. Here, \textit{In vitro} release tests were used as a pre-exclusive parameters. The release of cur from NanoCur formulations consisted of 12% oil phase was highly significant \((p<0.01)\) compared to the NanoCur formulations consisted of 10% oil phase (Fig. 2). Drug release from NanoCur 12 was rapid, as 101.16 ± 1.023% of drug was released in just 6 h. When we compared ≥ 80% drug release graph (Fig. 2), it was very clear that most of the formulations with 12% oil composition release approx 80% drug content within 8 h, only two formulations i.e. NanoCur 06 and NanoCur 11 consisted of 10% oil composition able to meet the approx 80% drug release. The order of drug release pattern in formulation with 12% oil phase were NanoCur 12 > NanoCur 07 > NanoCur 02 > NanoCur 27 > NanoCur 22 > NanoCur 17 in 12 h. Among them formulations, NanoCur 12, NanoCur 07, NanoCur 02 and NanoCur 27 release their major drug contents in just 6 h (Fig. 2). Similarly, drug release order in formulation with 10% oil phase were NanoCur 11 > NanoCur 06 > NanoCur 26 > NanoCur 21 > NanoCur 01 > NanoCur 16. This finding contributes that, those formulations with 10% oil phase falls under metastable nanogel, which interfere in proper emulsification leading to varied drug release pattern, where as NanoCur formulations consisted of 12% oil phase showed uniform droplet size and hence smooth drug release pattern (Fig. 2).

#### 3.4.3 Percentage Transmittance and Refractive Index

The percentage transmittance is a UV-spectrophotometer based study which indirectly gives an idea about the size of the droplet. It was very clear that droplet size is inversely proportional to percentage transmittance; therefore higher the value of %T means lower the droplet size of the formulation\(^17\). %T also indicates the isotropy of formulations with the dispersion medium. The %T approaching 100% indicates isotropy with the water. The results showed that %T of all selected NanoCur formulation ranges between 91.86 to 97.46 (\textit{Table 2}). The order of per-
Percentage transmittance were NanoCur 12 > NanoCur 07 > NanoCur 02 > NanoCur 22 > NanoCur 27 > NanoCur 17, with maximum 97.46 observed for NanoCur 12. This result could be used as a predicted droplet size range.

Similar to %T, Refractive index (Rf) was used to characterize the isotropic nature of the nanoformulations and also marked any colour chemical interaction, turbidity, precipitation among drug and excipients. The Rf value for all the formulations proved their isotropicity and free from colour interactions (Table 2). The least value of refractive index was observed for NanoCur 12 which was 1.361 ± 0.024.

3.4.4 Droplet diameter and surface charge

The droplet size distribution and surface charge analysis was performed by Malvern Zetasizer (Nano ZS-90 UK). The statistical distribution of droplet size and zeta potential are shown in Fig. 3 and Table 2. The summarized mean droplet size was provided in Table 2.
The size of optimized formulations were ranging from 104 to 157 nm. The order of average droplet size were followed as: NanoCur 12 (104.60 ± 9.21 nm) > NanoCur 07 (124.77 ± 7.45 nm) > NanoCur 02 (130.79 ± 8.91 nm) > NanoCur 27 (153.59 ± 12.44 nm) > NanoCur 22 (154.66 ± 9.87 nm) > NanoCur 17 (157.57 ± 10.76 nm). The results showed that size of the particles varied significantly, when the ratio of the three component mixture varied during formulation process. In droplet size analysis it was observed that size of NanoCur increased with increase in the contribution of co-surfactant. Although, co-surfactant gives flexible interface that is required for emulsification, but sometime lower than required HLB leading to inefficient nanosizing. Similarly, the increase in surfactant concentration also produced large droplet size, which may be the consequences of rigid interfacial film leading to improper emulsification. For proper emulsification and stability, a flexible interface with required HLB is always pre-requisite. Therefore, for every kind of oil-drug mixture, a specific ratio of Smix is most desirable to obtained small droplet size and narrow size distribution. The value of polydispersity index (PI) was found to be ≤ 1 for all formulations indicating uniform droplet distribution (Table 2). Apart from the interfacial tension, zeta potential or surface charge is one of the important parameters which deiced the fate of formulation stability. The stable zeta potential range leading to least contact angle and hence chances of flocculation are minimum. Generally it is accepted that ZP values approaching to ± 25 mV characterize a stable formulation. The zeta potential results of all the optimized formulations varied between 24.86 to 33.08 mV (Table 2) which comes under stability range.

3.4.5 Rheological measures

The viscosity of the optimized formulations was determined as shown in Table 2. The viscosity results can be correlated with the different contribution of the surfactants as well as cosurfactant consumed in stabilization of selected formulation. It was observed that the viscosity of all the formulations was less than 160 mPas. Formulation, NanoCur 12 and had the minimum viscosity (122.90 ± 10.73 and 123.31 ± 6.81 mPas respectively) which was significant (p < 0.01) as compared to the other formulations (Table 2). The low viscosities of all formulations meet the expected characteristics as required for better emulsification.

3.4.6 Transmission electron microscopy

The morphology of droplet determined by TEM was shown in Fig. 4, was found nearly spherical with no extra edges. TEM imaging photograph of NanoCur 12 had shown a better correlation with the droplet size distribution data (Fig. 4, Table 2). It was finally concluded that the droplet

Fig. 3 Dynamic light scattering technique for determining the particle size distribution (A) and zeta potential (B) of the optimized formulation (NanoCur12).

Fig. 4 Transmission electron microscopic positive image of optimized NanoCur revealing spheroid of the droplet.
were spherical in shape, surface was smooth and uniformly distributed justifying the narrow polydispersity index.

3.5 Ex vivo skin permeation studies

Ex vivo skin permeation studies were done to compare the permeation profile of curcumin from different NanoCur formulations and curcumin suspension (CrysCur) using abdominal rat skin as a permeation barrier (Fig. 5). In order to maintain the sink condition, acetate buffer (pH 5.4) was added along with tween 80 (1.5 % v/v) in the receiver compartment of the Franz diffusion cell. Highest ex vivo skin permeation exhibited for NanoCur 12 and lowest in CrysCur (Fig. 5). These studies were found in correlation with in vitro release studies. Maximum permeation was shown in NanoCur 12, perhaps the combined results of small droplet size, least polydispersity index and viscosity and larger surface area for partitioning. The possible mechanism by which nanoformulation could enhance permeability of drugs is the lipid pathway of the stratum corneum, in which neutral lipids are arranged as bilayers with their hydrophobic chains facing each other to form a lipophilic bimolecular leaflet and the drug dissolved in the lipid domain of nanogels to easily penetrate into the stratum corneum by destabilizing its bilayer structure with the help of interfacial surfactant. The formulation also contain the traces of ethanol and water, which was supposed to be favorable in increasing diffusion potential across the skin. Water is a natural penetration enhancer for both hydrophilic and hydrophobic molecules which alter stratum corneum and thereby modify partitioning of drug from the vehicle into the membrane.

Permeability parameters like steady state flux \( (J_{ss}) \), permeability coefficient \( (K_p) \) and permeation enhancement ratio \( (Er) \) were significantly increased in NanoCur as compared to CrysCur \( (p<0.01) \). Although, the permeation profile of NanoCur 12 was highest but the order of flux obtained was different (Fig. 5). The order of steady state flux were NanoCur 2 \( (7.637 \pm 0.298 \, \mu g/cm^2/h) \) > NanoCur 27 \( (7.033 \pm 0.431 \, \mu g/cm^2/h) \) > NanoCur 07 \( (6.852 \pm 0.406 \, \mu g/cm^2/h) \) > NanoCur 12 \( (6.840 \pm 0.372 \, \mu g/cm^2/h) \) > NanoCur 22 \( (6.552 \pm 0.169 \, \mu g/cm^2/h) \) > NanoCur 17 \( (6.519 \pm 0.281 \, \mu g/cm^2/h) \). Other permeability parameters of different formulations are depicted in Table 3.

3.6 Short term storage stability studies at regulated temperature and humidity

The stability of formulations was evaluated by size distribution measurements by dynamic light scattering; visual observation of macroscopic changes including phase separation or creaming, drug precipitation and changes in zeta

![Fig. 5](image-url) Ex vivo permeation profile of curcumin from different optimized NanoCur formulation using rat abdominal skin in Franz diffusion cell. Here receiver chamber of Franz diffusion cell contain acetate buffer (pH 5.4) with Tween 80 (1.5% w/v) as a permeation medium. Data represented as the mean ± standard deviation of triplicate trial (n=3).

Table 3 Comparative Ex vivo permeability parameter of different NanoCur formulation and CrysCur formulation.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Flux ( (J_{ss}, sd, , \mu g/cm^2/h) )</th>
<th>Permeability constant ( (K_p, sd, , cm/h) )</th>
<th>Enhancement ratio ( (Er) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>NanoCur 02</td>
<td>7.637 ± 0.298**</td>
<td>0.139 ± 0.035</td>
<td>4.46**</td>
</tr>
<tr>
<td>NanoCur 07</td>
<td>6.852 ± 0.406**</td>
<td>0.125 ± 0.028</td>
<td>4.0 **</td>
</tr>
<tr>
<td>NanoCur 12</td>
<td>6.840 ± 0.372**</td>
<td>0.125 ± 0.059</td>
<td>3.99**</td>
</tr>
<tr>
<td>NanoCur 17</td>
<td>6.519 ± 0.281*</td>
<td>0.119 ± 0.031</td>
<td>3.81*</td>
</tr>
<tr>
<td>NanoCur 22</td>
<td>6.552 ± 0.169*</td>
<td>0.119 ± 0.084</td>
<td>3.83*</td>
</tr>
<tr>
<td>NanoCur 27</td>
<td>7.033 ± 0.431**</td>
<td>0.128 ± 0.067</td>
<td>4.11**</td>
</tr>
<tr>
<td>CrysCur</td>
<td>1.713 ± 0.312</td>
<td>0.031 ± 0.092</td>
<td>-</td>
</tr>
</tbody>
</table>

All data were obtained from three experiments and expressed as the mean ± standard deviation. Here \( p*<0.05, p**<0.01 \) and \( p***<0.001 \).

potential. After 90 days storage cycle at regulated temperature and humidity, the formulations were critically evaluated. The visual observation showed no phase separation, precipitation, and change in colour for all the formulations. Although, droplet size distribution study reveal very insignificant changes in size and zeta potential (Table 4). The change in average droplet size of NanoCur 12 was 104.60 (± 9.21) → 106.65 (± 7.56) nm whereas change in zeta potential was 26.55 (± 1.28) → 23.07 (± 5.36) mV proved its stability potential at storage condition.²⁸

### Table 4 Short term storage stability study at regulated temperature and humidity.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Previous day sampling</th>
<th>90th day sampling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Σd ± sd (nm)</td>
<td>ζ ± sd (mV)</td>
</tr>
<tr>
<td>NanoCur 02</td>
<td>130.79 ± 8.91</td>
<td>26.14 ± 2.63</td>
</tr>
<tr>
<td>NanoCur 07</td>
<td>124.77 ± 7.45</td>
<td>24.86 ± 3.74</td>
</tr>
<tr>
<td>NanoCur 12</td>
<td>104.60 ± 9.21</td>
<td>26.55 ± 1.28</td>
</tr>
<tr>
<td>NanoCur 17</td>
<td>157.57 ± 10.76</td>
<td>33.08 ± 4.76</td>
</tr>
<tr>
<td>NanoCur 27</td>
<td>153.59 ± 12.44</td>
<td>27.69 ± 1.84</td>
</tr>
</tbody>
</table>

All data were obtained from three experiments and expressed as the mean ± standard deviation (n=3) Particle size distribution (Σd), Zeta potential (ζ), Standard deviation (sd), milli volt (mV)²⁸

### 3.7 In vivo study

#### 3.7.1 Anti-inflammatory study

Anti-inflammatory effects of curcumin in optimized NanoCur formulation were evaluated to prove its therapeutic efficacy. The % inhibition value after 12 h application was found to be insignificant for NanoCur 12 (78.54%) as compared to standard Diclofenac (81.13%) where as highly significant (p*<0.001) as compared to CrysCur (39.78%) as shown in Fig. 6.

In first 2 h, % inhibition by NanoCur 12 was dominating even in comparison to the standard diclofenac. After 3 h, there was parallel enhancement in anti-inflammatory activity. The anti-inflammatory effects of CrysCur gel was very poor after even after 12 h of application which indicated that curcumin because of big molecular size imposed permeation problem (Fig. 6). Anti-inflammatory potential of NanoCur was found equally effective compared to the standard diclofenac gel, therefore could be used for local as well as for transdermal drug (systemic) delivery system.

Curcumin has been reported to suppress the expression of various inflammatory cytokines, including TNF-α and IL-6.²⁹,⁴⁰ Because of big molecular size range, the permeation across the skin was limited and therefore the therapeutic inactivity. Studies also evaluated to increase the effectiveness of anti-inflammatory properties of curcumin using various penetration enhancer.¹¹-¹³ The enhanced anti-inflammatory effects of NanoCur 12 conclude that, the optimized formulation acts as a self penetration enhancer and therefore could attain high therapeutic level at local as well as in systemic pool by enhanced permeation of curcumin through skin. Although, anti-inflammatory activity of NanoCur 12 was lesser in comparison to standard diclofenac gel, but the differences was statistically insignificant (p<0.05). Further, to make the NanoCur 12 as effective comparable to diclofenac gel, a dose titration was pre-requisite.

#### 3.7.2 Histology of formulation treated skin

The histology of formulation treated skin of optimized formulation (NanoCur 12), Curcumin suspension (CrysCur); Diclofenac gel and saline control after 24 hours using various penetration enhancer.¹¹-¹³ The enhanced anti-inflammatory effects of NanoCur 12 conclude that, the optimized formulation acts as a self penetration enhancer and therefore could attain high therapeutic level at local as well as in systemic pool by enhanced permeation of curcumin through skin. Although, anti-inflammatory activity of NanoCur 12 was lesser in comparison to standard diclofenac gel, but the differences was statistically insignificant (p<0.05). Further, to make the NanoCur 12 as effective comparable to diclofenac gel, a dose titration was pre-requisite.
is shown in Fig. 7. The histological observations indicated that saline control treated group had no significant alteration on skin integrity. The local toxicity and permeation effect of NanoCur 12 showed slight changes in the skin integrity. The structural changes indicate the influences of penetrating enhancement potential of the formulations and its subordinate components like surface active agents and ethanol as a co-solubilizer. The group treated with CrystCur and Diclofenac gel showed similar changes in the skin integrity. There was no any inflammatory and toxic potential observed in formulation treated skin histology. This showed safe and effectiveness of the optimized NanoCur 12 formulation for local anti-inflammatory activity.

4 Conclusions

The compositional proportions of nanoformulations are playing an important role for an effective transdermal drug delivery system. On the basis of drug permeation, permeability parameters, drug release, droplet size, polydispersity index, viscosity and optimum surfactant & cosurfactant concentration, NanoCur 12 was selected as an optimized formulation. The composition of the optimized formulation was consisted of curcumin (0.154% w/w), Carbopol 934 (0.702% w/w), ethanolic oil phase [ethanol (0.013% w/w): Capryol 90 (0.015% w/w), Tween 20 (0.076% w/w) as surfactant, PEG 200 (0.038% w/w) as a co-surfactant, and q.s distilled water as an aqueous phase. The ethanol and water present in the formulation would help in increasing permeation potential of curcumin. The permeation rate as well as anti-inflammatory activity of the optimized NanoCur was conspicuously increased with the shortened lag times in comparison to CrystCur. Short term stability at regulated temperatures and humidity showed insignificant changes in mean diameters and zeta potential, which forecast the high shelf-life of the optimized formulation. The carrageenan induced rat paw oedema further potentiate NanoCur 12 as a promising vehicle for improved transdermal delivery of curcumin and in the management of local inflammations. The dose titration could make the optimized formulation as effective anti-inflammatory inhibitor.
Acknowledgements

The author would like to acknowledge the research group, College of Pharmacy, Al Dawadmi Campus, Shaqra University, KSA for their valuable suggestion and support.

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