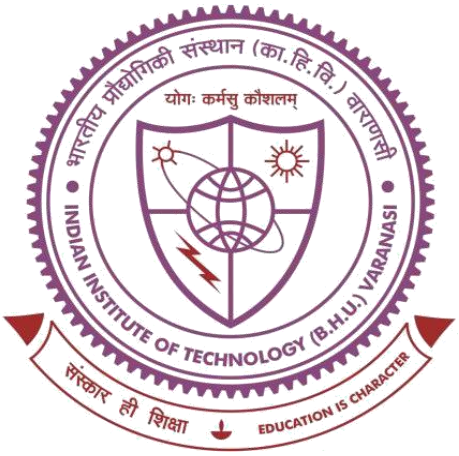
# DEVELOPMENT AND CHARACTERIZATION OF NOVEL PHYTOFORMULATIONS FOR MELANOMA THERAPY




**Thesis submitted in partial fulfillment for the Award of Degree**

## Doctor of Philosophy

**By**

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## CERTIFICATE

It is certified that the work contained in the thesis titled ***"Development and characterization of novel phytoformulations for melanoma therapy"*** by ***Mr. Debadatta Mohapatra*** has been carried out under my supervision and that this work has not been submitted elsewhere for a degree.

It is further certified that the student has fulfilled all the requirements of Comprehensive Examination, Candidacy and SOTA for the award of Ph.D. degree.

20.10.2023

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## DECLARATION BY THE CANDIDATE

I, ***"Debadatta Mohapatra"***, certify that the work embodied in this thesis is my own bonafide work and carried out by me under the supervision of ***"Dr. Alakh N Sahu"*** from ***"July 2019" to "October 2023",*** at the ***"Department of Pharmaceutical Engineering & Technology"***, Indian Institute of Technology (BHU), Varanasi. The matter embodied in this thesis has not been submitted for the award of any other degree/diploma. I declare that I have faithfully acknowledged and given credits to the research workers wherever their works have been cited in my work in this thesis. I further declare that I have not willfully copied any other's work, paragraphs, text, data, results, etc., reported in journals, books, magazines, reports dissertations, theses, *etc*., or available at websites and have not included them in this thesis and have not cited as my own work.

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# ACKNOWLEDGEMENT

*With humility, misty eyes, and folded hands, I sincerely acknowledge my gratitude to the Almighty for showering his blessings upon me. I would like to thank the God of wisdom, knowledge, and prosperity "**Lord Ganesha Ji**," Deva Deva Mahadeva "**Kashi Vishwanath Ji**," Lord of the Universe "**Lord Jagannath Ji**" for showering their continuous blessings. The Almighty constantly infused me with the strength and concentration to embark on and accomplish this work successfully. This research work would not be possible without their benediction and grace. I pray that this hidden force always blesses me with the strength, courage, wisdom, and willpower to face all the challenges of life.*

*I am grateful to all those guides of righteous path from whom the sources of actuality are alive even today. Gratitude is the fundamental value for progression towards awakening. Gratitude itself is the foundation and conserving value for culture and civilization.*

*One who is not grateful cannot produce evidence of bearing culture and civilization. One who does not bear culture and civilization cannot follow norms and orderliness.*

*Culture, Civilization, Norms, and Orderliness are mutually complementary. Without these, it is not possible to determine the Undivided society and Social harmony. Therefore –without gratitude, honour is not; without honour simplicity is not; without simplicity, coexistence is not; without coexistence, continuity of gratitude is not.*

*The human being who lives with gratitude, only his conduct is educative and inspiring for future generations. This is achievable only in the purview of Humanness. Gratitude is for all those who have been helpful, in whichever way, towards Consciousness Development – Value Education.*

*My first and foremost heartfelt gratitude and indebtedness would be towards "**Bharat Ratna Mahamana Pandit Madan Mohan Malviya Ji,"** the founder of Banaras Hindu University, for establishing a magnificent temple of learning. I am extremely grateful to "**Prof. Mahadeva Lal Schroff,**" the father of Indian pharmacy education, for his great contributions to the field of pharmacy towards the introduction of Bachelor's and Master Degree courses in the Discipline of Pharmaceutical Sciences in India.*

*I would like to express a deep sense of gratitude, appreciation, and indebtedness to my honourable supervisor* ***Dr. Alakh N. Sahu,*** *for providing his continuous support, motivation, and immense knowledge towards the conceptualization, experiment designing, project investigation, supervision, data interpretation, expert assistance, editing, drafting, and revision functions. His professional acumen, plausible and erudite thinking, and perfect counseling helped me in achieving my goal. His meticulous ways, utmost attention to all problems, continuous involvement, precious guidance, valued suggestions, and tireless efforts made this immense task come to a conclusion successfully. I am also thankful to him for providing the necessary facilities to carry out this research work. I feel fortunate to have been associated with and worked under such an accomplished personality.*

*I would also like to thank my Research Progress Evaluation Committee (RPEC) members,* ***Prof. Abha Mishra*** *(Professor, School of Biochemical Engineering, IIT (BHU), Varanasi) and* ***Dr. Ashish Kumar Agrawal*** *(Associate Professor, Department of Pharmaceutical Engineering and Technology, IIT (BHU), Varanasi) for their valuable guidance, supervision, expert assistance, scientific inputs, and suggestions throughout the experiments.*

*I express my gratitude to* ***Prof. Siva Hemalatha****, Head, Department of Pharmaceutical Engineering & Technology, I.I.T. (B.H.U.), Varanasi, for providing the necessary facilities and academic guidance during my Ph.D. work. I am immensely thankful to all former Head of the Department,* ***Prof. Brahmeshwar Mishra****,* ***Prof. Sushil Kumar Singh****,* ***Prof. Sanjay Singh****, and* ***Prof. Sushant Kumar Shrivastava,*** *for their cooperation during my research work.*

*I express my gratitude to all the faculty members and scientific officer of the Department of Pharmaceutical Engineering & Technology:* ***Prof. Sairam Krishnamurthy, Prof. Senthil Raja A., Dr. Ruchi Chawla, Prof. M. S. Muthu, Dr. Sunil K Mishra, Dr. Prasanta K. Nayak, Dr. Gyan Prakash Modi, Dr. Vinod Tiwari, Dr. Ashish K Agrawal, Dr. Rajnish, Dr. Deepak Kumar, Dr. Dinesh Kumar****,* ***Dr. Jairam Meena, Dr. Ashok Kumar, and Dr. Arun Khatri*** *for their co-operation, and scientific suggestions during my research work. My access to their Laboratory for various works during my Ph.D. works is highly acknowledged. Without their precious support, it would not have been possible to execute this research work.*

*The efforts of the non-teaching office staff* ***Mr. Yashwant Singh****,* ***Mr. Atul Kumar Gupta****,* ***Mr. Anand Kumar, Mr. Bipin Kumar Pandey, Mr. Surya Pratap Singh, Mr. Kapil Dev Rai, Smt. Archana Singh, Mr. Madan Lal, Mr. Virendra Kumar, Mr. Arun Kumar, Mr. Mohd. Jameel, Mr. Akhila Nand Upadhyay, Mr. Lakshminarayan Dhara, Mr. Sunil Kumar Singh, Mr. Amit Kumar, Mr. Ram Jiyawan, Km. Shyamali Ghosal, Mr. Nand Lal, Mr. Chhote Lal, Mr. Ram Hriday Pathak, and Mr. Rafique Ahmad*** *of the Department could never go unnoticed, who continuously lent their support and technical assistance during the tenure of the research.*

*I wish to express deep gratitude to* ***Dr. Avanish Singh Parmar*** *(Associate Professor, Department of Physics, IIT (BHU), Varanasi)****, Dr. Pawan K. Dubey*** *(Associate Professor, Centre for Genetics Disorders, Institute of Science (BHU), Varanasi),* ***Mr. Dhananjay Panigrahi*** *(Tech Lead, Dr. Reddy's Laboratories, Hyderabad, Telangana, India),* ***Dr. Pratap Chandra Acharya*** *(Assistant Professor, Department of Pharmacy, Tripura University (A Central University), Tripura),* ***Prof. Gopal Nath*** *(Department of Microbiology, IMS (BHU), Varanasi),* ***Prof. G. B. Jena*** *(Professor, Department of Pharmacology & Toxicology, NIPER, Mohali),* ***Dr. Narender Tadigoppula*** *(Chief Scientist, Division of Medicinal and Process Chemistry, CDRI, Lucknow),* ***Dr. Shantibhusan Senapati*** *(Scientist-E, Institute of Life Sciences, Bhubaneswar),* ***Dr. Sunday O. Otimenyin (****Department of Pharmacology, Faculty of Pharmaceutical Sciences, University of Jos, Jos, Plateau State, Nigeria), and* ***Prof. Subrat Kumar Bhattamisra*** *(Professor, GITAM (Deemed to be University), Andhra Pradesh, India) for providing the technical inputs during the research work.*

*I am thankful to* ***Mr. Dulla Naveen Kumar, Dr. Ravi Pratap, Dr. Md. Bayazeed Alam****, and* ***Dr. Vivek Pandey*** *for their collaborative work. My heartiest thanks to my lab mates at the Phytomedicine Research laboratory:* ***Mr. Dharmendra Yadav, Dr. Shreya H. Singh, Dr. Gaurav Gopal Naik, Mr. Sagar, Ms. Himani, Ms. Reena Madhavi, Mr. Pradeep Patel, Ms. Pooja Kathait, Ms. Soki Daeme Malang, Ms. Shambhavi, and Mr. Ankur*** *for making wonderful friendly environment in lab which made my work fruitful and enjoyable. They always had a friendly attitude and, at the same time, provided me with valuable suggestions, which helped me in carrying out my research work.*

*My thankful regards to my batch mates* ***Ms. Poorvi Saraf, Mr. K Venkateswarlu, Mr. Abhishesh Kumar Mehata, Mr. Akash Verma, Mr. Manish Kumar, Mr. Ravi Singh, Mr. Abhishek Jha, Mr. Gajendra T A, Ms. Neha Singh, Mr. Narendra, Ms. Nivedita Bhardwaj, Ms. Nancy Tripathi, Ms. Bhagwati Bhardwaj, Mr. Rangan Mitra, Ms. Gadepalli Anagha, Ms. Mohini Mishra, Mr. Swapan Maity, Ms. Divya Pareek, Ms. Sukanya Patra, and Ms. Shubhangi*** *for their pleasant company, cooperation, and help during research work.*

*I am also thankful to my respected seniors, friends, and beloved juniors of the Department:* ***Mr. Amit Singh, Mr. Tarkeshwar Dubey, Mrs. Sangita Hazarika, Dr. Kancharla Bhanukiran, Mrs. Vineeta Tiwari, Mrs. Deepa Dehari, Dr. Rinki Varma, Dr. Datta Pawde, Mr. Dharmanath Parbat, Mr. Himanshu Verma, Mr. Bhanuranjan Das, Mr. Anurag Tk Baidya, Mr. Alen T Mathew, Ms. Aiswarya Chaudhuri, Mr. Rohan Sahu, Ms. Abhipsa Mohapatra, Mr. Sanchit Arora, Ms. Udita Shiromani, Ms. Komal Rani, Ms. Sourav Bera, Ms. Vaishali Saini, Mr. Sunil Kumar, Mr. Himanshu Rai, Mr. Gaurav Singh, Mr. Kshirod Kumar Das, Ms. Km Deeksha Nigam, Ms. Aditi Tripathi, Ms. Karnika Tiwari, Mr. Govind Kumar Kachhava, Ms. Mayuri Purushottam Sawarkar, Mr. Gajanan Arjunrao Pawar, Mr. Jatin, Ms. Andarghiske Komal Rajesh, Mr. Manjeet Kumar Sah Gond, Ms. Rinki Jiwnani, Mr. Chinmaya Kumar Khuntia, Mr. Amit Maity, Mr. Chhatra Ram, Mr. Rohit Patil, Ms. Bharti Devi, Mr. Abhishek R. Mali, Mr. Souvik Mistri, and Mr. Gauransh Mishra** *for their healthy company, cooperation and devoting their valuable time in tailoring my work whenever required.*

*My heartiest thanks to* ***Mrs. Saibani Sahu, Late. Mr. Gadadhar Sahu****,* ***Mrs. Manasi Sahu****,* ***Ms. Aditi Sahu,*** *and* ***Ms. Anandi Sahu*** *for providing care, affection, and moral support as family members.*

*I express my gratitude to all of my respected B. Pharm and M. Pharm* ***Teachers, especially Prof. Prakash Chandra Senapati and Prof. Gyanada Sankar Dash, Sri Jayadev College of Pharmaceutical Sciences****, Bhubaneswar, Odisha, for their support and encouragement in studying for Ph.D. in this renowned Department of Pharmaceutical Engineering & Technology, IIT (BHU), Varanasi.*

*My special thanks to my B. Pharm and M. Pharm Batchmates, especially* ***Mr. Bijan Kumar Mohanty, Mr. Subhra Sailendra Mishra, Mr. Subir Kumar Patra, Mr. Satya Narayan Dash, Mr. Sarada Prasad Tripathy, Mr. Prasant Sagar, Mr. MD Sonu, Ms. Itishree Aradhana Mohanty, Mr. Suchindra Bishwal, Mr. Nigam Panda, Mr. Tushar Das, Mr. Sanat Kumar Mishra, and Mr. Jyotiranjan Parida*** *for their care, love, and mental support during my Ph.D. journey.*

*My heartiest thanks to all my respected seniors, especially* ***Mr. Satyabrat Sahoo, Mr. Priyabrata Panigrahi, Mr. Satyajeet Bishwal, Mr. Smruti Ranjan panda, Mr. Bishwajeeban Barik, Mrs. Aliva Minz****, and juniors* ***Mr. Abinash Samantray, Mr. Debasis Sahoo, Mr. Swadhin Kumar Lenka, Mr. Krushna Chandra Maharana, Mr. Sanat Kumar Dash, Mr. Pravas Ranjan Dash, Mr. Kalpataru Behera, Mr. Binaya Ranjan Dash, and Mr. Kuna Das****.*

*I am thankful to all of my 12$^{th}$ friends especially* ***Mr. Chitaranjan Roul, Mr. Sambit Choudhury, Mr. Sunil Samantaray, Ms. Sushree Nibedita Rout, Ms. Ankita Biswal, Ms. Madhusmita Sahoo, and Ms. Bhagyashree Pradhan****, my 10$^{th}$ school friends especially* ***Mr. Bishwaranjan Swain, Mr. Pravanjan Swain, Mr. Budhadev Rout, Mr. Tapan Das, Mr. Deepak Das, Mr. Akash Samal, Ms. Sunita Swain,*** *and* ***Ms. Ankita Pradhan*** *for providing support, care and mental strength during the journey.*

*I am very much thankful to all the members of Odia Society of Varanasi (Varanaseya Utkal Samaj) especially* ***Prof. Gopabandhu Mishra, Prof. Kalyan Ghadei, Dr. Amiya Kumar Samal, Mrs. Sonali Mohapatra, Dr. Soumya Ranjan Meher, Dr. Siva Prasad B N V, Mr. Saroj Kanta Behera, Mr. Biswajit Mishra, Mr. Anirbana Parida, Mr. Chinmaya Chiranjeeb Nayak, Ms. Sarita Lenka, Ms. Niharika Samantaray, Ms. Bhubaneswari Sahoo, Ms. Jyotirmayee, Ms. Madhumita Priyadarsini, Ms. Saswati Dehury, Mr. Vineet Bharadwaj, Ms. Anwesha Patnaik, Ms. Ankita Nayak, Ms. Sonali Priyadarsini Sahoo, Ms. Baishnabi Kishan, Ms. Niharika Mishra, Ms. Swetasoma Nayak, Mr. Soumya Ranjan Sahoo, Mr. Ritik Roshan Parida, Mr. Pratik Biswal, Mr. Tikun Dhal, Mr. Abhijit Sahoo, Mr. Bikash Behera, Mr. Balaram Sahoo, Mr. Abhinandan Sahoo, Ms. Aishwarya Rath, Ms. Anuska Beura, Ms. Suprava Mishra, Mrs. Jhuna Rani Ojha, and Ms. Twinkle Pradhan*** *for their care, affection, and moral support like my family members.*

*Finally, I shall always remain thankful and indebted to my parents,* ***Mr. Gouttam Mohapatra*** *and* ***Mrs. Jayalaxmi Mohapatra*** *and my brother* ***Mr. Shubhadatta Mohapatra****, and family members* ***Late Mr. Gopinath Mohapatra****,* ***Mrs. Diptimayi Pradhan, Mr. Kedar Swain, Mr. Chandrabhanu Gahan, Mrs. Padmini Gahan, Mr. Debaraj Pradhan, Ms. Pragyan Paramita Swain, and Ms. Pravati Swain****, my relatives* ***Ms. UB Bishnupriya, Mr. Asutosh Sahoo, Ms. Rosalin Swain, Mr. Omprakash Swain, Mrs. Meera Dubey, and Mrs. Shweta Dubey*** *for all their selflessly sacrifices, love, support, strength, and cooperation during the study. This work could not have been completed without their blessings. I have no words to thank them all.*

*Last but not least, I am grateful and would like to* ***thank everybody*** *who some or the other who contributed to this research work and motivated me to overcome hurdles during this journey.*

*The financial assistance for this work provided as a scholarship to me by the* ***Ministry of Human Resource Development (MHRD)****, Government of India, is highly acknowledged. I greatly acknowledge the* ***Central Instrumentation Facility*** *at* ***IIT (BHU), Varanasi,*** *for providing the facility for various analysis.*

*I am very much thankful to the* ***instrumental and infrastructure facilities*** *provided by the* ***Department of Pharmaceutical Engineering & Technology, IIT (BHU), Varanasi; Central Instrument Facility, IIT (BHU)****, Varanasi; Department of Physics, IIT (BHU), Varanasi; and Centre for Genetics Disorders, Institute of Science, Banaras Hindu University,*** *Varanasi, India.*

*Lastly, I pray to Almighty for the '****Moksha****' of all the* ***experimental animals*** *utilized in my experiments during the tenure of my research work.*

*Ultimately, I would like to bow down and pray to the* ***Almighty*** *for always giving me enough strength and courage to fulfill all my dreams and goals.*

**Date:** 20·10·2023

**Place:** Varanasi

Debadatta Mohapatra

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**List of Abbreviations**

| Abbreviations | Description |
|---|---|
| 2FI | Two-factor interaction model |
| ABC | ATP-binding cassette |
| ABCD | Asymmetry, border irregularity, color variation, and large diameter |
| Akt | Ak strain transforming kinase |
| ALM | Acral lentiginous melanoma |
| ALP | Alkaline phosphatase |
| ANOVA | Analysis of variance |
| AP-1 | Activated protein-1 |
| ATR-FTIR | Attenuated total reflectance-Fourier transform infrared spectroscopy |
| AUC | Area under the curve |
| b.wt | Body weight |
| BCC | Basal-cell carcinoma |
| BLAST | Basic Local Alignment Search Tool |
| BM | Branching myocytes |
| BRAF | Serine/threonine-protein kinase B-Rapidly Accelerated Fibrosarcoma |
| C.V. | Co-efficient of variation |
| CAP | Cellulose acetate phthalate |
| CCD | Central composite design |
| CDER | Center for Drug Evaluation and Research |
| CDK4 | Cyclin-dependent kinase 4 |
| CDKN1A | Cyclin-dependent kinase inhibitor 1A |
| CDKN2A | Cyclin-dependent kinase inhibitor 2A |
| CFT | Capillary flow technology |

| | |
|---|---|
| CI | Carr’s compressibility index |
| CLSM | Confocal laser scanning microscopy |
| CM | Cutaneous melanoma |
| CM | Carrier Matrices |
| CMA | Critical material attributes |
| $C_{max}$ | Maximum plasma concentration |
| CMC | Carboxy methylcellulose |
| CMC | Critical micelle concentration |
| CMEC | Carboxymethylethylcellulose |
| CMs | Carrier matrices |
| CMT | Critical micelle temperature |
| COX-2 | Cyclooxygenase 2 |
| CPCSEA | Committee for the Purpose of Control and Supervision of Experiments on Animals |
| CPP | Critical process parameters |
| CQA | Critical quality attributes |
| CREB | Cyclic AMP response element-binding protein |
| CTLA-4 | Cytotoxic T-lymphocyte–associated antigen 4 |
| CU-6-TFs | CU-6 loaded optimized TFs |
| CV | Central vein |
| CVM | Center for Veterinary Medicine |
| DAD | Diode array detector |
| DM | Desmoplastic melanoma |
| DMBA | Dimethylbenz[a]anthracene |
| DMEM/F-12 | Dulbecco's Modified Eagle's Medium |
| dNTPs | Deoxynucleoside triphosphate |
| DSC | Differential scanning calorimetry |

| | |
|---|---|
| DTIC | Dacarbazine |
| EA | Edge activators |
| EE | Entrapment efficiency |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| Er | Enhancement ratio |
| ERK1/2 | Extracellular signal-regulated kinases 1 and 2 |
| ETD | Everhart–Thornley detector |
| FbD | Formulation by design |
| FBS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| FGF | Fibroblast growth factor |
| FGFR | Fibroblast growth factor receptor |
| $F_{rel}$ | Relative bioavailability |
| GC-HS | Headspace gas chromatography |
| GCO | Global Cancer Observatory |
| GDP | Guanosine diphosphate |
| GEM | Genetically engineered models |
| GGT | γ-glutamyl transpeptidase |
| GHS | Globally Harmonised System |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| GRAS | Generally Recognized as Safe |
| GSH | Glutathione |
| GTP | Guanosine triphosphate |
| HAADF | High angle annular dark field |
| HCT | Hematocrit test |

| | |
|---|---|
| HEK 293 | Human embryonic kidney 293 cells |
| HIF-1α | Hypoxia-inducible factor-1α |
| HLB | Hydrophilic–lipophilic balance |
| HME | Hot-melt extrusion |
| HPC | Hydroxypropyl cellulose |
| HPLC | High-performance liquid chromatography |
| HPMC | Hydroxy propyl methylcellulose |
| HPMCP | Hydroxypropylmethylcellulose phthalate |
| HPTLC | High-performance thin layer chromatography |
| HR | Hausner ratio |
| HRAS | Harvey rat sarcoma viral oncogene homolog |
| HRSEM | High-resolution scanning electron microscopy |
| HRTEM | High-resolution transmission electron microscopy |
| i.p. | Intra peritonial |
| IAEC | Institutional Animal Ethics Committee |
| ICH | International Conference on Harmonization |
| IFN | Interferon |
| IL | Interleukin |
| ILS | Increase in life span |
| iNOS | Inducible nitric oxide synthase |
| IS | Internal standard |
| $J_{max}$ | Maximum flux |
| $J_{ss}$ | Steady-state Flux |
| $K_0$ | Zero order rate constant |
| $K_1$ | $1^{st}$ order rate constant |
| KDR/FIK-1 | Kinase insert domain receptor/fetal liver kinase 1 |

| | |
|---|---|
| $K_{el}$ | Elimination rate constant |
| $K_H$ | Higuchi dissolution constant |
| $K_{HC}$ | Hixson-Crowell rate constant |
| $K_{kp}$ | Korsmeyer-Peppas release rate constant |
| $K_p$ | Permeability coefficient |
| $K_p$ | Permeation coefficient |
| LC | Loading Capacity |
| $LD_{50}$ | Lethal Dose 50 |
| LLOD | Lower limit of detection |
| LLOQ | Lower limit of quantification |
| LMM | Lentigo maligna melanoma |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| LTC | Live tumor cells |
| MAPK | Mitogen-activated protein kinase |
| MC | Methyl cellulose |
| MCH | Mean corpuscular haemoglobin |
| MCHC | Mean corpuscular haemoglobin concentration |
| MCV | Mean corpuscular volume |
| MEK | Mitogen-activated protein kinase kinase |
| MEK1/2 | MAP kinase extracellular signal regulated kinases 1 and 2 |
| MHC | Major histocompatibility complex |
| MMP | Matrix metalloproteinases |
| MRT | Mean residence time |
| n | Number of repetitions |
| NCCS | National Centre for Cell Science |
| NCI | National Cancer Institute |

| | |
|---|---|
| NDDS | Novel drug delivery systems |
| NF-κβ | Nuclear factor kappa β |
| NK | Natural killers |
| NO | Nitric oxide |
| NR | Necrotic regions |
| NRAS | Neuroblastoma RAS viral oncogene homolog |
| OECD | Organization for Economic Co-operation and Development |
| OFAT | One-factor-at-a-time |
| OPM | Optimized physical mixture |
| OSD | Optimized solid dispersion |
| p.o. | Per oral |
| PAMAM | Polyamidoamine |
| PBS | Phosphate-buffered saline |
| PCNA | Proliferating cell nuclear antigen |
| PCV | Packed cell volume |
| PD-1 | Programmed cell death protein 1 |
| PDGFR | Platelet-derived growth factor receptor |
| PD-L1 | Programmed cell death ligand 1 |
| p-DMAB | p-dimethylaminobenzaldehyde |
| PDTXs | Patient-derived tumor xenografts |
| PEG | Polyethylene glycol |
| PER | Percent extraction recovery |
| P-gp | P-glycoprotein |
| PI3K | Phosphatidylinositol-3-kinase |
| PII | Primary irritation index |
| PIP | Piperine |

| | |
|---|---|
| PIP2 | Phosphatidylinositol- 4, 5-biphosphate |
| PIP3 | Phosphatidylinositol-3, 4, 5-triphosphate |
| PK | Pharmacokinetic |
| PKCα | Protein kinase C alpha |
| PL-90 H | Phospholipon® 90 H |
| PLFEE | *Piper longum* fruits ethanolic extract |
| PLGN | Piperlonguminine |
| PLM | Polarization microscopy |
| PM | Physical mixture |
| PPI | Pea protein isolate |
| PRESS | Predicted Residual Sum of Squares |
| PTEN | Phosphatase and tensin homolog deleted on chromosome 10 |
| PVA | Polyvinylalcohol |
| PVDF | Polyvinylidene fluoride |
| PVP | Polyvinylpyrrolidone |
| PVP/VA | Poly (vinylpyrrolidone-co-vinyl acetate) |
| PVP-CL | Crospovidone |
| PVPPVA | Polyvinylpyrrolidone-polyvinylacetate copolymer |
| QbD | Quality by design |
| QC | Quality control |
| QTPP | Quality target product profile |
| Raf | Rapidly accelerated fibrosarcoma |
| Ras | Rat sarcoma virus |
| RDW | Red cell distribution width |
| RET | Receptor tyrosine kinase |
| RP | Red pulp |

| | |
|---|---|
| RSD | Relative standard deviation |
| RSM | Response surface methodology |
| Rt | Retention time |
| RTK | Receptor tyrosine kinase |
| SAED | Selected area electron diffraction |
| SC | Stratum corneum |
| SCC | Squamous-cell carcinoma |
| SCID | Severe combined immune-deficient |
| SD | Solid dispersion |
| SD | Standard deviation |
| SDC | Sodium deoxycholate |
| SGOT | Serum glutamic-oxaloacetic transaminase |
| SGPT | Serum glutamic pyruvic transaminase |
| SLS | Sodium lauryl sulfate |
| SPM | Scanning probe microscopy |
| $t_{1/2}$ | Elimination half-life |
| T-80 | Tween® 80 |
| TBE | Tris-borate-Ethylenediaminetetraacetic acid |
| TCR | T-cell receptor |
| TDDS | Transdermal drug delivery system |
| TFG | Transgelosome |
| TFs | Transferosomes |
| TFs FD | Freeze-dried optimized TFs |
| TGA | Thermogravimetric analysis |
| TGF | Transforming growth factor |
| TGI | Tumor growth inhibition |
| TILs | Tumor infiltrating T lymphocytes |

| | |
|---|---|
| TIMP | Tissue Inhibitor of Metalloprotease |
| TLC | Total leucocyte count |
| $T_{max}$ | Time to reach maximum concentration |
| TMF | 5,7,4′trimethoxyflavone |
| TNF | Tumor necrosis factor |
| TO | Turmeric Oleoresin |
| TPA | 12-O-Tetradecanoylphorbol-13-acetate |
| TRBCs | Total red blood cell counts |
| TV | Tumor volume |
| UDP | Up-and-down-procedure |
| US-FDA | United States Food Drug Administration |
| UV | Ultraviolet |
| VDT | Volume doubling time |
| VEGF | Vascular endothelial growth factor |
| WBC | White blood cells |
| WP | White pulp |
| XRD | X-ray diffraction |
| $Z_{avg}$ | Vesicular size |
| ΔGt | Gibbs free energy transfer |

**List of symbols**

| Symbols | Description |
|---|---|
| % | Percentage |
| ° | Degree |
| α | Alpha |
| μ | Micro |
| β | Beta |
| γ | Gamma |
| μg | Microgram |
| mm | Milli meter |
| cm | Centi meter |
| > | Greater than |
| < | Less than |
| ↑ | Increase |
| ↓ | Decrease |
| °C | Degree Celsius |
| $T_g$ | Glass transition temperature |
| MW | Molecular weight |
| ® | Registered trademark |
| TM | Trademark |
| μL | Micro liter |
| mAU | Milli-absorbance unit |
| ppm | Parts per million |
| v | Volume |
| nm | Nano meter |
| $\lambda_{max}$ | Absorption maxima |

| | |
|---|---|
| bp | Base pair |
| kH | Kilohertz |
| W | Watt |
| min | Minute |
| # | Mesh size |
| rpm | Rotation per minute |
| $\theta$ | Theta |
| g | Gram |
| mg | Milli gram |
| cm | Centi meter |
| w | Weight |
| s | Second |
| h | Hour |
| ζ | Zeta potential |
| mV | Milli volt |
| mg/dL | Milligram/deciliter |
| U/L | Unit/liter |
| g/dL | Gram/deciliter |
| fL | Femtoliters |
| pg | Picogram |

**Preface**

The extract of the *Piper longum* fruit (Long pepper, Family- Piperaceae) and its phytoconstituents have been well investigated to possess anticancer activity against melanoma through *in-vitro* and *in-vivo* tumor models. Irrespective of reported anticancer activity, the poor aqueous solubility of the majority of contained phytoconstituents and the obstruction by major skin barrier, i.e., stratum corneum (SC) of skin restrict their therapeutic use through oral and transdermal routes, respectively. To improve its therapeutic properties against melanoma, two formulations, solid dispersion (SD) and transgelosome (TFG) of standardized *Piper longum* fruits ethanolic extract (PLFEE) were developed and optimized using Quality-by-Design (QbD). To maintain batch-to-batch consistency and provide dose uniformity, the PLFEE was standardized with respect to piperine (PIP) and piperlonguminine (PLGN) by high-performance liquid chromatography (HPLC). Acute oral toxicity and acute dermal toxicity of standardized PLFEE were carried out using healthy nulliparous and non-pregnant female C57BL/6 mice as per Organization for Economic Co-operation and Development (OECD) 425 and OECD 402 guidelines, respectively. The influence of various independent formulation factors on responses were statistically analyzed by response surface methodology (RSM) using 3-factor, 5 levels rotatable central composite design (CCD). The optimized SD and TFG was investigated for pharmaceutical and therapeutic activity against melanoma (B16F10) bearing C57BL/6 female mice. The anticancer activities of novel phytoformulations were also investigated with the standard anticancer drug dacarbazine (DTIC) as an adjuvant therapy. Further, the simultaneous administration of per oral SD and topical TFG formulations was carried out to investigate the *in-vivo* anticancer activity.

The thesis is divided into **nine chapters,** which are as follows:

**Chapter 1:** Introduction

**Chapter 2:** Literature review

**Chapter 3:** Rationale and objectives

**Chapter 4:** Materials, instruments, and software used in the experiments

**Chapter 5** Collection, authentication, extraction of *Piper longum* fruits, and marker-based standardization by validated HPLC method

**Chapter 6**: Development, optimization, and characterization of fourth-generation ternary solid dispersion of standardized *Piper longum* fruit extract for melanoma therapy

**Chapter 7:** Development, and characterization of transgelosome of standardized *Piper longum* fruit extract for melanoma therapy

**Chapter 8:** Combined *in-vivo* anticancer activity evaluation of optimized SD and TFG against melanoma

**Chapter 9:** Summary and conclusion

**Chapter 5, 6, 7 and 8 have "Summary points", that summarize the key outcomes of the respective chapters.**