2

Review of literature

The review of literature pertaining to the study entitled **"Comparative Evaluation of Liposome Encapsulated *Hygrophila auriculata* (Schumach.) Heine Root and Betulin on Wound Healing Activity through *In silico*, *In vitro* and *In vivo* approaches"** is furnished below

2.1. Skin: A structural elucidation

The skin is the most significant organ of the body in vertebrates which occupies an area of approximately 2 m^2, representing about one-tenth of the body mass. This complex organ has highly specialized functions. Based on the anatomy and function, the skin has two distinctive layers: the epidermis and dermis. A few authors quote a third layer, the hypodermis or subcutaneous layer, which mainly comprises of fat and a layer of loose connective tissue as shown in Fig. 1 (Oomens *et al.,* 2015). It protects the organism from toxins and microbes, allows regulation of body temperature, provides support to the blood vessels and nerves, and prohibits dehydration of all non-aquatic animals (Morgado *et al.,* 2014). Other critical functions of this organ are associated with immune surveillance and sensory detection.

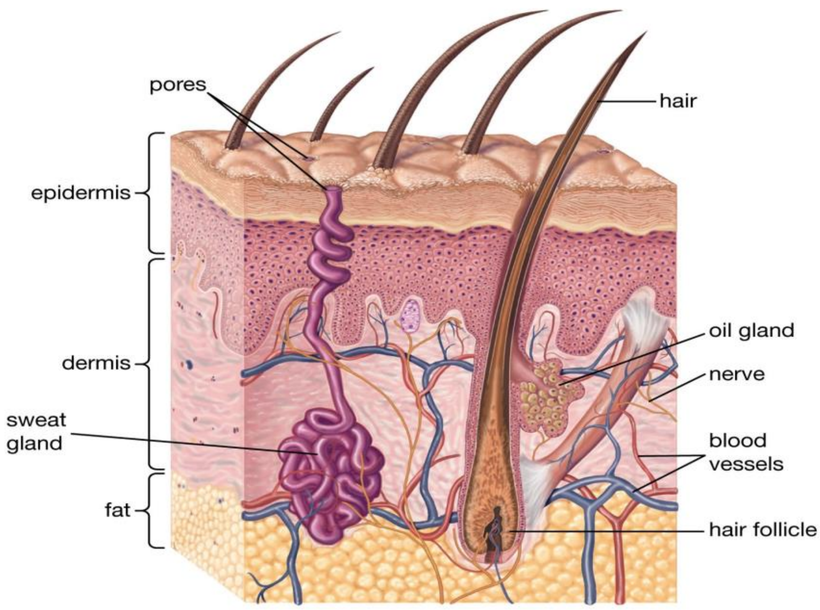

Fig. 1. Structure of skin (Géhin *et al.*, 2023)

Importantly, the skin also protects the underlying organs, a function necessary for the survival of the organism. As the cells in the deepest layers of the epidermis have higher replication rate, they are responsible for the constant renewal of cells. With the renewal of cells, older cells are pushed to the surface where they slough off. According to these stages, the different epidermal cells are divided into strata or layers, which are classified from the top layer to the deepest into the cornea, translucent, granular, spinous and basal This process causes a change in cell shape and its chemical composition, passing to secrete and accumulate keratin, and this process is known as keratinization (Monteiro-Riviere, 2020).

2.2. Wound formation and healing process

Wound, a break in the continuity of any bodily tissue due to an external action, typified by a cut, a bruise, or a hematoma. According to the World Health Organization (WHO), burns and wounds have been a serious public health problem due to the global increase in burn mortality rates. In South Africa, over 19500 fire-related deaths are reported annually and they rank among the 15 leading causes of death in children and young adults between the age of 5–29 years (Mabaso *et al.,* 2022). Wound healing is a complex process that occurs in almost all tissues after damage, aiming at repairing a lost or injured tissue.

The various processes of acute tissue repair, which are triggered by tissue injury, may be united into a sequence of four time-dependent phases: (i) coagulation and haemostasis, beginning immediately after injury; (ii) inflammation, which begins shortly thereafter; (iii) proliferation, which starts within days of the injury and encompasses the major healing processes; and (iv) wound remodelling, in which scar tissue formation takes place, and which may last up to a year or more (Fig.2) (Velnar and Gradisnik, 2018).

In everyday pathology, wounds remain a challenging clinical problem, with early and late complications presenting a frequent cause of morbidity and mortality. In an attempt to reduce the wound burden, much effort has focused on understanding the physiology of healing and wound care with an emphasis on new therapeutic approaches and the continuing development of technologies for acute and long-term wound management.

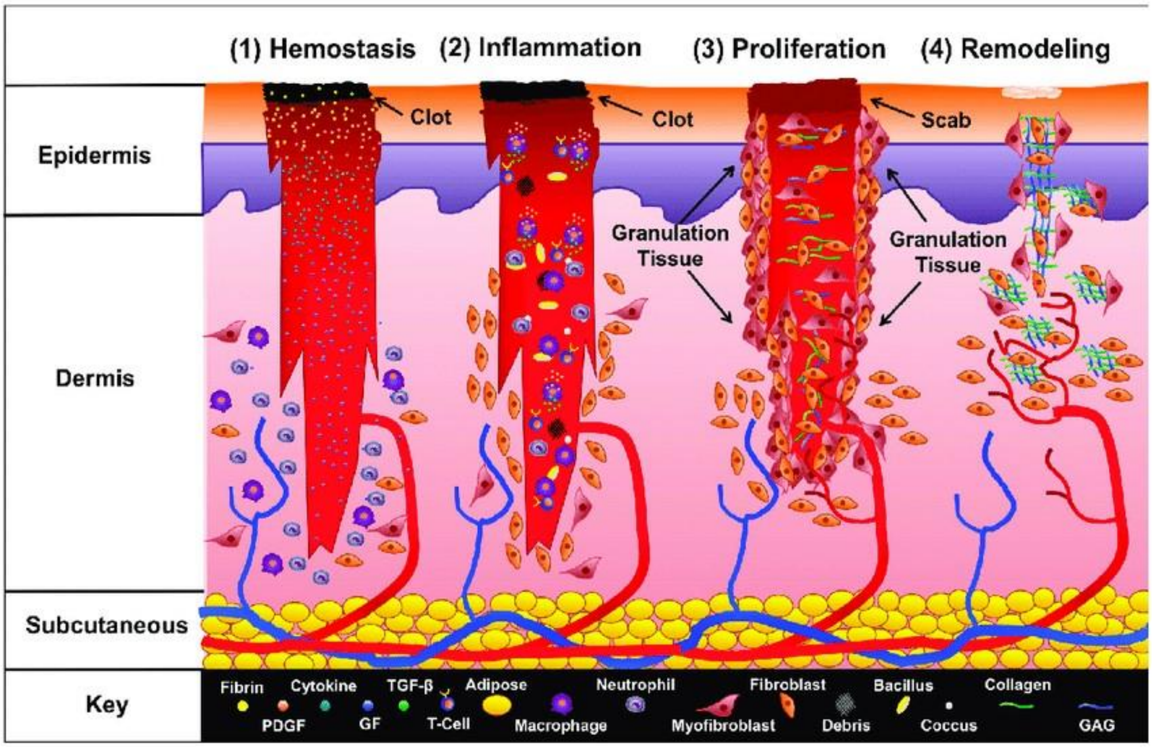

Fig.2. Stages of Wound Healing (Mellott *et al*., 2016)

2.2.1. Coagulation and Haemostasis phase

Immediately after injury, coagulation and haemostasis take place in the wound. The principal aim of these mechanisms is to prevent exsanguination (Repetto and De Re, 2017). Owing to the neuronal reflex mechanism, injured vessels constrict rapidly due to contraction of vascular smooth muscle cells in the circular muscle layer. The process is, however, only effective in transversally interrupted vessels and may cause complete cessation of blood leakage. Reflex vasoconstriction can temporarily reduce or even stop the amount of bleeding. The vascular smooth muscle tone is, however, only useful for a few minutes until hypoxia and acidosis in the wound wall cause their passive relaxation, and bleeding resumes. Hemostasis involves several events, such as vascular constriction, platelet aggregation, and fibrin clot formation, with subsequent development of a scab that provides strength, protection, and support to the damaged tissue (Han, 2023).

Together with haemostatic events, the coagulation cascade is activated through extrinsic and intrinsic pathways, leading to platelet aggregation and clot formation in order to limit blood loss. The blood clot and platelets trapped within it are not only important for haemostasis, as the clot also provides a provisional matrix for cell migration in the subsequent phases of the haemostatic and inflammatory phases (Opneja *et al.,* 2019).

2.2.2. Inflammatory phase

After bleeding, the healing process involves the migration and infiltration of inflammatory cells into the wound. At this phase, neutrophils, macrophages, and lymphocytes are responsible for multiple functions, including the promotion of the inflammatory response, inhibition of the penetration of exogenous microorganisms, elimination of microbes, and stimulation of keratinocytes, fibroblasts, and angiogenesis. The inflammatory phase, occurring simultaneously with the hemostasis, is characterized by the release of several pro inflammatory cytokines, cationic peptides, proteases, reactive oxygen species, and growth factors, allowing the wound cleaning. The cytoplasm of platelets contains α-granules filled with growth factors and cytokines, such as platelet derived growth factor (PDGF), transforming growth factor-β (TGF-β), epidermal growth factor and insulin-like growth factors which are responsible for the activation of fibroblasts, endothelial cells, and macrophages in the surrounding environment. These molecules act as promoters in the wound healing cascade by activating and attracting neutrophils and, later, macrophages, endothelial cells and fibroblasts (Opneja *et al.,* 2019).

2.2.3. Proliferative phase

The third wound healing stage which occurs 2−10 days after injury is characterized by fibroblast migration, ECM deposition, and granulation tissue formation. This phase initiates with keratinocytes migration over the injured dermis. Further to it, the formation of new blood vessels or angiogenesis occur, and the sprouts of capillaries related to fibroblast and macrophages restore fibrin matrix with granulation tissue, resulting in the formation of a new substrate for keratinocyte migration at subsequent stages of the repair process. These cells proliferate, mature, and restore the epithelial barrier function. In the final part of this stage, fibroblasts are attracted to the wound by numerous factors, including PDGF and TGF-β, and a few differentiate into myofibroblasts which is a type of contractile cells that bring the wound edges together (Spielman *et al.,* 2023)

2.2.4. Remodelling of wound tissues

Remodelling is the final step of acute wound healing which is also known as maturation, during which all the processes that are activated after injury are discontinued. It starts 2−3 weeks after onset of injury and lasts for a year or more. Majority of the endothelial cells, macrophages, and myofibroblasts undergo programmed cell death or exit from the wound, leaving a mass that comprises of a few cells and consists mostly of collagen and other ECM components. During this stage, the initial granulation tissue is weak which gradually gain strength over time because of gradual replacement of immature type III collagen by mature type I collagen (Wu *et al.,* 2021). This process is carried out by the matrix metalloproteinase secreted by fibroblasts, macrophages, and endothelial cells. Finally, granulation tissue forms a vascular scar composed of inactive fibroblasts, fragments of elastic tissue, dense collagen, and other components of the ECM. (Xue and Jackson, 2015).

2.3. Traditional medicine and ayurveda in wound healing

Natural product research is often based on ethno-botanical information and many of the drugs used today were developed from medicinal plants employed in indigenous societies. Regardless of finding new methods to enable the process of wound healing, wound care has found solace in the roots of medicine and is embracing some of the traditional therapies used ages ago. Ayurveda and folk medicinal systems encompass many such entities that traditionally accelerate healing. Healers yearn for traditional medicines for their high acceptability rate and real toleration (Beyene *et al.,* 2016).

According to the Ayurveda, Vrana (wounds or ulcers) is the discontinuation of lining membrane that after healing leaves a scar for life closely resembling the modern definition. (Gautam *et al.,* 2015). Classical management of wounds according to Sushruta Samhita follows 60 therapeutic steps, starting with an aseptic dressing of the affected part and ending with the rehabilitation of the normal structure and function. These therapeutic measures were aimed not only to accelerate the healing process but also to maintain the quality and aesthetics of the healing.

As described in different Ayurvedic classics like Charaka Samhita (ca. 5000 b.c.), Sushruta Samhita (ca. 1000 b.c.), Astamga Hridaya (ca. a.d. 600), Bhavaprakash Nighantu (ca. a.d. 1500), Dhanwantari Nighantu (ca. a.d. 1800), and Ayurveda Siksha (a.d. 20th century), it has been estimated that 70% of the wound healing Ayurvedic drugs are of plant origin, 20% of mineral origin, and the remaining 10% consisting of animal products. Scientific investigations have been carried out to assess the wound healing properties of some these drugs (Gupta and Nautiyal, 2016).

2.4. Patents on herbal formulation for wound healing

Phillip Roy *et al*. 2010 patented that honey could be used in dressings. The dressing consists of an alginate fiber sheet with honey completely impregnated into the fiber sheet. As a result, the dressing has porous surfaces and the dressing becomes gel-like when the exudate gets absorbed upon application to the wound. This patent includes 11 claims describing how honey is impregnated into the dressings. It can be used for treating acute as well as chronic wounds. Michael Koganov *et al*. 2013 patented "Bioactive compositions from theacea plants for the treatment of wounds and cuts." The invention relates to the bioactive topical formulation containing the bioactive fractions from theacea plants. The bioactive fraction from theacea plants shows anti-inflammatory action on the skin and normalizing skin damage or tissue injury.

Suresh Balkrishna *et al*. 2013 patented a "Novel herbal composition for the treatment of wound healing." Their innovation includes a new, synergistic, herbal composition as a regenerative medicine consisting of a mixture of therapeutically efficient quantities of extracts obtained as a basis *from Curcuma longa, Glycyrrhiza glabara, Hamil tonia suaveolens, Tipha angustifolia,* and *Azadirachta indica,* as well as an optional basis consisting of pig fatin *Sesamum indicum* (Til) oil, useful for wound cure care. Parveen Walia *et al* 2014. patented a "Multifunctional natural wound healing matrix" which consists of a wound pad made of hydrophilic cotton fabric coated on one side with zwitterionic low molecular weight chitosan and lined with organic-synthesized silver nanoparticles on top. Curcumin particles and tulsi extracts are used to further improve their properties with herbal medicinal principles and to have a synergistic impact with all the ingredients working together to provide better results for healing.

Suresh Balkrishna *et al*. 2013 also patented "A regenerative medicine, the herbal composition for the treatment of wound healing." This herbal composition is a combination of therapeutically effective amounts of extracts obtained from *Curcuma longa, Glycyrrhiza glabara, Hamil tonia suaveolens, Tipha angustifolia,* and *Azadirachta indica*, mainly used for the treatment of wounds and wound therapy. This includes 27 claims and 6 drawing sheets shows testing on different wounds. The invention shows novel synergism and effective composition of herbs as a regenerative medicine. This also offers a preparation method for the herbal composition.

Another scientist, Melikoglu *et al*. 2015 patented "Herbal formulation for topical wound treatment" in this interval with new herbal formulas, which have proved beneficial for the topical treatment of skin wounds and oral mucosal wounds. For the preparation, a solution or gel composed of poly hexamethylene biguanide as an anti-microbial agent and poloxamer as an emulsifier and a product includes at least one herbal ingredient (*Comfrey Symphytum* officinale L. extract and/or *Commiphora molmol* tincture) with analgesic, antibacterial, antifungal, and anti-inflammatory effects can be used to enhance analgesic, antibacterial, and anti-inflammatory effects. Poly hexamethylene biguanide (PHMB) was used as a protective agent, algaecide, bactericide/bacteriostatic, fungicide/fungistatic, and microbicide/microbiostatic.

Kerri-Anne *et al*. 2017 patented a "Topical herbal formulation" particularly suitable for the treatment of wounds and skin disorders. This comprises of Gotu kola (*Centella asiatica*), Figwot (*Scrophularia nodosa*), yarrow (*Achillea millefolium*), Plantago major, and *Echinacea purpurea*. The formulation has both anti-inflammatory and anti-microbial properties. It was found particularly effective as a synergistic healing agent in the treatment of wounds, prevention of scar formation, and promotion of hair regrowth in the wound area. It was also found suitable for the treatment of general skin disorders in humans including eczema and nappy rash.

Sabacinsk *et al*. 2019 invented and patented "Buckwheat honey and bacitracin wound-healing dressing." The invention has been found efficacious in the treatment of acute and chronic wounds and skin conditions and regeneration of skin and/or dermal tissue in a chronic wound. The product comprises a composition or formulation mixture of buckwheat honey and bacitracin. In one unique embodiment, the composition is gelled. The composition is applied directly to a wound or a patient's skin or is impregnated on gauze or other similar material on a bandage or dressing for application to an exuding or non-exuding acute or chronic wound or skin condition.

Dinesh Upendra *et al*. 2019patented "Herbal oil formulation for topical use and medicinal applications thereof." The invention includes a herbal oil solution based on *Heterophragma roxburghii* bark extract, which can be used topically and can be used to manage and repair numerous skin abnormalities and infections, all types of wounds, and other medical problems associated with diminished human and animal blood supply. The topical herbal oil formulation is an important natural curing therapy for different medical conditions such as, but not limited to, diabetic gangrene, dry gangrene, wet gangrene, athlete's foot, burn wounds, diabetic foot ulcer, bedsore, untreated open wounds, snakebite wounds, and cellulite-formed gangrene.

Mikolaj Tomulewicz *et al*. 2019 patented "Herbal preparation for accelerating wounds and skin inflammations healing and its application." The invention involves a medicinal preparation that can be used in the treatment of wounds and skin inflammation. The herbal preparation is distinguished by the fact that the preparation includes emulsified or suspended *Melittis melissophyllum* L. organic medium extract. Vaseline album was used as an organic medium in the case of an ointment from 40% to 70% w/w, 2% w/w triethylamine 2% w/w, hydroxy cellulose 1% w/w and filtered water, aqua purificata, from 30% to 35% w/w.

Omkar *et al*. 2020 patented "Wakeri (*Wagatea spicata* Dalzell) for wound healing." The invention comprises Wakeri-fortified Kampillakadi Tailam/oil. The Wakeri fortification comprises oil extract of root bark powder of Wakeri being a component in the Kampillakadi oil. Kampillakadi oil being a medicinal oil comprising oil extract of Vavding, Kutaj, Kapilla, Trifala, Patolpatra, Bala, Nimsal, Lodhra, Nagarmotha, Charolya, Khadirsal, Dhayatiphul, Agaru, and Chandanadded with Sarjaras. The invention also includes a composition comprising Wakeri-fortified Kampillakadi oil for topical application; the compositions comprise (a) a tulle, (b) an ointment, (c) a liniment, (d) a capsule, (e) a wound healing spray, (f) a cream, and (g) a gel.

The invention pertains to wound healing properties of Wakeri (*Wagatea spicata* Dalzell) Wight synonym of *Moullava spicata* (Dalzell Nicolson) with Kampillakadi Tailam (charak samhita chikitsa sthanam). New formulas, dressings, and medicinal plant composition are being explored by researchers for developing cost effective, efficient, stable, and sustainable delivery system for the management/treatment of wounds

2.5. Phytochemicals and antioxidants

Free radicals are an essential part of aerobic life and metabolism. They are highly indispensable to any biochemical process and are implicated in the etiology of many diseases such as cancer, Alzheimer's disease, Parkinson's disease, inflammatory disease, lipid peroxidation, DNA damage, celiac disease, stroke, cardiovascular disease, protein oxidation, and diabetes (Voituron *et al*., 2017; Usunobun *et al*., 2015; Atta *et al*., 2017; Bouzid *et al*., 2018). Antioxidants protect cells from damage caused by free radicals. Antioxidants have been shown to slow down or prevent the oxidation of other molecules. They possess the ability to terminate chain reactions and inhibit oxidation reactions via the removal of radical intermediates and by becoming oxidized themselves (Yu *et al*., 2018; Goodarzi *et al*., 2018).

The body system is rich with substances that have the ability to stop free radical formation or limit their damage. These antioxidants can be sourced internally and externally. Internally made antioxidants are generated via the activity of body enzymes which includes superoxide dismutase (SOD), catalase (Cat) and Glutathione peroxidize (Yasueda *et al*., 2016). In contrast, they are sourced externally from foods containing vitamins A, E (alpha tocopherol), C (ascorbic acid), minerals, and polyphenols which are predominantly plant based (Awuchi *et al*., 2022).

Plants contain numerous antioxidants which help to confer protection against free radicals associated diseases (Ofoedu *et al*., 2022). The antioxidant compounds are mostly produced in plants in the form of secondary metabolites. Phytochemicals can be literally referred to as 'plant-chemicals.' They are the non-nutritive chemical components of plants that possess numerous health benefits and disease prevention properties. The nutrients they contain are non-essential, i.e, they are not required by the human body for sustaining life (Rahim *et al*., 2022). These chemicals are produced by plants to sustain life which in turn confer health benefits to humans upon consumption. There are over a thousand known phytochemicals classified as primary or secondary constituents based on their role in plant metabolism (Ahaotu *et al*., 2020).

Phytochemicals classified as primary constituents includes the common sugars, amino acids, chlorophyll, purines and pyrimidines of nucleic acids and proteins etc. Others classified as the secondary constituents are the chemicals consisting of alkaloids, flavonoids, terpenes, phenolics, lignans, plant steroids, curcumines, saponins, glucosides. Of these secondary constituents, phenolics are seen to be the most numerous consisting of 45% of the secondary phytochemical constituents of plants, terpenoids and steroids 27%, alkaloids 18% and others 10% (Awuchi *et al*., 2021). Phytochemicals possess nutraceutical importance. They are the bioactive constituents that main-tain health and serve as a bridge between the food and pharmaceutical industries. Phytochemicals perform numerous functions (Zahnit *et al*., 2022). They possess unique pharmacological effects such as anti- inflammatory, antiplasmodic, anti-allergic, antioxidants, antibacterial, antifungals, chemo preventive, neuroprotective, hypotensive, antiaging, etc (Nazer *et al*., 2019; Khatoon *et al*., 2018).

They stimulate the immune system, block the formation of carcinogens, reduce oxidation, slow the growth rate of cancer cells, reduce inflammation, trigger apoptosis, prevent DNA damage, regulate hormones such as estrogen and insulin. Polyphenols are major dietary phenolics comprising the polyphenols (hydrolysable and condensed tannins), phenolic acids (hydroxybenzoic and hydroxycinnamic acids) and flavonoids. Flavonoids are the most extensively studied group of polyphenols. The major dietary sources of polyphenols are legumes (pulses and beans), cereals (corn, barley, oats, sorghum, rice and wheat), nuts, oilseeds (rapseed, flaxseed, olive seeds and canola) beverages (fruit juices, tea, coffee, beer, wine and cocoa), fruits and vegetables (Karamac *et al*., 2019).

The subclasses of phenols include flavones, flavanols and minor flavonoids (flavanones and dihydroflavonols). They exhibit their antioxidant potentials by preventing the decom-position of hydroperoxide into free radicals and by inactivating free radicals. Flavonoids play impor-tant roles in preventing diseases associated with oxidative stress. It has the capacity to transport electrons to free radicals, inhibit oxidases, reduce radicals of alpha tocopherol, activate antioxidant enzymes and chelate metals. They help to block angiotensin converting enzyme (ACE) that raises blood pressure. They have also been found to block enzymens that produce estrogen implicated in breast cancer and inhibit cyclooxygenase which has been known to form prostaglandins (Lawal *et al*., 2014).

2.6. Antioxidants in wound healing

Reactive Oxygen Species (ROS) are small oxygen-derived molecules mainly produced by the respiratory chain in mitochondria; some of them are hydrogen peroxide H_2O_2, superoxide anion O−2 or peroxide O2−2. They are oxidizing agents and major contributors to cell damage, but also have beneficial roles and, in particular, play a crucial role in the preparation of the normal wound healing response (Zhao *et al*., 2019). Therefore, a suitable balance between low or high levels of ROS is essential. Low levels of ROS are beneficial in protecting tissues against infection and stimulating effective wound healing by production of cell surviving signalling but, when present in excess, produce oxidative stress leading to cell damage and a pro-inflammatory status (Cano *et al*., 2018).

Redox imbalance occurs whenever the levels of ROS exceed the capacity of endogenous antioxidants to scavenge them, which dysregulates the healing process. There is no clear cut-off point for ROS level in tissues, but for the hydrogen peroxide (the most common ROS) the range 100–250 µM is considered as cut-off point for normal wounds (Ponugoti *et al*., 2013). In addition, some studies have reported that level of 10 µM of hydrogen peroxide act as chemo-attractant and stimulates the proliferation of fibroblasts and endothelial cells; at 100 µM stimulates angiogenesis via the production of vascular endothelial growth factor; but at 500 µM led to a pro-inflammatory status through the production of macrophage inflammatory protein 1-α (Johnson *et al*., 2021).

Healthy cells are essential for effective wound healing, and antioxidants help maintain their integrity by preventing oxidative stress-induced cell injury or death. Antioxidants neutralize ROS, reducing oxidative stress and creating a more favorable environment for healing. They can support the formation of new blood vessels, ensuring efficient nutrient and oxygen delivery. Common antioxidants involved in wound healing include vitamins C and E, beta-carotene, zinc, and selenium. These antioxidants can be obtained through a balanced diet or, in some cases, through supplements. However, it's important to note that excessive antioxidant supplementation may not always be beneficial and could potentially have adverse effects. Therefore, it's crucial to maintain a balanced and varied diet to support overall health and wound healing (Andre-Levigne. *et al*., 2017; Klyubin *et al*., 1996)

2.7. Nanotechnology in medicine

Nanobiotechnology is a novel concept and area of nanotechnology that has attracted worldwide interest. Nanoparticles are particles between 1 and 100 nanometres in size and are made up of carbon, metal, metal oxides or organic matter (El Shafey, 2020; Rzayev *et al*., 2021; Ramalingam *et al*., 2021). Chemical conditions, reaction circumstances e.g., temperature and pH which can change the structural attributes of nanoparticles such as size and shape. Nanomedicine is the term used to refer to the applications of nanotechnologies in medicine and healthcare.

Nanoparticles (also sometimes referred to as nanocarriers) have been investigated for various biomedical applications for over a decade. In general, the use of nano-sized particles offers several advantages over other drug delivery systems. They are used to (i) enhance the solubility of highly hydrophobic drugs; (ii) provide sustained and controlled release of encapsulated drugs; (iii) increase the stability of therapeutic agents by chemical or physical means; (iv) deliver higher concentrations of drugs to target areas due to an Enhanced Permeation and Retention (EPR) effect; and (v) provide targeted treatments when modified with cell-specific ligands (Murthy *et al*., 2021).

Due to their biocompatibility, anti-inflammatory and antimicrobial action, effective drug delivery, bioactivity, bioavailability, tumor targeting, and biological absorption, NPs are frequently utilized in biological, medical and environmental applications (Magdalane *et al*., 2018; Al-Dhabi *et al*., 2019; Salem and Fouda, 2021). Drug-loaded nanoparticles often accumulate in hair follicles and thereby facilitate the penetration of drug molecules through the superficial layers of the skin, followed by drug release into the deeper layers of the skin (Khalith *et al*., 2021).

2.8. Nanoparticles in wound healing

Nanotechnology has presented an excellent method to accelerate acute and chronic wound healing via stimulating appropriate movement through the diverse healing stages. Among various nanomaterials, nanoparticles (NPs) have been spotlighted as an efficient treatment strategy for wound healing due to their ability to act as both a therapeutic and carrier system. Their small size and high surface area to volume ratio enhance the probability of bio-interaction and penetration at the wound area aiding cell–cell interactions, the proliferation of cells, cell signaling, and vascularization. Their small size and physicochemical properties allow the intracellular delivery of these biomolecules or drugs, protect these agents from degradation, and enhance the drug penetration into the wound. All together allow the topical administration and increase the half-life of these agents, lowering the number of applications and costs. In addition, the encapsulation of drugs and biomolecules inside nanocarriers enables different drug release profiles that can match the wound healing requirements (Kushwaha *et al*., 2022).

2.9. Types of nanomaterials

Nanoparticles can be organized into four material-based categories; they are as follows:

- Carbon-based nanomaterials: Generally, these Nanomaterials contain carbon, and are found in morphologies such as hollow tubes, ellipsoids or spheres. Fullerenes (C60), carbon nanotubes (CNTs), carbon nanofibers, carbon black, graphene (Gr), and carbon anions (Kumar and Kumbhat, 2016).

- Inorganic-based nanomaterials: These nanomaterials include metal and metal oxide nanoparticles. They can be synthesized using various metals such as Au or Ag NPs, metal oxides such as TiO_2 and ZnO NPs, and semiconductors such as silicon and ceramics.

- Organic-based nanomaterials: These include nanomaterials made mostly from organic matter. The utilization of noncovalent (weak) interactions for the self-assembly and design of molecules helps to transform the organic nanomaterials into desired structures such as dendrimers, micelles, liposomes and polymer nanoparticles.

- Composite-based nanomaterials: Composite nanomaterials are multiphase nanoparticles and nanostructured materials with one phase on the nanoscale dimension that can either combine nanoparticles with other nanoparticles or nanoparticles combined with larger or with bulk-type materials (e.g. hybrid nanofibers) or more complicated structures, such as metal-organic frameworks. The composites may be any combinations of carbon-based, metal-based, or organic-based nanomaterials with any form of metal, ceramic, or polymer bulk materials (Jeevanandam *et al*., 2018).

2.10. Liposomes

Liposomes are small spherical vesicles prepared based on natural phospholipids, such as lecithin, with a structure similar to the major component of the cell membrane (Salimi, 2018). The phosphatidylcholine represents another frequently used phospholipid, which is obtained from soybean or egg yolk. The physicochemical properties of liposomes, such as the lipid composition, particle size, membrane rigidity or elasticity, surface charge, number of lamellae and drug storing potential, determine their stability and drug delivery abilities. The average diameter of liposomes varies from 50 to 300 nm. To date, about 600 clinical trials have involved lipid-derived drug delivery systems (Bozzuto and Molinari, 2015).

Liposomes can be prepared using: (i) thin film hydration, (ii) reverse-phase evaporation, (iii) solvent injection, (iv) detergent depletion, (v) supercritical fluid, (vi) size reduction sonication, (vii) high-pressure homogenization, and (viii) low-pressure extrusion (Lombardo and Kiselev, 2022). The stability of the liposomes is fragile, being influenced by several factors such as the composition of the lipid bilayer, the quantity of external water or the interaction between the drug and the bilayer. In addition, the shelf life of liposomes can be affected during flocculation or aggregation processes, modifying their size and stimulating the fusion of the vesicles. Lyophilization, or storage of the liposome in a dry state, is an effective technique to overcome the instability of the liposomes, specifically to develop stable boundaries between the vesicles (Yadav *et al*., 2011; Payton *et al*., 2014).

2.10.1 Liposome – wound healing

Liposomes are bilayer vesicles built by amphiphilic molecules such as phospholipids, emerging as one of promising nano-carriers for topical drug delivery (Wang *et al.,* 2019). They are nontoxic, biodegradable, biocompatible with skin, and capable of accommodating both hydrophilic drugs (e.g., growth factors) in inner water cavity and hydrophobic agent in bilayer. In this way, liposomes provide protection for encapsulated drug and sustain the drug release. Furthermore, liposomes effectively cover wound and create moist environment on wound surface after application, which is very conducive to wound healing. Taking advantage of all these merits, liposomes have been universally applied in wound treatment and skin regeneration. Wang *et al.,* (2021) prepared a novel liposome with hydrogel core of silk fbroin which effectively encapsulated Basic fibroblast growth factor (bFGF). The vehicles remarkably improved the stability of bFGF in wound fuids and maintained cell proliferation activity with respect to traditional liposomes. Furthermore, the liposomes with hydrogel core efficiently accelerated wound healing, particularly in inducing angiogenesis. Nunes *et al.* in 2016 evaluated the promoting efect of a gelatin-based membrane containing usnic acid-loaded liposome on wound healing. The experiments on a porcine model indicated that the liposomal membrane conspicuously controlled the secondary infection. In addition, more exuberant and cellularized granulation tissue with better collagen deposition was observed in the liposomal membrane treated group, therefore the special membrane boasted a comparable capacity to commercial product Duo derme with regard to enhanced maturation of granulation tissue and scar repair (Nunes *et al.,* 2016).

A wide range of nanosized-lipid-based delivery systems such as liposomes transfersomes, ethosomes and lipid nanoparticles as shown in Fig. 3 has been explored to overcome infections and enhance skin regeneration in burn wounds, with promising results (Xu *et al*., 2017; Li *et al*., 2016; Wasef *et al*., 2020; Saporito *et al*., 2018). Presented as a new generation of liposomes, deformable liposomes, also called transfersomes, mainly consist of

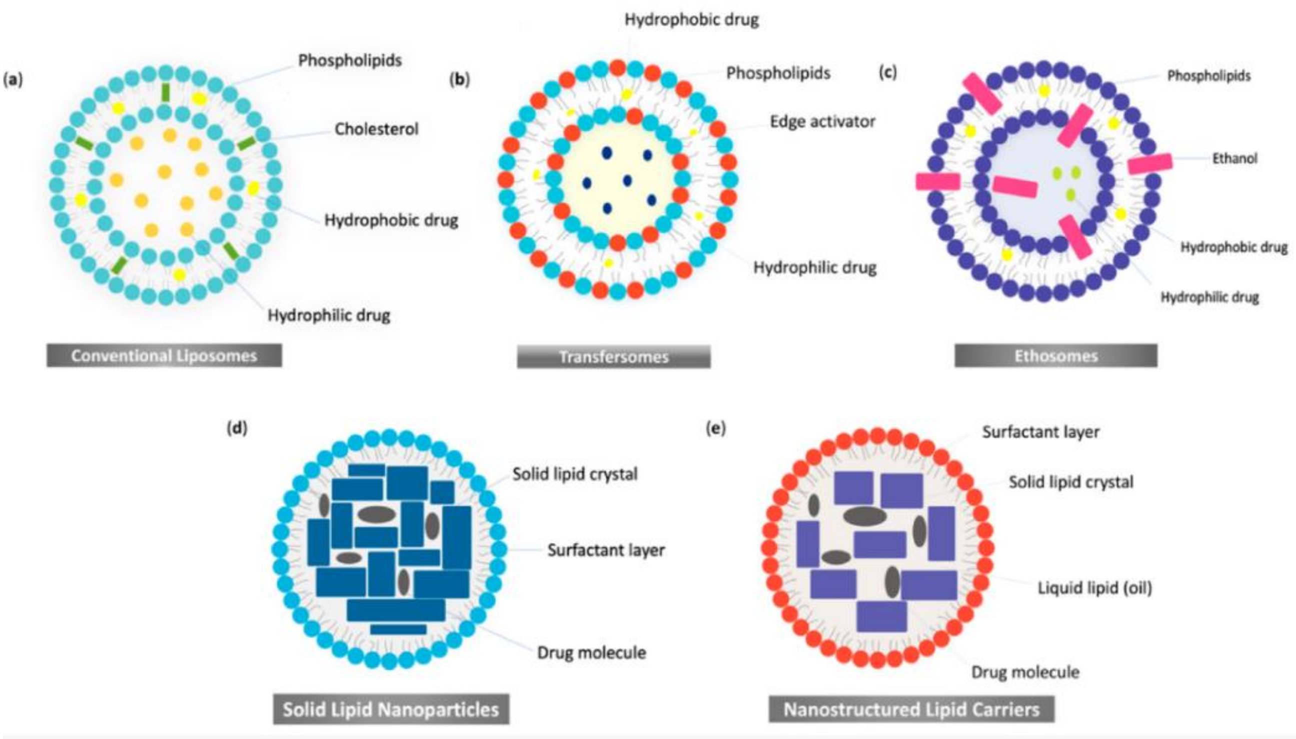

Fig. 3. Types of liposome (Matei *et al*., 2021)

phospholipids and an edge activator (such as sodium cholate, sodium deoxycholate and Tween-80), bringing new insight into topical drug delivery. These novel carriers not only integrate the benefits of traditional liposomes, but show more merits in topical application. The presence of edge activator renders high flexibility of deformable liposomes and enables them to cross the stratum corneum and reach the viable epidermis (Wang *et al*., 2019). The small sized liposomes were shown to be able to travel through the intracellular gaps inside the "brick and mortar" like stratum corneum structure.

After crossing the stratum corneum barrier, the liposomes distributed in the epidermis and dermis layer and gradually release the drug there.as shown in Fig.4. Choi *et al.,* (2017) conjugated low-molecular-weight protamine (LMWP) to the N-termini of EGF, PDGF-A and IGF-1, these molecules were subsequently complexed with hyaluronic acid and eventually incorporated into cationic deformable liposomes. The results showed that the cationic elastic liposomes containing the growth factor complex significantly accelerated the wound closure rate in the diabetic mouse model, with the maximal shrink of wound size by 58% compared with the native growth factor complex. It was fully confirmed that the elastic liposomes cooperated with growth factor complex, synergistically exerting both rapid and prolonged effects on promoting chronic wound healing.

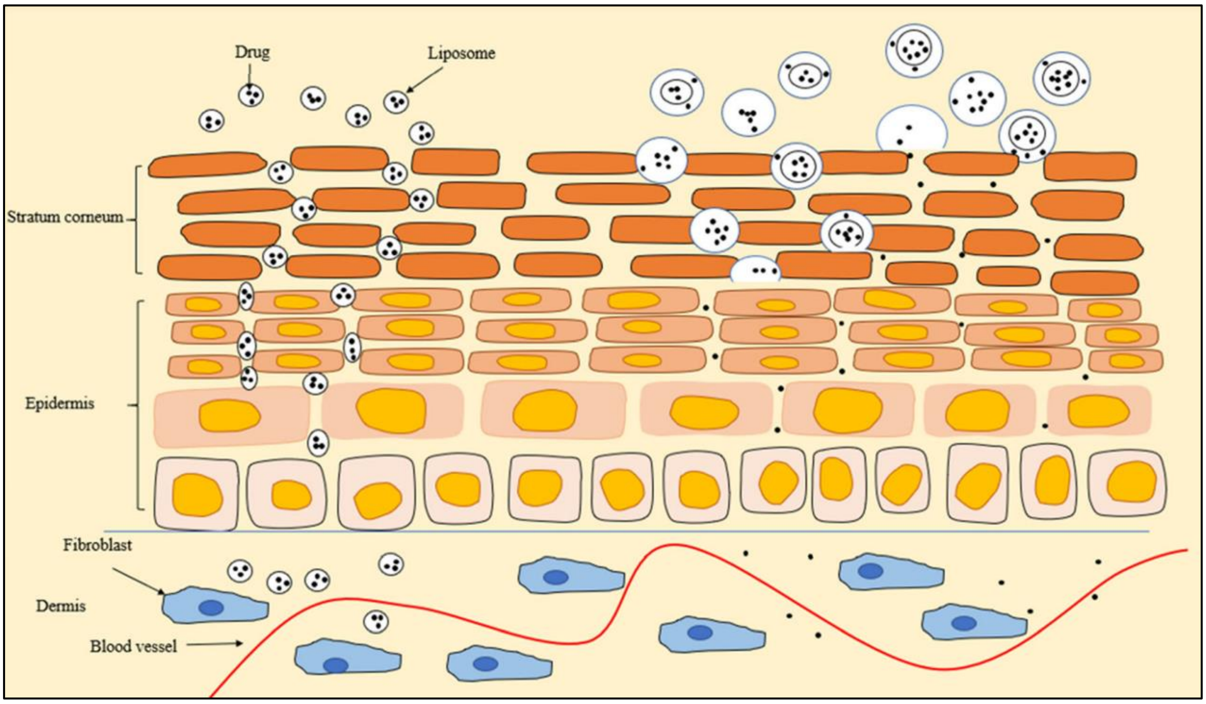

Fig. 4. Schematic drawing of the percutaneous absorption mechanism of liposomes with different sizes in drug delivery (Liu *et al*., 2021)

A new self-assembling core–shell gellan-transfersome loading baicalin was designed by Manconi *et al.,* (2018), they found out that novel transfersomes showed a relatively high skin drug deposition, about 11% of baicalin was retained in the whole skin, 8% of which was in the dermis, considered to be quite efficient. Daily application of baicalin transfersomes in mice model brought about complete skin restoration and inhibition of inflammatory markers such as oedema, TNF-α and IL-1β. Kianvash *et al.,* (2017) also noticed that their newly prepared propylene glycol nano liposomes containing curcumin not only featured by preferential physiochemical properties (small size, sustained drug release, good stability and biocompatibility), but promoted second degree burns in rat model in terms of avoiding infections and elevating wound contraction. Nevertheless, liposomes also exhibit some demerits in application: drug leakage in liposomes sometimes can be unavoidable and rapid; the low reproducibility and stability of liposomes remains a major obstacle for its expansion in clinical use.

2.11. *Hydrophilla auriculata* – an overview

Hygrophila auriculata (K. Schum) Heine, which has been used in traditional practice for many years. *Hygrophila auriculata* (K. Schum) Heine (synonym: *Asteracantha longifolia* Nees, *Barleria auriculata* schum, *Barleria longifolia* linn) Acanthaceae, is a wild herb commonly found in moist places on the banks of rivers, ditches and paddy fields throughout India) (Sethiya *et al*., 2018). The plant is an Ayurvedic herb used to make medicines for several gastrointestinal, kidney, reproductive, liver, and bone disorders. Kokilaksha is native to India and also to other places like Srilanka, Malaysia, Nepal and Myanmar. (Nikam *et al*., 2010; Kshirsagar *et al*., 2016)

The plant is a sub shrub, usually growing in marshy places along water courses. The stem is reddish brown and the shoot has 8 leaves and six thorns at each node. It is an annual, spiny, robust and erect herb with maximum height of 1.5 m, more or less hispid with long hairs, usually with numerous unbranched stems, containing subquadrangular thickened nodes. Leaves are subsessile, having oblong or linear lanceolate structure, with yellowish brown spines of 2–3 cm long in axil; flowers are pale to purple-blue in color, generally densely clustered in axil; and fruits are oblong having 4 to 8 seeds with glabrous capsules. The plant, when soaked in water, is immediately coated with mucilage, which is light brown in color, having slightly bitter taste without any distinct odour (Dash) *et al*., 2012; Rajalakshmi *et al*., 2016)

2.11.1. Total metabolites and phytoconstituents

H. auriculata has been investigated for its content of various metabolites. Phytochemical investigations of various plant parts have reported major classes such as flavonoids, alkaloids, triterpenes, aliphatic esters, sterols, minerals, amino acids, fatty acids and essential oils (Mandal *et al*., 2010). Specific compounds identified or isolated, to date, are reported in the Table I and II. The seeds were also reported to contain large amounts of tenacious mucilage and potassium salts, which may be responsible for their diuretic property. Various chemical structures of the phytocompounds were depicted in Fig. 5.

Table I. Total metabolites and cytological contents of *H. auriculata*

Parameter	Method adopted	Total Metabolic content	References
Phenolics	Folin Ciocalteu method	(17.75 ±0.30) g GAE per 100 g of plant extracts (80.86 ± 0.20) mg GAE per 100 mg of dried extract 20.83 mg GAE/g of dried leaf extract	Ouattara *et al*., 2013; Prasanna *et al*., 2017
Flavonoids	Dowd method	(0.033 ±0.002) g QE per 100 g of plant extracts (12.30 ± 0.71) mg QE per 100 mg of dried extracts	
	Aluminium chloride colorimetric method	3.59 mg QE/g of dried leaf extract	
Flavonols	Aluminium chloride method	(0.65 ± 0.03) mg QE per 100 mg of dried extracts	Ouattara *et al*., 2013
Tannins	European community method	(1.31 ± 0.31) mg TAE per 100 mg of dried extracts;	Ouattara *et al*., 2013; Prasanna *et al*., 2016
	Folin-Ciocalteu's and saturated sodium carbonate	7.1 mg TAE/g of dry material	
Alkaloids	Thin-layer chromatography	Steroidal alkaloid identification	Raj *et al*., 2010
Cytological studies	Karyotype analysis	Total chromosome was found to be 32	Behera *et al*., 2010

GAE: Gallic acid equivalents; QE: Quercetin equivalents; TAE: Tannic acid equivalents

Table II. Phytochemicals of *H. auriculata*

Class	Phytochemicals	References
Flavonoids	Apigenin 7-O glucuronide, apigenin 7-O-glucoside, luteolin, luteolin-7-O-rutinosides, ellagic acid, gallic acid and quercetin	Kshirsagar *et al*., 2010;
Alkaloids	Asteracanthine and asteracanthicine	Patra *et al*., 2009
Triterpenes	Lupeol, lupenone, hentricontane and betulin	Mazumdar *et al*., 1999
Aliphatic esters	25-oxo-hentriacontyl acetate and methyl 8-*n*-hexyltetracosanoate	Misra *et al*., 2001
Sterols	Stigmasterol and asterols I, II, III and IV	Quasim and Dutta *et al*., 1967
Minerals	Fe, Cu, Co, Mn, Mg, Zn, Ca, Fe, Ni, Cr, Na, K, Al and Sr	Sondhi and Agarwal, 1995
Amino acids	Histidine, lysine and phenyl-alanine	Chauhan *et al*., 2010
Fatty acids	72% of linoleic, 10% of oleic, 12% of stearic and 6% of palmitic and myristic acids	
Essential oils	Volatile oil	Kshirsagar *et al*., 2010

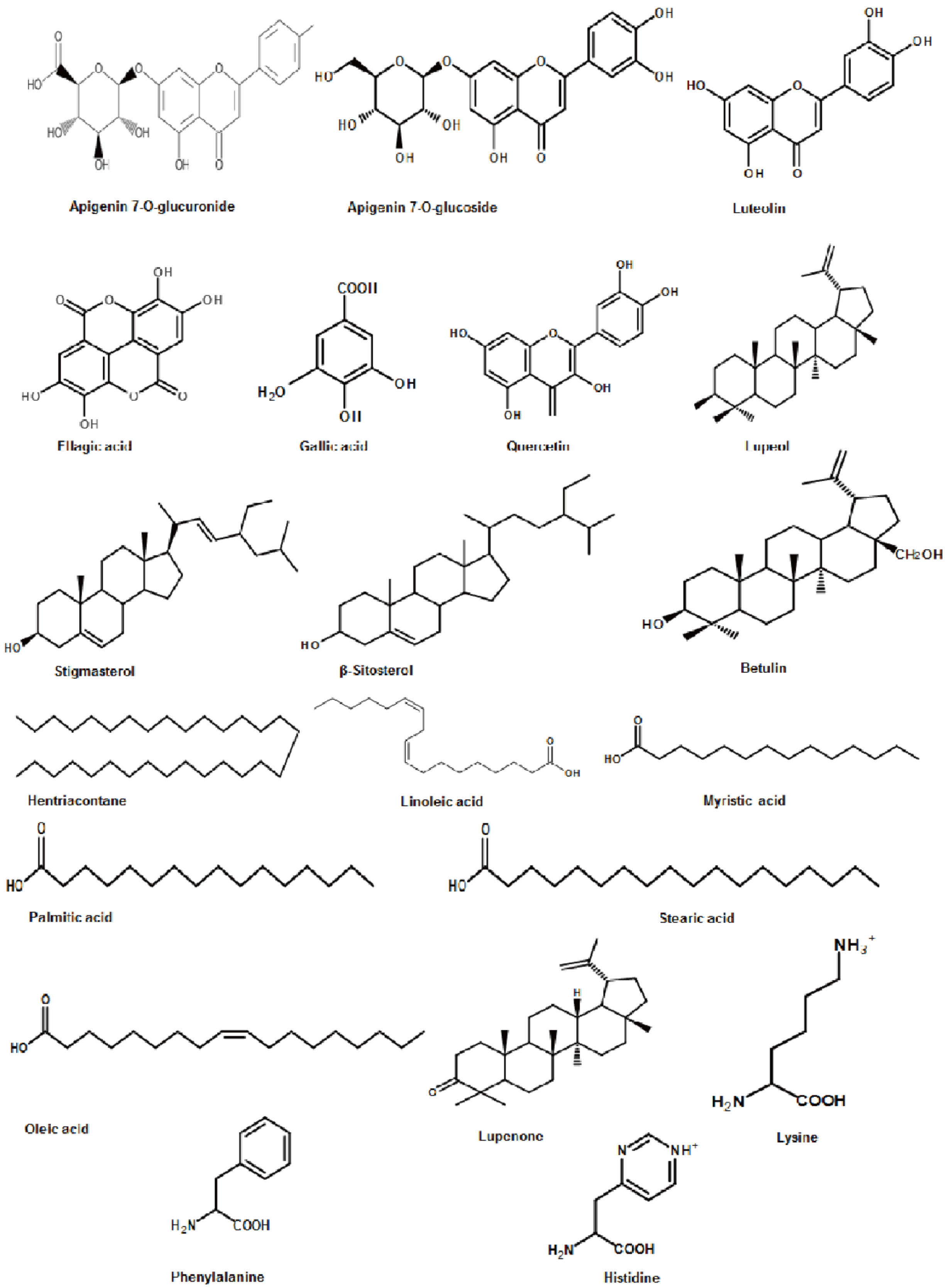

Fig. 5. Chemical structures of some phytocompounds identified in *H. auriculata*

2.11.2. Ethnobotanical uses

A survey of the ethnobotanical literature shows that the roots, seeds, and aerial parts of the plant are widely used in the traditional system of medicine for the treatment of jaundice, hepatic obstruction, rheumatism, inflammation, pain, urinary infection, edema, gout, malaria, and impotence and also as an aphrodisiac (Janghel *et al.,* 2019). Seeds are recognized as remedies for arthritis and diseases of the blood and biliousness. Bitter seeds are considered as aphrodisiac and tonic and used as a uterine sedative for pregnant women. The seeds are given with sugar or milk in the treatment of impotency, gonorrhea and spermatorrhoea. A paste of seeds, mixed with buttermilk, is given for diarrhea (Kshirsagar *et al*., 2010).

Root decoction is used to treat the condition of rheumatism and also act as a diuretic. Leaves are tonic and used for hypnotic and aphrodisiac effects. In Unani system of medicine, *H. auriculata* is utilized to improve the adaptability, reliability and energy. In this system, it is believed to have the power to properly improve and modify body dysfunction. It is also documented to be used for diseases like jaundice, kidney stone, nocturnal emission, seminal debility, sexual debility, spermatorrhoea and erectile dysfunction. In Siddha medicine, a decoction of *H. auriculata* leaves was prescribed twice a day for controlling blood pressure. In Homeopathy, it is being used to treat jaundice, diseases of the urogenital tract, arthritis and constipation (Rajalakshmi *et al*., 2016).

2.11.3. Major pharmacological actions

2.11.3.1. Toxicity studies

Petroleum ether extract of *H. auriculata* (40 and 80 mg/kg body weight (b.w.)), given intraperitoneally (i.p.), was reported to affect haematological parameters, metabolism and liver and kidney functions of mice. However, weekly doses up to 20 mg/kg and daily dose up to 4 mg/kg do not produce any symptoms or signs of toxicity (Mazumdar *et al*., 1996). Moreover, in another study, no sign of toxicity was observed in animals receiving fixed doses of up to 2000 mg/kg b.w. methanol extract of *H. auriculata* leaves for 14 days (Neharkar *et al*., 2015). Clinically, a multicenter double-blind homoeopathic pathogenetic trial on 48 patients suggested its safe therapeutic use for treatment of urticaria, frontal sinusitis, conjunctivitis, stomatitis, gastroenteritis, nausea (morning sickness) and intermittent fever (Rakshit *et al*., 2014).

2.11.3.2. Antimicrobial activities

Methanol extract (30 µg/mL) of leaves was reported to possess antibacterial activity against *Enterobacter aerogenes, S. aureus* and *Burkholderia pseudomallei* (Samy, 2005). Subsequently, in another study, alcohol and chloroform extracts of leaves were also reported to possess significant antibacterial activity against *Escherichia coli, S. aureus, B. subtilis* and *Pseudomonas aeruginosa*. It was also observed that treatment of extract caused growth inhibition of only two bacterial strains, i.e., *E. coli* and *Klebsiella pneumonia* (Arjun *et al*., 2008). However, there was no antibacterial activity against *Carnobacterium divergens, Enterococcus faecalis* and *Serratia marcescens* (Christibai *et al*., 2012). Aqueous leave extract was found to inhibit growth of *B. cereus, S. aureus, S. pneumonia, E. coli, P. aeruginosa, K. pneumonia, Salmonella typhi, Proteus vulgaris, Shigella flexneri, Aspergillus niger* and *C. albicans* (Doss and Anand, 2013).

In another study by Hussain and Kumaresan in 2013, diethyl ether extract was tested against pathogens such as *S. epidermidis, E. coli, Corneybacterium spp., Vibrio cholerae, E. fecalis, S. typi, C. albicans* and *A. niger*. However, inhibitory activity was found against *S. epidermidis* and *C. albicans* only. Petroleum ether, chloroform and n-butanol extracts obtained from aerial parts of *H. auriculata* were also reported to possess antibacterial activity against *V. cholerae, S. aureus* and *S. typhi.* Christibai *et al*. in 2012 reported that ethanol extract of leaves only inhibited *A. niger*, out of other tested fungal species viz. *A. flavus, A. fumigatous, Rhododendron indicum* and *Fusarium sps*. However, there was no antiviral activity was not detected by ethanol extract obtained from leaves, stem and root against poliomyelitis, coxsackie, measles and herpes simplex virus (Vlietinck *et al*., 1995).

2.11.3.3. Nephroprotective activities

Proximal tubular necrosis in the gentamicin-induced nephrotoxic model of kidney injury in male Sprague-Dawley rats was therapeutically recovered, which was verified by histopathological examination and kidney function tests after daily doses of 250 mg/kg ethanol extract of *H. spinosa*, whereas in another study, methanol extract of *H. spinosa* significantly reduced blood urea and serum creatinine levels and rectified adverse histopathological changes and renal enzyme levels induced by cisplatin. This action may be due to improvement in enzyme function in the kidney through antioxidant activity of secondary metabolites present in the *H. spinosa* extract (Ingale *et al*., 2013).

2.11.3.4. Hepatoprotective activities

In an *in vitro* study, total alkaloid fractions (40–80 µg/mL) obtained from methanol extract of *H. auriculata* leaves improved viability of HepG2 cells intoxicated with carbon tetrachloride (CCl_4) in a dose-dependent manner (Raj *et al*., 2010). In another study, methanol extract of the seeds was found to have significant hepatoprotective activity through improvement in liver functions and protection of necrosis against paracetamol and thioacetamide intoxication in rats (Singh and Handa 1995). Whole-plant aqueous extract had significant hepatoprotective activity as shown by return to normal architecture of liver cells and biochemical parameters after damage from CCl_4 and paracetamol intoxication. Crude extract of this plant directly interfered with the formation of free radicals, which is a suggested mechanism behind this hepatoprotective activity (Hewawasam *et al*., 2003).

Protective effect of the methanol extract of *A. longifolia* seeds was established against acetaminophen-induced liver damage in rats (Shivashangari *et al*., 2004). Whole-plant slurry of *A. longifolia* possessed significant hepatoprotective activity due to its antioxidant property against CCl_4-induced changes in biochemical parameters of hepatic enzyme activity (Shailajan *et al*., 2005). In another study, aqueous extract obtained from *H. auriculata* roots exhibited potent hepatoprotection through an antioxidant-based mechanism against CCl_4-induced liver damage in rats (Shanmugasundaram *et al*., 2006). This was further supported by testing in vitro antioxidant activity using ferric thiocyanate and thiobarbituric acid methods.

However, one similar experiment of whole-plant and root aqueous extracts supported the earlier results and showed the role of antioxidants from the extracts in heptoprotection against CCl4 and paracetamol-induced hepatic damage (Usha *et al*., 2007). In another study, ethanol extract of roots normalized serum enzyme levels and morphological parameters as shown by histopathological observations in CCl4-induced toxicity in albino rats (Raju *et al*., 2011).

Slurry, aqueous and ethanol extracts of whole plant each produce hepatoprotective effects against galactosamine-induced hepatotoxicity as evidenced by biochemical and histopathological findings (Shailajan *et al*., 2007). The hepatoprotective property of active components obtained from ethanol extract of aerial parts was also examined against isoniazid- and rifampicin-induced toxicities in biochemical and histopathological studies (Lina *et al*., 2012).

2.11.3.5. Antitumor/anticancer activities

According to an *in vitro* study, aqueous extract of *H. auriculata* seeds showed selective cancerous cell cytotoxicity, with a 50% inhibitory concentration (IC_{50}) of 0.22 mg/mL against colon cancer cells (Uddin *et al*., 2011). Another *in vitro* study on petroleum ether extract of *H. auriculata* roots supports antitumor activity due to nuclear factor-kB inhibition (Lampronti *et al*., 2005). Moreover, in vitro inhibitory activity of whole-plant methanol extract against U373 cancer cells was due to the presence of phenolic compounds (Nabèrè *et al*., 2012).

Antitumor activity was demonstrated by methanol extract of seeds against experimental hepatocarcinogenesis in rats and was due to the action of antioxidant enzymes, glutathione peroxidase and catalase, in a dose-dependent manner (Ahmed *et al*., 2001). The hydro-alcohol extract of the whole plant was reported to possess significant antitumor activity against 7,12-dimethylbenz (a) anthracene (DMBA)-induced mammary tumors in female rats, which was comparable with a standard drug, tamoxifen (Pattanayak *et al*., 2008). Moreover, in another study, whole-plant methanol extract inhibited tumor growth against DMBA-induced mammary tumor due to its high flavonoid content, which significantly decreased the levels of lipid peroxidation (Nair *et al*., 2005).

2.11.3.6. Antidiabetic activities

The rats administered with aqueous extract of the plant had a significant increase in the glycogen content of liver and muscle and a significant increase in triacylglycerol content of adipose tissue in comparison with control rats. However, the plant extract had no effect on the gluconeogenic capacity of the kidney and intestinal glucose absorption (Fernando *et al*., 1998). In addition, 3- week regular administration of ethanol extract from aerial parts was reported to significantly reduce blood glucose levels in streptozotocin-induced diabetic rats.

The treatment further demonstrated antioxidant activity and significantly increased the level of glutathione, glutathione peroxidase, glutathione S-transferase and catalase in the drug-treated group (Vijayakumar *et al*., 2006). Oral administration of aqueous extract, at doses of 100, 200 and 400 mg/kg, per oral (p.o.), to alloxan-induced diabetic rats was effective as seen in various measured parameters. It was concluded that activity of the aqueous extract might be due to increase in insulin secretion and antioxidant action (Muthulingam, 2010).

Some *in vitro* studies suggested that methanolic extract of the plant possessed antioxidant activity, α-amylase inhibition and glucose diffusion inhibition. The extract was reported to be effective in controlling chloride levels which had its role in glucose uptake (Rastogi *et al*., 2014). It was also revealed that the extract effectively controlled the activity of alkaline phosphatase, resulting in increased activity of adenosine monophosphate activated protein kinase, having a beneficial effect for diabetics. Moreover, α-amylase inhibitory activity of the extract prevented a sharp postprandial increase in blood glucose in diabetic patients (Rajalakshmi and Cathrine, 2015).

2.11.3.7. Antioxidant and free radical scavenging activities

The free radicalscavenging potential of 50% hydro-alcohol extract of *H. auriculata* (HAEt) was studied for 2,2 diphenyl, 1-picrylhydrazyl (DPPH) scavenging activity, nitric oxide, hydroxyl radical and ferryl bipyridyl complex-scavenging activity along with lipid peroxidation and total antioxidant capacity inhibition, using thiobarbituric acid reactive substances in rat liver homogenate. HAEt showed good radical-scavenging activity at various concentrations (200– 1000 μg/mL) against DPPH (32.32%–77.02%) with moderate scavenging activity against nitric oxide (12.46%–52.84%), hydroxyl radical (11.69%–55.26%), ferryl bipyridyl complex (17.66%–58.67%) and lipid peroxidation (0.829–0.416 nmol/mg protein) (Vijayakumar *et al*., 2005).

Sawadogo *et al*. in 2006 emphasized that the higher content of phenolics and flavonoids in methanol extract of leaves could be responsible for its promising antioxidant activity. Ethanol extract of whole plant was reported to improve the levels of antioxidant enzymes, which protect against oxidative damage caused by mercuric chloride intoxication (Sridhar *et al*., 2013). Aqueous extract of leaves was reported to have significant *in vitro* antioxidant and free radical-scavenging activity in all studied parameters (Dasgupta and De 2007).

In another study, the terpenoid-rich fraction (10–100 μg/mL) obtained from n-butanol extract exhibited high-DPPH radical-scavenging and lipid peroxidation inhibition activity. A dose-dependent inhibition of nitric oxide and hydroxyl radical suggested an antioxidant activity (Hussain *et al*., 2009). According to one comparative study, methanol extract obtained from *H. auriculata* possessed significantly higher antioxidant activity than the *Pergularia daemia* in various *in vitro* tests (Doss 2013).

Total phenol and flavonoid content in aqueous, ethanol, acetone, chloroform and petroleum ether extracts of leaves from three geographical regions of Tamil Nadu (Kanchipuram, Gummidipoondi and Chengalpattu) was examined, and it was found that the acetone extract of leaves from Gummidipoondi possessed more potent antioxidant activity than others due to having higher phenol and flavonoid content (Prasanna and Sridhar 2017). The root extract of *H. auriculata* exhibited significant ($P < 0.001$) *in vitro* antioxidant activity by inhibiting the oxidation of linoleic acid in both ferric thiocyanate and thiobarbituric acid methods. The activity of extract was found to be greater than the standards, vitamins E and C, used in the methods (Shanmugasundaram and Venkatraman 2006).

2.11.3.8. Erythropoietic / haematinic effect

The ethanol extract of the aerial parts, at doses of 100 and 200 mg/kg (p.o.), significantly increased the haemoglobin, haematocrit and red blood cell count in anaemic male rats, indicating the haematinic effect of the extract (Gomes *et al*., 2001). Petroleum ether and chloroform extracts of leaves showed haematopoietic activity, significantly increasing erythrocyte count, leukocyte count, and haemoglobin count (Pawar *et al*., 2006). Administration of ethanolic extract at the doses of 100 and 200 mg/kg b.w., i.p., demonstrated a significant increase in erythrocyte count, haemoglobin count, serum iron and serum protein. This effect may be due to the presence of iron (622 µg per 50 mg) in the extract, estimated by spectrophotometric method. The presence of higher iron content and a potent haematinic effect in the extract justified its use as a main ingredient of any herbal formulation designed for the management of iron deficiency such as anaemia (Pawar *et al*., 2010).

2.11.3.9. Diuretic activities

It has been claimed that the plant has diuretic activity by Kumari and Iyer in 1967. It was shown that the n-butanol fraction obtained from whole plant increased the urine output, at a dose of 200 mg/kg, more than other extracts and fractions studied (Hussain *et al*., 2009). The alcohol extract of seeds, at doses of 300 and 500 mg/kg, p.o., significantly increased electrolyte excretions (sodium, potassium and chloride). However only the 500 mg/kg dose increased urine volume (Preethi *et al*., 2012).

2.11.3.10. Antinociceptive activity

Aqueous extract of aerial parts and roots, at an oral dose of 200 mg/kg, had a potent antinociceptive activity in a mouse model of thermally induced analgesia (Shanmugasundaram and Venkatraman 2005). Abdominal constriction produced by acetic acid in mice was reduced by chloroform, alcoholic and aqueous extracts, administered at doses of 200 and 400 mg/kg b.w. These extracts dose dependently increased the pain threshold of mice to thermal stimuli (Patra *et al*., 2008).

2.11.3.11. Anti-inflammatory and antipyretic activity

Petroleum ether, chloroform, alcohol and aqueous extracts obtained from leaves were examined for anti-inflammatory activity using the carrageenan-induced paw oedema model in rats and antipyretic activity on Brewer's yeast-induced pyrexia in rats. Extracts obtained from chloroform and alcohol exhibited significant anti-inflammatory and antipyretic activities in a dose-dependent manner. The maximum anti-inflammatory activity was produced by the chloroform and alcoholic extracts at a dose of 400 mg/kg b.w. (Patra *et al*., 2009).

In another study, chloroform and alcoholic extracts exhibited anti-inflammatory activity, both in cotton pellet induced granuloma and Freund's adjuvant-induced arthritis, in a dose-dependent manner. The decrease in body weight, generally due to the injection of complete Freund's adjuvant, was improved positively by the extract. The results demonstrated that *H. spinosa* has anti-inflammatory activity in chronic models of inflammation, which supports the traditional use of *H. spinosa* in the treatment of rheumatism (Patra *et al*., 2017).

2.11.3.12. Wound healing activity

The northeastern region of India is inhabited by a large number of tribal communities of different ethnic groups. These ethnic communities have vast knowledge of curative properties of various traditional medicines. Interaction with rural people of Assam revealed that the people are using different medicinal plants for treating different types of wounds. *Hygrophila auriculata* Schumach. is one such plant, roots of which is used by the rural people as traditional wound healing agent. Dev *et al*., in 2019 reported a wound-healing potential of roots of *Hygrophila auriculata* schumach in swiss albino mice. Healing was assessed by measuring wound area, histo-morphological observations, estimation of protein and DNA content. The results showed that the wound area of the extract treated group was lesser than the control group. The epithelialization was faster in the treated group when compared with control group. The amount of protein and DNA were also more in treated mice than the control. Extent of healing in the treated animals was quite comparable to that of the positive control group.

2.11.3.13. Anti-urolithiatic and anti-infective activity

Oral administration of aqueous extract at a dose of 200 mg/ kg b.w. has been reported to prevent and treat urolithiasis in a rat model of the ethylene glycol-induced nephro-urolithiasis (Hussain *et al*., 2009). Treatment significantly reduced the elevated urinary oxalate, urinary calcium and serum uric acid with an increase in reduced urinary magnesium content. The increased deposition of stone-forming constituents in the kidneys of ethylene glycol-treated rats was significantly lowered by treatment with methanol extract of *H. spinosa* (Sathish *et al*., 2010). Additionally, syrup Uricitral, an herbal formulation containing *H. auriculata*, was studied in the management of childhood urinary tract infection on 50 patients (6 months to 16 years); it significantly reduced the clinical symptoms of disease, caused by various pathogens, by the 14 days of treatment (Chaturvedi *et al*., 2016).

2.12. Betulin – an overview

Betulin, chemically known as lup-20(29)-ene-3β,28-diol, is a naturally occurring triterpene characterized by a five-membered ring and an isopropylidene group. Betulin (3b,28-dixydroxy-20-(29)-lupane) and betulinic acid (3b-Hydroxylup-20(29)-en-(28)-oic acid) are pentacyclic lupane type triterpenes, together found widely in the bark extracts of the many plant species in varying amount. The terpenes are the largest groups of phytochemical consisting of more than 40,000 individual compounds, with many new compounds are discovered from time to time. “Isoprenoid” are the building blocks of terpenes.

The biosynthesis of triterpenes includes an amalgamation of triterpene hydrocarbon, cyclization of squalene, and precursor of all steroids (Cruz and Pereira, 2023). Though *Betula spp*. have been the largest source for betulin that leads to chemical synthesis of betulinic acid but now recent literature highlighted that these can be isolated from other different plants also. Betulin occurs naturally in foods, for example in olives and lingonberries (Reina *et al*., 1999; Szakiel *et al*., 2012).

Previously, the terpenes were not considered more effective as far as pharmacological point of view but after the recognition of antitumor activity of betulinic acid ignited the interest of a huge numbers of researchers considering its pharmacological activity and low toxicity with a larger range. Betulin is regarded as one of the important pentacyclic triterpene used for a drug screened by National Cancer Institute which has shown diverse pharmacological properties. Betulin can be extracted upto 30 % while betulinic acid is available only 0.5- 1.5% (Fiani *et al.,* 2020).

According to the available literature, it is evident that betulin has a broad spectrum of biological activities, including anti-HIV, anti-fungal, anti-bacterial, anti-inflammatory, anti-tumor, anti-leishmania, and immune regulatory effects (Amiri *et al*., 2020). However, most of these studies have focused on derivatives of betulin. Recent studies have shown that betulin exerts significant pharmacological effects once its insolubility is resolved. From these studies, betulin is shown to exert multitarget activities in different organs and disease states.

Betulin inhibits proinflammatory cytokines (IL-6, IL-1β, TNFα), HMGB-1, NFκB, and MAPK, which results in the reduction of lung and liver injuries in septic rats. Even though the exact molecular mechanism of these activities is still unknown, studies have purported that the anti-inflammatory properties of betulin are at the heart of its different biological functions (Zhao *et al*., 2016). Many diseases, including infection, cancer, allergies, diabetes, asthma, arthritis, and atherosclerosis, are characterized by chronic inflammation. Since inflammation is a key process in the development and occurrence of many chronic diseases, compounds with multitarget properties are the direction of the search for cure (Chen *et al*., 2018).

2.13. Betulin in wound healing

Betulin is a natural triterpene, usually from birch bark, known for its potential woundhealing properties. An *in vitro* model of fibroblasts and keratinocytes obtained from both diabetic and non-diabetic donors evaluated for betulin-enhancing wound-healing effects led to enhanced mRNA levels of proinflammatory cytokines, chemokines, and mediators crucial for wound healing such as IL-6, TNF, IL-8 and RANTES- Regulated upon Activation Normal T Cell Expressed and Presumably Secreted (Wardecki *et al*., 2016).

Other studies demonstrated that betulin decreased levels of pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α), matrix metalloproteinases (MMP-2 and 9), and interleukins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-13, and IL-17) (Kamaraj *et al*., 2021; Ouyang *et al*., 2022). According to the authors, betulin significantly upregulated RANTES, TNF-α, IFNγ, MIP-1α and β, and IP-10, all of which are proinflammatory mediators involved in the inflammatory phase of the wound healing process and enhanced migration of keratinocytes, which is essential for the second phase of wound healing (Ebeling *et al*., 2014).

Betulin may exert its anti-inflammatory effects via a multi-target mechanism, including inhibition of ROS production, inhibition of TNF-α, inhibition of pro-inflammatory cytokines, activation of the Nrf2-associated signaling pathway, post-transcriptional inhibition of iNOS, inhibition of the NFκB pathway, upregulation of PPAR-γ expression, and modulation of the STAT3 signaling pathway. Earlier research conducted to investigate the effects of betulin on inflammatory damage induced by *S. aureus*-mastitis in female BALB/c mice showed that betulin ameliorated histopathological changes and suppressed the expression of IL-1β, TNF-α, and IL-6 initiated by the inflammatory injury, acting as a protective anti-inflammatory agent against mastitis (Guo *et al*., 2015). Additionally, betulin prevented AML-12 or RAW 264.7 cells' activation of P2X7r-NLRP3 in response to EtOH- or lipopolysaccharide-induced inflammation (Dou *et al*., 2022)

According to a study on the effects of oleogel formulations on skin injuries, botulin based oleogel significantly accelerated the healing of wounds by encouraging the migration of immortalized human keratinocytes (Pârvănescu *et al*., 2021). Oleogel-S10 is a sterile wound gelfor topical use containing 10% refined triterpene dry extract from birch bark (quantified to 72%–88% betulin) and 90% refined sunflower oil. Additional components of birch bark extractinclude betulinic acid, lupeol, oleanolic acid, and erythrodiol.

The active pharmaceutical ingredient of Oleogel-S10 modulates chemokines in the inflammation phase of wound healing and promotes the migration and differentiation of keratinocytes, thus accelerating reepithelialisation and wound closure (Woelfle *et al*., 2010; Ebeling *et al*., 2014). More recently it was shown that fibroblasts too are stimulated by birch bark extract and its main constituents (Wardecki *et al*., 2016). For triterpenes present in birch bark extract, namely betulin, betulinic acid and oleanolic acid, antiviral, antibacterial, antimycotic, and anti-inflammatory effects have been described (Rastogi *et al*., 2015).

Also, a phase II pilot clinical trial of OleogelS10 (which contains a betulin-rich triterpene extract) on acute and chronic wounds of 10 patients with dystrophic epidermolysis bullosa showed faster wound healing and reepithelization than in the untreated groups (Schwieger-Briel *et al*., 2017). Other clinical studies on actinic keratoses, skin graft transplants, and burns revealed that betulin enhanced wound healing and reepithelization (Metelmann *et al*., 2015; Frew *et al*., 2019).

Additionally, most clinical trials on betulin have focused on its ability to treat skin diseases and heal wounds since inflammation is a key process in wound healing. At the time of the study, Oleogel-S10 was still an investigational medicinal product. Recently it has received regulatory approval by the European Medicines Agency as a new medicine for the treatment of partial thickness wounds in adults (tradename: Episalvan). Therefore, in addition to its wound-healing capacity, there is a need for preliminary clinical trials, randomized large cohort groups, and controlled long-term trials for specific clinical applications of betulin since not all results from preclinical studies are applicable to humans (Adepoju *et al*., 2023).