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Introduction

Over the course of millennia, the intricate evolution of our skin has yielded a remarkably adaptive and multifunctional organ, serving as a formidable barrier against the daily barrage of challenges posed by chemical and physical substances, and ultraviolet radiation. Any infringement upon the integrity of living tissue is designated as a wound. Such wounds manifest when the protective epidermal layer of the skin is breached, exposing the underlying dermis to the external environment. Depending on the depth and extent of the skin damage, the exposed tissues may range from blood vessels to bone. Consequently, wounds are broadly categorized into three classifications. A superficial wound pertains to injuries limited to the epidermal skin surface, while a partial-thickness wound involves deeper dermal layers, encompassing blood vessels, sweat glands, and hair follicles. In the case of a full-thickness wound, the underlying subcutaneous fat or deeper tissues become compromised (Wilkinson and Hardman, 2020)

The challenging external conditions frequently expose our skin to injuries and therefore, it is anticipated that our skin is endowed with sophisticated reparative mechanisms facilitating swift and efficient healing. The wound healing process unfolds as a meticulously orchestrated cascade of events, each phase seamlessly interacting with the next. These phases include coagulation, immune response and inflammation, proliferation, and remodelling (Takeo *et al*., 2015) The healing of cutaneous wounds is a crucial physiological process that requires coordinated actions from different cell types and their substances. The commencement of the restoration process for injuries resulting from local damage occurs in the early stages of inflammation, ultimately progressing towards repair and regeneration (Almadani *et al*., 2021)

Repair involves the substitution of specialized structures through collagen deposition, while regeneration entails the proliferation and subsequent differentiation of cells within the tissue and/or stem cells. Disruption of this highly regulated healing process results in the cessation of healing, leading to the development of chronic wounds. Addressing the intricate symptoms that emerge from the metabolic disorder in the wound microenvironment is a

significant medical need for effectively treating chronic wounds. This requirement remains substantial but yet un fulfilled in the field of comprehensive wound care (Comino *et al*., 2021).

Plant-based materials used in medicine, along with the herbal remedies derived from them, form a substantial portion of the worldwide medicinal market. Across the history of mankind, herbal remedies and medications have been pivotal in the treatment of diseases. Over the course of nearly a millennium, diverse plant species have been investigated as potential sources for developing therapeutic agents. Even in the present day, a significant proportion of the drugs in use are derived from natural products obtained from plants. Ancient records, including written drafts dating back to 2600 BC detailing the medicinal values of herbs and the documentation of medicinal plant usage in ancient Mesopotamia, have paved the way for potential drug development based on plants and natural products (Chen *et al.,* 2014).

Natural drugs have a rich history of being employed in the prevention and treatment of various human ailments. Their continued use has significantly impacted modern medical and healthcare services on a global scale. While the pharmaceutical industry traditionally relies on synthetic compound libraries and high-throughput screening for novel drug discovery, the complexity of natural-product based libraries has led to a decline in the introduction of new drugs. As a result, there is an increasing acknowledgment of the necessity for comprehensive interdisciplinary approaches in developing new drugs based on natural products. Despite an abundance of literature highlighting the curative properties of medicinal plants, standardized procedures for quality control of plant materials pharmacological properties, and therapeutic activity are currently lacking (Atanasov *et al.,* 2015).

Hygrophila auriculata (K. Schum) Heine, also known by synonyms such as *Asteracantha longifolia* Nees, *H. schulli* (Ham.) MR & SM Almeida, *H. spinosa* T. Anders, *Barleria auriculata* Schum, and *B. longifolia* Linn, is a member of the Acanthaceae family. This erect semiwoody plant is commonly found in moist habitats across India, particularly along the banks of fresh or stagnant water ditches and in swampy grounds, often intertwined with marshy grasses and sedges. In Ayurveda literature, specifically the Sushruta Samhita and Charak Samhita, *Hygrophila auriculata* is referred to as seethaveryam and mathuravipaka. Morphologically, the plant is characterized as ikshura, ikshagandha, and

kokilasha, reminiscent of the eyes of the Indian cuckoo (kokila). It is documented as a remedy for premeham (diabetes) and athisaram (dysentery). Ethno medicinally, the plant has been recognized for its diverse applications in treating and preventing various health conditions. These include allergy, anaemia, arthritis, hypertension, body pain, cancer, renal dysfunction, oedema, inflammations, jaundice, liver disorders, leprosy, skin diseases, tuberculosis, urogenital disorders, and venereal diseases (Sethiya *et al*., 2018 and Dhanalakshmi *et al*., 2020)

Phytochemicals, which are bioactive compounds present in plants, serve as the foundation for many contemporary pharmaceuticals. They are commonly employed in primary disease prevention or as complements to traditional therapies (Ulbricht and Chao, 2010). There is a need for cost-effective and efficient plant-derived chemotherapeutic agents to regulate the development and progression of wound healing. Natural products, including plant-derived compounds, have been utilized in traditional medicines worldwide for thousands of years to combat various diseases. Exploring the preclinical and clinical aspects of these compounds may result in the development of innovative therapies for wound healing. Additionally, ongoing research on established phytochemicals holds the potential to enhance evidence-based decision-making in integrative wound healing studies (Sharma *et al*., 2021).

Reactive Oxygen Species (ROS) are small oxygen-derived molecules primarily generated by the respiratory chain in mitochondria. Some examples include hydrogen peroxide (H_2O_2), superoxide anion (O_2), or peroxide (O_2). While reactive oxygen species (ROS) function as oxidants and the important factors in cell damage, they also play advantageous roles, especially in triggering the typical wound healing response. Striking a delicate balance between low and high ROS levels is vital. Low levels of ROS safeguards tissues against infection and facilitate efficient wound healing by generating signals that support cell survival. Conversely, an overabundance of ROS can induce oxidative stress, resulting in cell damage and fostering a pro-inflammatory condition.

The discrepancy occurs when levels of ROS surpass the capacity of endogenous antioxidants to neutralize them, disrupting the healing process. Antioxidants, which are chemical compounds with the ability to provide electrons to molecules like ROS, hinder the extraction of electrons from crucial biological molecules like proteins or DNA by these reactive species. Adjusting levels of ROS and antioxidants to control redox balance represents a focal point for novel therapeutic approaches.

Phytochemicals, as potential natural antioxidants, may be more effective than synthetic ones. Employing antioxidant substances to sustain non-toxic levels of reactive oxygen species (ROS) in wound tissues has the potential to improve the healing process. This has sparked growing interest in utilizing antioxidant compounds for wound treatment, resulting in the exploration and evaluation of diverse biomaterials. Nevertheless, many of these innovative compounds lack widespread recognition among clinicians, and their characteristics, along with their actual impact on the healing process, remain uncertain (Comino *et al*., 2021). Therefore, the current study aims to confirm the presence of biologically active phytochemicals and assess the antioxidant potential of the roots and leaves of *Hygrophila auriculata*.

A ligand is a substance that forms a complex with a biomolecule, serving a biological purpose. In molecular docking, natural compounds derived from plants were assessed for ADMET properties to identify a functional ligand. With a substantial increase in the capacity for biological screening, there is a growing demand for extensive information on ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) data. Predicting ADMET properties is crucial in the drug design process, as these properties contribute to 60% of drug failures in clinical phases. ADMET is applied early in the drug development process to eliminate molecules with unfavourable ADMET properties, resulting in significant cost savings in research and development (Jayasimha *et al.,* 2013).

Molecular docking is a method used to predict the binding orientation of drug candidates with their protein targets. This technique aids in forecasting the affinity and activity of these molecules. In recent times, there has been an upsurge in the application of docking ligands to receptors using rational drug design due to limitations in traditional drug design methods. As a result, molecular docking assumes a crucial role in the rational design of drugs. Given the biological and pharmaceutical importance of molecular docking, considerable efforts have been dedicated to improving the predictive methods employed in docking.

Compounds showing promising performance in *in silico* predictions can serve as potential starting materials for experimental investigations (Kitchen *et al.,* 2004). In this study, specific proteins associated with wound healing targets (Elastase, Glycogen synthase kinase-3β, Gelatinase, Collagenase) were subjected to docking with natural compounds obtained from *H. auriculata*. This holds significant promise for identifying potential lead

compounds in the context of wound healing. This exploration involves LCMS analysis coupled with *in silico* docking techniques.

Researchers can respond to the active components responsible for observed biological effects by extracting a specific bioactive compound from herbal plants. This is particularly crucial in fields like natural product research or drug discovery, where intricate mixtures are frequently encountered. The isolation of the active compound allows researchers to precisely identify the molecule possessing the desired properties. This, in turn, facilitates the exploration of its mechanism of action, enabling researchers to investigate how the bioactive compound interacts with specific targets or pathways in living systems. Such insights contribute to a deeper understanding of the underlying biological processes, which is essential for grasping disease mechanisms, devising therapeutic interventions, and optimizing drug design (McKinney *et al*., 2000).

However, the traditional pharmaceutical approach to develop medicines and treatment strategies has long followed the "one drug, one target, one disease" model. Over the past decade, there has been a gradual shift away from this mono-substance therapy approach towards the adoption of integrated therapies, incorporating numerous active components. This transition is influenced, to some extent, by the constrained efficacy of the traditional model in dealing with chronic diseases, treatment resistance, and the adverse effects linked to synthetic mono-drugs. Frequently, these drugs do not demonstrate equivalent activity to the unprocessed extract at similar concentrations or doses of the active component. This disparity is attributed to the lack of interacting substances found in the extract (Zhou *et al*., 2016).

Furthermore, progress in analytical chemistry and molecular biology techniques has broadened our comprehension of therapeutic targets in diseases, creating opportunities for potential treatment approaches targeting multiple factors. In summary, research on the synergy of herbal medicine is still in its early stages. The current methods available for studying synergy in herbal medicines have significant limitations, necessitating future methodological development. While diverse pharmacological studies reveal synergistic effects, these results do not consistently translate into clinical efficacy. As a result, the clinical advantages of multi-component combinations require meticulous validation through rigorous clinical trials (Yue *et al*., 2014).

Nanotechnology has become significantly influential within the scientific community in the past century, sparking numerous advancements in the field. Particles with dimensions

below 100nm are classified as nanoparticles. The primary advantage of these nanoparticles lies in their size, as it profoundly influences the physiochemical properties of substances. Consequently, nanoparticles of varying sizes exhibit differences in shape, size, and color (Khan *et al.,* 2019). Nanoparticles can be synthesized in diverse shapes, sizes, and dimensions, showcasing a wide range of physiochemical properties. Depending on the intended application, nanoparticles can be tailored into various forms, such as wires, rods, sheets, and particles (Modi *et al.*, 2022). They are categorized into four types based on the chemical composition used for their preparation: i) Organic nanomaterials, including nanoconjugates, hydrogels, polymerosomes, dendrimers, and micelles ii) Inorganic nanomaterials, encompassing ceramic nanomaterials, metal oxides, and metals iii) Composite nanostructures iv) Carbon-based nanomaterials, such as graphene, carbon nanotubes, carbon nanofibers, and fullerenes (Gaur *et al.,* 2021 and Sawy *et al.*, 2021).

Nanoparticles possess extensive applications by transforming bulk materials into nano-sized particles, thereby altering their physiochemical properties. Their broad utility spans across diverse fields such as medical and pharmaceutical sciences, molecular biology, material science, and physical, chemical, and biological sciences (Ulwali *et al.,* 2021). Nanomaterials, characterized by a high surface-to-volume ratio, have the capability to interact effectively with cellular and molecular processes. This distinctive property has drawn the interest of biomedical scientists, leading to their application in various biomedical research endeavours (Chandrakala *et al.,* 2022).

Vesicular systems, including liposomes, ethosomes and ultra-deformable liposomes are composed of amphipathic molecules characterized by both hydrophilic and lipophilic regions. Studies suggest that these vesicular systems, comprising liposomes, niosomes, transferosomes, penetration enhancer-containing vesicles, and ethosomes, have the potential to improve the therapeutic effectiveness of wound treatment medications. They contribute to extending the durability of both hydrophilic and hydrophobic drugs, alleviating notable side effects such as skin irritability, and function as depots for controlled drug release. Moreover, these vesicular systems enhance the skin penetration of medications. The main classifications of vesicular systems encompass solid vesicles, exemplified by liposomes and niosomes, and pliable or highly flexible vesicles, represented by transferosomes (Xu *et al*., 2017).

Liposomes are spherical vesicles comprised of a lipid bilayer, with cholesterol and phospholipids being their primary components. They have the capability to encapsulate both

hydrophilic and hydrophobic drugs (Beltran *et al.,* 2019). Alec D. Bangham discovered liposomes in 1965, marking them as the first approved therapeutic nanocarrier for cancer treatment (Bourquin *et al.,* 2018). Due to their non-immunogenic, biocompatible, nontoxic, and biodegradable nature, liposomes are extensively utilized in nano therapeutics (Zamani *et al.,* 2018). Liposomes serve as successful carriers because their phospholipid bilayer closely resembles the mammalian cell membrane, facilitating efficient cellular uptake. This characteristic enables liposome to reach cells with high concentrations, thereby reducing unnecessary side effects and enhancing efficacy (Gonda *et al.,* 2019).

Liposomes are categorized into four types based on the number of phospholipid bilayers present: unilamellar, multilamellar, oligolamellar, and multi vesicular vesicles. Various methods, such as the injection technique, microfluidic method, heating method, reverse phase evaporation technique, hydration methods, detergent depletion, thin film hydration method, membrane extrusion, and freeze-drying, are well-documented for liposome preparation (Has and Sunthar, 2019). Liposomes with a size ranging between 50 to 200 nm are preferred for drug delivery applications. The size of liposomes plays a pivotal role in influencing drug delivery to targeted cells. Liposomes smaller than 200 nm exhibit enhanced drug release, prolonged circulation time, and accumulation at the target site. Liposomes can be administered through various routes, including oral, ocular, and intravenous, for treating a variety of diseases (Leitgeb *et al.,* 2020).

Liposome-encapsulated antibiotics exhibit reduced toxicity, heightened target specificity, increased efficacy in treating bacterial infections, and improvements in pharmacokinetics and pharmacodynamics. Notably, there is an amplified action against external pathogens resistant to conventional treatments and enhanced activity against intracellular pathogens. In the context of burn wounds and chronic wounds, lipid nanoparticles may be preferable due to their occlusive effect on the stratum corneum, preventing trans-epidermal water loss and maintaining optimal lesion moisture. Liposomes, upon topical administration, can elicit various reactions. The predominant focus has been on the antibacterial efficacy of topical treatments, which has been hindered by the escalating antibiotic resistance. Nevertheless, liposomes can impede systemic absorption, minimize side effects, deliver a localized impact, facilitate targeted distribution to skin appendages, and improve drug deposition at the action site. Additionally, these vesicles play a pivotal role in the process of wound healing.

Moreover, when contrasted with vesicular systems, nanostructured lipid carriers stand out as the best among other nano delivery methods, providing outstanding stability, less toxicity, a high capacity for drug loading, and sustained drug release. These characteristics contribute to hastening the process of wound healing and diminishing the volume of administered drugs (Tiwari and Pathak, 2023).

By moistening the stratum corneum and increasing hydration, the integrity of the epidermal barrier can be compromised or diminished, causing a reconfiguration of the array and structural changes among skin cells. This leads to increased permeation of liposomes through the interstitial spaces between cells and their merging with skin cells for drug release. The phospholipid bilayer membrane of liposomes demonstrates enhanced flexibility and a tendency to fuse with the cell membrane (Reimer *et al*., 2000).

Utilizing liposomes on the skin presents numerous benefits. First, liposomes are composed of materials that are non-toxic and environmentally friendly, with component structures typically resembling or identical to endogenous substances. Second, liposomes display remarkable compatibility and affinity with the skin, promoting increased permeability of the stratum corneum and facilitating rapid skin penetration. Thirdly, when used as excipients or local drug depots, liposomes can be directly applied to skin lesions, ensuring the sustained long-term release of drugs. This approach reduces drug toxicity and side effects associated with the massive release of a one-time use drug in conventional drug administration. The presence of an edge activator enhances the flexibility of deformable liposomes, allowing them to traverse the stratum corneum and reach the viable epidermis. Numerous studies have utilized deformable liposomes to load various drugs and facilitate drug distribution into the skin. Notably, they can also serve as vehicles for delivering large biogenetic molecules into the skin (Li *et al*., 2016).

Moreover, as of now, there have been no studies investigating how liposome-based drug carrier systems can enhance the delivery of *Hygrophila auriculata* root to wound sites. Despite their recognized capability to improve drug permeation through the skin and localize in the dermis, this aspect has not been explored. Hence, the present study was undertaken using liposome-encapsulated *Hygrophila auriculata* root and its active compound betulin. The aim was to compare their efficacy in wound healing and evaluate their potential synergistic effects compared to the effects of a single active compound.

Skin and soft tissue infections (SSTIs) pose significant risks of morbidity and mortality. While some SSTIs respond well to treatment, those impacting subcutaneous tissue, fascia, or muscle can impede the healing process and escalate to life-threatening conditions. Consequently, there is a persistent need for more efficacious treatments to address such challenging pathological scenarios. Additionally, the growing prevalence of antibiotic-resistant bacteria, notably Methicillin-resistant *Staphylococcus aureus* bacteria (MRSA), in skin infections intensifies the urgency for more successful therapies. MRSA can spread to deeper soft tissues, leading to complications like cellulitis, abscesses, or even necrotizing fasciitis. In response to this, numerous research studies have devised liposomal formulations for skin infections, aiming not only to effectively clear pathogens but also to contribute to skin regeneration and wound healing (Wang *et al*., 2019).

Certainly, in the initial stage of infection, gram-positive bacteria such as *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pyogenes* (*S. pyogenes*) are the primary culprits, while gram-negative bacteria like *Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) tend to inhabit the evolving wound, potentially leading to sepsis if they infiltrate the lymphatic system and blood vessels (Cardona and Wilson 2015). During this phase, neutrophils and macrophages diligently work to eradicate all foreign particles and tissue debris, effectively preventing infections (Broughton *et al*., 2006). Additionally, the release of cytokines and enzymes stimulates fibroblasts and myofibroblasts, while the presence of wound exudate ensures the necessary moisture for healing (Das and Baker, 2016; Suarato *et al*., 2018). In the current study, the synthesized liposomes are scrutinized for their antimicrobial properties against both gram-positive and gram-negative bacteria.

Replicating the wound healing process *in vitro* presents challenges due to the absence of cell debris and intricate interactions among diverse cell types. Nevertheless, assessing an *in vitro* capability of peptide to accelerate pseudo-wound closure by promoting epithelial cell migration serves as an indicator of its potential in healing compromised epithelium. Cell migration, an important event in wound healing, becomes a central focus for research aimed at enhancing therapies for improved wound healing. This study introduces a highly reproducible experimental assay that utilizes specialized silicone culture to evaluate *in vitro* cell migration. This method represents progress compared to traditional assays, where a scratch is physically created by incising confluent cell monolayers with a sterile needle or pipette tip. Thus, the described approach holds substantial potential to significantly advance

our understanding of migratory characteristics in various adherent animal cells and epithelial tissues (Cappiello *et al*., 2018). Hence, the present study focuses on experimenting with the *in vitro* scratch assay using the synthesized liposomes.

In the realm of biomedical research, the superiority of *in vivo* assays over *in vitro* assays becomes evident in their capacity to deliver a more encompassing and contextually relevant understanding of the interactions within living organisms. While *in vitro* assays, conducted in controlled laboratory environments, provide valuable insights into cellular mechanisms, *in vivo* assays involving experiments within living organisms, present numerous critical advantages. These studies not only consider the intricacies of pharmacokinetic processes, such as drug metabolism, distribution, and absorption within the body, but also afford researchers the opportunity to observe systemic effects that involve multiple organs and physiological systems (Graudejus *et al*., 2018).

Additionally, *in vivo* assays enable the undertaking of long-term and chronic studies, facilitating the observation of delayed effects, cumulative toxicity, and other phenomena that may not become apparent in short-term *in vitro* experiments. The present study deals with analysing the efficacy of synthesized liposomes using the Swiss albino rat model. The excision wound was created and evaluated with the treatment of liposomes. With this background, the study was formulated with the following objectives:

- To evaluate the phytochemical and antioxidant potential of leaves and roots of *H. auriculata*
- To identify the lead molecules against wound healing target proteins from *H. auriculata* root extract using LCMS for *in silico* docking
- To characterize liposomes encapsulated *H. auriculata* root extract and the bio active compound betulin
- To evaluate the antibacterial potential of the liposomes encapsulated *H. auriculata* root extract and betulin
- To study the *in vitro* cytotoxicity and wound healing potential of the liposomes encapsulated *H. auriculata* root extract and betulin in HACAT– Human Keratinocyte cell lines
- To assess the *in vivo* wound healing activity of liposome encapsulated *H. auriculata* root extract and betulin using swiss albino rat model