Chapter 1

Introduction

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Melanoma is one of the most aggressive and lethal forms of skin cancer, originating from the malignant transformation of melanocytes, the pigment-producing cells in the skin [1]. According to the National Cancer Institute (NCI) epidemiological data, it is estimated that in 2023, there will be approximately 97,610 new diagnoses of melanoma in the United States, accompanied by an alarming 7,990 fatalities. Individuals of fair complexion, particularly those within Caucasian populations, exhibit a significantly higher susceptibility to this disease [2]. However, cases have also been observed among pigmented populations in regions such as Asia and Africa, albeit at a lower incidence rate [3, 4]. The most prevalent form of melanoma is cutaneous melanoma, which appears on the skin's surface. While its development is influenced by multiple factors, excessive exposure to ultraviolet (UV) radiation is recognized as the leading risk factor. UV radiation causes DNA damage and genetic mutations, and also triggers inflammatory responses that can contribute to the onset of the disease [5, 6].

Several additional factors increase an individual's susceptibility to melanoma. These include having numerous freckles, a higher number or larger size of melanocytic nevi (moles), and pre-existing dysplastic nevi, which are abnormal moles associated with a greater risk of melanoma [7, 8]. Other risk factors include impaired DNA repair mechanisms, poor tanning ability, a weakened immune system, and certain genetic mutations [9]. Notably, mutations in the cyclin-dependent kinase 4 (CDK4) gene and the cyclin-dependent kinase inhibitor 2A (CDKN2A, also known as p16) gene are closely linked to melanoma development and progression [6].

Moreover, the hyperactivation of mitogen-activated protein kinase (MAPK) signaling pathways, often resulting from mutations in the BRAF gene (Serine/threonine-protein kinase B-Rapidly Accelerated Fibrosarcoma) and the NRAS gene (Neuroblastoma RAS viral oncogene homolog), plays a crucial role in melanoma pathogenesis [10]. Additionally,

alterations in the phosphatidylinositol-3-kinase (PI3K) pathway due to various factors further contribute to the oncogenic processes leading to melanoma development [11]. Thus, understanding the intricate interplay of genetic, environmental, and biological factors is essential for elucidating the mechanisms underlying melanoma progression and for developing more effective therapeutic strategies [12].

Many of the chemotherapeutic agents currently employed in clinical practice exhibit a limited therapeutic index, resulting in various toxicities and adverse effects [10]. These include immune system suppression, tissue damage caused by extravasation, and the potential for developing drug resistance [13]. Additionally, the high financial burden associated with these treatments presents a significant barrier to their broader application and accessibility [14].

In this context, plant-derived compounds offer a promising alternative or adjunctive therapeutic strategy for the treatment of melanoma [15]. A variety of plant extracts and their bioactive constituents have been extensively investigated for their antitumor properties, particularly for their capacity to inhibit melanoma progression [16]. These natural products have been shown to modulate oxidative stress, enhance immune responses, correct aberrant cellular replication, induce programmed cell death (apoptosis), and prevent processes such as invasion, angiogenesis, and metastasis [17-19].

The process of drug development is widely recognized as an arduous and complex endeavor, characterized by a low probability of successfully identifying a singular optimal compound from an initial lead [20]. This inherent challenge is compounded by the high attrition rates observed during the preclinical evaluation phases, where many candidate drugs are ultimately abandoned due to inadequate safety and efficacy profiles [21]. Recent studies indicate that approximately 40-45% of new chemical entities (NCEs) undergoing investigation in both clinical and preclinical settings exhibit poor water solubility. This physicochemical limitation

significantly undermines a crucial aspect of drug formulation: bioavailability. Consequently, these NCEs' therapeutic potential and overall effectiveness may be adversely affected [22, 23]. Considering these challenges, there is a pressing need for innovative drug delivery systems that leverage advancements in nanotechnology to enhance the solubility of poorly water-soluble pharmaceuticals. Various nanotechnology-based formulations, such as nanoemulsions, microemulsions, liposomes, solid lipid nanoparticles, and polymeric micelles, have been developed to address these solubility issues and improve the pharmacokinetic profiles of these compounds [24, 25].

Additionally, drug repurposing has emerged as a compelling strategy in the pharmaceutical landscape, garnering significant interest due to the availability of comprehensive clinical trial data on existing medications [26]. This wealth of information includes detailed insights into drug safety, pharmacokinetics, and pharmacodynamics, facilitating the identification of alternative therapeutic applications for established compounds [27]. By repurposing drugs that have already undergone extensive testing, researchers can potentially expedite the development timeline and enhance treatment options for various diseases, including cancer [28].

DHA is a semi-synthetic derivative derived from artemisinin (AT), a compound extracted from the plant Artemisia annua L. Initially recognized for its efficacy in the treatment of malaria, recent research has illuminated DHA's potential beyond its antiparasitic properties [29, 30]. Emerging evidence indicates that DHA possesses significant anticancer activity across a diverse range of malignancies, including but not limited to melanoma, prostate cancer, breast cancer, colorectal cancer, and ovarian cancer [29, 31].

The mechanisms through which DHA exerts its antitumor effects are being actively investigated, with studies suggesting that it may induce apoptosis, inhibit cell proliferation, and disrupt the processes of metastasis in various cancer cell lines [29, 32-34]. This broad spectrum of anticancer activity positions DHA as a promising candidate for further exploration in

oncology, particularly in the context of developing novel therapeutic strategies aimed at improving treatment outcomes for patients afflicted with these challenging cancers [32]. As the scientific community continues to delve into the multifaceted applications of DHA, its role in cancer therapy appears increasingly significant, warranting comprehensive studies to elucidate its full therapeutic potential.

On the other hand, flavonoids, which are secondary metabolites derived from plants and belong to the polyphenolic compound family, have increasingly attracted scientific interest due to their multifaceted mechanisms of action and their potential as therapeutic agents [35, 36]. These compounds are not only cost-effective but also exhibit a favorable safety profile with minimal adverse effects, making them appealing candidates for various medical applications [37]. Among these, hesperidin (HES) stands out as a particularly significant flavonoid, predominantly found in citrus fruits [38]. This compound has been shown to possess a wide range of pharmacological activities, including but not limited to anticancer effects, anti-inflammatory properties, and therapeutic benefits for conditions such as hemorrhoids and varicose veins [38].

The anticancer mechanisms of HES are notably complex, involving both intrinsic and extrinsic pathways of apoptosis, which lead to programmed cell death in cancer cells [39, 40]. Furthermore, hesperidin promotes the generation of reactive oxygen species (ROS), contributing to oxidative stress that can inhibit tumor growth [41]. Additionally, it plays a pivotal role in the modulation of various inflammatory mediators, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) [42]. The efficacy of hesperidin in these domains has been supported by a wealth of research, including numerous *in vitro* and *in vivo* studies that have demonstrated its potential across a spectrum of cancer types [43, 44]. Collectively, these

findings underscore the therapeutic promise of hesperidin and its capacity to serve as a valuable component in the development of novel cancer treatments.

Although DHA and HES demonstrate notable anticancer properties along with a range of pharmacological benefits, their therapeutic effectiveness is significantly hindered by several factors. These include poor oral bioavailability, extremely limited water solubility, and a short plasma half-life. To address these limitations, researchers have investigated various formulation strategies aimed at enhancing the solubility and bioavailability of both DHA and HES [45-49]. Approaches such as the utilization of inorganic gold nanoparticles, poly(lactic-co-glycolic acid) (PLGA) nanoparticles, and polymeric micelles have been explored [50-53]. However, these existing drug delivery systems are not without their challenges; for instance, inorganic nanoparticles often lack specificity for tumor targeting, while polymeric nanoparticles face issues related to rapid clearance by the reticuloendothelial system (RES) [50, 53]. Consequently, there is an urgent need for the development of innovative drug-delivery systems that can more effectively transport DHA and HES directly to tumor sites, thereby improving their therapeutic outcomes.

Exosomes are biologically derived nanovesicles that typically measure less than 200 nanometers in diameter [54]. These nanoparticles have the remarkable capacity to transport a wide array of molecular cargo, including but not limited to DNA, RNA, proteins, and microRNAs [55, 56]. They play a pivotal role in intercellular communication, facilitating the exchange of biological information and contributing to a variety of physiological processes [57].

One of the primary advantages of using exosomes as vehicles for drug delivery lies in their unique surface characteristics [58]. They express CD44 proteins, which enhance their ability to evade rapid clearance from the circulatory system, thereby prolonging their circulation time and minimizing the degradation of their therapeutic agents. Additionally, the biocompatibility

of exosomes ensures that they can integrate seamlessly with biological systems, enabling them to effectively navigate through various physiological barriers, including the blood-brain barrier (BBB) [59, 60].

Furthermore, exosomes exhibit a non-immunogenic profile, coupled with low production costs and minimal toxicity, making them an exceptionally attractive option for drug delivery applications [61]. Capitalizing on these significant benefits, exosomes have been harnessed as effective delivery vehicles over the past two decades. This positions them as a highly promising method for delivering DHA and HES in the context of melanoma treatment. The potential to utilize exosomes for targeted and efficient drug delivery could enhance therapeutic outcomes significantly while reducing side effects associated with traditional delivery methods.

The **first objective** is to isolate the exosomes from the bovine milk and load it with DHA, characterize its pharmaceutical properties, and evaluate its anti-cancer efficacy in melanoma. Exosomes were isolated from bovine milk and loaded with DHA using the sonication method. The resulting Exo-DHA formulation was characterized for particle size, polydispersity index (PDI), zeta potential, and morphology. *In vitro* experiments assessed Exo-DHA's anti-cancer activity through cytotoxicity, qualitative and quantitative cell uptake assay, reactive oxygen species assay, acridine orange apoptosis assay, mitochondrial membrane potential assay, and cell migration assay, along with examining its impact on oncogene expression via immunoblotting. Additionally, *in vivo* efficacy and anti-metastasis effects were evaluated using a B16F10-induced melanoma model, while histopathology and biochemical analyses assessed toxicity profiles. This comprehensive approach aims to demonstrate the potential of Exo-DHA as a safe and effective therapy for melanoma, offering improved drug delivery, reduced toxicity, and enhanced therapeutic outcomes.

The **second objective** is to isolate the exosomes from the bovine milk and load it with HES, characterize its pharmaceutical properties, and evaluate its anti-cancer efficacy in melanoma.

HES-loaded exosomes were prepared by sonication method followed by characterization (morphology, particle size, XRD, and drug release), and *in vitro* (cytotoxicity, ROS estimation, MMP-assay, and cell migration assay) and *in vivo* efficacy testing in B16F10 induced melanoma in swiss mice. To the best of our knowledge and based on a literature survey, this study marks the inaugural report on delivering HES through bovine milk exosomes for the treatment of melanoma.