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Summary and Conclusion

Hygrophila auriculata (Schumach.) Heine, a member of the Acanthaceae family, is a medicinal plant commonly grown in moist environments. As a traditional medicine, it is employed to address various liver disorders. The roots and seeds of this plant hold particular significance in formulations aimed at treating jaundice and other hepatic obstructions. Additionally, the roots of *Hygrophila auriculata* are recognized for their therapeutic application in managing various types of wounds. Esteemed for its manifold medicinal attributes, this plant is acclaimed for its anti-diabetic, antitumor, hypoglycemic, antimicrobial, and free radical scavenging properties. Notably, in contrast to certain other herbal plants, there is no documented evidence of side effects or reported toxicity linked to the utilization of *Hygrophila auriculata*. The primary constituents of this plant encompass betulin, flavonoids, lupeol, terpenoids, and fatty acids.

The cutaneous wound healing process involves the natural self-repair of the skin and is typically classified into four phases: haemostasis, inflammation, proliferation, and remodelling. In humans, the rapid regeneration of a functional epidermis through keratinocytes, referred to as reepithelialisation, is essential for closing the wound and restoring tissue balance. Dermal fibroblasts play an important role in the process of wound healing by migrating into the wound bed, proliferating, and creating granulation tissue rich in extracellular matrix proteins. This tissue supports the growth of new blood vessels. As time progresses, the tissue undergoes remodelling, gradually restoring the injured area to a state similar to its pre-injury condition. Disruption in the systematic advancement of wound healing phases may result in delayed recovery and could lead to diverse skin pathologies and persistent non-healing ulcers.

To facilitate effective wound healing, there is a requirement for intelligent delivery systems capable of sustainably releasing antioxidant and anti-inflammatory ingredients. Nano carriers, characterized by a substantial surface area in comparison to larger particles, can be modified to encapsulate a significant amount of drugs, leading to enhanced circulation in the bloodstream. Liposomes and phytosomes prove valuable in encapsulating and delivering both lipophilic and hydrophilic therapeutic agents in larger quantities. Liposomes, in particular,

offer a versatile platform for drug delivery and wound management, ensuring targeted and efficient delivery of therapeutic agents to improve the wound healing process.

The research encompassed four distinct phases. Phase I involved conducting phytochemical studies and assessing the free radical scavenging activity of different extracts from *H. auriculata* leaves and roots, followed by estimating enzymic and non-enzymic antioxidants. In Phase II, *in silico* studies were performed, identifying lead compounds against wound targets from the secondary metabolites of *H. auriculata* roots using ligand-based molecular docking. Phase III entailed the synthesis and characterization of liposomes using EEHA and the active compound betulin. In the final phase, the synthesized liposomes were assessed for their antibacterial activity against both gram-positive and gram-negative bacteria species. The *in vitro* wound healing potential of the liposomes was assessed using the human keratinocyte cell line, HACAT. Finally, *in vivo* studies on Swiss albino rats were conducted to evaluate the wound healing activity.

The relevant features of the present study are summarized as follows:

Phase I- Preliminary phytochemical and antioxidant studies

- The initial screening and qualitative phytochemical analysis of various solvents (ethanol, chloroform, ethyl acetate, water) shown the presence of eight major phytoconstituents: alkaloids, phenols, tannins, flavonoids, proteins, carbohydrates, cardiac glycosides, and terpenoids.
- Among the four solvents analysed, the ethanol extract exhibited the highest intensity of phytochemicals.
- The natural antioxidants present in plant cells function as powerful eliminators of free radicals. Free radical scavenging assays demonstrated that the ethanol extract of *H. auriculata* exhibited remarkable scavenging effects on DPPH, FRAP, and ABTS compared to other solvent extracts.
- Consequently, for further investigation, the ethanol extract of *H. auriculata* was analyzed to identify potential sources of natural antioxidants.
- The levels of antioxidant in the ethanol extract were determined by evaluating enzymic antioxidants (catalase, superoxide dismutase, peroxidase, polyphenol oxidase, and glutathione S transferase) and non-enzymic antioxidants (α-tocopherol, polyphenols and ascorbic acid).

- Notably, among the roots and leaves, the highest concentration of antioxidants was observed in the roots and hence the root was utilized for further studies.

Phase-II- *In silico* studies

- LC-MS analysis is a widely utilized method for identifying potential bioactive components within plant extracts and herbal preparations, serving as potential lead molecules in the development of new pharmaceutical drugs.
- The LC-MS analysis of *H. auriculata* root showed the presence of 15 significant compounds, including hydroxyl and carbonyl groups. This information is valuable in establishing parameters for the development of phytotherapeutic products, ensuring their quality, safety, and efficacy.
- Ligand optimization, based on the LC-MS profile, identified 10 ligands that were subsequently analysed for their docking characteristics with proteins such as elastase (1HNE), gelatinase (1QIB), collagenase (2Y6I), and glycogen synthase kinase 3β (1Q5K), which play a crucial role in wound prognosis.
- Among the eight complexes showing the highest docking scores, betulin exhibited the highest affinity.
- Among the docked compounds the compounds such as betulin (-9.65 kcal/mol), chlorogenic acid (9.12 kcal/mol), Kaempferol-7-O-Glucoside (-8.19 kcal/mol), Linoleic acid (-7.96 kcal/mol), and Epiafzelechin (-6.62 kcal/mol) displayed the highest binding affinity toward the target proteins.
- Based on this observation, it can be concluded that betulin may serve as an effective inhibitor for wound target proteins.
- Consequently, betulin was selected for further *in vitro* and *in vivo* studies to explore the comparative efficiency between a single bioactive compound and the entire root extract.
- Furthermore, the terpenoid betulin was also compared with the *H. auriculata* root for its potential in wound healing studies.

Phase III- Liposome synthesis and characterization

- The liposome was formulated using both the compound betulin and the *H. auriculata* root extract for a comparative analysis of their wound healing properties.

- The synthesized liposomes exhibited favourable spectral characteristics in FTIR, XRD and enhanced stability as observed through TEM and ZETA potential measurements.
- Followed by that, the antibacterial effectiveness of the bioactive compound betulin and the ethanol extract of *H. auriculata* was evaluated both before and after encapsulation in liposomes.
- The *H. auriculata* root encapsulated liposome demonstrated superior inhibition against all bacterial species compared to other samples.
- The zone of inhibition values ranged from 12.3 ± 0.57 mm to 22.2 ± 2.3 mm, indicating a significantly more pronounced effect than the control, which ranged from 18.6 ± 1.15 mm to 27.6 ± 3.2 mm.

Phase IV- Antibacterial activity, *in vitro* cytotoxicity, wound healing activity and *in vivo* studies

- The cytotoxicity of the synthesized liposomes on HACAT skin cells was assessed through the MTT assay.
- In this investigation, the cytotoxicity indices of HACAT cells were determined based on the percentage of cell mortality.
- Notably, the cell viability experienced a significant decline when HACAT cells were exposed to liposome concentrations exceeding 100 µg/ml.
- This indicates that liposomes influenced cell viability but remained non-toxic to HACAT cells at concentrations below 100 µg/ml.
- In the examination of cell morphology, liposomes induced noticeable cell shrinkage, reducing cell volume and resulting in a decrease in the number of viable cells.
- The IC_{50} values for LHA and LB (LHA - Liposome of *H. auriculata*, LB - Liposome of betulin) were determined to be 125.77 µg/ml and 97.65 µg/ml, respectively.
- Liposomes formulated with *H. auriculata* root exhibited enhanced cell viability compared to those synthesized using betulin.
- The findings from the Wound Healing assay indicate that both liposomes exhibited effective wound-healing potential on Human Skin Keratinocytes (HACAT), leading to a reduction in the wound area in an incubation-dependent manner.
- Notably, there was a raise in the cell migration percentage over time in HACAT cells treated with liposomes synthesized using *H. auriculata* root compared to those synthesized using betulin.

- Cells treated with liposomes formulated from *H. auriculata* root extract showed significantly enhanced migration after 48 hours of treatment compared to that of the cells at 0-hour time point.
- As a result, the expression of Collagen 1 in HACAT cells treated with liposomes synthesized using *H. auriculata* root extract, as well as control drug-treated cells, was assessed through flow cytometry.
- Following a 48-hour treatment with the liposomes, HACAT cells exhibited a significant increase in Collagen 1 expression, similar to the expression observed in cells treated with the positive control, represented by hEGF.
- Specifically, 78.35% of HACAT cells treated with liposomes expressed Collagen 1, closely resembling the percentage of cells treated with the standard control expressing Collagen 1, which was 84.89%.
- This similarity suggests the induction of Collagen 1 expression in HACAT cells by the liposomes.
- The current study sought to evaluate the comparative effectiveness of liposomes in promoting wound healing in an excision-wounded rat model.
- The wound-healing properties of liposomes encapsulating *H. auriculata* root and betulin were assessed *in vivo* using an excision wound-healing model in Swiss albino rats.
- The application of 10% (w/w) LHA liposome ointment showed remarkable wound contraction ($p < 0.01$) on day 12 and on the following post-wounding days ($p < 0.01$ and $p < 0.05$) in comparison to the standard ointment.
- Similarly, on treatment with LB ointment resulted in significant wound contraction on all post-wounding days, ($p < 0.01$) when compared to the standard ointment.
- Histological examination of the healed wounds treated with liposomes revealed a normal appearance of both epidermis and dermis.
- The levels of IL-6 and CRP were observed to be reduced in excision-wounded treated rats in comparison with the normal untreated rats.
- Upon treatment with LHA ointment, the IL-6 and CRP levels significantly decreased compared to LB and the positive control.
- Similar outcomes were noted for procalcitonin. Hematological parameters, including macrophages, T lymphocytes, platelets, and ESR, exhibited a more favorable decline in LHA-treated rats than in LB-treated rats.

In conclusion, this research throws light on the potential of liposomes produced from *H. auriculata* as a wound healing agent, owing to its remarkable radical scavenging, antioxidant, and wound healing characteristics, surpassing those of the bioactive compound betulin. The results indicated that liposomes synthesized using *H. auriculata* have cytotoxic effects against HACAT cells and demonstrated superior wound healing effects in rats compared to betulin liposomes. Traditional knowledge underscores the importance of synergistic effects in enhancing efficiency, especially when compared to the impact of a single bioactive compound. Overall, there is a need for targeted research with specific hypotheses to maximize the success rate of future investigations.

Future Directions

- Clinical trials can be initiated to validate the efficacy and safety of *H. auriculata*-derived liposomes for the healing of wound in patients.
- Exploration of different materials for encapsulation to enhance stability, bioavailability, and sustained release of wound healing drugs, and growth factors.
- Experiments can be undertaken to understand the unique challenges associated with each type of chronic wound and develop dressings that address those challenges effectively.
- The immunomodulatory mechanism of action of drugs on wound healing can be explored.

Recommendations

- Launch awareness campaigns to inform the public and healthcare professionals about the potential benefits of liposome encapsulated *H. auriculata* ointment for wound healing.
- Collaboration can be made with pharmaceutical and biotech industries for the development, production, and commercialization of liposome encapsulated *H. auriculata* ointment for wound healing.