5

Discussion

The skin serves as the primary defense against infections and injury. Infections and tissue injuries cause harm or disrupt the integrity of the skin, which may disturb the body homeostasis. The loss of skin integrity can lead to morbidity and even death in severe cases, as skin is involved in maintaining the fluid homeostasis (Rosique *et al*., 2015). An injury to the skin results in a wound. The healing of the wound is an essential phenomenon that restores the skin integrity. The innate immune system constantly monitors the body for the presence of foreign bodies and triggers a localized inflammatory response in order to eliminate them (Tracey, 2002). Wound healing is a complex and well-regulated process, typically divided into three main phases: the initial inflammatory phase, the proliferative phase, and the final remodeling phase (Wu and Chen, 2014; Wilkinson *et al.,* 2002).

Several ongoing researches are focusing on the development of better and safer wound healing drugs from natural products due to their affordability and potential pharmacological properties. In recent times, the nanotechnology is extensively applied the in medicine field due to the unique properties of the nanomaterials including chemical, physical and optical properties (Patil and Patil, 2016). Liposomes are lipid vesicles that serve as carriers for targeted drug delivery. They offer several advantages, including biodegradability and biocompatibility. Moreover, liposomes can encapsulate both hydrophilic and hydrophobic drugs, that serves as protective vesicles that shield the drug from physiological degradation. This not only prolongs the shelf life of the encapsulated drug but also allows for controlled release. Additionally, liposomes enable targeted drug delivery, minimizing unwanted side effects (Liu *et al.,* 2022).

In accordance with the above cited literature, this study focuses on synthesizing liposomes, one loaded with betulin and the other loaded with the ethanol extract of *H. auriculata* (*Hygrophila auriculata*) roots, for wound healing. In this current investigation, the levels of phytochemicals and antioxidants were examined in the fresh leaves and roots of *H. auriculata* at the pilot level. The study encompassed an analysis of wound healing activities through *in silico, in vitro,* and *in vivo* approaches, utilizing liposomes encapsulated with *H. auriculata* roots and its active compound betulin chosen based on the findings from the *in silico* pilot study. The study was conducted in four distinct phases. In Phase I,

phytochemical studies and the assessment of free radical scavenging activity in various extracts of *H. auriculata* leaves and roots were conducted, followed by the determination of enzymatic and non-enzymatic antioxidant levels. Phase II involved *in silico* studies, where lead compounds targeting wound pathogenesis were retrieved from the secondary active constituents of *H. auriculata* roots using ligand-based molecular docking. In Phase III, liposomes were synthesized and characterized using the ethanol extract of *Hygrophila auriculata* and the active compound betulin, which exhibited the highest docking score. Antimicrobial studies were conducted with the synthesized liposomes. The final phase of the study involved *in vitro* and *in vivo* assessments of wound healing activities using cell lines and the Swiss albino rat model.

Many conventional medications commonly used in modern medicine are derived from bioactive compounds found in plants, known as phytochemicals. Conducting preliminary phytochemical screening is crucial for predicting the nature of the drugs. Typically, the percentage of active chemical constituents is reported on air dried basis. Therefore, it is important to determine the loss on drying of plant materials and control their moisture content. The loss on drying test measures both water and volatile substances. In the case of *H.auriculata* leaves and roots, the low loss on drying values (0.54% and 0.42%) indicate a minimal moisture content. The acid-insoluble ash serves as an indicator of the mineral content present in the plant material, whereas the water-soluble ash is utilized to evaluate the quantity of inorganic elements in medicinal substances (Namdeo and Kale, 2015). In the current study, the total ash, acid-insoluble ash, and water-soluble ash of *H. auriculata* leaves and roots were found to be at appreciable levels, measuring 4.17%, 2.81%, 3.26%, and 3.24%, 3.66%, and 4.66%, respectively.

Through a qualitative phytochemical analysis of *H. auriculata* leaves and roots using various solvents, the presence phytoconstituents such as alkaloids, flavonoids, phenols, tannins, proteins, carbohydrates, terpenoids, and cardiac glycosides was identified, with the highest intensity observed in the ethanol extract compared to other extracts. This underscores its superior extraction capability. Significantly, alkaloids were not detected in the aqueous extract but were found in other organic extracts, attributed to their limited solubility in water (Ding *et al*., 2010). These findings align with prior reports indicating the presence of tannins, saponins, phenols, flavonoids, cardiac glycosides, terpenoids, alkaloids, and steroids in various solvents of *H. auriculata* leaves (Prasanna and Sridhar, 2016). This indicates that ethanol is highly capable of extracting secondary metabolites of *H. auriculata* compared with

all other solvents since the high polarity of the solvent accounts for the extraction of a wide range of compounds.

Free radicals, characterized by unpaired electrons, can trigger a harmful chain reaction in cells. The targeted molecule, in turn, becomes a free radical, triggering a cascade of events that can result in cellular damage. To counter this, assessing free radical scavenging is crucial. Natural antioxidants found in plants, like phenolic compounds and flavonoids, neutralize free radicals such as peroxide, hydroperoxide or lipid peroxyl, preventing oxidative damage and diseases. Antioxidants operate through hydrogen atom transfer, where the antioxidant donates a hydrogen atom, or electron transfer, where the antioxidant donates an electron, both processes turning the antioxidant into a radical (Di Meo and Venditti, 2020)

The capacity of the plant extracts to scavenge the free radicals such as DPPH (2,2- di (4-test-octyl phenyl) -1-picrylhydrazyl radical) and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation followed by the ability to reduce ferric (III) iron to ferrous (II) iron were assessed by performing the *in vitro* free radical scavenging assays. In the present investigation, the ethanol extract of the roots showed the highest free radical scavenging activity than the leaves indicating a correlation between antioxidant content and DPPH reduction. Our findings highlight varying activities among different extracts, with ethanol extract showing the highest activity, followed by ethyl acetate and aqueous extracts. The solvent polarity significantly affects the presence of secondary metabolites and their antioxidant potential (Huyut *et al*., 2017).

In the DPPH assay, the antioxidant from leaves and roots of *H. auriculata* effectively converted the violet DPPH radical into the stable yellow compound, 1,1-diphenyl-1,2-picryl hydrazine, by donating a hydrogen atom. This transformation indicates a reduction in DPPH reactivity. A similar process occurs in the ABTS assay, where antioxidants interact with the blue ABTS radical, restoring its color to a decolorized state. The method is based on the reaction between ABTS and potassium per sulfate, forming a blue-green ABTS radical cation. Our findings revealed that the order of ABTS radical scavenging activity in all extracts matched that observed in the DPPH assay. Our findings indicate that various extracts exhibit varying degrees of activity, with the highest level of activity observed in the ethanol extract, followed by the ethyl acetate, aqueous, and chloroform extracts. The observed antioxidative properties of these extracts may be attributed to either hydrogen atom transfer or electron

transfer mechanisms, while their scavenging effects are likely attributable to the presence of bioactive phytoconstituents within them (Takatsuka *et al*., 2022).

The FRAP assay is commonly linked to the presence of reductones, which have demonstrated antioxidant activity by contributing a hydrogen atom and disrupting the free radical chain. In the course of the reducing power assay, reductants (antioxidants) within *H. auriculata* leaves and roots would convert the Fe3+/ferricyanide complex into its ferrous form (Fe2+). The quantity of Fe2+ can be assessed by measuring the generation of Perl's Prussian blue at 700 nm, which is directly proportional to its antioxidant capacity. Consequently, the observed antioxidant effect of the extract may arise from either the donation of a hydrogen atom or the transfer of an electron, and the scavenging effect can be ascribed to the presence of active phytoconstituents in the plant material.

Natural phenols and flavonoids act as antioxidants, protecting against oxidative stress and lipid peroxidation by neutralizing reactive oxygen species (Mutha *et al*., 2021). In this study *H. auriculata* leaves and roots contain phyto compounds, including flavonoids, phenolic acids, and phenolic diterpenes, which enhance radical scavenging activities due to their hydroxyl groups, especially the o-dihydroxy group, providing potent antioxidant effects. Hydroxyl groups play a crucial role in donating hydrogen bonds, facilitating the neutralization of free radicals, reducing metal ions, and interacting with biomolecules (Sarker *et al*., 2020).

A prior study on *H. auriculata* roots found that petroleum ether extract displayed a 93.91±6.57% DPPH radical scavenging ability at 120 µg/mL, followed by 62.07±4.34% ferric reducing power at the same concentration (Murugan and Kumar, 2018). Similarly, methanol leaf extract exhibited significant DPPH and ABTS radical scavenging activities, with values of 72.82±0.19% at 120 µg/mL and 88.93±0.23% at 30 µg/mL, respectively (Raaman, 2015). The current investigation has shown that the ethanolic extracts of *H. auriculata* roots and leaves displayed remarkable radical scavenging activity against DPPH and ABTS radicals. As a result, the subsequent research assessed the antioxidant potential of ethanol extracts from *H. auriculata* leaves and roots by measuring enzymic and non-enzymic parameters.

Endogenous cellular defense mechanisms effectively neutralize harmful free radicals, protecting biomolecules from oxidative damage. These cellular defense compounds are categorized as enzymatic and non-enzymatic antioxidants. Plant-derived phytochemicals

accelerate the scavenging and disruption of free radical chains within the body by promoting the production of enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), polyphenol oxidase, Glutathione-S-transferase (GST), as well as exogenous non-enzymatic antioxidants like flavonoids, α-tocopherol, and vitamin C. The ethanolic extracts of *H. auriculata* exhibited significant accumulations of enzymatic antioxidants, including superoxide dismutase, peroxidase, catalase, polyphenol oxidase, and glutathione-S-transferase, along with non-enzymatic antioxidants such as flavonoids, α-tocopherol, and vitamin C. Notably, the ethanol extract from *H. auriculata* roots exhibited higher activity compared to the leaves. Enzymatic antioxidants play a crucial role in the initial defense against reactive oxygen species (ROS) and superoxide anions, converting them into hydrogen peroxide (H_2O_2) and hydro peroxides. Catalase enzyme further breaks down hydrogen peroxide and hydro peroxides into water and oxygen, aided by metal ions such as zinc (Zn), copper (Cu), and manganese (Mn) (Bacha *et al*., 2017).

Polyphenol oxidase, a copper enzyme also known as catechol oxidase or tyrosinase, acts as a potent antioxidant by scavenging hydrogen peroxide (H_2O_2), thereby mitigating oxidative stress and regulating other oxidases in the body. The phenolic hydroxyl structure's electrons weaken hydrogen ion binding, enhancing their dissociation risk. Consequently, active hydrogen ions suppress reactive oxygen species and other oxidants, stabilizing themselves (De Oliveira and Orlanda *et al*., 2017). Additionally, glutathione reduction enhances antioxidant defense by neutralizing cellular hydrogen peroxide, aided by detoxification enzyme glutathione S-transferases (GSTs) that conjugate glutathione with various electrophilic compounds, increasing their solubility (Vaish *et al*., 2020).

The secondary defense mechanism acts to inhibit the generation of damaged cellular species and the progression of harmless free radicals, thereby minimizing the impact of oxidative reactions. This effect is achieved through non-enzymatic antioxidants like flavonoids, α-tocopherol, and Vitamin C (Haida and Hakiman 2019). In the qualitative phytochemical analysis of *H. auriculata* root ethanol extract, the presence of flavonoids corresponds with their high accumulation in the quantitative analysis. The roots exhibit a significant flavonoid content of 2.40 ± 1.23 µg/g, surpassing that of the leaves.

Flavonoids, a subgroup of polyphenols, are closely associated with antioxidant potential, as they inhibit free radical production by complexing with chelating metal ions, thereby neutralizing them and preventing DNA damage. Moreover, vitamin E, a lipid-soluble

antioxidant, is highly effective α-tocopherol, and the most bioactive variation for humans. It acts as a safeguard for cell membranes, shielding them from free radicals by interrupting lipid peroxyl radicals (LOOc) and halting lipid peroxidation reactions. When α-tocopherol reacts with lipid peroxyl radicals, it generates tocopheroxyl radicals, which, while relatively stable, do not trigger or initiate further lipid peroxidation, a critical characteristic of a potent antioxidant (Moussa *et al.,* 2019).

Similar to vitamin E, vitamin C disrupts the lipid peroxidation chain reaction by donating an electron to lipid radicals and transforming into an ascorbate radical. It also serves as a substrate for the enzyme ascorbate peroxidase, converting hydrogen peroxide (H_2O_2) to water (H_2O) (Kojo, 2004). The combined action of vitamins C and E effectively suppresses hydro peroxide and other radical formation. The increased levels of SOD, CAT, POD, polyphenol oxidase, and GST observed in *H. auriculata* indicate its ability to counteract oxidative stress effectively.

The preliminary research on *H. auriculata*, focusing on its phytochemical and antioxidant properties, has highlighted the remarkable biological characteristics of its root extract. This discovery underscores the need for further exploration of its potential. Several researchers have utilized LC-MS analysis to identify potential bioactive components in plant extracts, aiding in the development of new pharmaceutical drugs. LC–MS-based studies are valuable for identifying and quantifying targeted metabolites in herbal medicine, offering valuable structural information on chemical constituents. These techniques have been extensively employed to analyze bioactive components in various plants.

In the current study, the bioactive compounds in *Hygrophila auriculata* root were qualitatively identified through LCMS analysis in both positive (+) and negative (-) modes. These compounds were classified as secondary metabolites, including terpenoids, alkaloids, aliphatic compounds, and phenolics. These findings partially align with previous research conducted by Sethiya *et al.,* 2018 who identified flavonoids (such as luteolin, quercetin, gallic acid, apigenin and ellagic acid), alkaloids (asteracanthicine and asteracanthine), triterpenes (betulin, lupenone, hentricontane, and lupeol), sterols (asterol and stigmasterol), minerals, fatty acids, essential oils, aliphatic esters, and amino acids in *H. auriculata*.

Furthermore, he stated that extracts and bioactive compounds from this plant have exhibited a wide array of beneficial properties, including antimicrobial, antitermite, hepatoprotective, anthelmintic, nephroprotective, central nervous system protective,

antidiabetic, antitumor, haematopoietic, anticataract, antioxidant, diuretic, antinociceptive, antipyretic, antimotility, antiinflammatory, aphrodisiac, anti-endotoxin, neuroprotection and antiurolithiatic activities (Sethiya *et al.,* 2018).

For *in silico* analysis, ligand optimization was carried out. The total 15 bio active molecules obtained from LC-MS analysis, were retrieved without any ambiguities. All the 15 molecules have CAS number from the Pubchem. This approach stems from the awareness, dating back to the late 1990s, that poor pharmacokinetics and toxicity played significant roles in the costly failures of drug development at later stages. Consequently, it has become a widely recognized imperative to address these aspects early in the drug discovery process.

The biological effects of compounds are significantly influenced by their physicochemical properties, impacting pharmacokinetic and pharmacodynamics features. Utilizing the Swiss ADME tool, compounds adhering to Lipinski rule of five were scrutinized, an important criterion for drug likeness and the development of orally active drugs in humans. Approximately ten compounds obeyed the Lipinski rule of five, a significant indicator of their suitability for drug development. Furthermore, the analysis considered parameters like XLOGP3, WLOGP, and MLOGP, which collectively reflect lipophilicity. The LOGP of a molecule within the n-octanol and water system serves as a quantitative descriptor of its lipophilicity, with n-octanol serving as an excellent mimic of phospholipid membrane properties due to its amphiphilic nature (Liu *et al*., 2011; Daina *et al*., 2014).

A strong bioactivity score signifies excellent *in vivo* pharmacological activity (Sreejaya and Santhy, 2015). The findings of this study indicate that the examined compounds exhibited biological activity, exerting physiological effects through interactions with GPCR ligands, nuclear receptor ligands, and inhibition of proteases and other enzymes. Bioactivity scores were assessed using a signaling cascade involving GPCR ligands, ion channel modulators, protein kinase inhibitors, nuclear receptor ligands, and protease inhibitor ligands. The GPCR ligand-based cascade contributes to the progress of functional drugs with improved binding selectivity.

Targeting ion channel modulators is crucial for therapeutic interventions, as they govern the transport of charged particles across cellular membranes. Kinase inhibitors, on the other hand, one of the promising approach for drug development by selectively blocking or modulating disease-related signaling pathways. Bioactive scores fall into three categories:

active (> 0), moderately active (−5.0 to 0.0), and inactive (< -5.0). Based on physicochemical and bioactivity analysis, compounds like epiafzelechin, betulin, caffeic anhydride, quercetin, palmitic acid, linoleic acid, chlorogenic acid, and Coumaroyl quinic acid have demonstrated high bioactivity and drug-like characteristics (Sivanandan S. and Pimple, 2018).

A favorable ADMET profile is necessary for the molecules in new drug discovery. Properties like adsorption, permeability, and interactions with cellular barriers are vital considerations. Caco-2 cells, mimicking small intestine epithelial cells, serve as a standard model for studying absorption efficiency (Shou, 2020). Compounds with Caco-2 permeability greater than 8×10^{-6} are considered permeable; the phytocompounds in this study displayed high permeability (77.207 to 81.13 percent), indicating easy gut absorption. Additionally, their Log Kp values below -2.5 suggest high skin permeability. Quercetin and Epiafzelechin were identified as P-glycoprotein substrates, while other compounds acted as P-glycoprotein inhibitors, potentially preventing excessive drug efflux.

In the distribution analysis, our findings indicate that quercetin and Epiafzelechin are likely to be present in tissues rather than plasma due to their steady-state volume distribution (VDss) being higher than 0.45, while other compounds are expected to be in plasma with log VDss values below -0.15. The blood-brain barrier blocks molecules from entering the central nervous system, allowing only water and lipid-soluble and selective transport molecules like plasma glycoprotein and glucose transporters to pass through (Pires *et al.*, 2015).

Certain molecules like kaempferol-7-O-Glucoside, linoleic acid, and chlorogenic acid can cross the blood-brain barrier (BBB) as their log BB > 0.3, making them permeable to the central nervous system (CNS). Epiafzelechin and betulin moderately penetrate the BBB and CNS. In a study, CYP2D6 and CYP3A4, the isoforms of CYP450, is a detoxifying enzyme that aids xenobiotic metabolism by oxidizing and facilitating detoxification (Issa *et al.*, 2012). In metabolism analysis, betulin and linoleic acid are substrates for both CYP2D6 and CYP3A4 enzymes, while epiafzelechin, kaempferol-7-O-Glucoside, and chlorogenic acid are substrates for one of these enzymes. Additionally, epiafzelechin, kaempferol-7-O-Glucoside, and chlorogenic acid act as inhibitors of specific CYP450 isoforms.

Total clearance plays a crucial role in a drug's pharmacokinetics, influencing factors like its half-life, bioavailability, dosage, and frequency (Wang *et al.*, 2012). In the analysis of excretion, all examined phytocompounds demonstrated significant total clearance values and renal clearance as substrates for renal OCT2. In terms of toxicity, various tests indicated that

all studied drug candidates were non-toxic according to AMES toxicity, Hepatotoxicity, and skin sensitivity assessments. The hERG potassium channel (human Ether-a-Go-go-related gene) is the biomarker for the cardiotoxicity. Blocking the hERG potassium channel in humans leads to QT interval prolongation and major cardiovascular complications, a significant issue in pharmacological trials (Wu *et al*., 2020). The compounds showed no inhibition of the hERG potassium channel, which is associated with cardiac complications. Overall, the phytocompounds exhibited low toxicity, were non-carcinogenic to mice, and posed minimal risks in terms of lethality and hepatotoxicity.

Following the initial examinations for drug-like properties, all the ligands underwent docking with the target proteins. Totally 40 *in silico* docking analyses were performed. The present computational study aimed to find an efficient drug candidate from the *H. auriculata* root which can inhibit the target proteins responsible for the pathogenesis of the wound. The interaction patterns between proteins and ligands vary based on the ligand's nature. In molecular docking analyses, the strongest binding affinity is indicated by the most negative value.

Effective protein-ligand interactions occur when the ligand fits well into the receptor pockets. Elastase exhibited the best interaction with betulin and epiafzelechin, forming 1 and 2 hydrogen bonds, respectively. Collagenase showed favorable docking with betulin and kaempferol-7-O-Glucoside, forming 2 and 5 hydrogen bonds, respectively, with binding energies of -9.65 kcal/mol and -7.48 kcal/mol. Chlorogenic acid and kaempferol-7-o-glucoside displayed strong binding affinity with glycogen synthase kinase-3β protein, forming 5 and 2 hydrogen bonds, respectively. Gelatinase exhibited excellent interactions with kaempferol-7-o-glucoside and linoleic acid, forming 6 hydrogen bonds and alkyl/nonbonded interactions, with binding energies of -8.05 kcal/mol and -7.96 kcal/mol, respectively.

Wound formation triggers changes in intracellular signaling pathways, resulting in the destruction of the extracellular matrix (ECM), breakdown of collagen, and an upsurge in matrix metalloproteinases (MMPs). Increased MMP levels contribute to a hindered wound healing which is linked with the breakdown of newly formed ECM, possibly due to oxidative stress and inflammation (Potekaev *et al*., 2021).

The present study explored how bioactive compounds from *H. auriculata* interact with MMPs, which are involved in ECM degradation and delayed wound healing.

Compounds like epiafzelechin, 5-P coumaroyl quinic acid, quercetin, betulin, palmitic acid, kaempferol-7-O-Glucoside, linoleic acid, and chlorogenic acid displayed strong binding energies above -4 kcal/mol with collagenase, gelatinase, and elastase. These compounds may enhance wound healing by inhibiting MMPs, increasing ECM components, reducing platelet aggregation, and decreasing pro-inflammatory factors. Inhibiting collagenase deactivation at the wound site can alleviate inflammation and promote healing, while inhibiting metalloelastase enzymes can improve wound healing. Additionally, Betulin and Kaempferol-7-O-Glucoside demonstrated strong binding affinity with GSK 3β in molecular docking, potentially promoting cutaneous wound healing through the beta-catenin-dependent Wnt pathway by inhibiting GSK 3β.

Computational analysis indicated betulin as potential for wound healing, warranting further *in vitro* and *in vivo* investigations. Commercial betulin was used in the subsequent research which involved a comparative analysis of wound healing efficiency using liposome-encapsulated *Hygrophila auriculata* root and betulin.

The utilization of nanotechnology in wound healing treatment presents several notable advantages in contrast to traditional cutaneous therapies like occlusive dressings and readily permeable ointments. These benefits encompass the safeguarding of active therapeutic components, improved drug penetration, facilitation of localized drug effects, and diminished undesirable systemic absorption. A significant advantage of these nanocarriers lies in their capacity to encapsulate lipophilic, amphiphilic, and hydrophilic substances owing to their biphasic nature (Kushwaha *et al*., 2022).

Liposomes were synthesized by the thin film hydration method and assessed using TEM analysis, XRD, and Zeta potential analysis. The encapsulation efficiency, signifying the successful entrapment of drug or nanoparticles within lipid vesicles, is crucial for the therapeutic impact of the drug delivery system. In the present study the percentage of EEHA (Ethanol Extract of *H. auriculata*) entrapped in the liposome was 79.2% and for betulin-encapsulated liposome, the efficiency was found to be 75%. This efficiency depends on factors like incubation time, lipid composition, lipid-to-drug ratio, and the pH of the aqueous phase (Karimi *et al.,* 2019).

The appropriate ratios of lecithin, cholesterol, and *H. auriculata* root allowed for allows for efficient drug binding and increased encapsulation efficiency than with betulin. In the case of herceptin-conjugated liposomes loaded with Simvastatin and Doxorubicin, the

encapsulation efficiency was 81.7% and 84.32%, respectively (Li *et al*., 2019). Likewise, Hardiansyah *et al*. (2017) incorporated curcumin into both PEGylated magnetic liposomes and PEGylated liposomes, with the latter exhibiting a superior encapsulation efficiency of curcumin (78.06 ± 0.57%). Ng *et al*. (2018) achieved high encapsulation efficiencies of curcumin and salbutamol at 81.1% and 83.6%, respectively, due to the appropriate lipid-to-drug ratios. The present study suggests that the superior encapsulation of *H. auriculata* root and betulin into liposomes results from the well-balanced lipid-nanoparticle ratios, adequate incubation, and lipid composition.

FTIR, a widely utilized technique among researchers and industrialists, serves as a conventional method for analyzing the composition and structure of molecules. It proves to be a rapid and steady tool for identifying the functional groups present in substances. Operating on reflectance and absorption spectroscopy in the infrared region, FTIR is extensively employed for material characterization by researchers (Fahelelbom *et al*., 2022).

In the present investigation the peak intensity of *H. auriculata* encapsulated liposome was found to be sharper when compared to that of the peak obtained for the non-encapsulated ethanol extract. This phenomenon indicates the successful entrapment of *H. auriculata* within the liposomes. Our findings align with Ben-Fadhel *et al*. 2022, where natural extracts-loaded food grade nanoliposomes were prepared and analyzed for functional groups. The analysis of those functional groups affirmed the encapsulation of the drug into the lipid bilayer. A structural shift between blank liposomes and those loaded with natural extracts confirmed the encapsulation of the drug into the lipid bilayer.

Similar outcomes were noted by Wang *et al*. in 2021, who, through FTIR analysis, detected changes in functional groups in the synthesis of blueberry anthocyanin liposomes. The interaction between liposomal functional groups and anthocyanin facilitated successful encapsulation, strengthened by hydrogen bonding or hydrophobic interactions. Hence the FTIR results underscore that various functional groups in the hydroethanolic extract of *H. auriculata* contribute to enhancing the stability of synthesized nanoparticles. Notably, the disparity in functional groups between plain extract and liposomes signifies the successful encapsulation of *H. auriculata* and betulin into the liposomes.

XRD is a conventional method used to characterize liposome nanoparticles, determining their crystalline structure, size, and phase nature (Mourdikoudis *et al.,* 2021). In the present study, *H. auriculata* and betulin-loaded liposomes were analyzed. *H. auriculata*-

loaded liposomes exhibited a face-centered cubic crystalline structure with an average size of approximately 30.98 nm, influenced by nanoparticle size. Plant extract constituents acted as capping agents, stabilizing liposome nanoparticles and imparting a crystalline nature (Gabriel *et al.,* 2018). In contrast, betulin-loaded liposomes had an amorphous nature.

Zeta potential analysis assesses the surface charge and colloidal stability of *H. auriculata* and betulin-loaded liposomes. When an electric field is applied, particles in the solution move toward the positive or negative electrode, determining their velocity (Silva *et al.,* 2019). High negative or positive zeta potential values indicate repulsion among particles, preventing agglomeration and resulting in enhanced stability. Conversely, low zeta potential values result in particle attraction and flocculation (Sharma *et al.,* 2022).

The zeta potential of the liposomes synthesized using *H. auriculata* root extract was -51.7 mV and for liposomes synthesized using betulin, it was seen to be -0.475 mV. The result with a single peak signified that the presence of repulsion among the synthesized nano particles. Similar findings were recorded by Luo *et al*., (2020) who prepared nano liposomes loaded with procyandins from lychee pericarp and observed that the zeta potential was −32.8 mV indicating that the nanoliposome are relatively stable

Effective treatment of chronic wounds is challenging due to the complex symptoms caused by microorganisms in the wound area. In our study, liposome-encapsulated EEHA showed better antibacterial activity than liposome-encapsulated betulin, with inhibition zones ranging from 12.3 ± 0.57 mm to 22.2 ± 2.3 mm. The spherical lipid bilayer of liposomes holds hydrophobic compounds from the ethanol extract, interacting with the bacterial cell membrane through hydrophobic interactions. This disrupts the outer membrane, making the inner membrane permeable, leading to the leakage of ions and cell constituents and ultimately causing cell death.

Previous research has explored various antimicrobial actions, such as inhibiting cell membrane enzymes, disrupting electron transport systems, altering membrane permeability, increasing membrane fluidity, and damaging cell walls through hydrophobic and hydrogen bonding (Vaou *et al*., 2021). The negatively charged aminoglycosides in liposomes usually enhances antibacterial activity against *Pseudomonas aeruginosa* (Eleraky *et al*., 2020). Alhariri *et al*., in 2017 demonstrated that negatively charged liposomes carrying meropenem and gentamicin were more effective against selected gram-positive and gram-negative bacteria. Liposomes with negative surface charge containing azithromycin or ciprofloxacin

strongly inhibited *Mycobacterium avium* compared to their non-liposomal forms (Ferreira *et al*., 2021). These findings emphasize the efficacy of liposomes for delivering antibacterial agents like EEHA and betulin.

The liposomes were subjected to *in vitro* assessments of cytotoxicity and wound healing activity. The study employed antiproliferative indices, which quantify the percentage of cell mortality via MTT assay. Keratinocytes constitute the primary cellular components of the epidermis and play pivotal roles in the initiation, maintenance, and completion of the wound healing process (Vang and Jenssen, 2018). Cell viability tests on HaCaT cells (human keratinocyte cell lines) indicated significant viability, with a slight decrease observed between concentrations of 12.5 and 200 µg/mL. At high concentrations, a slight decrease in viability was observed, particularly at 100 µg/mL, indicating low toxicity for liposome-encapsulated *H. auriculata* compared to betulin. Cell migration rate refers to the speed at which cells move over time and it is influenced by the environment. The results of the migration test revealed that liposome-encapsulated *H. auriculata* increased cell migration rate, approaching that of standard etoposide. The present study demonstrated that liposome-encapsulated *H. auriculata* and betulin specifically promoted keratinocyte proliferation and migration. IC_{50} values indicated that both liposome-encapsulated *H. auriculata* and betulin were non-cytotoxic to normal keratinocyte cells. The differential effects between liposome-encapsulated *H. auriculata* and betulin may be attributed to the lipid carrier, which reduces toxicity and enhances bioavailability.

An *in vitro* wound healing assay was conducted to observe the effect of liposome-encapsulated *H. auriculata* and betulin on HACAT cells, simulating wound healing by covering a scratch on cell culture plates. The scratch wound healing assay is a widely utilized and adapted method to investigate the impact of various experimental conditions, such as gene knockdown or chemical exposure, on mammalian cell migration and proliferation. In a typical procedure, a "wound gap" is created in a cell monolayer by scratching, and the subsequent "healing" of this gap through cell migration and growth towards the center is observed and often quantified.

The healing percentage, calculated based on the area covered in the scratch after forty-eight hours, indicated significant improvement in wound closure compared to untreated cells. The healing results were influenced by the initial gap size; narrower gaps had higher chances of achieving 100% healing. Several tests were performed, and the presented data

reflect outcomes from the identical batch to reduce standard deviation values. Evaluating migration rates at specific intervals provided insights into how treatments influenced cell movement. Diminished migration rates in later hours often occur as a result of reduced supplies or a narrowing of the wound gap, constraining the available space for cell migration (Soliman *et al*., 2021)

Cells subjected to liposome-encapsulated *H. auriculata* and betulin displayed comparable migration patterns, primarily differing in migration rates at specific intervals, leading to varying cell migration rates at the conclusion of the 40-hour period. This was supported by the experiments which was done by using tocotrienol-based nanoemulsified (NE) systems on keratinocyte cell lines. The MTT assay revealed cell viability exceeding 100% at lower concentrations. Furthermore, following 24 hours of treatment, the keratinocyte wound closure exhibited a significant acceleration, with a 73.76%, 63.37%, and 35.56% rise observed in the groups treated with 3.50 μg/ml and 1.75 μg/ml of NE as the control group (Chong *et al*.,2022).

Collagen I, characterized by a triple helix structure composed of two alpha-1 chains and one alpha-2 chain, is a Type I collagen belonging to group I collagen (fibril-forming collagen) present in various connective tissues, notably abundant in bone, cornea, dermis, and tendon. This plays a crucial role in several cellular processes during wound healing, including extracellular matrix (ECM) remodeling and angiogenesis, influencing the strength and integrity of newly formed blood vessels (Hwang *et al*., 2020).

Our investigation focused on evaluating the expression of Collagen type 1 in HACAT cells treated with LHA, LB, and control substances. The HACAT cells treated with LHA and LB upregulated Collagen type 1 expression, suggesting that liposomes may enhance Collagen type 1 expression, facilitating fibroblast migration and wound healing. This indicates potential wound healing of LHA than LB due to their synergistic effect and it may be beneficial in the search for many wound healing agents. It was also identified that the bioactive compounds responsible for inducing collagen expression, indicated that plants stimulate collagen production through these compounds.

Examination of phytochemicals in other plants associated with wound healing indicates the involvement of substances such as flavonoids and triterpenoids. These compounds are recognized for their astringent qualities, as well as their antioxidant and free radical scavenging properties, contributing to the process of wound healing. Research on

plants with wound healing properties has demonstrated enhanced collagen production, as indicated by increased levels of DNA, total protein, and total collagen in rat models treated with plant extracts (Asante *et al*., 2021). This underscores the potential effectiveness of these extracts in promoting wound healing. In an experiment conducted by Shirwaikar *et al*., 2003 it was observed that *A. bracteolate* heightened the process of wound healing by elevating DNA, total protein, total collagen content, and hydroxyproline levels in granulation tissues of wounded rat models.

In vitro experiments stand as invaluable initial investigations, shedding light upon intricate cellular responses and mechanisms at the fundamental level. These experiments serve to unveil the potential mechanisms of action and intricate cellular interactions that underlie the complex process of wound healing. The collaboration between *in vitro* and *in vivo* research is pivotal in advancing the development of innovative wound healing therapies, providing optimism for enhanced clinical outcomes within the realm of regenerative medicine.

Wound contraction is the process of shrinkage of wound area that relies on tissue repair capabilities, damage extent, and overall tissue health. This process involves intricate interactions among cells, extracellular matrix, and cytokines to mobilize healthy skin to cover the wounded area. In the present animal study, ointments containing liposome-encapsulated EEHA and liposome-encapsulated betulin were assessed using excision wound models. Application of LHA 10% exhibited notable wound contraction ($p < 0.01$) on day 12 and on subsequent post-wounding days ($p < 0.01$ and $p < 0.05$) when compared to the standard ointment.

Enhanced wound contraction in liposome-encapsulated *H. auriculata* (LHA) and liposome-encapsulated betulin (LB) treated animals might have resulted from increased fibroblast proliferation and transformation into myofibroblasts. The application of LHA resulted in faster wound contraction and enhanced skin restoration compared to LB treatment, potentially attributed to the occurrence of flavonoids and saponins in the herbal ointment. These compounds are known to stimulate the release of cytokines, collagen synthesis, and angiogenesis, collectively playing a crucial role in promoting healing.

Additionally, liposomes contribute to creating an optimal moist environment for wound healing and facilitate wound closure as seen in the rat model using cationic elastic liposomes constituting a growth factor complex, showing maximal reduction in wound size

compared to the native growth factor complex. Through the hydration and moistening of the stratum corneum, liposomes have the capacity to diminish or eliminate the epidermal barrier. This facilitates their permeation through extracellular spaces to the skin cells, allowing for effective drug release (Wang *et al*., 2019). The pliable phospholipid bilayer of liposomes readily integrates with the bilayer of the cell membrane. Furthermore, the inclusion of an edge activator augments the flexibility of deformable liposomes, enabling them to navigate the stratum corneum and reach the viable epidermis. These deformable liposomes have been used in numerous studies to transport various drugs into the skin, including large biogenetic molecules (Nayak and Tippavajhala, 2021). In 2019, Gunal studied wound healing using various concentrations of liposomal trans-resveratrol formulations in rats. The healing rates were comparable between the 5% Resveratrol group and a commercial product containing 1% *Centella asiatica* extract, suggesting similar effectiveness in wound healing by the 10th and 12th days.

In a rat model, the consistent use of LHA ointment led to full skin restoration and the reduction of inflammatory markers such as IL-6, CRP, and procalcitonin. In excision-wounded rats, these markers were observed to be higher when compared to normal rats. Significantly, the overexpression of TNF-α, CRP, and IL-6 can have detrimental effects on wound healing and various skin-related medical conditions. Upon the onset of a wound, these cytokines become activated, triggering macrophages to release increased levels of IL-6 and C-reactive protein. The excessive production of these biomarkers induces oxidative stress within the wound area. Consequently, this oxidative stress impedes the movement and growth of fibroblasts and keratinocytes at the wound site, resulting in delayed wound healing (Sproston and Ashworth, 2018).

Upon treatment with LHA and LB ointments, there was a significant reduction in the levels of IL-6, C-reactive protein, and procalcitonin. The inflammatory phase marks the initial and pivotal stage in the wound healing process. Nevertheless, prolonged inflammation can lead to an excessive release of cytokines such as IL-1ß, IL-6, and TNF-α, causing significant disruptions in the healing process and an increase in fibrosis and scarring. The prolonged and heightened activity of pro-inflammatory cytokines is linked to tissue damage, resulting from the production and generation of proteolytic enzymes and arachidonic acid metabolites. This ultimately hinders the initiation of the repair phase. Inhibiting these mediators may offer a means to regulate the progression of cutaneous wound healing, representing a promising therapeutic target (Nirenjen *et al*., 2023).

Followed by these, hematological parameters such as macrophages, T lymphocytes, neutrophils, platelets and erythrocyte sedimentation rate were recorded. T cells are essential in wound healing research because they have the potential to coordinate the various responses to tissue injury. They can influence the balance between inflammation and the formation of fibrous tissue in a wound, either promoting a regenerative or fibrotic outcome. These cells play a role in reducing excessive inflammation, which can prevent harm to the tissue caused by an overly aggressive immune response (Boothby *et al*., 2020)

T lymphocytes release signaling molecules, like cytokines, that activate fibroblasts, important cells for tissue repair. In the initial phases of wound healing, there is an elevated ratio of CD4 to CD8 cells among T-lymphocytes, but this ratio diminishes as the healing process advances. This alteration arises from an augmentation in the count of CD8+ T lymphocytes and a reduction in the count of CD4+ T lymphocytes. The simultaneous rise in the expression of CD25 and CD27, which are markers of lymphocyte activation and proliferation, suggests that the increase in CD8+ cells may be linked to lymphocyte growth at the wound site. If CD8+ cells have a regulatory role in human wounds, then the initial low levels of these cells may allow other subsets of lymphocytes, possibly CD4+ cells, to positively influence healing and expedite wound closure (Landén *et al*., 2016; Chen *et al*., 2014; Kumar *et al*., 2022)

As the healing process advances, it becomes necessary to slow down cell growth and movement. The increasing numbers of CD8+ T lymphocytes may provide the right environment by releasing certain cytokines to facilitate this process, effectively switching off the healing mechanism (Wang *et al*., 2019). In the present study, having fewer T lymphocytes was more effective in LHA compared to LB, indicating that this lower T cell presence may contribute to an improved healing process in LHA. Therefore, the lowered T lymphocytes in LHA as compared indicates the better healing process in LHA.

In addition to that, macrophages and neutrophils also show declined range in LHA than LB which was quiet nearer to the standard. Fibroblasts respond to cytokines and growth factors secreted by platelets and macrophages by migrating from the wound edge to the injured area. Therefore, the substantial decrease in the number of macrophages results in a reduction in inflammatory cytokines and promotes the healing process. Neutrophils, are the foremost inflammatory cells to attain at the injury site, play an essential role in defending the body against infections. They are attracted to the injury within 24-36 hours after the injury

occurs. Neutrophils are highly recruited to eliminate debris, microorganisms, and reactive oxygen species (ROS). They also contribute to the early stages of the inflammatory response by releasing signals that enhance inflammation. However, they also serve as signals to terminate this process (Janakiram *et al*., 2021).

In normal wound healing, neutrophils undergo apoptosis (cell death) after fulfilling their function and are subsequently engulfed by macrophages, providing essential signals to resolve inflammation. This step facilitates the transition to the next phase of healing. Continued recruitment of active neutrophils and the accumulation of apoptotic cells due to inadequate regulation of healing processes or inefficient macrophage clearance can lead to prolonged inflammation, leading to the growth of chronic wounds. Furthermore, the phagocytic ability of neutrophils is essential for cleaning potentially infectious wounds, regulating inflammation, and producing mediators required to activate other key cells in the repair process. Thus, a significant reduction in macrophages and neutrophils after the healing process promotes the early healing of wounds (Filep and Ariel, 2020).

The significant decrease in platelet levels observed in the LHA-treated animal group, in comparison to the LB-treated group, is positive indicator for wound healing. Platelets, commonly referred to as thrombocytes, are diminutive blood cells derived from megakaryocytes. They rapidly accumulate in injured tissues, playing a pivotal role in halting bleeding or promoting hemostasis. Activated platelets are instrumental in commencing the clotting process and the generation of fibrin, which aids in sealing damaged blood vessels and preventing further blood loss. The fibrin produced within the wound during the initial stages facilitates the migration of various cells into the wound site, contributing to tissue repair (Wang and Yang., 2023).

Beyond their hemostatic functions, platelets also possess diverse immune functions, releasing growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor-beta (TGF-β). They enhance the activity of other cell types participating in the repair process. These growth factors contribute to tissue regeneration and the overall healing of the wound (Chen *et al*., 2019). So, the reduced platelet count observed in the LHA-treated group is a sign that these animals may be experiencing improved wound healing.

ESR serves as a general indicator of inflammation. A heightened ESR can signal the presence of inflammation, which is a normal and vital aspect of early wound healing. In the

current study, treated rats exhibited ESR rates of 15 mm/hr in the LB group and 13 mm/hr in the LHA group where the treatment with LHA revealed reduced ESR rate. The connection between ESR and wound healing is indirect. ESR acts as a marker for inflammation, and its increase during the initial stages of wound healing reflects the body's natural reaction to injury. ESR changes can be observed over time to gauge the response of body to the wound healing process. A declining ESR suggests that the initial inflammatory phase is diminishing as the wound heals, marking the transition to subsequent stages of tissue repair and remodeling (Song *et al.,* 2022).

In a prior study conducted by Chen *et al*. in 2019 using Sea bass (*Lateolabrax maculatus*), wound healing was accelerated, and there was a decrease in the levels of lymphocytes, neutrophils, and platelets in the mouse model. Yadav *et al*. in 2017 conducted research with the title "Attenuation of dermal wounds via downregulating oxidative stress and inflammatory markers by protocatechuic acid-rich n-butanol fraction of *Trianthema portulacastrum* Linn. in Wistar albino rats". With the treatment, it was observed that the CRP content was significantly lower in the standard group and nBuTP10 group, followed by nBuTP5 group as compared to control group (nBuTP5- n-butanol fraction of *Trianthema portulacastrum* Linn 5% ointment; nBuTP10- n-butanol fraction of *Trianthema portulacastrum* Linn 10% ointment).

Following the completion of the experimental treatment, skin tissue was examined for histopathological analysis. The histological findings revealed full recovery of the epithelial tissue on the epidermis and the existence of scar tissue in the dermis. Furthermore, the collagen fibers around the wound area displayed a similar pattern in all groups. This was attributed to the existence of macrophages and lymphocytes, which are associated with the generation of fibroblasts and the formation of new blood vessels. In the LHA-treated group, the epidermis underwent complete re-epithelialization, and the underlying dermal tissue exhibited a well-organized structure. It displayed dense, thick, and mature collagen fibers neatly aligned in parallel with the regenerated epidermis, in contrast to the standard group.

The results of this study emphasize the remarkable properties of liposomes synthesized from *H. auriculata* roots, including radical scavenging, antioxidant, antimicrobial, and wound healing capabilities when compared to liposomes produced from its active component, betulin. This suggests that the liposomes derived from *H. auriculata* roots exhibit more effective wound healing activity than those made from betulin. The study also

revealed that *H. auriculata* roots are rich in biologically active phytoconstituents like phenols and flavonoids, which likely contribute to the wound healing effects of the synthesized liposomes. Additionally, liposomes played a crucial role as potential drug carriers. The combined effects of *H. auriculata* roots were found to be more potent than liposomes synthesized from betulin. Docking studies further indicated the presence of promising chemotherapeutic drug candidates for targeting wound-related pathogens.

This discrepancy in efficacy can be attributed to the complex interplay of various components present in the *H. auriculata* root extract. The root extract, comprising a diverse array of phytochemicals, appears to engage in a synergistic action that enhances its overall wound healing potential. This synergistic effect is likely to involve a combination of compounds working together in a coordinated manner, amplifying their individual therapeutic benefits and resulting in a more robust response than what betulin alone could achieve.

While betulin is recognized for its inherent wound healing properties, its isolated application may not fully justify the intricate dynamics present in the natural composition of *H. auriculata* root extract. The observed superiority of the root extract suggests that other constituents in the extract contribute significantly to the enhanced wound healing effects. This highlights the importance of considering the holistic composition of plant extracts in therapeutic applications, as isolated compounds may not always reflect the full spectrum of benefits provided by the entire plant matrix.

These results provide substantial evidence supporting the use of liposomes derived from *H. auriculata* roots in innovative wound healing treatments. As a nut shell, the study highlights the significance of synergistic interactions among compounds within *H. auriculata* root extract, highlighting the complexity of plant-based therapeutic interventions. The findings advocate for a more nuanced approach in harnessing the healing potential of natural substances, emphasizing the need to explore and understand the synergistic effects that may be crucial for maximizing therapeutic outcomes.