2 Chapter 2: Literature review

2.1 Skin cancer and melanoma

The skin is the largest organ of the body that protects against sunlight, heat, infection, and injury. It also regulates the body temperature and stores fat, water, and vitamin D. The skin is composed of three main layers: epidermis (upper or outer layer), dermis (lower or inner layer), and subcutaneous tissue (Figure 2. 1). Skin cancer mainly originates at the epidermis, which is made up of three kinds of cells, such as (i) squamous cells: thin, & flat cells that constitute the top layer of the epidermis, (ii) basal cells: round cells beneath the squamous cells, and (iii) melanocytes: melanin-producing cells and are found in the lower part of the epidermis. When skin is exposed to the sun or artificial light, melanocytes secrete more pigment (melanin) and darken the skin.

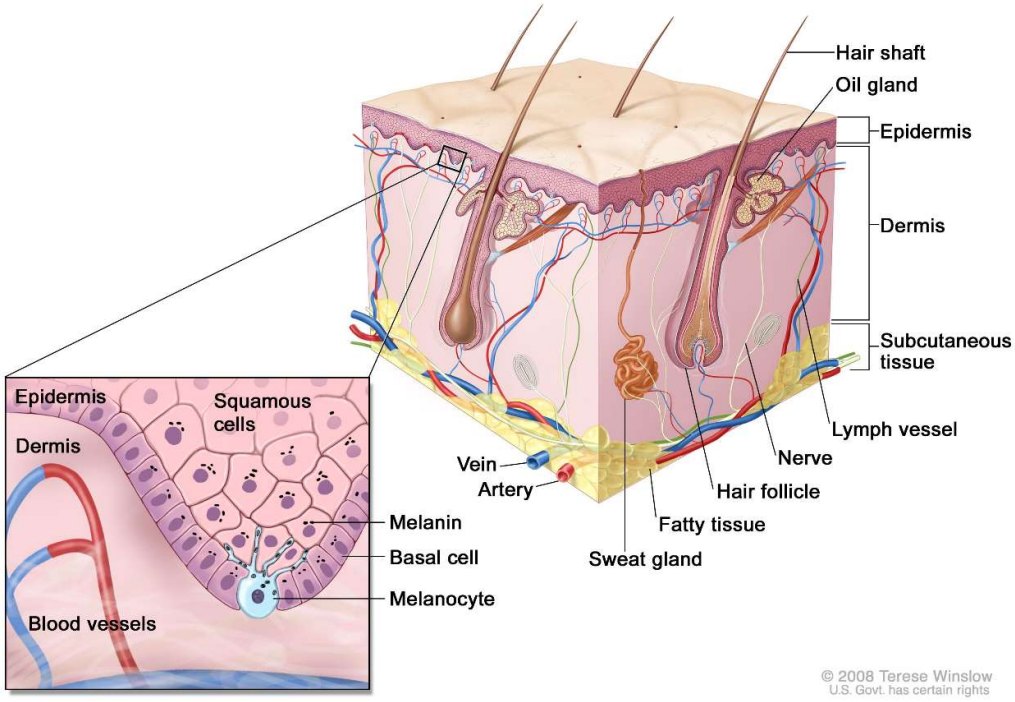

Figure 2. 1 Anatomy of the skin, showing the epidermis, dermis, and subcutaneous tissue. Melanocytes are in the layer of basal cells at the deepest part of the epidermis. *Figure adapted from National Cancer Institute (NCI), USA*

Chapter 2

Skin cancers include basal-cell carcinoma (BCC), squamous-cell carcinoma (SCC), and melanoma. BCC and SCC known as non-melanoma skin cancers which, account for 80 and 16% of all skin cancers, respectively, whereas melanomas account for only 4% of all skin cancers. BCCs are slow-growing and rarely metastasize, whereas SCCs can be highly invasive and may metastasize. In contrast, melanoma is derived from melanocytes, and the mortality associated with melanoma is high [36].

Melanoma is the most aggressive and deadly form of skin cancer that arises from the malignant transformation of melanocytes. 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]. As per National Cancer Institute (NCI) epidemiology survey reports, melanoma is the 5th most common cancer in USA, with 97,610 estimated new cases and 7,990 deaths in 2023 [1]. Data from the Global Cancer Observatory (GCO) shows that the annual incidence of melanoma cancer in both sexes in 2020 was 3,24,635 cases worldwide, with the highest number recorded in Europe (150627), followed by Northern America (105172), Asia (23753), Oceania (19239), Latin America and the Caribbean (18881), and Africa (6963) (Figure 2. 2a). The estimated no of new cases of melanoma of skin in male and female at all ages is graphically represented in Figure 2. 2b.

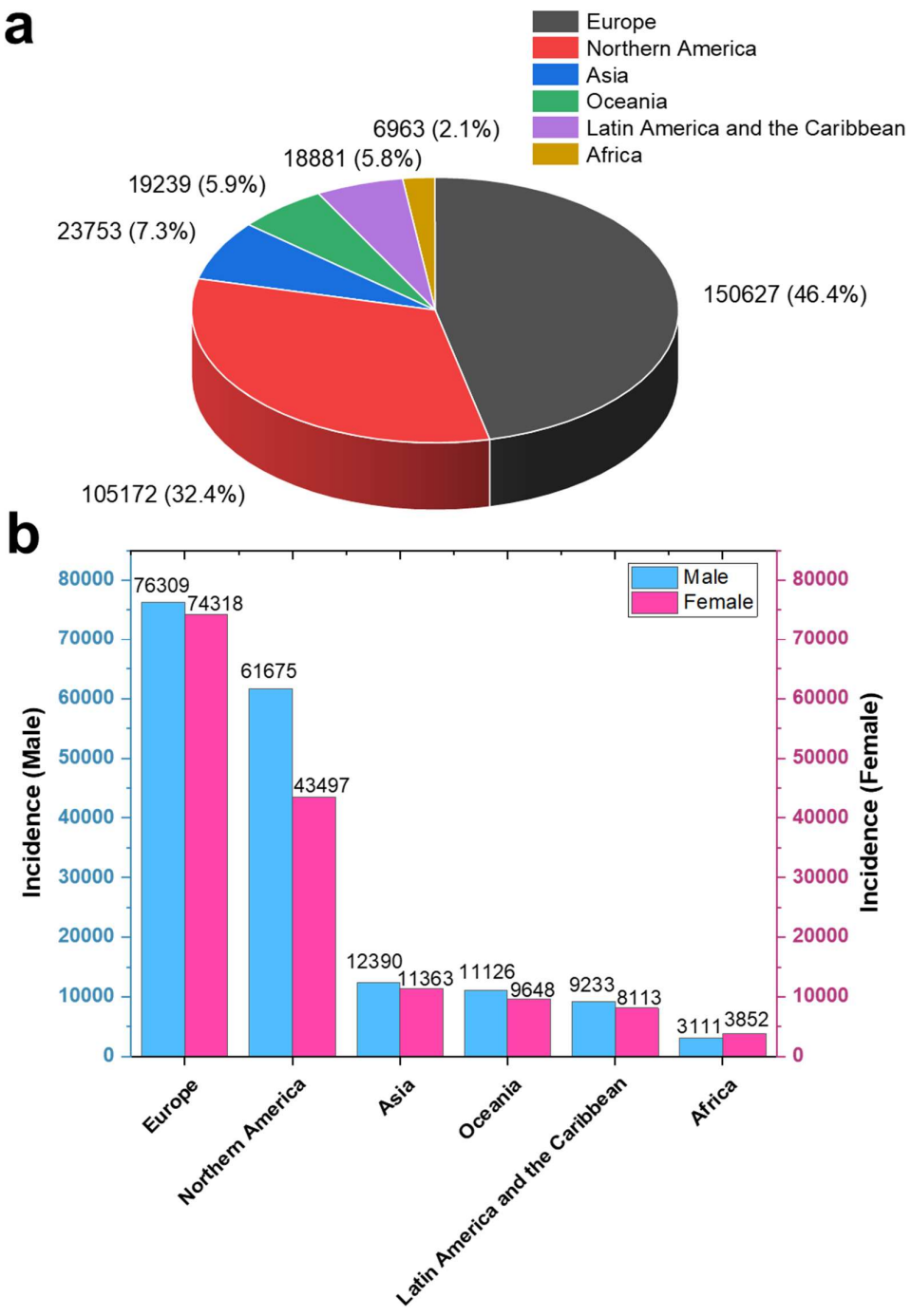

Figure 2. 2 Estimated No. of new cases of melanoma of skin in 2020 as per Global Cancer Observatory (GCO)-2020 data base (a) overall estimated no of new cases of melanoma of skin in both sexes at all ages and (b) estimated no of new cases of melanoma of skin in male and female at all ages.

The overall estimated no of mortality due to melanoma of skin in 2020 in world among both sexes at all ages as per GCO-2020 data base is shown in Figure 2. 3a. The individual reports of estimated no of death in case of male and female is shown in Figure 2. 3b.

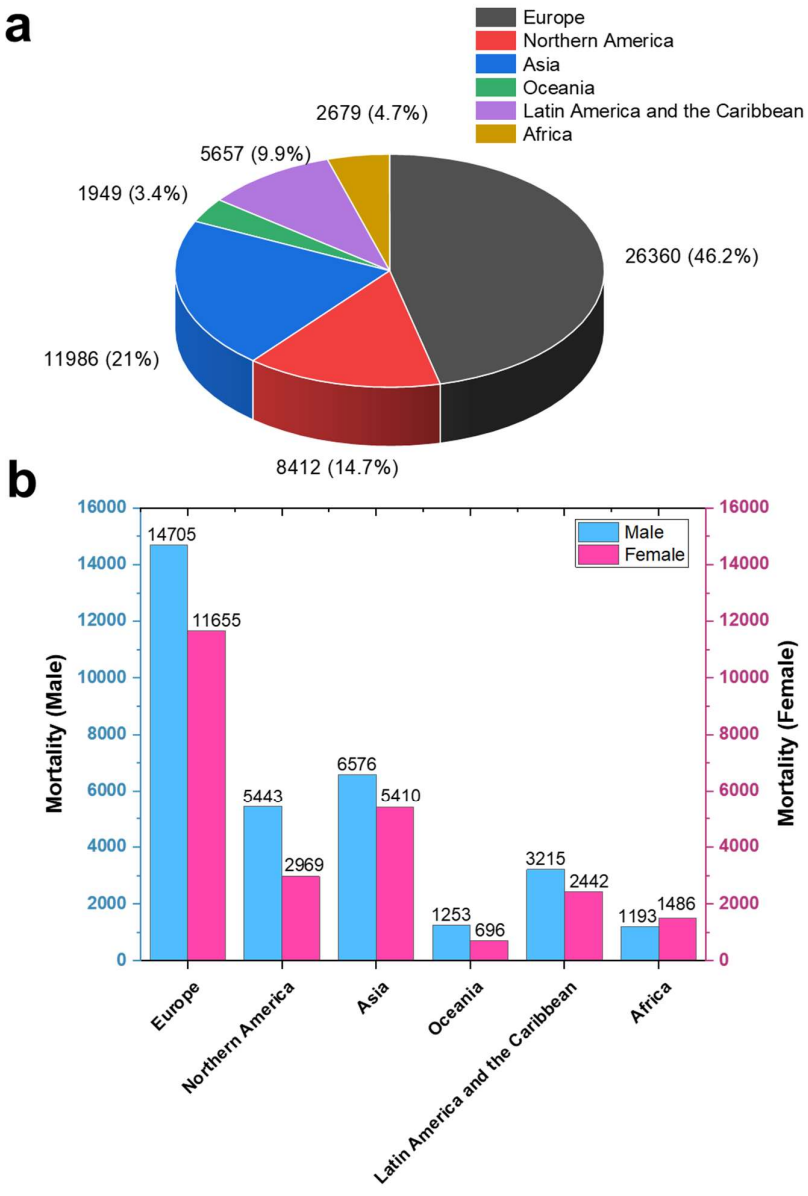

Figure 2. 3 Estimated No. of death due to melanoma of skin in 2020 in world as per GCO-2020 data base (a) overall estimated no of death due to melanoma of skin in both sexes at all ages and (b) estimated no of death due to melanoma of skin in male and female at all ages.

2.1.1 Risk Factors

Melanoma is considered a multi-factorial disease arising from an interaction between genetic susceptibility and environmental exposure. Although it 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, such as sunburn, genetic susceptibility, family history with cancer syndromes, numerous freckles, increased number or size of melanocytic nevi (benign accumulations of melanocytes), pre-existing dysplastic nevus, decreased DNA repair ability, tanning inability, aging, suppressed immune system, mutations in cyclin-dependent kinase inhibitor 2A (CDKN2A or p16) and cyclin-dependent kinase 4 (CDK4) participate in development and progression of melanoma [3, 5]. Certain phenotypic characteristics, such as fair skin, red hair, light eyes, numerous freckles, sun sensitivity, and an inability to tan, raise the risk of developing melanoma by approximately 50% [5].

2.1.2 Diagnosis

Various diagnosis include (i) skin self-examination, (ii) dermoscopy, and (iii) total-body photographic images and short-term surveillance [5]. Skin self-examination offers a lot of potential as a quick, easy way to check for precancerous lesions and melanoma. The “ABCD” criteria were developed in 1985 for the diagnosis of melanoma (Figure 2. 4). The “ABCD” acronym stands for “Asymmetry,” “Border irregularity,” “Color variegation,” and “Diameter >6 mm”, respectively. Later, the letter “E” was added, which represents “Evolving,” which is especially important for the diagnosis of nodular melanomas. These criteria act as a simple tool to alert both the public and non-dermatologists in differentiating common moles from cutaneous lesions most suspicious for early melanoma. Other clinical approaches have been

established to improve early diagnosis, such as the "Glasgow 7-point checklist", which includes 3 major criteria (change in size, shape, color) and 4 minor criteria (sensory change, diameter of 7 mm or greater; and the presence of inflammation, crusting or bleeding) [5].

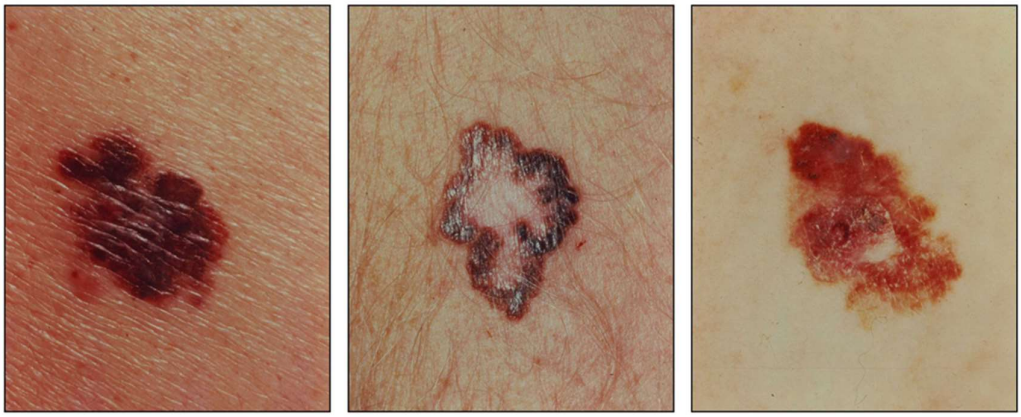

Figure 2. 4 Melanomas with characteristic asymmetry, border irregularity, color variation, and large diameter (ABCD).
Image adapted from NCI, USA

Dermoscopy is a non-invasive diagnostic technique that includes the use of various assistive optical devices to visualize morphological structures for the diagnosis of melanoma [5]. Some melanomas are difficult to diagnose, both visually and dermoscopically. But, it is possible to produce images that can be electronically captured, archived, retrieved, and analyzed. Reflectance confocal microscopy has been shown to be a valuable imaging tool in the diagnosis of malignant melanocytic lesions [5].

2.1.3 Progression of melanoma

The multistep developmental phases of melanoma include acquired nevi formation due to amplified proliferation of melanocytes, abnormal differentiation with dysplastic nevi, radial growth phase to develop primary tumor at the epidermis, vertical growth phase with invading potential to deeper dermis layer, and metastatic lesion formation

at distant visceral organs leads to malignancy [37]. Melanoma is classified into various stages (Stage 0, Stage I, Stage II, Stage III, Stage IV) based on the thickness of the tumor, its spreading to lymph nodes or other parts of the body, and other factors.

2.1.4 Types of melanomas

There are two main types of melanomas: (i) cutaneous melanoma and (ii) mucosal melanoma. Based on clinical and histological characteristics, CM is divided into superficial spreading melanoma, lentigo maligna melanoma, nodular melanoma, acral lentiginous melanoma, and desmoplastic melanoma [5]. Certain other types of melanomas have also been reported, especially head and neck melanoma, anorectal, vulvovaginal, myxoid melanoma, balloon cell melanoma, rhabdoid melanoma, and osteogenic melanoma [5].

2.1.5 Pathogenesis

Excessive exposure to ultraviolet (UV) radiation causes genetic mutations, DNA damage, and melanoma [2, 3, 5]. Mutations in CDKN2A or p16 and CDK4 participate in the development and progression of melanoma [3, 5]. The hyperactivation of major signal transduction pathways, such as mitogen-activated protein kinase (MAPK) due to mutation in BRAF and NRAS genes and phosphatidylinositol-3-kinase (PI3K) by multiple factors leads to the development of melanoma (Figure 2. 5) [2, 6, 7]. The activation of oncogenic NRAS causes the downstream activation of both MAPK (Ras/Raf/MEK/ERK) and PI3K/Akt pathway [37].

MAPK pathway is a signal transduction cascade forwarding extracellular signals to the nucleus through a series of consecutive phosphorylation events, while attachment of various growth factors occurs to plasma membrane growth factor receptors, such as fibroblast growth factor receptor (FGFR), epidermal growth factor receptor (EGFR),

or Platelet-derived growth factor receptors (PDGFR) [7, 37]. After receiving growth stimuli by plasma membrane receptor tyrosine kinase (RTK), the activation of proto-oncogene RAS occurs by converting inactive RAS-guanosine diphosphate (RAS-GDP) to active RAS-guanosine triphosphate (RAS-GTP) [38]. The active form of RAS recruits RAF from the cytosolic region to the cell membrane, where it becomes activated by phosphorylation [39]. Activated RAF then phosphorylates MAP kinase extracellular signal-regulated kinases 1 and 2 (MEK1/2), which in turn activates extracellular signal-regulated kinases 1 and 2 (ERK1/2) by phosphorylation at specific Thr and Tyr residues [7, 40]. The ultimate ERK in the MAPK pathway translocates to the nuclear region and phosphorylates several nuclear transcription factors (CREB, Elk-1, Myc, Fos, and others), boosting the proliferation, differentiation, and survival of multiple cell types. In most of the melanoma, abnormal activation of the MAPK cascade and extracellular signal-regulated kinases (ERK) hyperphosphorylation occurs due to mutations of BRAF (50-70%) and NRAS (15-30%) genes [7, 41]. BRAF mutation through substitution of glutamic acid for valine at codon 600 in exon 15 occurs (Val600Glu; B-RafV600E) and is very common in case of cutaneous melanoma; however, its incidence is rare in conjunctival, mucosal, and uveal melanomas [42]. BRAF, also referred to as the proto-oncogene B-RAF, is a serine/threonine-protein kinase. B-RAF, A-RAF, and C-RAF (also known as RAF-1) constitute the RAF kinase family. However, the BRAF mutation is very rare in mucosal, acral, conjunctival, and uveal melanomas [43]. Mutation in both NRAS and BRAF rarely coexist in melanoma, conveying that mutation in BRAF or NRAS alone is capable of activating the MAPK pathway [7].

Again, in response to activated RTK receptors, the PI3K causes the phosphorylation of phosphatidylinositol- 4, 5-biphosphate (PIP2) to phosphatidylinositol-3, 4, 5-

triphosphate (PIP3). The PIP3 causes the activation of Akt (Ak strain transforming kinase), which phosphorylates and activates the major downstream effector proteins of the PI3K pathway that promote cell proliferation and survival [44, 45]. The lipid phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) negatively regulates this cascade through dephosphorylation of PIP3 [45]. The deregulation of this pathway due to the deletion of PTEN or through oncogenic RAS leads to the development of melanoma [7]. It has been observed that elevated levels of Akt in nearly 70 % of cutaneous melanomas in comparison to healthy melanocytes [37]. There are three Akt family members, Akt, Akt 2, and Akt 3, known as important downstream effectors that relay the signal transduction cascade coming from PI3K. Akt 3 plays a major role in melanoma genesis. Earlier studies reported that ~70 % of cutaneous melanomas have elevated Akt expression compared to normal melanocytes.

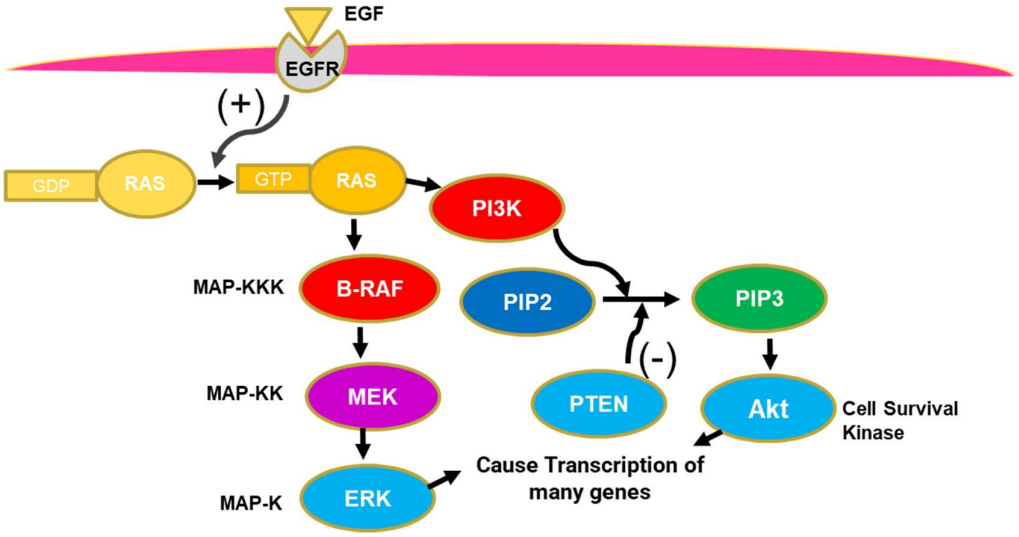

Figure 2. 5 Mitogen-activated protein kinase (MAPK pathway) and phosphoinositide-3-OH kinase (PI3K/Akt)

Moreover, immune suppression and immune escape also contribute to melanomagenesis. Tumor infiltrating T lymphocytes (TILs) are important effector cells that have the ability to recognize and kill tumor cells. Melanoma cells can

upregulate the expression of programmed death ligand-1 (PD-L1), which specifically binds to the PD-1 receptors expressed on the surface of TILs and further inhibits the effector function of TILs through the PD-1/PD-L1 interaction.

2.1.6 Current treatment strategies for melanoma

There are various types of treatment for melanoma patients, out of which some treatments are standard (the currently used treatment), and some are being investigated in clinical trials. Mainly, 5 types of standard treatments are currently used, such as (i) surgery, (ii) chemotherapy, (iii) radiation therapy, (iv) immunotherapy, and (v) targeted therapy. Certain new types of treatment (e.g., vaccine therapy) are being tested in clinical trials.

Chemotherapy uses chemotherapeutics to stop the growth of cancer cells, either by killing the cells or by stopping them from dividing, and the course of chemotherapy depends on the type and stage of the melanoma being treated. Certain chemotherapeutics approved by the Food and Drug Administration (FDA) for the treatment of melanoma include dacarbazine, interferon alpha (IFN-α), and interleukin-2 (IL-2). Dacarbazine has been used as first-line therapy for the treatment of melanoma since its US FDA approval in 1976. IFN-α is used for adjuvant immunotherapy in advanced melanoma. High-dose IL-2 was approved in 1998 for melanoma treatment [43].

Immunotherapy uses the patient's immune system to fight against melanoma. They boost, direct, or restore the body's natural defenses system against melanoma. Immune checkpoint inhibitors block proteins (i.e., the checkpoints) that are made by some types of immune cells (T cells) and some cancer cells. During the blocking of these checkpoints, T lymphocytes attack cancer cells more effectively. They are effective in

the treatment of some patients with advanced melanoma or tumors that cannot be removed by surgery. There are two types of immune checkpoint inhibitor therapy: cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4 inhibitor) therapy and programmed cell death protein 1 (PD-1) and programmed cell death ligand 1 (PD-L1) inhibitor therapy [46]. Ipilimumab is a type of CTLA-4 inhibitor. Atezolizumab is a PD-L1 inhibitor that is being studied in combination with cobimetinib and vemurafenib for melanoma treatment. Other immune therapies, such as Interleukin-2 (IL-2) and tumor necrosis factor (TNF) are also used for the treatment of melanoma. The IL-2 boosts the growth and activity of many immune cells, especially lymphocytes that can attack and kill cancer cells [46]. White blood cells produce the protein TNF in response to an antigen or infection. The TNF is being reported in the treatment of melanoma [46].

Targeted therapies usually cause less harm to normal cells than chemotherapy or radiation therapy. It includes signal transduction inhibitor therapy and oncolytic virus therapy. Inhibitors of signal transduction stop the transmission of signals within cells from one molecule to another, which is necessary for the survival, growth, and replication of cells. Blocking these signals may kill cancer cells [46]. *BRAF inhibitors (*e.g., dabrafenib, vemurafenib, encorafenib) block the activity of proteins made by mutation of BRAF genes. *MEK inhibitors* (e.g., trametinib, cobimetinib, and binimetinib) block proteins called MEK1 and MEK2, which affect the growth and survival of cancer cells. Virus is used in oncolytic virus therapy that infects and destroys cancer cells but not healthy cells. Chemotherapy and radiation therapy may be given after oncolytic virus therapy. "*Talimogene laherparepvec*" is a type of oncolytic virus therapy produced with a form of the herpesvirus that has been modified

in the laboratory. During treatment, it is injected directly into the tumors site at the skin and lymph nodes [46].

Certain new treatments (vaccine therapy) are being tested in clinical trials. Vaccine therapy is a cancer treatment that uses a substance or group of substances to stimulate the immune system to find the tumor and kill it. A melanoma peptide antigen vaccine (gp100) was investigated in combination with IL-2 [43].

2.1.7 Various *in-vivo* tumor models with special reference to the syngeneic tumor model

There are various pre-clinical melanoma models that have been developed to achieve an in-depth understanding of tumor biology and have great translational value in the diagnosis, treatment, and prevention of melanoma. These *in vivo* models reflect the true melanoma microenvironment. Xenograft, syngeneic, and genetically engineered models (GEM) are the most widely used pre-clinical murine models that provide a significant understanding of melanoma progression [43, 47].

Xenograft models are of two types, namely cell line xenografts and patient-derived tumor xenografts (PDTXs). Cell line xenograft models involve the inoculation of human melanoma cells (e.g., WM164 and WM793B) into an immune-deficient murine model, such as nude athymic (nu/nu) mice that lack T-lymphocytes or severe combined immune-deficient (SCID/SCID) mice. In the PDTXs model, patient-derived tumors are implanted into immunocompromised mice, such as athymic nude or NOD/SCID IL-2 receptor gamma chain knockout mice.

Syngeneic models are established by inoculation of melanoma cells into the same species and genetic background. The most widely utilized cell lines (B16 cell line) were generated from C57BL/6J mice, which were induced by specific chemical

carcinogens. B16F1 and B16F10 cell lines are two well-established sub-clones by *in vivo* passaging of B16 cells. This model offers the advantages of (i) rapid growth and development of tumors, (ii) interaction of melanoma cells with immunocompetent T and B lymphocytes appearing naturally in the human melanoma microenvironment. Thus, provides valuable insights into melanoma immunology studies, as well as immunotherapy strategies. However, compared to human melanoma, the growth factors and adhesion proteins of murine cell lines are quite dissimilar.

Genetically engineered models (GEMs) develop upon transgenic mice with engineered gene expression specific to melanoma-genesis. GEMs include CDKN2A models, RAS model (Rat sarcoma virus), PTEN/BRAF models, and RET model (Receptor tyrosine kinase).

Physically, melanoma is induced by UV radiation. This model is similar to the natural human melanoma-genesis by UV radiation. Chemicals, such as 7,12-Dimethylbenz[a]anthracene (DMBA) and 12-O-Tetradecanoylphorbol-13-acetate (TPA) can both be applied topically to induce skin irritation and black lesions that develop into melanoma. They are mostly used in combination with UV and other genetically engineered models for the development of melanoma.

2.2 Role of plant extracts and phytoconstituents against melanoma

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 (Figure 2. 6) [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 rather than a single constituent or drug molecule.

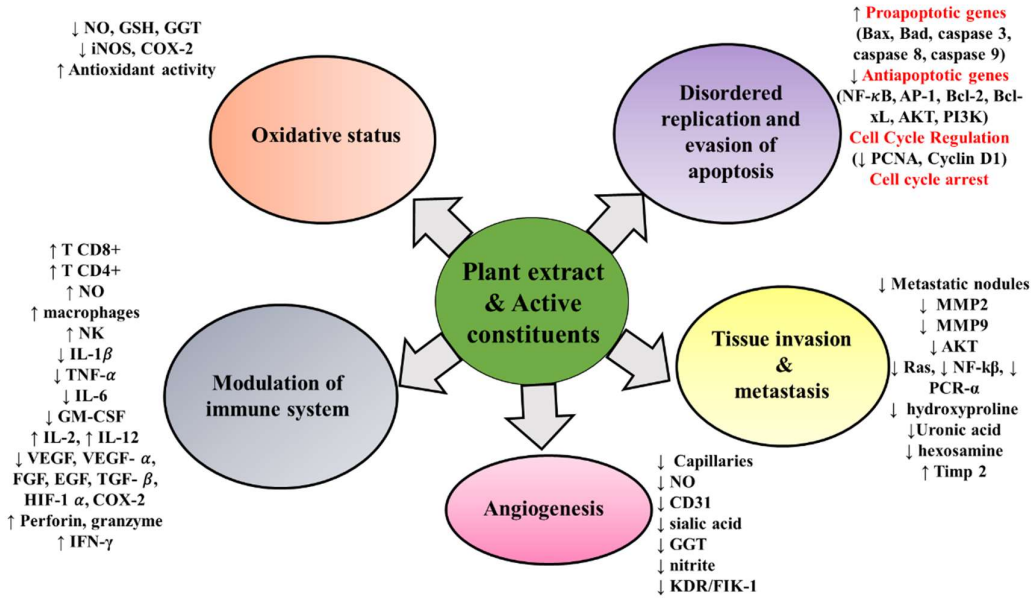

Figure 2. 6 Mechanism of action of various plant extracts and active phytoconstituents against melanoma

NF-κβ (nuclear factor kappa β), AP-1 (activator protein 1), Akt (Ak strain transforming protein kinase), PI3K (phosphatidylinositol-3-kinase), PCNA (proliferating cell nuclear antigen), MMP-2 and MMP-9 (metalloproteinases 2 and 9), PKCα (protein kinase C alpha), TIMP-1 and TIMP-2 (metallopeptidase inhibitors 1 and 2), NO (nitric oxide), GGT (gama-glutamil transpeptidase), KDR/FIK-1 (kinase insert domain receptor/fetal liver kinase 1), NK (natural killers), IL (interleukin), IFN-γ (interferon gama), TNF-α (tumor necrosis factor alfa),GM-CSF (granulocit-monocit colony-stimulating factor), VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor), EGF (epidermal growth factor), TGF-β (transforming growth factor-beta), HIF-1α (hypoxia-inducible factor-1α), COX-2 (cyclooxygenase 2), GSH (glutathione), and iNOS (inducible nitric oxide synthase)

2.3 Plant profile of *Piper longum*

Piper longum Linn (Indian long pepper) is one of the commonly used herbs and has been extensively used as various indigenous medicines, specifically in the traditional Indian Ayurvedic system of medicine. The whole plant and plant parts, such as the fruit, are traditionally used for various therapeutic uses. The fruits contain alkaloids, which contribute to their pungency. *Piper longum* was first written by Hippocrates, who described it as a medicament rather than a spice [11].

2.3.1 Geographical distribution

The plant grows in evergreen forests of India and is cultivated in Tamil Nadu, Assam, and Andhra Pradesh. In places with limestone soil, frequent rainfall, and high relative humidity, long pepper is grown on a huge scale [11].

2.3.2 Plant description

Piper longum is a small shrub with a large woody root and numerous creeping, jointed stems that are thickened at the nodes (Figure 2. 7a). The fruits, which grow in fleshy spikes 2.5−3.5 cm long and 5 mm thick, are oblong, blunt, and blackish-green (Figure 2. 7b). The leaves are alternating, spreading, stipule-free, and have a wide range of leaf sizes [11].

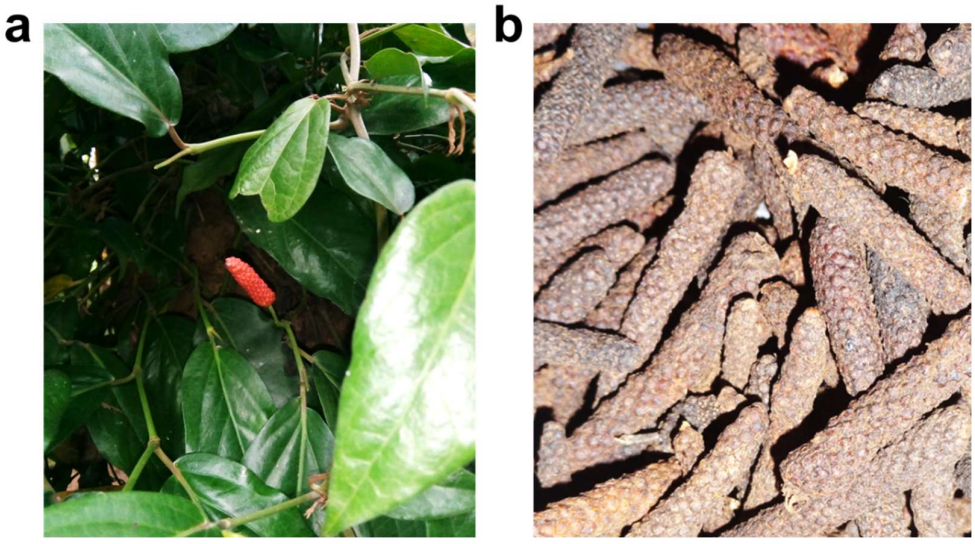

Figure 2. 7 Photograph of (a) *Piper longum* plant and (b) fruits

2.3.3 Scientific classification

- Botanical name: *Piper longum*
- Kingdom: Plantae
- Division: Magnoliophyta
- Class: Magnoliopsida
- Order: Piperales
- Family: Piperaceae
- Genus: *Piper*
- Species: *longum*

2.3.4 Synonyms

- Arabic: Dâr fulful (دار فِلْفِل)
- Bengali: Piplamor (পিপুল)
- Chinese: Bi bo (碧波), Bi ba gen (毕巴根)
- Dutch: Langwerpige peper
- English: Indian long pepper, Jaborandi pepper, Long pepper
- French: Poivre long
- German: Bengalischer Pfeffer, Jaborandi Pfeffer, Langer Pfeffer
- Gujuarati: Pipli (પીપલી)
- Hindi: Pipar, piplamul (पिप्पली)
- Hungarian: Bengáli bors
- Italian: Pepe lungo
- Kannada: Thippili (ತಿಪ್ಪಲಿ)
- Malaya: Magadhi, Pippali, Thippili, Tippili (തിപ്പലീ)
- Marathi: Pimpli (पिंपळी)
- Nepalese: Gaj pipla (गज पिपला), Saano pipla (सानो पिपला)

- Odia: Pipali (ପିପ୍ପଳୀ)
- Portuguese: Pimenta-longa
- Sanskrit: Pippali (पिप्पली)
- Swedish: Långpeppar
- Tamil: Kandan lippilli, pippili (பிப்லி), sirumulam, tippili, thippili
- Telugu: Pippallu (పిప్పళ్లతో)
- Turkish: Dar biberi

2.3.5 Chemical profile of fruit

The chemical profile of *P.longum* fruit is represented in Table 2. 1 [11].

Table 2. 1 Chemical profile of *P. longum* fruit

Alkaloids and related compounds	Piperine, methyl piperine, piperlongumine, piperlonguminine, pellitorine, pipernonaline, piperettine, asarinine, piperundecalidine, retrofractamide A, Retrofractamide C, pergumidiene, piperrolein B, brachystamide-B, desmethoxypiplartine, N-isobutyl decadienamide, brachyamide-A, brachystine, pipercide, piperderidine, longamide, dehydropipernonaline piperidine, tetrahydro piperine. tetrahydropiperlongumine, trimethoxy cinnamoyl-piperidine, 1-(3',4'-methylenedioxyphenyl)-1E-tetradecene, 3-(3',4'-methylenedioxophenyl)-propenal, piperoic acid, 3',4'-di-hydroxy-biabola-1, 10-diene, eudesm-4(15)-ene-1beta, 6-alpha-diol, 7-epi-eudesm-4(15)-ene-1beta, 6beta-diol, guineesine, and 2E,4E-dienamide, (2E, 4E, 8E) - Nisobutylhenicosa-2,4,8-trienamide
Lignans	Sesamin, fargesin, pulviatilol
Esters	Tridecyl-dihydro-p-coumarate, eicosanyl-(E)-p-coumarate, and Z-12-octadecenoicglycerol-monoester
Volatile oils	Caryophyllene, pentadecane, and bisabolene, thujone, terpinolene, zingiberene, pmethoxyacetophenone, dihydrocarveol, p-cymene, and vitamins A and E
Organic acids	Palmitic acid and tetrahydropiperic

2.3.6 Pharmacological profile

The fruits of the *P. longum* was reported to possess anticancer, melanin-inhibiting, immunomodulatory, antioxidant, anti-inflammatory, hepatoprotective, antimicrobial, antihyperlipidemic, analgesic, antifertility, antidepressant, antifungal, antiamoebic,

cardioprotective, coronary vasodilation, antiobesity, bioavailability-enhancing property activities [11].

2.3.7 Role of *Piper longum* fruit extract and contained phytoconstituents against melanoma

The extract of 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 (piperine: 0.04 mg/mL and piperlonguminine: 0.006 mg/mL) [48], limited dissolution, and *in-vivo* oral bioavailability of the majority of active constituents (piperine: 37.7±11.7% [49], piperlongumine: 50.08%) [19-21, 50]. Thus, the use of appropriate techniques to evade the solubility, dissolution, and

bioavailability issues is extremely crucial to realize the actual therapeutic effectiveness. The anticancer mechanism of *P. longum* extract and its active phytoconstituents against melanoma are shown in Figure 2. 8 & Table 2. 2.

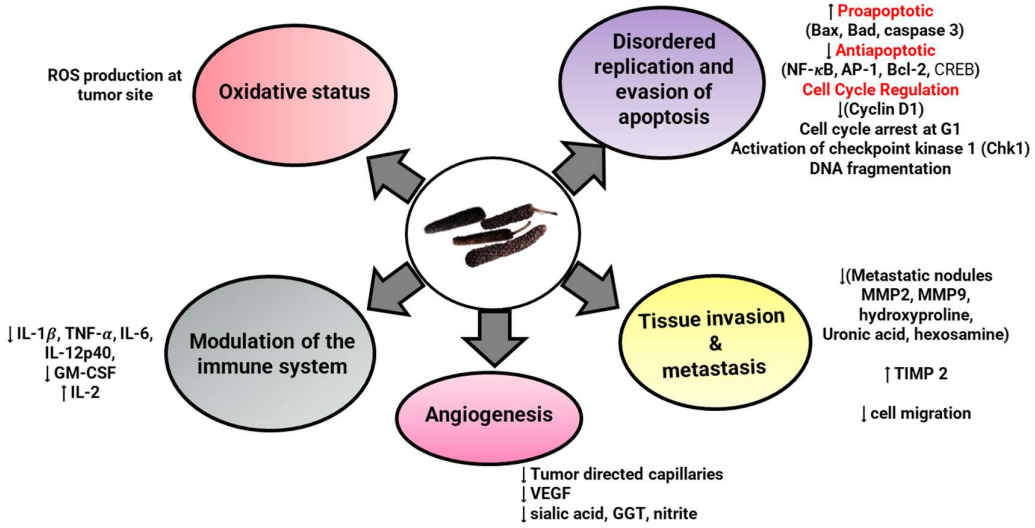

Figure 2. 8 Role of *Piper longum* in melanoma therapy

Table 2. 2 Studies on *Piper longum* and their constituents for melanoma therapy.

Plant species	**Anticancer activity and the reported mechanism**	**References**
Piper longum (Ethanolic extract)	Antiangiogenic activity in C57BL/6 mice: • ↓ Number of tumor-directed capillaries • ↓ Proinflammatory cytokine and VEGF • Increases IL-2 and TIMP-2 • Decreased cell migration of VEGF-induced migration of endothelial cells • Modulation of immune system	[18]
Piperine	• ↓ Proinflammatory cytokines (IL-1β, IL-6, TNF-α, IL-12p40, and GM-CSF) in B16F10 cells • Inhibition of NF-kB, ATF2, c-Fos, CREB • Inhibit MMP (MMP2 & MMP9)	[12]
Piperine	Inhibition of lungs metastasis in C57BL/6 mice: • Suppress tumor cell growth • ↓ Metastatic nodule formation	[14]

Piperine	• ↑ Survival rate and ILS • ↓ sialic acid, GGT, HP, Uronic acid • ROS production at tumor site • Imbalance of calcium homeostasis • Loss of mitochondrial membrane potential, caspase activations, and DNA fragmentation • Cell cycle arrest at G1 • Increase in the ratio of Bax to Bcl-2 • Upregulate the expression of apoptosis-inducing factor (AIF) • Alterations in the expression of MAPK family proteins and PI3K–Akt survival signals • Disrupt NF-kB signaling • Reversed multi drug resistance (MDR) by reducing UVB-induced p-glycoprotein activity	[51]
Piperlongumine	• Induce apoptosis via reactive oxygen species-mediated disruption of mitochondria in human melanoma (A375, A875) and murine melanoma (B16F10)	[15]
Piperlonguminine	• Suppress melanogenesis	[16, 17]

2.4 Solid dispersion (SD)

Out of various routes of administration, the oral route is mainly preferred due to better patient compliance, avoidance of pain, reduced therapy cost, etc. However, most phytoconstituents possess limited aqueous solubility, poor membrane permeability, and gastric instability during oral administration, thus producing poor bioavailability [52, 53]. Several attempts have been made to overcome the above issues through F&D of Novel drug delivery systems (NDDS), such as solid dispersions, nanoparticles, nanocapsule, microparticles, microspheres, emulsion, phospholipid complex, liposomes, phytosomes, cyclodextrin, etc. [52, 54, 55]. These NDDS improve solubility, enhance drug absorption, increase bioavailability, reduce the required dose as well as side effects, offer control release characteristics, enhance the stability of

constituents inside body fluid and gastric environment, and improve therapeutic activity [52, 53]. From a stability point of view, solid dosage forms are considered more stable than liquid or semisolid dosage forms [56]. Thus, NDDS with more excellent stability, scale-up potential, and ease of manufacturing ability should be adapted to present multiconstituent-based herbal extract or enriched fractions to amplify their inherent therapeutic efficacy.

One of the interesting NDDS is solid dispersion (SD), which can enhance the solubility and oral absorption of poorly soluble candidates. SD allows the homogeneous dispersion of drugs (in the form of solid, liquid, or gas) in the carrier matrix (CM), stabilizes unstable drugs, enhances solubility, absorption, bioavailability, and also offers fast, prolonged, or sustained release profile of incorporated drug candidates [27]. SD was widely exploited with the incorporation of herbal extracts or enriched fractions or isolated bioactive, thereby enhancing their solubility, stability, dissolution, absorption, bioavailability, and ultimate therapeutic response [57-60].

Solid dispersion, developed by Sekiguchi and Obi in 1961, describes the dispersion of one or more drugs into inert CM at a solid state formulated by melting, solvent, or melt-solvent technique rather than simple dispersion of drugs in the solid carrier by physical mixing [27]. Being a simple, efficacious dosage form, it offers the successful delivery of poorly water-soluble drugs using a wide variety of excipients via multiple formulation approaches [24, 29]. The enhanced solubility, dissolution, and greater bioavailability of the drug in SDs are ascribed to various reasons shown in Figure 2. 9 [22, 24, 27]. The enhanced dissolution of SD can be understood through the "spring and parachute" concept. The attainment of supersaturation (spring) is achieved during the dissolution of the drug along with CM, and the supersaturation is maintained for a

long time (parachute), thereby providing a greater concentration of dissolved drug for drug absorption [24, 28, 29]. The presence of CM hinders the nucleation and crystal growth of supersaturated solution, hence maintaining it for a prolonged time. The reduced particle size in SDs provides greater effective surface area and decreased thickness of the stagnant layer, increasing the solubility and dissolution. The improved solubility and dissolution lead to rise maximum absorbable dose (MAD), absorption rate, and, ultimately the bioavailability [29].

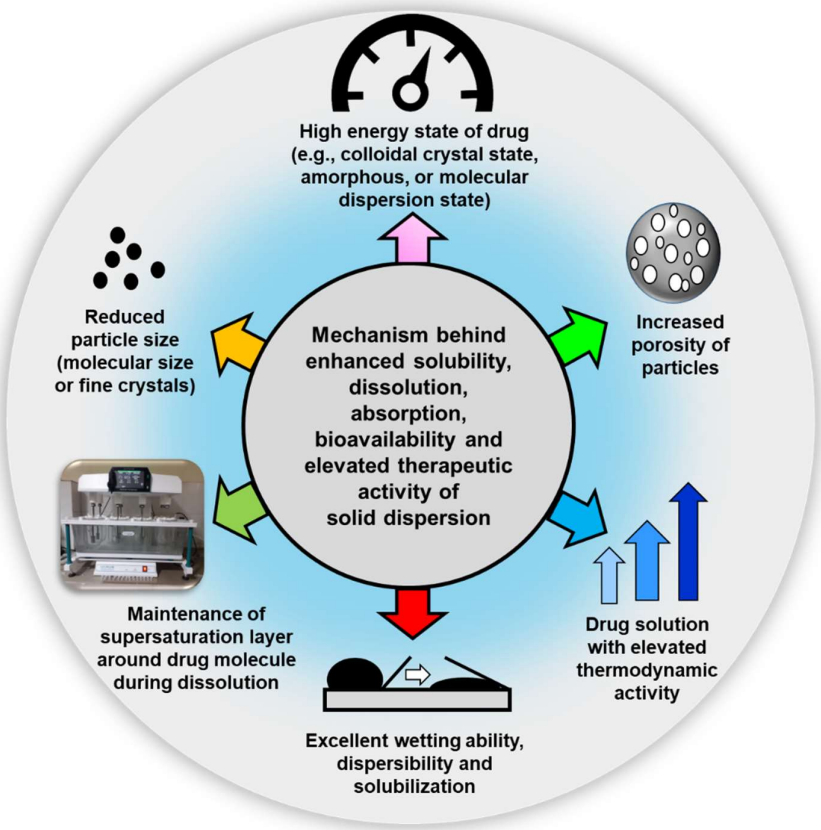

Figure 2. 9 The mechanism behind enhanced solubility, dissolution, absorption, bioavailability, and elevated therapeutic activity of solid dispersion

2.4.1 Advantages & disadvantages

- Improved solubility, dissolution, and bioavailability
- Capacity to load solid, liquid, or gaseous drugs

- Improved stability
- Taste masking
- Control over the release of drugs from the dosage form
- Enhanced therapeutic activity

However, some limitations such as moisture absorbing capacity & phase separation, toxicity associated with residual solvents, chances of physical instability due to conversion to more stable crystalline form during prolonged storage, and difficulty in attending to the reproducibility of physicochemical properties should be considered for designing stable SD [30, 61, 62].

2.4.2 Classification of solid dispersion

SDs are classified as per the arrangement of the drug, the physical state of the drug & CM, and as per generation. Considering the arrangement of the drug in CM, SD is mainly divided into four types: (i) partially dissolved with excess drug existing in crystalline form, (ii) partially dissolved with excess drug existing in amorphous form, (iii) completely dissolved or molecularly dispersed form & (iv) colloidal dispersion [22, 24, 28]. Based upon the physical state of the drug and CM, SD is classified into six major groups, such as simple eutectic mixture (Type-1), solid amorphous suspension/ amorphous precipitations (Type-2), solid solution/ mixed crystal (Type-3), glass suspension (Type-4), glass amorphous suspension (Type-5) & glass solution (Type-6) [24, 27]. Besides, compound or complex formations and combinations of these also exist sometimes [27]. Based on knowledge, the complexity of the system & type of CM used, the SDs are classified into first-generation SDs (e.g., simple eutectic mixture) contain low molecular weight crystalline CM (e.g., urea, sugars, etc.); 2nd generation SDs (e.g., glass solutions) contain amorphous polymeric CM (e.g., Polyvinylpyrrolidone, polyethylene glycol, crospovidone, etc.); 3rd generation SDs use polymers with surfactant/emulsifier as CM (Gelucire 44/14, poloxamer, etc.) &

4th generation SDs use Eudragit® RS, RL, Carbopol®, Soluplus®, etc. to produce controlled release formulation for drugs having poor water solubility, short biological half-life, narrow therapeutic window [61].

2.4.3 Excipients used for SD

Commonly employed excipients in SD are shown in Table 2. 3 [22, 61, 62].

Table 2. 3 Commonly employed excipients/carrier matrix in solid dispersion

Types	Example of carrier matrix (CM)
Polymers	Polyvinylpyrrolidone (PVP), Polyvinylpyrrolidone-polyvinylacetate copolymer (PVPPVA), poly(vinylpyrrolidone-co-vinyl acetate) (PVP/VA), Polyvinylcaprolactam–Polyvinyl Acetate–Polyethyelne Glycol Graft Copolymer (Soluplus®), Polyvinylalcohol (PVA), Crospovidone (PVP-CL), Carboxymethylcellulose (CMC), Polyethylene glycol-4000 (PEG-4000), PEG-6000, Guar gum, Xanthan gum, Sodium alginate, Methyl cellulose (MC), Hydroxypropylcellulose (HPC),Hydroxypropylmethylcellulose(HPMC), Eudragit® L100, Carboxymethylethylcellulose (CMEC), cellulose acetate phthalate (CAP), Hydroxypropylmethylcellulose phthalate (HPMCP), etc.
Sugars	Sucrose, Mannitol, Sorbitol, Dextrose, Maltose, Galactose, Lactose, Soluble starch, D- glucose (Chitosan), Galactose, Xylitol, British gum, Galactomannan, Amylodextrin, Dextrin, ß-CD, HPß-CD
Acids	Phosphoric acid, Citric acid, Succinic acid, Tartaric acid
Surfactants	Sodium lauryl sulfate (SLS), Tweens and Spans, Polyoxyethylene stearate, Poloxamer (Poloxamer 188, Poloxamer 407), Deoxycholic acid, Docusate sodium, Myrj-52, Gelucire 44/14, Gelucire 50/13, Pluronic- F68, Tocopheryl Polyethylene Glycol Succinate (TPGS), Compritol 888 ATO, Inutec SP1
Carboxypoly methylene	Carbopol 947, Carbopol 907
Dendrimers	Starburst, Polyamidoamine (PAMAM)
Hydrotropes	Sodium p-hydroxy benzoate, Sodium-o-hydroxy benzoate, Sodium acetate, Sodium citrate, Ascorbic acid, Resorcinol
Others	Skimmed milk, Hydroxyalkyl xanthenes, Pentaerythritol, Urea, Urethane, Dicalcium phosphate, Microcrystalline cellulose, Silica gel, Sodium chloride

2.4.4 The need for SD formulation of herbal extracts, enriched fractions, and isolated bioactive

The need for SD formulation of herbal extracts, enriched fractions, and isolated bioactives is presented in Figure 2. 10.

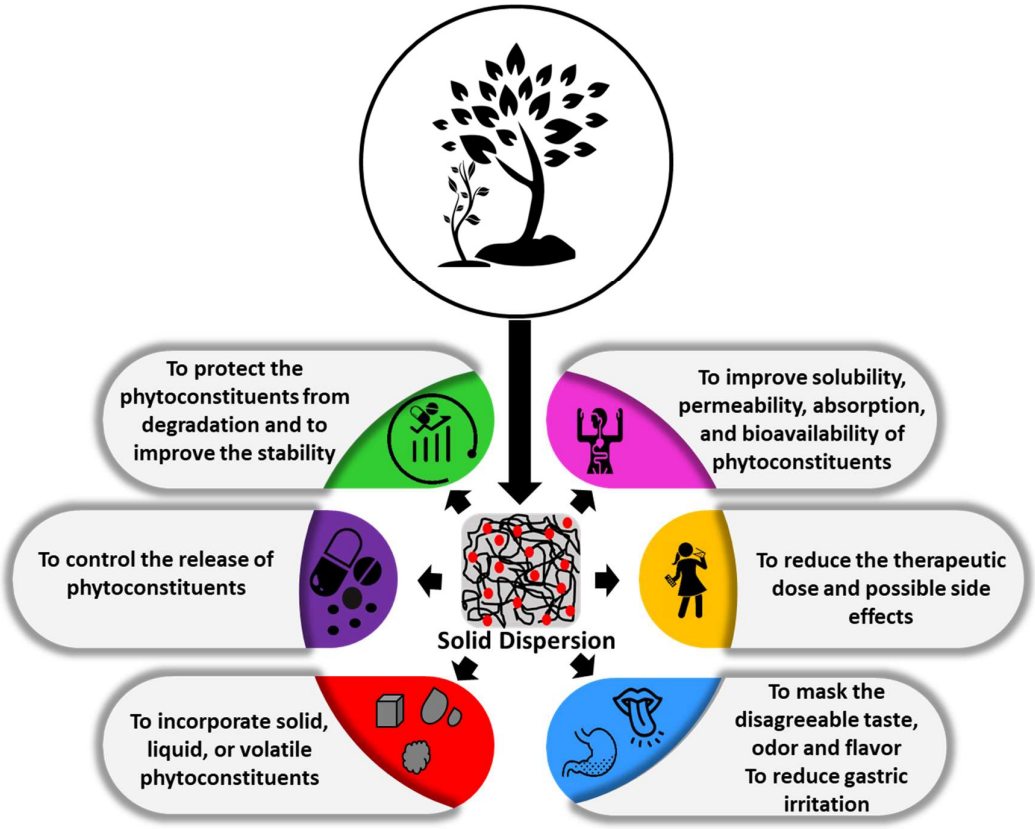

Figure 2. 10 Need for SD formulation of herbal extracts, enriched fractions, and isolated bioactives

2.4.5 Method of preparation with special reference to solvent method

The methodologies (Figure 2. 11) are broadly classified as melting or fusion, solvent method, and melting-solvent method [24, 27]. Other advanced methods are also used nowadays to improve product performance. Out of various methods, the methods that are easy, highly scalable, and have a lower degrading ability to incorporate phytoconstituents should be considered.

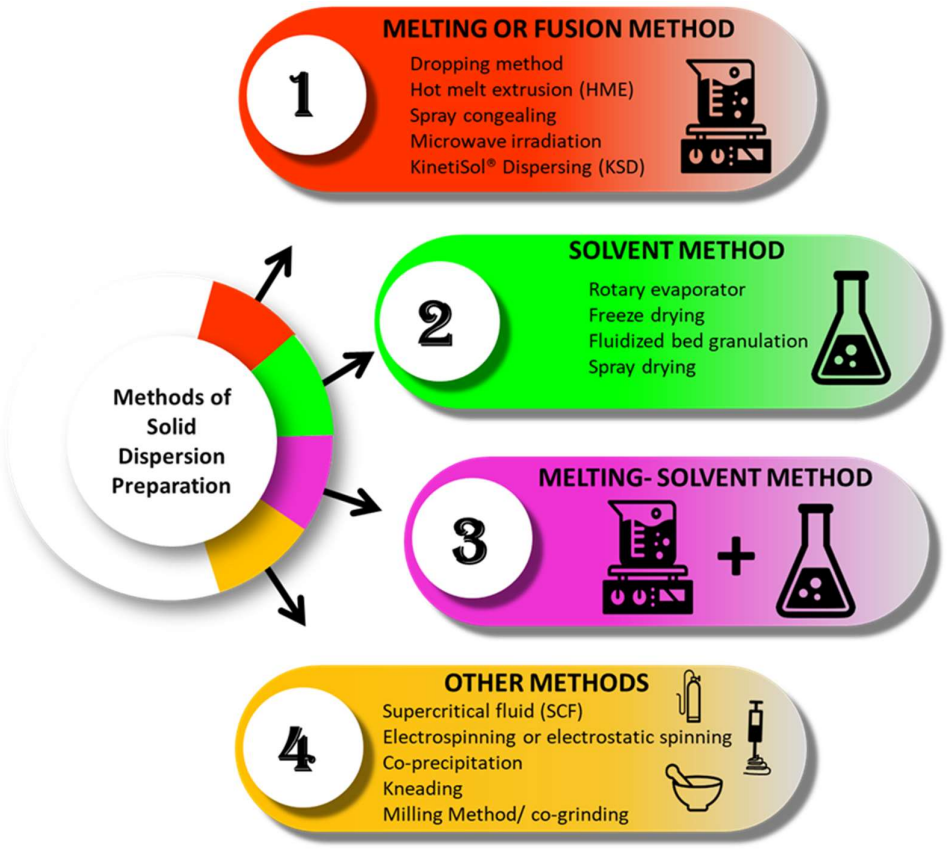

Figure 2. 11 Various methods of preparation of SD

2.4.5.1 Solvent method

SD is prepared by solubilizing the physical mixture of both drug and CM in a common organic solvent followed by removal of the solvent by suitable evaporation technique (rotary evaporator, freeze-drying, fluidized bed granulation, spray drying) [22, 24, 27]. The solvent with low toxic potential, mainly class III solvents (e.g., Ethanol, 1-propanol, 2-propanol, 1-butanol, Acetic acid, 1-pentanol, Isopropyl acetate) & class IV solvents (e.g., isopropyl ether, Petroleum ether) commonly used instead of toxic solvents[30]. The temperature (25-65°C) used for the removal of the solvent is lower than that used in the fusion method [24]. Thermal decomposition of the drug or CM could be avoided, hence suitable for thermolabile drugs and some phytoconstituents [27]. Again, the selection of a common organic solvent is quite easier than choosing a

common melting point of herbal extract/enriched fraction & CM; hence, it is suitable for incorporating multiconstituent extract/enriched fraction. Among other methods, the rotary-based solvent evaporation method was widely studied for the incorporation of multiconstituent-based standardized herbal extract/enriched fraction [58, 63-65] and isolated phytoconstituents [66-69]. Among them, rotary-based evaporation is widely explored for the lab-scale formulation of SDs, owing to advantages like simplicity, economical, safety, suitability for heat-sensitive phytoconstituents, requiring a short period, and avoiding the use of sophisticated instruments. Sometimes, difficulty in the complete removal of solvent leads to chemical instability, plasticity & toxicity of the finished product [24, 27].

2.4.6 Chemistry of CM-phytoconstituents interaction in SD

The drug/phytomolecule interacts with CM through weak interaction, such as hydrogen bonding, hydrophobic interactions, electrostatic, ionic & Van der Waals forces [70, 71]. During the inclusion of the drug (amorphous form) into the cross-linked 3-dimensional polymer matrix, these weak interactions restrict the molecular mobility of the drug, prevent recrystallization & offer stability to the finished product. Various underlying mechanisms for stabilization include increased configurational entropy, decreased molecular mobility, increased Tg, increased drug-CM interaction, reduced chemical potential, and the presence of a physical barrier [70].

2.4.7 SD of herbal extracts and isolated plant bioactives

Recent research works carried out with SD of herbal extract are summarized in our review article [25]. The SD of various plant extracts and isolated bioactives showed improved solubility, dissolution, absorption, bioavailability, and therapeutic outcomes [25]. In one of the recently reported works on SD of standardized *Polygonum cuspidatum* extract (PCE), the improved dissolution and oral bioavailability of

"resveratrol" as a representative marker was reported. The SD of standardized ethanolic PCE was prepared by hot melt extrusion using hydroxypropyl acetate methylcellulose succinate, which demonstrated excellent pharmaceutical possessions, improved dissolution of resveratrol (46.75 ± 0.47% to 130.06 ± 0.12%.), and a 1.44-fold increase in oral bioavailability [72].

2.5 Transdermal drug delivery system (TDDS)

A transdermal drug delivery system (TDDS) is a promising strategy for the delivery of plant bioactives through the skin.

Advantages and hurdles of TDDS

The TDDS offers the following advantages [31]:

- Non-invasive site-specific drug delivery
- Better patient convenience
- Prolonged drug release
- Extended duration of the activity
- Reduced side effects
- Avoids gastric degradation, gastric irritation, and first-pass metabolism

Hurdles of TDDS

- 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 (Figure 2. 12).
- 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].

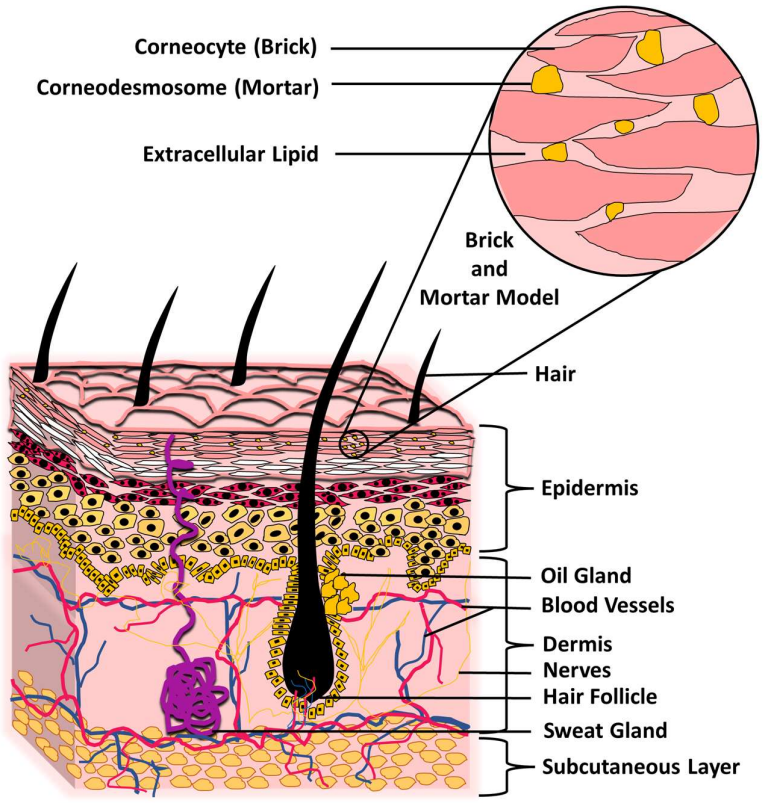

Figure 2. 12 Structure of skin

2.5.1 Novel formulation strategies to avoid the hurdles of transdermal permeation

The issues of TDDS may be solved through novel topical formulations, such as transferosomes (TFs), phytosomes, liposomes, ethosomes, noisomes, 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].

2.5.2 Transferosome (TFs)

These are ultradeformable nanovesicles with an average vesicular diameter within 100 to 200 nm [35], primarily composed of three basic components, such as phospholipids, surfactant/edge activators (EA), and water Figure 2. 13 [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]. Due to the ultradeformability, they have the capacity to cross 5 to 10 times smaller pores than their vesicular diameter. The maintenance of a transdermal osmotic gradient is the driving force behind the transportation of TFs across the skin [34]. The advantages of TFs were shown in Figure 2. 14 [34]:

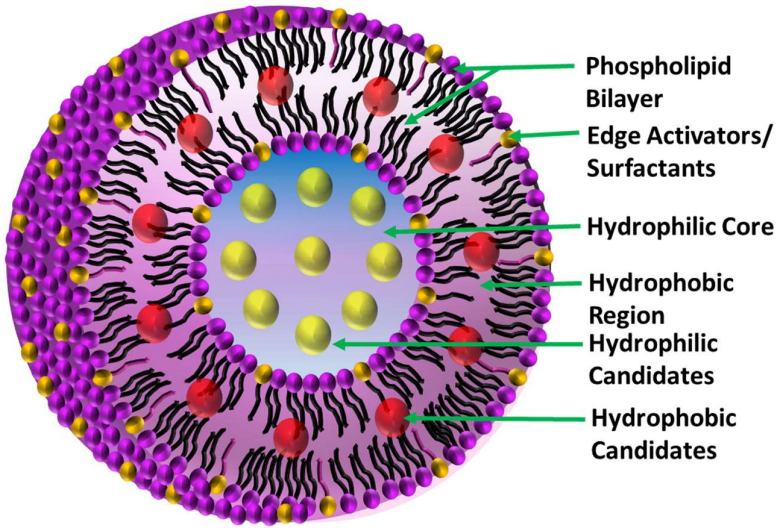

Figure 2. 13 Composition & structure of transferosome

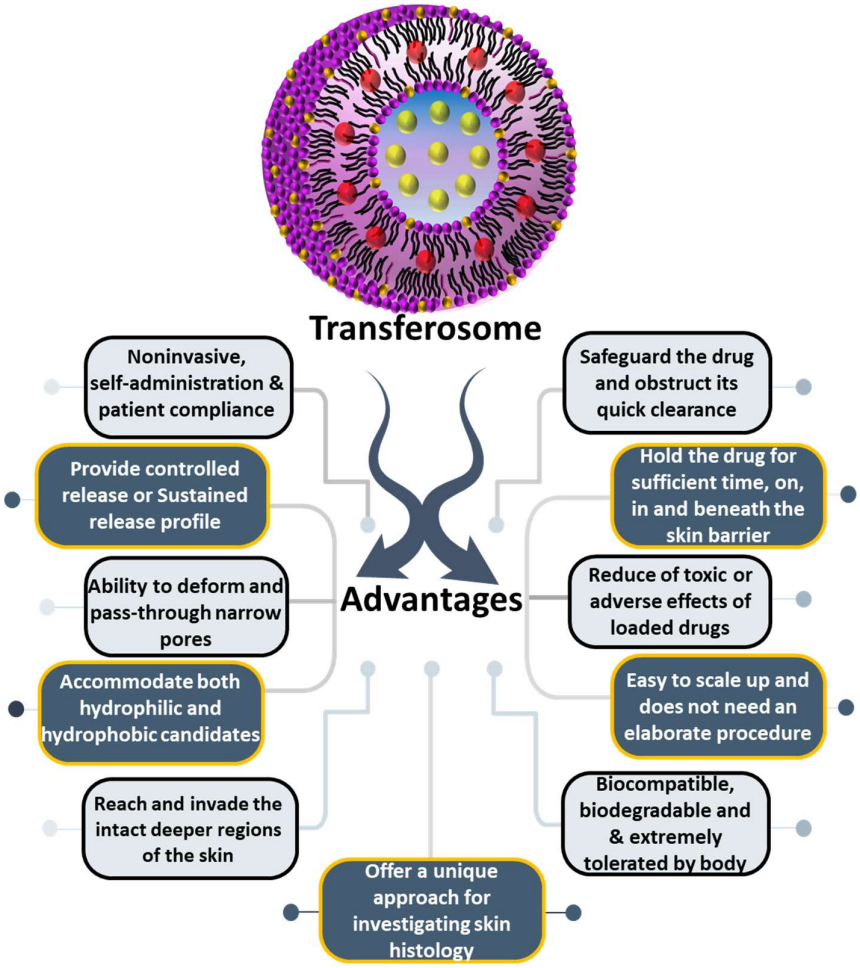

Figure 2. 14 Advantages of TFs

2.5.3 Methods for formulation of TFs

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 (Figure 2. 15) [33, 34]. Among them, the thin-film hydration-based rotary evaporation is widely explored for the lab-scale formulation of TFs, owing to advantages like simplicity, economical, safety, suitability for heat-sensitive phytoconstituents, requiring a short period, and avoiding the use of sophisticated instruments [33].

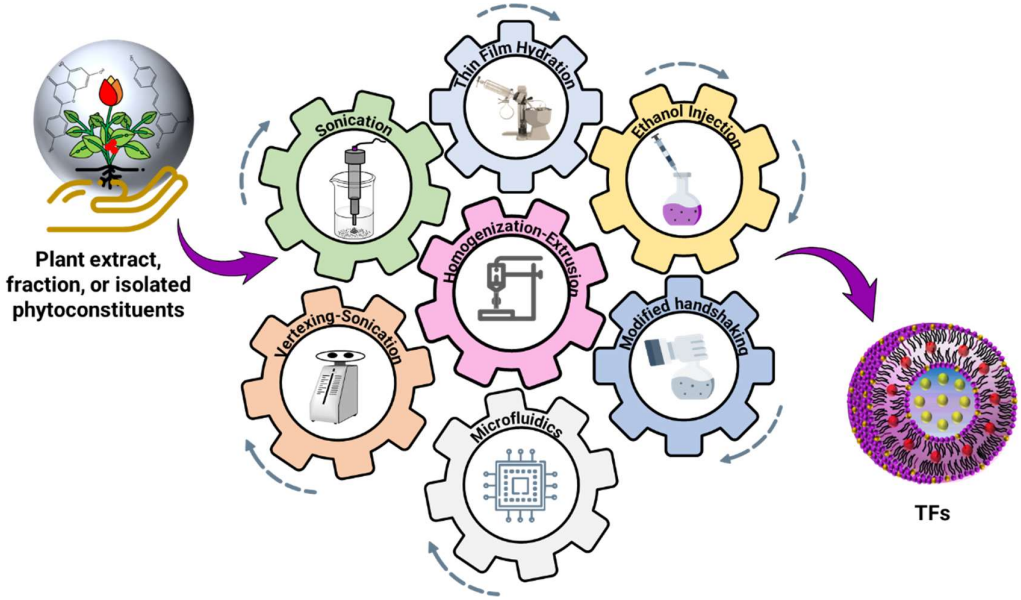

Figure 2. 15 Formulation methods of TFs

2.5.4 Transgelosome

TFs possess low viscosity, so they can be incorporated into suitable gelling agents to increase the viscosity to achieve prolonged residence time at the application site and improved patient compliance. The incorporation of TFs into gel develops transferosomal gel (TFG) or transgelosome, which offers the advantage of both TFs and gel.

The TFG offers the following advantages:

- Deeper skin penetration and retention
- Greaseless
- Thixotropic
- Easily spreadable and removable
- Prolonged retention at application site
- Provide sustained release or controlled release
- Suitable for delivery of hydrophilic and hydrophobic drug candidate
- Good patient compliance

2.5.5 TFG of multiconstituent-based herbal extract and isolated bioactives

Phytoconstituents, either in their purified form or in the mixture, have been widely investigated and loaded into TFG for better transdermal delivery to maximize their efficacy. The TFG and TFs of herbal extract and isolated phytoconstituents showed improved pharmaceutical possessions, *in-vitro* or *ex-vivo* skin permeation, and therapeutic properties compared to unformulated neat extract and constituents [34].

In one of the recent reports (in 2024), TFG of standardized methanolic extract of *Solanum xanthocarpum* was reported for its improved skin permeability. The TFG was prepared using phospholipon 90G, cholesterol, sodium cholate, and carbopol 934 as gelling agent. Chlorogenic acid was considered a representative marker in the experiment. The *ex-vivo* skin permeation tests revealed significantly higher permeation of TFG (82.86 ± 2.38%) compared to the extract-loaded plain gel (35.28 ± 1.62%). The dermal kinetic study revealed significantly improved results of TFG (710.87 ± 17.00 μg/cm^{2}h) compared to the extract-loaded plain gel (399.07 ± 11.00 μg/cm^{2}h). The confocal laser scanning microscopy photomicrographs also revealed the significant deeper skin permeation across the skin of Wistar rats using rhodamine-B as a fluorescent marker [73].