1 Chapter 1: Introduction

Melanoma, a most aggressive and deadly form of skin cancer, arises from the malignant transformation of melanocytes. As per the National Cancer Institute (NCI) epidemiology survey, 97,610 new cases and 7,990 deaths in USA are reported in 2023 [1]. Fair-skinned Caucasian populations are more prone to melanoma; however, its occurrence in pigmented populations in Asia and Africa has also been noticed on the nail beds, mucous membranes, and soles of the feet at a low incidence rate [2, 3]. The most prevalent type of melanoma is cutaneous melanoma, which appears on the cutaneous surface [4, 5]. Although melanoma is regarded as multifactorial, the major risk factor is excessive exposure to ultraviolet (UV) radiation, which causes genetic mutations, DNA damage and mediates inflammatory responses [2, 3, 5]. In addition, other factors like numerous freckles, increased number or size of melanocytic nevi, pre-existing dysplastic nevus, decreased DNA repairability, tanning inability, suppressed immune system, mutations in cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase inhibitor 2A (CDKN2A or p16) participate in development and progression of melanoma [3, 5]. The hyperactivation of mitogen-activated protein kinase (MAPK) kinases due to mutation in BRAF (Serine/threonine-protein kinase B-Rapidly Accelerated Fibrosarcoma) and NRAS genes (Neuroblastoma RAS viral oncogene homolog) and phosphatidylinositol-3-kinase (PI3K) by multiple factors leads to the development of melanoma [2, 6, 7].

The majority of currently used chemotherapeutics possess narrow therapeutic window, induce toxicities, unwanted adverse events, suppression of the immune system, tissue damage (extravasation), and induce resistance [6, 8, 9]. High cost is another problem that restricts their widespread use. The plant-derived medicaments can be used as an alternative and supportive therapy to the current chemotherapeutics for melanoma.

Numerous plant extracts and phytoconstituents have been well exploited for melanoma therapy for their ability to suppress melanoma through the regulation of oxidative status, modulation of immunity, correction of disordered replication and induction of apoptosis, prevention of invasion, angiogenesis, and metastasis [2, 10]. Multiple mechanisms are involved in the development, progression, invasion, angiogenesis, and metastasis of melanoma. Hence, it is rational to use plant extract or fraction (comprising numerous phytoconstituents) that may act synergistically in a multi-targeting manner.

The fruits of *Piper longum* (Long pepper, Family: Piperaceae) have several medicinal properties, including anticancer, immunomodulatory, antiinflammatory, hepatoprotective, and melanin-inhibiting activity [11]. Major constituents of fruit include alkaloids (e.g., piperine (PIP), piperlonguminine (PLGN), piperlongumine, pellitorine, etc.), lignans, esters, volatile oils, and organic acids [11]. The extract of the fruit and its constituents have shown their anticancer activity against melanoma. PIP inhibits transcription factors, such as cyclic AMP response element-binding protein (CREB), activated protein-1 (AP-1), nuclear factor-kβ (NF- kβ), and proinflammatory cytokine gene expression (IL-6, IL-1β, GM-CSF, and TNF-α) in B16F10 (melanoma) cells [12]. It also causes G1 phase arrest and apoptosis induction in B16F0 and SK MEL 28 melanoma cells through activation of checkpoint kinase-1 [13]. The PIP was also studied for inhibition of lung metastasis in the B16F10 cell-induced tumor model in C57BL/6 mice [14]. Piperlongumine was reported to produce cytotoxicity against human melanoma (A375, A875) and murine melanoma (B16F10) and induce apoptosis via reactive oxygen species-mediated disruption of mitochondria [15]. The PLGN was also reported to suppress melanogenesis via the downregulation of tyrosinase expression in the melanin synthesis pathway [16], and inhibition of

melanogenesis seems a rational adjuvant approach for the treatment of metastatic melanoma [17]. The ethanolic extract of fruit was also examined both *in-vitro* and *in-vivo* for antiangiogenic properties via inhibition of vascular endothelial growth factor (VEGF), tumor-directed capillary formation, and inhibition of proinflammatory cytokines [18]. Irrespective of wide significance, its therapeutic utility is restricted due to the low water solubility, limited dissolution, and *in-vivo* oral bioavailability of the majority of active constituents [19-21]. Thus, the use of appropriate techniques to evade the solubility, dissolution, and bioavailability issues is extremely crucial to realize the actual therapeutic effectiveness.

Multiple formulation strategies, such as solid dispersion, micronization, salt formation, nanocrystalization, micro/nanoemulsion, pH modification, use of cosolvent, surfactant, micellar solubilization, lipid-based formulation, cyclodextrin complexation, co-crystallization, liquisolid technique, and nanoparticles encapsulation have been well exploited to avoid solubility issues [22-24]. Solid dispersion (SD) is one of the solid units of oral dosage form that possess the ability to overcome the solubility and bioavailability-related issues of poorly water-soluble drug candidates [25]. Oral administration of anticancer medications is most frequently used due to better patient compliance (easier administration, feasibility for repeated drug administration, independence, pain-free administration), home-based therapy, decreased risk of infections, better tolerability, limited severity of toxicity, and lower cost of therapy [26]. The SD allows homogeneous dispersion of drug candidates in the carrier matrix (CM) in a molecular, amorphous, microcrystalline, or colloidal state, which leads to the increased effective surface area, dispersibility, wetting ability, porosity, maintenance of supersaturation, dissolution, and oral bioavailability [24, 26-29]. Formulation methods, such as fusion or melting, solvent methods (e.g., freeze

drying, spray drying, and rotary evaporation), and melting-solvent methods are widely used for SD preparation; however, other advanced techniques like KinetiSol® dispersing, hot melt extraction, supercritical fluid method, electrospinning spinning, co-precipitation technique, microwave irradiation also used nowadays in industrial scale [24, 27, 29, 30]. The process of the formulation should be affordable, safe, and rid of the usage of expensive machinery. Considering these factors, the solvent method via rotary evaporation is more beneficial for the lab-scale formulation of SDs. The use of a non-toxic solvent (Class-III and Class-IV) is essential for avoiding solvent-related toxicities during the use of the solvent evaporation method [25].

A transdermal drug delivery system (TDDS) is a promising strategy for the delivery of plant bioactives through the skin. The transdermal route offers non-invasive site-specific drug delivery, better patient convenience, prolonged drug release, extended duration of the activity, reduced side effects, and avoids gastric degradation, gastric irritation, and first-pass metabolism [31]. However, the anatomical architecture of the skin is a major challenge of the transdermal route, which prevents the loss of water, electrolytes, and other body constituents and averts the entry of therapeutic as well as harmful substances from the outer milieu [32]. The uppermost stratum corneum (SC) is a major obstacle to dermal and transdermal drug delivery, which limits drug transport across the skin and makes the topical route inefficient for medical use. The SC comprises proteins and lipids layers, which are structurally arranged as “bricks and mortar” that obstruct the entry of drug candidates during topical administration [33]. Such issues may be solved through novel topical formulations, such as transferosomes (TFs), phytosomes, liposomes, ethosomes, niosomes, cubosomes, micro or nanoemulsions, nanostructured lipid carriers, nanofibers, and solid lipid nanoparticles. Among them, the TFs (ultradeformable nanovesicles) have been perceived as the most

promising novel topical vesicular formulations for improving transdermal permeability and therapeutic activity of plant bioactives [34]. They have an average vesicular diameter in the range of 100 to 200 nm [35]. These ultradeformable nanovesicles are primarily composed of three basic components, namely phospholipids, surfactant/edge activators (EA), and water [34]. The incorporation of EA makes the membrane ultradeformable, and increases the penetration across the skin by acting as a penetration enhancer [34]. TFs hold a self-optimizing deformability behavior, which penetrates through the tight junctions of the intact skin easily and rapidly by changing their shape and size [34]. They own the advantages of non-invasiveness, self-administration ability, ultradeformability (capacity to cross 5 to 10 times smaller pores than their vesicular diameter), excellent skin tolerability (due to the phospholipids), provide sustained-release and accommodation for both hydrophobic and hydrophilic candidates [34]. The maintenance of a transdermal osmotic gradient is the driving force behind the transportation of TFs across the skin [34]. Phytoconstituents, either in their purified form or in the mixture, have been widely investigated and loaded into TFs for better transdermal delivery to maximize their efficacy [34]. Various techniques, such as thin-film hydration, ethanol injection, homogenization/extrusion, sonication, modified handshaking, high-pressure homogenization, suspension homogenization, and microfluidics are used for the preparation of TFs [33, 34]. Among them, the thin-film hydration-based rotary evaporation is widely explored for the lab-scale formulation of TFs, owing to the advantages like simplicity, economical, safe, suitability for heat-sensitive phytoconstituents, requiring a short period, and avoiding the use of sophisticated instruments [33]. TFs possess low viscosity, so they can be incorporated into suitable gelling agents to increase the viscosity for achieving prolonged residence time at the

application site and improving topical applicability. The incorporation of TFs to gel develops transferosomal gel (TFG) or transgelosome, which offers the advantage of both TFs and gel.

The **1st objective** is to prepare ternary SD of standardized *Piper longum* extract via solvent-based rotary evaporation technique and characterize its pharmaceutical properties and evaluate its anticancer activity against melanoma. High-performance liquid chromatography (HPLC) was used to achieve the marker-based standardization of the PLFEE concerning PIP and PLGN to maintain batch-to-batch consistency and provide dose uniformity. The response surface methodology (RSM) and optimization were implemented to achieve highly efficient formulation with high saturation solubility. The proposed formula was prepared, and the outcome was verified to validate the optimized formula predicted by the software. The optimized *Piper longum* fruits ethanolic extract solid dispersion was characterized for percentage yield, drug content, content uniformity, moisture content, micromeritics properties (density, flow property), surface morphology (high resolution scanning electron microscopy (HRSEM)), crystallinity (X-ray diffraction (XRD) and polarization microscopy (PLM)), thermal behavior (differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)), drug-excipient compatibility (Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) and High-Performance Thin Layer Chromatography (HPTLC)), *in-vitro* dissolution, stability (long term and accelerated), *in-vivo* oral bioavailability, and for anticancer activity against melanoma (B16F10) bearing C57BL/6 mice.

The **2nd objective** is the Quality-by-Design (QbD)-based development and optimization of ultradeformable nanovesicular transgelosome of standardized *Piper*

longum fruits ethanolic extract (PLFEE) via thin-film hydration method for melanoma therapy. The rationale for selecting such ultradeformable nanovesicular formulation was to attain optimum skin delivery of hydrophobic extract (PLFEE) by a non-invasive approach for the treatment of melanoma. To our knowledge, no reports are available for the standardized PLFEE-loaded transgelosome for melanoma cancer therapy. Further, the combination of hydrogenated phosphatidylcholine (Phospholipon® 90 H) as membrane former, Tween® 80 as edge activator, and Xanthan gum as gelling agent is not explored for the development of TFG. The ultradeformable vesicular TFs formulation has been designed, statistically analyzed, and optimized using Box-Wilson's Central Composite Design (CCD) based QbD approach to obtain the best formulation with low hydrodynamic vesicular size (Z_{avg}), maximum % entrapment efficiency (% EE), and optimum flexibility. For better topical applicability, the optimized TFs were loaded into the Xanthan gum-based hydrogel to produce TFG and characterized for their physicochemical properties, pharmaceutical properties, and *in-vivo* anticancer activity in melanoma (B16F10) bearing C57BL/6 mice. Also, the *in-vivo* anticancer activity of transgelosome was scientifically and statistically compared with a standard anticancer drug (Dacarbazine).

The **3rd objective** is to investigate the anticancer effect of both formulations when simultaneously administered in melanoma (B16F10) bearing C57BL/6 mice.