

## SPECIMEN FORMAT FOR THESES OF MONTH

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**Branch/ Area:** : Zoology

**Sub Subject Heading:** : Phytotherapeutics

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**Title of the thesis** : Comparative Evaluation of Liposome Encapsulated  
*Hygrophila auriculata* (Schumach.) Heine Root and  
Betulin on Wound Healing Activity through *In  
silico*, *In vitro* and *In vivo* approaches

(i) In Roman Script -

(ii) In roman Script -

**Nomenclature of Degree:** : PhD.,

**Month & Year of Enrolment:** : January 2021

**Month & Year of Registration:** : January 2021

**Month & Year of Submission:** : December 2023

**Month & Year of Award** : July 2024

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**Designation of Supervisor** : Professor

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which research was conducted** : School of Biosciences,  
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### **Abstract within 300 words:**

The study evaluated the wound healing properties of liposome-encapsulated *Hygrophila auriculata* (K. Schum.) Heine root extract and betulin through a four-phase process. Initially, various extracts from the leaves and roots were analyzed for phytochemical content and free radical scavenging activities. Phytochemical analysis revealed eight major constituents, and the ethanol extract showed significant scavenging effects in DPPH, FRAP, and ABTS assays. The ethanol extract, which exhibited superior antioxidant activity, was selected for further research. Enzymic and non-enzymic antioxidants were particularly abundant in the roots, making them the focus of subsequent studies. In the second phase, *in silico* studies identified 10 lead compounds from the root extract for docking with proteins target to wound healing. Betulin emerged as the most promising compound. Subsequently, liposomes were synthesized using the ethanol extract of *H. auriculata* (LHA) and betulin (LB). These liposomes were characterized through FTIR, XRD, TEM, and ZETA potential measurements, confirming their stability. The antibacterial activity of the liposomes, especially those from *H. auriculata* roots, showed superior inhibition against various bacterial species. Cytotoxicity assessments on HACAT cells indicated low toxicity, with IC<sub>50</sub> values of 125.77 µg/ml for LHA and 97.65 µg/ml for LB. Both LHA and LB liposomes promoted wound healing in HACAT cells, reducing wound area in a time-dependent manner. Flow cytometry revealed that LHA treatment significantly increased Collagen 1 expression, close to the standard control of human Epidermal Growth Factor (hEGF). *In vivo* studies demonstrated that 10% LHA liposome ointment achieved the highest wound contraction by day 16, surpassing the standard ointment (Povidone). LB ointment also showed significant wound contraction. Histological analysis showed normal healing, with reduced inflammatory markers (IL-6, CRP, procalcitonin) in treated rats. Haematological parameters indicated a better decline in inflammatory cells in LHA-treated rats compared to LB-treated rats. The study highlights the enhanced wound healing potential of LHA due to the synergistic effects of multiple bioactive compounds, compared to LB, which contains a single active ingredient.

#### **i) Major 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

**ii) Methodology:**

The materials used and methods adopted in the present study entitled “Comparative Evaluation of Liposome Encapsulated *Hygrophila auriculata* (Schumach.) Heine Root and Betulin on Wound Healing Activity through *In silico*, *In vitro* and *In vivo* approaches” are furnished below.

**PHASE I- Preliminary phytochemical and antioxidant studies**

**Collection of plants and authentication**

Healthy plants of *H. auriculata* were collected from the areas of Coimbatore, Tamil nadu, India. The plant material was identified and authenticated at the herbarium of Siddha regional research institute, Kerala. A voucher specimen of the plant has been preserved for future reference in the Department of Zoology, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore (001-3001921001).

**Preparation of plant powder and extraction**

The roots and leaves of *H. auriculata* were washed entirely and let dry for 5-7 days at room temperature. The dried-out leaves were ground to powder and stored in screw-cap bottles until further analysis. A 50 g of sample was dissolved in 500 ml of various solvents (ethanol, ethyl acetate, water, and chloroform). It was then filtered and further concentrated by evaporation

**Determination of physicochemical parameters**

One part of the powder was analyzed for physicochemical parameters such as moisture content, total ash value, acid insoluble ash value, water soluble ash value, alcohol soluble extractive and water-soluble extractive values.

### **Qualitative phytochemical analysis (Kokate *et al.*, 2008)**

All the extracts were subjected to different phytochemical tests to determine the phytochemical constituents present in the extract. Various tests to determine the presence of alkaloids, flavonoids, sterols, triterpenoids, carbohydrates, tannins, proteins, amino acids, saponins, phenols and glycosides were performed as per the standard procedure.

### **Free radical scavenging activities DPPH radical scavenging activity (Mensor *et al.*, 2001)**

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Aldrich Co., St. Louis, USA. The diluted working solutions of the test extracts were prepared in methanol. About 3ml of graded concentration (20 - 100µg/ml) of extracts were taken in different test tubes, 1 ml of 0.3mM DPPH methanol solution was added to these test tubes and shaken vigorously. Methanol served as the blank and DPPH in methanol, without the rhizome extracts, served as the positive control. After 30 min incubation of samples at 25°C in the dark, the absorption was measured at 517 nm. The radical scavenging activity was calculated as follows

Inhibition percentage (%) =  $\frac{\text{Abs (control)} - \text{Abs (sample)}}{\text{Abs (control)}} \times 100$ ,

Where, Abs (control) - absorbance of control with methanol and Abs (sample)- absorbance of the sample extract.

### **ABTS radical scavenging activity (Shirwaiker *et al.*, 2006)**

ABTS radical cations (ABTS<sup>+</sup>) were produced by reacting ABTS solution (7mM) with 2.45mM ammonium persulfate. The mixture was allowed to stand in the dark at room temperature for 12-16 hours before use. Aliquots (5µl) of the different extracts were added to 0.3ml of ABTS solution and the final volume was made up to 1ml with ethanol. The absorbance was read at 745nm in a spectrophotometer and the percent scavenging was calculated using the formula

Inhibition percentage (%) =  $\frac{\text{Abs (control)} - \text{Abs (sample)}}{\text{Abs (control)}} \times 100$ ,

Where, Abs (control) - absorbance of control with methanol and Abs (sample)- absorbance of the sample extract.

### **Ferric reducing power assay (FRAP) (Rice-Evans *et al.*, 1991)**

Reaction mixtures were prepared by adding 2.5 ml of phosphate buffer (0.2 M, pH 6.6), 2.5 ml potassium ferricyanide (1%), and varying concentrations of extracts (20 - 100µg/ml). After the reaction, mixtures were incubated at 50°C in a water bath for 30 min, allowed to cool at

room temperature (28°C), and 2.5 ml of 10% TCA (Trichloroacetic acid) was mixed into each reaction mixture, followed by the centrifugation at 2000 rpm for 10 min. The supernatant (2.5 ml) was separated in the test tube, added with 2.5 ml of distilled water and 0.5 ml FeCl<sub>3</sub> (1.0%), and allowed to react for 10 min and absorbed at 700 nm.

### **Antioxidant activity**

The antioxidant status of the roots of *H. auriculata* was estimated by analyzing various enzymic antioxidants such as SOD (superoxide dismutase), POD (peroxidase), CAT (catalase), polyphenol oxidase and GST (glutathione S transferase) and non enzymic antioxidants such as flavonoids,  $\alpha$ -tocopherol and vitamin – C.

### **Estimation of catalase activity (Luck, 1974)**

H<sub>2</sub>O<sub>2</sub>-phosphate buffer (3.0ml) was taken in an experimental cuvette, followed by the rapid addition of enzyme extract (0.01 - 0.04 ml), and mixed thoroughly. The time taken for a decrease in absorbance for 0.5 units is noted. This value was used for calculations. If 't' was more than 60 seconds, repeated the measurement with a more concentrated sample solution.

### **Estimation of peroxidase activity (Reddy *et al.*, 1995)**

Three milliliters of 0.05M pyrogallol solution and 0.5 to 1.0 ml of enzyme extract were taken in a test tube. 0.5 ml of 1% hydrogen peroxide was added to the test cuvette. The spectrophotometer was adjusted to read '0' at 400nm. Changes in absorbance were recorded every 30 seconds up to 3 min.

### **Estimation of superoxide dismutase activity (Misra and Fridovich, 1972)**

The incubation medium contained 300  $\mu$ l of each reagent (50mM potassium phosphate buffer (pH 7.8), 45mM Methionine, 5.3mM Riboflavin, 84mM Nitro Blue Tetrazolium (NBT), and 20 mM potassium cyanide. 300  $\mu$ l of the sample was added to this mixture, and the final volume was made up to 3ml with water. The tubes were placed in an aluminum foil lined box maintained at 25°C and equipped with 15W fluorescent lamps. The NBT reduction was measured at 600nm after 10 min of exposure to light. The maximum reduction was evaluated in the absence of an enzyme giving 50% inhibition of the reduction of NBT.

### **Estimation of polyphenol oxidase activity (Esterbauer *et al.*, 1977)**

2.5 ml of 0.2M phosphate buffer (pH 6.5) and 0.3 ml of catechol solution (0.01 M) were taken into the cuvette and added the enzyme extract (0.2 ml). The spectrophotometer was set at 495nm and recorded the change in absorbance every 30 seconds up to 5 min.

#### **Estimation of Glutathione-S-transferase (Habig *et al.*, 1974)**

A total of 1 ml of buffer, 1.7 ml of water, and 0.1 ml of CDNB were added to the 0.1 ml of sample and incubated for 5 min at 37°C. This was followed by the addition of 0.1 ml of glutathione S transferase. At 340 nm, the optical density was calculated and compared to a blank.

#### **Estimation of vitamin C (Roe and Kuether, 1953)**

The assay volumes were made up of 2 ml with 4% TCA. 0.2 to 1 ml of the working standard solution containing 20-100 µg of ascorbate, respectively, were pipetted out into a clean, dry test tube, the volume of which was also made up to 2 ml with 4% TCA. Added 0.5ml of DNPH reagent to all the test tubes, followed by two drops of 10% thiourea solution. The sample was incubated at 37°C for 3 hours. The osazones formed were dissolved in 2.5ml of 85% sulfuric acid in cold, drop by drop, with no appreciable rise in temperature. The DNPH reagent and thiourea were added to the blank alone after adding H<sub>2</sub>SO<sub>4</sub>. The tubes were incubated for 30 min at room temperature, and the absorbance was read spectrophotometrically at 540nm. The ascorbic acid content in the sample was calculated using the standard graph.

#### **Estimation of α-tocopherol (Rosenberg, 1992)**

Into three stoppered centrifuge tubes (test, standard and blank), pipetted out 1.5 ml of extract, 1.5 ml of standard (10mg of α-tocopherol was dissolved in 10ml of absolute alcohol), and 1.5ml of water, respectively. To the test and blank, 1.5 ml of ethanol was added, and to the standard, 1.5 ml of water was added. Added 1.5 ml xylene to all the test tubes, stoppered, mixed well, and centrifuged. From this, 1.0 ml of the xylene layer was transferred into another stoppered tube. Added 1.0 ml of 2, 2'- dipyridyl reagent to each tube, stoppered, and mixed well. 1.5 ml of this mixture was pipetted into colorimeter cuvettes, and noted the extinction and standard against the blank at 460nm. 0.33 ml of ferric chloride solution was added to all the test tubes, including the blank. The amount of vitamin E can be calculated using the formula,

Amount of tocopherols in  $\mu\text{g}$  = reading at 520 nm- reading at 460 nm/reading of standard at 520 nm  $\times$  0.24  $\times$  15

### **Estimation of flavonoids (Liu *et al.*, 2007)**

An aliquot of the extract was pipetted out and evaporated to dryness. Different volumes of standard catechin (0.2 to 1.0ml) were taken and made up to 1 ml with distilled water. An aliquot of 4ml of vanillin reagent was added, and the tubes were heated for 15 min in a boiling water bath and cooled. The optical density of the solution was read at 340 nm. The standard curve was constructed in an electronic calculator set to the linear regression mode, and the concentration of flavonoids was calculated. The values are expressed as mg flavonoids/g tissue.

## **PHASE II- *In silico* studies**

### **LCMS analysis**

LCMS analysis was conducted using XEVO-TQS micro #QEA0592. A reversed-phase C18 analytical column (HSS T3, C18 Column from Waters, USA) with dimensions 2.1 $\times$ 100 mm and a particle size of 2.5  $\mu\text{m}$  was employed at 40 °C. The mobile phase consisted of a binary gradient involving water and acetonitrile, maintaining a steady flow rate of 0.4 mL/min. The gradient elution programming was as follows: 5% B (0 seconds), 10% B (60 seconds), 23% B (90 seconds), 28% B (3.8 min), 35% B (6.5 min), 43% B (8 min), 45% B (9.5 min), 95% B (12 min), 5% B (20 min). Data collection and processing were performed using Empower3 software.

### **Retrieval of ligands and proteins**

The chemical structures of the selected ligands were obtained from the pub chem database. The X-ray crystal structures of the receptors Elastase (1HNE), Glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ;1Q5K), Gelatinase (1QIB), Collagenase (2Y6I) were retrieved from the Protein Data Bank.

### **Drug likeliness and Physicochemical properties**

The SWISS ADME online server was employed for the computation of drug likeliness parameters, adhering to Lipinski rules. The SMILES notations for each compound were sourced from the PubChem database to calculate their respective properties (Mahanthesh *et al.*, 2020 and Han *et al.*, 2019)

### **ADMET properties**

ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) prediction provides valuable facts about the compound that could be evidenced for drug design. The computational pkCSM tool (<http://biosig.unimelb.edu.au/pkcsm/prediction>) was employed to conduct ADMET studies. The molecules were fed in the canonical SMILE format for calculating the ADMET properties (Pires *et al.*, 2015)

### **Bioactivity score**

The SMILES notations for the chosen compounds were input into the Molinspiration software version 2011.06 ([www.molinspiration.com](http://www.molinspiration.com)) to forecast bioactivity scores, encompassing GPCR ligands, kinase inhibitors, ion channel modulators, enzymes, and nuclear receptors.

### **Molecular docking**

Docking simulations were conducted using Autodock4 (version 4.2.8) (Trott and Olson *et al.*, 2010). A scoring function reliant on energy was employed to prioritize the receptor-ligand poses obtained during the docking calculations. Subsequently, the optimal docked confirmation was visualized using the Discovery Studio Visualizer (BIOVIA), allowing for the identification of the docking site, binding interactions, and bond lengths.

## **PHASE III- Liposome synthesis and characterization**

### **Synthesis of liposomes (Fathy *et al.*, 2019)**

The *H. auriculata* and betulin encapsulated liposomes were developed by thin-film hydration method.

### **Encapsulation efficiency of liposomes (Nayer *et al.*, 2019)**

The *H. auriculata* and betulin encapsulation efficiency was determined using the indirect spectrophotometric method.

### **Spectral studies**

#### **Fourier Transform Infrared (FTIR) Spectroscopy**

The functional group present in the synthesized liposomes were analyzed using SHIMADZU, FTIR spectroscopy- miracle 10, and was scanned within a range of 600-3600 cm<sup>-1</sup>.

## **X-Ray Diffraction**

The crystalline nature of the synthesized liposomes was examined using X-ray Diffraction utilizing the X'pert Pro X-ray diffractometer. Particle characterization involved determining the crystallite size through line broadening analyses, utilizing the Debey–Scherrer formula while accounting for instrumental broadening.

## **Transmission electron microscopy (TEM)**

Transmission electron microscopy was utilized for liposome imaging. In a brief procedure, 10 µL of each liposome suspension was applied to a strip of para-film. A formvar/carbon 200 mesh copper grid from Electron Microscopy Sciences in Hatfield, PA, USA, was positioned on the liposome drop and allowed to sit for 5 min. Subsequently, the copper grid was transferred to the surface of a uranyl acetate drop for an additional 5 min. To eliminate any excess stain, the grid was gently dabbed with an adsorbent pad. TEM images were captured using a JEM-1010 microscope from JEOL Inc. in Peabody, MA, USA.

## **Zeta potential**

To ascertain the stability of the synthesized *H. auriculata* root and betulin loaded liposomes Zeta potential analysis was carried out. The analysis was performed using Malvern Panalytical, Zetasizer Ver. 7.13.

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

### **Microorganisms**

The bacterial strains used in the study are clinical isolates obtained from P.S.G. Hospitals, Coimbatore. The microbial strains were sub cultured before the experiment. The bacterial species used in the study were *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Proteus* sp.,

### **Antibacterial activity (Valgas *et al.*, 2007)**

The antibacterial assay adopted the agar well diffusion method against selected bacterial species. The sterile muller hinton agar medium were prepared and swabbed with selected bacterial isolates. The respective samples and standard (Amoxicillin) were added to separate wells. The bacterial plates were incubated at 37°C for 24 hours and room temperature for five days, and the zone of inhibition was measured.

## **Cytotoxicity study**

### **MTT assay (Igarashi and Miyazawa, 2001)**

In the assay, 200 µl of cell suspension was seeded in a 96-well plate at the desired cell density (20,000 cells per well) without the test agent. The cells were allowed to grow for approximately 24 hours. The sample at appropriate concentrations (12.5, 25 50, 100 and 200 µg/ml) was added and incubated for 24 hours at 37°C in a 5% CO<sub>2</sub> atmosphere. After 48 hours of incubation, 15 µl of MTT was added in phosphate-buffered saline (PBS) to each well and incubated at 37°C for 4 hours, achieving a final concentration of 0.5 mg/mL in the total volume. The medium was removed with MTT, and the formed formazan crystals were solubilized in 100 µl of DMSO. The absorbance at 570 nm was measured using a microplate reader. The percentage of cell inhibition was calculated using the following formula.

% Cell viability is calculated using below formula:

$$\% \text{ cell viability} = [\text{Mean abs of treated cells} / \text{Mean abs of untreated cells}] \times 100$$

The IC<sub>50</sub> value was determined by using linear regression equation i.e.  $Y = Mx + C$ .

Here,  $Y = 50$ ,  $M$  and  $C$  values were derived from the viability graph.

### **Scratch assay (Fronza *et al.*, 2009)**

To perform the assay, the cells were grown in high-glucose DMEM media supplemented with 10% FBS until reaching 70-80 % confluence. The cells were seeded into a 12-well tissue culture plate at a density of 0.25 million cells per well, allowing them to reach ~80-100 % confluence as a monolayer during a 24-hours incubation period without changing the medium. Using a new 200 µl pipette tip, the monolayer was scratched gently and slowly across the centre of the well, ensuring that the long axis of the tip is always perpendicular to the bottom of the well. The resulting gap distance is equal to the outer diameter of the tip end, and this distance can be adjusted by using different types of tips. A cross in each well was created by scratching a straight line in one direction and another perpendicular to the first line. After scratching, the well was washed twice with medium to remove detached cells. The cells were then treated with the desired concentrations of the sample prepared and incubated at 37°C with 5% CO<sub>2</sub>. The cells were allowed to grow for an additional 48 hours (or the required time for different cells). The cell images at different time intervals (e.g., 0, 24 hr, 48 hr, etc.) was

captured maintaining the same microscope configurations for different views of the monolayer. Quantitatively the gap distance was evaluated using software such as Image J.

Formula used for the analysis:

% Wound Healing Score = (Initial Area - Final area) / Initial area \* 100

### **Collagen I expression using flow cytometry (Krishan, 1995)**

For experimental procedures, the cells were cultured in a 6-well plate at a density of  $0.5 \times 10^6$  cells/2 ml and incubated in a CO<sub>2</sub> incubator overnight at 37°C for 24 hours. The spent medium was removed, treated the cells with the sample and control in 2 ml of culture medium, and incubated the cells for 24 hours.

a. Negative control – Untreated cells.

b. Positive Control - Cells treated with 5 ng/ml of human Epidermal Growth Factor (hEGF).

c. Test Compound - Cells treated with 25 µg/ml.

After treatment, the medium from all wells were removed into 12 x 75 mm polystyrene tubes and washed with 500 µl PBS. The PBS was removed, added with 250 µl of trypsin-EDTA solution, and incubated at 37°C for 3-4 min. The culture medium was poured back into their respective wells, harvested the cells into 12 x 75 mm polystyrene tubes, and centrifuged the tubes for five min at 25°C. The supernatant was decanted carefully and washed with PBS twice, and decanted the PBS completely. The cells were stained with 5 µg/ml FITC-conjugated Collagen Type I antibody at a density of  $1 \times 10^6$  cells/ml and incubated at 37°C for 30 min, protected from light. The tubes were subjected to centrifuge for 5 min, removed the supernatant, and gently suspended cells in 400 µl pre-warmed DPBS. The flow cytometry was analysed by using the 488 nm laser for excitation and detection at 535 nm.

### ***In vivo* studies**

#### **Excision model wound healing activity (Mortone and Malone, 1972)**

The animals were divided into four groups as given below. Each group containing of five animals.

Group I served as excision wounded animals without treatment,

Group II and III served as excision wounded animals treated with liposome encapsulated betulin and liposome encapsulated *H. auriculata* extract ointment applied topically for 16 days

Group IV served as excision wounded animals treated with standard drug povidone ointment topically for 16 days.

The mice were anaesthetized using ketamine prior to creation of wounds. The dorsal fur of the animals was shaved with an electric clipper and wound was created on dorsal thoracic region. The full thickness of the excision wound was created around 2.5cm in length, 0.2cm in depth by using surgical blade, pointed scissors and toothed forceps. The wound was left open. Haemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. All the rats were given regular dressing changes and kept for observation. The wound closure rate was assessed by tracing the wounds on days 0, 4, 8, 12 and 16.

#### **Measurement of wound contraction**

The excision wound was monitored by systematically tracking the evolving changes in wound area plan metrically, excluding the day of wounding. The dimensions of wounds were documented daily on transparent paper throughout the study period. Subsequently, the tracings were transferred to graph paper, enabling the assessment of the wound surface area. The percentage of wound contraction was determined using the following formula:

Percentage wound contraction = Healed Area / Total Area X 100

#### **Parameters studied** (Grellner *et al.*, 2000; Talekar *et al.*, 2017)

Following the experimental period, the animals were euthanized through cardiac puncture, and blood and tissue samples were obtained for the analysis of biomarker parameters, including IL-6, procalcitonin and C-reactive protein. Additionally, haematological parameters, such as macrophages, neutrophils, T-lymphocytes, platelets, and erythrocyte sedimentation rate, were assessed. The collected tissues underwent histopathological analysis

#### **Statistical analysis**

All numerical data is presented as the mean value  $\pm$  standard deviation. All the *in vitro* experiments were done in triplicate, and the experiments were repeated at least thrice. The statistical software SPSS version 17.0 was used for the analysis. p value <0.05 was considered significant. The Inhibitory Concentration (IC<sub>50</sub>) was determined by plotting a nonlinear

regression graph between % call inhibition and Log10 concentration using Graph Pad Prism software. *In vivo* wound healing activity was determined by applying One-way ANOVA followed by Duncan's multiple range test. Statistical difference was considered significant if p value was less than 0.05 and 0.01.

### iii) Findings:

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 ( $\alpha$ -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 3 $\beta$  (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 Epiarzelechin (-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  $12.3 \pm 0.57$  mm to  $22.2 \pm 2.3$  mm, indicating a significantly more pronounced effect than the control, which ranged from  $18.6 \pm 1.15$  mm to  $27.6 \pm 3.2$  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<sub>50</sub> 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.

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