
**“DEVELOPMENT AND EVALUATION OF
DIACEREIN AND ACECLOFENAC LOADED
TOPICAL NANOEMULGEL FOR
OSTEOARTHRITIS: AN EXPERIMENTAL STUDY”**

**Thesis submitted to
THE KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH,
BELAGAVI**

(KLE DEEMED UNIVERSITY)

**[Declared as Deemed-to-be-University u/s 3 & 12B of the UGC Act, 1956 vide
Govt. of India Notification No.F.9-19/2000-U.3 (A)]**

(Accredited ‘A+’ Grade by NAAC) (3rd Cycle)

[Placed in Category ‘A’ by MoE (GoI)]



***For the award of the degree of
DOCTOR OF PHILOSOPHY
In the Faculty of Pharmacy***

By

Mr. TAUFIK MUBARAK KAZI M.Pharm.

(Registration No: KLEU/Ph.D./19-20/ DO1219012)

Under the Guidance of

Prof. (Dr.) PANCHAXARI M. DANDAGI Ph.D.

Professor and Head,

Department of Pharmaceutics,

KLE College of Pharmacy, Belagavi,

KLE Academy of Higher Education and Research,

Belagavi - 590010, Karnataka, INDIA.

2025

UNDERTAKING

I, **Mr. TAUFIK MUBARAK KAZI**, hereby declare that the information and the data mentioned in my thesis entitled “**DEVELOPMENT AND EVALUATION OF DIACEREIN AND ACECLOFENAC LOADED TOPICAL NANOEMULGEL FOR OSTEOARTHRITIS: AN EXPERIMENTAL STUDY**” belongs to me and is original.

I am aware of definition of plagiarism as detailed below:

- An act or instance of using or closely imitating the language and thoughts of another author without authorization and the representation of that author's work as one's own, as by not crediting the original author.
- A piece of writing or other work reflecting such unauthorized use or imitation.
- The deliberate or reckless representation of another's words, thoughts or ideas as one's own without attribution in connection with submission of academic work, whether graded or otherwise.

I hereby declare that the thesis prepared by me is original-one and does not involve plagiarism anywhere. In case at a later stage it is found that I have indulged in plagiarism, then I am solely responsible for the same and the Institution is at liberty to take any disciplinary action against me including cancellation of dissertation or any other penalties imposed by the University.

Date: 05/03/2025
Place: Belagavi


Signature of the Research Scholar
Mr. TAUFIK MUBARAK KAZI
Full time Ph.D. Research Scholar
Reg. No. D01219012
KAHER, Belagavi-590010

PLAGIARISM REPORT



Ref. No. KAHER/AA/24-25/D-676

3rd February 2025

Sir,

The soft copy of Ph.D. research thesis of **Mr. Taufik M. Kazi, Faculty of Pharmacy, KAHER, Belagavi** has been submitted for anti-plagiarism check at the office of the undersigned through "Turn-it-in" package. The scan has been carried out and the scanned output reveals a match percentage of **9%** which is within the acceptable limit of 10%.

To obtain the comprehensive report of the plagiarism test, research scholar can send a mail to **diracademic@kledeemeduniversity.edu.in** along with the Registration Number, Name of the Scholar, Name of Guide/Co-guide and title of the thesis.




Dr.(Mrs.) Jyoti M Nagamoti
Director, Academic Affairs

To,
Mr. Taufik M. Kazi,
Full-Time Ph.D. Scholar, 2019-20 Batch
Faculty of Pharmacy,
KLE College of Pharmacy,
KAHER, **Belagavi.**

Cc to :

1. The Principal, KLE College of Pharmacy, Belagavi
2. Prof. (Dr.) Panchaxari M. Dandagi, Professor & Head of Pharmaceutics, KLE College of Pharmacy, Belagavi - Guide

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH

(Deemed-to-be-University established u/s 3 & 12B of the UGC Act, 1956)
Accredited **A* Grade** by NAAC (3rd Cycle) Placed in **Category A** by MoE (GoI)

📍 JNMC Campus, Nehru Nagar, Belagavi - 590 010, Karnataka, India 📞 0831-2444444
✉ info@kledeemeduniversity.edu.in 🌐 www.kledeemeduniversity.edu.in

**KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH,
(KLE DEEMED UNIVERSITY)**

[Declared as Deemed-to-be-University u/s 3 & 12B of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

(Accredited 'A⁺' Grade by NAAC) (3rd Cycle)

[Placed in Category 'A' by MoE (GoI)]

BELAGAVI



Copyright Declaration

*We hereby declare that **KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI, KARNATAKA**, shall have the rights to preserve, use and disseminate this thesis in print or electronic format for academic/research purpose.*

Signature

Mr. TAUFIK M. KAZI

Full -Time Ph.D Research Scholar

Reg. No: DO1219012

KAHER –Belagavi-10

Date: 05/03/2025

Place: Belagavi

Signature

Prof. (Dr.) PANCHAXARI M. DANDAGI

Professor and Head,

Department of Pharmaceutics,

KLE College of Pharmacy, KAHER,

Belagavi-590010.

Date: 05/03/2025

Place: Belagavi

© KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH, BELAGAVI

**KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH,
(KLE DEEMED UNIVERSITY)**

[Declared as Deemed-to-be-University u/s 3 & 12B of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

(Accredited 'A⁺' Grade by NAAC) (3rd Cycle)

[Placed in Category 'A' by MoE (GoI)]

BELAGAVI



I hereby declare that the thesis entitled **“DEVELOPMENT AND EVALUATION OF DIACEREIN AND ACECLOFENAC LOADED TOPICAL NANOEMULGEL FOR OSTEOARTHRITIS: AN EXPERIMENTAL STUDY”** is a bonafide and original research carried out by me under the guidance of **Prof.(Dr.)PANCHAXARI M. DANDAGI**, Professor and Head, Department of Pharmaceutics, KLE College of Pharmacy, Belagavi- 590010. The thesis or any part thereof has not formed the basis for the award of any degree/fellowship or similar title to any candidate of any University.

Date: 05/03/2025

Place: Belagavi



Signature of the Research Scholar

Mr. TAUFIK MUBARAK KAZI

Full time Ph.D. Research Scholar

Reg. No. D01219012

KAHER, Belagavi-590010

**KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH,
(KLE DEEMED UNIVERSITY)**

[Declared as Deemed-to-be-University u/s 3 & 12B of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

(Accredited 'A⁺' Grade by NAAC) (3rd Cycle)

[Placed in Category 'A' by MoE (GoI)]

BELAGAVI



This is to certify that the thesis entitled **“DEVELOPMENT AND EVALUATION OF DIACEREIN AND ACECLOFENAC LOADED TOPICAL NANOEMULGEL FOR OSTEOARTHRITIS: AN EXPERIMENTAL STUDY”** is a bonafide and genuine research carried out by **Mr. TAUFIK M. KAZI** under the guidance of **Prof. (Dr.) PANCHAXARI M. DANDAGI**, Professor and Head, Department of Pharmaceutics, KLE College of Pharmacy, Belagavi-590010.

Signature

Prof. (Dr.) V. S. MASTIHOIMATH

Dean, Faculty of Pharmacy,
KAHER, Belagavi- 590010.

Date: 05/03/2025

Place: Belagavi

**KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH,
(KLE DEEMED UNIVERSITY)**

[Declared as Deemed-to-be-University u/s 3 & 12B of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

(Accredited 'A⁺' Grade by NAAC) (3rd Cycle)

[Placed in Category 'A' by MoE (GoI)]

BELAGAVI



This is to certify that the thesis entitled **“DEVELOPMENT AND EVALUATION OF DIACEREIN AND ACECLOFENAC LOADED TOPICAL NANOEMULGEL FOR OSTEOARTHRITIS: AN EXPERIMENTAL STUDY”** is a bonafide and genuine research carried out by **Mr. TAUFIK M. KAZI** under the guidance of **Prof. (Dr.) PANCHAXARI M. DANDAGI**, Professor and Head, Department of Pharmaceutics, KLE College of Pharmacy, Belagavi-590010.

Date: 05/03/2025

Place: Belagavi



Signature

Prof. (Dr.) SUNIL S. JALALPURE

Principal,

KLE College of Pharmacy,

KAHER, Belagavi – 590010.

**KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH,
(KLE DEEMED UNIVERSITY)**

[Declared as Deemed-to-be-University u/s 3 & 12B of the UGC Act, 1956 vide Govt. of India Notification No.F.9-19/2000-U.3 (A)]

(Accredited 'A⁺' Grade by NAAC) (3rd Cycle)

[Placed in Category 'A' by MoE (GoI)]

BELAGAVI



This is to certify that the thesis entitled **“DEVELOPMENT AND EVALUATION OF DIACEREIN AND ACECLOFENAC LOADED TOPICAL NANOEMULGEL FOR OSTEOARTHRITIS: AN EXPERIMENTAL STUDY”** is a bonafide record of original research carried out by **Mr. TAUFIK M. KAZI** for the award of the degree of **DOCTOR OF PHILOSOPHY IN FACULTY OF PHARMACY** under my supervision and guidance.

Date: 05/03/2025
Place: Belagavi

Signature of Guide 
Prof. (Dr.) PANCHAXARI M. DANDAGI
Professor and Head,
Department of Pharmaceutics,
KLE College of Pharmacy, Belagavi,
KAHER, Belagavi-590010.

ACKNOWLEDGMENT

I express my sincere thanks to the Almighty and the many individuals whose blessings, unwavering support, and enthusiasm sustained my vision throughout this challenging Ph.D. journey.

First and foremost, I express my deepest gratitude to my esteemed research guide, **Prof. (Dr.) Panchaxari M. Dandagi**, for his invaluable mentorship, insightful guidance, and unwavering support. From the initial stages of refining my research proposal to the final submission of my thesis, his unwavering presence and profound wisdom have been instrumental in shaping my academic growth. I am deeply grateful for the freedom he afforded me in my research and for his unwavering support.

My sincere gratitude extends to our esteemed former Vice-Chancellor, **Prof. (Dr.) Vivek A. Saoji** and esteemed Vice-Chancellor **Dr. Nitin M. Gangane** for providing me with the necessary resources and opportunities to embark upon and complete the Ph.D. program as a full-time research scholar at KLE College of Pharmacy, Belagavi.

I would like to express my sincere gratitude to **Mr. Prakash R. Biradar**, Assistant Professor in the Department of Pharmacology and Toxicology, for his invaluable assistance with the animal studies that were integral to my Ph.D. research. I also extend my sincere thanks to **Professor (Dr.) Mrs. Bhagyashri R. Hungund** and **Mr. Shivanand G. Hiremath**, junior laboratory technician in the Department of Pathology at KAHER's JNMC, Belagavi, for their invaluable assistance with the histopathology studies. Their expertise and support were essential to the successful completion of this project.

I also express my deepest gratitude to the respected **Prof. (Dr.) Sunil S. Jalalpure**, Principal and AICTE QIP coordinator, and **Prof. Mrs. Rajashree Masareddy**, former Head of Department, KLE College of Pharmacy, KAHER, Belagavi, for their unwavering support, cooperation, and constant availability.

I am immensely grateful to the AICTE, New Delhi; the Directorate of Technical Education, Maharashtra State, Mumbai; and KLE Academy of Higher Education and Research, Belagavi, for providing me with the opportunity to pursue a Ph.D. under the QIP program.

I am deeply indebted to the former Director of Academic Affairs, **Dr. (Mrs.) Daksha Dixit**, **Dr. (Mrs.) Roopa Bellad**, and the esteemed Director of Academic Affairs, **Dr. (Mrs.) Jyoti Nagamoti**, KLE Academy of Higher Education and Research, for their valuable support and cooperation.

My special thanks go to **Prof. (Dr.) Ramesh S. Paranjape**, Distinguished Professor & In-charge Director, Dr. Prabhakar Kore Basic Science Research Centre, for generously sharing his knowledge and expertise, and for providing me with the necessary infrastructure and motivation to conduct my research work.

I am grateful to all HODs and staff members of KLE College of Pharmacy, Belagavi, including **Prof. (Dr.) Vinayak Mastiholimath**, **Prof. (Dr.) Nayeem Khatib**, **Prof. (Dr.) Shankar Alegaon**, and **Prof. (Dr.) Mrs. Meenaxi Maste**, for permitting me to work in their respective laboratories and for their invaluable support throughout my research.

I am particularly grateful to respected **Dr. Remeth J. Dias**, HOD (diploma in pharmacy), Government College of Pharmacy, Karad, Maharashtra, for his

unwavering motivation and support throughout my Ph.D. journey. I am also grateful to **Dr. Niraj Gokhale, Mr. Masood Ahmed, Dr. Bhaskar Kurangi, Mr. Abhijit Bhatkal, Dr. Vijay Kumbar, Dr. Suneel Dodamani, Mrs. Dhanashree Patil,** and **Ms. Geetanjali Mastiholimath,** for their valuable assistance during my research.

I am very thankful to my colleague and friend, **Mr. Tukaram Kedar,** for his constant encouragement and support throughout this long journey and to **Mrs. Prasiddhi Raikar, Ms. Shivani Tendulkar, Ms. Supriya Chimagave, and Mr. Roshan Sharma** for their valuable support during my research.

Finally, I express my immense gratitude and love to my greatest source of inspiration, my parents, for their endless encouragement, guidance, and support throughout these years. Most importantly, I wish to thank my loving and supportive wife, **Mrs. Shahista,** and my sons, **Mohd. Uzair and Mohd. Izahan,** for their unwavering mental and emotional support.

Thank you, one and all.

Date:
Place: Belagavi

Signature of the Research Scholar
Mr. TAUFIK MUBARAK KAZI
Full time Ph.D. Research Scholar
Reg. No. D01219012
KAHER, Belagavi-590010

LIST OF ABBREVIATIONS USED

ACE	Aceclofenac
ACN	Acetonitrile
ACR	American College of Rheumatology
ANOVA	Analysis of variance
API	Active pharmaceutical ingredient
AUC	Area under curve
BA	Bioavailability
BBD	Box-Behnken Design
BCS	Biopharmaceutics classification system
CC	Calcified cartilage
CCD	Central composite design
CDER	Centre for drug evaluation and research
CDR	Cumulative drug release
CH	Chondrocyte
cm	Centimetre
C _{max}	Maximum plasma concentration of drug
CMC	Carboxymethyl cellulose
COX	Cyclooxygenase
cP	Centipoises
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CV	Coefficient of variation
DCN	Diacerein
DL	Diclofenac
DLS	Diclofenac sodium

DMA	Dimethyl acetamide
DMOAD	Disease-modifying osteoarthritis drug
DMSO	Dimethyl sulfoxide
DoE	Design of experiment
DSC	Differential scanning calorimetry
EDTA	Ethylene diamine tetra acetic acid
EE	Entrapment efficiency
FA	Fenofibric acid
FDA	Food and Drug Administration
FDC	Fixed-dose combination
FE-SEM	Field emission-scanning electron microscopy
FFD	Fractional factorial design
FR	Flow rate
FTIR	Fourier transform infrared spectroscopy
GI	Gastrointestinal
GS	Globule size
H&E	Hematoxylin and eosin
HETP	Height equivalent to a theoretical plate
HLB	Hydrophilic-Lipophilic Balance
HPMC	Hydroxy propyl methyl cellulose
h/ hrs	Hours
HR-TEM	High resolution transmission electron microscopy
IAEC	Institutional Animal Ethics Committee
ICH	International Council for Harmonization
IL	Interleukin
IS	Internal standard

K ₀	Zero-order rate constant
K ₁	First-order rate constant
KBr	Potassium bromide
K _e	Elimination rate constant
K _H	Higuchi dissolution constant
kV	Kilovolts
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
MDS	Mean droplet size
ME	Microemulsion
Mg	Milligram
Min.	Minute
mL	Millilitre
mm	Millimetre
mV	Millivolt
NC	No change
NE	Nanoemulsion
NEG	Nanoemulgel
ng	Nanogram
nm	Nanometer
NP	Nanoparticle
ns	non- significant
NSAIDs	Nonsteroidal anti-inflammatory drugs
o/w	Oil in water
OA	Osteoarthritis

OPA	Orthophosphoric acid
Opt.	Optimized
P	Probability
PBS	Phosphate buffer saline
PDA	Photodiode array detector
PDI	Polydispersity index
pKa	The negative logarithm of the dissociation constant
ppm	Parts per million
PS	Particle size
PTFE	Polytetrafluoroethylene
QC	Quality control
R ²	Correlation coefficient
RA	Rheumatoid arthritis
RH	Rhein
RP-HPLC	Reverse phase high performance liquid chromatography
rpm	Revolution per minute
RSD	Relative standard deviation
SD	Standard deviation
SLN	Solid lipid nanoparticles
Smix	Surfactant and cosurfactant mixture
SNEDDS	Self-nanoemulsifying drug delivery systems
Sod. MIA	Sodium monoiodoacetate
T	Transmittance
t _{1/2}	Half life
TDDS	Transdermal drug delivery system
TEA	Triethylamine

TF	Tailing factor
T _{max}	Time of peak plasma concentration
TP	Theoretical plate
USFDA	United states food and drug administration
v/v	Volume by volume
VAS	Visual Analogue Scale
w/o	Water in oil
w/w	Weight by weight
WOMAC	Western Ontario and McMaster Universities Osteoarthritis Index
XRD	X-ray diffraction
ZP	Zeta potential
λ_{max}	Absorption maxima
%	Percentage
<	Less than
>	Greater than
°C	Degree centigrade
μg	Microgram
μm	Micrometre

TABLE OF CONTENTS

SL. NO.	PARTICULARS	PAGE NO.
1.	INTRODUCTION	1-17
1.1	Background	1-7
1.2	Literature Review	8-13
1.3	Justification	14-16
1.4	Objective	17
2.	MATERIAL AND METHODS	18-51
3.	STATISTICAL ANALYSIS	52
4.	RESULTS	53-113
5.	DISCUSSION	114-132
6.	SUMMARY	133-137
7.	CONCLUSION	138-139
8.	BIBLIOGRAPHY	140-165
9.	ANNEXURES	166-172
	A. Animal Ethical Clearance Letters	166-167
	B. Publications and Certificates	168-172

LIST OF TABLES

SL. NO.	TABLE CONTENT	PAGE NO
1.	List of equipment/instruments/devices used in the study.	18
2.	List of chemicals/polymers/API used.	19-20
3.	Variables and their design constraints in D-optimal mixture design.	30
4.	Composition of DCN-and ACE-loaded NE, different NEG, and gel formulations.	36
5.	Animal grouping and treatment details (study design) for <i>in vivo</i> anti-OA study.	47
6.	Drug: excipients compatibility study result based on the visual observation.	55
7.	Results of the transmittance and titration study for different surfactants and co-surfactants.	63
8.	Experimental run of 2-level D-optimal mixture design for independent variables and responses (n=3).	69
9.	Summary of ANOVA for the studied responses.	69
10.	Two-sided composition and confirmation report by design for optimized NE.(Confidence 95% & n=1)	70
11.	Predictive and actual values of NE formulations suggested by the design.	70
12.	Characterization of the NE formulations suggested by the design.	72
13.	Characterization of DCN-and ACE-loaded NEG and plain gel formulations.	77

14.	<i>In vitro</i> DCN and ACE release kinetics from different NEG, gels and opt-NE.	81-82
15.	Results of rat skin permeation and deposition study of NE, NEG, and plain gel formulation in different media.	85
16.	Results of the stability study of optimized NEG formulation.	90
17.	Mean Erythema and Edema scores after application of formulation on rat skin (Draize method).	93
18.	Results of system suitability parameters in spiked plasma samples.	99
19.	Stability results of quality controls of RH, ACE, and DL in rat plasma at different conditions.	100-101
20.	Pharmacokinetic parameters of RH, ACE, and DL in Wistar rats following transdermal and oral dose.	103
21.	Details of the histological scoring (modified Mankin) after 3 weeks.	110
22.	Details of the histological scoring (modified Mankin) after 5 weeks.	110

LIST OF FIGURES

Sl No	Figure Name	Page No
1.	Chemical structure for (a) DCN, (b) RH, (c) ACE, and (d) DL.	3
2.	Schematic representation depicting the various methods for the preparation of NE.	6
3.	Overlay UV spectra of DCN and ACE in (a) methanol, (b) THF: phosphate buffer pH 6.8 (20:80), and (c) ACN: 0.1% OPA buffer pH 3 with an Isosbestic point at 264 nm.	24
4.	Permeation study setup: Franz diffusion cell apparatus (PremeGear, USA) and RP-HPLC instrument (Shimadzu, Japan).	34
5.	Images depicting anesthetized rats and the intra-articular injection of MIA solution.	49
6.	Schematic representation depicting the modified Mankin grading system for osteoarthritis.	50
7.	Absorption spectra and maxima of a) ACE in methanol, and b) DCN in DMSO: Methanol (10:90).	53
8.	DSC thermograms, FTIR spectra, and particle size of ACE (a, c, and e), and DCN (b, d, and f).	54
9.	Desirability bar graph (a) for all individual factors, responses, and combination of all responses and overlay plot (b-d) of the selected responses.	57
10.	Chromatogram of (a) Blank solution, (b) DCN standard solution, (c) ACE standard solution, (d) RH standard solution, (e) DLS standard solution, (f) Mixed DCN and ACE standard solution, (g) Mixed DCN, RH, ACE, and DLS standard solution, (h) Dycerin A tablet, (i) Hilin-Ace Tablet, (j)	59

	Placebo nanoemulgel, (k) DCN and ACE loaded nanoemulsion and (l) Nanoemulgel formulation.	
11.	Linearity graph of (a) DCN, (b) RH, (c) ACE, and (d) DLS.	60
12.	Individual solubility profile of DCN and ACE in different oils (a and b), surfactants (c and d), and, co-surfactants (e and f), respectively.	61
13.	Combined solubility profile of DCN and ACE in different oils (a and b), surfactants (c), and, co-surfactants (d and e).	62
14.	Pseudoternary phase diagram for (a) Transcutol P, and (b) PEG 400.	64
15.	Pseudoternary phase diagram of oil mix (Triacetin: Capmul MCM C8, 2:1), Smix (Kolliphor-EL: Transcutol P, 1:1), and water with NE regions (yellow shaded area) at various Smix ratios 1:0 to 4:1.	65
16.	Two-dimensional contour plots and three-dimensional response surface plots indicating the interaction effect of the independent variable on a) GS and b) % T. The desirability (c) and overlay plot (d) for the optimized NE-1 suggested by design.	71
17.	Globule size, PDI, and electrokinetic (zeta) potential of (a) NE-1, and (b) NE-2 formulation.	73
18.	<i>In vitro</i> DCN and ACE permeation profile from NE formulations (NE-1 and NE-2) suggested by design in PBS pH 5.7 (a-b) and mixture of PBS pH 6.8: methanol (7:3) (c-d).	76
19.	FE-SEM (a) and TEM (b) image of optimized DANE with x=0.10.	77
20.	<i>In vitro</i> permeation of DCN and ACE from optimized NE, DCN-and ACE-loaded NEG, and Plain gel in PBS pH 5.7 (a-b) and methanolic PBS pH 6.8 (c-d).	79
21.	<i>Ex vivo</i> permeation profile of DCN and ACE from NEG-1, NEG-2, and Plain gel-2 in PBS pH 5.7 (a and b), and methanolic PBS pH 6.8 (c and d).	84

22.	DSC thermograms for (a) ACE API, (b) DCN API, (c) DCN, ACE, Physical mixture of DCN and ACE with Capmul MCM C-8 (A) , Kolliphor-EL (B) , Transcutol-P (C) , DMA (D) , Triacetin (E) , and optimized NE, and (d) DCN, ACE, optimized NE, and optimized NEG.	86
23.	FTIR spectra of (a) DCN, (b) ACE, (c) Capmul MCM C8, (d) DCN+ACE + Capmul MCM C8, (e) Triacetin, (f) DCN + ACE + Triacetin, (g) Kolliphor-EL, (h) DCN + ACE + Kolliphor-EL, (i) Transcutol P, (j) DCN + ACE + Transcutol P.	88
24.	FTIR spectra of (a) NE placebo, (b) Opt. NE, (c) NEG placebo, and (d) Opt. NEG formulation.	89
25.	FTIR spectra of initial sample (a) and stability samples (b-c) of proposed NEG formulation.	91
26.	Histopathological examination (H&E staining) of rat skin after 7 days of application for the (a) normal control, (b) Formalin (0.8%v/v) treated, and (c) Opt. NEG-treated group. (x10 and x20).	93
27.	Recovery of rhein, aceclofenac, and diclofenac sodium in different protein precipitating solvents along with methanol as extracting agent.	94
28.	Pareto chart displaying the effect of independent factors on the responses (a) R1 (RH-ACE), (b) R2 (ACE-FA), (c) R3 (FA –DL), and (d) RT (DL).	95
29.	Desirability bar graph (a) for all individual factors, responses, and combination of all responses and overlay plot (b-d) of the selected responses.	96
30.	Chromatogram of (a) FA standard solution (IS) 10 µg/mL, (b) Mixed RH (10 µg/mL), ACE (20 µg/mL), FA (10 µg/mL), and DL (20 µg/mL) standard solution, (c) Blank rat plasma, (d) Blank plasma spiked with IS (500 ng/mL), (e) Blank plasma spiked with RH, ACE, DL & IS at LQC, (f) Blank plasma spiked with RH, ACE, DL & IS at MQC, (g) Blank plasma spiked with RH, ACE, DL & IS at LLOQ.	98

31.	Linearity graph of (a) RH, (b) ACE, and (c) DLS.	99
32.	Mean plasma concentration vs time profile with standard error of the mean bars for RH, ACE, and DL after oral single-dose administration of Dycerin A tablet and topical administration of optimized NEG to Wistar rat (DCN: 15mg/kg and ACE 30 mg/kg body weight) (n=6).	102
33.	Anti-inflammatory study in rats. The image depicts the experimental setup for subplantar injection and paw edema measurement in rats.	104
34.	Differences in rat paw volume (mL) recorded at different time intervals from different groups post-treatment. *: $p < 0.0001$, compared to the normal control group; †: $p < 0.01$, ††: $p < 0.0001$, in the marketed tablet, NEG (low dose) and NEG (high dose) treated groups compared to the MIA treated group; #: $p < 0.05$, ##: $p < 0.001$, in the marketed tablet, NEG (low dose) and NEG (high dose) treated groups compared to the plain gel treated group; ns: non-significant.	105
35.	(a) Measurement of rat knee thickness, and (b) Rat knee thickness (mm) recorded weekly for five weeks post-treatment.	106
36.	Histopathological examination of OA-induced rat knee joints (H & E staining) for protective cartilage degeneration and bone cellular abnormalities after 14 days (A-B), 35 days (C-G) and 49 days (H-M) of MIA injection and drug treatment. Different groups represented as Normal control (A, C, H), MIA treated (B, D, and I), Marketed tablet treated (E, J), NEG (low dose) treated (F, K), NEG (high dose) treated (G, L), and Plain gel treated (M). (x20).	107
37.	Histopathological examination of MIA-induced OA rat knee joints (toluidine blue staining) for matrix staining after 14 days (A-B), 35 days (C-G), and 49 days (H-M) of MIA injection and drug treatment. Different groups represented as Normal control (A, C, H), MIA treated (B, D, and I), Marketed tablet treated (E, J), NEG (low dose) treated (F, K), NEG (high dose) treated (G, L), and Plain gel treated (M). (x20).	108

38.	Histopathological examination of MIA-induced OA rat knee joints (safranin-O staining) for matrix staining after 14 days (A-B), 35 days (C-G), and 49 days (H-M) of MIA injection and drug treatment. Different groups represented as Normal control (A, C, H), MIA treated (B, D, and I), Marketed tablet treated (E, J), NEG (low dose) treated (F, K), NEG (high dose) treated (G, L), and Plain gel treated (M). (x20).	109
------------	--	------------

ABSTRACT

Background

Osteoarthritis (OA) affects millions globally, causing chronic pain and disability. Current oral therapies, like nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 enzyme inhibitors, have limited effectiveness and significant side effects. Nowadays, the focus of OA treatment has shifted towards modifying and slowing disease progression along with symptomatic management. Diacerein (DCN) and aceclofenac (ACE), frequently prescribed for OA treatment due to their disease-modifying and analgesic properties, are available in fixed-dose combination tablets (DCN 50 mg/ACE 100 mg) for OA and rheumatoid arthritis. However, the chronic oral delivery of DCN and ACE remains challenging due to their poor aqueous solubility, low bioavailability (BA), and undesirable side effects, which collectively cause patient noncompliance and decreased adherence to their oral regimens. Hence, it is essential to develop a new formulation that can increase the rate and extent of DCN and ACE absorption, resulting in enhanced BA and a significant reduction in side effects. Since poor BA and side effects may occur due to oral administration, novel, non-oral DCN and ACE formulations have been proposed to overcome these problems.

Objectives

The objectives of the study are: (i) To develop, optimize, and evaluate diacerein- and aceclofenac-loaded topical nanoemulgel for osteoarthritis. (ii) To study the effect of diacerein and aceclofenac-loaded nanoemulgel as anti-inflammatory and anti-osteoarthritis by topical route.

Methodology

A DCN-and ACE-loaded NE was prepared by a spontaneous emulsification using an oil mix (Triacetin: Capmul MCM C8, 2:1 w/w) and a Smix; surfactant/co-surfactant mixture (Kolliphor-EL: Transcutol P, 1:1 w/w). The NE formulation was optimized using a pseudoternary phase diagram and a 3-factor, 2-level D-optimal mixture design, generating 16 formulations based on varying oil phase ratios, Smix ratio, and water content. Globule size (GS) and percentage transmittance (%T) were the measured responses. The optimized NE suggested by the design expert was characterized for GS, %T, zeta potential, polydispersity index (PDI), pH, and thermodynamic stability. Field-emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM) were used to examine globule size, shape, and surface morphology. The optimized NE was then converted into an NEG using hydroxypropyl methylcellulose (HPMC) K15M and characterized for pH, rheological properties, *in-vitro* drug release along with the release kinetic, stability, acute toxicity study, *ex-vivo* permeation through rat skin, rat skin deposition, pharmacokinetics, and *in vivo* anti-inflammatory and anti-osteoarthritic activity in male Wistar rats.

Results

The GS, PDI, %T, and zeta potential of optimized NE was found to be 10.20 ± 0.70 nm, 0.20 ± 0.12 , 98.03 ± 0.03 , and -28.40 ± 2.77 mV, respectively. The optimized NE was found to be thermodynamically stable. The FE-SEM and TEM confirmed the nano-size (< 20 nm) and spherical shape of the NE globules. The optimized NE was converted into an NEG using 4% w/w HPMC K15M as the gelling agent and 2.5% w/w DMSO as the penetration enhancer. An *in vitro* release study showed the optimized NE and NEG exhibited superior drug release compared to plain NEG and

plain gel with and without a DMSO. An *ex vivo* permeation study using Wistar rat skin demonstrated significantly higher drug release, flux, and skin deposition for the optimized NEG than plain NEG and plain gel with and without a DMSO. An *in vitro* and *ex vivo* release kinetic profiles of DCN and ACE from optimized NEG followed the Korsmeyer-Peppas model, indicating non-Fickian diffusion. The optimized NEG demonstrated stability for 90 days and was found to be non-irritating and non-toxic in acute skin toxicity and histopathology studies. An *in vivo* pharmacokinetic study in Wistar rats revealed enhanced bioavailability of DCN and ACE from the optimized NEG compared to a marketed tablet with the relative bioavailability of 369% for rhein (the active metabolite of DCN), 247% for ACE, and 106% for DL (the active metabolite of ACE). Furthermore, the optimized NEG exhibited superior *in vivo* anti-inflammatory and anti-osteoarthritic activity in the carrageenan-induced rat paw edema model and sodium monoiodoacetate-induced osteoarthritic rat model, respectively.

Conclusion

The outcomes of this study suggest a potential role for this novel nanoemulgel formulation in the management of osteoarthritis. This nanoemulgel platform offers a promising technique for the transdermal delivery of drugs with poor oral and transdermal bioavailability and significant oral side effects by improving patient compliance through reduced dosing frequency and enhancing therapeutic effectiveness.

Keywords: Diacerein, Aceclofenac; Rhein; D-optimal mixture design; Pseudoternary phase diagram; Nanoemulsion; Nanoemulgel; Anti-inflammatory; Anti-osteoarthritis.

1. INTRODUCTION

1.1 BACKGROUND

Osteoarthritis

Osteoarthritis (OA), caused by the deterioration of joint protective cartilage with inflammation, affects more than two hundred fifty million older adults globally, causing chronic pain, inflammation, and disability⁽¹⁻³⁾. The progressive cartilage damage and the growth of osteophytes are hallmarks of OA ^(4,5). The World Health Organization reports that among adults over the age of 60, approximately 10 percent of men and 18 percent of women suffer from symptomatic OA. Of those affected, 80 percent face mobility restrictions, and 25 percent cannot perform daily activities ⁽⁶⁾. The American College of Rheumatology defines OA as a broad category of disorders with joint problems and signs linked to bone changes and cartilage degradation ⁽⁷⁾. As life expectancy continues to increase, the occurrence of OA is also on the rise, resulting in a substantial burden on society ^(8,9). Osteoarthritis, the second most prevalent rheumatic disease and the most common joint illness, impacts around 22% to 39% of the Indian population ⁽¹⁰⁻¹²⁾. Although 80% of persons may have radiographic indications of OA by the time they are 65 years old, only 60% actually experience symptoms ⁽¹³⁾. While OA can affect any joint, it predominantly affects the knees and hips. This condition plays a substantial role in causing global disability and chronic pain ^(3,14). Osteoarthritis has no known cure; instead, treatment aims to minimize functional impairment, maintain or increase joint mobility, and alleviate pain ⁽¹⁵⁾.

Currently, the primary clinical approach for OA management involves nonsteroidal anti-inflammatory drugs (NSAIDs), analgesics, cyclooxygenase-2

(COX-2) enzyme inhibitors, and hyaluronan, which primarily alleviate symptoms but do not modify disease progression ^(16–18).

Among the studies of OA therapy, NSAIDs are the most commonly recommended medication for OA, although they often cause dissatisfaction in the management of pain ⁽¹⁹⁾. The traditional oral administration of NSAIDs is known to cause gastrointestinal (GI) damage and significant fluctuation in plasma drug concentrations, leading to the risk of overdose. Furthermore, the majority of NSAIDs require high dosing frequency for prolonged pain and inflammation relief ⁽²⁰⁾. However, COX-2 enzyme inhibitors may have substantial renal and cardiovascular toxicities despite the fact that they have decreased the frequency of GI adverse events ⁽²¹⁾.

Consequently, oral NSAIDs are contraindicated or used cautiously in many individuals due to comorbidities or concomitant medications ⁽²²⁾. Topical analgesics, such as topical NSAIDs, are often prescribed alone or in combination, particularly for hand or knee OA. However, NSAIDs, including COX-2 inhibitors, do not address the underlying cause of OA, limiting their ability to modify disease progression and improve long-term quality of life ⁽²³⁾.

Recently, there has been an increased use of disease-modifying osteoarthritis drugs (DMOADs) such as chondroitin sulfate, glucosamine sulfate, and diacerein (DCN). These agents have the potential to alter the course of joint degeneration, slowing its progression and possibly making it asymptomatic. These DMOADs exhibit a slow onset of action and exert their effect after 28 to 42 days. However, they maintained their symptomatic effects for 28 to 56 days after discontinuing the treatment ⁽²⁴⁾.

Diacerein is an anthraquinone derivative commonly used as a DMOAD and besides, possesses anti-inflammatory activity⁽²⁵⁾. DCN; 4,5-diacetoxy-9,10-dihydro-9,10-dioxo-2-anthracene-carboxylic acid (Fig. 1a) is a prodrug of rhein (RH) (Fig. 1b) and exhibits its disease-modifying OA effect by inhibiting the synthesis of interleukin-1 β (IL-1 β), superoxide, and other cartilage degrading enzymes expressed in OA. It also increases the synthesis of proteoglycans and hyaluronic acid, an essential cartilage component⁽²⁵⁻²⁷⁾. Its disease-modifying effects become evident within 2-4 weeks, reaching their peak at 4-6 weeks and persisting for several weeks post-treatment⁽²⁵⁾. Diacerein was reported to have slow-acting properties. Because of their extended onset of action, DCN must be administered in combination with other analgesics, including NSAIDs, during the initial months of treatment⁽¹⁹⁾. Combination therapy of DCN and NSAIDs (e.g., Aceclofenac (ACE), Celecoxib, Meloxicam, etc.) shows a more satisfactory effect on OA as compared with that of DCN or NSAIDs alone^(19,28,29). Fixed-dose combination tablets containing DCN 50 mg and ACE 100 mg are available in the market for rheumatoid arthritis (RA) and OA treatment.

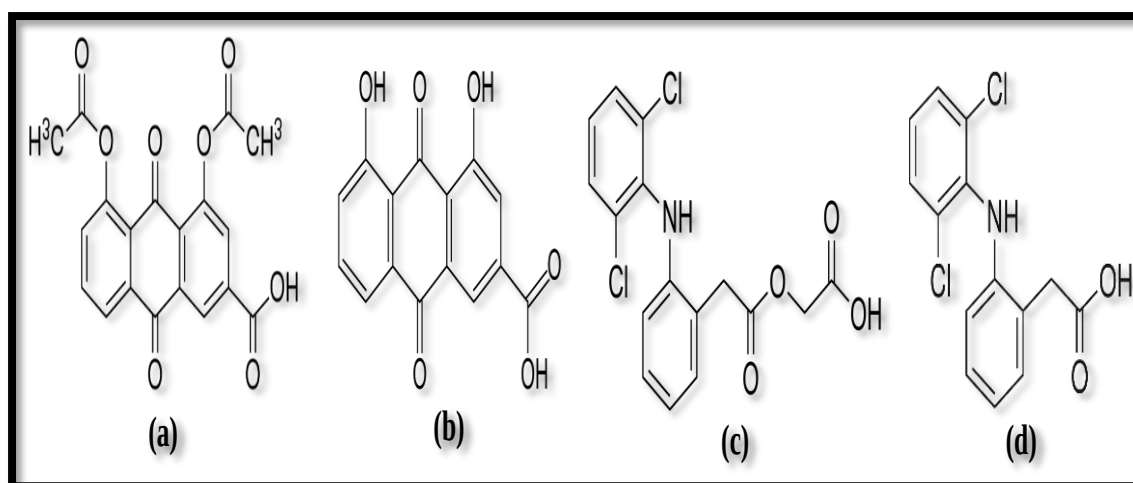


Figure 1. Chemical structure for (a) DCN, (b) RH, (c) ACE, and (d) DL.

Aceclofenac, 2- [(2, 6-dichlorophenyl) amino] phenylacetoxy acetic acid (Fig. 1c), is a preferential COX-2 inhibitor and most commonly prescribed NSAID to relieve the pain associated with ankylosing spondylitis, RA and OA ⁽³⁰⁾. It is the congener of diclofenac (DL) (2-[(2, 6-dichloro-phenyl) amino] benzene acetic acid; Fig.1d) with improved GI tolerance than other NSAIDs ⁽³¹⁾. Aceclofenac exhibits potent analgesic and anti-inflammatory effects by selectively inhibiting COX-2, an essential catalyst in synthesizing pro-inflammatory prostaglandins and cytokines ^(30,32).

However, the oral delivery of DCN and ACE remains challenging due to their poor aqueous solubility (DCN: 3.197 µg/mL; ACE: 60 µg/mL for ACE, classified as BCS Class II drugs), low bioavailability (35-56% for DCN and 40-50% for ACE), short half-life (4 h for ACE and 7-8 h for DCN), and extensive hepatic first-pass metabolism. Furthermore, DCN and ACE require more frequent oral administration due to their first-pass effect and short half-life ^(20,33-35). After oral administration, DCN shows undesirable side effects like soft stools, abdominal pain, and severe diarrhea due to unabsorbed DCN in the stomach ⁽³³⁾. In the case of ACE, after long-term oral administration, it shows undesirable side effects like peptic ulcers, dyspepsia, and GI hemorrhage due to prostaglandin inhibition. It also causes local irritation and GI mucosal lesions due to its acidic character ^(20,34,35).

Unfortunately, poor bioavailability, frequent dosing, and oral side effects collectively cause patient noncompliance and decreased adherence to DCN and ACE oral regimens ^(20,33-35). Hence, it is utmost essential to develop a new formulations or compositions that can increase rate and extent of DCN and ACE absorption, resulting in enhanced bioavailability and a significant reduction in side effects such as dyspepsia, abdominal pain, diarrhea, soft stools, etc. Since poor bioavailability and

side effects may occur due to oral administration, novel, non-oral DCN, and ACE formulations or compositions have been proposed to overcome these problems.

Transdermal Drug Delivery System (TDDS)

The TDDS offers an alternative to orally administered drugs that circumvents GI side effects and hepatic first-pass effects. Transdermal drug delivery system offers several benefits over the use of conventional oral or parenteral routes, such as non-invasive and painless administration, higher site-specific delivery, a low risk of drug overdose, controlled and predetermined drug release, reduced frequent dosing, controlled therapeutic responses, and improved patient compliance. It is thus ideally suited to long-term therapy, such as chronic pain management in arthritis ^(33,36,37). Consequently, TDDS is anticipated to serve as a viable alternate method for delivering ACE and DCN to improve their effectiveness and avoid oral side effects ^(26,33,38–41). However, designing TDDS is challenging due to the restricted permeation of drugs through the stratum corneum ^(33,42). As a result, most conventional topical formulations on the market (ointments, creams, and gels) have poor penetration and therapeutic potential ^(33,35). As per the literature review, different micro- or nanocarrier systems like ethosomes, liposomes, microemulsions, nanoemulsions (NEs), and lipid nanoparticles have been devised to enhance medication penetration through the skin ^(32,43–45). Prior research has demonstrated that nanoemulsion (NE) is a highly effective and promising approach for improving transdermal delivery and bioavailability of a wide range of lipophilic (BCS II) drugs ^(38–40,46–49).

Nanoemulsion

Nanoemulsions are monophasic, transparent, and thermodynamically stable dispersions in which usually oil globules (globule diameter less than 200 nm) are uniformly distributed throughout the aqueous phase (continuous phase) through the use of a Smix (surfactant & co-surfactant mixture) ^(50–52). Nanoemulsions are typically prepared using two primary methods (Fig. 2): high and low-energy emulsification techniques ⁽⁵³⁾. The oil and water components are reduced into nano-sized droplets using high-energy techniques, which utilize substantial mechanical energy. In contrast, low-energy approaches depend on precise conditions to naturally create NEs without requiring high levels of energy input ⁽⁵³⁾.

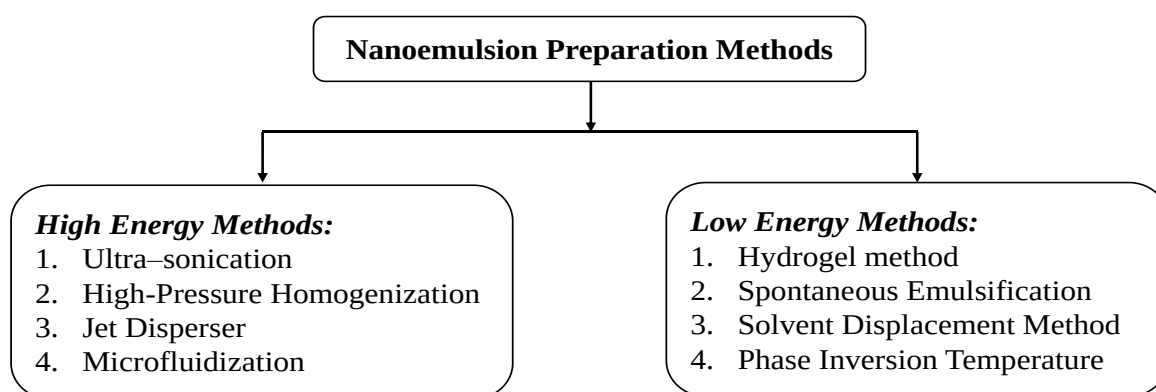


Figure 2. Schematic representation depicting the various methods for the preparation of NE.

Nanoemulsions have numerous benefits over microemulsions, ethosomes, niosomes, liposomes, and other nanoparticles. Some of these benefits include improved bioavailability, smaller droplet size, lower viscosity, ease of preparation, higher solubilization capacity, and physical stability ⁽⁵⁴⁾. Despite its many advantages, NE's low viscosity and spreadability make topical application challenging ^(55–58).

Nanoemulgel

To address the challenges of NE application, a new transdermal delivery technique called nanoemulgel (NEG) was developed by thickening NE with hydrogel^(57,59–61). Upon contact with intact skin, NEG releases oily droplets from its gel structure. After penetrating the stratum corneum, these droplets immediately transport drug molecules without the need for a transfer through the aqueous phase of NEs⁽⁵⁵⁾. The NEG exhibits improved NE properties for transdermal application, including good adhesion properties, low skin sensitivity, increased permeability, and high drug-carrying capacity⁽⁶²⁾. The NEG system's improved thixotropic properties, non-greasy, non-staining, emollient, superior spreadability, ease of removal, and appealing look all contribute to increased patient compliance^(57,59).

Therefore, this study aimed to develop a fixed dose combination of DCN- and ACE-loaded NEG and evaluate its effectiveness in treating OA.

1.2 REVIEW OF LITERATURE

Heena et al., (2016) conducted an extensive analysis of conventional therapies (oral, topical, and injectable) for OA and compared them to emerging nanoscale drug delivery systems. The authors contrasted the therapeutic outcomes of traditional treatments with those achieved through topical and parenteral nanotechnological approaches. This review underscores the potential of nanotechnology for topical drug delivery, emphasizing enhanced permeability, targeted delivery, and sustained drug release. Topical nanoscale systems, such as microemulsions, vesicular systems, and nanocarriers, demonstrated superior drug delivery compared to conventional methods, offering targeted treatment, reduced dosing, minimized side effects, and prolonged drug release. Consequently, nanotechnology-based drug delivery systems showed immense promise for effectively managing OA ⁽⁶³⁾.

Achnit Jain et al., (2013) formulated stearic acid-based DCN-loaded solid lipid nanoparticles (SLN) using homogenization and ultrasonication methods to improve their bioavailability and avoid their oral side effects. The particle size, drug content, polydispersity index (PDI), entrapment efficiency, and zeta potential of the optimized batch were found to be 387 nm, 98.1%, 0.191, 88.9%, and -19.89 mV, respectively. The surface morphology study using DSC, TEM, and XRD revealed the spherical shape of nanoparticles. The *in vivo* pharmacokinetic and anti-diarrheal studies of developed SLN and pure drug dispersion were conducted in Charles foster rats. It was found that SLN produced a 2.7 times improvement in bioavailability with a reduction in diarrhoeal side effects of DCN compared to plain drug dispersion. The reduced diarrhoeal side effects of DCN may be due to its increased absorption, which results in less availability of DCN or its metabolite, rhein, at the colonic site. This study concluded that the diarrhoeal side effect of DCN can be avoided/reduced by increasing its oral absorption or bypassing the oral route ⁽²⁶⁾.

Diana E. Aziz et al., (2018) developed DCN-loaded elastosomes using a full factorial design approach to enhance efficacy and circumvent oral side effects. The elastosomes were prepared as per experimental design using different edge activators and evaluated for entrapment efficiency, deformability index, particle size, and PDI. The optimized elastosomes formulation with the highest desirability showed 96.25% entrapment efficiency, 506.35 nm particle size, 0.46 PDI, -38.65mV zeta potential, and a 12.74 deformability index. Both the DCN-loaded elastosomes and bilosomes demonstrated superior flux and skin permeability compared to DCN suspension. A pharmacokinetic study in albino rabbits revealed no significant differences between DCN oral suspension and DCN topical elastosomes. These results suggested that TDDS can be considered as a promising alternative mode for DCN delivery with improving effectiveness and avoiding its oral side effects ⁽³³⁾.

Bazla Siddiqui et al., (2020) developed a NEG containing encapsulated DCN for transdermal delivery, utilizing biodegradable polymers to achieve sustained release and circumvent oral side effects. The NEG was formulated using chitosan, chondroitin sulfate, Tween 80, HPMC, and argan oil. The NE was prepared via ionic gelation and optimized using Design-Expert software with a central composite design. *In vitro* release studies revealed a 30% drug release within 2 h, followed by a cumulative release of 95% within 72 h through a dialysis bag at pH 7.4. The mean particle size of the optimized formulation was 321 ± 3.0 nm, and the zeta potential was 39.63 ± 0.3 mV. These findings suggested that the transdermal delivery of DCN in an NEG formulation utilizing biodegradable polymers and penetration enhancers can consistently release the drug up to 96 h ⁽³⁸⁾.

Nishal S et al., (2022) developed a Design of Experiment (DoE)- based DCN-loaded NEG for topical delivery to enhance permeation and avoid oral side effects. The NEG

was formulated using the ultrasonication method. Based on solubility analysis, NE components (surfactant, co-surfactant, and oil) were selected. The formulation was optimized using the Box-Behnken design. The NE components, Tween 80, propylene glycol, and oleic acid, were chosen as factors, and particle size and drug loading efficiency as responses. The prepared NEs were characterized, and responses were recorded. A 1% carbopol 934P hydrogel was used to convert the optimized NE into an NEG, which was subsequently evaluated for pH, rheological properties, spreadability, extrudability, drug content, and *in vitro* release. *In vitro* release studies at pH 7.4 phosphate buffer using a cellophane membrane diffusion method revealed a drug release of $93.61 \pm 0.57\%$ within 24 h, with a flux of 0.574 ± 0.016 . These findings suggest that the NEG can effectively improve the topical delivery of DCN, a BCS Class II drug while avoiding oral side effects ⁽³⁹⁾.

Shakeel F et al., (2009) compared the pharmacokinetic profiles of ACE-loaded NE and nanoemulsion gels to a commercially available Aceclofar® tablet. The Labrafil: Triacetin (2:1) based ACE-loaded NE was produced through spontaneous emulsification. The NE was characterized for PDI, surface morphology, globule size, and refractive index. By adding the NE to 1% w/w carbopol 940 hydrogels, the NEG was later created. The pharmacokinetic study of optimized NE, NEG, and Aceclofar® tablets was conducted in male Wistar rats. The results demonstrated significantly higher absorption of ACE from the topical NE and NEG compared to the oral tablet formulation. The bioavailability of ACE increased by 2.95 and 2.60 times for NE and NEG, respectively, relative to the oral tablet. These results showed that topical NEs are a viable method for increasing ACE bioavailability ⁽⁴⁰⁾.

Alam et al., (2020) formulated ACE-loaded NE and NEG for prolonged pain relief and reduced inflammation in arthritis and OA. The Labrafil: Triacetin-based ACE-

loaded NE was produced through high-pressure homogenization. The prepared NE underwent physicochemical evaluation and tests for thermodynamic stability. The average globule size of the NE was found to be 141 nm. The ACE-loaded NEG was formulated by incorporating the optimized NE into a Carbopol 940 gel base, with triethanolamine used to adjust the final pH. The final formulation exhibited a pH of 5.8, a viscosity of 502 cP, and a drug content of 99.81%. *In vitro* drug diffusion studies compared the performance of the prepared NE, NEG, and a commercially available gel. The results showed that the ACE-loaded NE had a higher permeation rate and coefficient than the NEG and the marketed gel. However, the prepared NEG demonstrated a greater drug flux ($68.88 \mu\text{g}/\text{cm}^2/\text{hr}$) compared to the marketed gel ($33.43 \mu\text{g}/\text{cm}^2/\text{hr}$). This study confirms the feasibility of producing stable NE with droplet sizes below 200 nm using minimal surfactant concentrations through high-pressure homogenization ⁽⁴¹⁾.

Loitongbam L SS et al., (2013) compared the effectiveness and adverse effects of DCN when combined with ACE versus ACE alone in OA patients. Seventy-six OA patients were evenly allocated into Group A and Group B. Group A administered DCN once a day orally for initial 28 days, followed by twice a day for 56 days, along with ACE tablet twice a day for 7 days and subsequent ACE as needed. Group B received ACE tablet twice a day for 7 days and subsequent ACE as needed. The dose of DCN and ACE was 50 mg and 100 mg, respectively. Using the VAS and WOMAC scale, symptoms and functional impairments were evaluated at baseline, 42 days, and 91 days. Results indicated that Group A, receiving DCN and ACE, exhibited significantly improved activities of daily living, reduced pain, and decreased stiffness compared to Group B. The study concluded that DCN is an effective drug for knee OA and shows a more satisfactory effect on OA when combined with ACE ⁽²⁸⁾.

Li Zhifu et al., (2015) investigated the therapeutic efficacy of a combined treatment using celecoxib (a COX-2 inhibitor) and DCN in a type II collagenase induced OA rat model. The results were compared to those obtained with individual drug treatments. The study was performed on male Sprague-Dawley rats. Osteoarthritis was induced in the right knee joint of rats. Experimental groups received daily injections of either celecoxib, DCN, or a combination of both for four weeks. A control group received normal saline. Plasma and joint tissues were analyzed post-treatment. The study demonstrated that the combination therapy of celecoxib and DCN effectively attenuated OA progression compared to single-drug treatments. This combination reduced inflammatory markers such as IL-1 β and nitric oxide, improved cartilage metabolism, and inhibited chondrocyte apoptosis. The findings suggest that the combined treatment offers superior therapeutic benefits for OA compared to either drug alone⁽²⁹⁾.

Dwicandra NMO et al., (2017) conducted a study comparing pain outcomes in OA patients treated orally with either a combination of DCN and meloxicam or meloxicam alone. The study included 68 patients with knee OA, allocated into two groups. One group administered a daily combination of 50 mg DCN and 15 mg meloxicam, while the other received 15 mg meloxicam daily for four weeks. Pain intensity was assessed using a combination of the Wong-Baker Faces Rating Scale and the Numeric Rating Scale. The combination therapy demonstrated a significantly lower pain intensity compared to meloxicam alone at both the third and fourth weeks ($p < 0.05$). The study concluded that combining DCN and meloxicam was more effective in treating OA pain than meloxicam alone⁽¹⁹⁾.

Helen Chattopadhyay et al., (2020) developed a topical NE-loaded hydrogel containing DCN and glucosamine sulfate to assess their chondroprotective and

disease-modifying properties while avoiding oral side effects. The NE was produced through ultrasonication and high-pressure homogenization and evaluated for TEM, cryo-SEM analysis, and globule size. This optimized NE was incorporated into a xanthan gum hydrogel to produce NEG. The resulting NE exhibited a spherical shape globule of 81.95 nm in size. *Ex vivo* permeation studies on rat skin demonstrated a controlled release profile for NEG with reduced transdermal flux compared to plain xanthan gum hydrogel. An *in vivo* study was conducted on a sodium-monoiodoacetate (MIA)-induced OA rat model, evaluating gross morphology, biochemistry, and histology. The animal study concluded that transdermal delivery of DCN and glucosamine sulfate via the nanoemulsion hydrogel exhibited superior effectiveness and fewer adverse effects than oral administration ⁽⁶⁴⁾.

Tripti et al., (2021) developed a ketorolac tromethamine-loaded w/o microemulsion for transdermal delivery. The microemulsion incorporated eucalyptus oil and methyl salicylate, known for their counter-irritant, anti-inflammatory, and analgesic properties, to enhance skin permeation and avoid oral side effects. Tween 80 and Labrafil 1944M were used as surfactants, with butanol as the co-surfactant. The microemulsion components concentration was determined using a ternary phase diagram, which was subsequently optimized using a mixture design. The microemulsion with 180 nm globule size and 95.7% T was selected as the optimized formulation. An *in vitro* release study at pH 7.4 PBS using goat skin indicated a drug release of 90-95% within 25 hours. The results of anti-inflammatory efficacy and analgesic activity in mice demonstrated superior performance of the optimized microemulsion compared to a control solution. These findings suggest that ketorolac tromethamine-loaded microemulsions offer a promising alternative for transdermal drug delivery ⁽⁶⁵⁾.

1.3 JUSTIFICATION

Osteoarthritis (OA), caused by the deterioration of joint protective cartilage with inflammation, affects more than two hundred fifty million older adults globally, causing chronic pain, inflammation, and disability ^(1–3). The progressive cartilage damage and the growth of osteophytes are hallmarks of OA. Cartilage, with its slow turnover rate, is particularly vulnerable. When it degenerates, it triggers the release of inflammatory molecules. These, in turn, further break down cartilage and accelerate disease progression ^(4,5). Therefore, therapies that simultaneously alleviate pain, suppress inflammation and stimulate cartilage regeneration are essential for effectively preventing, delaying, and treating this debilitating disease.

Unfortunately, current treatment strategies for OA primarily focus on symptom management, offering limited relief without addressing the underlying disease process. Available options such as NSAIDs, analgesics, opioid analgesics, newer COX-2 inhibitors, intra-articular steroids, and hyaluronic acid injections provide symptomatic relief but have no apparent disease-modifying effects ^(16–18). This has led to a growing interest in emerging DMOADs. These drugs can alter the course of joint degeneration, slow its progression, and potentially prevent symptom onset ⁽²⁴⁾. Diacerein, a prodrug of rhein (RH) and a slow-acting DMOAD, has gained popularity for its anti-inflammatory, anti-catabolic (protecting cartilage breakdown), and pro-anabolic (promoting cartilage synthesis) properties on cartilage and synovial membranes. It also exerts chondroprotective effects, shielding cartilage from further damage ^(25–27). However, due to its slow onset of action, DCN is often combined with NSAIDs like celecoxib, meloxicam, or ACE during the initial treatment phase of OA. This combination therapy has demonstrated superior efficacy compared to using DCN or NSAIDs alone ^(19,25,29). Among the NSAIDs, ACE, a preferential COX-2 inhibitor,

is primarily prescribed for the management of pain and inflammation allied with various conditions like ankylosing spondylitis, OA, and RA⁽³⁰⁾. Fixed-dose combination tablets containing DCN 50 mg and ACE 100 mg are available in the market for RA and OA treatment. However, oral delivery of DCN and ACE presents significant challenges due to their poor water solubility (BCS Class II), low bioavailability, rapid elimination, higher first-pass effect, and associated adverse effects^(20,33–35).

To overcome the limitations associated with oral administration, a combined transdermal delivery of DCN and ACE could be a promising approach. However, designing such a system is difficult due to the limited penetration of these drugs through the skin's outer layer (stratum corneum)^(33,42). Hence, to deliver the lipophilic drugs DCN and ACE locally to the target site with enhanced skin permeation and bioavailability, a promising nanocarrier-based transdermal system, such as an NE, has been explored^(38,40,46–49). Nanoemulsions offer several advantages, including improved bioavailability, smaller globule size, lower viscosity, ease of preparation, enhanced physical stability, and increased ability to dissolve lipophilic and hydrophilic drugs.

These characteristics make them potentially superior to other nanocarriers for treating OA⁽⁵⁴⁾. While NE offers advantages for drug delivery, their low viscosity and spreadability make topical application challenging^(55–58). Nanoemulgels were developed to address this issue by combining NE with hydrogel^(57,59–61). Nanoemulgels exhibit improved properties suitable for transdermal delivery, including enhanced adhesion, reduced skin irritation, increased permeability, and higher drug-loading capacity. Additionally, NEGs possess desirable characteristics such as thixotropy (gel-like consistency), a non-greasy and non-staining nature,

superior spreadability, easy removal, and aesthetic appeal, all contributing to improved patient compliance ^(57–59). Surprisingly, no topical formulation combining DCN and ACE for OA treatment has been produced to date. This gap in the market underscores the need for new approaches. Therefore, this study aims to develop a fixed-dose combination of a DCN and ACE-loaded NEG formulation and evaluate its efficacy in treating OA.

1.4 OBJECTIVES

- To develop, optimize, and evaluate diacerein- and aceclofenac-loaded topical nanoemulgel for osteoarthritis.
- To study the effect of diacerein- and aceclofenac-loaded nanoemulgel as anti-inflammatory and anti-osteoarthritis by topical route.

2. MATERIALS AND METHODS

2.1 Materials

The table below mentions the list of equipment/instruments/devices used from our laboratory/outsourced from recognized institutes.

Table 1. List of equipment/instruments/devices used in the study.

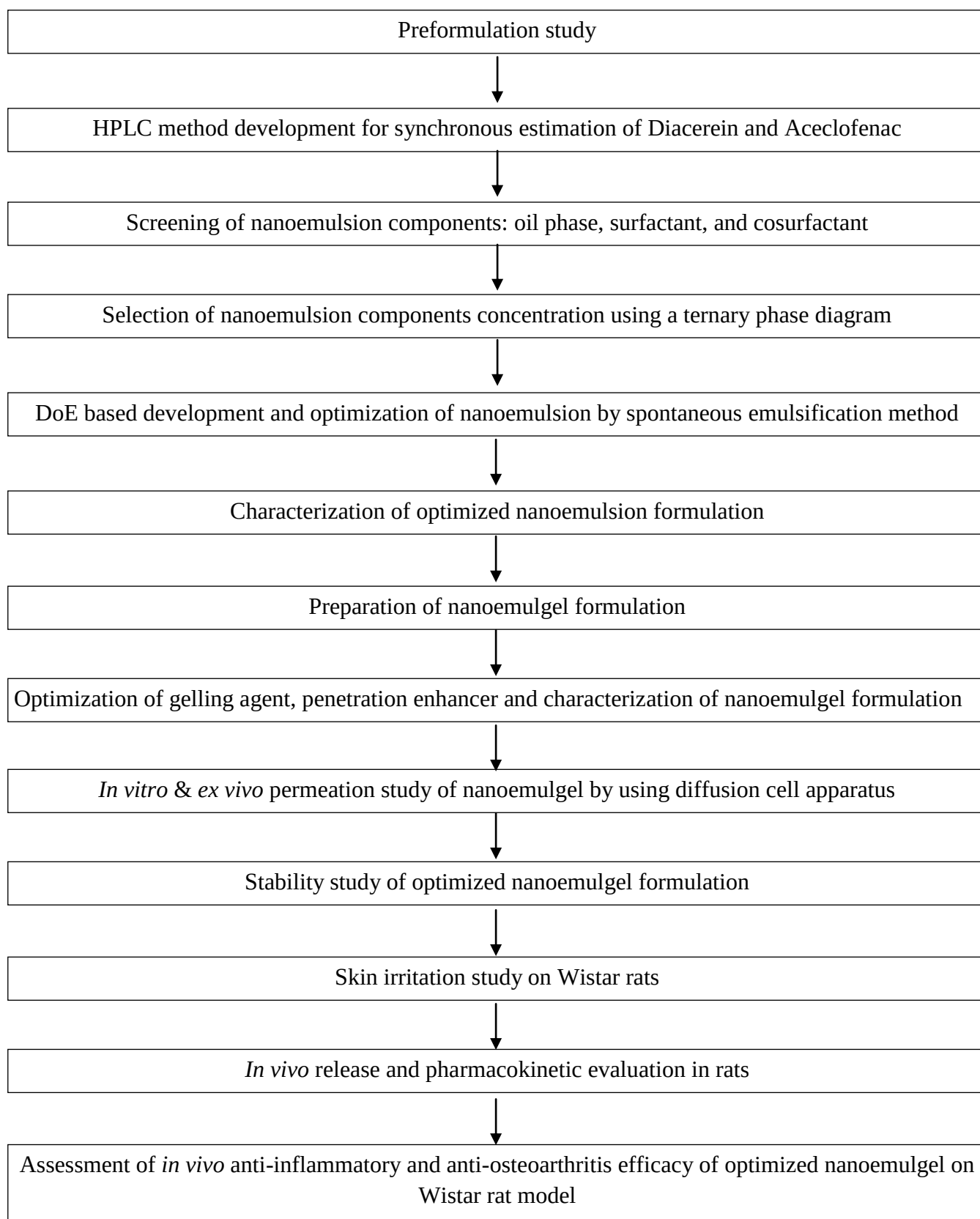
Sr. No.	Equipment/instrument name	Model and make
1	Cellulose dialysis membrane	150, LA401-5MT, HiMedia Lab Pvt. Ltd., Mumbai, India.
2	Centrifuge	Eppendorf AG, 5424R, Hamburg, Germany.
3	DSC	TA 60, Shimadzu, Kyoto, Japan.
4	Digital Plethysmometer	PLM-01 Plus, Orchid Scientific & Innovative India Pvt. Ltd., Nashik, India.
5	Electronic balance	AUW 220D, Shimadzu, Kyoto, Japan.
6	Franz diffusion cell	V6B-02 and 31011, Preme Gear, USA.
7	FTIR	IR Affinity-1S, Shimadzu, Kyoto, Japan.
8	Refrigerated centrifuge	Kubota 7000, Japan.
9	High-speed homogenizer	T25 digital Ultra Turax, IKA, Germany.
10	Homogenizer	RQT-127A, Remi Laboratory Instruments.
11	High-performance liquid chromatography	CBM 22A, Prominence, Shimadzu corporation, Japan.
12	HR-TEM	JEM, 2100, Jeol.
13	Incubated shaker	New Brunswick, Excella [®] E24 Inc Shaker, Eppendorf, U.S.A.
14	Magnetic stirrer	RCT Basic, IKA, Germany.
15	Microcentrifuge	Spinwin MC-02, Tarson.
16	Nanotracs	R-15 Ultra, Microcrack Inc. PA, U.S.A.
17	Olympus biological microscope	CX33, Olympus corporation, Tokyo, Japan.
18	pH meter	CyberScan pH 510, Eutech Instruments, India.
19	Ultrasonic bath	CPX 1800 H-E, Branson, Danbury, U.S.A.
20	UV-Vis spectrophotometer	UV-1800, Shimadzu, Kyoto, Japan.
21	Viscometer	CAP 2000+, Brookfield, U.S.A.
22	Vortex shaker	Eppendorf AG, 22331, Hamburg, Germany.
23	Zetasizer	Horiba Scientific SZ-100, HORIBA, Ltd., Kyoto, Japan.

Following are the chemicals, polymers, and API procured as gift samples or purchased from authentic / approved suppliers/vendors.

Table 2. List of chemicals/polymers/API used.

Sr. No.	Chemical/polymer/API name	Supplier
1	Acconon MC8-2 EP/NF	Abitec Corporation Ltd., Mumbai, India.
2	Aceclofenac IP	Wockhardt Research Centre, Chhatrapati Sambhaji Nagar, India.
3	Acetonitrile	Fisher Scientific, Mumbai, India.
4	Capmul MCM C8 NF	Abitec Corporation Ltd., Mumbai, India.
5	Capmul PG-12 EP/ NF	Abitec Corporation Ltd., Mumbai, India.
6	Carbopol 940	HiMedia Lab Pvt. Ltd., Mumbai, India.
7	Carrageenan (Low viscosity)	TCI Chemicals (India) Pvt. Ltd., Chennai, India.
8	Castor oil	CDH Pvt. Ltd., Mumbai, India.
9	Cremophor RH 40	BASF India Ltd., Mumbai, India.
10	Diacerein IP	Ami Life Sciences Pvt. Ltd., Thane, India.
11	Diclofenac sodium IP	Zuventus Healthcare Ltd., Pune, India.
12	Dimethyl sulfoxide	Qualigens, India.
13	Ethanol	Changshu Yangyuan Chemical, China.
14	Fenofibric acid	TCI Chemicals (India) Pvt. Ltd., Chennai, India.
15	HPMC K15M	Yarrow Chem Products, Mumbai, India.
16	Isopropyl myristate	Otto Chemie Pvt. Ltd., Mumbai, India.
17	Kolliphor PS 80 (Tween 80)	BASF India Ltd., Mumbai, India.
18	Kolliphor-EL USP/NF	BASF India Ltd., Mumbai, India.
19	Kollisolv ® GTA (Triacetin)	BASF India Ltd., Mumbai, India.

Sr. No.	Chemical/polymer/API name	Supplier
20	Labrafac PG EP/USP/ NF	Gattefosse India Pvt. Ltd., Mumbai, India.
21	Labrafil M 1944 CS EP/USP NF	Gattefosse India Pvt. Ltd., Mumbai, India.
22	Labrasol	Gattefosse India Pvt. Ltd., Mumbai, India.
23	Lauroglycol FCC	Gattefosse India Pvt. Ltd., Mumbai, India.
24	Methanol	Fisher Scientific, Mumbai, India.
25	Oleic acid	CDH Pvt. Ltd., Mumbai, India.
26	Orthophosphoric acid	Fisher Scientific, Mumbai, India.
27	Peceol	Gattefosse India Pvt. Ltd., Mumbai, India.
28	Polyethylene glycol 200	BASF India Ltd., Mumbai, India.
29	Polyethylene glycol 300	BASF India Ltd., Mumbai, India.
30	Polyethylene glycol 400	BASF India Ltd., Mumbai, India.
31	Polyethylene glycol 600	BASF India Ltd., Mumbai, India.
32	Propylene glycol	CDH Pvt. Ltd., Mumbai, India.
33	Rhein	Yucca Enterprises, Mumbai, India.
34	Sodium Monoiodoacetate	TCI Chemicals (India) Pvt. Ltd., Chennai, India.
35	Soybean oil	AOS Products Pvt. Ltd., UP, India.
36	Span 80	Mohini Organics Pvt. Ltd., Mumbai, India.
37	Transcutol P	Gattefosse India Pvt. Ltd., Mumbai, India.
38	Tween 20	CDH Pvt. Ltd., Mumbai, India.
39	Tween 60	CDH Pvt. Ltd., Mumbai, India.

2.2 Flow chart for the plan of work

2.3 Preformulation study

2.3.1 Identification and characterization of API

Preformulation is the research and development phase in which a drug's physical, chemical, and mechanical properties are characterized alone and in combination with excipients to create a stable, safe, and effective dosage form ^(66,67). Aceclofenac IP (ACE) and Diacerein IP (DCN) were received from Wockhardt Research Centre, Chhatrapati Sambhaji Nagar, and Ami Lifesciences Pvt. Ltd, Thane, India, respectively. The identification and characterization of ACE and DCN were performed by using UV-Vis spectroscopy, Nanotrak particle size analyzer, FTIR spectroscopy and Differential Scanning Calorimetry (DSC) thermal analysis methods. The absorption maxima of ACE and DCN were determined using a UV-1800 spectrophotometer (Shimadzu) following established protocols. Briefly, standard stock solutions of 100 µg/mL of each DCN and ACE were prepared separately in 50 mL volumetric flasks. Aceclofenac was dissolved in methanol, while DCN was first dissolved in 5 ml dimethyl sulfoxide and then diluted with methanol. Individual working standard solutions of 10 and 20 µg/mL of each DCN and ACE were prepared with a proper dilution of standard stock solution of respective analytes with the methanol. The working standard solutions of ACE (20 µg /mL) and DCN (10 µg /mL) were scanned between 200–800 nm, and their respective absorption maxima were determined ^(68,69). DSC analysis was performed using a Shimadzu DSC-TA 60 (temperature range 25°C to 420°C, nitrogen flow rate 20 mL/min), and FTIR analysis was conducted with a Shimadzu IR Affinity-1s (scanning range 400–4000 cm⁻¹ and resolution 4 cm⁻¹,) on pure drugs, DCN and ACE. The particle size of DCN and ACE were measured using a Nanotrak R-15 Ultra (Microtrac Inc. PA, USA) particle size analyzer.

2.3.2 DCN, ACE, and excipients compatibility study

The physical and chemical interaction of DCN and ACE with various excipients used for the study (based on a literature review) was conducted at 2-8°C (control conditions), room temperature, and at $40 \pm 2^\circ\text{C}/75 \pm 5\%\text{RH}$ (accelerated conditions). The drugs, along with different excipients in a 1:1 ratio, were mixed and packed in 'flint' glass vials (10 mL) and then stored at the conditions mentioned above. The samples were examined periodically to detect any change in appearance (66,67,70).

2.4 Analytical method for synchronous estimation of DCN and ACE

The quantification of DCN and ACE from the newly fabricated NEG formulation is crucial for the comprehensive evaluation of the formulation, including its properties, drug release, and product stability. Besides this, DCN is very sensitive to alkaline degradation. Its principal alkaline degradation product and the main active metabolite is RH ⁽⁴⁾. Rhein has low oral bioavailability compared with DCN ⁽⁷¹⁾ and is responsible for the laxative and severe diarrheal effect of DCN ^(26,33). In the case of ACE, diclofenac (DL) is the principal degradation product ⁽⁷²⁾. Diclofenac was found to be less effective and produces more GI adverse effects than ACE ^(73,74). Hence, it is utmost essential to develop a simple, robust, and effective RP-HPLC method for the evaluation of fabricated NEG formulation. Also, it is necessary to detect the presence of RH and DL in the commercial tablet dosage form to avoid patient noncompliance because of adverse side effects. A simple, robust, and effective RP-HPLC method for simultaneous quantification of DCN and ACE in a novel NEG formulation and the marketed tablet formulation in the presence of their major degradation product was developed using the design of experiment (DoE) approach and validated as per ICH guidelines.

2.4.1 HPLC instrumentation

The analytical method development and validation were performed on a PDA detector (SPD-M20A) based RP-HPLC (CBM-22A, Shimadzu Corporation, Kyoto, Japan) outfitted with a mobile phase delivery LC-20AD pump (Shimadzu), CTO-10AS VP, column oven (Shimadzu), SIL-20AC HT autosampler (Shimadzu) and LabSolution software (LC solution V 1.25).

2.4.2 Chromatographic condition

The chromatographic separation was carried out on Phenomenex C18 column (Size: 250 x 4.6 mm, Luna 5 μ m particle size, 100A) using a mobile phase consisting of acetonitrile (ACN) and phosphate buffer pH 3 (0.1% v/v orthophosphoric acid (OPA)) in the proportion of 60: 40 v/v. The pH of the phosphate buffer was adjusted to 3 by triethylamine (TEA). The mobile phase flow rate (FR) was 1 mL/min, with a 20 μ L injection volume and a column oven temperature of 35°C. The analytes were detected at their isosbestic point, i.e., at 264 nm (Fig.3).

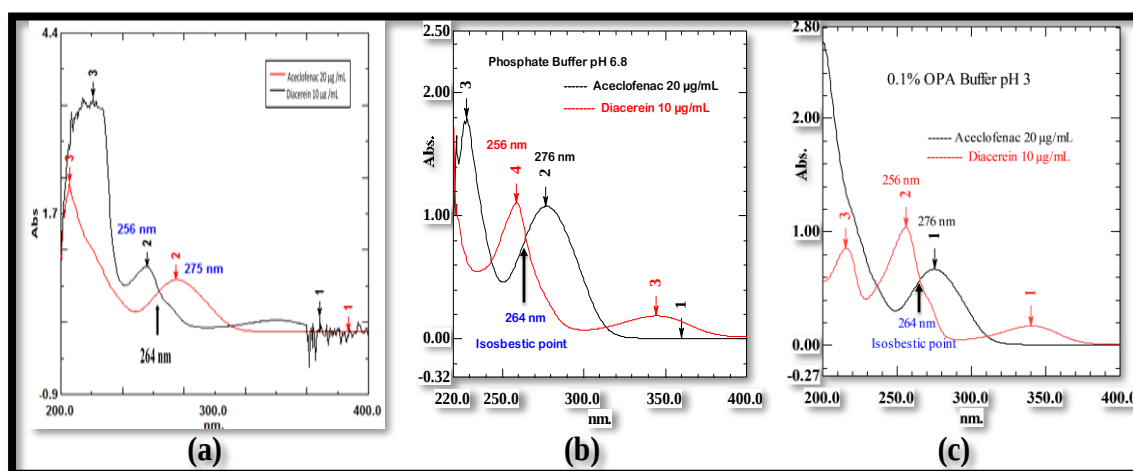


Figure 3. Overlay UV spectra of DCN and ACE in (a) methanol, (b) THF: phosphate buffer pH 6.8 (20:80), and (c) ACN: 0.1% OPA buffer pH 3 with an isosbestic point at 264 nm.

2.4.3 Preparation of standard and sample solutions

Standard stock solutions of 200 µg/mL of each DCN, ACE, diclofenac sodium (DLS), and 100 µg/mL of RH were prepared separately in 50 mL volumetric flasks using methanol. Individual working standard solutions of 10 µg/mL of each of the four analytes were prepared with a proper dilution of standard stock solution of respective analytes with the mobile phase. For mixed working standard solution (10 µg/mL of each), a standard stock solution of DCN (0.5 mL), RH (1 mL), ACE (0.5mL), and DLS (0.5mL) were transferred to a 10 ml volumetric flask and further diluted with the mobile phase. For the linearity study, individual working standard solutions of ACE, DCN, DLS, and RH were prepared in the range of 0.1-64 µg/mL by sequentially diluting an appropriate quantity of respective drug stock solutions with mobile phase in a separate 10 ml volumetric flask.

2.4.4 Method development

Optimal chromatographic conditions were determined through extensive testing. Various mobile phases were initially evaluated, followed by a fractional factorial design (FFD) to identify key factors influencing peak separation and retention. In the FFD screening total 8 experiments were carried out in randomized order ⁽⁷⁵⁾ to study the effects of four independent variables at two levels- pH of OPA buffer ⁽³⁻⁴⁾, flow rate (FR, 0.8-1.2 mL/min), percentage of ACN (55-65%v/v), and injection volume (10-20 µL) were evaluated on four response variables: a theoretical plate of DCN (TP-DCN), peak resolution between DCN and RH (R1), peak resolution between ACE and DLS (R3), and retention time of DLS (RT-DLS). The effect of independent variables on considered responses was evaluated by analysis of variance (ANOVA) and Pareto chart analysis. The variables found significant in the screening study were used for further optimization using a three-factor rotatable central

composite design (CCD) ^(75,76). Flow rate (0.8-1.2 mL/min), ACN content (55-65 % v/v), and pH of 0.1%v/v OPA buffer ⁽³⁻⁴⁾ were selected as independent variables, whereas TP of DCN (Y1), peak resolution between DCN-RH, R1 (Y2), peak resolution between ACE-DLS, R3 (Y3), and RT of DLS (Y4) were considered as responses. A total of 19 experiments suggested by CCD were performed, and the responses were recorded. The graphical and regression analyses were performed on the obtained data. Based on the desirability, the optimal experimental conditions were selected and further used for method validity.

2.4.5 Method validation

The established method was further validated according to ICH guidelines for specificity, linearity and sensitivity, precision, accuracy, and forced degradation. For the linearity and sensitivity study, the individual working standard solution (0.1 - 64 µg/mL) was analyzed in triplicate. The linearity graph of concentration against peak area responses was plotted separately for each analyte. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation (SD) of response and slope of the calibration curve. The system suitability was determined by injecting a mixed working standard solution (10 µg/mL) six times consecutively, and variables like peak area, retention time (RT), peak resolution, therapeutic plate (TP), tailing factor (TF) were evaluated for all four analytes. Accuracy (% recovery) was determined by spiking a known amount of analytes to a predetermined Dycerin A tablet solution at three quality control (QC) levels, i.e., 5, 10, and 15 µg/mL, and comparing the measured and expected concentrations. Intra-day and inter-day precision of the developed analytical method was determined by injecting the six mixed test solutions of each DCN, RH, ACE, and DLS (10 µg/mL of each) on the same day and successive three days, respectively. The % relative

standard deviation (RSD) of the assay on the same day and successive three days were calculated.

2.5 Screening of NE components

The right selection of oil phase, surfactant, and co-surfactant is an important factor for forming and stabilizing NE ^(77–79). The NE components were selected based on their solubilizing capacity for combined DCN and ACE and their emulsification ability as follows:

2.5.1 Solubility of the DCN and ACE in various excipients

The individual and combined saturation solubility of DCN and ACE in different oils, surfactants, and co-surfactants is carried out using the Shake Flask Method ^(77–81). An excess amount of individual drug, DCN (300 mg) or ACE (300 mg), or a combination of both (300 mg each of DCN and ACE) was added to 2 ml of each oil, surfactant, and cosurfactant in separate screw-capped Greiner tubes and mixed well using a vortex mixture for 10 minutes, followed by shaking for 72 h in an isothermal mechanical shaker with 100 rpm at 40°C ± 2°C. After 72 h, the samples were centrifuged for 10 minutes at 5000 rpm. The clear supernatant was then collected, filtered through 0.22 µm PTFE syringe filters, and diluted with the required amount of methanol, followed by the mobile phase to determine drug concentration using the developed HPLC method.

2.5.2 Screening of surfactants

In the current study, the surfactant was selected following two criteria such as their solubilizing capacity for the DCN and ACE, and miscibility with the selected oil. Based on the solubilizing capacity for DCN and ACE, three non-ionic surfactants,

namely, Tween 20, Kolliphor-EL, and Tween 80, were chosen and further evaluated for their emulsifying ability with the selected oil mix. The miscibility of surfactant with the selected oil mix, Triacetin: Capmul MCM C8 (2:1)), was assessed by both the % transmittance and titration method ^(79,82), surfactant was mixed with Triacetin: Capmul MCM C8 (2:1) in a 1:1 ratio and mildly heated (at 40-45°C) for thirty seconds. Further, 50 mg from the resultant mixture was taken and completed to 50 mL with double distilled water to get the fine emulsion. Count the number of inversions for homogenization and time of emulsification. The emulsion was allowed to stand for 2 h before testing the % transmittance (T) at 650 nm using double distilled water as blank. In the titration method ^(57,83), 2.5 mL of 15% w/w surfactant aqueous solution was titrated with oil mix (Triacetin: Capmul MCM C8 (2:1)). The oil mix was added in an increment of 5 µL with vigorous vertexing. The oil mix was added continuously until the mixture took on a turbid or hazy appearance. The surfactant solution with the best miscibility or emulsification capacity for the Triacetin: Capmul MCM C8 (2:1) was optimized for 24 h. After that, it was examined for phase separation or turbidity.

2.5.3 Screening of co-surfactants

Further, different co-surfactants were also examined for NE formulation. Based on the solubilizing capacity for DCN and ACE, four co-surfactants, namely Transcutol P, PEG (300, 400, and 600), were selected and evaluated for their miscibility/ emulsifying ability with selected surfactant and oil. The chosen co-surfactant, Kolliphor-EL, and Triacetin: Capmul MCM C8 (2:1) were mixed in a 1:2:3 ratio, respectively, and the miscibility/ emulsifying ability was assessed by the % transmittance method in a similar manner as that of the surfactant ^(79,82).

2.5.4 Selection of surfactant: co-surfactant mixture ratio using a pseudo-ternary phase diagram

Based on the results from saturation solubility of both drugs, % transmittance, and ease of miscibility/emulsification capacity, Triacetin: Capmul MCM C8 (2:1 w/w) and Kolliphor-EL: Transcutol P were selected as oil phase and surfactant: co-surfactant mixture (Smix), respectively. To determine the NE/ microemulsion (ME) area and finalize component concentration ratios, pseudo-ternary phase diagrams were constructed. For this, the different Smix (surfactant and co-surfactant mixture) ratios were prepared by mixing Kolliphor-EL and Transcutol P in 1:0, 1:1, 1:2, 1:3, 1:4, 4:1, 3:1, and 2:1 mass ratio. The Triacetin: Capmul MCM C8 (2:1 w/w) and each Smix ratio were combined in the mass ratio of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1. The resultant oil: Smix mixture was slowly titrated with water until turbidity. The amount of water added was calculated. The pseudo-ternary phase diagram was plotted, and the NE/ME area was calculated using OriginPro v 8.1 software ^(54, 84–87).

2.6 Optimization of NE using mixture design

The NE formulations were optimized by mixture experimental design using the Design-Expert (V 8.0.6, StatEase, Inc., USA) software. Mixture design, a specific variant of response surface methodology, is an effective approach for ascertaining the amounts of variables (ingredients) within a mixture. In the present study, the NE formulations were optimized using a 3-factor, 2-level D-optimal mixture design to investigate the relationship between three formulation components and two responses. The main reason this design was selected is because it minimizes the total variance of the anticipated regression coefficient by optimizing the value of the information matrix's determinant. D-optimal designs have fewer experimental runs than conventional factorial or fractional factorial designs, resulting in reduced

experimental time and costs. Following the established NE region in the ternary diagram, a Smix (Kolliphor-EL: Transcutol P) in a 1:1 ratio was chosen. Oil mix (A), Smix (B), and water (C) were chosen as independent factors, while globule size (GS) and percent transmittance (%T) at 650 nm were measured as response variables. The lower and upper limits of independent variables and responses and design constraints are presented in Table 3 ^(65,88–91). According to the experimental run suggested by a design expert, 16 formulations loaded with 0.5%w/w of DCN and 1 % w/w of ACE were prepared by spontaneous emulsification method in a randomized order and characterized. The GS and %T of the 16 formulations were fitted to a D-optimal mixture design and analyzed. Model validity was assessed using ANOVA. A numerical method identified the best formulation with minimum GS (nm), maximum %T, and maximum composite desirability. These suggested optimal experimental conditions with the smallest GS and highest T (%) were chosen as the optimized NE formulation. Randomly, two different NE formulations suggested by the numerical method were prepared and characterized for GS and %T. The experimental values and predicted values were used to validate the polynomial model ⁽⁹²⁾.

Table 3. Variables and their design constraints in D-optimal mixture design.

Independent variables	Levels		Design constraints
	Low	High	
A: Oil mix, (Triacetin: Capmul MCM C8, 2:1 w/w)	10	20	$10 \leq A \leq 20$
B: Smix, (Kolliphor-EL: Transcutol P, 1:1 w/w)	25	35	$25 \leq B \leq 35$
C: Water	45	65	$45 \leq C \leq 65$
			$A+B+C = 100$
			$35 \leq A+B \leq 55$
Responses (R)	Goals		
R1, GS: Globule size (nm)	Minimum		
R2, T: Transmittance (%) at 650nm	Maximum		

2.7 Preparation of DCN-and ACE-loaded NE

Based on the findings of the saturation solubility study of DCN and ACE in individual and combined forms, the emulsification capacity of Smix, pseudo-ternary phase diagram analysis, and optimization using D-optimal mixture design, the optimal composition of DCN- and ACE-loaded NE was determined. The DCN- and ACE-loaded NE formulation was prepared using the spontaneous emulsification method^(93–95). The DCN (0.5% w/w) and ACE (1 % w/w) were dissolved in an appropriate quantity of N, N-dimethylacetamide. The prepared oil mix (Triacetin: Capmul MCM C8, 2:1 w/w) and Smix (Kolliphor-EL: Transcutol P, 1:1 w/w) were then slowly added to the drug solution under constant magnetic stirring. Subsequently, a sufficient quantity of double-distilled water was gradually added to the oily mixture under continuous magnetic stirring. The pH of the prepared formulation was adjusted from 5.7 to 6.00 using a 2% (v/v) aqueous triethanolamine solution and further stirred for 20 minutes at 2000 rpm to get a clear and transparent NE. Finally, the prepared NE was allowed to optimize for 24 h at room temperature before further characterization. In addition, an NE-placebo formulation (without DCN and ACE) was prepared using the same method as the optimized NE formulation for FTIR analysis.

2.8 Characterization of optimized DCN-and ACE-loaded NE

The DCN-and ACE-loaded NE was evaluated for the following parameters:

2.8.1 Globule size and electrokinetic potential analysis

The average GS and polydispersity index (PDI) of the NE formulations were determined using a Horiba Scientific SZ-100 (Z-type, V 2.40) nanoparticle and zeta (electrokinetic) potential analyzer. This instrument employs dynamic light scattering

to determine the zeta (electrokinetic) potential, which is calculated based on the particle's electrophoretic mobility. The aliquots of the NE were dispersed in HPLC-grade water in a 1: 30 ratio and then transferred to a 45x12.5x12.5 mm round-bottom quartz cuvette with four polished sides ⁽⁶⁵⁾.

2.8.2 Percentage transmittance, viscosity, and pH

For the % transmittance measurement, the 100-fold diluted NE formulation samples were analyzed using distilled water as blank on a UV-1800 spectrophotometer, and the percent transmittance was recorded at 650 nm. A previously calibrated digital pH meter (MK-VI, Systronics) and Brookfield viscometer (CAP 2000+, USA) with spindle IV at 100 rpm were used to determine the pH and viscosity, respectively, at 25°C ± 0.5°C ^(65,96–98).

2.8.3 Drug content analysis of the DCN-and ACE-loaded NE

For the drug content analysis, 250 mg of NE was dissolved in up to 25 ml of methanol by sonication for 5 minutes. A 2 mL aliquot of this solution was diluted with mobile phase to achieve final concentrations of 10 µg/mL of DCN and 20 µg/mL of ACE. The subsequent solution was further filtered through a 0.22 µm PTFE filter and analyzed in triplicate using the developed RP-HPLC method at 264 nm.

2.8.4 Physical stability of DCN-and ACE-loaded NE

The cycling stress and thermodynamic stability of the DCN-and ACE-loaded NE formulations were determined using centrifugation stress, freeze-thaw, and heating-cooling cycles. In the centrifugation test, the formulation stability was assessed by centrifuging the NE formulation at high speed, 3500 rpm, for half an hour and examined for any breaking of NE or sedimentation. To study the impact of

temperature variation, the NE formulation was further tested for six consecutive heating-cooling cycles. Each cycle involved incubation for 48 h at 4°C, followed by incubation for 48 h at 45°C. The formulation was observed for any physical changes after each cycle. Similarly, the formulation underwent three freeze-thaw cycles. Each cycle was frozen for 48 h at -21°C and then thawed at 25°C for 48 h. The formulation was inspected for physical changes after each freeze-thaw cycle ^(46,57,93,99,100).

2.8.5 *In vitro* permeation study of DCN-and ACE-loaded NE

The *in vitro* permeation studies of DCN and ACE from DCN- and ACE-loaded NE (NE-1 and NE-2) formulations were conducted using jacketed Franz diffusion cells (1.77 cm² diffusion area, PremeGear, USA, 06 cells) (Fig. 4). The permeation study was performed at two different pH conditions, namely phosphate-buffer saline (PBS) pH 5.7 and a mixture of PBS pH 6.8 and methanol (7:3) through a dialysis membrane-150 (LA401, 12,000-14,000 Daltons) (41,101–105). Media was filled into the receptor chamber (12 mL cell volume) and maintained at 37 ± 0.5°C with 100 rpm stirring. The dialysis membrane was hydrated with corresponding dissolution media for 12 h prior to use. Equivalent amounts of prepared NEs (containing 2.5 mg DCN and 5 mg ACE) were applied to the donor compartment. At specified intervals (0, 1, 2, 4, 6, 8, 10, 12, and 24 h), a 0.3 mL sample was taken out of the receptor compartment and replaced with an equal volume of fresh respective media in order to preserve sink conditions. Samples were diluted with methanol and mobile phase, filtered through a 0.22 µm PTFE filter, and analyzed in triplicate using the developed RP-HPLC method at 264 nm.

The *in vitro* permeation profiles of both nanoformulations at different pH conditions were compared. The closeness between the two permeation profiles was determined using the similarity factor (f₂). The formula for similarity factor (f₂):

$$f2 = 50 \cdot \log \left(\left[1 + \frac{1}{n} \sum_{t=1}^n (R_t - T_t)^2 \right]^{-0.5} \times 100 \right)$$

Where n= number of time points, R_t = permeation value of the reference at time t, and T_t = permeation value of the test at time t.

The higher similarity factor value (close to 100) indicates greater similarity between the two profiles, whereas the f2 value of less than 50 indicates significant differences between the two profiles⁽¹⁰⁶⁾.



Figure 4. Permeation study setup: Franz diffusion cell apparatus (PremeGear, USA) and RP-HPLC instrument (Shimadzu, Japan).

2.8.6 Morphological and structural behavior of optimized NE formulation

Transmission electron microscopy (TEM) (Tecnai T20, USA) and field-emission scanning electron microscopy (FE-SEM) (Supra 55, Germany) were used to examine the shape and surface morphology of globules of the optimized NE. For TEM analysis, two drops of the NE sample were deposited onto a copper grid coated with carbon. The sample was then dried before analysis under vacuum at an accelerating voltage of 120 kV. FE-SEM analysis employed a different preparation method. The NE sample was placed on a stem stub, followed by a sputter-coating of a

small layer of gold onto the sample under a vacuum. After that, the sample stub was put into the SEM chamber and run at 10 kV of accelerating voltage. Lastly, pictures at different magnifications were taken by scanning the material.

2.8.7 DSC and FTIR analysis of optimized NE

The FTIR and DSC analysis were performed on a DCN, ACE, the physical mixture of DCN and ACE with each excipient (Triacetin, Capmul MCM C-8, Kolliphor-EL, Transcutol P, DMA), and optimized NE formulation to determine the drug-excipients compatibility and to confirm the complete solubilization and incorporation of the DCN and ACE into the formulated NE (70,96). DSC analysis (Shimadzu DSC-TA 60, Japan) was performed in the 25°C to 420°C temperature range with 5°C/minute and 20 mL/min scanning rate and nitrogen atmosphere, respectively.

For the FTIR analysis of DCN and ACE, the potassium bromide (KBr) dispersion method (Shimadzu IR Affinity-1s) was utilized. Samples were prepared by thoroughly triturating 1 part of the drug with 99 parts of KBr. The scan range and resolution were set to 400-4000 cm^{-1} and 4 cm^{-1} , respectively. For liquid samples (drug-excipient physical mixtures and optimized NE) analysis using FTIR (Bruker alpha II), direct analysis was performed without sample preparation.

2.9 Preparation of DCN-and ACE-loaded NEG and gel

The optimized NE formulation was transformed into NEG using a suitable gelling agent. Based on the literature review, HPMC K15M was selected as a gelling agent^(58,107–110). To optimize the gelling agent, the different DCN- and ACE-loaded NEG formulations were prepared by direct dispersion method using HPMC K15M at varying concentrations (3%, 3.5%, 4%, and 4.5% w/w). The prepared NEG

formulations were visually observed, and the HPMC K15M with 4% w/w concentration was selected based on the desirable consistency, physical appearance, and viscosity. For further optimization of the NEG formulations to improve drug release, additional DCN- and ACE-loaded NEG formulations (NEG-1 to NEG-4) were prepared with and without dimethyl sulfoxide (DMSO) as a penetration enhancer using the direct dispersion method ^(111,112). An appropriate amount of DMSO (2.5-7.5% w/w), followed by HPMC K15M (4% w/w), was dispersed to the optimized NE and stirred continuously for up to 10 minutes at 1500 rpm to achieve a homogeneous gel. Finally, the pH was adjusted to 5.7 - 6.00 using a 2% (v/v) aqueous triethanolamine solution. The prepared NEGs were then allowed to rest for 24 hours for optimization. Plain gels with and without DMSO (2.5% w/w) were also prepared using HPMC K15M gel base to compare the permeation profiles of NEGs and plain gels. Table 4 summarizes the different topical DCN- and ACE-loaded formulation compositions. Furthermore, a NEG-placebo formulation (without DCN and ACE) was prepared using the same method as the optimized NEG formulation for FTIR analysis.

Table 4. Composition of DCN-and ACE-loaded NE, different NEG, and gel formulations.

Ingredients (%w/w)	Opt-NE (NE-1)	NEG-1 (without DMSO)	NEG-2 (with 2.5% DMSO)	NEG-3 (with 5% DMSO)	NEG-4 (with 7.5% DMSO)	Plain gel-1 (without DMSO)	Plain gel-2 (with 2.5% DMSO)
DCN	0.50	0.50	0.50	0.50	0.50	0.50	0.50
ACE	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Oil mix	13.34	13.34	13.34	13.34	13.34	---	---
Smix	31.77	31.77	31.77	31.77	31.77	---	---
HPMC K15M	---	4.00	4.00	4.00	4.00	4.00	4.00
DMSO	---	---	2.50	5.00	7.50	---	2.50
Water up to	100	100	100	100	100	100	100

2.10 Characterization of DCN-and ACE-loaded NEG and gel

2.10.1 Viscosity, spreadability, pH, and drug content analysis

The pH, and viscosity of the DCN-and ACE-loaded formulations were determined using a previously calibrated digital pH meter (MK-VI, Systronics) and Brookfield viscometer (CAP 2000+, USA) using spindle IV at 100 rpm, at 25°C± 0.5°C, respectively. The spreadability of the DCN-and ACE-loaded NEG and gel formulations was measured using the sandwich method. This method utilizes two glass plates. The first glass plate has a pre-marked circle at 15 cm diameter. The formulation was applied to the first glass plate in the proper amount (0.5 g). The second glass plate, which was unmarked, was then placed on top of the first. The upper plate was subjected to a 500-gm weight for a duration of one minute. Following this, the spread formulation's diameter on the lower plate was measured to determine its increase ^(32,113–115). The drug content analysis of formulated NEGs and plain gels was performed similarly to that of optimized NE formulation.

2.10.2 *In vitro* permeation study of DCN-and ACE-loaded NEG and plain gel

The *in vitro* permeation studies of DCN and ACE from DCN- and ACE-loaded NEGs and plain gel formulations were conducted similarly to that of the optimized NE (as described in section 2.8.5 above). The permeation profile of the different NEGs and plain gels were compared with the optimized NE to investigate the effect of the gelling agent and penetration enhancer. The gel formulation, which exhibits higher permeation, was chosen as the optimized formulation. Based on the permeation profile, NEG-2 with 2.5% DMSO was chosen as an optimized NEG (Opt. NEG).

2.10.3 *Ex vivo* release study of DCN-and ACE-loaded NEG and plain gel

Ex vivo permeation study of DCN and ACE from NEG-1 (without DMSO), NEG-2 (with 2.5% DMSO), and Plain gel-2 (with 2.5% DMSO) was performed using Wistar rats abdominal skin ⁽¹¹⁶⁾. The permeation study was performed at two different pH conditions, namely PBS pH 5.7 and a mixture of PBS pH 6.8 and methanol (7:3). Before being sacrificed, the rats were anesthetized, and the hairs on their abdominal skin were delicately removed with an electric clipper to prevent any harm to the skin. Subsequently, the rats were euthanized and fixed. Full-thickness skin from the abdomen was excised, and the adipose tissue was removed. The excised skin was hydrated with corresponding phosphate buffer to stabilize the membrane for 4 h before the experiment began. Afterwards, the excised rat skin was secured on the cell with the stratum corneum side towards the donor compartment. Further study was carried out in a similar manner to the *in vitro* study. To compare the skin permeability of different formulations, various parameters were estimated. Also, the skin deposition of DCN and ACE was investigated at the end of the release study ⁽¹¹⁷⁾. The skin samples were unclipped from the Franz diffusion cells, rinsed with respective PBS solutions three times, chopped, and then homogenized RQT-127A, Remi) with methanol. After that, the mixture was centrifuged (3000 rpm, 15 min). As previously described, the final aliquot was filtered (0.22 µm PTFE) and analyzed for DCN and ACE concentration by HPLC.

2.10.4 DSC and FTIR analysis of optimized DCN-and ACE-loaded NEG

DSC and FTIR analyses of the optimized NEG formulation were conducted following the same procedure used for the optimized NE. The DSC thermograms and FTIR spectra of the DCN, ACE, and the optimized NEG formulation were compared

to assess drug-excipient compatibility and to verify the complete solubilization and incorporation of DCN and ACE within the formulated NEG.

2.10.5 Stability study of optimized NEG

The optimized NEG and placebo formulation were stored in aluminum collapsible tubes (5g) at room temperature and $40^{\circ}\text{C} \pm 2^{\circ}\text{C}/75\% \pm 5\% \text{ RH}$ for a maximum of 3 months. Samples were routinely withdrawn at one-month intervals and assessed for visual appearance, viscosity, spreadability, pH, FTIR spectra, drug content, and drug permeation^(38,41,70).

2.11 *In vivo* evaluations of DCN-and ACE-loaded NEG

2.11.1 Experimental animals

Healthy male Wistar rats (160-220 g) were purchased from Invivo Biosciences Bengaluru, Karnataka, and Global Bioresearch Solution Pvt Ltd., Pune, Maharashtra, India. The rats were housed in cages with free access to food and water in a temperature-controlled ($22\text{-}25^{\circ}\text{C}$) room and maintained at a 12 h cycle of light and darkness. After a 14-day acclimatization period, the rats were used for an *in vivo* release study, skin irritation, and anti-inflammatory study. The experimental protocol (proposal no. 12 and 15) was approved by the Institute Animal Ethics Committee (IAEC) of KLE College of Pharmacy, Belagavi, Reg. No.221/Po/Re/S/2000/CPCSEA.

2.11.2 Skin irritation study

Wistar male rats were used in a skin irritation test to assess the dermal toxicity and skin sensitization potential of the developed NEG^(118–120). The hairs on the rat's dorsal surface were closely clipped without damaging the skin 24 h before the

experiment. After 24 h, each rat's skin was examined for any abrading, and only rats with normal skin were used for further study. A total of 09 rats were categorized into (n=3): Group 1 – normal control (without any application), Group 2 – positive control (treated with standard irritant; 0.8% v/v formalin solution), and Group 3 – optimized NEG treated group. The third-group rats' hairless skin received a 2 cm² application of the optimized NEG. After applying the formulation, the experimental animals' skin surfaces were inspected for one, twenty-four, forty-eight, and seventy-two hours, and rated for any dermal reaction, such as erythema or edema. The mean erythema and edema scores were recorded based on the degree of severity, as per the Draize scale⁽¹¹⁷⁾. The application of formalin solution and optimized NEG was continued for 7 days. After this, the experimental animals were sacrificed, skin sections were collected and processed for histology study using hematoxylin and eosin (H&E) staining to check the toxicity of the developed NEG formulation⁽¹¹⁷⁾.

2.12 Bioanalytical method for estimation of RH, ACE, and DL

For the pharmacokinetic study of the developed NEG formulation, the accurate quantification of RH (a primary metabolite of diacerein), ACE, and DL (an active metabolite of ACE) at low concentrations in plasma is essential. Hence, a simple, fast, and sensitive HPLC method was developed and validated for synchronous quantification of RH, ACE, and DL in rat plasma using fenofibric acid (FA) as an internal standard (IS). The method was optimized using experimental design to achieve short run time and high sensitivity.

2.12.1 Instrumentation and chromatographic condition

The HPLC instrumentation employed in this analysis was identical to that described in the previously detailed *in vitro* analytical method in section 2.4.1. The

plasma component was separated using a mobile phase consisting of ACN and phosphate buffer pH 3.5 (0.1% v/v OPA) in the proportion of 60: 40 v/v on a Phenomenex Luna C₁₈ column (100A, 5 µm, 250 x 4.6 mm size). Triethylamine was used to adjust the pH of the buffer to 3.5. The separation was performed at a 1.0 mL/min FR, with a 20 µL injection volume and 35°C column oven temperature. The detection of analytes was carried out at 264 nm.

2.12.2 Preparation of standard and working solutions

Individual standard stock solutions of RH (100 µg/mL), ACE (200 µg/mL), DL (200 µg/mL), FA (100 µg/mL), and mixed standard stock solution (100 µg/mL of RH, 200 µg/mL of each of ACE, and DL) were prepared separately in methanol. A mixed working standard solution containing RH (2 to 10 µg/mL), ACE (4 to 20 µg/mL), and DL (4 to 20 µg/mL) was prepared by mixing an appropriate amount of mixed standard stock solution of RH, ACE, and DL in a separate 10 ml volumetric flask and further diluted with the mobile phase.

2.12.3 Preparation of calibration curve and quality control plasma samples

Rhein, ACE, and DL were extracted from the plasma samples using the protein precipitation technique. In a 2 ml Eppendorf tube, a 50 µL aliquot of rat plasma was spiked with an appropriate amount of mixed standard stock and working solutions of analytes and 100 µL of FA (IS) (100 µg/mL), followed by vortexing for 2 minutes. After that, the mixture was precipitated with 100 µL of ACN and an appropriate quantity of methanol (quantity sufficient to produce 2 mL). The mixture was vortexed for 2 minutes before being centrifuged at 12000 rpm for 15 minutes at 4°C. The clear supernatant was then collected, filtered through 0.22 µm PTFE syringe filters, and diluted with the required amount of mobile phase to produce calibration

standard solutions with concentrations of 25 to 3000 ng/mL for RH, 50-6000 ng/mL for ACE and DL, respectively. A plasma sample solution containing (25, 75, 500, and 1000 ng/mL) RH, (50, 150, 1000, and 2000 ng/mL) ACE, and (50, 150, 1000, and 2000 ng/mL) DL were also prepared and used as a lower limit of quantification (LLOQ), low (L), mid (M) and high (H) quality control samples (QCs), respectively.

2.12.4 Method development and optimization

The mobile phase of the earlier developed analytical method was used for the quantification of analytes in rat plasma using FA as an IS. However, it was found that the ACE and FA peaks overlapped. Hence, we developed a new method using FFD and Box-Behnken design (BBD). The FFD screening was performed in a manner similar to that described for the *in vitro* analytical method in section 2.4. In the FFD screening ⁽⁷⁵⁾, a total of eight experiments were carried out in randomized order to study the effects of four independent variables (pH of OPA buffer, flow rate, % of ACN, and temperature) at two levels and four response variables: RH-ACE peak resolution (R1), ACE-FA peak resolution (R2), FA-DL peak resolution (R3), and RT-DL retention time. The variables that were found significant in the screening study were used for further optimization by using a three-factor BBD design ^(121,122). FR (0.8-1.2 mL/min), ACN content (55-65 % v/v), and pH of 0.1%v/v OPA buffer (pH 3-4) were selected as independent variables, whereas peak resolution between RH-ACE (R1), peak resolution between ACE-FA (R2), peak resolution between FA-DL (R3), and RT of DL was considered as responses. A total of 17 experiments suggested by BBD were performed, and the responses were recorded. The graphical and regression analyses were performed on the obtained data. Based on the desirability, the optimal experimental conditions were selected and further used for method validity.

2.12.5 Method validation

The proposed method was validated as per the U.S. Food and Drug Administration (USFDA) guidelines (2018) for bioanalytical method validation. For the linearity and sensitivity study, the individual working standard solution (25-3000 ng/mL for RH, 50-6000 ng/mL for ACE and DL) was analyzed in triplicate. The standard curve of the peak area ratio of analyte/IS vs. nominal concentration of analyte was plotted separately for each analyte. The proposed method's sensitivity was assessed by evaluating the lower limit of quantification (LLOQ), the smallest amount of an analyte on the calibration curve that can be quantifiable with sufficient precision and accuracy. The extraction efficiency of the analytical method was evaluated by calculating the percentage recovery of RH, ACE, and DL from three QC samples (LQC, MQC, and HQC). The recovery was assessed by comparing the peak area of each analyte from extracted mixed plasma samples versus the mean peak area of corresponding extracts of blank plasma spiked with the analyte post-extraction. The proposed analytical method's intra-day and inter-day accuracy and precision were established using four QCs (LLOQ, LQC, MQC, and HQC levels) with IS (500 ng/mL). Five replicates of each QC level were injected and analyzed in a single run on the same day and three successive days. The mean concentration, % coefficient of variation (CV), and accuracy (percent bias) of the assay within-run and between runs were calculated. The short-term (bench-top), freeze-thaw, and long-term stability study were performed on low and high QC samples of RH, ACE, and DL by storing at room temperature for 24 h, at -20°C for 72 h, and -20°C for 15 days, respectively. The mean concentration, % CV, and accuracy were determined after a specific time interval. In all storage conditions, the acceptance criteria for the accuracy at each level were within $\pm 15\%$ of the nominal concentration.

2.13 *In vivo* release study of optimized DCN- and ACE-loaded NEG and marketed tablet

An *In vivo* release / pharmacokinetic study of the optimized NEG and marketed tablet formulation was performed in Wistar rats. The developed bioanalytical HPLC method was used to evaluate the pharmacokinetics of the formulations in Wistar rats. A total of 12 rats (160–210 g) were randomly allocated to two groups (n=6): group 1: marketed tablet (Dycerin A tablet) and group 2: optimized NEG. In the case of group 2, 24 h before the experiment, the rats were anesthetized, and the hairs on the rat's dorsal surface were closely clipped without damaging the skin. After overnight fasting, group-1 Wistar rats were dosed orally with a Dycerin A tablet (DCN: 15 mg/kg and ACE 30 mg/kg of body weight) ⁽²⁶⁾. An appropriate quantity (DCN: 15 mg/kg and ACE 30 mg/kg of body weight) of optimized NEG formulation was applied to the hairless skin of 2nd group of animals, covering an area of 4 cm². After a single dose administration, blood samples were collected from the retro-orbital vein at given time intervals in K3 EDTA tubes. The plasma was separated immediately using centrifugation at 12000 rpm at 4°C for 15 minutes and stored at -20°C. An appropriate quantity of standard stock solution of FA was added to a 50µL aliquot of rat plasma and processed as per plasma sample preparation. The plasma samples of each time interval were injected and analyzed in triplicate. Mean plasma concentration vs. time curves for RH, ACE, and DL were plotted and analyzed using a non-compartmental analysis program with WinNonlin software (version 8.3.4.295). All pharmacokinetic parameters were reported as mean ± standard deviation (SD). Statistical analysis utilized GraphPad Prism (version 8.0.2) at a 95% confidence level. Pharmacokinetic parameters of NEG were compared using both parametric unpaired t-tests and non-parametric tests (Mann-Whitney and Wilcoxon Signed Rank Test).

2.14 *In vivo* anti-inflammatory efficacy of DCN-and ACE-loaded NEG and marketed tablet formulation

The anti-inflammatory activity of optimized NEG at low and high doses, plain gel (with 2.5% DMSO), and marketed tablet formulations were assessed using the hind paw edema method ^(35,115,123). The hairs on the rat's dorsal surface were closely clipped without damaging the skin 24 h before the experiment. All 30 Wistar rats were fasted overnight and weighed before the experiment commenced. A total of six groups were prepared, with six rats in each group. The carrageenan suspension was prepared by dispersing 80 mg of carrageenan in up to 8 mL of normal saline under continuous stirring. The prepared carrageenan suspension was used to induce hind paw edema in the right hind paws of the rats from groups II to VI through subplantar injection (100 μ L). The Group-I rats served as the normal control, injected subplantarly a 100 μ L of saline solution. Group II rats served as positive control and were kept untreated. Half an hour before the carrageenan challenge, group III received a single dose of a Dycerin A tablet orally (tablet blend dispersed in 0.5% sodium carboxymethylcellulose (CMC)), group IV received plain gel (containing 2.5% DMSO), and Groups V and VI received optimized NEG at low dose and high doses, respectively. Rats in groups II, IV, and VI received doses of 15 mg/kg of DCN and 30 mg/kg of ACE ^(26,124), while group V rats received optimized NEG with low doses of DCN (7.5 mg/kg) and ACE (15 mg/kg). Paw volume was measured at 0 hr and up to 24 h post-carrageenan or normal saline injection using a digital plethysmometer (PLM-01 Plus). The difference in the right and left paw volume of each rat (representing an increase in paw edema volume) was calculated at each time point, and the graph was plotted for the difference in paw volume vs. time for each group. The data were analyzed statistically using two-way ANOVA and post hoc Tukey's

multiple comparisons tests. Also, the % inhibition of paw volume between drug (oral/transdermal) treated and positive control groups at a particular time was calculated as follows:

$$Inhibition(\%) = \left(\frac{V_{Positive\ control\ group} - V_{Test\ group}}{V_{Positive\ control\ group}} \right) \times 100$$

Where, $V_{Positive\ control\ group}$ and $V_{Test\ group}$ denote an increase in paw volume in the carrageenan-induced group and treated group at corresponding times, respectively (124,125).

2.15 Evaluation of anti-osteoarthritis efficacy of optimized DCN-and ACE-loaded NEG and marketed tablet formulation

2.15.1 Experimental design and induction of OA

The anti-osteoarthritis efficacy of DCN-and ACE-loaded NEG formulation was studied on the OA-induced male Wistar rat model. Osteoarthritis was induced in rats by intra-articular injection of sodium monoiodoacetate (MIA) (64,126–128). Monoiodoacetate, a glyceraldehyde 3-phosphate dehydrogenase enzyme inhibitor, interrupts glycolysis, leading to chondrocyte death and cartilage degeneration. This model replicates human OA, characterized by cartilage lesions, proteoglycan matrix loss, and functional joint impairment (126,127,129). For this study, 75 rats weighing 200-220 grams were used. Healthy male Wistar rats were purchased from Invivo Biosciences Bengaluru, Karnataka, and Global Bioresearch Solution Pvt. Ltd., Pune, Maharashtra, India. The rats were housed in cages with free access to food and water in a temperature-controlled (22-25°C) room and maintained at 12h cycle of light and darkness. After a 14-day acclimatization period, the rats were used for further study. The experimental protocol (proposals no. 12 and 15) was approved by the Institute

Animal Ethics Committee (IAEC) of KLE College of Pharmacy, Belagavi, Reg. No.221/Po/Re/S/2000/CPCSEA. Twenty-four hours before the commencement of the experiment, animals were anesthetized, and the knee hair was carefully clipped without skin damage. OA was induced into the right knees of 60 rats by injecting 30 μ L of a 3.33%w/v sodium MIA solution in saline using a 0.5-inch, 27-gauge needle (Fig.5) ^(126,128,129). The left knee was used as a control. A normal group of 15 rats (n=5) received intra-articular injections of 30 μ L of normal saline solution in both knees. The rats were then categorized into six groups (Table 5) as follows:

Table 5. Animal grouping and treatment details (study design) for *in vivo* anti-OA study.

Group no.	Group and treatment details	Number of rats required
I	Negative (normal) control group: Received intra-articular injection of 30 μ L of normal saline solution in both knees.	15
II	MIA-treated group: (Untreated OA group) OA-induced by injecting intra-articularly sodium MIA in the right knee. OA will be induced 2 weeks after the MIA injection.	18
III	NEG (low dose) treated group: OA-induced rats were treated with optimized NEG (150 mg bid). (Dose: 7.5 mg/kg of DCN and 15 mg/kg of ACE)	12
IV	NEG (high dose) treated group: OA-induced rats were treated with optimized NEG (300 mg bid). (Dose: 15 mg/kg of DCN and 30 mg/kg of ACE).	12
V	Plain gel treated group: OA-induced rats were treated with plain gel (with 2.5% DMSO) (300 mg bid). (Dose: 15 mg/kg of DCN and 30 mg/kg of ACE).	06
VI	Marketed tablet treated group: OA-induced rats were treated with a marketed tablet (Dycerin A Tab). (Dose: 15 mg/kg of DCN and 30 mg/kg of ACE).	12

The OA induction period was 2 weeks post MIA injection ^(126,128,129). Treatment with optimized NEG, plain gel, and the marketed tablet began two weeks after the MIA injection and continued for further five weeks. The OA-induced rats in group II were kept untreated, whereas the OA-induced rats in groups III and group IV received optimized NEG at low and high doses, respectively. Group V rats with induced OA were treated with plain gel. The optimized NEG and plain gel were applied twice daily to the osteoarthritic (right) knee. Group VI, with induced OA, received a single dose of a Dycerin A tablet orally (tablet blend dispersed in 0.5% sodium CMC). Due to the unavailability of a commercial transdermal medication containing both drugs, we used a marketed tablet formulation as a comparator in this anti-OA study of the developed NEG formulation ⁽⁶⁴⁾. The high dose of optimized NEG was equal to the oral dose of DCN and ACE. Rats in groups IV, V, and VI received doses of 15 mg/kg of DCN and 30 mg/kg of ACE ^(26,124), while group III rats received optimized NEG with low doses of 7.5 mg/kg of DCN and 15 mg/kg of ACE. Throughout the experiment, the thickness of the right (osteoarthritic) knee after treatment was measured ⁽⁶⁴⁾. For thickness measurements, six animals from each group (except the normal group where n=5), scheduled for sacrifice on the 49th day (end of the experimental period), were used. These animals were identified by tail-marking with different colors using a permanent marker and housed in a separate cage. The thickness of the right (osteoarthritic) knee was measured weekly for five weeks following treatment using a calibrated vernier caliper. The relationship between the changes in knee thickness among the groups was determined using two-way ANOVA multiple comparison and post hoc Tukey's multiple comparison test.

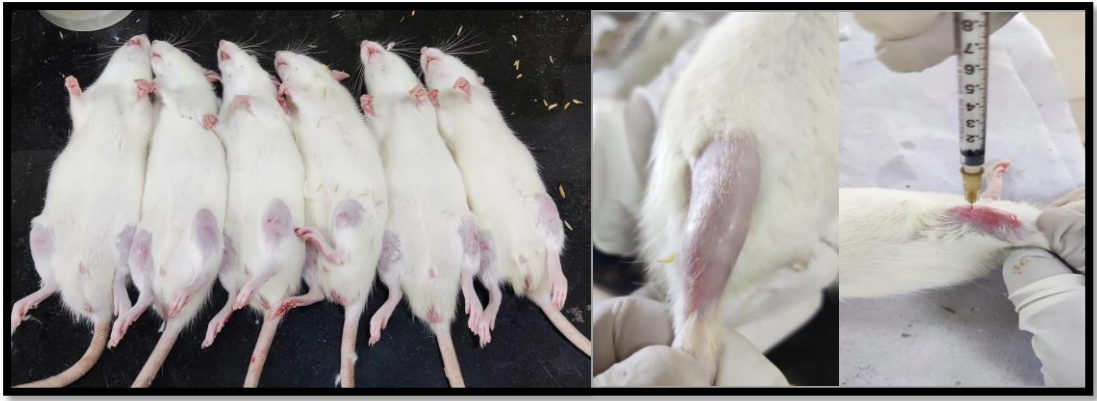


Figure 5. Images depicting anesthetized rats and the intra-articular injection of MIA solution.

2.15.2 Histological staining and scoring

After 2 weeks of post-MIA injection, five rats from Group I and six rats from Group II were sacrificed to observe the joint deformities. Six rats from each group (Except group V) were decapitated after 3 weeks and 5 weeks of treatment. In the case of group V, the rats were sacrificed after 5 weeks of treatment. The entire right knee joints were removed by carefully dissecting away the surrounding soft tissues. All the samples were fixed in 10 % formalin, followed by decalcification in 20% formic acid for 15 days and 10% nitric acid for 2 days. After decalcification, the samples were dehydrated using 20% formalin for 4 h, followed by ascending ethanol concentrations (70%,80%, 95%, and absolute alcohol). After the dehydration, samples were immersed in a clearing agent (xylene) for 2 h and then infiltrated with paraffin wax for 4 hrs. After infiltration, the samples were embedded in paraffin wax to form a paraffin block using tissue mold. The tissue sections (7 μ m thick) were prepared by cutting a paraffin block using a Leica RM2245 rotary microtome. The sections were stained with three different dyes: Hematoxylin and Eosin (H & E), Toluidine blue (Sigma-Aldrich), and Safranin-O (TCI chemicals, Tamilnadu, India) staining for the general histological evaluation ^(128,130).

Hematoxylin and eosin staining gives the cell nuclei a blue-purple color and a pinkish tint to the cartilage matrix, exhibiting a bluish shade in regions with elevated proteoglycan concentration. Toluidine blue and Safranin O, both cationic dyes, are employed to stain glycosaminoglycans and proteoglycans within the cartilage matrix. Toluidine blue imparts a dark violet hue to the cartilage matrix and nuclei, whilst the cytoplasm and other tissue components exhibit varying hues of pale blue. Safranin O imparts an orange-to-red hue to the cartilage matrix, whilst the adjacent bone and cytoplasm exhibit a green-to-gray-green coloration ⁽¹³⁰⁾. The prepared slides were observed under the CX33 Olympus biological microscope (Olympus Corporation, Shinjuku-Ku, Tokyo, Japan) at the Department of Pathology, JNMC, KAHER, Belagavi. The images were taken and analyzed using the Jenoptik Gryphax® microscope camera and Jenoptik Gryphax® software (Jenoptik AG, Germany). Each slide section was scored using a previously published modified Mankin grading system ^(64,128,130) for the assessment of cartilage structure, cellular abnormalities, and matrix staining in joint sections, as summarized below (Fig. 6):

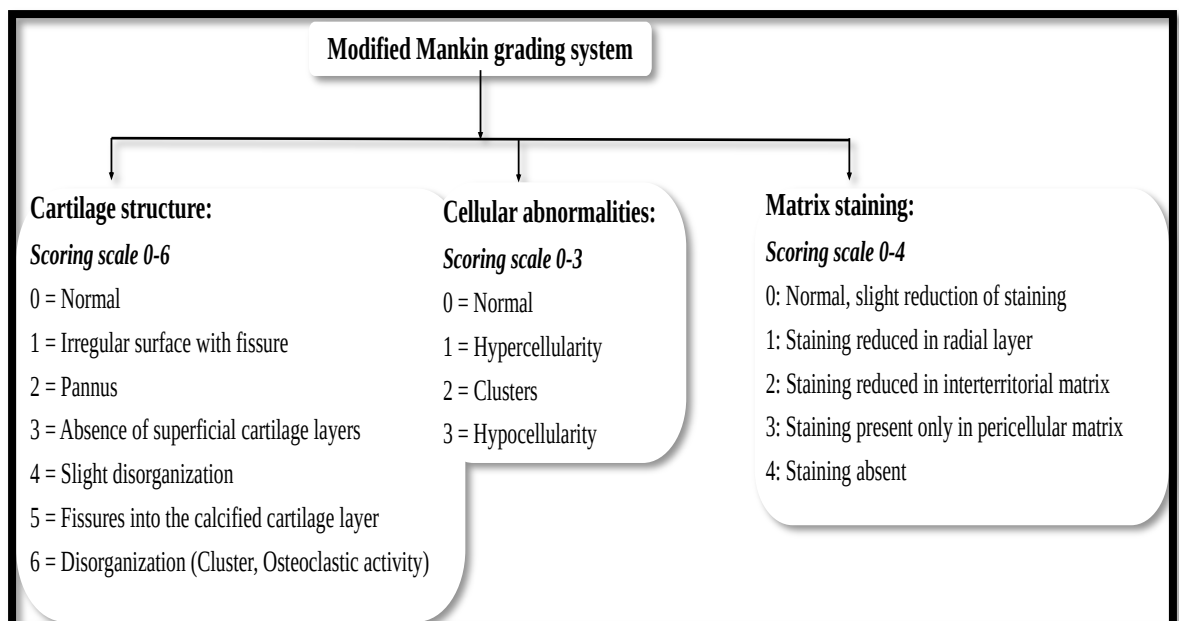


Figure 6. Schematic representation depicting the modified Mankin grading system for osteoarthritis.

The aggregate score for each group was computed and represented as the mean. An observer from the pathology department, unaware of the group allocations (control versus experimental), independently assessed the sections. Statistical differences among experimental groups were evaluated using nonparametric one-way analysis of variance (ANOVA).

3. STATISTICAL ANALYSIS

OriginPro v8.1 software (OriginLab Corporation, USA) was utilized to develop pseudoternary phase diagrams and determine the optimal NE components ratio. Subsequently, Design-Expert (V 8.0.6, StatEase, Inc., USA) software facilitated the optimization of NE. The validity of the developed model was assessed through ANOVA. The statistical analysis of *in vitro*, *ex vivo*, and *in vivo* pharmacodynamic and pharmacokinetic studies were conducted with DD solver, PK solver software, PCP DISSO V3 Software, and GraphPad Prism (V 8.0.2, GraphPad Software Inc., USA) software.

4. RESULTS

4.1 Preformulation studies

4.1.1 Identification and characterization of API

Aceclofenac was observed as a white powder, while DCN appeared as a yellow powder. Figure 7a-b and Figure 8a-f present the absorption maxima, DSC thermograms, FTIR spectra, and particle size distribution curves of ACE and DCN, respectively. The absorption maxima ($\lambda_{\max}$) of ACE and DCN were confirmed at 275 nm and 256 nm, respectively, aligning with previously reported literature^(68,69).

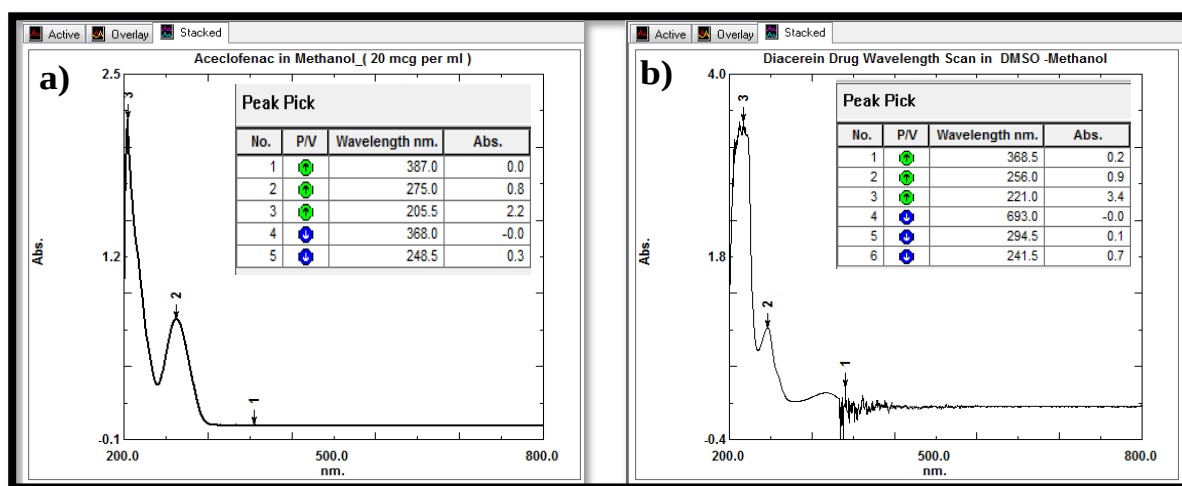


Figure 7. Absorption spectra and maxima of a) ACE in methanol, and b) DCN in DMSO: Methanol (10:90).

DSC thermogram (Fig. 8a-b) exhibited sharp endothermic peaks for ACE and DCN at 155.40°C and 260.82°C, respectively, relating to their melting points. These results confirmed the crystalline nature of both drugs, which is consistent with prior findings^(131,132). The FTIR spectrum (Fig. 8c-d) of ACE in KBr displayed characteristic absorption bands at approximately 3320, 1772, 1453, 1439, 1419, and 750 cm^{-1} , while DCN exhibited maxima at approximately 3070.81, 2986.9, 1669.46, 1463.07, 1369.52, and 1028.1 cm^{-1} , consistent with previous reports^(131,132). Aceclofenac and DCN exhibited average particle sizes of 290.4 nm and 735 nm, respectively (Fig. 8e-f).

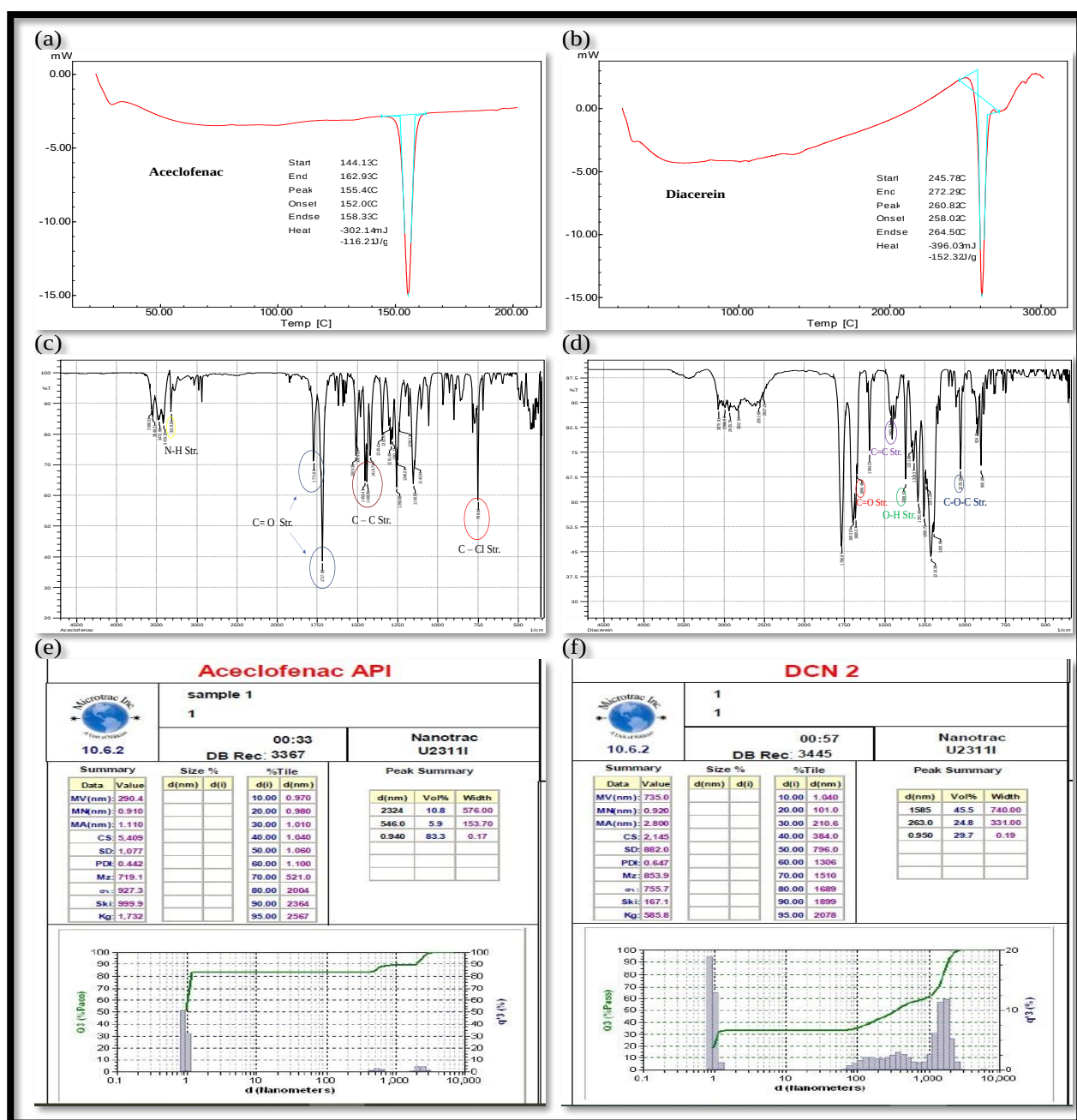


Figure 8. DSC thermograms, FTIR spectra, and particle size of ACE (a, c, and e) and DCN (b, d, and f).

4.1.2 DCN, ACE, and excipients compatibility study

The DCN, ACE, and excipients compatibility study results, summarized in Table 6, which showed no significant changes in the DCN, ACE, mixture of DCN and ACE, and physical mixture of combined drugs with excipient under any of the tested storage conditions.

Table 6. Drug: excipients compatibility study result based on the visual observation.

Sr. No.	Physical admixture	Initial description	2 - 8°C	25 ± 2°C/ 60 ± 5% RH	40 ± 2°C / 75 ± 5% RH	
			1 Month	1 Month	15 Days	1 Month
1	Diacerein (DCN)	Yellow powder, free-flowing, no aggregates.				
2	Aceclofenac (ACE)	White to almost white powder, free-flowing, no aggregates.				
3	DCN + ACE (Drug mixture)	Light yellow powder, free-flowing, no aggregates				
4	DCN +ACE + Capmul MCM C8	Very light yellow	NC	NC	NC	NC
5	DCN +ACE + Capmul PG-12	Very light yellow	NC	NC	NC	NC
6	DCN +ACE + Castor oil	Very light yellow	NC	NC	NC	NC
7	DCN +ACE + Labrafil M 1944CS	Very light yellow	NC	NC	NC	NC
8	DCN +ACE + Labrafac PG	Very light yellow	NC	NC	NC	NC
9	DCN +ACE + Triacetin	Very light yellow	NC	NC	NC	NC
10	DCN +ACE + Kolliphor-EL	Light yellow	NC	NC	NC	NC
11	DCN +ACE + Labrasol	Transparent/Clear	NC	NC	NC	NC
12	DCN +ACE + Tween 20	Very light yellow	NC	NC	NC	NC
13	DCN +ACE + Tween 60	Transparent	NC	NC	NC	NC
14	DCN +ACE + Tween 80	Very light yellow	NC	NC	NC	NC
15	DCN +ACE + PEG 200	Transparent	NC	NC	NC	NC
16	DCN +ACE + PEG 300	Transparent	NC	NC	NC	NC
17	DCN +ACE + PEG 400	Transparent	NC	NC	NC	NC
18	DCN +ACE + PEG 600	Transparent	NC	NC	NC	NC
19	DCN +ACE + Transcutol P	Very light yellow	NC	NC	NC	NC
20	DCN +ACE + Propylene glycol	Transparent	NC	NC	NC	NC

NC: No change (in color/ physical appearance), RH: Relative humidity

This finding suggests good physical compatibility between the DCN, ACE, and the tested excipients. Amongst all excipients, further screening of surfactant, co-surfactant, and oil was performed using saturated solubility and miscibility or emulsification ability study. The selected excipients compatibility study was further confirmed by conducting additional studies using analytical techniques like DSC and FTIR. A detailed discussion of DSC and FTIR analyses of ACE, DCN, physical mixture, and the proposed formulation is provided in “section 4.6.4: DSC and FTIR analysis of DCN, ACE, physical mixture, optimized NE, and NEG.”

4.2 Analytical method for synchronous estimation of DCN and ACE

4.2.1 Optimization of chromatographic method

Screening of the mobile phase was carried out to achieve the optimum chromatographic parameter with the best separation of four analytes. Initially, different mobile phases consisting of water, phosphate buffer (0.01%, 0.1%, and 0.2% v/v OPA) pH 2 to 5 adjusted with TEA, and formic acid buffer (0.1% v/v) were tried with methanol and ACN in different ratios. Phosphate buffer (0.1% v/v OPA) demonstrated a superior peak shape compared to formic acid buffer and water. Subsequent evaluation of different combinations of phosphate buffer pH 3 (0.1% v/v orthophosphoric acid (OPA)) with methanol and ACN revealed that a mobile phase composed of phosphate buffer pH 3 (0.1% v/v OPA) and ACN provided optimal peak shape with baseline separation and acceptable retention times. Based on the results of the FFD, followed by statistical analysis (ANOVA) and numerical optimization using CCD, to achieve $TP\text{-}DCN \geq 8300$, $R1 \geq 6.3$, $R3 \geq 2$, and $RT\text{-}DLS = 9.2$, the software recommended optimal experimental conditions: a flow rate of 1 mL/min, a buffer pH of 3, and an acetonitrile content of 60%, with maximum desirability of 0.740 (Fig. 9).

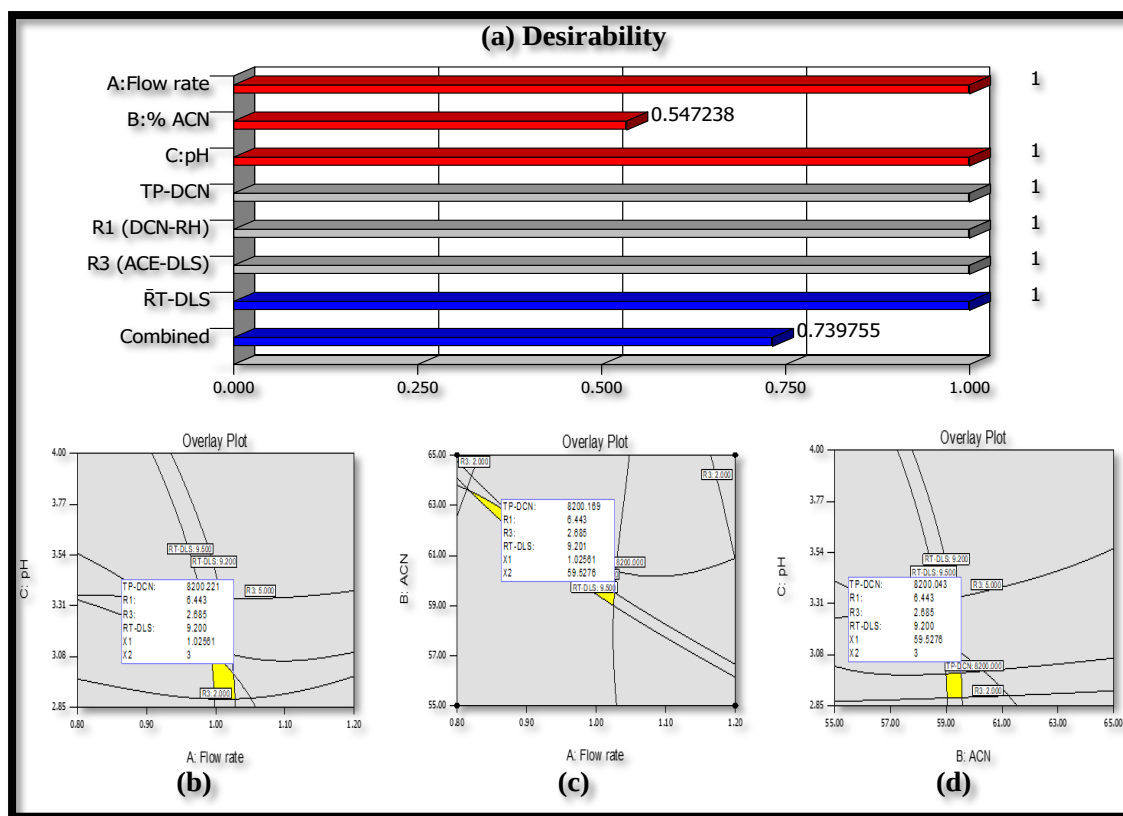


Figure 9. Desirability bar graph (a) for all individual factors, responses, and combination of all responses and overlay plot (b-d) of the selected responses.

4.2.2 Method validation

The developed RP-HPLC method was validated according to the ICH guidelines. The chromatogram obtained from blank solution (methanol and mobile phase), mixed working standard solutions, individual drug solutions, commercial tablet dosage form (Dycerin A tablet and Hilin Ace tablet), placebo NEG (without DCN and ACE), developed NE and NEG formulation are shown in Fig.10. Diacerein, ACE, RH, and DLS were found to have retention times of 4.32 ± 0.15 , 5.77 ± 0.07 , 8.28 ± 0.20 , and 9.10 ± 0.18 min, respectively. Figure 10 demonstrated consistent retention times for all analytes without any co-elution with DCN, RH, ACE, or DLS peaks, confirming the method's specificity and selectivity. System suitability parameters were found within acceptable limits, as the % RSD for retention time and

peak area were less than 2% for all analytes. Calibration curves for all four analytes exhibited linearity within the range of 0.1-64 µg/mL, with regression coefficients exceeding 0.9998 (Fig.11). The LOD for DCN, RH, and DLS was determined to be 3 ng/mL, while it was 12 ng/mL for ACE. The LOQ values were 10 ng/mL for DCN, RH, and DLS, and 40 ng/mL for ACE. The average % recovery of all four analytes at each concentration level was found between 98 and 102 %. The developed method is determined to be precise and reliable as the % RSD of the assay for intra-day and inter-day precision was found to be less than 2.0. The developed method was successfully applied to assay commercial tablet dosage forms. The mean DCN content was determined to be $98.59 \pm 0.48\%$ and $104.88 \pm 0.07\%$ for Dycerin A and Hilin Ace tablets, respectively. The corresponding ACE content values were $100.88 \pm 1.67\%$ and $97.03 \pm 0.36\%$.

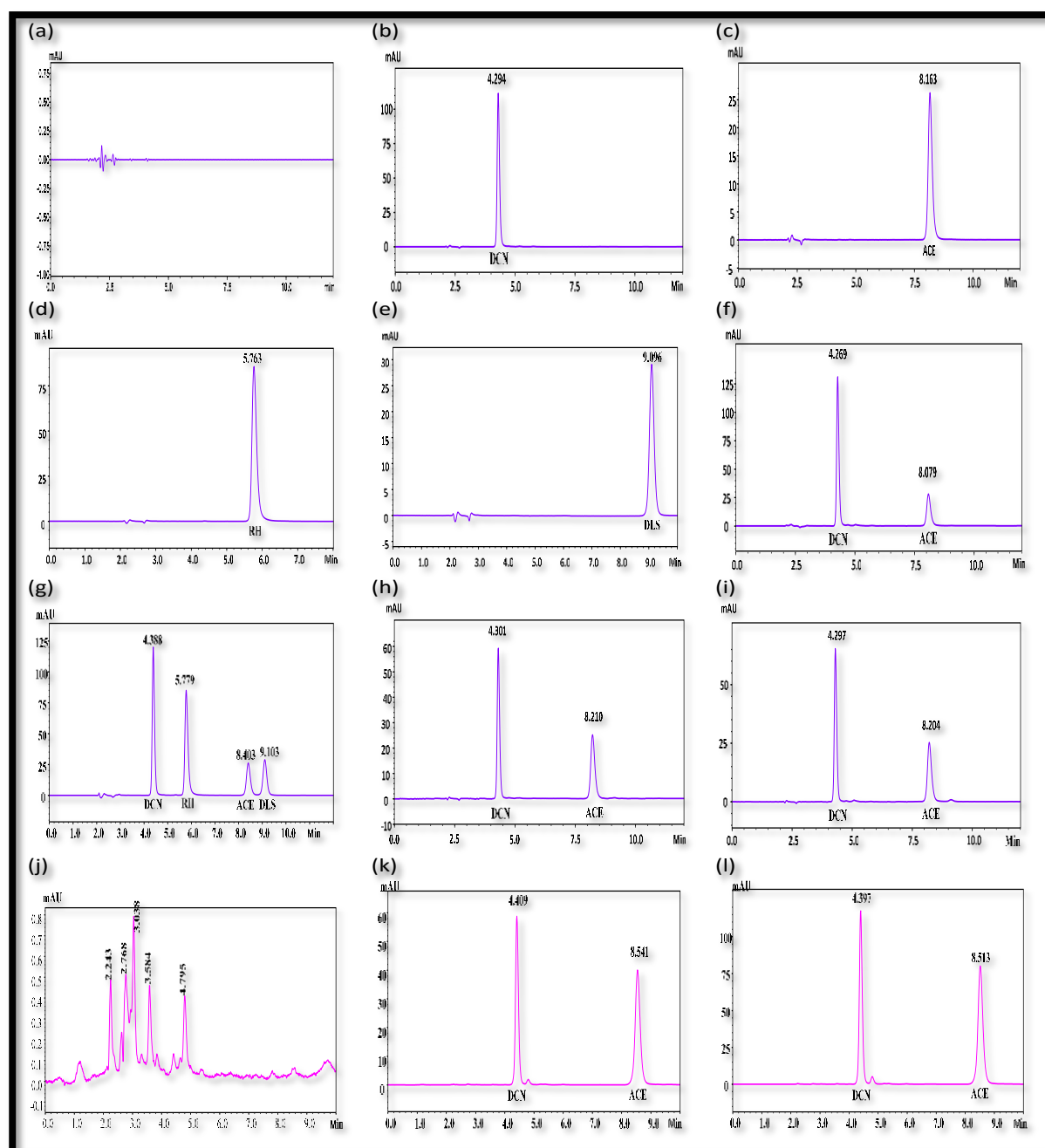


Figure 10. Chromatogram of (a) Blank solution, (b) DCN standard solution, (c) ACE standard solution, (d) RH standard solution, (e) DLS standard solution, (f) Mixed DCN and ACE standard solution, (g) Mixed DCN, RH, ACE, and DLS standard solution, (h) Dycerin A tablet, (i) Hilin-Ace Tablet, (j) Placebo nanoemulgel, (k) DCN and ACE loaded nanoemulsion and (l) Nanoemulgel formulation.

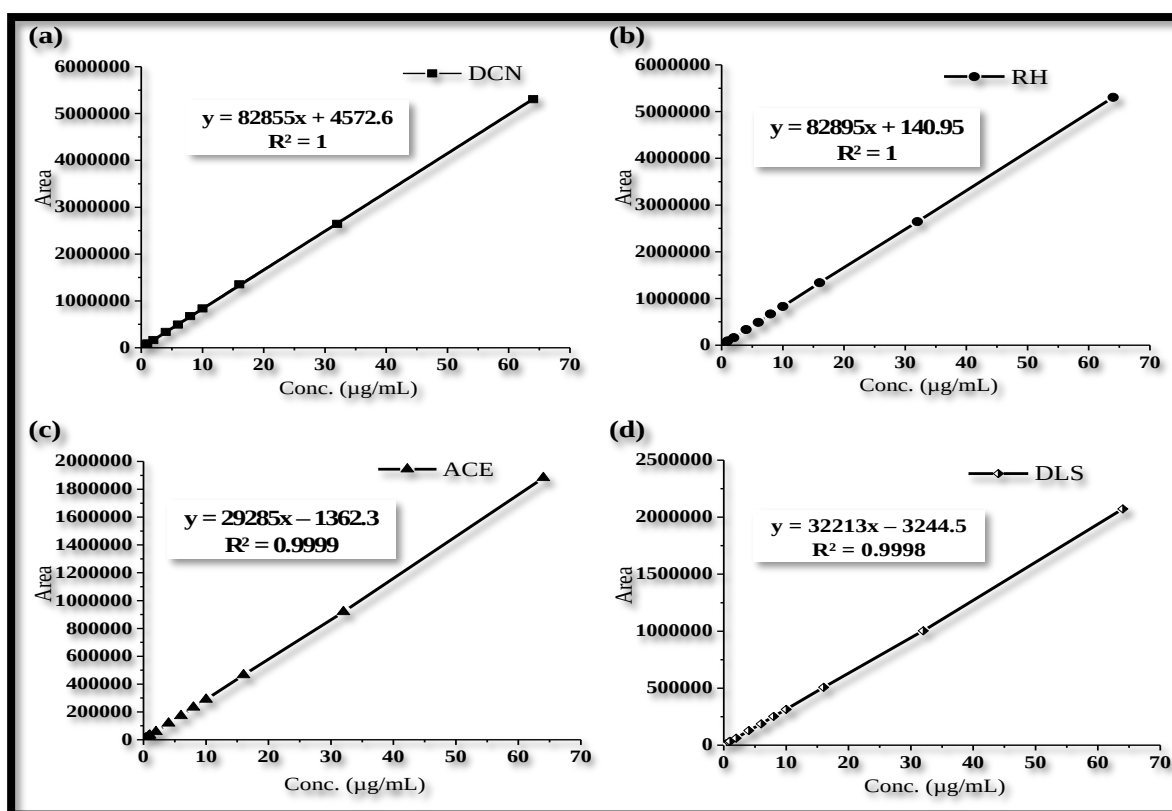


Figure 11. Linearity graph of (a) DCN, (b) RH, (c) ACE, and (d) DLS.

4.3 Screening of NE components

4.3.1 Solubility of the DCN and ACE in various excipients

The results of individual and combined saturated solubility of DCN and ACE in different oils, surfactants, and co-surfactants are shown in Fig. 12a-f and Fig. 13a-e, respectively. Individually, both DCN and ACE exhibited highest solubility in Capmul MCM C8, followed by Triacetin (Fig.12a-b). However, when combined, their solubility decreased in Capmul MCM C8 but increased in Triacetin (Fig.13a-b). Solubility studies revealed that the combined solubility (Fig. 11a-b) of DCN and ACE was highest in an oil mixture of Triacetin: Capmul MCM C8 (2:1) (0.48 ± 0.05 mg/mL for DCN and 87.25 ± 2.45 mg/mL for ACE) compared to other oil ratios and individual oils. Among surfactants (Figure 12c-d), DCN exhibited the highest solubility in Tween 20 (4.33 mg/mL) and Kolliphor-EL (4.16 mg/mL), while ACE

demonstrated the highest solubility in Acconon MC8-2 (144.84 ± 6.25 mg/mL) followed by Labrasol (56.70 ± 1.64 mg/mL). However, the combined solubility of both drugs was optimal in Tween 20 and Kolliphor-EL (Fig.13c). In the case of co-surfactants (Fig.12e-f), ACE exhibited the highest solubility in Transcutol P (203.86 ± 3.57 mg/mL), whereas DCN showed the highest solubility in PEG 300 (2.93 ± 0.3 mg/mL). In combination, both drugs displayed optimal solubility in Transcutol P, followed by PEG 400 (Fig.13e-f).

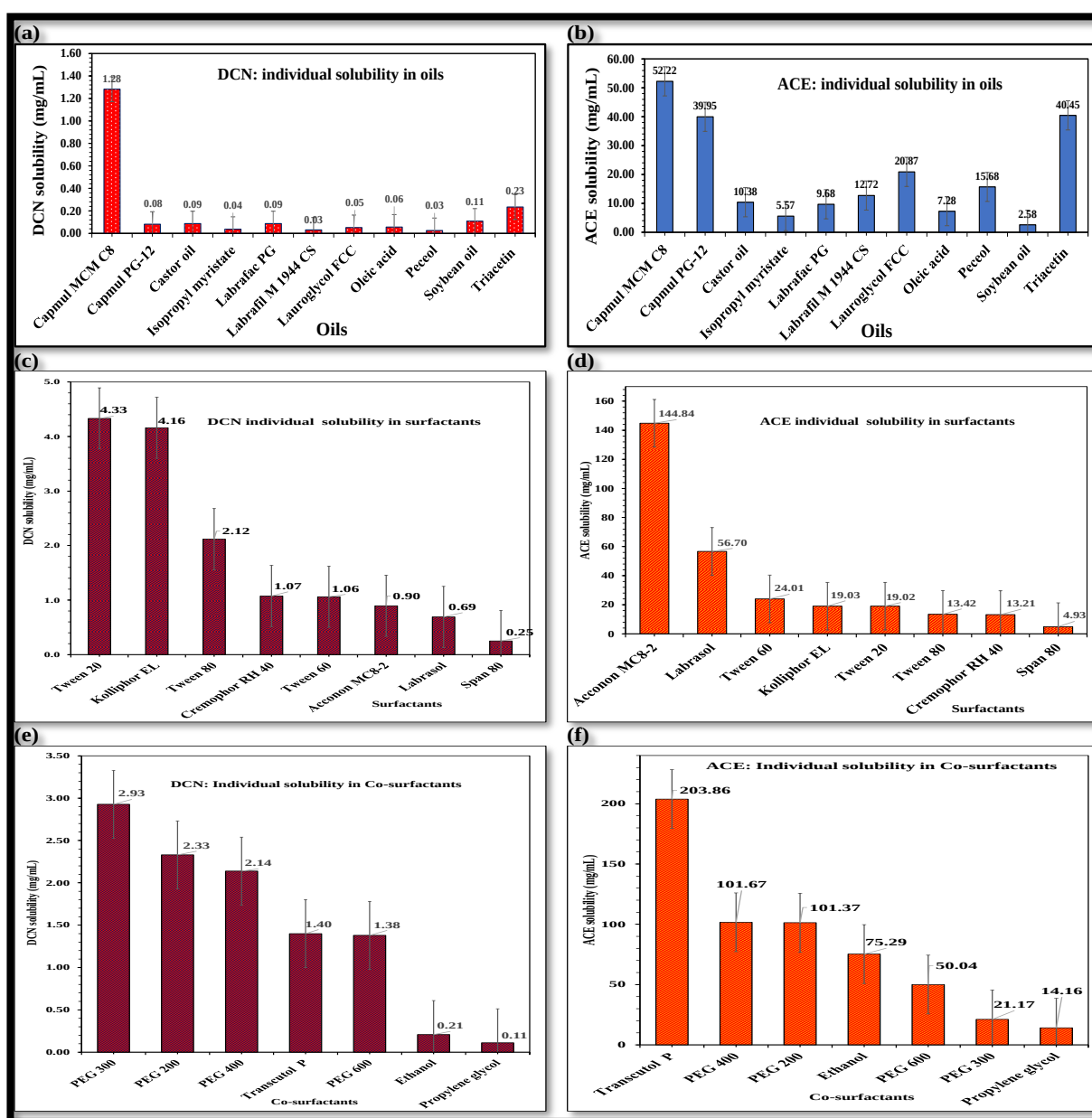


Figure 12. Individual solubility profile of DCN and ACE in different oils (a and b), surfactants (c and d), and, co-surfactants (e and f), respectively.

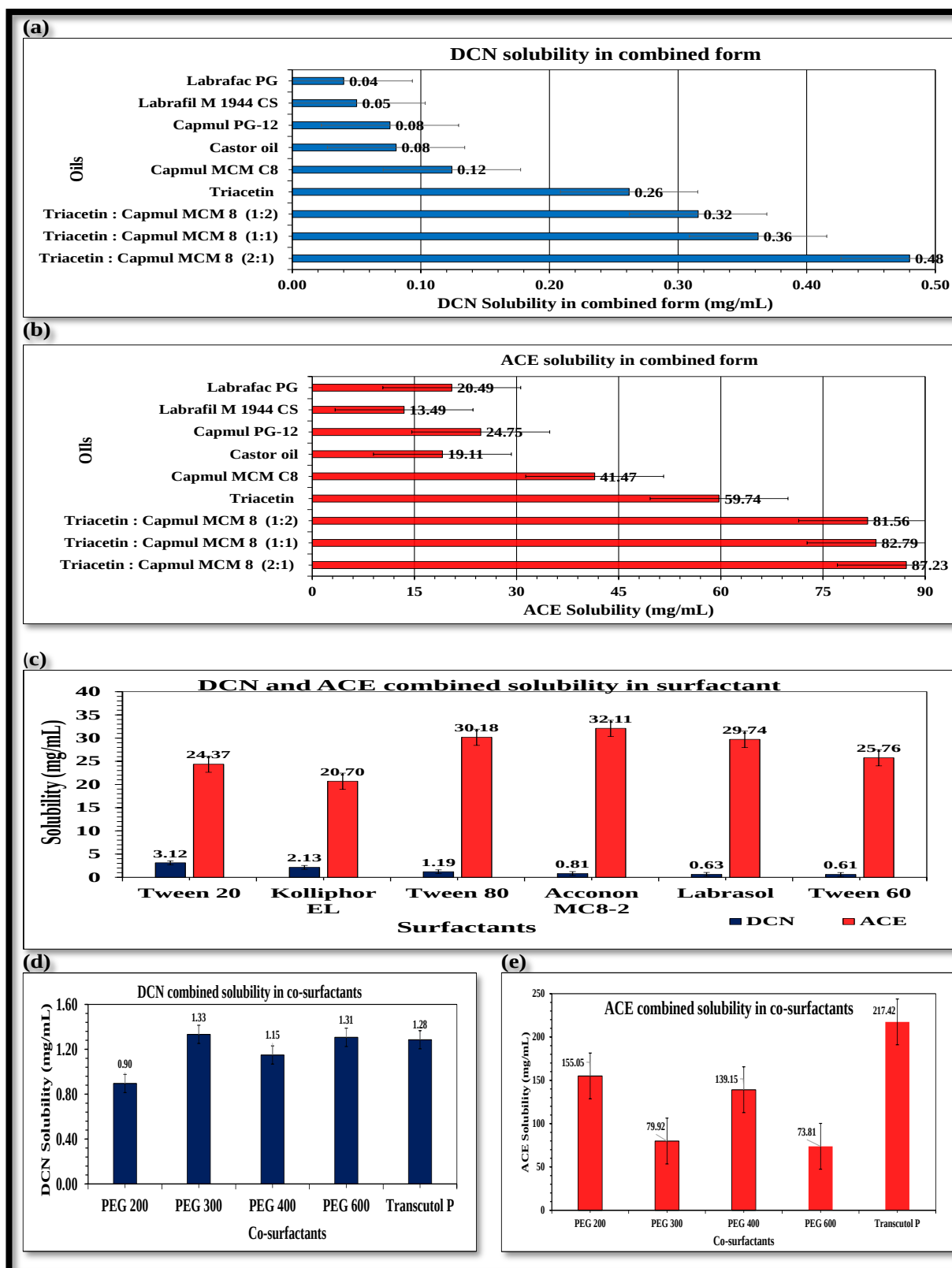


Figure 13. Combined solubility profile of DCN and ACE in different oils (a and b), surfactants (c), and, co-surfactants (d and e).

4.3.2 Screening of surfactant and co-surfactant

Based on the findings of titration method and % transmittance studies (Table 7), it was found that among the three surfactants, Kolliphor-EL showed a higher % T of 99.47 ± 0.03 with the highest miscibility (12.13 ± 0.15 %) with oil mix followed by Tween 80 (%T: 95.96 ± 0.09 ; miscibility: 10.31 ± 0.11 %) and Tween 20 (%T: 92.25 ± 0.19 ; miscibility 9.86 ± 0.07 %).

Table 7. Results of the transmittance and titration study for different surfactants and co-surfactants.

Sr. No.	Surfactant/ Co-surfactant	% Transmittance method			Titration method
		Number of inversions	Emulsification time (Seconds)	Transmittance at 650 nm (% ± SD)	% of oil solubilized (% ± SD)
Surfactant					
1	Tween 20	04	05	92.25 ± 0.19	9.86 ± 0.07
2	Kolliphor-EL	03	05	99.47 ± 0.03	12.13 ± 0.15
3	Tween 80	02	03	95.96 ± 0.09	10.31 ± 0.11
Co-surfactant					
1	Transcutol P	02	05	98.29 ± 0.23	--
2	PEG 300	02	04	88.93 ± 0.06	--
3	PEG 400	03	05	98.09 ± 0.10	--
4	PEG 600	03	06	94.08 ± 0.13	--

SD: standard deviation; Mean $\pm$ SD, n=3.

As a result, Kolliphor-EL was chosen as a surfactant for the preparation of a stable NE, with Triacetin: Capmul MCM C8 (2:1) serving as the oil mix. In the case of co-surfactant, Transcutol P and PEG 400 showed the highest %T of 98.29 ± 0.23 and 98.09 ± 0.10 , followed by PEG 600 (94.08 ± 0.13) and PEG 300 (88.93 ± 0.06). As the Transcutol P and PEG 400 showed nearly the same % T, further investigation was done by preparing a pseudoternary phase diagram. Briefly, the separate mixture (Smix) of Transcutol P and PEG 400 was prepared with a selected surfactant (Kolliphor-EL) in a 1:1 ratio. The Smix (1:1) was mixed with the selected oil in the mass ratio of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1 ratio, separately. The resultant oil: Smix mixture was slowly titrated with water until turbidity. A pseudoternary phase diagram was plotted, and the area of the NE/ME region was compared. Co-surfactants that gave maximum NE/ME region were selected for further development. The pseudoternary phase diagram (Fig.14a-b) revealed that co-surfactant Transcutol P (HLB 4) gives maximum NE/ME region (shaded area) compared to PEG 400 (HLB 8). Hence, Transcutol P was chosen as a co-surfactant for further formulation optimization.

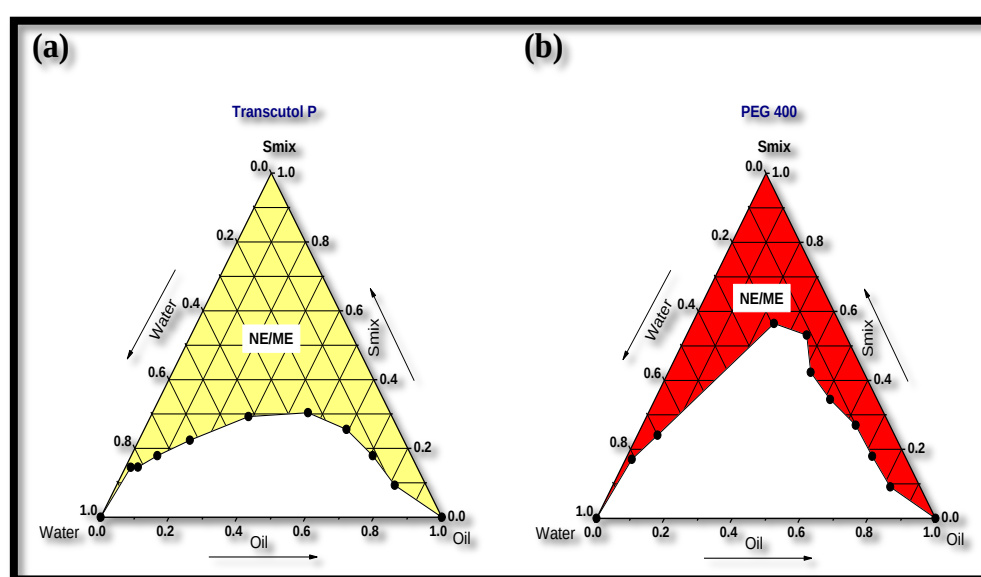


Figure 14. Pseudo-ternary phase diagram for (a) Transcutol P, and (b) PEG 400.

4.3.3 Selection of Smix ratio using a pseudoternary phase diagram

Pseudoternary phase diagrams were plotted for every Smix ratio (Fig. 15). The area encompassing the NE region, represented by a yellow-colored zone, was subsequently quantified using OriginPro v8.1 software. The findings revealed a minimal NE area (55%) at 100% surfactant concentration (Smix 1:0). A significant expansion of the NE area (60.31%) was observed with a gradual increase in the concentration of co-surfactant from 1:0 to 1:1 in Smix. However, further increments from 1:2 to 1:4 did not yield any substantial change in the NE area. Interestingly, despite possessing a higher HLB value, a further increase in the surfactant concentration from 2:1 to 4:1 resulted in a decrease in the NE area. This suggests no substantial enhancement in emulsification, perhaps attributable to the reduced fluidity of Kolliphor-EL at higher concentrations^(93,96,133). Therefore, based on the analysis of these diagrams, Kolliphor-EL: Transcutol P in 1:1 ratio was chosen for further development studies.

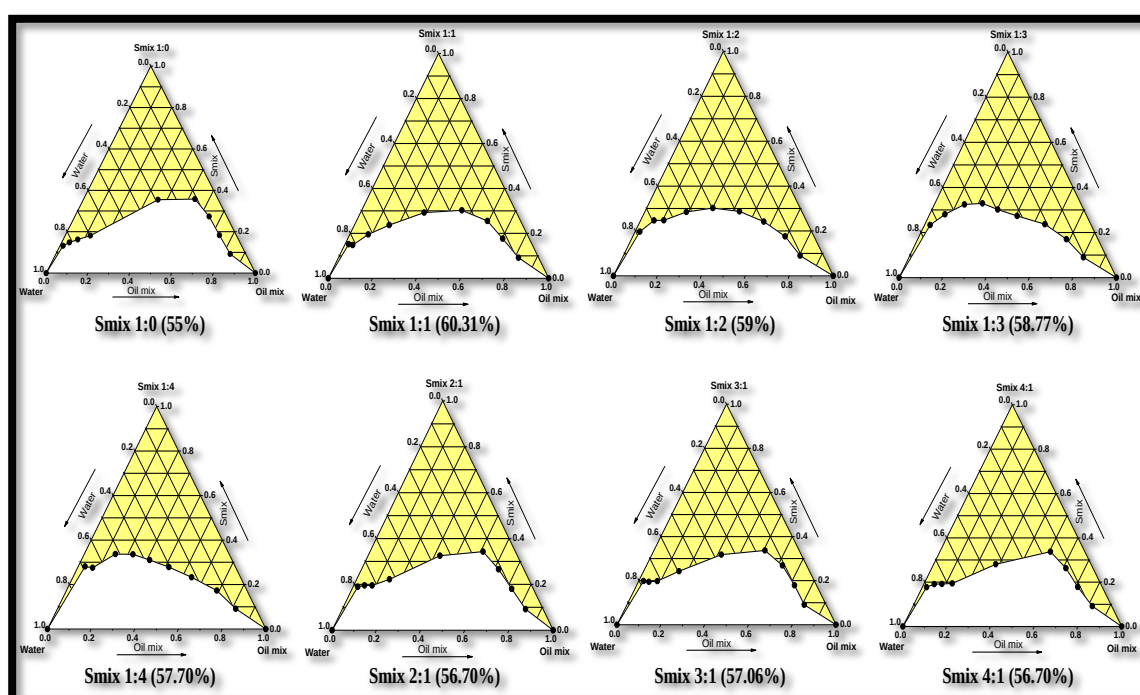


Figure 15. Pseudoternary phase diagram of oil mix (Triacetin: Capmul MCM C8, 2:1), Smix (Kolliphor-EL: Transcutol P, 1:1), and water with NE regions (yellow shaded area) at various Smix ratios 1:0 to 4:1.

4.4 Optimization of NE using mixture design

In the present study, a 3-factor, 2-level D-optimal mixture design was successfully employed to optimize the NE formulation. This design allowed us to determine the main and interaction effects of three formulation components (oil mix (A) %, Smix (B), and water (C) %) on two responses- R1: globule size (GS) and R2: % transmittance (%T). Details of the sixteen experimental runs and their respective responses are presented in Table 8. Analysis of variance (ANOVA) results in Table 9 revealed that all suggested models were statistically significant ($p < 0.05$). Notably, a cubic model and a reduced cubic model were recommended for the response GS and %T, respectively. Importantly, no transformation was required for either response during the analysis. These proposed models exhibited a lack of fit p -values of 0.8120 for GS and 0.1068 for %T, demonstrating no significant lack of fit and adequate precision (>4), indicating their suitability for navigating the design space. The *Scheffe* polynomial equation established the relationship between factors and responses. The equations for GS and %T were derived from the relevant cubic mixture design model, as indicated in (1) and (2).

$$(1) \mathbf{R1 (GS)} = -214.42 *A - 36.27 *B + 12.06*C + 560.86*AB + 453.33*AC + 136.23*BC - 858.20*ABC + 474.45*A B (A-B) + 224.39*A C (A-C) + 182.53*BC (B-C)$$

$$(2) \mathbf{R2 (T)} = 47.45 *A + 36.98 *B + 91.65*C + 211.23*AB + 100.81*AC + 124.04*BC - 273.17*ABC + 36.06*A C (A-C) + 76.53*BC (B-C)$$

The obtained polynomial equations (1) and (2) describe the relationship between the formulation components (A, B, and C) and the responses (GS and %T). A positive coefficient in these equations indicates a synergistic effect, meaning the

factors work together to enhance the response. Conversely, a coefficient with a negative sign suggests an antagonistic effect, where factors counteract each other's impact. Examining equations (1) and (2), we can see that factor A (oil mix) has a negative influence on GS, followed by factor B (Smix). In contrast, factor C (water) has a positive impact on GS. For %T, factor C (water) has the most significant positive effect, followed by factors A and B. Interestingly, the interaction term AB (oil mix and Smix) exhibits the greatest positive influence on both responses, followed by AC (oil mix and water) and BC (Smix and water). However, for the GS response, the interaction term BC ($p = 0.2076$) has a lower F-value (1.42), suggesting a minimal effect on reducing the GS of the NE. Conversely, the interaction terms ABC ($p = 0.0003$) for GS and AC and ABC ($p = 0.0001$) for %T show high F-values (GS: 58.71, %T: 87.98 for AC, & 71.81 for ABC). These high F-values indicate a significant impact: lowering GS and increasing %T of the NE, respectively.

The impact of independent variables on the responses was further visualized using both contour plots (2D) and 3D response surface plots (Fig. 16a-b). Figure 16a shows that the GS of NEs decreased within a specific range (from 18.39 nm to 6.08 nm, indicated by the dark blue color) as the oil concentration decreased and the Smix concentration increased. However, this trend reversed at higher Smix concentrations (above 32.5% w/w), where a further increase in Smix led to an increase in GS. The concentration of water and Smix significantly impacted the T (Eq. 2 & Fig. 16b). The %T initially increased with increasing Smix concentration, likely due to reduced interfacial tension and increased fluidity of the surfactant. However, further increases in Smix concentration led to a decrease in %T, possibly due to the low fluidity of the surfactant at high concentrations. These findings are consistent with previously reported data.

A numerical method was employed to identify the optimal formulation for NEs with minimum GS (nm), maximum T (%), and maximum composite desirability. Among the suggested solutions (NE-1 and NE-2), the design expert selected NE-1 as the optimized NE (Opt-NE) formulation based on its experimental conditions (13.34% w/w for oil mix, 31.77% w/w for Smix, 54.89% w/w for water) and high composite desirability of 0.908 (Fig. 16c). To determine the design space, an overlay plot (a graphical method) was used. Figure 16d depicts this overlay plot, where each response's maximum and minimum limits were set. The yellow-colored region, also known as the "sweet spot," highlights the desirable ranges for both factors and responses. The design suggested composition and confirmation report for the optimized NE are provided in Fig. 16a-d and Table 10. The formulation with the highest desirability (NE-1 and NE-2) was prepared and characterized for GS and T to validate the model. The details of this formulation and the obtained results are presented in Table 11. The obtained results showed no significant difference between the actual and predicted values, confirming the predictive ability of the polynomial model suggested by the design expert.

Table 8. Experimental run of 2-level D-optimal mixture design for independent variables and responses (n=3).

Run	Batch	Independent variables			Responses		PDI $\pm$ SD
		Oil (%w/w)	Smix (%w/w)	Water (%w/w)	Globule size (GS) (nm) $\pm$ SD	Transmittance (T) (%) $\pm$ SD	
1	F1	10.00	35.00	55.00	21.65 $\pm$ 3.18	95.42 $\pm$ 1.09	0.33 $\pm$ 0.03
2	F2	14.50	31.00	54.50	11.69 $\pm$ 1.40	96.03 $\pm$ 1.16	0.20 $\pm$ 0.08
3	F3	10.00	30.81	59.19	10.40 $\pm$ 0.20	94.73 $\pm$ 0.04	0.13 $\pm$ 0.11
4	F4	18.08	25.00	56.92	20.00 $\pm$ 1.31	96.49 $\pm$ 1.58	0.22 $\pm$ 0.07
5	F5	10.00	25.00	65.00	11.55 $\pm$ 0.78	91.55 $\pm$ 0.67	0.28 $\pm$ 0.12
6	F6	20.00	35.00	45.00	16.00 $\pm$ 2.69	95.12 $\pm$ 0.56	0.11 $\pm$ 0.12
7	F7	10.00	35.00	55.00	22.23 $\pm$ 5.19	95.28 $\pm$ 1.32	0.30 $\pm$ 0.18
8	F8	20.00	29.12	50.88	15.70 $\pm$ 0.85	94.98 $\pm$ 0.86	0.24 $\pm$ 0.17
9	F9	14.34	27.83	57.83	11.43 $\pm$ 0.64	95.37 $\pm$ 1.25	0.20 $\pm$ 0.09
10	F10	20.00	35.00	45.00	13.70 $\pm$ 0.57	94.86 $\pm$ 0.90	0.26 $\pm$ 0.10
11	F11	13.60	35.00	51.40	10.63 $\pm$ 1.10	95.04 $\pm$ 1.30	0.15 $\pm$ 0.01
12	F12	20.00	29.12	50.88	15.77 $\pm$ 2.72	94.59 $\pm$ 0.73	0.37 $\pm$ 0.06
13	F13	10.00	25.00	65.00	12.53 $\pm$ 0.35	91.73 $\pm$ 0.83	0.37 $\pm$ 0.07
14	F14	13.31	25.00	61.69	16.54 $\pm$ 5.06	94.93 $\pm$ 0.50	0.30 $\pm$ 0.07
15	F15	17.67	33.15	49.18	17.60 $\pm$ 1.13	97.17 $\pm$ 0.88	0.16 $\pm$ 0.08
16	F16	18.08	25.00	56.92	18.65 $\pm$ 0.06	96.25 $\pm$ 2.16	0.11 $\pm$ 0.02

SD: standard deviation; Mean $\pm$ SD.

Table 9. Summary of ANOVA for the studied responses.

Sr. No.	Response	Model	Lack of Fit (<i>p</i> -value)	R ²	Adjusted R ²	Predicted R ²	Adequate precision
1	GS (nm)	Cubic mixture	0.812	0.9812	0.953	0.8807	17.438
2	T (%)	Reduced cubic mixture	0.1068	0.9874	0.973	0.9237	29.053

Table 10. Two-sided composition and confirmation report by design for optimized NE.(Confidence 95% & n=1)

Component	Name	Level	Low level	High level	Std. Dev.	Coding
A	Oil	13.34	10.00	20.00	0.000	Actual
B	Smix	31.76	25.00	35.00	0.000	Actual
C	Water	54.89	45.00	65.00	0.000	Actual
		Total=100				
Response	Prediction	Std. Dev.	SE (n=1)	95% PI low	95% PI high	
GS (nm)	10.40	0.843401	1.2364	7.37465	13.4254	
T (%)	96.18	0.243179	0.282653	95.5124	96.8491	

SE: standard error

Table 11. Predictive and actual values of NE formulations suggested by the design.

Ingredients/ parameters	NE-1		NE-2	
Ingredients (%w/w)				
Oil	13.34		14.70	
Smix	31.77		35.00	
Water	54.89		50.30	
Parameters				
Desirability	0.908		0.804	
	Predicted value	Actual value	Predicted value	Actual value
GS (nm)	10.4	10.20 ± 0.70	10.63	11.10 ± 0.20
T (%)	96.18	98.03 ± 0.03	95.27	97.78 ± 0.29

SD: standard deviation. Actual values expressed in Mean ± SD.

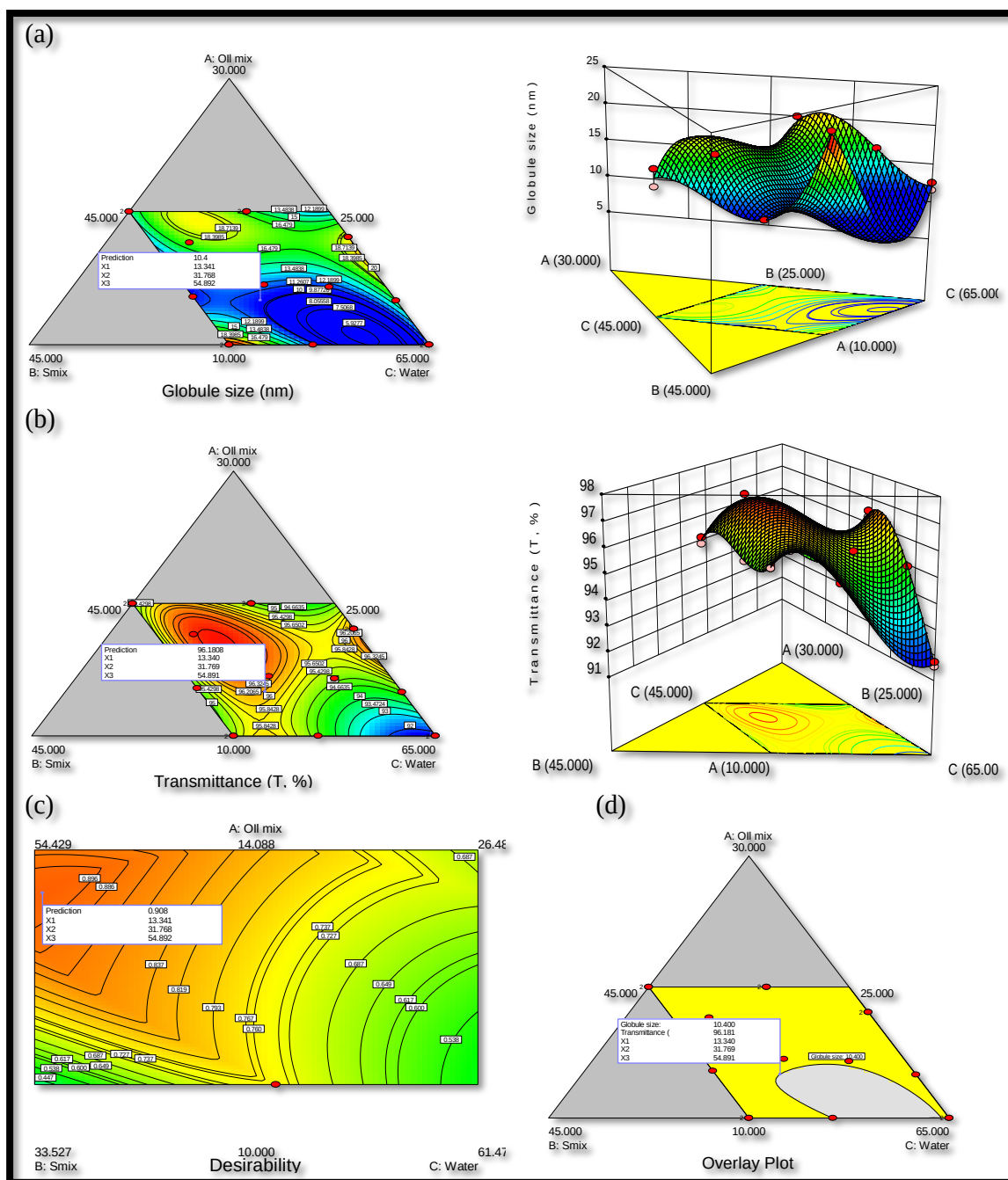


Figure 16. Two-dimensional contour plots and three-dimensional response surface plots indicating the interaction effect of the independent variable on a) GS and b) % T. The desirability (c) and overlay plot (d) for the optimized NE-1 suggested by design.

4.5 Characterization of optimized DCN -and ACE-loaded NE

The NEs suggested by design were prepared and evaluated for the following parameters:

4.5.1 Globule size and electrokinetic potential analysis

Design experts suggested two nanoemulsions (NE-1 and NE-2), whose average GS, electrokinetic potential (zeta potential), and PDI are summarized in Tables 11 & 12.

Table 12. Characterization of the NE formulations suggested by the design.

Characterization parameter	Formulation code	
	NE-1	NE-2
pH $\pm$ SD	5.81 $\pm$ 0.03	5.78 $\pm$ 0.01
PDI $\pm$ SD	0.20 $\pm$ 0.12	0.22 $\pm$ 0.08
Zeta potential (mV) $\pm$ SD	-28.40 $\pm$ 2.77	-30.67 $\pm$ 1.62
Viscosity at 100 rpm $\pm$ SD (cP)	36.00 $\pm$ 2.00	39.67 $\pm$ 4.73
Thermodynamic stability study		
Centrifugation stress	✓	✓
Heating-cooling cycle	✓	✓
Freeze-thaw cycle	✓	✓

✓indicate passed the test.

The GS increased with increasing oil mix and Smix concentration in the formulations. The formulation NE-1, containing 13.34% w/w of oil mix and 31.77 % of Smix, exhibited the lowest GS (10.20 $\pm$ 0.70 nm) compared to NE-2 (11.10 $\pm$ 0.20 nm) with 14.70% w/w oil mix and 35% of Smix. However, both NEs achieved the

desired GS of ≤ 100 nm. Figure 17a-b shows the GS, PDI, and zeta potential of the suggested NE-1 and NE-2 formulation. The PDI reflects the homogeneity or uniformity of the GS distribution within the formulation. A lower PDI value (closer to 0) indicates a more uniform GS distribution. The mean PDI of all formulated NEs ranged from 0.20 ± 0.12 to 0.22 ± 0.08 (Table 12), indicating good uniformity in GS distribution (134). Zeta potential (Electrokinetic potential) is a crucial parameter for predicting the stability of a formulation and understanding the surface properties of nanosized globules. A zeta potential value exceeding ± 25 mV indicates a stable NE formulation with minimal agglomeration or flocculation(65,135). Higher negative or positive zeta potential values result in repulsive forces between globules that surpass attractive forces, reducing the likelihood of aggregation and coalescence (136). All formulated NEs displayed a mean zeta potential between -30.67 ± 1.62 mV and -28.40 ± 2.77 mV, suggesting long-term stability (Table 12).

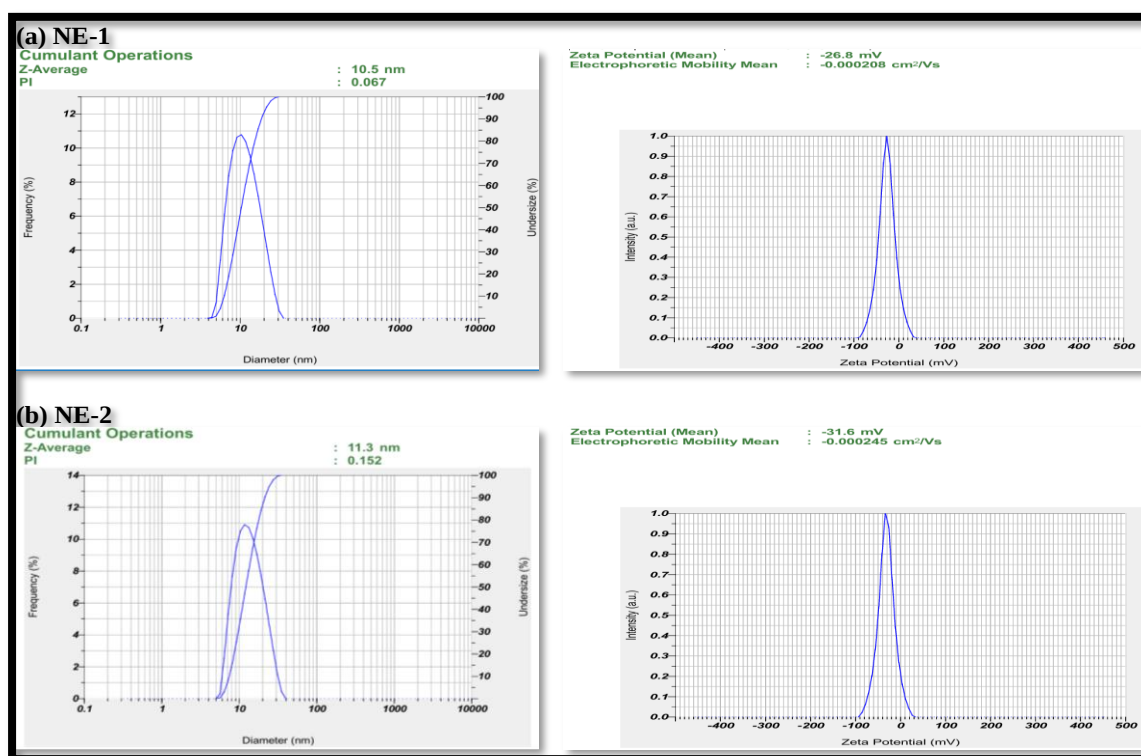


Figure 17. Globule size, PDI, and zeta potential of (a) NE-1 and (b) NE-2 formulation.

4.5.2 Percentage transmittance, viscosity, and pH

The results of %T, pH, and viscosity of the NEs suggested by the design expert were summarized in Tables 11 and 12. The % T of NE-1 and NE-2 at 650 nm were found to be 98.03 ± 0.03 and 97.78 ± 0.29 , respectively. Results closer to 100% T indicate that all NE formulations appear clear and transparent (97). The pH and viscosity of formulation NE-1 were 5.81 ± 0.03 and 36.00 ± 2.00 cP, respectively, while those of NE-2 were 5.78 ± 0.01 and 39.67 ± 4.73 cP. During the checkpoint analysis, it was observed that the viscosity of the NE formulation increases with an increase in oil and Smix concentration, as revealed in Table 12.

4.5.3 Drug content analysis of the DCN-and ACE-loaded NE

The average % drug content of the NE-1 and NE-2 formulation, determined by HPLC in triplicate at 264 nm, was found in the range of 95-105. The DCN content was $98.52 \pm 0.85\%$ for NE-1 and $101.72 \pm 0.97\%$ for NE-2. Whereas the ACE content for NE-1 and NE-2 was found to be $98.78 \pm 1.01\%$ and $97.83 \pm 0.41\%$, respectively. This result indicates good drug-loading capacity, an important factor for effective NEs.

4.5.4 Physical stability of DCN-and ACE-loaded NE

Table 12 presents the results of the cycling stress and thermodynamic stability study for the two proposed NE formulations. As previously mentioned, improved thermodynamic stability translates to a longer shelf life for NEs compared to conventional emulsions (93,96). This key difference stems from the enhanced kinetic stability of these emulsions, which minimizes the risk of phase separation. Encouragingly, none of the proposed NE formulations displayed any signs of creaming, cracking, or phase separation, indicating exceptional physical stability.

4.5.5 *In vitro* permeation study of DCN-and ACE-loaded NE

The *in vitro* permeation profile of DCN-and ACE-loaded NEs (NE-1 & NE-2) at PBS pH 5.7 and methanolic PBS pH 6.8 are presented in Fig. 18a-d. At pH 5.7, NE-1 released 63% (891.34 $\mu\text{g}/\text{cm}^2$) of the total DCN content after 24 h, while NE-2 released 59% (834.75 $\mu\text{g}/\text{cm}^2$). Similarly, for ACE, NE-1 released 65% (2758.91 $\mu\text{g}/\text{cm}^2$) and NE-2 released 62% (2631.58 $\mu\text{g}/\text{cm}^2$) after 24 h. The release behavior differed at pH 6.8. NE-1 released 50% (707.40 $\mu\text{g}/\text{cm}^2$) of the total DCN content after 24 h, compared to 44% (622.52 $\mu\text{g}/\text{cm}^2$) for NE-2. For ACE, NE-1 achieved a significantly higher release of 98% (4159.59 $\mu\text{g}/\text{cm}^2$) compared to NE-2's 91% (3862.48 $\mu\text{g}/\text{cm}^2$) after 24 h.

Overall, NE-1 consistently demonstrated a slightly higher release rate for both DCN and ACE than NE-2 at both pH levels. This slight variation in drug permeation could be due to changes in the composition and the GS of the NEs. Based on the results of the numerical optimization study, desirability, and permeation study, NE-1 was selected as the optimized nanoemulsion (Opt. NE) and used for further evaluation and development.

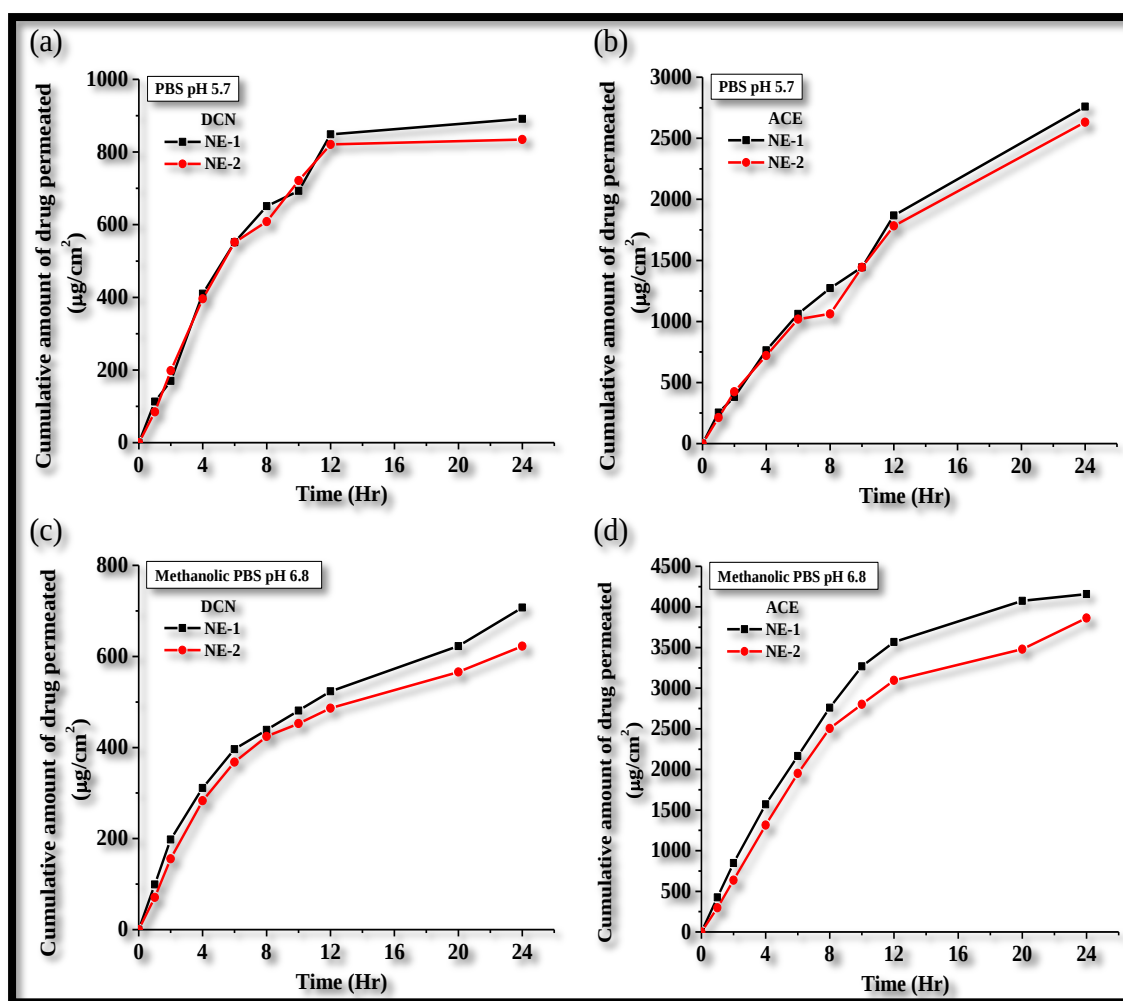


Figure 18. *In vitro* DCN and ACE permeation profile from NE formulations (NE-1 and NE-2) suggested by design in PBS pH 5.7 (a-b) and mixture of PBS pH 6.8: methanol (7:3) (c-d).

4.5.6 Morphological and structural behavior of optimized NE

The proposed NE's GS, shape, and surface morphology were investigated using FE-SEM and TEM. FE-SEM and TEM images of the optimized NE are shown in Fig. 19a-b. The obtained images revealed that all the globules exhibited a spherical shape and smooth layer and were confirmed to be within the nano-size range (< 20 nm). The GS agreed with the results obtained from the GS analysis using nanoparticle and zeta potential analyzer.

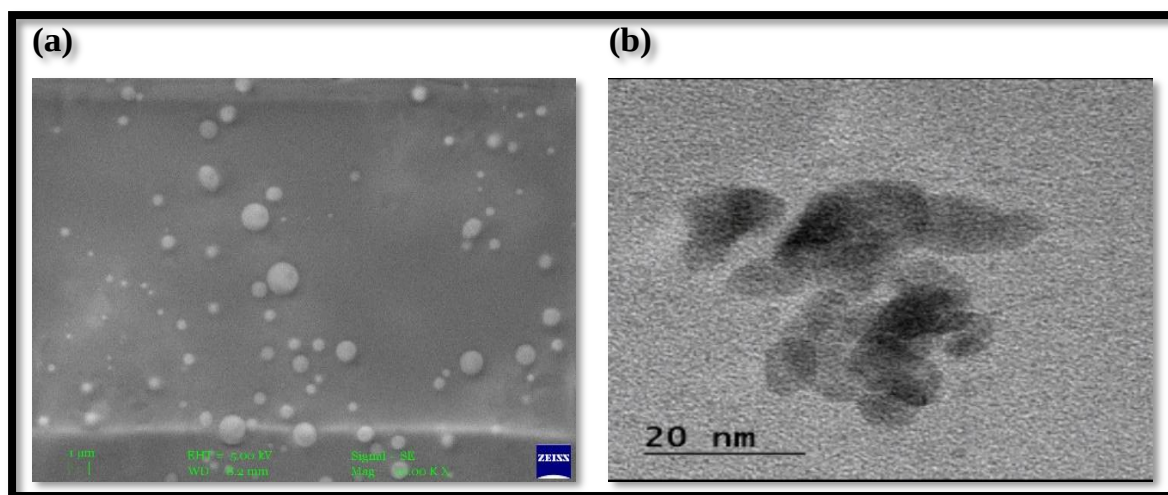


Figure 19. FE-SEM (a) and TEM (b) image of optimized DANE with $x=0.10$.

4.6 Characterization of DCN-and ACE-loaded NEG and gel.

4.6.1 Viscosity, spreadability, pH, and drug content analysis

The pH values, % drug content, viscosities, and spreadability values of all DCN-and ACE-loaded NEG and plain gel batches are summarized in Table 13.

Table 13. Characterization of DCN-and ACE-loaded NEG and plain gel formulations.

Formulation	pH $\pm$ SD	Viscosity (cP) $\pm$ SD	Spreadability (g cm/s) $\pm$ SD	Drug content (%) $\pm$ SD	
				DCN	ACE
NEG-1 (without DMSO)	5.97 $\pm$ 0.03	582.47 $\pm$ 12.01	35.42 $\pm$ 1.30	99.38 $\pm$ 0.48	101.65 $\pm$ 1.01
NEG-2 (with 2.5% DMSO)	5.91 $\pm$ 0.03	476.83 $\pm$ 3.23	37.75 $\pm$ 0.68	99.85 $\pm$ 0.39	100.68 $\pm$ 0.67
NEG-3 (with 5% DMSO)	5.93 $\pm$ 0.09	444.00 $\pm$ 7.94	38.19 $\pm$ 0.46	96.17 $\pm$ 0.41	101.37 $\pm$ 0.82
NEG-4 (with 7.5% DMSO)	5.86 $\pm$ 0.02	436.33 $\pm$ 11.06	39.22 $\pm$ 1.53	97.63 $\pm$ 0.58	102.82 $\pm$ 0.91
Plain gel-1 (without DMSO)	5.93 $\pm$ 0.10	534.54 $\pm$ 7.76	36.11 $\pm$ 1.13	97.97 $\pm$ 1.61	101.56 $\pm$ 1.18
Plain gel-2 (with 2.5% DMSO)	5.95 $\pm$ 0.37	515.65 $\pm$ 10.23	36.83 $\pm$ 1.08	99.88 $\pm$ 0.34	102.78 $\pm$ 2.02

SD: standard deviation, Mean $\pm$ SD, n=3.

The pH of all formulations ranged from 5.86 to 5.97. NEG-1 exhibited significantly higher viscosity than plain gel formulations (Plain gel-1 and Plain gel-2). All formulations demonstrated good spreadability, with values between 35.42 and 39.22 g cm/s. The DCN and ACE drug content in all formulations was close to 100%, ranging from 96.17% to 99.88% for DCN and 100.68% to 102.82% for ACE.

4.6.2 *In vitro* permeation study of optimized NE, DCN-and ACE-loaded NEG and plain gel

Figure 20a-d illustrates the *in vitro* permeation of Opt-NE, DCN- and ACE-loaded NEG (NEG-1 to NEG-4), Plain gel-1 & 2 across two different media: PBS pH 5.7 and Methanolic PBS pH 6.8. The results demonstrated that NE-1 (Opt-NE) exhibited the highest permeation rate for both drugs, while Plain gel-1 displayed the lowest permeation rate for both drugs compared to all other formulations in both media.

At pH 5.7 (Fig. 20a-b), Opt-NE released 63% of the total DCN content and 65% of the total ACE content after 24 h, whereas NEG-1(without DMSO) released 44% of DCN and 50% of ACE. Plain gel-1 (without DMSO) exhibited significantly lower release with only 5% of DCN and 15% of ACE, while Plain gel-2, prepared with 2.5% DMSO, demonstrated slightly higher permeation with 6% DCN and 20% ACE release after 24 h. However, no significant change in permeation of DCN and ACE was detected between Plain gel-1 and Plain gel-2, as evidenced by similarity factors (f_2) of 96.5 and 71.1 for DCN and ACE, respectively. The NEG formulations, NEG-2, NEG-3, and NEG-4, all formulated with DMSO, exhibited cumulative drug release of 48%, 49%, and 49% for DCN and 58%, 59%, and 55% for ACE, respectively, after 24 h.

The release behavior differed at methanolic PBS pH 6.8 (Fig. 20c-d). Opt-NE and NEG-2 (with 2.5% DMSO) formulations exhibited substantially higher DCN and ACE permeation than all other formulations. After 24 h, the Opt-NE, Plain gel-1, Plain gel-2, NEG-1, NEG-2, NEG-3, and NEG-4 exhibited 50%, 25%, 25%, 35%, 37%, 34%, and 35% permeation of DCN, respectively.

Similarly, ACE permeation from Opt NE, Plain gel-1, Plain gel-2, NEG-1, NEG-2, NEG-3, and NEG-4 was 98%, 35%, 41%, 75%, 89%, 75%, and 79%, respectively. The results demonstrated that NE formulation enhances drug permeation. On the other hand, adding NE or drug to a gel matrix might slow release a little bit, probably because the gels become more viscous.

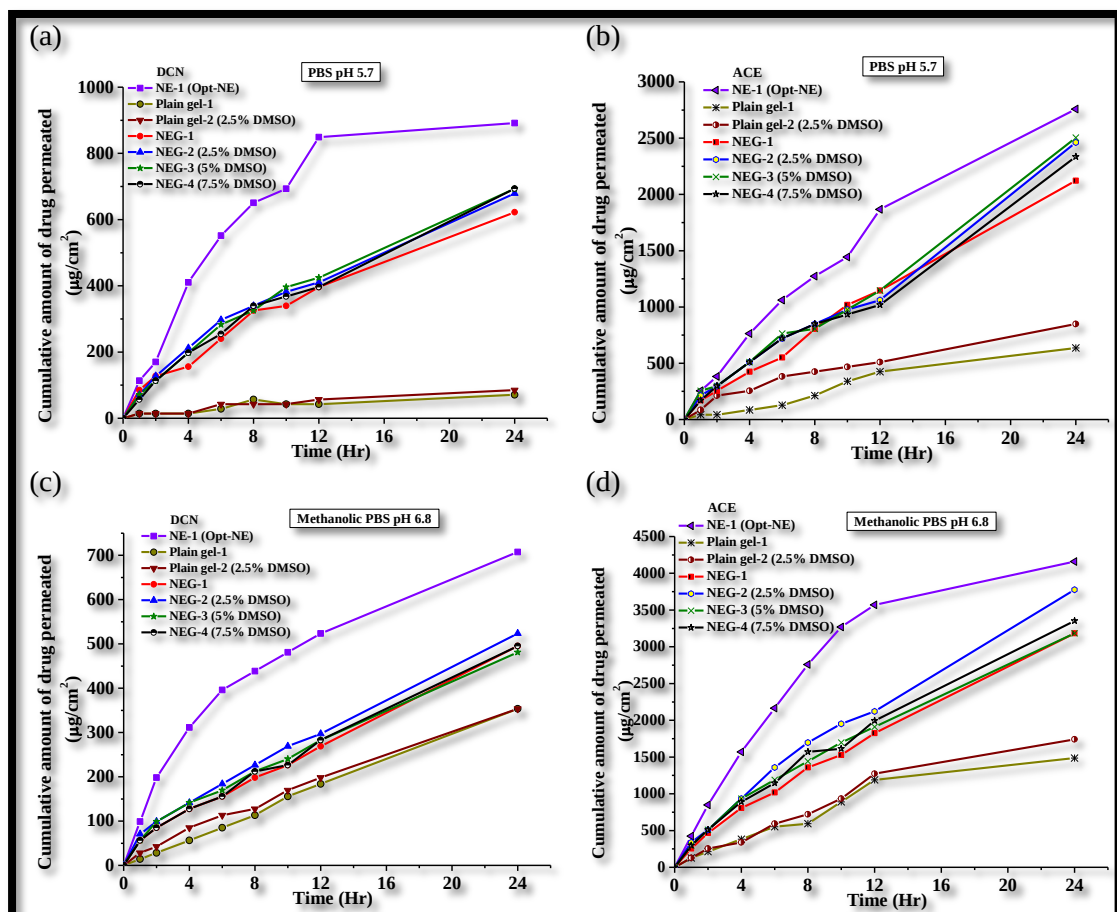


Figure 20. *In vitro* permeation of DCN and ACE from optimized NE, DCN- and ACE-loaded NEG, and Plain gel in PBS pH 5.7 (a-b) and methanolic PBS pH 6.8 (c-d).

Nanoemulgels prepared with DMSO as a penetration enhancer showed increased permeation of both drugs compared to Plain gel-1, Plain gel-2, and NEG-1 in both media. However, no significant difference (similarity factor (f_2) > 50) in the permeation profile of either drug was observed among the NEG formulations prepared with DMSO. The overall permeation profile of NEG-3 (with 5% DMSO) and NEG-4 (with 7.5% DMSO) was found to be similar to or slightly higher than NEG-2 (with 2.5% DMSO).

The permeation values for the optimized NE, Plain gels, and NEG formulations were fitted into different mathematical models. The results of release kinetics are presented in Table 14. The software analysis suggested that DCN and ACE release from all these formulations, except Opt-NE, followed the Korsmeyer-Peppas model of drug diffusion, with maximum R^2 values ranging from 0.9247-0.9985 and 0.9569 to 0.9993 in both media, respectively. However, the DCN and ACE release from the Opt-NE followed Korsmeyer-Peppas model and first-order release kinetics in both media with maximum R^2 values ranging from 0.9247-0.9880 and 0.9489 and 0.9932, respectively.

In both media, the diffusional exponent (n) value for ACE release from all formulations was more than 0.45 and less than 1, suggesting the ACE permeation follows anomalous (non-Fickian case II) diffusion involving both dissolution and diffusion processes. The diffusional exponent (n) value for DCN release from Opt-NE, Plain Gel-1, and all NEGs in both media was found to be more than 0.45 and less than 1, which suggests that the release follows anomalous (non-Fickian case II) diffusion involving both dissolution and diffusion processes. The DCN release from Plain Gel- 2 showed n value of 0.681 and 1 at PBS pH 5.7 and methanolic PBS pH 6.8, respectively. This indicates that the DCN release mechanism from Plain Gel-2

follows anomalous (non-Fickian) diffusion at PBS pH 5.7 and super case II transport mechanism at methanolic PBS pH 6.8. Based on the *in vitro* drug release results, NEG-2 with 2.5 % DMSO was finalized as the optimal NEG and used for further evaluation.

Table 14. *In vitro* DCN and ACE release kinetics from different NEGs, gels and opt-NE.

Batches	Zero order		First order		Higuchi model		Korsmeyer-Peppas model	
	R^2	K_0	R^2	K_1	R^2	K_H	R^2	n
At PBS pH 5.7								
Opt-NE (ACE)	0.9227	3.097	0.9946	0.045	0.9340	11.630	0.9932	0.720
Plain gel -1 (ACE)	0.9581	0.668	0.9603	0.007	0.8013	2.408	0.9625	0.941
Plain gel -2 (ACE)	0.8512	0.961	0.8910	0.011	0.9660	3.668	0.9905	0.631
NEG-1 (ACE)	0.9889	2.179	0.9938	0.027	0.8644	7.947	0.9974	0.887
NEG-2 (ACE)	0.9827	2.392	0.9621	0.030	0.8306	8.665	0.9841	0.950
NEG-3 (ACE)	0.9829	2.442	0.9618	0.031	0.8320	8.851	0.9845	0.947
NEG-4 (ACE)	0.9794	2.291	0.9674	0.029	0.8424	8.332	0.9832	0.920
Opt-NE (DCN)	0.5660	3.690	0.9064	0.068	0.9246	14.627	0.9247	0.495
Plain gel -1 (DCN)	0.7073	0.252	0.7194	0.003	0.8637	0.970	0.8759	0.593
Plain gel -2 (DCN)	0.8599	0.288	0.8700	0.003	0.9116	1.089	0.9531	0.681
NEG-1 (DCN)	0.8882	2.111	0.9607	0.027	0.9540	7.998	0.9929	0.671
NEG-2 (DCN)	0.8131	2.205	0.9265	0.029	0.9791	8.484	0.9946	0.599
NEG-3 (DCN)	0.8022	2.156	0.9210	0.028	0.9735	8.307	0.9887	0.597
NEG-4 (DCN)	0.8160	2.079	0.9238	0.027	0.9712	7.990	0.9891	0.607

Batches	Zero order		First order		Higuchi model		Korsmeyer-Peppas model	
	R^2	K_0	R^2	K_1	R^2	K_H	R^2	n
At Methanolic PBS pH 6.8								
Opt-NE (ACE)	0.6810	5.477	0.9912	0.131	0.9455	21.427	0.9489	0.544
Plain gel -1 (ACE)	0.8884	1.727	0.9423	0.021	0.8974	6.483	0.9569	0.722
Plain gel -2 (ACE)	0.9336	1.946	0.9739	0.024	0.8900	7.227	0.9747	0.777
NEG-1 (ACE)	0.9660	3.379	0.9878	0.050	0.9088	12.524	0.9989	0.793
NEG-2 (ACE)	0.9526	4.068	0.9791	0.066	0.9204	15.152	0.9980	0.764
NEG-3 (ACE)	0.9280	3.506	0.9929	0.054	0.9420	13.172	0.9993	0.717
NEG-4 (ACE)	0.9468	3.627	0.9867	0.056	0.9237	13.534	0.9968	0.754
Opt-NE (DCN)	0.6279	2.681	0.8495	0.039	0.9874	10.563	0.9880	0.495
Plain gel -1 (DCN)	0.9794	1.105	0.9918	0.012	0.8861	4.063	0.9975	0.841
Plain gel -2 (DCN)	0.9983	1.049	0.9956	0.012	0.8121	3.753	0.9985	1.000
NEG-1 (DCN)	0.9611	1.549	0.9809	0.018	0.9048	5.745	0.9927	0.795
NEG-2 (DCN)	0.9300	1.695	0.9691	0.020	0.9365	6.360	0.9951	0.724
NEG-3 (DCN)	0.9208	1.569	0.9611	0.019	0.9424	5.900	0.9950	0.709
NEG-4 (DCN)	0.9554	1.571	0.9803	0.019	0.9123	5.839	0.9937	0.778

K_0 : Zero-order rate constant; K_1 : First-order rate constant; K_H : Higuchi dissolution constant.

4.6.3 Ex vivo release study of DCN-and ACE-loaded NEG and plain gel

An ex vivo permeation study of NEG-1 (without DMSO), NEG-2 (with 2.5% DMSO), and Plain gel-2 (with 2.5% DMSO) was performed using Wistar rat skin. Cumulative drug permeation over time was plotted as shown in Fig. 21a-d, and the transdermal flux, permeability coefficient, and enhancement ratio of DCN and ACE were calculated from each formulation and shown in Table 15. Results demonstrated

(Fig. 21a-d) significantly higher drug release from NEG-1 and NEG-2 compared to Plain gel-2 in both PBS pH 5.7 and methanolic PBS pH 6.8. At PBS pH 5.7 (Fig. 21a-b), NEG-1, NEG-2 and Plain gel-2 released 38%, 44%, 4% of DCN and 44%, 56%, and 21% of ACE, respectively. Similarly, at methanolic PBS pH 6.8 (Fig. 21c-d), NEG-1, NEG-2, and Plain gel-2 released 33%, 37%, and 6% of DCN and 81%, 90%, and 42% of ACE, respectively.

Nanoemulgel (NEG-2) formulation demonstrated higher DCN and ACE release in both media than NEG-1, likely attributable to the permeation-enhancing effect of DMSO. This translated to a significantly higher transdermal flux for NEG-2 than NEG-1 and Plain gel-2 ($P < 0.05$). Table 15 shows that both NEG-2 followed NEG-1 formulations demonstrated higher permeation parameters and skin deposition for both drugs compared to the plain gel in both media. The mean transdermal flux of DCN and ACE from NEG-1 in PBS pH 5.7 was 9.69 and 17.71 $\mu\text{g}/\text{cm}^2/\text{h}$, respectively, while NEG-2 exhibited higher values at 10.73 and 20.87 $\mu\text{g}/\text{cm}^2/\text{h}$, respectively. In methanolic PBS pH 6.8, the mean transdermal flux of DCN and ACE from NEG-1 increased to 11.08 and 34.61 $\mu\text{g}/\text{cm}^2/\text{h}$, respectively, whereas NEG-2 showed further enhancement with values of 12.03 and 36.31 $\mu\text{g}/\text{cm}^2/\text{h}$, respectively. Notably, both flux and skin deposition of DCN and ACE were significantly higher in methanolic PBS pH 6.8 compared to PBS pH 5.7 for all formulations.

Furthermore, NEG-2 resulted in significantly greater drug deposition on the skin compared to NEG-1 and the plain gel. Specifically, skin deposition for DCN was $26.30 \pm 3.58 \mu\text{g}/\text{cm}^2$ and for ACE was $53.15 \pm 6.54 \mu\text{g}/\text{cm}^2$ in PBS pH 5.7 for NEG-2. In methanolic PBS pH 6.8, these values increased to $29.22 \pm 2.14 \mu\text{g}/\text{cm}^2$ for DCN and $64.31 \pm 5.09 \mu\text{g}/\text{cm}^2$ for ACE, further emphasizing the superior performance of NEG-2.

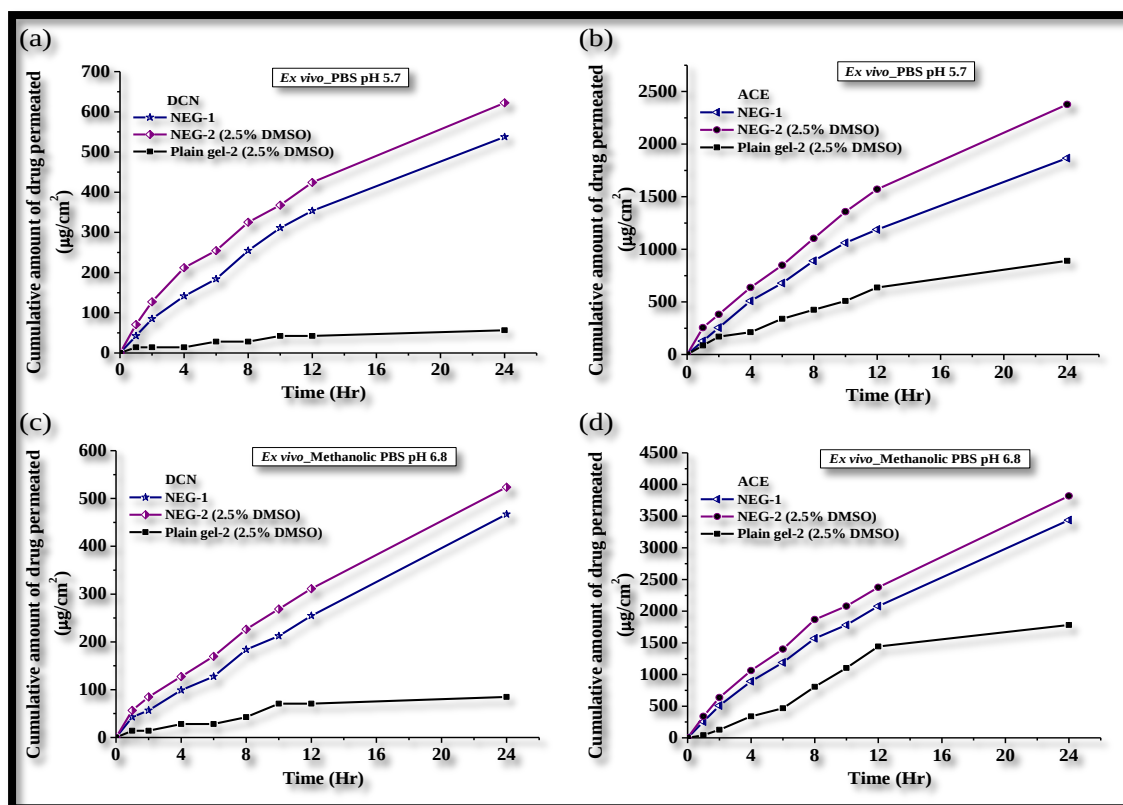


Figure 21. *Ex vivo* permeation profile of DCN and ACE from NEG-1, NEG-2, and Plain gel-2 in PBS pH 5.7 (a and b), and methanolic PBS pH 6.8 (c and d).

To understand the release patterns of the NEG formulations and plain gel, *ex vivo* release data were fitted to various mathematical models. The model which shows the maximum correlation coefficient value (R^2) was chosen as the best match to describe drug release kinetics⁽¹³⁷⁾. The R^2 value revealed that all formulations matched the Korsmeyer-Peppas model, which describes drug diffusion. The formulations, NEG-1, NEG-2, and Plain gel-2 achieved R^2 values of 0.9111 to 0.9984 for DCN and 0.9312 to 0.9989 for ACE in both media, indicating excellent fit. Additionally, the diffusional exponent (n) for DCN and ACE from all three formulations was in the range of 0.553 to 0.872. Since all n values fall between 0.45 and 1, the drug release mechanism for all formulations is likely a (anomalous transport) combination of dissolution and diffusion. The optimized NEG formulation (NEG-2) demonstrates the most promising potential for effective drug delivery based on its superior permeation and skin deposition characteristics.

Table 15. Results of rat skin permeation and deposition study of NE, NEG, and plain gel formulation in different media.

Formulation	Drug	Transdermal flux ($\mu\text{g}/\text{cm}^2/\text{h}$)	Permeability coefficient (cm/hr)	Enhancement ratio (%)	Skin deposition ($\mu\text{g}/\text{cm}^2$)
At PBS pH 5.7					
NEG-1	DCN	9.69 ± 0.14	3.87 ± 0.05	5.47	11.29 ± 0.92
	ACE	17.71 ± 0.22	3.54 ± 0.04	13.64	20.29 ± 2.12
NEG-2	DCN	10.73 ± 0.16	4.29 ± 0.06	6.14	26.30 ± 3.58
	ACE	20.87 ± 0.13	4.17 ± 0.03	16.07	53.15 ± 6.54
Plain gel-2	DCN	1.74 ± 0.01	0.69 ± 0.01	1.00	4.48 ± 0.58
	ACE	1.28 ± 0.02	0.26 ± 0.03	1.00	11.78 ± 1.72
At methanolic PBS pH 6.8					
NEG-1	DCN	11.08 ± 0.05	4.43 ± 0.02	6.05	16.66 ± 1.03
	ACE	34.61 ± 0.25	6.92 ± 0.05	19.33	38.33 ± 2.07
NEG-2	DCN	12.03 ± 0.02	4.81 ± 0.01	6.30	29.22 ± 2.14
	ACE	36.31 ± 0.18	7.26 ± 0.04	20.23	64.31 ± 5.09
Plain gel-2	DCN	1.84 ± 0.02	0.73 ± 0.02	1.00	10.38 ± 1.48
	ACE	1.80 ± 0.02	0.36 ± 0.02	1.00	22.41 ± 2.50

SD: standard deviation; Mean $\pm$ SD, n=6.

4.6.4 DSC and FTIR analysis of DCN, ACE, physical mixture, optimized NE, and NEG

DSC thermogram of DCN, ACE, physical mixture, optimized NE, and NEG formulation are shown in Fig. 22a-d. DSC thermograms exhibited sharp endothermic peaks at 155.40°C and 260.82°C for ACE and DCN, respectively, confirming their

crystalline nature and aligning with previously reported melting points (Fig. 22a-b) (131,132). DSC was also performed on a physical mixture (Fig. 22c) of combined drugs (DCN and ACE) with Triacetin (283.29°C), Capmul MCM C8 (196.75°C), Kolliphor-EL (133.89°C), Transcutol P (167.35°C, 212.17°C), and DMA (153.93°C). Optimized NE and NEG formulation (Fig. 22d) showed endotherm at 133.10°C and 116.71°C, respectively. No peaks were observed for DCN and ACE in the physical mixture, optimized NE, and optimized NEG formulation DSC thermograms. This indicates that DCN and ACE were compatible with all excipients, did not exist in a solid state, and was completely entrapped within the core of NE and NEG.

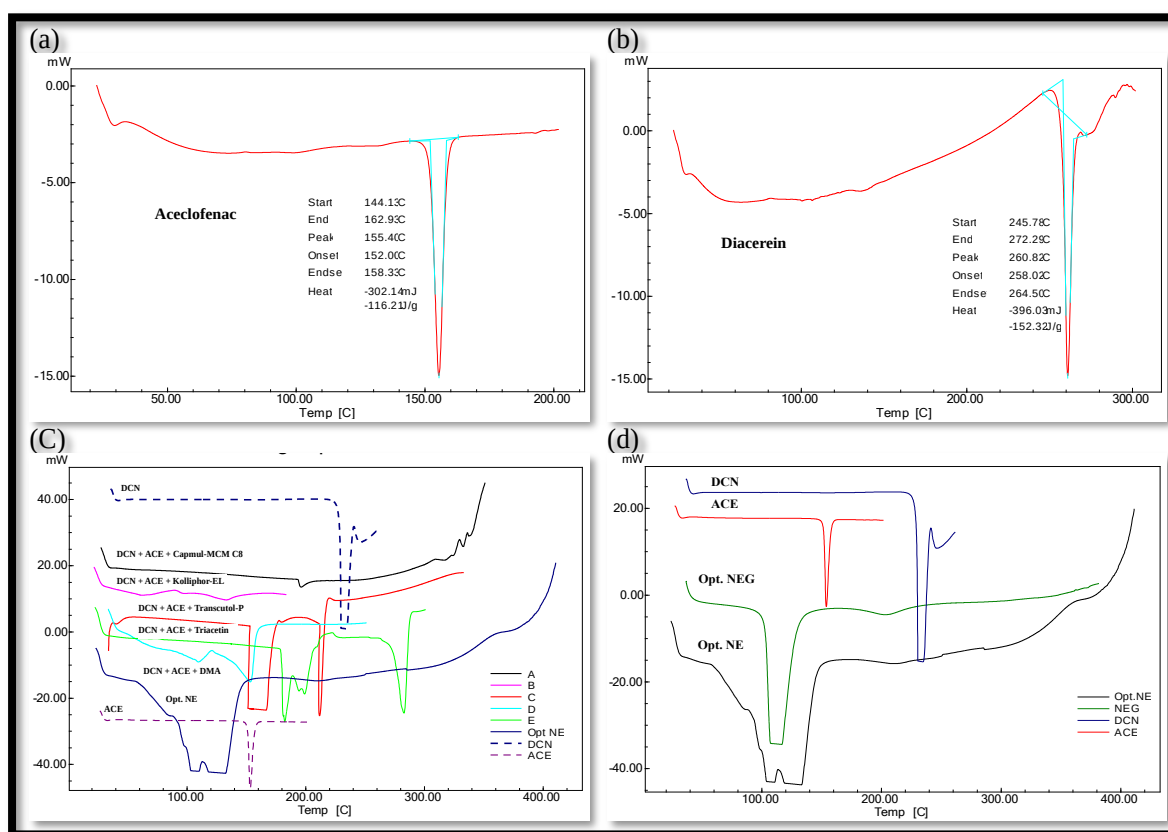


Figure 22. DSC thermograms for (a) ACE API, (b) DCN API, (c) DCN, ACE, Physical mixture of DCN and ACE with Capmul MCM C-8 (A), Kolliphor-EL (B), Transcutol-P (C), DMA (D), Triacetin (E), and optimized NE, and (d) DCN, ACE, optimized NE, and optimized NEG.

The FTIR spectrum of DCN, ACE, individual excipients, and a mixture of excipients with DCN and ACE are shown in Fig. 23a-j. The FTIR spectra of DCN (Fig. 23a) in KBr exhibit characteristic absorption peaks at wave numbers of 3071 cm^{-1} (aromatic, C-H), 2986.9 cm^{-1} (aliphatic, C-H), 1766.87 cm^{-1} (ester group, C=O), 1669.46 cm^{-1} , (carboxylic, C=O), 1594.23 cm^{-1} (aromatic, C=C), 1463.07 cm^{-1} (aliphatic, C-C), 1369.52 cm^{-1} (O-H), 1193.99 cm^{-1} , (ester, C-O), and 1028.1 cm^{-1} (ester, C-O-C) and found to be aligned with the published data. Similarly, ACE (Fig. 23b) in KBr exhibit characteristic absorption peaks at wave numbers of 3319.63 cm^{-1} (N-H), 1771.69 (ester group, C=O), 1717.68 cm^{-1} (carbonyl group, C=O), 1463.43 & 1438.96 cm^{-1} (C-C), and 750.34 cm^{-1} (C-Cl). The obtained peaks were found to be allied with published data.

Also, the FTIR spectra of optimized NE and NEG formulation, along with their placebo formulation, are shown in Fig. 24a-d. The FTIR spectra of optimized NE (Fig. 24b) showed peaks at 3391.18 , 2938.65 , 2139.69 , 1734.35 , 1612.4 , 1505.3 , 1410.48 , 1231.58 , 1097.98 , 1064.39 , 945.67 , 834.08 , and 732.54 cm^{-1} , whereas the optimized NE placebo formulation (without DCN and ACE) (Fig. 24a) showed peaks at 3393.98 , 2879.83 , 2341.07 , 1734.85 , 1613.62 , 1505.27 , 1409.4 , 1230.85 , 1098.7 , 1062.47 , 945.78 , 835.16 , 691.57 cm^{-1} .

The FTIR spectra (Fig. 24d) of optimized NEG showed peaks at 3402.68 , 2935.67 , 2876.36 , 2142.45 , 1734.37 , 1613.51 , 1506.63 , 1452.84 , 1420.07 , 1373.52 , 1100.63 , 1061.91 , 946.13 , 839.28 , and 742.08 cm^{-1} . whereas the optimized NEG placebo formulation (without DCN and ACE) (Fig. 24c) showed peaks at 3402.79 , 2938.26 , 2877.8 , 2138.68 , 1734.97 , 1614.35 , 1506.78 , 1419.74 , 1403.01 , 1374.13 , 741.74 cm^{-1} . The FTIR spectra of the physical mixture, optimized NE, and optimized NEG do not show any characteristic peaks of DCN and ACE. Also, comparing the

FTIR spectra of DCN, ACE, optimized NE and NEG formulation with the respective placebo formulations (without DCN and ACE), no difference was shown in the position and trend of the absorption bands indicating the compatibility of DCN and ACE with the excipients used. Also, the FTIR results correlate well with DSC results, demonstrating that DCN and ACE were completely solubilized and incorporated into the formulated NE without any physical and chemical incompatibility with the excipients.

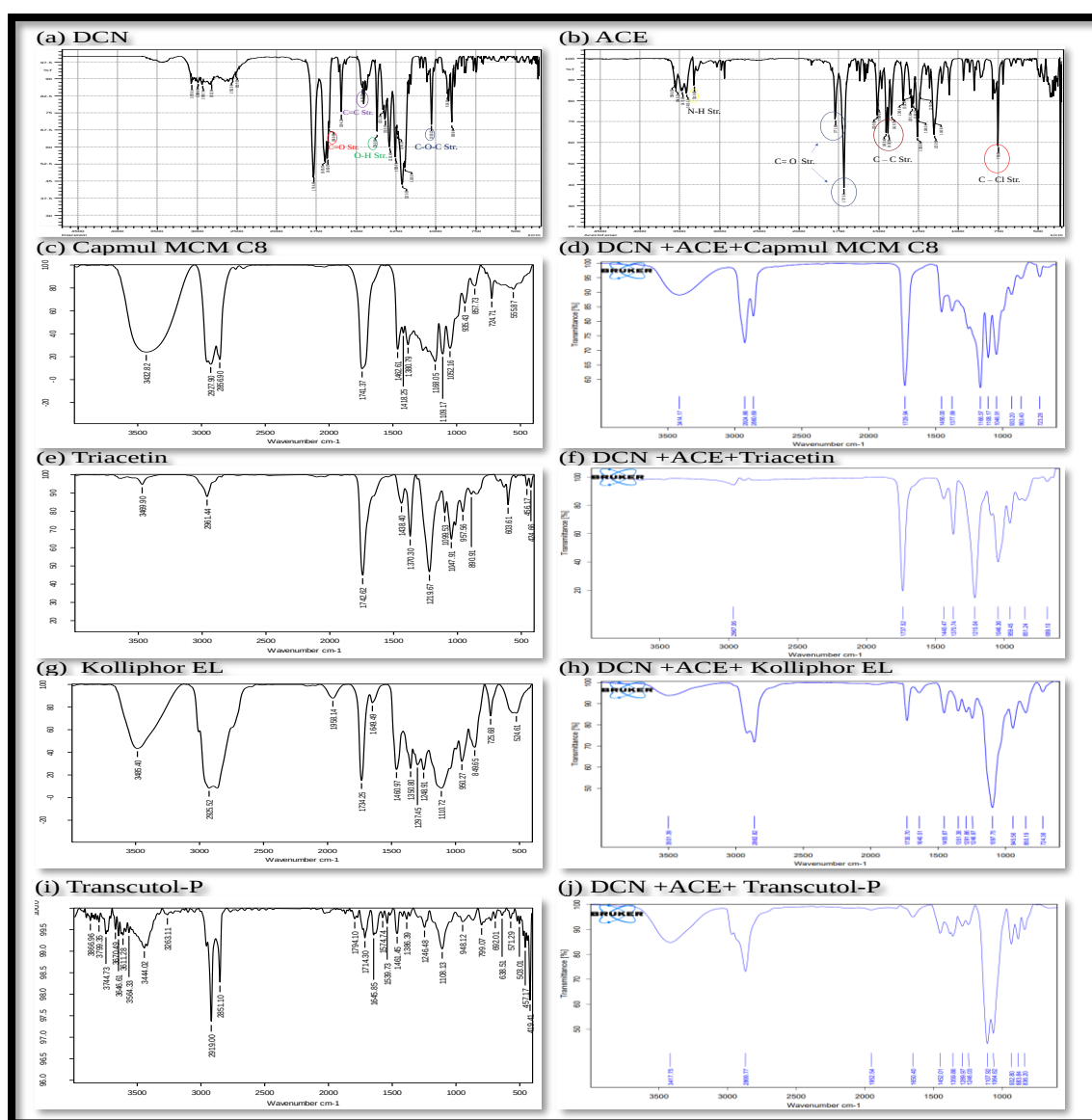


Figure 23. FTIR spectra of (a) DCN, (b) ACE, (c) Capmul MCM C8, (d) DCN+ACE + Capmul MCM C8, (e) Triacetin, (f) DCN + ACE + Triacetin, (g) Kolliphor-EL, (h) DCN + ACE + Kolliphor-EL, (i) Transcutol P, (j) DCN + ACE + Transcutol P.

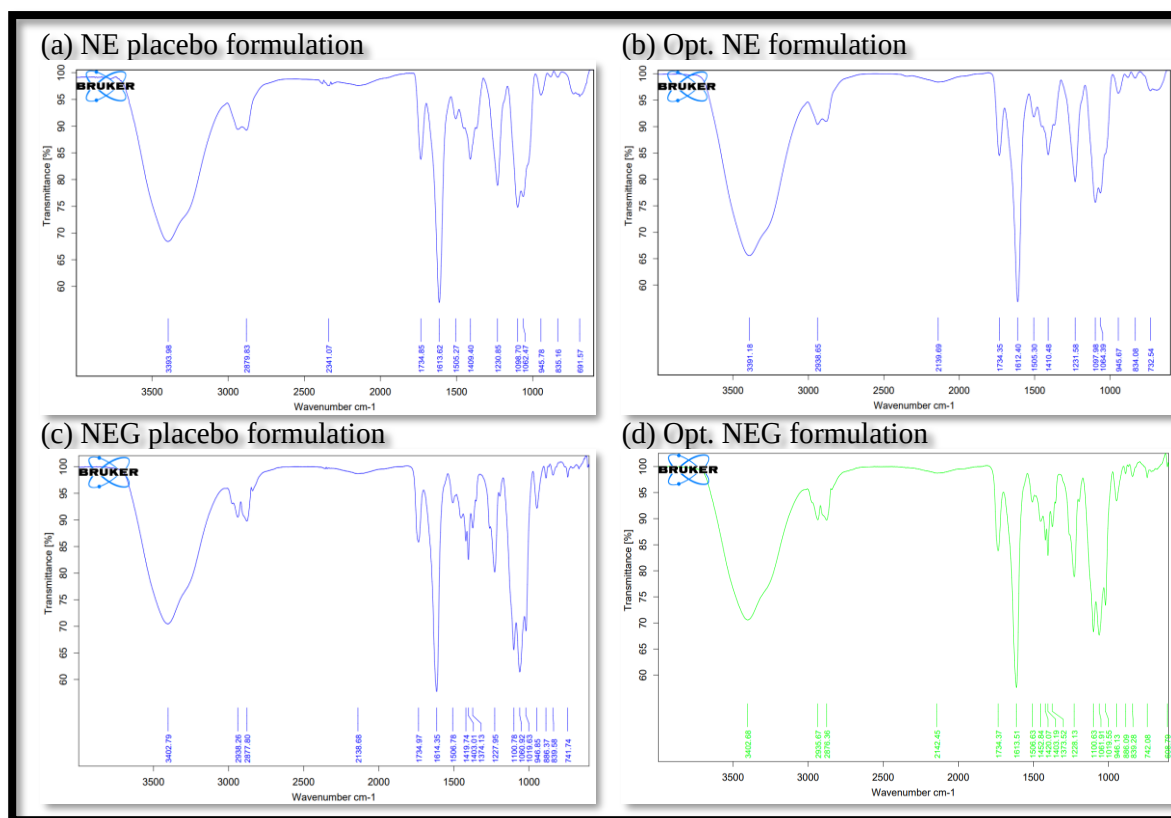


Figure 24. FTIR spectra of (a) NE placebo, (b) Opt. NE, (c) NEG placebo, and (d) Opt. NEG formulation.

4.6.5 Stability study of optimized NEG formulation

The proposed NEG exhibited good physico-chemical stability for 3 months across all storage conditions based on the results of the evaluated parameters (Table 16). As per the FTIR spectra (Fig. 25a-c), no alteration in the peak was seen between the initial level and after 3 months in both storage environments, as indicated by the consistent position and trend of the absorption bands. Overall, no substantial changes were noticed in appearance, pH values, viscosity, FTIR spectra, % drug content, and drug permeation over the 3 months study period.

Table 16. Results of the stability study of optimized NEG formulation.

Parameter	Initial	Room temperature	40 ± 2°C / 75 ± 5% RH			
		3M	1M	2M	3M	
Appearance	Yellowish translucent gel					
pH ± SD	5.97 ± 0.04	5.96 ± 0.03	5.98 ± 0.11	5.96 ± 0.20	5.99 ± 0.07	
Viscosity at 100 rpm ± SD (cps)	484.66± 6.01	479.11±10.50	474±8.58	472±5.38	466.33±12.44	
Spreadability ± SD (g.cm2)	36.67 ± 0.26	36.83 ± 0.14	37.57 ± 1.05	37.18 ± 1.54	38.07 ± 0.42	
Drug content (%) ± SD	DCN:	DCN:	DCN:	DCN:	DCN:	
	98.87 ± 0.54	94.93 ± 0.38	95.70 ± 0.14	93.68 ± 0.27	91.07 ± 0.41	
	ACE:	ACE:	ACE:	ACE:	ACE:	
	98.25 ± 0.48	95.08 ± 0.52	95.88 ± 0.12	92.52 ± 0.36	90.34 ± 0.15	
In vitro % cumulative drug permeation (after 24 hrs)						
Methanolic PBS pH 6.8	DCN:	DCN:			DCN:	
	36.89 ± 0.67	34.78 ± 0.61	--	--	30.29 ± 0.54	
	ACE:	ACE:			ACE:	
	89.10 ± 0.49	85.52 ± 0.45	--	--	80.99 ± 0.41	
PBS pH 5.7	DCN:	DCN:			DCN:	
	44.18 ± 0.27	43.64 ± 0.32	--	--	36.94 ± 0.22	
	ACE:	ACE:			ACE:	
	58.02 ± 0.42	56.37 ± 0.69	--	--	53.38 ± 0.56	

PBS: Phosphate buffer solution; SD: standard deviation; Mean± SD, n=3.

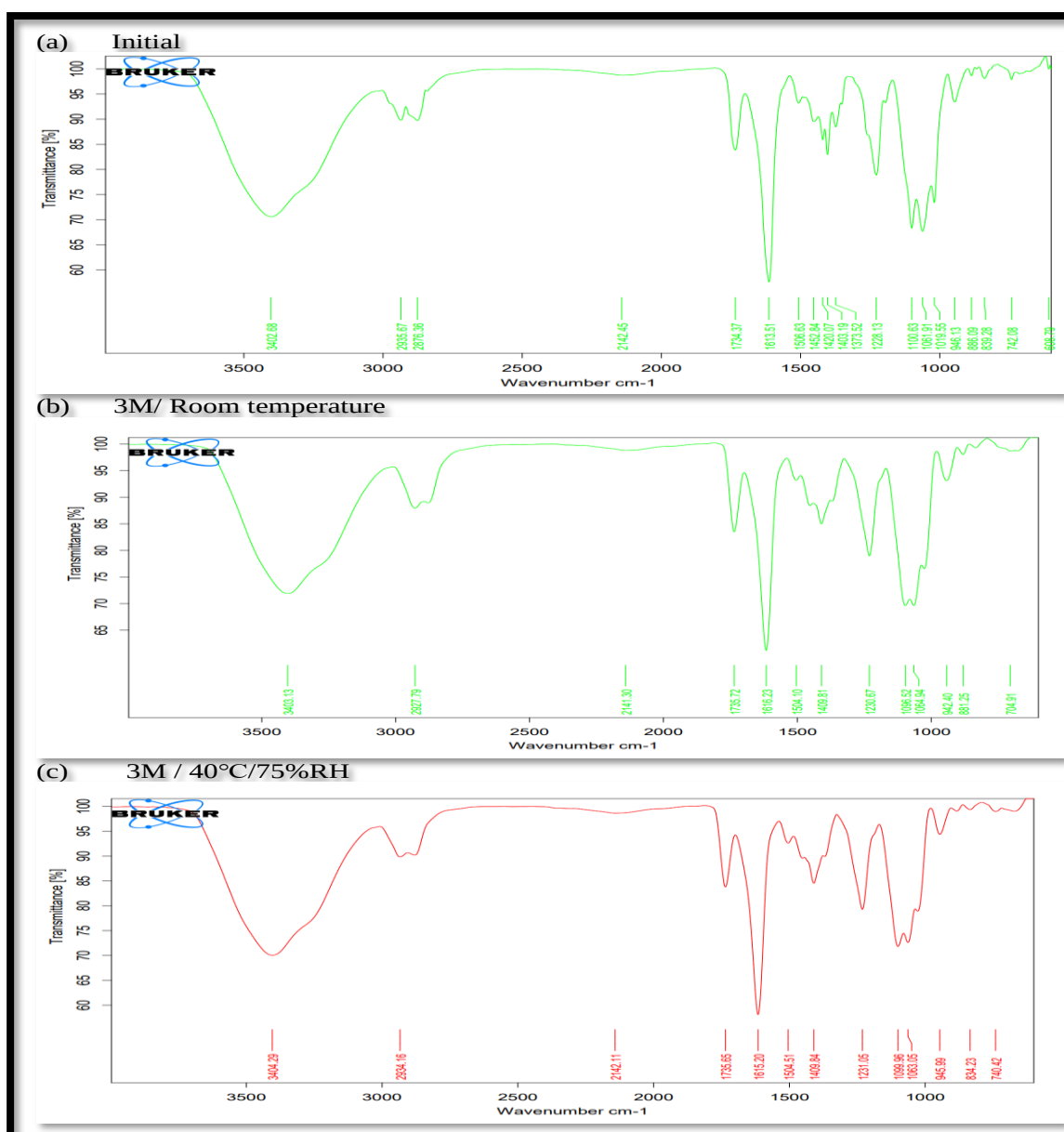


Figure 25. FTIR spectra of initial sample (a) and stability samples (b-c) of proposed NEG formulation.

4.7 Skin irritation study of optimized NEG formulation

A rat skin irritation study was conducted to evaluate the safety profile of the NEG formulation. The Draize scores (a scoring system for irritation) for the optimized NEG formulation and control groups are summarized in Table 17. The optimized NEG did not cause any signs of erythema (reddening) or edema (swelling) during the 72 h observation period of the acute toxicity study. In contrast, the positive

control, aqueous formalin (0.8% v/v) solution, induced significant skin reactivity characterized by moderate to severe erythema (scores ranging from 1-3) and edema (scores ranging from 1-2) at 24 and 72 h. These findings demonstrate that the optimized NEG formulation is free from skin irritation.

4.7.1 Histopathological examination

The possibility of skin toxicity of the formulated NEG was further investigated through a histopathology study on treated rat skin after seven days of application. Figure 26 displays representative images (a-c) from each group: normal control, positive control (aqueous formalin), and optimized NEG. The normal control group (Fig. 26a) showed an intact epidermal layer with no signs of spongiosis, congestion, or inflammation, as expected. In contrast, the formalin-treated (positive control) group (Fig. 26b) displayed significant damage to the epidermal layer. This damage included exocytosis (movement of cells out of tissues), congestion, inflammation, and separation of collagen fibres, confirming the irritant potential of formalin. Notably, the skin treated with the optimized NEG (Fig. 26c) exhibited an intact epidermal layer with no signs of erosion, spongiosis, collagen fibre separation, or inflammation. The optimized NEG showed similar results to the normal skin, with no observable changes. Founded on the acute toxicity study and histopathology results, the optimized NEG formulation demonstrated acceptable safety for topical application.

Table 17. Mean Erythema and Edema scores after application of formulation on rat skin (Draize method).

Groups/formulations	Erythema (reddening) score				Edema (swelling) score			
	1h	24h	48h	72h	1h	24h	48h	72h
Normal control	0	0	0	0	0	0	0	0
Formalin solution (0.8%v/v)	0	1	2.67	3	0	1	2	2
Opt. NEG-treated groups	0	0	0	0	0	0	0	0

n=3.

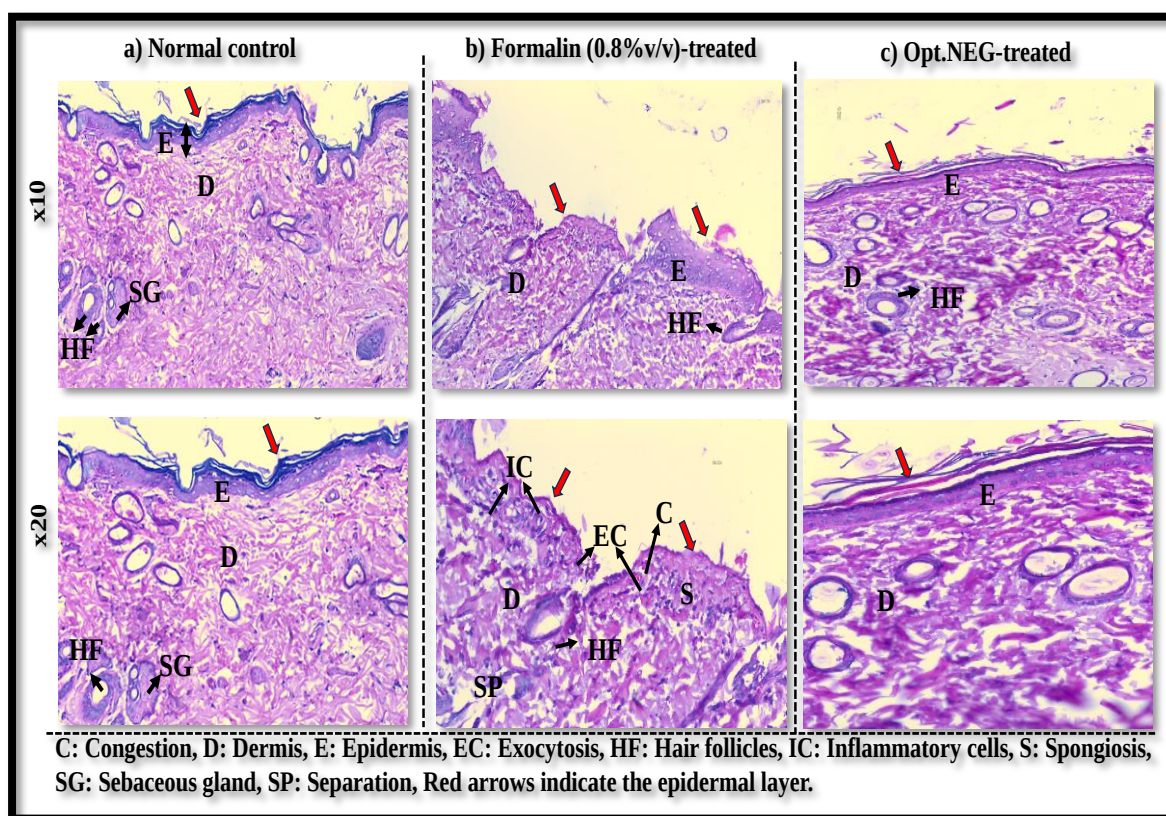


Figure 26. Histopathological examination (H&E staining) of rat skin after 7 days of application for the (a) Normal control, (b) Formalin (0.8%v/v) treated, and (c) Opt. NEG-treated group. (x10 and x20).

4.8 Bioanalytical method for estimation of RH, ACE, and DL

4.8.1 Method development and optimization of chromatographic method

Based on the development and optimization data of previously developed *in vitro* HPLC method (described in section 2.4) and additional study using two C_{18} columns (Phenomenex Luna and Inertsil ODS-3V), the mobile phase (mixture of 0.1% v/v OPA buffer and ACN) and Phenomenex C_{18} column was selected for chromatographic separation. Further, ACN and methanol were selected as the precipitating and recovery solvents, respectively, for extracting all analytes from plasma (Fig. 27).

Pareto chart analysis (Fig. 28 a-d) indicated that buffer pH and %ACN significantly influenced all responses. Interaction between these factors impacted resolutions R2 and R3. Flow rate and column temperature affected RT-DL and resolution R2. The blue and orange bars in the Pareto chart represent a negative and positive effect on the selected response, respectively.

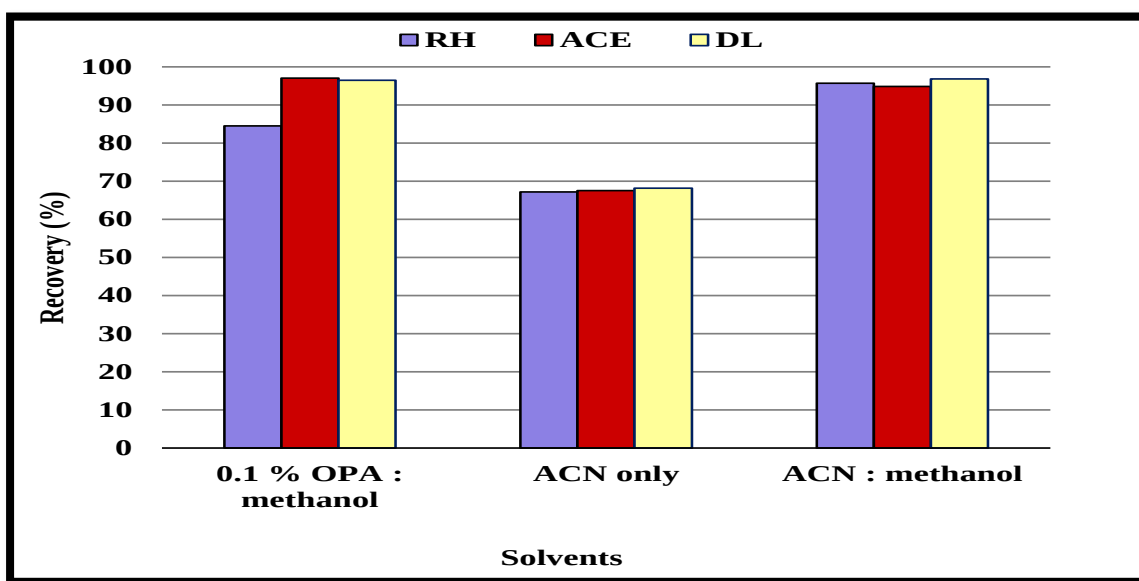


Figure 27. Recovery of rhein, aceclofenac, and diclofenac sodium in different protein precipitating solvents along with methanol as extracting agent.

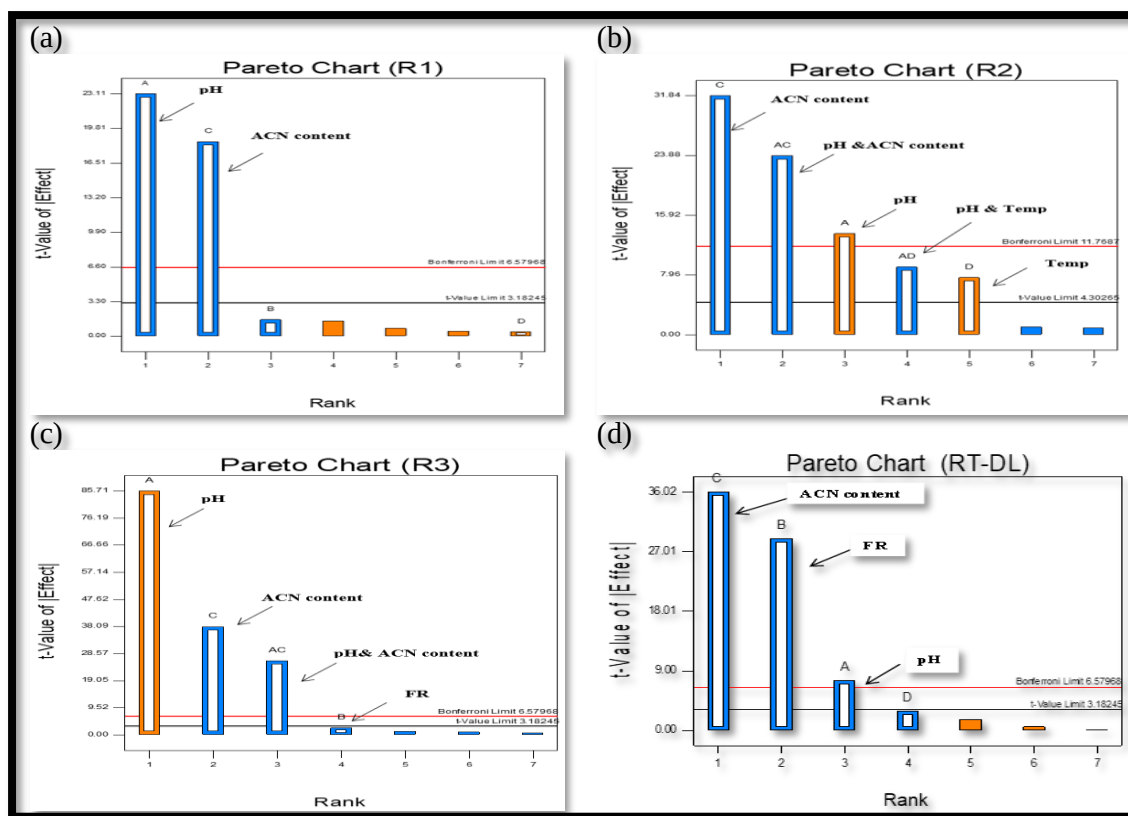


Figure 28. Pareto chart displaying the effect of independent factors on the responses (a) R1 (RH-ACE), (b) R2 (ACE-FA), (c) R3 (FA –DL), and (d) RT (DL).

Based on the FFD results, followed by statistical analysis and numerical optimization using BBD, to achieve R1 (≥ 6.725), R2 (≥ 2.658), R3 (≥ 3.384) and RT-DL (8-9), the software recommended optimal experimental conditions: a flow rate of 1 mL/min, a buffer pH of 3.5, and an acetonitrile content of 60%, with maximum desirability of 0.938 (Fig. 29).

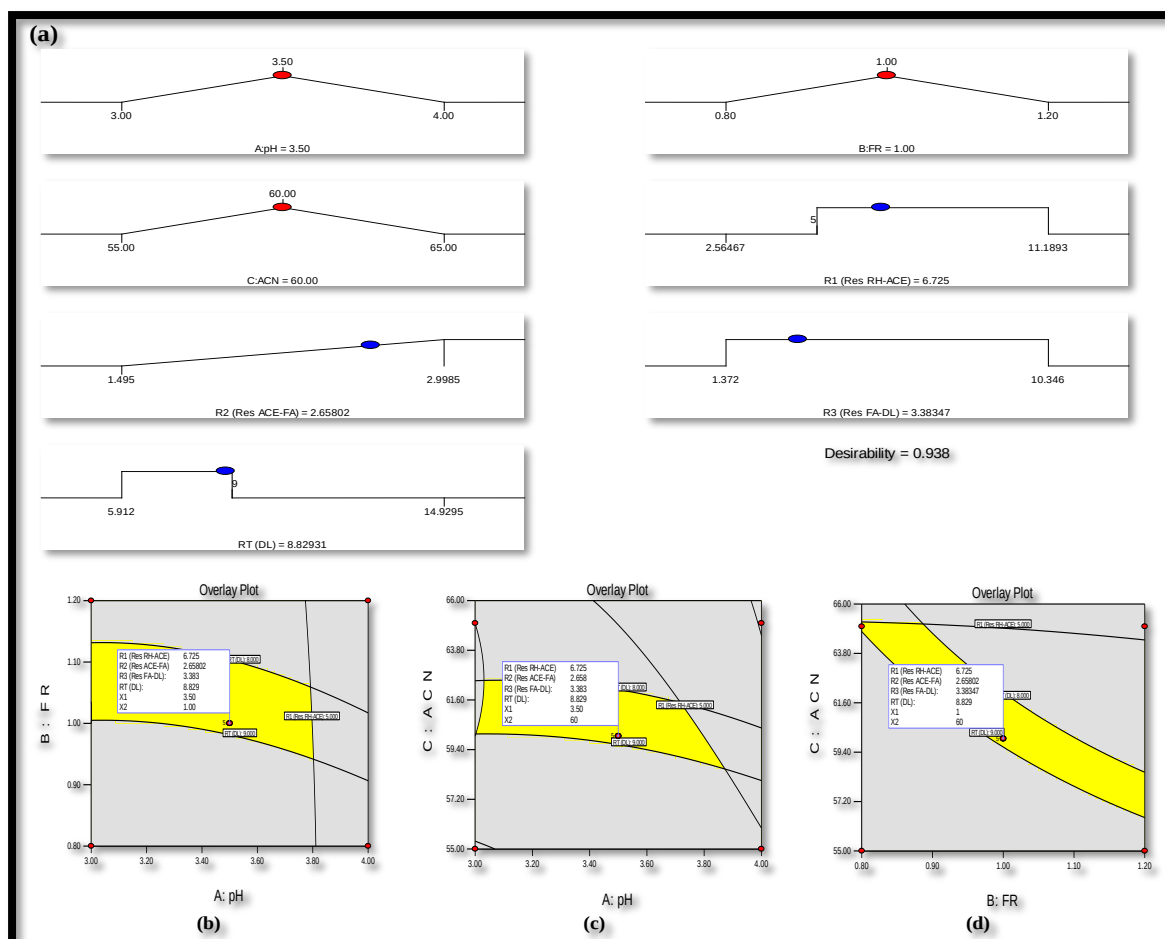


Figure 29. Desirability bar graph (a) for all individual factors, responses, and combination of all responses and overlay plot (b-d) of the selected responses.

4.8.2 Method validation

The proposed method was validated as per the US Food and Drug Administration (USFDA) guidelines for bioanalytical method validation (U.S. Department of Health and Human Services and USFDA 2018). The chromatogram (Fig. 30) obtained from the internal standard (IS) solution, mixed working standard solution, blank rat plasma sample, rat plasma spiked with IS, plasma samples spiked with three analytes and IS (LLOQ, LQC & MQC), and rat plasma sample after 30 min of oral administration of Dycerin A tablet were compared. The RT for RH, ACE, FA, and DL were found to be 5.02 ± 0.01 , 7.03 ± 0.02 , 7.72 ± 0.02 , and 8.93 ± 0.03 min,

respectively. Figure 30 confirmed the method's specificity and selectivity by demonstrating consistent retention times for all analytes without any co-elution with RH, ACE, FA, or DL peaks.

The MQC plasma sample was injected six consecutive times, and system suitability variables like RT, peak area, theoretical plates (TP), peak resolution, tailing factor (TF), and height equivalent to a theoretical plate (HETP) were evaluated from the obtained chromatogram (Table 18). All system suitability variables were well within the specified limits as per FDA guidelines. The calibration curve obtained was linear ($R^2 > 0.999$) over the concentration range of 25-3000 ng/mL for RH, 50-6000 ng/mL for ACE, and 50-6000 ng/mL for DL, demonstrating its suitability and adequate sensitivity for analysis (Fig. 31). The LLOQ for RH, ACE, and DL was determined to be 25, 50, and 50 ng/mL, respectively. The average recovery $\pm$ RSD was found to be 95.63 ± 3.22 % for RH, 96.10 ± 2.367 % for ACE, 92.27 ± 8.18 % for DL, and 91.85 ± 5.08 % for FA. Whereas, at LLOQ, the mean percentage recovery was found to be 91.25 for RH, 95.00 for ACE, and 80.11 for DL. The developed method is determined to be precise and reliable as the % CV (precision) in intra-day and inter-day plasma samples was found to be less than 5.0. The accuracy results for intra-day and inter-day were -7.13 to 18.88 % and -6.66 to 17.94 % for RH, ACE, and DL.

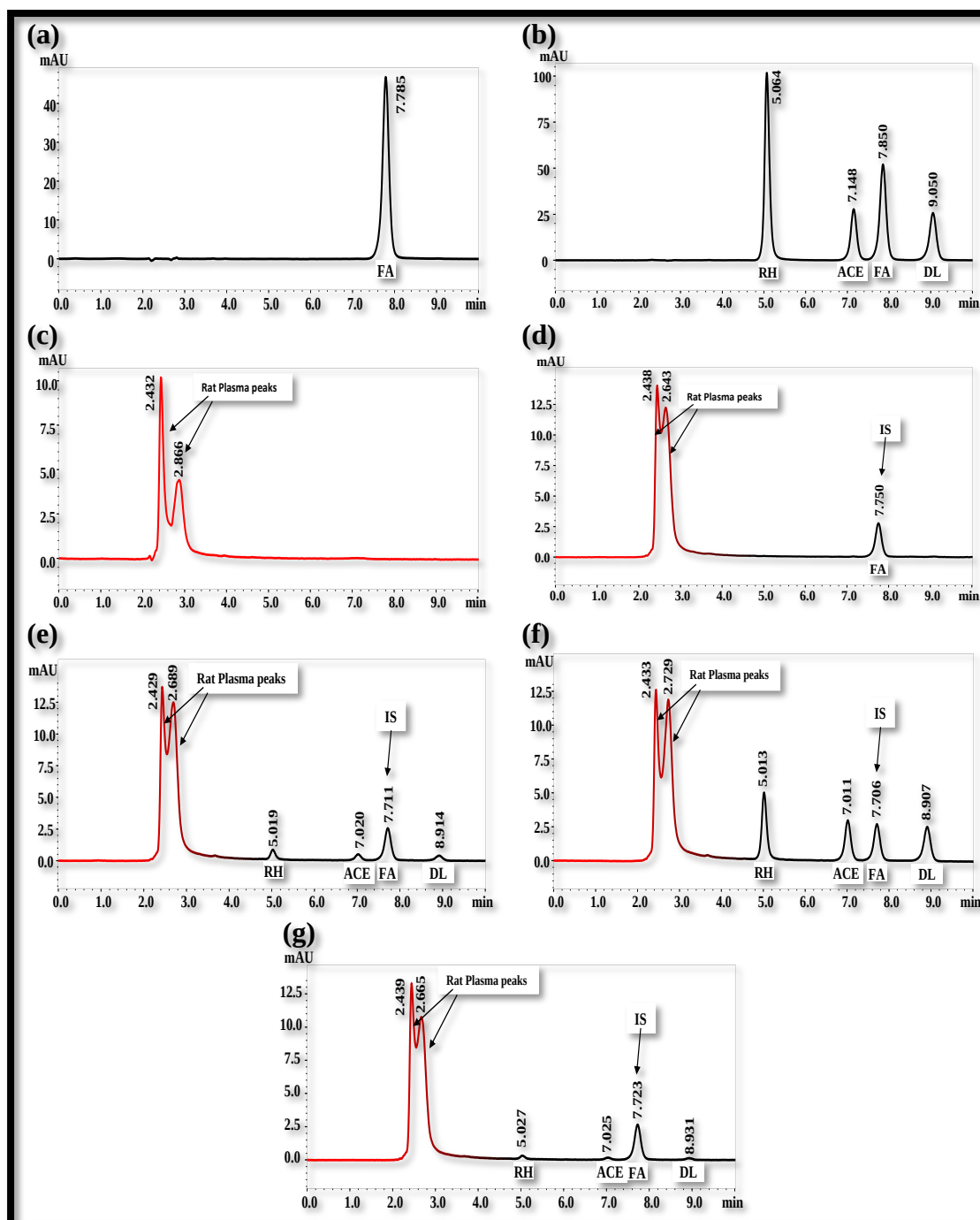
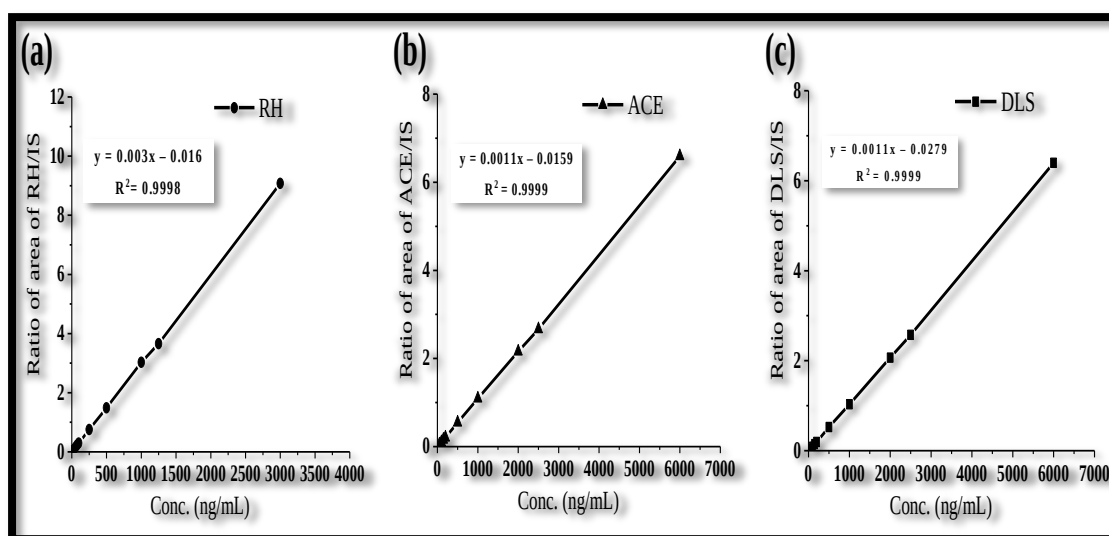


Figure 30. Chromatogram of (a) FA standard solution (IS) 10 $\mu\text{g/mL}$, (b) Mixed RH (10 $\mu\text{g/mL}$), ACE (20 $\mu\text{g/mL}$), FA (10 $\mu\text{g/mL}$), and DL (20 $\mu\text{g/mL}$) standard solution, (c) Blank rat plasma, (d) Blank plasma spiked with IS (500 ng/mL), (e) Blank plasma spiked with RH, ACE, DL & IS at LQC, (f) Blank plasma spiked with RH, ACE, DL & IS at MQC, (g) Blank plasma spiked with RH, ACE, DL & IS at LLOQ.

Table 18. Results of system suitability parameters in spiked plasma samples.

Parameters	RH	ACE	FA	DL
Retention time, RT (min), (Mean $\pm$ SD)	5.02 ($\pm$ 0.01)	7.03 ($\pm$ 0.02)	7.72 ($\pm$ 0.02)	8.93 ($\pm$ 0.03)
Peak area (Mean $\pm$ SD)	42888.83 ($\pm$ 295.54)	31424.00 ($\pm$ 249.98)	30206.00 ($\pm$ 352.81)	29699.17 ($\pm$ 205.15)
Theoretical plates (TP) (Mean $\pm$ SD)	7278.92 ($\pm$ 36.20)	10049.28 ($\pm$ 21.00)	10874.28 ($\pm$ 51.14)	12778.24 ($\pm$ 61.61)
Tailing factor (TF) (Mean $\pm$ SD)	1.16 ($\pm$ 0.01)	0.97 ($\pm$ 0.0.01)	0.94 ($\pm$ 0.01)	0.92 ($\pm$ 0.01)
Peak resolution (Mean $\pm$ SD)	---	7.78 ($\pm$ 0.02)	2.41 ($\pm$ 0.01)	3.94 ($\pm$ 0.01)
HETP (mm) (Mean $\pm$ SD)	20.61 ($\pm$ 0.10)	14.93 ($\pm$ 0.03)	13.79 ($\pm$ 0.06)	11.74 ($\pm$ 0.06)

Mean $\pm$ SD, n=6.**Figure 31. Linearity graph of (a) RH, (b) ACE, and (c) DLS.**

The stability of RH, ACE, and DL was evaluated under various conditions. Results (Table 19) indicate that all three analytes exhibited acceptable stability at room temperature for 24 h and at -20°C for 72 h. While freeze-thaw cycles and long-term storage at -20°C resulted in slight degradation, the overall stability of all analytes met USFDA guidelines. Rhein demonstrated greater stability compared to ACE and DL. Additionally, DL showed increased degradation at higher concentrations, whereas ACE degradation was consistent across concentration levels.

Table 19. Stability results of quality controls of RH, ACE, and DL in rat plasma at different conditions.

Stability conditions	Time / Cycle	Analyte	At low QC (RH: 75 ng/mL, ACE & DL: 150 ng/mL)			At high QC (RH: 1000 ng/mL, ACE & DL: 2000 ng/mL)		
			Mean conc. (ng/mL) $\pm$ SD, (n=5)	CV (%)	Bias (%)	Mean conc. (ng/mL) $\pm$ SD, (n=5)	CV (%)	Bias (%)
Initial	Zero time	RH	76.43 $\pm$ 3.70	4.33	1.90	990.03 $\pm$ 2.26	0.20	-1.00
		ACE	147.11 $\pm$ 1.70	1.03	-1.93	1979.18 $\pm$ 2.51	0.11	-1.04
		DL	155.67 $\pm$ 12.8	7.34	3.78	1967.37 $\pm$ 7.71	0.36	-1.63
Short-term stability	24 h (RT)	RH	74.18 $\pm$ 0.60	0.73	-1.10	992.46 $\pm$ 4.08	0.37	-0.75
		ACE	143.77 $\pm$ 6.14	3.82	-4.15	1931.46 $\pm$ 12.6	0.58	-3.43
		DL	147.14 $\pm$ 4.49	2.73	-1.91	1946.46 $\pm$ 10.6	0.48	-2.68
Freeze-thaw (-20°C) stability	1 st cycle, (24 h)	RH	74.43 $\pm$ 1.88	2.26	-0.76	979.28 $\pm$ 8.96	0.82	-2.07
		ACE	146.16 $\pm$ 3.86	2.36	-2.56	1943.62 $\pm$ 11.0	0.51	-2.82
		DL	142.70 $\pm$ 7.54	4.73	-4.87	1916.33 $\pm$ 5.70	0.27	-4.18
Freeze-thaw (-20°C) stability	2 nd cycle, (48 h)	RH	72.15 $\pm$ 0.71	0.88	-3.79	961.97 $\pm$ 1.59	0.15	-3.80
		ACE	144.41 $\pm$ 5.67	3.51	-3.72	1945.12 $\pm$ 4.63	0.21	-2.74
		DL	148.57 $\pm$ 6.37	3.83	-0.96	1959.26 $\pm$ 10.0	0.46	-2.04

Stability conditions	Time / Cycle	Analyte	At low QC (RH: 75 ng/mL, ACE & DL: 150 ng/mL)			At high QC (RH: 1000 ng/mL, ACE & DL: 2000 ng/mL)		
			Mean conc. (ng/mL) $\pm$ SD, (n=5)	CV (%)	Bias (%)	Mean conc. (ng/mL) $\pm$ SD, (n=5)	CV (%)	Bias (%)
Freeze-thaw (-20°C) stability	3 rd cycle, (72 h)	RH	75.26 $\pm$ 1.85	2.20	0.35	992.77 $\pm$ 3.41	0.31	-0.72
		ACE	147.30 $\pm$ 4.88	2.96	-1.80	1960.18 $\pm$ 8.77	0.40	-1.99
		DL	150.10 $\pm$ 5.99	3.57	0.07	1939.54 $\pm$ 3.77	0.17	-3.02
Freeze-thaw (-20°C) stability	15 days	RH	75.50 $\pm$ 2.39	2.84	0.66	1006.48 $\pm$ 0.83	0.05	0.65
		ACE	147.57 $\pm$ 4.86	2.94	-1.62	1964.09 $\pm$ 6.04	0.28	-1.80
		DL	155.27 $\pm$ 12.3	7.10	3.51	1958.81 $\pm$ 9.76	0.45	-2.06

SD: standard deviation; Mean $\pm$ SD, n=5.

4.9 *In vivo* release study of optimized DCN- and ACE-loaded NEG and marketed tablet formulation

An *in vivo* release study of an optimized NEG and marketed tablet formulation was conducted in Wistar rats. Plasma concentrations of RH, ACE, and DL were determined at various time points using a developed HPLC method. Mean plasma concentration vs. time curves for RH, ACE, and DL were plotted (Fig. 32) and analyzed using a non-compartmental analysis program with WinNonlin software (version 8.3.4.295) (Table 20). For the tablet formulation, pharmacokinetic profiling was limited to 10 h due to analyte concentrations falling below the lower limit of quantification (LLOQ) for RH (25 ng/mL) and ACE and DL (50 ng/mL). Consequently, the pharmacokinetic profiles of the two formulations were not entirely superimposable.

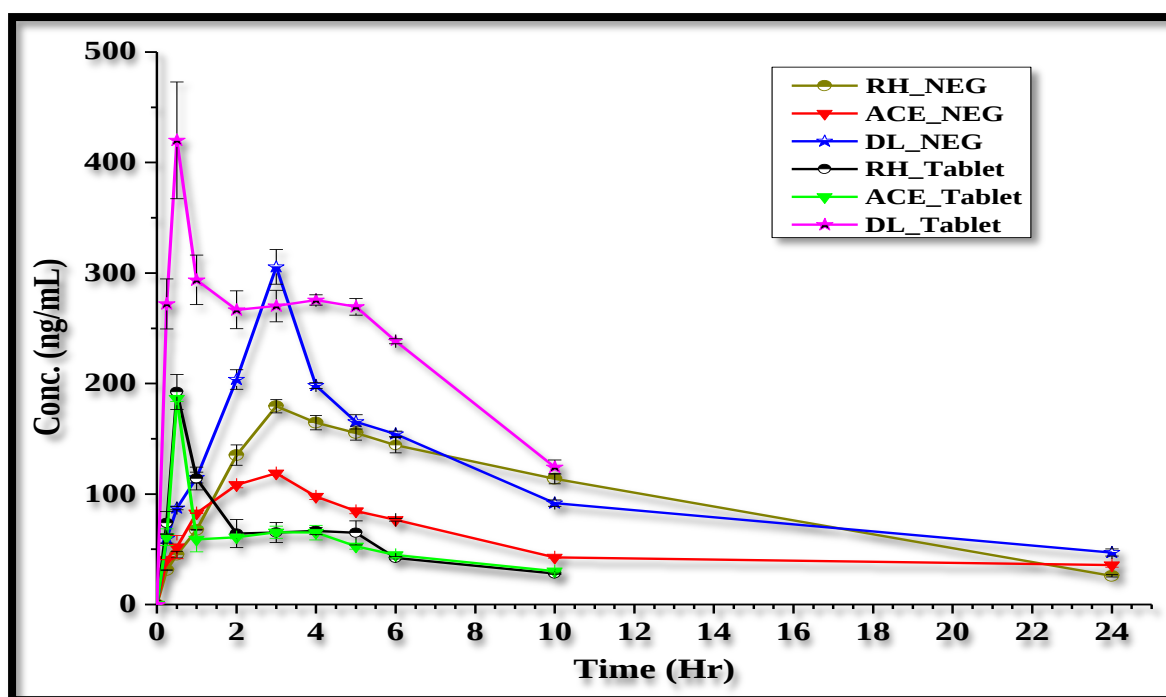


Figure 32. Mean plasma concentration vs time profile with standard error of the mean bars for RH, ACE, and DL after oral single-dose administration of Dycerin A tablet and topical administration of optimized NEG to Wistar rat (DCN: 15mg/kg and ACE 30 mg/kg body weight) (n=6).

The results (Table 20) indicated that the mean C_{max} (maximum concentration) of RH, ACE, and DL from the optimized NEG formulation was 179.42 ± 10.23 , 118.94 ± 3.36 , and 305.52 ± 27.24 ng/mL, and that of the marketed tablet was 192.30 ± 27.44 , 186.44 ± 4.69 , and 420.12 ± 91.59 ng/mL, respectively. The optimized NEG demonstrated a higher area under the curves (AUC_{0-t} , $AUC_{0-\infty}$) and a longer half-life ($t_{1/2}$) for RH, ACE, and DL than the tablet formulation. However, no significant difference was observed in the AUC_{0-t} and $AUC_{0-\infty}$ of DL between the two formulations. The relative bioavailability of RH, ACE, and DL from NEG was 369%, 247%, and 106%, respectively. Furthermore, the elimination rate constant of RH, ACE, and DL was found to be 0.09 ± 0.01 , 0.04 ± 0.02 , 0.05 ± 0.01 for NEG and 0.14 ± 0.01 , 0.10 ± 0.01 , 0.16 ± 0.02 for tablet formulation, respectively. The pharmacokinetic parameters of the marketed tablet were found to be in agreement with previously reported methods^(138–140).

Table 20. Pharmacokinetic parameters of RH, ACE, and DL in Wistar rats following transdermal and oral dose.

Pharmacokinetic parameters and observed values						
Drug	T _{max} (h)	C _{max} (ng/mL)	AUC _{0-∞} (h.ng/mL)	AUC _{0-t} (h.ng/mL)	t _{1/2} (h)	K _e (1/h)
Optimized NEG						
RH	3*	179.42 ± 10.23***	2530.35 ± 129.41***	2266.79 ± 152.63***	7.05 ± 0.65***	0.09 ± 0.01*
ACE	3*	118.94 ± 3.36***	2251.59 ± 393.69***	1316.19 ± 16.57***	18.36 ± 8.13**	0.04 ± 0.02***
DL	3*	305.52 ± 27.24*	3538.66 ± 378.81	2492.83 ± 144.52	15.26 ± 1.93***	0.05 ± 0.01***
Marketed Tablet						
RH	0.5	192.30 ± 27.44	811.96 ± 24.38	613.97 ± 32.03	4.90 ± 0.36	0.14 ± 0.01
ACE	0.5	186.44 ± 4.69	842.34 ± 11.39	533.78 ± 28.58	6.65 ± 0.64	0.10 ± 0.01
DL	0.5	420.12 ± 91.59	3168.92 ± 326.60	2356.86 ± 136.61	4.48 ± 0.73	0.16 ± 0.02

(Mean ± SD), n=6; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ compared with oral tablet.

4.10 In vivo anti-inflammatory efficacy of optimized DCN- and ACE-loaded NEG and marketed tablet formulation

The anti-inflammatory activity of optimized NEG (high and low dose), plain gel (containing 2.5% w/w DMSO), and a marketed tablet formulation was assessed using the hind paw edema method. (Fig. 33 & 34). The difference in the right and left paw volume of each rat within each group was calculated at each hour and is presented in Fig. 34. The results revealed a substantial increase in paw edema volume

in the edema-induced group compared to the saline-treated (normal) group ($p < 0.0001$). The plain gel-treated group IV rats demonstrated a substantial rise in right hind paw edema volume compared to normal control, NEG (high dose), NEG (low dose), and tablet-treated groups while showing no significant difference with respect to the positive control groups. The Group VI, NEG at high dose-treated group, exhibited a substantial reduction in edema volume. The peak anti-inflammatory activity was observed at 4 h, followed by a gradual decline in edema inhibition. The inhibition of edema after 24 h was 53.02% for the high-dose NEG group, 35.43% for the marketed tablet group, and 31.36% for the low-dose NEG group. However, no statistically substantial difference in the inhibition effect of the low-dose NEG group and the marketed tablet-treated group was observed. These increased inhibitions in optimized NEG can be attributed to higher drug concentrations at the target site and enhanced drug permeation through the skin, likely facilitated by the nano-sized formulation and the inclusion of a permeation enhancer.



Figure 33. Anti-inflammatory study in rats. The image depicts the experimental setup for subplantar injection and paw edema measurement in rats.

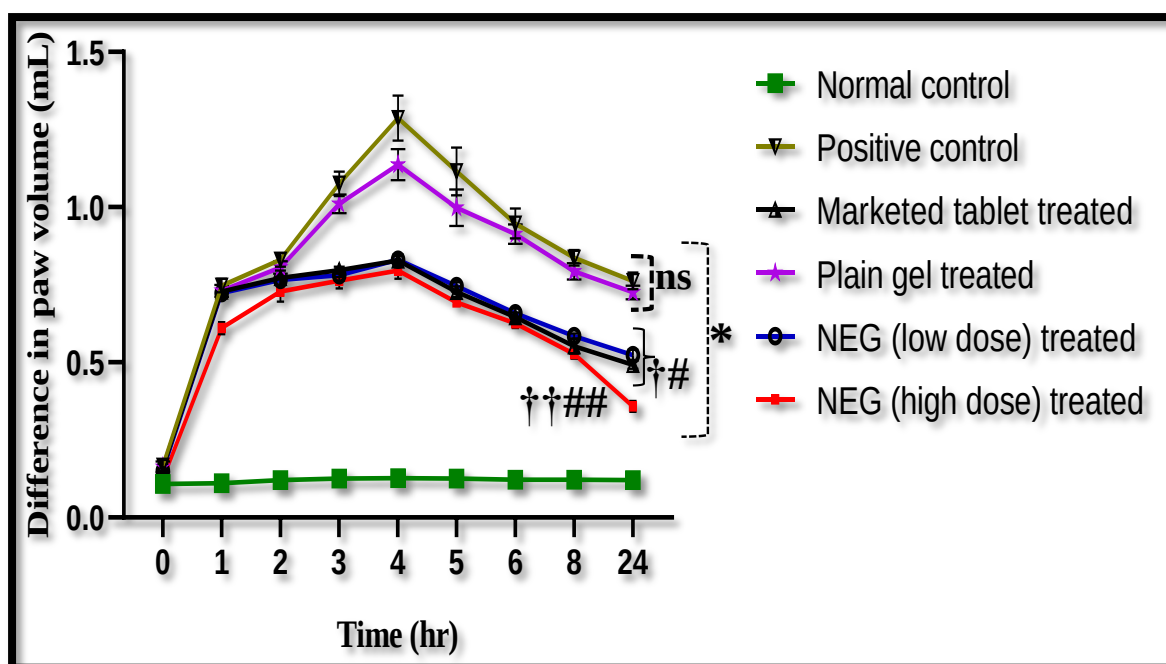


Figure 34. Differences in rat paw volume (mL) recorded at different time intervals from different groups post-treatment. *: $p < 0.0001$, compared to the normal control group; †: $p < 0.01$, ††: $p < 0.0001$, in the marketed tablet, NEG (low dose) and NEG (high dose) treated groups compared to the MIA treated group; #: $p < 0.05$, ##: $p < 0.001$, in the marketed tablet, NEG (low dose) and NEG (high dose) treated groups compared to the plain gel treated group; ns: non-significant.

4.11 Evaluation of anti-osteoarthritis efficacy of optimized DCN- and ACE-loaded NEG and marketed tablet formulation

The effect of optimized NEG at low and high doses, plain gel, and marketed tablet formulation on the knee thickness, protective cartilage structure, and bone cellular abnormalities in the OA rat model was investigated. The results of the kinetic changes in the right knee thickness over the treatment periods (weeks) are summarized in Figure 35. The results were statistically analyzed using ANOVA. The analysis revealed that the MIA-treated group had significantly thicker knees than the usual saline-treated group ($p < 0.0001$). The thickness of the knee was significantly increased in a plain gel-treated group than in normal control, NEG (high dose) treated,

NEG (low dose) treated, and tablet-treated groups while showing no significant difference with the MIA-treated rats. The NEG (high dose) exhibited a rapid recovery effect on knee thickness and was increased during weeks 2-5 post-treatment. The knee thickness was significantly reduced in the NEG (high dose), followed by NEG (low dose), against MIA treated during weeks 1-5 post-treatment ($p < 0.0001$). The marketed tablet-treated group also exhibited a significant reduction in knee thickness compared to the MIA-treated group ($p < 0.01$). However, no substantial change was found between the marketed tablet-treated and NEG (low-dose) treated groups.

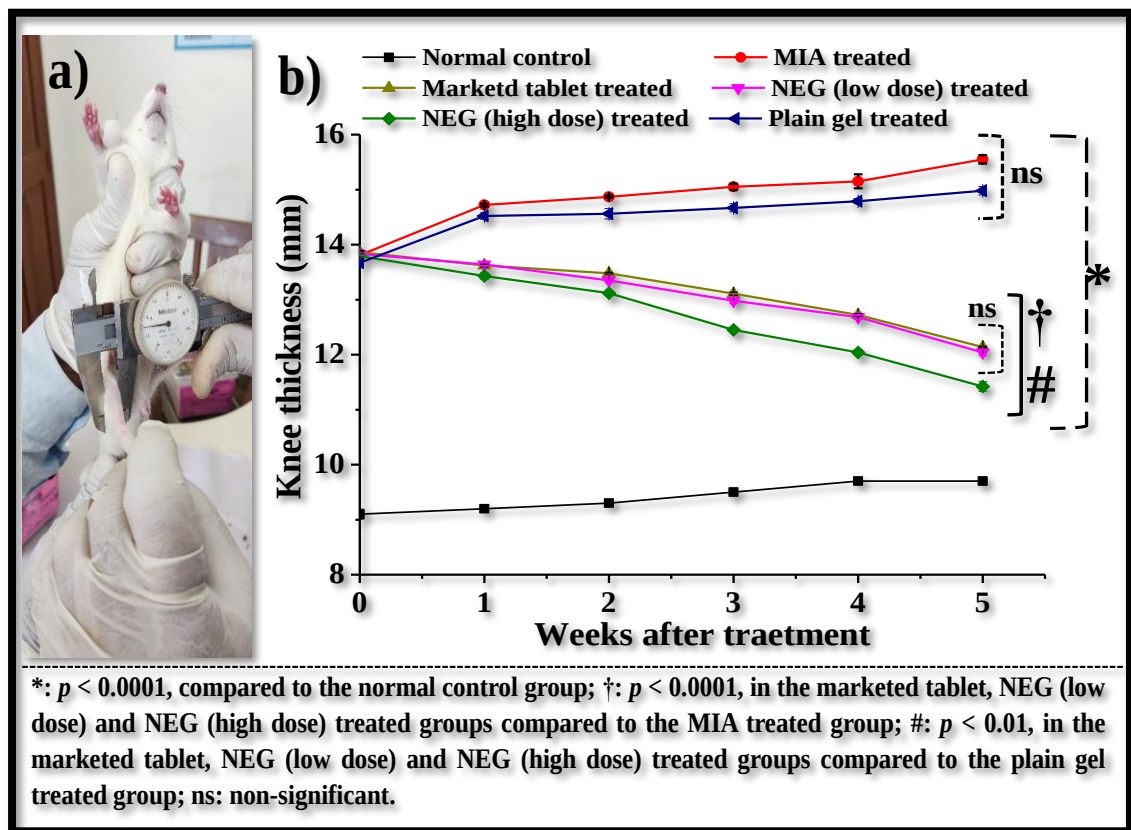


Figure 35. (a) Measurement of rat knee thickness, and (b) Rat knee thickness (mm) recorded weekly for five weeks post-treatment.

Histological analysis was conducted on sections of OA-induced rat knees with and without treatment. The histopathological examinations of OA-induced rat knees from each group were performed using three different staining (toluidine blue, H&E,

and safranin-O). The results of these examinations are presented in Figures 36A-M, 37A-M, and 38A-M, respectively. Additionally, Mankin scores for all groups were determined after 3 and 5 weeks of treatment and are summarized in Tables 21 and 22.

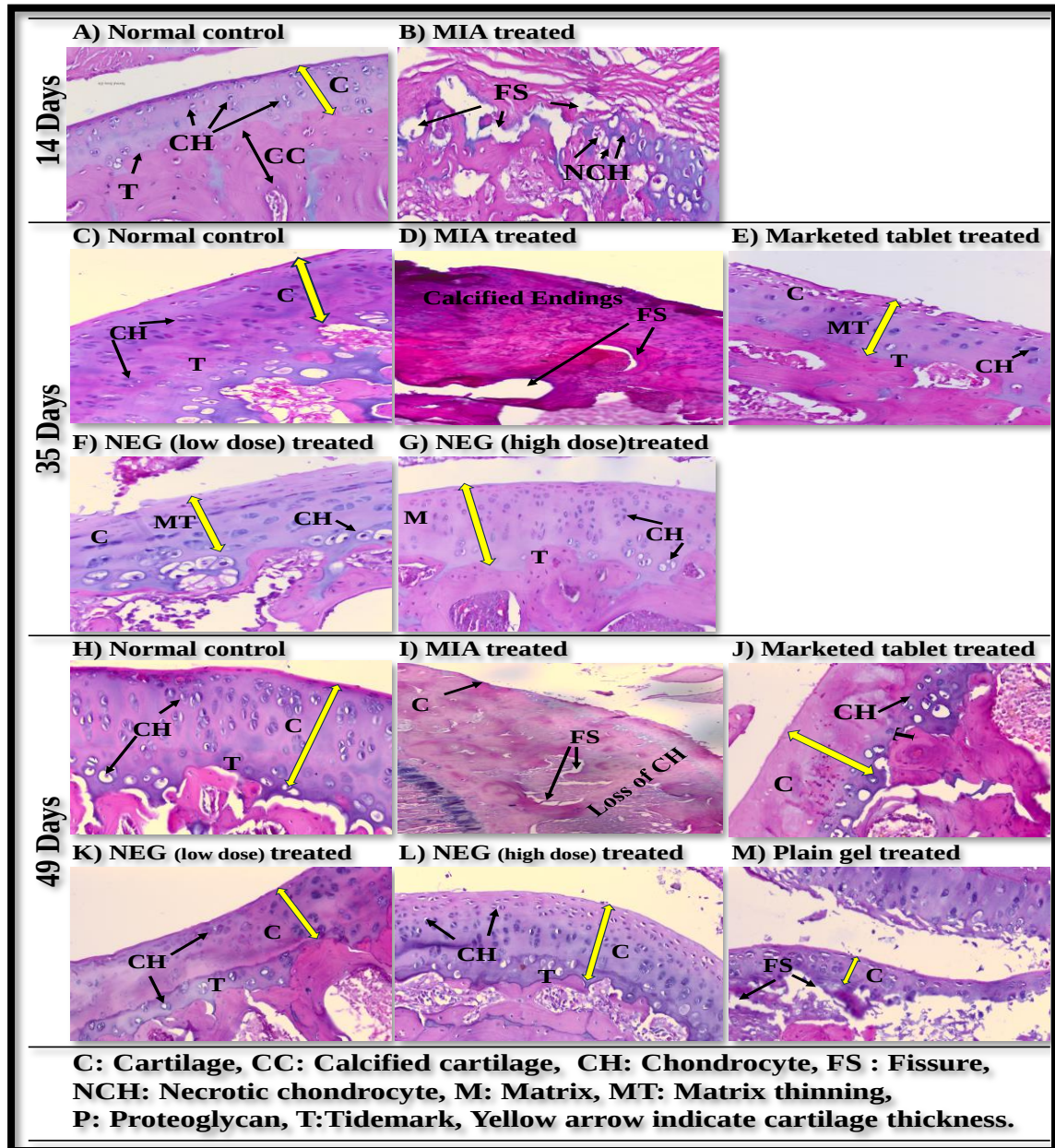


Figure 36. Histopathological examination of OA-induced rat knee joints (H & E staining) for protective cartilage degeneration and bone cellular abnormalities after 14 days (A-B), 35 days (C-G), and 49 days (H-M) of MIA injection and drug treatment. Different groups represented as Normal control (A, C, H), MIA treated (B, D, and I), Marketed tablet treated (E, J), NEG (low dose) treated (F, K), NEG (high dose) treated (G, L), and Plain gel treated (M). (x20).

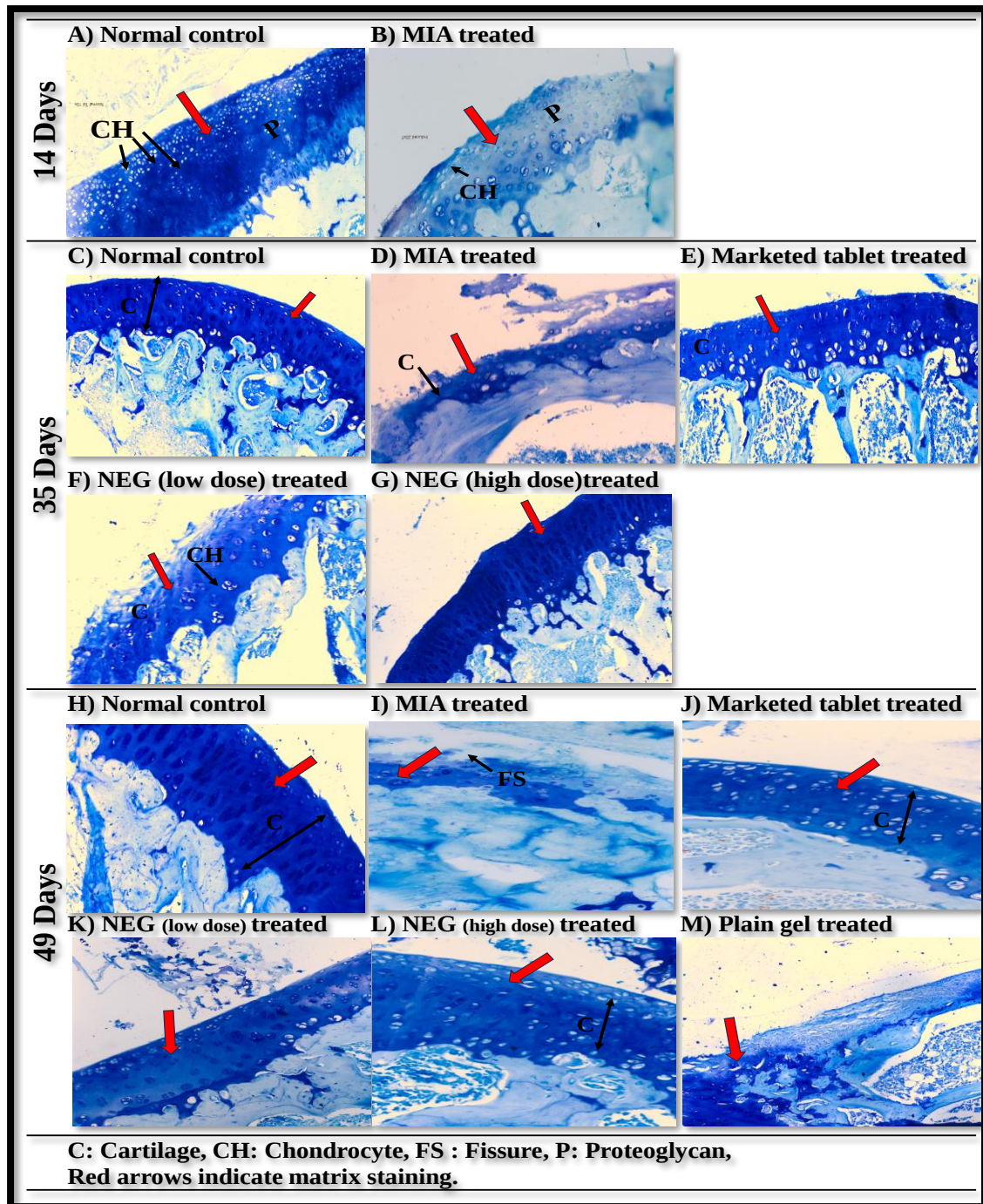


Figure 37. Histopathological examination of MIA-induced OA rat knee joints (toluidine blue staining) for matrix staining after 14 days (A-B), 35 days (C-G), and 49 days (H-M) of MIA injection and drug treatment. Different groups represented as Normal control (A, C, H), MIA treated (B, D, and I), Marketed tablet treated (E, J), NEG (low dose) treated (F, K), NEG (high dose) treated (G, L), and Plain gel treated (M). (x20).

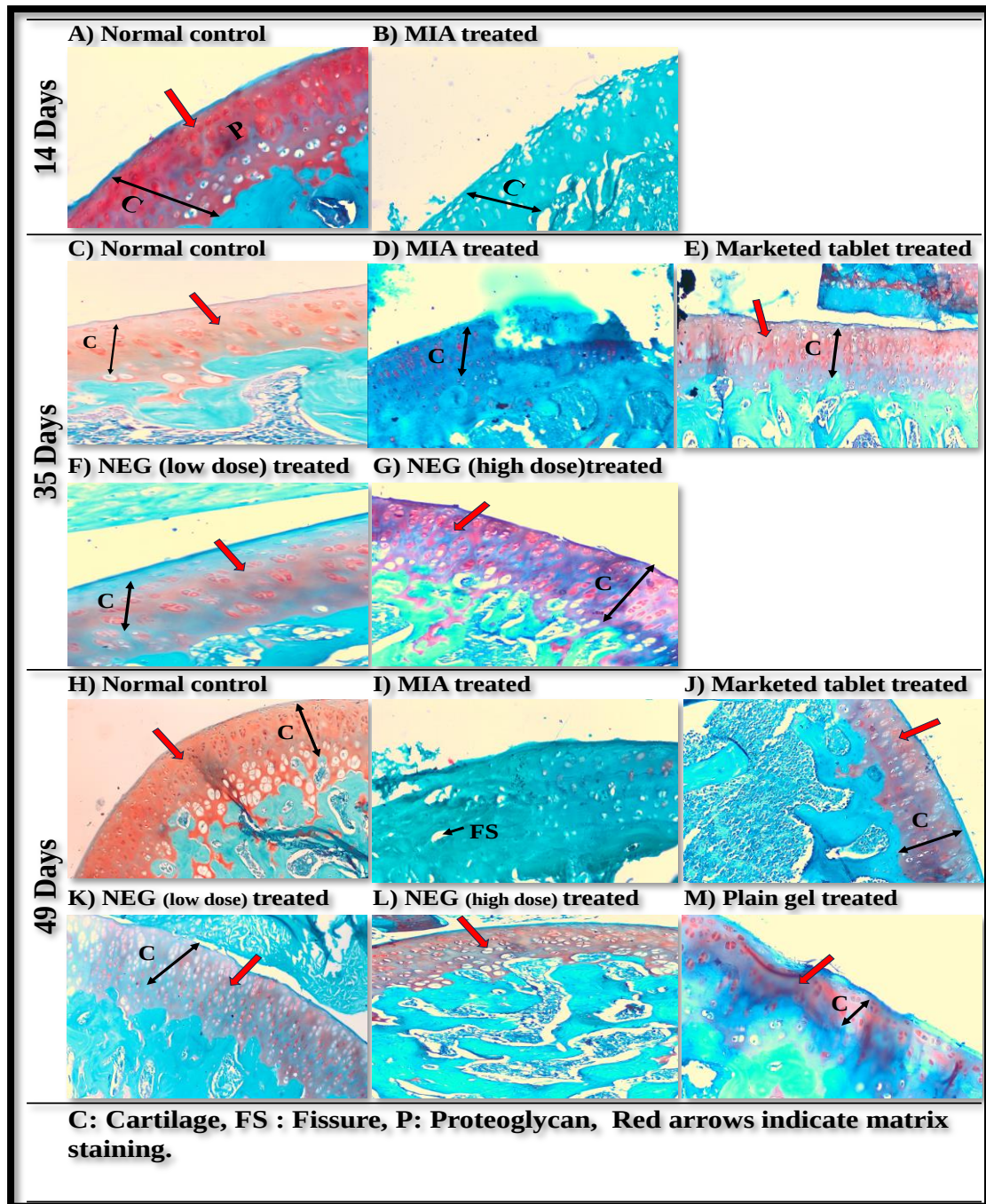


Figure 38. Histopathological examination of MIA-induced OA rat knee joints (safranin-O staining) for matrix staining after 14 days (A-B), 35 days (C-G), and 49 days (H-M) of MIA injection and drug treatment. Different groups represented as Normal control (A, C, H), MIA treated (B, D, and I), Marketed tablet treated (E, J), NEG (low dose) treated (F, K), NEG (high dose) treated (G, L), and Plain gel treated (M). (x20).

Table 21. Details of the histological scoring (modified Mankin) after 3 weeks.

Groups	Total Mankin	Structure	Cellularity	Matrix staining
Normal control	0.00	0.00	0.00	0.00
MIA treated	11.06 ± 1.04 [*]	5.17 ± 0.41	2.5 ± 0.55	3.39 ± 0.54
Marketed tablet treated	3.23 ± 0.21 ^{*†}	1.00 ± 0.63	1.5 ± 0.84	0.56 ± 0.10
NEG (high dose) treated	1.45 ± 0.26 ^{*†#}	0.67 ± 0.52	0.5 ± 0.55	0.28 ± 0.10
NEG (low dose) treated	5.83 ± 0.44 ^{*†#\$}	1.83 ± 0.98	2.17 ± 0.41	1.33 ± 0.44

Mean ± SD, for all groups n=6 except normal where n=5, $p < 0.0001$, symbols *, †, #, and \$ indicate comparison with normal, MIA treated, tablet treated, NEG with high dose treated group, respectively.

Table 22. Details of the histological scoring (modified Mankin) after 5 weeks.

Treatment/groups	Total Mankin	Structure	Cellularity	Matrix staining
Normal control	0.00	0.00	0.00	0.00
MIA treated	12.55 ± 0.58 [*]	5.83 ± 0.41	3.00 ± 0.00	3.72 ± 0.48
Marketed tablet treated	2.45 ± 0.39 ^{*†}	0.67 ± 0.52	0.50 ± 0.55	1.28 ± 0.67
NEG (high dose) treated	0.94 ± 0.15 ^{*†#}	0.33 ± 0.52	0.17 ± .041	0.44 ± 0.10
NEG (low dose) treated	3.06 ± 0.59 ^{*†\$}	0.83 ± 0.41	0.67 ± 0.52	1.56 ± 0.69
Plain gel treated	8.44 ± 0.99 ^{*†#S¥}	4.33 ± 1.63	2.00 ± 0.67	2.11 ± 0.54

Mean ± SD, for all groups n=6 except normal where n=5, $p < 0.0001$, Symbols *, †, #, \$, and ¥ indicate comparison with normal, MIA treated, tablet treated, NEG with high and low dose treated group, respectively.

At 14 days post sodium MIA injection, the joint sections from group-II (untreated OA) animals were compared to those from a control group (Fig. 36A-B). In normal control rats (Fig.36A), H&E staining revealed an intact and smooth superficial zone of articular cartilage. Flattened chondrocytes were observed within the tangential zone. The tidemark remained intact, clearly demarcating the calcified and uncalcified cartilage boundary. In contrast, histopathological images from MIA-treated rats (Fig. 36B) demonstrated significant cartilage matrix erosion with necrotic chondrocytes, deep fissures, and fibrillation. The yellow-colored arrows indicate the change in the matrix thickness due to matrix erosion (Fig. 34A-M). In the MIA-treated group, cartilage matrix staining (Fig. 36B_H&E, and Fig. 37B_toluidine blue) was reduced in both interterritorial and pericellular matrices. Safranin-O staining was completely absent (Fig. 38B), indicating a severe reduction or absence of proteoglycan and glycosaminoglycan content within the matrix. The total Mankin score for MIA-treated group was 8.88 ± 0.96 , while scores for matrix staining, cellularity, and cartilage structure were 3.22 ± 0.67 , 2.33 ± 0.52 , and 3.33 ± 0.52 , respectively.

After 3 weeks of treatment, the NEG (high-dose) treated rat (Fig. 36G) exhibited intact and smoother cartilage than the marketed tablet-treated group (Fig. 36E). However, the articular cartilage matrix was thinner than in the normal control group (Fig. 36C), with a noticeable reduction in the pink-staining area. The Mankin score of NEG (high dose) was substantially lower than the MIA-treated, marketed tablet-treated, and NEG (low dose) treated groups (Table 21). In comparison to the groups treated with NEG (low dose) or marketed tablets, the results for the NEG (high dose) group indicate that chondrocyte regeneration and repair of the articular surface was improved. Intense dark violet staining (Fig. 37C, G) and orange-to-red staining (Fig. 38C, G), along with the lowest Mankin score of 0.28 ± 0.10 , indicated a higher

proteoglycan content and better preservation of the cartilage matrix in the NEG (high dose) treated group. In contrast, the rat knee treated with the low dose of NEG (Fig. 36F) exhibited more erosion and significant matrix thinning with a significantly higher Mankin score compared to the normal control, NEG (high dose) treated, and marketed tablet-treated groups (Fig. 36E, Table 21). Additionally, the tidemark was less distinct in the NEG (low dose) than in the other groups: normal, NEG (high dose), and marketed tablet-treated groups. In the rat knee treated with the marketed tablet (Fig. 36E), the cartilage matrix appeared thinner than in the normal and NEG (high dose) treated groups, with cracked cartilage and a noticeable reduction in the pink-staining area. The number of chondrocytes was decreased, suggesting cell loss or death. The matrix was thinned and calcified. However, both the NEG and tablet-treated groups demonstrated a more prominent healing effect on the osteoarthritic rat knee than the MIA-treated group.

After five weeks of oral and topical treatment, the MIA-treated group (Fig. 36I) exhibited a thinner and irregular cartilage surface with fibrillation and chondrocyte loss. Vertical fissures extended into the deeper layers of the cartilage, indicating structural disruption. The subchondral bone appeared sclerotic. Additionally, mild inflammation was observed, characterized by increased cellularity and vascularity. This resulted in an increased Mankin score (Table 22). After five weeks of treatment, the Mankin score was higher in the MIA-treated group, while it decreased in the groups treated with another drug (Table 22). The group treated with NEG at a high dose demonstrated a significant ($p < 0.0001$) healing effect on the osteoarthritic rat knee than the marketed treatment group and the group treated with NEG at a low dose. The cartilage structure appeared smoother and more intact, with minimal evidence of erosion or fibrillation in NEG (high-dose) than the marketed

treatment and low-dose NEG groups (Fig. 36J-L). The matrix exhibited more intense staining with all three dyes in NEG (high dose) than in the marketed tablet and low-dose NEG groups. Conversely, the marketed tablet group displayed a less severe degree of cartilage thinning with less extensive fibrillation (clefts and fissures) and less chondrocyte disorganization compared to the low-dose NEG group (Fig. 36J-K). However, the scoring results indicated no substantial difference among the low-dose NEG and marketed tablet-treated groups. The plain gel-treated group (Fig. 36M) showed severe cartilage erosion against the normal control and other drug-treated group. However, the plain gel-treated group demonstrated less severe cartilage erosion and minimal subchondral bone changes compared to the MIA-treated group (Fig. 36I). Furthermore, a severe reduction in staining intensity with toluidine blue and safranin-O demonstrated proteoglycan loss (Fig. 37M and 38M). These results demonstrate that the extensive healing of cartilage structure and the mitigation of cellular abnormalities may be due to the improved permeation and bioavailability of DCN and ACE from the NEG formulation.

5. DISCUSSION

5.1 Preformulation study

5.1.1 Identification and characterization of API

The identity and characterization of ACE and DCN were established using UV-Vis spectroscopy, Nanotracer particle size analyzer, DSC, and FTIR spectroscopy. UV-Vis spectroscopy revealed absorption maxima (λ_{max}) for ACE and DCN at 275 nm and 256 nm, respectively, consistent with previously reported literature values^(68,69). DSC thermograms exhibited sharp endothermic peaks at 155.40°C and 260.82°C for ACE and DCN, respectively, confirming their crystalline nature and aligning with previously reported melting points^(131,132). FTIR spectra of ACE and DCN in KBr exhibited characteristic absorption bands: ACE at approximately 3320, 1772, 1453, 1439, 1419, and 750 cm⁻¹; and DCN at approximately 1669, 1463, 1370, and 1028 cm⁻¹. The analytical results, therefore, confirmed the identity and authenticity of the received DCN and ACE drug samples.

5.1.2 DCN, ACE, and excipients compatibility study

The FTIR analysis and thermal analysis (DSC) were carried out to determine the drug-excipient compatibility and to confirm the complete solubilization and incorporation of the DCN and ACE into the formulated NE and NEG. The DSC thermogram and FTIR spectra obtained from DCN, ACE, physical mixture of combined drugs, optimized nanoemulsion (Opt. NE), and optimized nanoemulgel (Opt. NEG) formulation were compared. It was found that no sharp endothermic peaks were observed for DCN and ACE in the physical mixture, optimized NE, and optimized NEG formulation DSC thermograms. Similarly, the FTIR spectra of these formulations did not reveal any characteristic peaks of DCN and ACE. This indicates

that DCN and ACE were completely solubilized and incorporated into the formulated NE and NEG without any physical and chemical incompatibility with the excipients (70,131,132,141).

5.2 Analytical method development for synchronous estimation of DCN and ACE

A stability-indicating RP-HPLC method with run time < 10 minutes was developed using the experimental design for the simultaneous determination of DCN and ACE in developed NEG formulation and commercial tablet dosage form in the presence of their major degradation product: RH and DL respectively. The established method was validated as per ICH guidelines for various parameters, including specificity, system suitability, linearity and sensitivity, accuracy, and precision. The percent recovery for all four analytes was found to be between 98 and 102, and the procedure was discovered to be linear in the range of 0.1-64 µg/mL with R² value > 0.999. All the validation parameter was found to be within specified limits as per ICH guidelines. The method effectively separated DCN and ACE from their degradation products under various stress conditions, as well as from the complex matrices of the marketed tablet formulation and the developed NEG. The mean percent assay values for DCN and ACE in both formulations ranged from 95-105. The results demonstrated that the developed method is superior to other existing methods^(142–145) on its low RT, wide linearity range, and complete validation parameters, including stability-indicating parameters, and can be effectively employed for the routine analysis of DCN and ACE, both individually and in combination, within a laboratory setting.

5.3 Screening of NE components

5.3.1 Solubility of the DCN and ACE in various excipients

The appropriate selection of oily phase, surfactant, and co-surfactant is important for forming and stabilizing NE. The NE components were selected based on their solubilizing capacity for DCN and ACE and their emulsification ability. The solubility results revealed that, individually, both DCN and ACE exhibited higher solubility in Capmul MCM C8, followed by Triacetin, which is consistent with previously published data^(80,81,101,146,147).

Based on these findings, oils with higher individual solubilities, including a combination of Triacetin and Capmul MCM C8, were further investigated for their ability to solubilize the drug combination. The results revealed that the combination of DCN and ACE showed the highest solubility in an oil phase composed of Triacetin: Capmul MCM C8 (2:1) compared to other oil ratios and types. Triacetin, a monoglyceride, and Capmul® MCM C8, a mixture of mono- and diglycerides of medium-chain fatty acids, are well-known for their excellent solubilizing capacities for various BCS class-II drugs^(148–150). Consequently, Triacetin and Capmul MCM C8 (2:1) mixture was chosen as the optimal oil mix for the subsequent fabrication of nanoemulsions.

For surfactants, both drugs in combined form show optimum solubility in Kolliphor-EL, followed by Tween 20 and Tween 80. In the case of co-surfactants, in combination, both drugs were found to be more soluble in Transcutol P, followed by PEG 400. The individual solubility results of DCN and ACE in surfactants and co-surfactants were found to be in agreement with previously reported findings^(80,151–153).

5.3.2 Screening of surfactant and co-surfactant

Beyond assessing solubilization capacity, the surfactant and co-surfactant were further evaluated for their emulsification potential with the selected oil phase (Triacetin: Capmul MCM C 8, 2:1), a critical factor for stable NE formation. This step was crucial as surfactants with high solubilization capacity may not necessarily exhibit optimal emulsification properties ⁽¹³³⁾. Although DCN and ACE in combination exhibited optimum solubility in Tween 20, Kolliphor-EL (polyoxy 35 castor oil) demonstrated higher T and superior miscibility with the oil mix, leading to its selection ⁽¹³³⁾. Similarly, Transcutol P (diethylene glycol monoethyl ether), exhibiting the highest T (%) and emulsification capacity with the oil mix and the selected surfactant (Kolliphor-EL), was picked as the suitable co-surfactant for NE formulation. The observed T (%) and miscibility of Kolliphor-EL and Transcutol P with Triacetin: Capmul MCM C8 (2:1) were consistent with previously reported literature ^(133,137). Both Kolliphor-EL and Transcutol P are non-ionic compounds with HLB values of 13.5 and 4.2, respectively. They exhibit high compatibility with other components and are commonly used to improve the bioavailability of BCS-II / lipophilic drugs ^(51,80,133,154). Therefore, Kolliphor-EL and Transcutol P were chosen for the development of NE based on the Triacetin: Capmul MCM C8 (2:1) oil phase.

5.3.3 Selection of Smix ratio using a pseudo-ternary phase diagram

Pseudoternary phase diagrams were developed by titrating a mixture of Triacetin: Capmul MCM C8 (2:1), Kolliphor EL, and Transcutol P with water to delineate the NE region and determine optimal component ratios. Results indicated that a minimum NE area was observed at 100% surfactant concentration (Smix 1:0). This likely resulted from the surfactant's (Kolliphor EL) limited ability to sufficiently reduce o/w interfacial tension on its own, hindering NE formation ^(133,155). However,

increasing the Transcutol P concentration in the Smix from 1:0 to 1:1 significantly expanded the NE area. This expansion is attributed to decreased interfacial tension and increased interfacial film fluidity facilitated by Kolliphor-EL at this ratio. Furthermore, the inclusion of Transcutol P reduces interfacial tension, enhances surfactant film flexibility, and increases the system's entropy^(133,156). However, further increases in Transcutol P concentration beyond a 1:2 ratio (up to 1:4) did not substantially increase the NE area. This may result from the increased concentration of Transcutol P, which expands the interfacial film^(133,157,158). Interestingly, despite its higher HLB value, increasing Kolliphor-EL concentration beyond a 2:1 ratio (up to 4:1) ratio resulted in a decrease in the NE area. This suggests that further increases in Kolliphor-EL concentration may not significantly enhance emulsification, possibly due to a reduction in surfactant film fluidity at higher levels of concentrations^(93,96,133). Based on the analysis of the phase diagrams, Kolliphor-EL: Transcutol P in 1:1 ratio was chosen for further development studies.

5.4 Optimization of NE using mixture design

This study successfully employed a 3-factor, 2-level D-optimal mixture design to optimize the DCN-and ACE-loaded NE formulations. The design effectively investigated the main and interaction effects of three key formulation components: oil mix (A), surfactant-co-surfactant mixture (Smix) (B), and water (C) on two critical responses: globule size (GS) and % transmittance (%T). The results demonstrated that the GS and %T of all 16 batches ranged from 10.40 ± 0.20 nm to 22.23 ± 5.19 nm and 91.55 ± 0.67 to 97.17 ± 0.88 , respectively. The observed range of globule sizes (<100 nm) within the design space highlights the significant influence of the selected independent variables on this critical parameter. Furthermore, a higher T value consistently indicates favourable emulsification conditions⁽¹⁵⁹⁾. ANOVA results

revealed statistically significant (p value less than 0.05) cubic and reduced cubic models for GS and %T, respectively. The lack-of-fit tests for both models were non-significant (F -value = 0.06, p = 0.8120), supporting the model's validity and ability to predict the system's behavior within the experimental domain accurately⁽⁹²⁾.

The obtained *Scheffe* polynomial equations revealed that instead of the individual impact of oil mix and Smix, the interaction effect was more significant. The oil mix concentration exerted a negative impact on GS and a positive impact on %T. Whereas, Smix exerted a negative impact on GS and a positive impact on %T. Also, the interaction terms, particularly AB (oil mix and Smix) and AC (oil mix and water), demonstrated significant positive influences on both responses. These findings align with previously reported data^(65,88). Contour two-dimensional and response surface three-dimensional plots visually represented the relationships between the factors and responses. These plots corroborated the findings from the polynomial equations, demonstrating that the GS of NEs decreased within a specific range (from 18.39 nm to 6.08 nm) as the oil concentration decreased and the Smix concentration increased. This trend is likely attributable to a reduction in interfacial tension and an increase in surfactant fluidity. However, this trend reversed at higher Smix concentrations (above 32.5% w/w), where a further increase in Smix led to an increase in GS, potentially due to a decrease in surfactant fluidity at elevated concentrations^(65,88,93). The numerical optimization method suggested an optimal NE formulation with 13.34%, 31.77% w/w, and 54.89% w/w of oil mix, Smix, and water, respectively, exhibiting a high composite desirability of 0.908. This optimized formulation was selected for further investigation.

5.5 Characterization of optimized DCN -and ACE-loaded NE

5.5.1 Globule size and electrokinetic potential analysis

Globule size is one of the most crucial factors in NE, affecting thermodynamic stability and efficacy^(65,158,159). Nanoemulsions with small droplet sizes (20-200nm) exhibit a large surface area, enhancing solubility, skin permeation, and, ultimately, the bioavailability of BCS class II drugs^(160–163). In the present study, the mean GS of the NE formulations was less than 50 nm, indicating their kinetic stability and potential for effective skin permeation^(65,158,159). These results corroborate the positive interactive effect of the oil mix and Smix on GS, as suggested by the 2D and 3D response surface plots. The polydispersity index (PDI) reflects the homogeneity or uniformity of the GS distribution within the formulation. A lower PDI value (closer to 0) indicates a more uniform GS distribution. The mean PDI of all formulated NEs ranged from 0.20 ± 0.12 to 0.22 ± 0.08 (Table 12), indicating good uniformity in GS distribution⁽¹³⁴⁾. Zeta potential is a critical parameter for predicting the stability of a formulation and understanding the surface properties of nanosized globules. A zeta potential value exceeding ± 25 mV indicates a stable NE formulation with minimal agglomeration or flocculation^(65,135). Higher negative or positive zeta potential values result in repulsive forces between globules that surpass attractive forces, reducing the likelihood of aggregation and coalescence⁽¹³⁶⁾. All formulated NEs displayed a mean zeta potential between -30.67 ± 1.62 mV and -28.40 ± 2.77 mV, suggesting long-term stability (Table 12)^(92,164). The negative zeta potential indicates the presence of adequate electrostatic repulsion to stabilize the oil-in-water (o/w) system⁽⁹²⁾. Furthermore, according to Kohli and Alpar (2004), only negatively charged particles (≤ 50 nm) can effectively permeate the skin. This permeation occurs through channels created by the repulsive interactions of negatively charged particles and negatively

charged lipids in the skin. Consequently, negatively charged globules are expected to exhibit enhanced transdermal permeation ⁽¹⁶⁵⁾. Moreover, the observed negative zeta potential in the fabricated formulations can be attributed to the presence of fatty acid esters and alcohol moiety within Kolliphor EL and Transcutol P, respectively ^(95,166,167).

5.5.2 Percentage transmittance, viscosity, pH and drug content analysis of the DCN-and ACE-loaded NE

Transmittance is a crucial parameter that reflects the quality of NE. A higher T value (closer to 100) is generally desirable as it indicates a clear, stable, and well-formed NE with smaller globule sizes ^(96,97,168). The % T of NE 1 and NE 2 at 650 nm were found to be 98.03 ± 0.03 and 97.78 ± 0.29 , respectively, indicating good stability and optical clarity ^(96-98,168). The pH of the NE formulation was 5.78 to 5.81 which falls within acceptable limits for transdermal administration ^(65,137). The viscosity of the NE formulation ranged from 36.00 to 39.67 cP. According to Saurabh Jain et al., (2018) DCN, with a pKa value of 4.74, exhibits pH-dependent solubility in water ⁽¹⁰⁴⁾. It exhibits reduced solubility at acidic pH and undergoes degradation at alkaline pH. During NE preparation, the pH of the formulation decreased to the range of 4.2-4.5, leading to DCN precipitation. To maintain DCN in a dissolved state and ensure formulation stability, the pH of the NE was subsequently adjusted to 5.7-6.0 using a 2% (v/v) aqueous solution of triethanolamine ⁽¹⁰⁴⁾. Further, the average DCN and ACE content for the NE-1 and NE-2 formulation, determined by HPLC in triplicate at 264 nm, was found in the range of 97-102%. This result indicates good drug-loading capacity, an important factor for effective NEs.

5.5.3 Physical stability of DCN-and ACE-loaded NE

The cycling stress and thermodynamic stability study results demonstrated that both proposed NE formulations (NE-1 and NE-2) exhibited excellent kinetic stability with no sign of creaming, cracking, or phase separation. As previously mentioned, this enhanced thermodynamic stability translates into a prolonged *shelf life* for NEs compared to conventional emulsions^(93,96).

5.5.4 *In vitro* permeation study of DCN-and ACE-loaded NE

The *in vitro* permeation study of the NE was conducted under two distinct pH conditions: PBS at pH 5.7 and a mixture of PBS at pH 6.8 and methanol (7:3). In transdermal drug permeation studies of nanocarriers, drug release is often considered a multi-step process. Initially, the drug is released from the formulation and deposited on the skin surface before penetrating deeper skin layers and ultimately reaching systemic circulation^(62,103). For transdermal dosage forms containing pH-sensitive drugs like DCN, accurately interpreting the formulation's performance at systemic circulation pH (7.4) can be challenging^(103,104). To better simulate physiological skin conditions and obtain more meaningful results, the permeation study was performed using PBS at pH 5.7. Furthermore, numerous studies have investigated the permeation of ACE-loaded nanoformulations (including nanoemulsions, nanoemulgels, and transferosomes) using various media, such as PBS pH 7.4, with or without adding methanol or ethanol^(41,46,101,105,151,169). The methanol /ethanol was added to solubilize the released hydrophobic drugs and facilitate their subsequent assay. Additionally, several studies have employed different pH media (e.g., PBS pH 5.5, 6.8, and 7.4) for the permeation studies of pH-sensitive drugs^(102,103,170–172). Considering the solubility and stability of both DCN and ACE and to gain a comprehensive understanding of the developed NE and NEG formulations, we utilized two distinct media for the

permeation study: PBS pH 5.7 and a mixture of PBS pH 6.8 (70%v/v): methanol (30%v/v). *In vitro* permeation studies revealed that NE-1 consistently demonstrated a slightly higher release rate for both DCN and ACE than NE-2 at both pH levels. This subtle variation in drug permeation may be due to changes in the composition and GS of the two NEs. Diacerein exhibited higher permeation at pH 5.7 compared to that in methanolic PBS 6.8. In contrast, ACE demonstrated higher permeation from both formulations in methanolic PBS 6.8. This variation in permeation profiles may be attributed to the potential instability of DCN at a higher pH and the higher solubility of ACE at this pH. Based on the outcomes of numerical optimization study, desirability and permeation study, NE-1 was selected as the optimized nanoemulsion (Opt. NE).

5.5.5 TEM and FE-SEM analysis of optimized NE

The globule shape, size, and surface morphology of the proposed NE were investigated using TEM and FE-SEM. The images obtained from TEM and FE-SEM study, revealed that all globules exhibited a spherical shape and were confirmed to be within the nano-size range (< 20 nm). The GS agreed with the results obtained from the GS analysis using nanoparticle and zeta potential analyzer. The findings of the present study are found to be in agreement with the reported data of Sharma et al. ⁽¹³⁷⁾

5.6 Characterization of DCN-and ACE-loaded NEG and gel

5.6.1 Viscosity, spreadability, pH, and drug content analysis

The pH value of the DCN-and ACE-loaded plain gels and NEG ranged from 5.86 to 5.97, falling within acceptable limits for transdermal administration. Viscosity is another critical parameter significantly impacting transdermal

permeation. Results revealed that NEG prepared without DMSO exhibited significantly higher viscosity than plain gel formulations (Plain gel-1 and Plain gel-2). This higher viscosity in NEG can be attributed to the presence of surfactant at higher concentrations (above 2%) ⁽¹⁷³⁾. As per Cao et al., ⁽¹⁷⁴⁾ DMSO effectively decreases the viscosity of cellulose by swelling the cellulose coils and causing them to spread apart. Consistent with this finding, the present study demonstrated decreased NEG viscosity with increasing DMSO concentration. Similarly, the viscosity of plain gel containing DMSO was lower compared to those without DMSO. However, all formulations exhibited sufficient viscosity to ensure their suitability for application to the skin surface and extrusion from collapsible tubes. Moreover, all formulations demonstrated good spreadability, with values ranging from 35.42 to 39.22 g cm/s, indicating that they could be easily spread with minimal shear. The DCN and ACE drug content in all formulations was close to 100%, ranging from 96.17% to 99.88% for DCN and 100.68% to 102.82% for ACE, indicating excellent drug loading capacity of the formulations.

5.6.2 *In vitro* permeation study of optimized NE, DCN-and ACE-loaded NEG and plain gel formulation

In vitro permeation profiles of the optimized NE, various NEG formulations, and plain gel formulations were compared across two media: PBS pH 5.7 and Methanolic PBS pH 6.8. The results demonstrated that both DCN and ACE exhibited significantly higher cumulative permeation from the optimized NE and NEG-1 (without DMSO) compared to the plain gels, both with and without DMSO, across all conditions. This enhanced permeation from the optimized NE and NEG-1 can be attributed to the nano-sized globules of the NE and the penetration-enhancing properties of the surfactants and co-surfactants within the formulations ^(54,133,175).

However, permeation from NEG-1 was lower than that from the optimized NE, suggesting that incorporating the NE within the gel matrix may hinder drug release, potentially due to an increased gel viscosity. Dimethyl sulfoxide (DMSO) is a well-established penetration enhancer for both BCS class II and III drug molecules. It acts by reversibly altering the structure of proteins within the stratum corneum, thereby enhancing skin permeability^(111,112).

Nanoemulgels prepared with DMSO (NEG-2, NEG-3, and NEG-4) demonstrated increased permeation of both drugs compared to NEG-1 (without DMSO) in both media. In methanolic PBS pH 6.8, after 24 h, the permeation of DCN and ACE from NEG-2 (with 2.5% DMSO) increased by 6% and 19%, respectively, compared to NEG-1. Similarly, in PBS pH 5.7, the permeation of DCN and ACE from NEG-2 increased by 9% and 16%, respectively, compared to NEG-1. These findings are in line with previously reported data^(64,111,112). However, no substantial variation in the permeation profiles of either drug was detected among the NEG formulations prepared with 2.5%, 5%, and 7.5% DMSO (similarity factor (f_2) > 50), indicating that increasing DMSO concentration beyond 2.5% did not significantly enhance permeation. The NEG formulation containing 2.5% DMSO (NEG-2) consistently demonstrated the highest cumulative drug permeation across all conditions. This suggests that the combination of NE with a small amount of DMSO within a gel matrix provides the most effective drug delivery system. Kinetic analysis revealed that DCN and ACE release from all formulations, except Opt-NE, best fit the Korsmeyer-Peppas model of drug diffusion, which is consistent with previous findings^(64,176,177). In contrast, DCN and ACE release from Opt-NE followed both the Korsmeyer-Peppas model and first-order release kinetics in both media, with high R^2 values ranging from 0.9247 to 0.9880 and 0.9489 to 0.9932, respectively^(65,178,179).

In both media, the diffusional exponent (n) values for DCN and ACE release from all NEG formulations were more than 0.45 and less than 1, suggesting that the ACE release mechanism follows anomalous diffusion involving dissolution and diffusion processes ^(64,176,177,179). Based on these *in vitro* drug permeation results, NEG-2 with a minimum quantity of DMSO (2.5%) was finalized for further *ex vivo* and *in vivo* characterization.

5.6.3 *Ex vivo* release study optimized DCN-and ACE-loaded NEG formulation

Ex vivo permeation studies demonstrated that NEG-2 consistently exhibited significantly higher cumulative DCN and ACE permeation than NEG-1 and plain gel-2 across all experimental conditions. Notably, the *ex vivo* and *in vitro* permeation profiles of both drugs from all formulations did not exhibit any significant differences (similarity factor (f₂) > 50) across all media. Furthermore, NEG-2, followed by NEG-1, demonstrated higher permeation parameters (flux, permeation-coefficient) and skin deposition for both drugs compared to the plain gel in both media. Specifically, for NEG-2, skin deposition for DCN was $26.30 \pm 3.58 \mu\text{g}/\text{cm}^2$ and $29.22 \pm 2.14 \mu\text{g}/\text{cm}^2$ and for ACE was $53.15 \pm 6.54 \mu\text{g}/\text{cm}^2$ and $64.31 \pm 5.09 \mu\text{g}/\text{cm}^2$ in PBS pH 5.7 and methanolic PBS pH 6.8, respectively. These results further highlight the superior performance of NEG-2. This indicates that NEG-2 may function as a drug reservoir in the skin, thereby extending the duration of drug effects ⁽⁶²⁾. The permeation enhancement ratio (ER) of DCN and ACE was significantly higher for NEG-2 than for other formulations. This enhanced permeation from the NEG-2 formulation can be attributed to the combined impact of numerous factors: the globules nano size of the incorporated NE, the permeation boosting effect of DMSO, and the unique properties of the Smix (mixture of surfactants and co-surfactants). The permeation enhancer, DMSO, acts by reversibly altering the structure of proteins within the stratum

corneum, whereas the surfactant, Kolliphor® EL, is known to temporarily disrupt the tight junctions in the stratum corneum, facilitating drug penetration. Additionally, Transcutol P, a co-surfactant, acts as a powerful solubilizing agent, further enhancing the permeation effect of Kolliphor-EL. Furthermore, its solubility in both water and oil may contribute to the formation of an intracutaneous depot for drugs, potentially prolonging and intensifying drug delivery. These findings corroborate those published by EI-Zahaby et al. and Prasanthi et al. ^(54,175,180,181).

The kinetic study results of both drugs from NEG-1, NEG-2, and plain gel-2 were also consistent with the *in vitro* kinetic data. The R² values, ranging from 0.9111 to 0.9984 for DCN and 0.9312 to 0.9989 for ACE in both media, indicated excellent fit to the Korsmeyer-Peppas model, suggesting drug diffusion as the primary release mechanism. The diffusional exponent (n) for DCN and ACE from all three formulations fell within the range of 0.553 to 0.872. Since all n values fall between 0.45 and 1, the drug release mechanism for all formulations is likely a combination of dissolution and diffusion. Similar findings were observed by Chattopadhyay et al. for glucosamine sulfate and diacerein ⁽⁶⁴⁾. This optimized formulation (NEG-2) demonstrates the most promising potential for effective drug delivery based on its superior permeation and skin deposition characteristics.

5.6.4 Stability study of optimized NEG formulation

The stability study results indicate that the DCN and ACE in the proposed NEG were stable at normal and accelerated temperatures, as insignificant changes were observed in the evaluated parameters at the initial level and after specific intervals of up to 3 months. The FTIR results confirmed the DCN and ACE compatibility with NEG components and the solubilization and incorporation into oily globules.

5.7 Skin irritation study

Skin irritation testing, performed by the Draize method, confirmed the non-irritant properties of the optimized NEG formulation. Throughout the 72 h observation period of the acute toxicity investigation, the optimized NEG did not elicit any indications of erythema or edema. The results demonstrate that the optimized NEG adheres to acceptable skin tolerance thresholds and is deemed safe for transdermal use⁽¹⁸²⁾.

A histopathological study revealed that 0.8% (v/v) formalin triggered significant damage to the epidermal layer, including exocytosis, congestion, inflammation, and separation of collagen fibres, confirming its irritant potential. In contrast, skin treated with the optimized NEG exhibited an intact epidermal layer with insignificant skin irritation. This reduced irritation potential may be attributed to the increased viscosity of the gel, which likely minimized direct contact between the NE and the skin layers. The optimized NEG demonstrated minimal observable changes compared to normal skin. As per the acute toxicity study and histopathology outcomes, the optimized NEG formulation exhibited acceptable safety for topical use.

5.8 Bioanalytical method for estimation of RH, ACE, and DL

We developed a simple, rapid, sensitive, and cost-effective bioanalytical method for simultaneous assessment of RH, ACE, and DL in rat plasma within 10 minutes was developed using Box-Behnken design and validated according to USFDA guidelines for bioanalytical method validation. A simple protein precipitation technique with acetonitrile and methanol was used to extract drugs from plasma. The analytes, RH, ACE, and DL showed good linearity in the 25-3000 ng/mL, 50-6000 ng/mL, and 50- 6000 ng/mL concentration ranges, respectively. The precision and

accuracy results were well within the acceptable range, with an average recovery between 92.27 and 96.10 %. The findings demonstrated that the developed method is precise, selective, reproducible, and superior to other current methods in its short analysis time (less than 10 min), and LLOQ ⁽³¹⁾. The developed method was found to be stable and successfully used to determine the pharmacokinetic parameters in Wistar rats after oral administration of the Dycerin A tablet. Furthermore, this analytical method was adequate and could be helpful for routine clinical analyses, pharmacokinetics, and bioequivalence investigations with a short running time.

5.9 *In vivo* release study of optimized DCN-and ACE-loaded NEG and marketed tablet formulation

To investigate the influence of formulation and route of administration on the pharmacokinetic parameters (drug absorption, bioavailability, elimination), the pharmacokinetic study of optimized NEG and marketed tablet formulations was performed in Wistar rats, and the obtained parameters were evaluated. For the tablet formulation, the pharmacokinetic profile was plotted up to 10 h only, as the analyte concentration fell below the LLOQ (25 ng/mL for RH and 50 ng/mL for ACE & DL). Hence, the pharmacokinetic profile of the two formulations was not superimposable. The marketed tablet formulation exhibited a higher C_{max} and a shorter T_{max} than the NEG formulation, possibly due to higher permeation into the blood after absorption through the GI membrane. The optimized NEG formulation achieved higher mean AUC_{0-t} (area under the curve) values for RH, ACE, and DL compared to the marketed tablet, indicating enhanced bioavailability. Additionally, the intrinsic pharmacokinetic properties, such as the elimination rate constant (K_e) of all three drugs, were higher in the tablet formulation than the NEG formulation, indicating faster drug clearance in the case of the tablet than in the NEG formulation ⁽¹⁸³⁾. The relative bioavailability of

RH, ACE, and DL from optimized NEG was 369%, 247%, and 106%, respectively. This improved bioavailability of optimized NEG is likely attributed to the no-first-pass metabolism, slow elimination rates, and enhanced skin permeation due to small GS and the presence of permeation enhancers (DMSO and Smix) ^(54,133,156,175,183). Statistical analysis demonstrated that the optimized NEG and marketed tablet formulations exhibited substantial differences (*p* value less than 0.05) in critical pharmacokinetic metrics (C_{max} , AUC).

5.10 *In vivo* anti-inflammatory efficacy of optimized DCN-and ACE-loaded NEG and marketed tablet formulation

The *in vivo* anti-inflammatory activity in the rat paw edema model demonstrated the highest edema inhibition by NEG at high and low doses compared to the plain gel (containing 2.5% DMSO), probably due to higher drug concentrations at the affected site and enhanced drug permeation through the skin, likely facilitated by the nano-sized formulation and the inclusion of Smix and DMSO as permeation enhancer. As per Dasgupta et al., ACE-loaded NE, ACE-loaded NEG, and marketed ACE gel showed 73.04%, 65.36%, and 41.74% of edema inhibition, respectively, after 24 h of administration, which further signified the role of nano-sized globules and the presence of Smix in enhancing the efficacy of the formulation ⁽¹⁰¹⁾. In our study, NEG at high and low doses exhibited 53.02% and 31.36% edema inhibition, respectively, after 24 h, consistent with the findings of Dasgupta et al. Notably, an insignificant difference was observed in the inhibitory effect between the marketed tablet and the low-dose NEG formulation, highlighting the effectiveness of NEG in improving transdermal permeability and bioavailability. Our study results align with those of Abdallah et al., ⁽¹⁸⁴⁾ who observed significantly higher inhibition of inflammation with brucine-loaded NEG compared to oral administration. Similarly,

Bhattacharya and Prajapati ⁽⁸⁵⁾ reported a greater reduction in inflammation with celecoxib-loaded NEG compared to the commercial gel, further supporting the possibility of NEG as an effective delivery system.

5.11 Evaluation of anti-osteoarthritis efficacy of optimized DCN-and ACE-loaded NEG and marketed tablet formulation

The effect of optimized NEG at low and high dose, plain gel and marketed tablet formulation on the knee thickness, protective cartilage structure, and bone cellular abnormalities in OA rat model was investigated. The drug treatment (oral and transdermal) was started from the 15 days of post-MIA injection. The results of the kinetic changes in the right knee thickness over the treatment periods (weeks) revealed that the knee thickness was significantly reduced in the NEG (high dose), followed by NEG (low dose) and marketed tablet-treated group, compared to MIA treated during weeks 1-5 post-treatment ($p < 0.0001$). However, no substantial change was observed among the marketed tablet-treated and NEG (low-dose) treated groups. The better effect observed for optimized NEG at high and low doses might be related to the no-first-pass metabolism, slow elimination rates, and enhanced skin permeation due to small GS and the presence of permeation enhancers (DMSO and Smix) ^(54,65,156,175). Chattopadhyay et al., ⁽⁶⁴⁾ studied the effect of diacerein and glucosamine sulfate-loaded nanoemulsion-laden hydrogel on the thickening of the joint capsule of OA induced rat model and revealed the fast-healing effect of the high-dose-treated group. The present study's findings agree with those of Chattopadhyay et al.

As per the literature review, numerous studies have successfully induced OA in animal models utilizing Monoiodoacetate (MIA). These studies have consistently demonstrated the development of OA-like changes within approximately 14 days,

including significant cartilage matrix erosion, necrotic chondrocytes, deep fissures, fibrillation, and proteoglycan loss ^(129,185,186). In the present study, histopathological examination of knee joint sections from the MIA-treated group at 14 days post-MIA injection revealed similar findings consistent with these previously reported observations ^(129,185,186). The effect of MIA further intensified at 35- and 49 days post-injection, with complete cartilage damage, chondrocyte loss, sclerosis, and mild inflammation, as evidenced by a consistent increase in total Mankin scores ⁽¹⁸⁵⁾. During the 1–5 weeks treatment period, the recovery of the articular surface with chondrocyte regeneration was most pronounced in the NEG at high dose, followed by the marketed tablet and NEG (low dose)-treated group. While the NEG (low dose) initially exhibited slightly lower efficacy than the marketed tablet at 3 weeks, its effectiveness increased by 5 weeks, becoming comparable to the marketed tablet. This enhanced efficacy was further supported by a decrease in the total Mankin score at 5 weeks. The delayed effectiveness of NEG at low dose may be due to the low plasma concentration of DCN and ACE and depot formation in the skin as confirmed by the *ex vivo* release study ^(62,187–189).

In contrast, plain gel treatments demonstrated limited efficacy in mitigating OA progression. The higher effectiveness of NEG (high dose) is likely attributed to increased permeation, enhanced relative bioavailability, higher localized drug concentrations, and prolonged drug delivery, as evidenced by the pharmacokinetic study ^(54,133,156,175). These findings indicate that NEG (at high and low doses) may be a viable treatment option for controlling OA due to its ability to improve cartilage regeneration and slow disease progression.

6. SUMMARY

Osteoarthritis (OA), caused by the deterioration of joint protective cartilage with inflammation, affects more than two hundred fifty million older adults globally, causing chronic pain, inflammation, and disability. Currently, the management of OA presents significant challenges. This is mainly due to the limited effectiveness and restricted long-term use of oral therapies such as NSAIDs, analgesics, COX-2 inhibitors, and hyaluronan, which often exhibit severe adverse effects. Nowadays, the focus of OA treatment has shifted towards modifying and slowing disease progression along with symptomatic management. Diacerein (DCN) and Aceclofenac (ACE) are among the most regularly prescribed medications for the OA treatment owing to their respective slow disease-modifying and analgesic properties. Fixed-dose combination tablets containing DCN 50 mg and ACE 100 mg are available in the market for rheumatoid arthritis and OA treatment. However, the chronic oral delivery of DCN and ACE remains challenging due to their poor aqueous solubility, low bioavailability, short half-life, extensive hepatic first-pass metabolism, and undesirable side effects, which collectively cause patient noncompliance and decreased adherence to DCN and ACE oral regimens. To enhance the bioavailability of DCN and ACE while significantly reducing oral side effects, this study explored a combinational nanoformulation approach for transdermal delivery. Hence, in the current study, we fabricated a NE and NEG formulation with improved transdermal codelivery of a DCN and ACE using a mixture design approach and evaluated their potential in treating OA.

A simple, reliable, accurate, sensitive, and effective stability-indicating RP-HPLC method was developed using a design of experiment (DoE) approach for the simultaneous determination of DCN and ACE in novel NEG formulation and

commercial tablet dosage form. The established method was further validated as per ICH guidelines. In the preformulation study, the DCN and ACE were characterized using UV spectroscopy, DSC, and FTIR methods. The DCN & ACE compatibility with excipients was confirmed by using DSC and FTIR analysis.

The critical components of NE, such as oily phase, surfactant, and co-surfactant, were chosen based on their compatibility with both DCN and ACE, solubilizing capacity for combined DCN and ACE, and emulsification ability. Further, based on the pseudoternary phase diagrams, the concentration of oil phase, aqueous phase, and Smix (mixture of surfactant & co-surfactant) ratio, which gives maximum NE area, was finalized.

The DCN- and ACE-loaded NE was successfully formulated using a low-energy method and optimized using a 3-factor, 2-level D-optimal mixture design implemented with Design-Expert (V 8.0.6, StatEase, Inc., USA) software. As per the experimental run, 16 formulations loaded with DCN and ACE were prepared and characterized for globule size (GS) and % transmittance (T). Further, the obtained response values were fitted to a mixture design and analyzed. The model's validity was assessed using the analysis of variance, and the statistically substantial model ($p < 0.05$) with insignificant lack of fit, highest R^2 , and adequate precision value (>4) was selected. Further, the main and interaction effects of three formulation components (oil mix, Smix, and water) on the response (GS and %T) were visualized using both plots: contour two-dimensional and response surface three-dimensional. Based on the numerical and graphical optimization results, the NE formulation with minimum GS (nm), maximum %T, and the highest composite desirability was chosen. Further, to validate the polynomial model, the first two formulations with the highest desirability were prepared and characterized for their GS and %T. The obtained

experimental and predicted values were compared, and no substantial variation was seen, confirming the model's validity. The suggested first two DCN-and ACE-loaded NE formulations were further characterized for various parameters. The electrokinetic (zeta) potential and GS were determined using a zeta sizer analyzer. The pH, viscosity, and T of NE formulations were measured using a pH meter, Brookfield viscometer, and UV-Vis spectrophotometer, respectively. The % drug content of NE was determined utilizing the developed RP-HPLC method. The cycling stress and thermodynamic stability of the NE were determined.

The *in vitro* permeation studies of DCN and ACE from design expert suggested NE formulations (NE-1 and NE-2) were determined using jacketed Franz diffusion cells apparatus with dialysis membrane at two different pH conditions, namely PBS pH 5.7 and a mixture of PBS pH 6.8 and methanol (7:3). The permeation profile of DCN and ACE from the NE-1 formulation was higher than the permeation of the NE-2 formulation in both media. Based on the desirability, other characteristics, and *in vitro* drug release results, NE-1 was finalized. The globule shape and globule surface morphology of the proposed NE were confirmed by using TEM and FE-SEM analysis. The optimized NE formulation was transformed into NEG using HPMC K15M (4%) as a gelling agent. For further optimization of the NEG formulations to improve drug release, various HPMC K15M (4%w/w) based NEG formulations were prepared with DMSO (2.5-7.5% w/w). Plain gels with and without DMSO (2.5% w/w) were also prepared using HPMC K15M gel base to compare the permeation profiles with NEG. The prepared DCN-and ACE-loaded NEG formulations and plain gels were characterized by pH, viscosity, spreadability, drug content, and *in vitro* permeation. The obtained *in vitro* permeation profile demonstrated the highest permeation of NEG with 2.5% DMSO compared to NEG

without DMSO and all plain gel formulations. The drug release kinetic study revealed that all NEG formulations followed the Korsmeyer-Peppas model with anomalous transport. Hence, NEG with 2.5% DMSO (NEG-2) was finalized for further evaluation.

An *ex vivo* cumulative drug permeation through rat skin demonstrated significantly higher permeation, transdermal flux, permeability coefficient, enhancement ratio, and skin deposition for both drugs from the optimized NEG formulation than the plain NEG and plain gel in both media. The drug release kinetic study revealed that all NEG and plain gel formulations followed the Korsmeyer-Peppas model with anomalous transport.

The complete solubilization and incorporation of the DCN and ACE into the formulated NE and NEG were confirmed using DSC and FTIR analysis. The stability study results demonstrated the stability of DCN and ACE in NEG at normal and accelerated conditions for up to 3 months with insignificant changes in the evaluated parameters. Skin irritation testing, performed by the Draize method, confirmed the non-irritant properties of the optimized NEG formulation. A histopathological study revealed that the optimized NEG formulation exhibited acceptable safety for topical use with insignificant skin irritation. A simple, rapid, sensitive, and cost-effective bioanalytical method for simultaneous assessment of RH, ACE, and DL in rat plasma within 10 minutes was developed using BBD and validated according to USFDA guidelines for bioanalytical method validation. The pharmacokinetic study of the optimized NEG and marketed tablet formulation was performed in Wistar rats, and the obtained results confirmed the enhanced bioavailability of DCN and ACE from the optimized NEG formulation compared to the marketed tablet formulation. The optimized NEG demonstrated superior anti-inflammatory activity compared to plain

gel and a marketed tablet in a rat hind paw edema model induced by carrageenan. The anti-osteoarthritis study demonstrated that topical application of the optimized NEG (high dose) to the OA-induced knee joints of rats for five weeks exhibited a substantial reduction in knee joint thickness than the plain gel and the marketed tablet-treated groups. The histopathological examination further corroborated these findings, revealing significantly greater cartilage regeneration and healing of the articular surface in the high-dose NEG-treated group than in the plain gel and marketed tablet-treated groups. However, the low-dose NEG formulation did not significantly differ in knee thickness reduction or articular surface healing compared to the marketed tablet treatment.

Overall, the increased *in vitro* & *ex vivo* permeation and *in vivo* release, coupled with higher anti-inflammatory activity and rapid cartilage healing properties of the NEG formulation, are likely attributed to enhanced permeation, increased relative bioavailability, higher localized drug concentrations, and prolonged drug delivery. These results collectively suggest that the NEG formulation holds significant promise as a transdermal drug delivery system for the treatment of OA.

7. CONCLUSION

This research aimed to develop and evaluate a novel NEG formulation for the transdermal codelivery of DCN and ACE to enhance the efficacy of OA treatment. Diacerein- and ACE-loaded NE was successfully formulated and optimized using a 3-factor, 2-level D-optimal mixture design. The optimized NE formulation (NE-1) with 13.34 % w/w oil mix and 31.77% w/w Smix exhibited desirable GS (<20 nm) and the highest T (98.03%) with excellent thermodynamic stability. The DSC and FTIR studies demonstrated complete solubilization and incorporation of DCN and ACE into the formulated NE without any physical and chemical incompatibility with the excipients. The TEM and FE-SEM images revealed that all the globules were spherical and confirmed to be within the nano-size range (< 20 nm). *In vitro* drug release results demonstrated that DCN and ACE from NE formulation followed the Korsmeyer-Peppas model and first-order release kinetics, respectively, with anomalous (non-Fickian case II) diffusion involving both dissolution and diffusion processes. The optimized NE was transferred into NEG using HPMC K15 gelling agent and characterized for viscosity, spreadability, pH, and drug content.

The results of *in vitro* drug permeation (using dialysis membrane) and *ex vivo* permeation through rat skin study demonstrated that DCN and ACE from optimized NEG (with 2.5%w/w DMSO) exhibited increased permeation compared to plain NEG (without DMSO) and all plain gel formulations with and without DMSO. The drug release kinetic study revealed that all NEG formulations followed the Korsmeyer-Peppas model with anomalous transport in both permeation studies. An *ex vivo* permeation study demonstrated significantly higher permeation, transdermal flux, permeability coefficient, enhancement ratio, and skin deposition for both drugs from the optimized NEG formulation than the plain NEG and plain gel. The DSC and FTIR

studies of optimized NEG demonstrated the physico-chemical stability of DCN and ACE with complete solubilization and incorporation into the formulated NEG. The optimized NEG was found stable at room temperature and accelerated condition for 3 months with no substantial difference in the permeation profile. Skin irritation and acute toxicity studies revealed that optimized NEG was non-irritating to rat skin, exhibiting no signs of edema or erythema. The histopathological examination results demonstrated acceptable safety of optimized NEG for topical application. Pharmacokinetic studies in Wistar rats revealed that the optimized NEG exhibited higher area under the curve values (AUC_{0-t} , $AUC_{0-\infty}$), a longer half-life ($t_{1/2}$), and lower elimination rate constant for RH, ACE, and DL compared to the marketed tablet formulation, indicating enhanced bioavailability with slow elimination rates. Furthermore, *in vivo*, anti-inflammatory, and anti-osteoarthritis studies in rat models demonstrated the superior therapeutic efficacy of the optimized NEG compared to the marketed tablet and plain gel formulations, with significant improvements in anti-inflammatory activity and cartilage protection.

The outcomes of this study suggest a potential role for this novel NEG formulation in the management of OA. This NEG platform offers a promising technique for the transdermal delivery of drugs with poor oral and transdermal bioavailability and significant oral side effects by improving patient compliance through reduced dosing frequency and enhancing therapeutic effectiveness. To thoroughly evaluate the therapeutic potential of this NEG formulation for OA, further studies employing more advanced preclinical models are necessary to bridge the gap toward clinical translation.

8. BIBLIOGRAPHY

1. Hunter DJ, Bierma-Zeinstra S. Osteoarthritis. *Lancet*. 2019;393(10182):1745–59.
2. Liem Y, Judge A, Kirwan J, Ourradi K, Li Y, Sharif M. Multivariable logistic and linear regression models for identification of clinically useful biomarkers for osteoarthritis. *Scientific Reports*. 2020;10(11328):1–13.
3. O'Neill TW, McCabe PS, McBeth J. Update on the epidemiology, risk factors and disease outcomes of osteoarthritis. *Best Practice & Research: Clinical Rheumatology*. 2018;32(2):312–26.
4. Tamura T, Kosaka N, Ishiwa J, Sato T, Nagase H, Ito A. Rhein, an active metabolite of diacerein, down-regulates the production of pro-matrix metalloproteinases-1, -3, -9 and -13 and up-regulates the production of tissue inhibitor of metalloproteinase-1 in cultured rabbit articular chondrocytes. *Osteoarthritis Cartilage*. 2001;9(3):257–63.
5. Abramoff B, Caldera FE. Osteoarthritis: Pathology, diagnosis, and treatment options. *Medical Clinics of North America*. 2020 Mar 1;104(2):293–311.
6. Woolf AD, Pfleger B. Burden of major musculoskeletal conditions. *Bull World Health Organ*. 2003;81(9):646–56.
7. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, et al. Development of criteria for the classification and reporting of osteoarthritis: Classification of osteoarthritis of the knee. *Arthritis and Rheumatology*. 1986 Aug;29(8):1039–49.
8. Sarzi-Puttini P, Cimmino MA, Scarpa R, Caporali R, Parazzini F, Zaninelli A, et al. Osteoarthritis: An overview of the disease and its treatment strategies. *Seminars in Arthritis and Rheumatism*. 2005 Aug;35(1 SUPPL.1):1–10.

9. Felson DT, Lawrence RC, Dieppe PA, Hirsch R, Helmick CG, Jordan JM, et al. Osteoarthritis: new insights. Part 1: the disease and its risk factors. *Annals of Internal Medicine*. 2000 Oct 17;133(8):635–46.
10. Pal CP, Singh P, Chaturvedi S, Pruthi KK, Vij A. Epidemiology of knee osteoarthritis in India and related factors. *Indian Journal of Orthopaedics*. 2016 Sep 1;50(5):518–22.
11. Silman AJ, Hochberg MC. *Epidemiology of the rheumatic diseases*. 2nd ed. Oxford: Oxford University Press; 2001.
12. Symmons D, Mathers C, Pflieger B. Global burden of osteoarthritis in year 2000: Global burden of disease 2000 study. *World health report*. 2002; 5:2.
13. Bhat TJ, Bhat JA, Bhat F. Comparative analysis of analgesic efficacy, safety and compliance of fixed dose combination (Aceclofenac+ Diacerein) versus free drug combination in 400 patients of early osteoarthritis knee. *International Journal of Pharmacological Research*. 2017;7(05):107–19.
14. Cross M, Smith E, Hoy D, Nolte S, Ackerman I, Fransen M, et al. The global burden of hip and knee osteoarthritis: estimates from the global burden of disease 2010 study. *Annals of the Rheumatic Diseases*. 2014 Jul;73(7):1323–30.
15. Altman R. Recommendations for the medical management of osteoarthritis of the hip and knee. American College of Rheumatology Subcommittee on Osteoarthritis, Guidelines. *Arthritis and Rheumatology*. 2000; 43:1905–15.
16. Bollet AJ. Analgesic and anti-inflammatory drugs in the therapy of osteoarthritis. *Seminars in Arthritis and Rheumatism*. 1981 Jan 1;11(1):130–2.
17. Pelletier JP, Martel-Pelletier J. Protective effects of corticosteroids on cartilage lesions and osteophyte formation in the Pond-Nuki dog model of osteoarthritis. *Arthritis and Rheumatology*. 1989 Feb;32(2):181–93.

18. Cao Y, Ma Y, Tao Y, Lin W, Wang P. Intra-articular drug delivery for osteoarthritis treatment. *Pharmaceutics*. 2021 Dec;13(12):2166.
19. Dwicandra NMO, Setiadi AAP. Comparison of pain score in osteoarthritis patients treated with a combination of diacerein and meloxicam and meloxicam alone. *International Journal of Pharmacy and Pharmaceutical Sciences*. 2017 Jun 1;9(6):69–73.
20. Wang Y, Zhao XP, Ruan JW. Transdermal drug delivery system of aceclofenac for rheumatoid arthritis and the effect of permeation enhancers: In vitro and in vivo characterization. *International Journal of Pharmacology*. 2015;11(5):456–62.
21. Shah J, Mirza N, Patel V. A comparative study for safety and efficacy of oxaceprol and diacerein in osteoarthritis of knee joints. *Pharma Tutor*. 2014;2(6):108–14.
22. Conaghan PG, Dickson J, Bolten W, Cevc G, Rother M. A multicentre, randomized, placebo- and active-controlled trial comparing the efficacy and safety of topical ketoprofen in transfersome gel (IDEA-033) with ketoprofen-free vehicle (TDT 064) and oral celecoxib for knee pain associated with osteoarthritis. *Rheumatology*. 2013;52(7):1303–12.
23. Colville-Nash PR, Willoughby DA. COX-1, COX-2 and articular joint disease: A role for chondroprotective agents. *Biorheology*. 2002 Jan 1;39(1–2):171–9.
24. Hochberg MC, Dougados M. Pharmacological therapy of osteoarthritis. *Best Practice and Research Clinical Rheumatology*. 2001;15(4):583–93.
25. Pelletier JP, Yaron M, Haraoui B, Cohen P, Nahir MA, Choquette D, et al. Efficacy and safety of diacerein in osteoarthritis of the knee: A double-blind, placebo-controlled trial. *Arthritis and Rheumatology*. 2000;43(10):2339–48.

26. Singh S, Jain A, Singh SK, Singh Y. Development of lipid nanoparticles of diacerein, an antiosteoarthritic drug for enhancement in bioavailability and reduction in its side effects. *Journal of Biomedical Nanotechnology*. 2013 May;9(5):891–900.
27. Nebesen M, Abd El-Rahman MK, Salem MY, El-Kosasy AM, El-Bardicy MG. Stability-indicating spectrophotometric and spectrodensitometric methods for the determination of diacerein in the presence of its degradation product. *Drug Testing and Analysis*. 2011;3(4):221–7.
28. Singh U, Singh LLS, Gita H, Yadav SL. Efficacy of diacerein in the treatment of osteo-arthritis of knee. *Indian Journal of Physical Medicine and Rehabilitation*. 2013;24(4):92–8.
29. Li Z, Meng D, Li G, Xu J, Tian K, Li Y. Celecoxib combined with diacerein effectively alleviates osteoarthritis in rats via regulating JNK and p38MAPK signaling pathways. *Inflammation*. 2015;38(4):1563–72.
30. Legrand E. Aceclofenac in the management of inflammatory pain. *Expert Opinion on Pharmacotherapy*. 2004;5(6):1347–57.
31. Ojha A, Rathod R, Padh H. Simultaneous HPLC-UV determination of rhein and aceclofenac in human plasma. *Journal of Chromatography B*. 2009 Apr 15;877(11–12):1145–8.
32. Kalra R, Mulik RS, Badgujar L, Paradkar AR, Mahadik KR, Bodhankar SL, et al. Development and characterization of micro-emulsion formulations for transdermal delivery of aceclofenac: A research. *International Journal of Drug Formulation and Research*. 2010;1(1):359–86.
33. Aziz DE, Abdelbary AA, Elassasy AI. Fabrication of novel elastosomes for boosting the transdermal delivery of diacerein: Statistical optimization, ex-

- vivo permeation, in-vivo skin deposition and pharmacokinetic assessment compared to oral formulation. *Drug Delivery*. 2018;25(1):815–26.
34. Raza K, Kumar M, Kumar P, Malik R, Sharma G, Kaur M, et al. Topical delivery of aceclofenac: Challenges and promises of novel drug delivery systems. *BioMed Research International*. 2014; 2014:1–11.
35. Godbole R, Invally M, Wairkar S, Bhullar S, Kaur G, Pratapbhai S. Formulation, development and evaluation of a novel polymer-based anti-Inflammatory emulgel of aceclofenac. *Science International*. 2018;6(2):71–9.
36. Kamboj S, Saini V, Bala S, Sharma G. Formulation and characterization of drug loaded niosomal gel for anti-Inflammatory activity. *International Journal of Pharmacological and Pharmaceutical Sciences*. 2013;7(12):877–81.
37. Quan LD, Thiele GM, Tian J, Wang D. The development of novel therapies for rheumatoid arthritis. *Expert Opinion on Therapeutic Patents*. 2008 Jul;18(7):723–38.
38. Siddiqui B, Rehman AU, Haq IU, Ahmad NM, Ahmed N. Development, optimization and evaluation of nanoencapsulated diacerein emulgel for potential use in osteoarthritis. *Journal of Microencapsulation*. 2020 Nov 16;37(8):595–608.
39. Nishal S, Jhawar V, Phaugat P, Dutt R. DoE Based Formulation Development and Characterization of Topical Nanoemulgel of Diacerein: In-Vitro Release and Permeation Studies. *Bionanoscience*. 2022 Jun 1;12(2):472–85.
40. Mohammed S FS, Sheikh Shafiq F. Comparative pharmacokinetic profile of aceclofenac from oral and transdermal application. *Journal of Bioequivalence and Bioavailability*. 2009;01(01):13–7.

41. Alam MS, Algahtani MS, Ahmad J, Kohli K, Shafiq-Un-Nabi S, Warsi MH, et al. Formulation design and evaluation of aceclofenac nanogel for topical application. *Therapeutic Delivery*. 2020 Dec 1;11(12):767–78.
42. Moghddam SRM, Ahad A, Aqil M, Imam SS, Sultana Y. Formulation and optimization of niosomes for topical diacerein delivery using 3-factor, 3-level Box-Behnken design for the management of psoriasis. *Materials Science and Engineering C*. 2016 Dec 1; 69:789–97.
43. Richard C, Cassel S, Blanzat M. Vesicular systems for dermal and transdermal drug delivery. *RSC Advances*. 2021 Dec 12;11(1):442–51.
44. Prausnitz MR, Mitragotri S, Langer R. Current status and future potential of transdermal drug delivery. *Nature Reviews Drug Discovery*. 2004;3(2): 115–24.
45. Paudel KS, Milewski M, Swadley C.L, Brogden NK, Ghosh P, Stinchcomb AL. Challenges and opportunities in dermal/transdermal delivery. *Therapeutic Delivery*. 2010;1(1):109–31.
46. Shakeel F, Baboota S, Ahuja A, Ali J, Aqil M, Shafiq S. Nanoemulsions as vehicles for transdermal delivery of aceclofenac. *AAPS PharmSciTech*. 2007 Oct;8(4):191–9.
47. Shakeel F, Shafiq S, Haq N, Alanazi FK, Alsarra IA. Nanoemulsions as potential vehicles for transdermal and dermal delivery of hydrophobic compounds: An overview. Vol. 9, *Expert Opinion on Drug Delivery*. 2012 Aug 1;9(8):953-74.
48. Gannu R, Palem CR, Yamsani VV, Yamsani SK, Yamsani MR. Enhanced bioavailability of lacidipine via microemulsion based transdermal gels: Formulation optimization, ex vivo and in vivo characterization. *International Journal of Pharmaceutics*. 2010 Mar 30;388(1–2):231–41.

49. Sengupta P, Chatterjee B. Potential and future scope of nanoemulgel formulation for topical delivery of lipophilic drugs. *International Journal of Pharmaceutics*. 2017 Jun 30;526(1–2):353–65.
50. Solans C, Solé I. Nano-emulsions: Formation by low-energy methods. *Current Opinion in Colloid and Interface Science*. 2012 Oct;17(5):246–54.
51. Singh B, Singh R, Bandyopadhyay S, Kapil R, Garg B. Optimized nanoemulsifying systems with enhanced bioavailability of carvedilol. *Colloids and Surfaces B: Biointerfaces*. 2013 Jan 1;101:465–74.
52. Somagoni J, Boakye CHA, Godugu C, Patel AR, Faria HAM, Zucolotto V, et al. Nanomiemgel - A novel drug delivery system for topical application - In vitro and in vivo evaluation. *PLoS One*. 2014 Dec 29;9(12):1–30.
53. Shaker DS, Ishak RAH, Ghoneim A, Elhuoni MA. Nanoemulsion: A review on mechanisms for the transdermal delivery of hydrophobic and hydrophilic drugs. *Scientia Pharmaceutica*. 2019;87(3):17.
54. Radhika PR, Guruprasad S. Nanoemulsion based emulgel formulation of lipophilic drug for topical delivery. *International Journal of PharmTech Research*. 2016;9(6):210–23.
55. Mou D, Chen H, Du D, Mao C, Wan J, Xu H, et al. Hydrogel-thickened nanoemulsion system for topical delivery of lipophilic drugs. *International Journal of Pharmaceutics*. 2008 Apr 2;353(1–2):270–6.
56. Khurana S, Jain NK, Bedi PMS. Nanoemulsion based gel for transdermal delivery of meloxicam: Physico-chemical, mechanistic investigation. *Life Sciences*. 2013 Mar 14;92(6–7):383–92.
57. Ahmad J, Gautam A, Komath S, Bano M, Garg A, Jain K. Topical nano-emulgel for skin disorders: Formulation approach and characterization. *Recent Patents on Anti-Infective Drug Discovery*. 2018;14(1):36–48.

58. Donthi MR, Munnangi SR, Krishna KV, Saha RN, Singhvi G, Dubey SK. Nanoemulgel: A novel nano carrier as a tool for topical drug delivery. *Pharmaceutics*. 2023 Jan 3;15(1):164.
59. Eid AM, Elmarzugi NA, El-Enshasy HA. Preparation and evaluation of olive oil nanoemulsion using sucrose monoester. *International Journal of Pharmacy and Pharmaceutical Sciences*. 2013 ; 5 (Suppl 3):434–40.
60. Chellapa P, Mohamed AT, Keleb EI, Elmahgoubi A, Eid AM, Issa YS, Elmarzugi NA. Nanoemulsion and nanoemulgel as a topical formulation. *IOSR Journal of Pharmacy*. 2015 Oct;5(10):43-7.
61. Singh RP, Parpani S, Narke R, Chavan R. Emulgel: A recent approach for topical drug delivery system. *Asian Journal of Pharmaceutical Research and Development*. 2014 May;2(2):112–23.
62. Rao SV, Vani DBS, Padmalatha K. Topical nanoemulgel: A novel approach for wound healing Efficacy. *Journal of Chemical and Pharmaceutical Research*. 2021;13(9):1–09.
63. Gada HJ, Sawarkar SP. Osteoarthritis: Overview and novel treatment approaches. *European Journal of Biomedical*. 2016;3(12):71.
64. Chattopadhyay H, Auddy B, Sur T, Gupta M, Datta S. Transdermal co-delivery of glucosamine sulfate and diacerein for the induction of chondroprotection in experimental osteoarthritis. *Drug Delivery and Translational Research*. 2020 Oct; 10:1327-40.
65. Shukla T, Pandey SP, Khare P, Upmanyu N. Development of ketorolac tromethamine loaded microemulsion for topical delivery using D-optimal experimental approach: Characterization and evaluation of analgesic and anti-

- inflammatory efficacy. *Journal of Drug Delivery Science and Technology*. 2021 Aug 1;64:102632.
66. Hirakant M, Nagoba SN. Design, development and evaluation of novel nanoemulgel for topical drug delivery system. *Journal of Biomechanical Science and Engineering*. 2023;(7):84–99.
67. Pimple S, Gurjar M, Shah M, Maurya P, Joshi A, Swami A, et al. Development and evaluation of low dose solid oral formulation of eszopiclone tablets 1mg, 2 mg AND 3 mg. *International Journal of Universal Pharmacy and Bio science*. 2014;3(4):40–50.
68. Bhure M V., Hemke AT, Gupta KR. UV-spectrophotometric methods for determination of aceclofenac and diacerein in pharmaceutical formulation. *Journal of Pharmaceutical Sciences and Research*. 2010;2(7):426–32.
69. Chitlange SS, Pawbake GR, Mulla AI, Wankhede SB. Simultaneous spectrophotometric estimation of diacerein and aceclofenac in tablet dosage form. *Der Pharma Chemica*. 2010;2(1):335-41.
70. Chadha R, Bhandari S. Drug-excipient compatibility screening-Role of thermoanalytical and spectroscopic techniques. *Journal of pharmaceutical and biomedical analysis*. 2014 Jan 18; 87:82–97.
71. Cheng L, Chen Q, Pi R, Chen J. A research update on the therapeutic potential of rehin and its derivatives. *European Journal of Pharmacology*. 2021 May 15; 899:173908.
72. Hinz B, Rau T, Auge D, Werner U, Ramer R, Rietbrock S, et al. Aceclofenac spares cyclooxygenase 1 as a result of limited but sustained biotransformation to diclofenac. *Clinical Pharmacology and Therapeutics*. 2003;74(3):222–35.
73. Pareek A, Chandurkar N. Comparison of gastrointestinal safety and tolerability of aceclofenac with diclofenac: A multicenter, randomized,
-

- double-blind study in patients with knee osteoarthritis. *Current Medical Research and Opinion*. 2013 Jul 1;29(7):849–59.
74. Kanaki AR, Patil RS, Santoshkumar J, Mala RD. Comparative study of safety, efficacy, and tolerability of aceclofenac versus diclofenac in osteoarthritis patients. *Journal of Evolution of Medical and Dental Sciences*. 2013 Apr 15;2(15):2432–9.
75. Hashem H, El-sayed HM. Quality by design approach for development and validation of a RP-HPLC method for simultaneous determination of co-administered levetiracetam and pyridoxine HCl in prepared tablets. *Microchemical Journal*. 2018 Dec 1; 143:55–63.
76. Ganorkar SB, Dhumal DM, Shirkhedkar AA. Development and validation of simple RP-HPLC-PDA analytical protocol for zileuton assisted with Design of Experiments for robustness determination. *Arabian Journal of Chemistry*. 2017;10(2):273–82.
77. Ernoviya E, Masfria M, Sinaga KR. Optimization and evaluation of topical ketoconazole nanoemulsion. *Asian Journal of Pharmaceutical and Clinical Research*. 2018 May 1;11(5):143–6.
78. Dixit RP, Nagarsenker MS. Self-nanoemulsifying granules of ezetimibe: Design, optimization and evaluation. *European Journal of Pharmaceutical Sciences*. 2008 Oct 2;35(3):183–92.
79. Nasr A, Gardouh A, Ghorab M. Novel solid self-nanoemulsifying drug delivery system (S-SNEDDS) for oral delivery of olmesartan medoxomil: Design, formulation, pharmacokinetic and bioavailability evaluation. *Pharmaceutics*. 2016 Jun 27;8(3):20.
80. El-Laithy HM, Basalious EB, El-Hoseiny BM, Adel MM. Novel self-nanoemulsifying self-nanosuspension (SNESNS) for enhancing oral

- bioavailability of diacerein: Simultaneous portal blood absorption and lymphatic delivery. *International Journal of Pharmaceutics*. 2015 May 30;490(1–2):146–54.
81. Malik MZ, Ahmad M, Minhas MU, Munir A. Solubility and permeability studies of aceclofenac in different oils. *Tropical Journal of Pharmaceutical Research*. 2014;13(3):327–30.
82. Arora R, Aggarwal G, Harikumar SL, Kaur K. Nanoemulsion based hydrogel for enhanced transdermal delivery of ketoprofen. *Advances in Pharmaceutics*. 2014;2014(1):468456.
83. Shakeel F. Criterion for excipients screening in the development of nanoemulsion formulation of three anti-inflammatory drugs. *Pharmaceutical Development and Technology*. 2010;15(2):131–8.
84. Kaur G. TPGS loaded topical nanoemulgel of mefenamic acid for the treatment of rheumatoid arthritis. *International Journal of Pharmacy and Pharmaceutical Research*. 2019;15(1):64–107.
85. Bhattacharya S, Prajapati BG. Formulation and optimization of celecoxib nanoemulgel. *Asian Journal of Pharmaceutical and Clinical Research*. 2017;10(8):353–65.
86. Shao H, Xi N, Zhang Y. Microemulsion formulation of a new biopesticide to control the diamondback moth (Lepidoptera: Plutellidae). *Scientific Reports*. 2018 Jul 12;8(1):10565.
87. Prajapati BG, Jivani M, Paliwal H. Formulation and optimization of topical nanoemulsion based gel of mometasone furoate using 32 full factorial design. *Indian Drugs*. 2021 Jun 1;58(6):19–29.
88. Yahya NA, Wahab RA, Attan N, Hashim SE, Abdul Hamid M, Mohamed Noor N, et al. Optimization of oil-in-water nanoemulsion system of Ananas

- comosus peels extract by D-optimal mixture design and its physicochemical properties. *Journal of Dispersion Science and Technology*. 2020;43(2):302–15.
89. Chouhan P, Saini TR. D-optimal design and development of microemulsion based transungual drug delivery formulation of ciclopirox olamine for treatment of onychomycosis. *Indian Journal of Pharmaceutical Sciences*. 2016 Jun 1;78(4):498–511.
90. Gohil R, Patel A, Pandya T, Dharamsi A. Optimization of brinzolamide loaded microemulsion using formulation by design approach: Characterization and in-vitro evaluation. *Current Drug Therapy*. 2019 Jan;14(1):1–18.
91. Holm R, Jensen IHM, Sonnergaard J. Optimization of self-microemulsifying drug delivery systems (SMEDDS) using a D-optimal design and the desirability function. *Drug Development and Industrial Pharmacy*. 2006 Oct 1;32(9):1025–32.
92. Romes NB, Wahab RA, Abdul Hamid M, Hashim SE. D-optimal design-assisted *Elaeis guineensis* leaves extract in olive oil-sunflower seed nanoemulsions: development, characterization, and physical stability. *Journal of Dispersion Science and Technology*. 2022 Feb 1;43(2):289–301.
93. Aqil M, Kamran M, Ahad A, Imam SS. Development of clove oil based nanoemulsion of olmesartan for transdermal delivery: Box-Behnken design optimization and pharmacokinetic evaluation. *Journal of Molecular Liquids*. 2016 Feb 1; 214:238–48.
94. Sita V G, Vavia P. Bromocriptine nanoemulsion-loaded transdermal gel: Optimization using factorial design, in Vitro and in Vivo evaluation. *AAPS PharmSciTech*. 2020 Jan 23;21(3):80.

95. Chin LY, Tan JYP, Choudhury H, Pandey M, Sisinty SP, Gorain B. Development and optimization of chitosan coated nanoemulgel of telmisartan for intranasal delivery: A comparative study. *Journal of Drug Delivery Science and Technology*. 2021 Apr 1; 62:102341.
96. Mittal S, Ali J, Baboota S. Enhanced anti-psoriatic activity of tacrolimus loaded nanoemulsion gel via omega 3 - Fatty acid (EPA and DHA) rich oils- fish oil and linseed oil. *Journal of Drug Delivery Science and Technology*. 2021 Jun 1; 63:102458.
97. Fadhel AY, Rajab NA. Tizanidine nano emulsion: Formulation and in-vitro characterization. *Journal of Pharmaceutical Negative Results*. 2022;13(3): 572–81.
98. Gaba B, Khan T, Haider MF, Alam T, Baboota S, Parvez S, et al. Vitamin E loaded naringenin nanoemulsion via intranasal delivery for the management of oxidative stress in a 6-OHDA Parkinson's disease model. *BioMed Research International*. 2019;2019(1):2382563.
99. Das S, Sharadha M, Venkatesh MP, Sahoo S, Tripathy J, Gowda D V. Formulation and evaluation of topical nanoemulgel of methotrexate for rheumatoid arthritis. *International Journal of Applied Pharmaceutics*. 2021 Sep 1;13(5):351–7.
100. Aparna C, Srinivas P, Rao Patnaik KSK. Enhanced transdermal permeability of telmisartan by a novel nanoemulsion gel. *International Journal of Pharmacy and Pharmaceutical Sciences*. 2015;7(4): 335–42.
101. Dasgupta S, Dey S, Choudhury S, Mazumder B. Topical delivery of aceclofenac as nanoemulsion comprising excipients having optimum

- emulsification capabilities: Preparation, characterization and in vivo evaluation. *Expert Opinion on Drug Delivery*. 2013 Apr;10(4):411–20.
102. Khan NR, Wong TW. Microwave-aided skin drug penetration and retention of 5-fluorouracil-loaded ethosomes. *Expert Opinion on Drug Delivery*. 2016 Sep 1;13(9):1209–19.
103. Sheshala R, Anuar NK, Abu Samah NH, Wong TW. In vitro drug dissolution/permeation testing of nanocarriers for skin application: A comprehensive review. *AAPS PharmSciTech*. 2019 Jul; 20:1–28.
104. Jain S, Nagori BP, Yadav SK. Improvement of the dissolution behavior of the poorly water-soluble drug diacerein by solid dispersion technology and its formulation into tablet dosage form. *Madridge Journal of Novel Drug Research*. 2018 Oct 12;2(1):79–89.
105. Garg NK, Tandel N, Bhadada SK, Tyagi RK. Nanostructured lipid carrier-mediated transdermal delivery of aceclofenac hydrogel present an effective therapeutic approach for inflammatory diseases. *Frontiers in Pharmacology*. 2021 Sep 20;12.
106. Alhakamy NA, Kotta S, Ali J, Alam MS, Hosny KM, Shaik RA, et al. Formulation development, statistical optimization, in vitro and in vivo evaluation of etoricoxib-loaded eucalyptus oil-based nanoemulgel for topical delivery. *Applied Sciences*. 2021 Aug 9;11(16):7294.
107. Pathan IB, Munde SJ, Shelke S, Ambekar W, Mallikarjuna Setty C. Curcumin loaded fish scale collagen-HPMC nanogel for wound healing application: Ex-vivo and In-vivo evaluation. *International Journal of Polymeric Materials and Polymeric Biomaterials*. 2019 Mar 4;68(4):165–74.

108. Paliwal S, Pandey K, Joshi H, Kaur G, Akbar N. An overview of nanoemulgel as a nanocarrier drug delivery. *Journal of Medical Pharmaceutical and Allied Sciences*. 2022 Jan 1;2(2):235–8.
109. Shehata TM, Elnahas HM, Elsewedy HS. Development, characterization and optimization of the anti-inflammatory influence of meloxicam loaded into a eucalyptus oil-based nanoemulgel. *Gels*. 2022 Apr 22;8(5):262.
110. Khalifa NE, Abdallah MH, Elghamry HA, Khojali WMA, Khafagy ES, El-Sayed El-Horany H, et al. Development of tea tree oil based nanoemulgel loaded with azithromycin for enhancing the antibacterial activity. *Processes*. 2023 Jun 1;11(6):1836.
111. Morteza-Semnani K, Saeedi M, Akbari J, Eghbali M, Babaei A, Hashemi SMH, et al. Development of a novel nanoemulgel formulation containing cumin essential oil as skin permeation enhancer. *Drug Delivery and Translational Research*. 2022 Jun 1;12(6):1455–65.
112. Kumar B, Jain SK, Prajapati SK. Effect of penetration enhancer DMSO on in-vitro skin permeation of acyclovir transdermal microemulsion formulation. *International Journal of Drug Delivery*. 2011;3(1):83–94.
113. Shukla A, Gupta MK, Dev R, Purohit D, Singh M, Pal P. Formulation and evaluation of the polyherbal nanoemulgel for the treatment of inflammation. *Neuro Quantology*. 2022 Nov;20(13):2773–83.
114. Wadile KA, Ige PP, Sonawane RO. Preparation of itraconazole nanoparticles and its topical nanogel: Physicochemical properties and stability studies. *International Journal of Pharmaceutical Sciences and Developmental Research*. 2019 Jan 3;5(1):001–8.
115. Bashir M, Ahmad J, Asif M, Khan SUD, Irfan M, Ibrahim AY, et al. Nanoemulgel, an innovative carrier for diflunisal topical delivery with

- profound anti-inflammatory effect: In vitro and in vivo evaluation. *International Journal of Nanomedicine*. 2021 Feb 22; 16:1457–72.
116. Morsy MA, Abdel-Latif RG, Nair AB, Venugopala KN, Ahmed AF, Elsewedy HS, et al. Preparation and evaluation of atorvastatin-loaded nanoemulgel on wound-healing efficacy. *Pharmaceutics*. 2019 Nov 13;11(11):609.
117. Kurangi B, Jalalpure S, Jagwani S. Formulation and evaluation of resveratrol loaded cubosomal nanoformulation for topical delivery. *Current Drug Delivery*. 2021 Jun 1;18(5):607-19.
118. Draize JH. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *Journal of Pharmacology and Experimental Therapeutics*. 1944; 82:377–90.
119. Qumbar M, Aameeduzzafar, Imam SS, Ali J, Ahmad J, Ali A. Formulation and optimization of lacidipine loaded niosomal gel for transdermal delivery: In-vitro characterization and in-vivo activity. *Biomedicine and Pharmacotherapy*. 2017 Sep 1;93:255–66.
120. Patel HK, Barot BS, Parejiya PB, Shelat PK, Shukla A. Topical delivery of clobetasol propionate loaded microemulsion based gel for effective treatment of vitiligo: Ex vivo permeation and skin irritation studies. *Colloids and Surfaces B: Biointerfaces*. 2013 Feb 1; 102:86–94.
121. Aameeduzzafar, El-Bagory I, Alruwaili NK, Imam SS, Alomar FA, Elkomy MH, et al. Quality by design (QbD) based development and validation of bioanalytical RP-HPLC method for dapagliflozin: Forced degradation and preclinical pharmacokinetic study. *Journal of Liquid Chromatography & Related Technologies*. 2020 Jan 20;43(1–2):53–65.

122. Bonde S, Bonde CG, Prabhakar B. Quality by design-based development and validation of HPLC method for simultaneous estimation of paclitaxel and vinorelbine tartrate in dual drug loaded liposomes. *Microchemical Journal*. 2019 Sep 1; 149:103982.
123. Jincy J, Sunil C. Exploring antiulcer and anti-inflammatory activities of methanolic leaves extract of an Indian mistletoe *Helicantes elasticus* (Desv.) Danser. *South African Journal of Botany*. 2020 Sep 1; 133:10–6.
124. Chiranjeevi G, Rajendar M, Dara H, Krishna RV. Aceclofenac-induced physicochemical alterations in toxicity among male Albino rats. *International Journal of pharma and bio sciences*. 2018;8(1):632–5.
125. Pathan IB, Dwivedi R, Ambekar W. Formulation and evaluation of ketoprofen loaded chitosan nanogel for pain management: Ex-vivo and in-vivo study. *Ars Pharmaceutica (Internet)*. 2019 Jun 20;60(2):101–8.
126. Jain A, Mishra SK, Vuddanda PR, Singh SK, Singh R, Singh S. Targeting of diacerein loaded lipid nanoparticles to intra-articular cartilage using chondroitin sulfate as homing carrier for treatment of osteoarthritis in rats. *Nanomedicine: Nanotechnology, Biology and Medicine*. 2014 Jul 1;10(5):1031-40.
127. Belkhodja H, Meddah B, Touil AMT, Slimani K, Tou A. Radiographic and histopathologic analysis on osteoarthritis rat model treated with essential oils of *rosmarinus officinalis* and *populus alba*. *Pharmaceutical Sciences*. 2017 Jan 5;23(1):12–7.
128. Niazvand F, Khorsandi L, Abbaspour M, Orazizadeh M, Varaa N, Maghzi M, et al. Curcumin-loaded poly lactic-co-glycolic acid nanoparticles effects on mono-iodoacetate-induced osteoarthritis in rats. *Veterinary Research Forum*. 2017 Jun 15;8(2):155–61.

129. Kobayashi K, Imaizumi R, Sumichika H, Tanaka H, Goda M, Fukunari A, et al. Sodium iodoacetate-induced experimental osteoarthritis and associated pain model in rats. *Journal of Veterinary Medical Science*. 2003;65(11):1195–9.
130. Schmitz N, Lavery S, Kraus VB, Aigner T. Basic methods in histopathology of joint tissues. *Osteoarthritis Cartilage*. 2010 Oct 1; 18:113–6.
131. Paun J, Soniwala MM, Parekh KK, Paun JS, Soniwala MM. Formulation and evaluation of nanosuspension to improve solubility and dissolution of diacerein. *International Journal of Pharmaceutical Sciences and Research*. 2017;8(4):1643–53.
132. Sipos E, Kósa N, Kzsoki A, Szabó ZI, Zelkó R. Formulation and characterization of aceclofenac-loaded nanofiber based orally dissolving webs. *Pharmaceutics*. 2019 Aug 17;11(8):417.
133. Patel MH, Sawant KK. Self microemulsifying drug delivery system of lurasidone hydrochloride for enhanced oral bioavailability by lymphatic targeting: In vitro, Caco-2 cell line and in vivo evaluation. *European Journal of Pharmaceutical Sciences*. 2019 Oct 1; 138:105027.
134. Alyoussef A, El-Gogary RI, Ahmed RF, Ahmed Farid OA, Bakeer RM, Nasr M. The beneficial activity of curcumin and resveratrol loaded in nanoemulgel for healing of burn-induced wounds. *Journal of Drug Delivery Science and Technology*. 2021 Apr 1; 62:102360.
135. Samson S, Basri M, Fard Masoumi HR, Abedi Karjiban R, Abdul Malek E. Design and development of a nanoemulsion system containing copper peptide by D-optimal mixture design and evaluation of its physicochemical properties. *RSC Adv*. 2016;6(22):17845–56.

136. Kong WY, Salim N, Masoumi HRF, Basri M, da Costa SS, Ahmad N. Optimization of hydrocortisone-loaded nanoemulsion formulation using D-optimal mixture design. *Asian Journal of Chemistry*. 2018;30(4):853–8.
137. Sharma A, Singh AP, Harikumar SL. Development and optimization of nanoemulsion based gel for enhanced transdermal delivery of nitrendipine using box-behnken statistical design. *Drug Development and Industrial Pharmacy*. 2020 Feb 1;46(2):329–42.
138. Hinz B, Auge D, Rau T, Rietbrock S, Brune K, Werner U. Simultaneous determination of aceclofenac and three of its metabolites in human plasma by high-performance liquid chromatography. *Biomedical Chromatography*. 2003;17(4):268–75.
139. Musmade P, Subramanian G, Srinivasan KK. High-performance liquid chromatography and pharmacokinetics of aceclofenac in rats. *Analytica Chimica Acta*. 2007 Feb 28;585(1):103–9.
140. Zhu C, Zheng Z, Chen Z, Liang X. Determination of rhein in plasma of rat by HPLC/MS. *Journal of Chinese Medicinal Materials*. 2002 Sep;25(9):646–9.
141. Tomar S, Chakraborti S, Jindal A, Grewal MK, Chadha R. Cocrystals of diacerein: Towards the development of improved biopharmaceutical parameters. *International Journal of Pharmaceutics*. 2020 Jan; 574:118942.
142. Suganthi A, Ravi TK. Stability indicating HPLC method for simultaneous determination of diacerein and aceclofenac. *Research Journal of Pharmacy and Technology*. 2010;3(2):600–3.
143. Gandhi SP, Dewani MG, Borole TC, Damle MC. Development and validation of stability indicating HPLC method for determination of diacerein and aceclofenac as bulk drugs and in tablet dosage form. *International Journal of Research in Pharmacy and Chemistry*. 2011;1(4):799–806.

144. Sharma MC, Sharma S. Stability and reverse phase HPLC assay method for determination of diacerein and aceclofenac in tablet dosage form - application to dissolution studies. *Asian Journal of Pharmaceutical and Clinical Research*. 2011;4(SUPPL. 1):31–4.
145. Kumar RS, Srisutherson N, Solomon WDSAM, Nallasivan PK, Venkatnarayanan R. Validated RP-HPLC method for the simultaneous estimation of aceclofenac and diacerein in bulk and formulation. *International Journal of PharmTech Research*. 2010;2(1):940–4.
146. Pol AS, Parel PA, Hegde D. Peppermint oil-based drug delivery system of aceclofenac with improved anti-inflammatory activity and reduced ulcerogenecity. *International Journal of Pharma Bioscience and Technology*. 2013;1(2):89–101.
147. Pawbake GR, Shirolkar S V. Formulation and evaluation of diacerein microemulgel topical drug delivery. *International Journal of Pharmaceutical Research*. 2020 Oct 1;12(4):422–37.
148. Bayanati M, Khosroshahi AG, Alvandi M, Mahboobian MM. Fabrication of a thermosensitive in situ gel nanoemulsion for nose to brain delivery of temozolomide. *Journal of Nanomaterials*. 2021;2021(1):1–11.
149. Thakkar HP, Khunt A, Dhande RD, Patel AA. Formulation and evaluation of Itraconazole nanoemulsion for enhanced oral bioavailability. *Journal of Microencapsulation*. 2015 Aug 18;32(6):559–69.
150. Nasiri MI, Vora LK, Ershaid JA, Peng K, Tekko IA, Donnelly RF. Nanoemulsion-based dissolving microneedle arrays for enhanced intradermal and transdermal delivery. *Drug Delivery and Translational Research*. 2022 Apr 1;12(4):881–96.

151. Gupta SK. Formulation and evaluation of nanoemulsion based nanoemulgel of aceclofenac. *Journal of Pharmaceutical Sciences and Research*. 2020 Apr 1;12(4):524–32.
152. Modi JD, Patel JK. Nanoemulsion-based gel formulation of aceclofenac for topical delivery. *International Journal of Pharmacy and Pharmaceutical Science Research*. 2011;1(1):6–12.
153. Jianxian C, Saleem K, Ijaz M, Ur-Rehman M, Murtaza G, Asim MH. Development and in vitro evaluation of gastro-protective aceclofenac-loaded self-emulsifying drug delivery system. *International Journal of Nanomedicine*. 2020 Jul 23; 15:5217–26.
154. Narang AS, Delmarre D, Gao D. Stable drug encapsulation in micelles and microemulsions. *International Journal of Pharmaceutics*. 2007 Dec 10;345(1-2):9-25.
155. Saini JK, Nautiyal U, Kumar M S, Singh D, Anwar F. Microemulsions: A potential novel drug delivery system. *International Journal of Pharmaceutical and Medical Research*. 2014 Jan;2(1):15–20.
156. Ammar HO, Salama HA, Ghorab M, Mahmoud AA. Nanoemulsion as a potential ophthalmic delivery system for dorzolamide hydrochloride. *AAPS PharmSciTech*. 2009 Sep;10(3):808–19.
157. Poudel BK, Marasini N, Tran TH, Choi HG, Yong CS, Kim JO. Formulation, characterization and optimization of valsartan self-microemulsifying drug delivery system using statistical design of experiment. *Chemical and Pharmaceutical Bulletin*. 2012 Nov 1;60(11):1409-18.
158. Wei Y, Ye X, Shang X, Peng X, Bao Q, Liu M, et al. Enhanced oral bioavailability of silybin by a supersaturatable self-emulsifying drug delivery

- system (S-SEDDS). *Colloids and Surfaces A: Physicochemical and Engineering Aspects*. 2012 Feb 20; 396:22–8.
159. Barot BS, Parejiya PB, Patel HK, Gohel MC, Shelat PK. Microemulsion-based gel of terbinafine for the treatment of onychomycosis: Optimization of formulation using D-optimal design. *AAPS PharmSciTech*. 2012 Mar;13(1):184–92.
160. Uluata S, Decker EA, McClements DJ. Optimization of Nanoemulsion Fabrication Using Microfluidization: Role of Surfactant Concentration on Formation and Stability. *Food Biophysics*. 2016 Mar 1;11(1):52–9.
161. Singh Y, Meher JG, Raval K, Khan FA, Chaurasia M, Jain NK, et al. Nanoemulsion: Concepts, development and applications in drug delivery. *Journal of Controlled Release*. 2017 Apr 28; 252:28-49.
162. Preeti, Sambhakar S, Malik R, Bhatia S, Al Harrasi A, Rani C, et al. Nanoemulsion: An emerging novel technology for improving the bioavailability of drugs. *Scientifica*. 2023;2023(1):1–25.
163. Prathvi S, Sandeep D, Jose J. Formulation and evaluation of luliconazole nanoemulgel using box-behnken design approach. *International Journal of Applied Pharmaceutics*. 2024 Nov 1;16(6):211–23.
164. Srivastava M, Kohli K, Ali M. Formulation development of novel in situ nanoemulgel (NEG) of ketoprofen for the treatment of periodontitis. *Drug Delivery*. 2016 Jan 2;23(1):154–66.
165. Kohli AK, Alpar HO. Potential use of nanoparticles for transcutaneous vaccine delivery: Effect of particle size and charge. *International Journal of Pharmaceutics*. 2004 May 4;275(1–2):13–7.

166. Rachmawati H, Budiputra DK, Mauludin R. Curcumin nanoemulsion for transdermal application: Formulation and evaluation. *Drug Development and Industrial Pharmacy*. 2015 Apr 1;41(4):560–6.
167. Marzuki NHC, Wahab RA, Hamid MA. An overview of nanoemulsion: Concepts of development and cosmeceutical applications. *Biotechnology and Biotechnological Equipment*. 2019 Jan 1;33(1):779–97.
168. Gurpreet K, Singh S. Review of nanoemulsion formulation and characterization techniques. *Indian Journal of Pharmaceutical Sciences*. 2018 Sep;80(5):781–9.
169. Dudhipala N, Phasha Mohammed R, Adel Ali Youssef A, Banala N. Effect of lipid and edge activator concentration on development of aceclofenac-loaded transfersomes gel for transdermal application: in vitro and ex vivo skin permeation. *Drug development and industrial pharmacy*. 2020 Aug 2;46(8):1334–44.
170. Al-Kassas R, Wen J, Cheng AEM, Kim AMJ, Liu SSM, Yu J. Transdermal delivery of propranolol hydrochloride through chitosan nanoparticles dispersed in mucoadhesive gel. *Carbohydrate Polymers*. 2016 Nov 20; 153:176–86.
171. Kaur A, Katiyar SS, Kushwah V, Jain S. Nanoemulsion loaded gel for topical co-delivery of clobetasol propionate and calcipotriol in psoriasis. *Nanomedicine*. 2017 May 1;13(4):1473–82.
172. Ranjan A, Mehta P, Namdeo P. Formulation and evaluation of niosomal gel loaded with boswellic acid. *European Chemical Bulletin*. 2023 Aug;12(11):431–50.

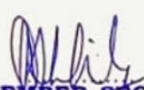
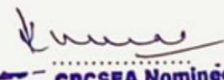
173. Jing J, Shan Y, Wang N, Sun J, Jiang C, Cao L, et al. The influencing factors and mechanism of anionic and zwitterionic surfactant on viscosity reduction in heavy o/w emulsions. *ACS Omega*. 2024 Sep;9(37):39259–76.
174. Cao HT, Song J, Fang XL. Effect of dimethyl sulfoxide (DMSO) on cellulose solution with high concentration. *Advanced Materials Research*. 2015 Mar 20; 1095:329–32.
175. Elmataeeshy ME, Sokar MS, Bahey-El-Din M, Shaker DS. Enhanced transdermal permeability of terbinafine through novel nanoemulgel formulation; Development, in vitro and in vivo characterization. *Future Journal of Pharmaceutical Sciences*. 2018 Jun;4(1):18–28.
176. Mali KK, Dhawale SC, Dias RJ. Microemulsion based bioadhesive gel of itraconazole using tamarind gum: In-vitro and ex-vivo evaluation. *Marmara Pharmaceutical Journal*. 2017 Jun 24;21(3):688–700.
177. Ullah N, Amin A, Farid A, Selim S, Rashid SA, Aziz MI, et al. Development and evaluation of essential oil-based nanoemulgel formulation for the treatment of oral bacterial infections. *Gels*. 2023 Mar 21;9(3):252.
178. Algahtani MS, Ahmad MZ, Ahmad J. Nanoemulsion loaded polymeric hydrogel for topical delivery of curcumin in psoriasis. *Journal of Drug Delivery Science and Technology*. 2020 Oct 1; 59:101847.
179. Jan Y, Al-Keridis LA, Malik M, Haq A, Ahmad S, Kaur J, et al. Preparation, modelling, characterization and release profile of vitamin D3 nanoemulsion. *LWT*. 2022 Nov 1; 169:113980.
180. El-Zahaby SA, AbouGhaly MHH, Abdelbary GA, El-Gazayerly ON. Zero-order release and bioavailability enhancement of poorly water soluble vinpocetine from self-nanoemulsifying osmotic pump tablet. *Pharmaceutical Development and Technology*. 2018 Oct 21;23(9):900–10.

181. Prasanthi D, Lakshmi PK. Effect of chemical enhancers in transdermal permeation of alfuzosin hydrochloride. *ISRN Pharmaceutics*. 2012;2012(1):1–8.
182. Mao Y, Chen X, Xu B, Shen Y, Ye Z, Chaurasiya B, et al. Eprinomectin nanoemulgel for transdermal delivery against endoparasites and ectoparasites: preparation, in vitro and in vivo evaluation. *Drug Delivery*. 2019 Jan 1;26(1):1104–14.
183. Mohamed EM, Dharani S, Khuroo T, Hamed R, Khan MA, Rahman Z. In vitro and in vivo characterization of the transdermal gel formulation of desloratadine for prevention of obesity and metabolic syndrome. *Pharmaceutics*. 2023 Apr;16(4):578.
184. Abdallah MH, Lila AAS, Unissa R, Elsewedy HS, Elghamry HA, Soliman MS. Preparation, characterization and evaluation of anti-inflammatory and anti-nociceptive effects of brucine-loaded nanoemulgel. *Colloids and Surfaces B: Biointerfaces*. 2021 Sep 1; 205:111868.
185. Guzman RE, Evans MG, Bove S, Morenko B, Kilgore K. Mono-iodoacetate-induced histologic changes in subchondral bone and articular cartilage of rat femorotibial joints: An animal model of osteoarthritis. *Toxicologic Pathology*. 2003 Oct;31(6):619–24.
186. Schuelert N, McDougall JJ. Grading of monosodium iodoacetate-induced osteoarthritis reveals a concentration-dependent sensitization of nociceptors in the knee joint of the rat. *Neuroscience Letters*. 2009 Nov 13;465(2):184–8.
187. Bajaj S, Whiteman A, Brandner B. Transdermal drug delivery in pain management. *Continuing Education in Anaesthesia, Critical Care and Pain*. 2011 Apr;11(2):39–43.

188. Margetts L, Sawyer R. Transdermal drug delivery: Principles and opioid therapy. *Continuing Education in Anaesthesia, Critical Care and Pain*. 2007 Oct;7(5):171–6.
189. Cai B, Soderkvist K, Engqvist H, Bredenberg S. A new drug release method in early development of transdermal drug delivery systems. *Pain research and treatment*. 2012;2012(1):953140.

9. ANNEXURES

A. ANIMAL ETHICAL CLEARANCE LETTERS

	KLE College of Pharmacy A Constituent Unit of KLE Academy of Higher Education and Research (Deemed to be University) DEPARTMENT OF PHARMACOLOGY JNMC Campus, Nehru Nagar, Belagavi - 590 010, Karnataka, India		
<u>INSTITUTIONAL ANIMAL ETHICS COMMITTEE</u> Reg.No.221/Po/Re/S/2000/CPCSEA			
CERTIFICATE		Date: 17/03/2021	
This is to certify that the project proposal no ...15... entitled, "Development and evaluation of Diacerein and Aceclofenac loaded topical nanoemulgel for Osteoarthritis: An experimental study" submitted by Dr./ Mr. / Ms., Taufik Mubarak Kazi under the guidance of Prof. (Dr.) P.M.Dandagi has been approved/recommended by the IAEC of KLE College of Pharmacy, Belagavi, Reg.No.221/Po/Re/S/2000/CPCSEA in its meeting dated 13/03/2021, resolution No. 30 has been sanctioned 36..... Rats/ Mice/ Rabbits/Guinea pig (animals) sex <u>male</u> under this proposal for a duration of next.....months.			
You are hereby informed to strictly adhere to the protocol submitted for approval. Further you are required to keep the account of animals used for the project in specified Performa, Form D.			
Authorized by	Name	Signature	Date
Member Secretary:	<u>Dr. N.A. Khatib</u>	 MEMBER SECRETARY Institutional Animal Ethical Committee, KLE's College of Pharmacy, BELGAUM - 590010	<u>17/03/2021</u>
Main Nominee of			
CPCSEA:	<u>Dr. Vinod Kumar C.S.</u>	 CPCSEA Nominee Institutional Animal Ethics Committee KLE's College of Pharmacy, BELGAUM.	<u>17-3-2021</u>



KLE College of Pharmacy
A Constituent Unit of
KLE Academy of Higher Education and Research
(Deemed to be University)
DEPARTMENT OF PHARMACOLOGY
JNMC Campus, Nehru Nagar, Belagavi - 590 010, Karnataka, India



INSTITUTIONAL ANIMAL ETHICS COMMITTEE
Reg.No.221/Po/Re/S/2000/CPCSEA

Date: 21/01/2022

CERTIFICATE

This is to certify that the project proposal no ¹²... entitled, "Development and evaluation of Diacerein and Aceclofenac loaded topical nanogel for osteoarthritis: An experimental study", submitted by Mr. / Ms. Taufik M. Kazi under the guidance of Dr. P.M. Dandagi has been approved/recommended by the IAEC of KLE College of Pharmacy, Belagavi, Reg.No.221/Po/Re/S/2000/CPCSEA in its meeting dated 21/1/2022, resolution No. 32 has been sanctioned ⁴⁸..... Rats/ ~~Mice~~/ ~~Rabbits~~/Guinea pig (animals) sex ^{male}..... under this proposal for a duration of next...⁰.....months.

You are hereby informed to strictly adhere to the protocol submitted for approval. Further you are required to keep the account of animals used for the project in specified Performa, Form D.

Authorized by Name Signature Date

Member Secretary: Dr. N. A. Khatib  22/01/2022
MEMBER SECRETARY
Institutional Animal Ethics Committee
KLE's College of Pharmacy
BELAGAVI

Main Nominee of
CPCSEA: Dr. Vinod Kumar CS  22/01/2022
CPCSEA Nominee
Institutional Animal Ethics Committee
KLE's College of Pharmacy,
BELAGAVI

B. PUBLICATIONS AND CERTIFICATES

i. List of publications

Sr. No.	Title	Journal Name / Patent details	Publisher	Indexing
1	A New Stability-Indicating RP-HPLC Method for Simultaneous Quantification of Diacerein and Aceclofenac in Novel Nanoemulgel Formulation and Commercial Tablet Dosage Form in the Presence of their Major Degradation Products Using DoE Approach.	Journal of Chromatographic Science	Oxford University Press	Scopus Q3 IF 1.6
2	A Doe-Based Fast and Synchronous Quantification of Rhein, Aceclofenac, and Diclofenac by RP HPLC-DAD Method in Spiked Wistar Rat Plasma: Application to Pharmacokinetic Studies.	Journal of Liquid Chromatography & Related Technologies	Taylor and Francis	Scopus Q3 IF 1.4
3	A Novel Nanoemulgel for the Enhanced Transdermal Co-Delivery of Diacerein and Aceclofenac.	Indian Patent Office; Application No.202541004416 A; Published date: 31/01/2025.		

(The front page of each article has been attached)

ii. List of poster presentations

Sr No.	Event Type	Event Details	Prizes/Awards received
1	National Conference (E-poster presentation)	National Conference on “Recent Advancement and Challenges in Analytical Chemistry” organized by the Department of Pharmaceutical Chemistry, JSS College of Pharmacy, Mysuru, Karnataka, on 20 th May 2022.	First Prize
2	National Pharmacy Week (E-poster presentation)	“National Pharmacy Week and World Antimicrobial Awareness Week” organized by the Department of Pharmaceutical Chemistry & Pharmacognosy, Sri Padmavathi School of Pharmacy, Tiruchanoor, in Association with Indian Pharmaceutical Association, (IPA) Tirupati Branch, Andhra Pradesh on 18th Nov. 2022.	First Prize

(Certificates are attached)

A New Stability-Indicating RP-HPLC Method for Simultaneous Quantification of Diacerein and Aceclofenac in Novel Nanoemulgel Formulation and Commercial Tablet Dosage Form in the Presence of their Major Degradation Products Using DoE Approach

Taufik M. Kazi^{1,2,3} and Panchaxari M. Dandagi^{1,*}

¹Department of Pharmaceutics, KLE College of Pharmacy, KLE Academy of Higher Education and Research, Nehru Nagar, Belagavi 590010, Karnataka, India

²Dr. Prabhakar Kore Basic Science Research Center, KLE Academy of Higher Education and Research, Nehru Nagar, Belagavi 590010, Karnataka, India

³Department of Pharmacy, Government Polytechnic Jalgaon, NH-06, Jalgaon 425001, Maharashtra, India

*Author to whom correspondence should be addressed. Email: pmdandagi@yahoo.com

The present study aimed to develop a simple, robust, sensitive and effective stability-indicating reversed-phase high-performance liquid chromatography method for simultaneous quantification of diacerein (DCN) and aceclofenac (ACE) in novel nanoemulgel formulation and commercial tablets in the presence of their main degradation product: rhein (RH) and diclofenac sodium (DLS), respectively. A fractional factorial design was used to screen the crucial independent factors, whereas a central composite design was used for the optimization of the chromatographic conditions. The separation was carried out on Phenomenex C18 column (5 μ m, 250 $\times$ 4.6 mm), using a mobile phase consisting of phosphate buffer pH 3 (0.1% v/v orthophosphoric acid) and acetonitrile (40:60 v/v) at a flow rate of 1 mL/min with detection at 264 nm. The analytes were exposed to a variety of stress conditions, including heat, alkali, acid, oxidation, photochemical, humidity and hydrolysis. DCN, ACE, RH and DLS were found to have retention times of 4.32 ± 0.15 , 5.77 ± 0.07 , 8.28 ± 0.20 and 9.10 ± 0.18 min, respectively. The percent recovery for all four analytes was found to be between 98 and 102, and the procedure was discovered to be linear in the range of 0.1–64 μ g/mL with R^2 value > 0.999. The established method was validated as per ICH guidelines and successfully used to assay DCN and ACE in their combined marketed tablet dosage form and developed nanoemulgel formulation.

Introduction

Diacerein (DCN) is an anthraquinone derivative commonly used as a disease-modifying osteoarthritis (OA) drug and besides, possesses anti-inflammatory activity. DCN; 4,5-diacetoxy-9,10-dihydro-9,10-dioxo-2-anthracene-carboxylic acid (Figure 1a) is a prodrug of rhein (RH) and exhibits its disease-modifying OA effect by inhibiting the synthesis of interleukin-1 β , superoxide and other cartilage degrading enzymes expressed in OA (1, 2). Fixed-dose combination (FDC) tablets containing DCN 50 mg and aceclofenac (ACE) 100 mg are available in the market for rheumatoid arthritis (RA) and OA treatment. ACE, 2-[(2, 6-dichlorophenyl) amino] phenylacetoxy acetic acid (Figure 1b), is a preferential cyclooxygenase-2 enzyme inhibitor and most commonly prescribed NSAID to relieve the pain associated with RA and OA (3). Unfortunately, DCN and ACE are Biopharmaceutics classification system Class II drugs with low oral bioavailability (BA) and need to be administered frequently because of their short half-life. In addition, after chronic oral administration, DCN and ACE show severe undesirable side effect (4, 5). Besides this, DCN is very sensitive to alkaline degradation. Its principal alkaline degradation product and the main active metabolite is RH (6). RH has low oral BA compared with DCN (7) and responsible for the laxative and severe diarrheal effect of DCN (2, 5). In the case of

ACE, diclofenac (DL) is the principal degradation product (8). DL was found to be less effective and produces more GI adverse effects than ACE (9, 10). Poor BA, frequent dosing and oral side effects collectively cause patient noncompliance and poor adherence to ACE and DCN oral regimens. Therefore, the FDC of DCN- and ACE-loaded nanoemulgel as the transdermal delivery system is formulated with the aim of improving its effectiveness and avoiding its oral side effects. Hence, it is utmost essential to develop a simple, robust and effective RP-HPLC method for the evaluation of fabricated nanoemulgel formulation. Also, it is necessary to detect the presence of RH and DL in the commercial tablet dosage form to avoid patient noncompliance because of adverse side effects.

As per the literature review, several analytical methods have been reported for the determination of DCN and ACE as a single component in pharmaceutical formulation and biological fluids (11–22) and in combination with other drugs using ultra-high-performance liquid chromatography (UHPLC) (23) and HPLC (24, 25) methods.

For simultaneous quantification of DCN and ACE, several analytical methods have been published, including spectrophotometric (26–28), high-performance thin-layer liquid chromatography (29, 30), HPLC-photo diode array (PDA) (31–34), HPLC-Ultraviolet-Visible (UV-Vis)

Received: February 26, 2022. Revised: January 12, 2023. Editorial decision: April 24, 2023

© The Author(s) 2023. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com

A DoE-based fast and synchronous quantification of rhein, aceclofenac, and diclofenac by RP-HPLC-DAD method in spiked Wistar rat plasma: application to pharmacokinetic studies

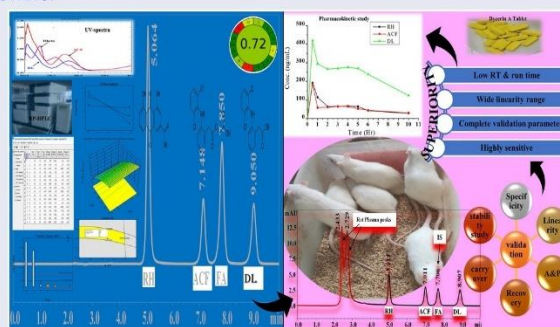
Taufik M. Kazi^{a,b,c} , Panchaxari M. Dandagi^a , and Prakash R. Biradar^a 

^aKLE College of Pharmacy, KLE Academy of Higher Education and Research, Belagavi, Karnataka, India; ^bDr. Prabhakar Kore Basic Science Research Center (BSRC), KLE Academy of Higher Education and Research, Belagavi, Karnataka, India; ^cGovernment Polytechnic Jalgaon, Jalgaon, Maharashtra, India

ABSTRACT

A fixed-dose combination of diacerein and aceclofenac (ACF) is commonly used in the treatment of osteoarthritis for their disease-modifying and analgesic effects, respectively. We introduce a simple, fast, sensitive, and green HPLC method for synchronous quantification of rhein (RH) (a primary metabolite of diacerein), ACF, and diclofenac (DL) (an active metabolite of ACF) in rat plasma using fenofibric acid as an internal standard (IS). The chromatographic conditions were optimized using Fractional Factorial and Box-Behnken design. Drugs were extracted by acetonitrile-methanol-based simple protein precipitation technique. The separation was performed on C₁₈ column (Phenomenex, 250 × 4.6 mm), using acetonitrile: phosphate buffer pH 3.5 (60:40, v/v) at a 1 mL/min flow rate in a 10 min run time and detected at 264 nm. As per the USFDA guidelines, the established method was validated. RH, ACF, and DL showed good linearity in the 25–3000, 50–6000, and 50–6000 ng/mL concentration ranges respectively. The precision and accuracy results were found to be well within the acceptable range, with average recovery ranging between 92.27 and 96.10%. The developed method was found to be stable and successfully used to determine the pharmacokinetic parameters in Wistar rats after oral administration of Dycerin A tablet.

GRAPHICAL ABSTRACT



KEYWORDS

Box-Behnken design; Dycerin A tablet; Green assessment; protein-precipitation; USFDA guidelines

Introduction

Diacerein; 4,5-diacetoxy-9, 10- anthraquinone-2-carboxylic acid, is a semisynthetic anthraquinone derivative widely used in the treatment of osteoarthritis (OA).^[1] Diacerein is a prodrug that is completely metabolized to its primary metabolite, RH before reaching systemic circulation.^[2] RH; ((4, 5-dihydroxy-9, 10-dihydro-9, 10-dioxo-anthracene-2-carboxylic acid); Figure 1a), is also a natural anthraquinone obtained from senna leaves and rheum species. The kidneys mostly eliminate RH as sulfate conjugate (20%), glucuronide conjugate (60%), and an unchanged drug (20%).^[3,4] ACF is

a preferential cyclooxygenase-2 enzyme inhibitor and the most prescribed non-steroidal anti-inflammatory drug (NSAID) for rheumatoid arthritis (RA) and OA pain.^[5] ACF, 2- [(2, 6-dichlorophenyl) amino] phenylacetoxy acetic acid (Figure 1b) is the congener of DL (2-[(2,6-dichlorophenyl) amino] benzene acetic acid; Figure 1c) with improved gastrointestinal tolerance than other NSAIDs. After oral administration, ACF is well absorbed as an unchanged drug. The urine excretes about two-thirds of the orally administered dose, mostly as glucuronidated and hydroxylated forms of ACF and DL.^[6] Diacerein and RH show an anti-cartilage effect by inhibiting the synthesis of

CONTACT Taufik M. Kazi  taufik.kazi@gmail.com  KLE College of Pharmacy, KLE Academy of Higher Education and Research, Belagavi, Karnataka, India.

 Supplemental data for this article can be accessed online at <https://doi.org/10.1080/10826076.2023.2202231>.

© 2023 Taylor & Francis Group, LLC

(12) PATENT APPLICATION PUBLICATION

(21) Application No.202541004416 A

(19) INDIA

(22) Date of filing of Application :20/01/2025

(43) Publication Date : 31/01/2025

(54) Title of the invention : A NOVEL NANOEMULGEL FOR THE ENHANCED TRANSDERMAL CO-DELIVERY OF DIACEREIN AND ACECLOFENAC

(51) International classification :A61K0009000000, A61K0031216000, A61K0009107000, A61K0009060000, A61K0047100000

(86) International Application No :NA
Filing Date :NA

(87) International Publication No : NA

(61) Patent of Addition to Application Number :NA
Filing Date :NA

(62) Divisional to Application Number :NA
Filing Date :NA

(71)Name of Applicant :

1)MR. TAUFIK MUBARAK KAZI

Address of Applicant :Ph. D. Research Scholar, KLE College of Pharmacy, Belagavi, Dr. Prabhakar Kore Basic Science Research Centre (BSRC), KLE Academy of Higher Education and Research, JNMC Campus, Nehru Nagar, Belagavi-590010, Karnataka, India.; Government Polytechnic Jalgaon, Department of Pharmacy, NH-06, zilha peth, Jalgaon 425001, Maharashtra, India. -----

2)DR. PANCHAXARI M. DANDAGI**3)MR. PRAKASH R. BIRADAR****4)DR. BHAGYASHRI R. HUNGUND**

Name of Applicant : NA

Address of Applicant : NA

(72)Name of Inventor :

1)MR. TAUFIK MUBARAK KAZI

Address of Applicant :Ph. D. Research Scholar, KLE College of Pharmacy, Belagavi, Dr. Prabhakar Kore Basic Science Research Centre (BSRC), KLE Academy of Higher Education and Research, JNMC Campus, Nehru Nagar, Belagavi-590010, Karnataka, India.; Government Polytechnic Jalgaon, Department of Pharmacy, NH-06, zilha peth, Jalgaon 425001, Maharashtra, India. -----

2)DR. PANCHAXARI M. DANDAGI

Address of Applicant :Professor & Head, Department of Pharmaceutics, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, JNMC Campus, Nehru Nagar, Belagavi-590010, Karnataka, India. -----

3)MR. PRAKASH R. BIRADAR

Address of Applicant :Associate Professor, Department of Pharmacology and Toxicology, KLE College of Pharmacy, Belagavi, KLE Academy of Higher Education and Research, JNMC Campus, Nehru Nagar, Belagavi-590010, Karnataka, India. -----

4)DR. BHAGYASHRI R. HUNGUND

Address of Applicant :Professor, Department of Pathology, KLE Academy of Higher Education and Research, JNMC Campus, Nehru Nagar, Belagavi-590010, Karnataka, India -----

(57) Abstract :

The present invention relates to a novel nanoemulgel system for the co-delivery of diacerein (DCN) and aceclofenac (ACE) via the transdermal route. The formulation leverages a nanoemulsion (NE) optimized using a 3-factor, 2-level D-optimal mixture design to enhance drug bioavailability and minimize systemic side effects. The optimized NE, featuring a well-balanced oil mix, surfactants, and water content, ensures high drug loading and stability. The NE is then transformed into a nanoemulgel (NEG) by incorporating HPMC K15M as a gelling agent, with or without dimethyl sulfoxide (DMSO) as a penetration enhancer. Characterization studies demonstrate superior drug release, nano-sized globules (<20 nm), and excellent physical stability under various stress conditions. The formulation offers sustained drug release, improved skin permeation, and minimal side effects, making it ideal for the effective and localized treatment of inflammatory conditions like arthritis.

No. of Pages : 28 No. of Claims : 4

