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Materials and methods

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.

3.1. PHASE I- Preliminary phytochemical and antioxidant studies

3.1.1. 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 - Appendix-III).

Fig. 6 *Hygrophila auriculata* in its habitat

3.1.2. 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

3.1.3. 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. The procedures are given in the Appendix I.

3.1.4. Qualitative phytochemical analysis

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 procedure as per the Appendix II.

3.1.5. Free radical scavenging activities

3.1.5.1. 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 (%) = Abs (control) - Abs (sample)/Abs (control) x 100,

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

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

ABTS radical cations (ABTS+) 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 (%) = Abs (control) - Abs (sample)/Abs (control) x 100,

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

of the sample extract.

3.1.5.3. 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 $FeCl_3$ (1.0%), and allowed to react for 10 min and absorbed at 700 nm.

3.1.6. 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, α-tocopherol and vitamin – C.

3.1.6.1. Estimation of catalase activity (Luck, 1974)

H_2O_2-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.

3.1.6.2. 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.

3.1.6.3. Estimation of superoxide dismutase activity (Misra and Fridovich, 1972)

The incubation medium contained 300 µl 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 µl 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.

3.1.6.4. 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.

3.1.6.5. 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 37^0C. 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.

3.1.6.6. 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 37^0C 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_2SO_4. 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.

3.1.6.7. 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 μg = reading at 520 nm- reading at 460 nm/reading of standard at 520 nm × 0.24 × 15

3.1.6.8. 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.

3.2. PHASE II- *In silico* studies

3.2.1. 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 2.1×100 mm and a particle size of 2.5 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.

3.2.2. 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-3β (GSK-3β;1Q5K), Gelatinase (1QIB), Collagenase (2Y6I) were retrieved from the Protein Data Bank.

3.2.3. 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)

3.2.4. 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)

3.2.5. Bioactivity score

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

3.2.6. 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.

3.3. PHASE III- Liposome synthesis and characterization

3.3.1. Synthesis of liposomes

The *H. auriculata* and betulin encapsulated liposomes were developed by thin-film hydration method as given in Appendix III.

3.3.2. Encapsulation efficiency of liposomes

The *H. auriculata* and betulin encapsulation efficiency was determined using the indirect spectrophotometric method as given in Appendix IV.

3.3.3. Spectral studies

3.3.3.1. 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^{-1}.

3.3.3.2. 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.

3.3.3.3. 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.

3.3.3.4. 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.

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

3.4.1. 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 subtitlis*, *Staphylococcus aureus*, *Escherichia coli*, and *Proteus sp*.,

3.4.2. Antibacterial activity

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 (Valgas *et al*., 2007)

3.4.3. Cytotoxicity study

3.4.3.1. 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_2 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:

% cell viability = [Mean abs of treated cells/Mean abs of untreated cells] x 100

The IC_{50} 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.

3.4.4. 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_2. 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.) were 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 Scored= (Initial Area-Final area)/Initial area *100

3.4.4.1. 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 x 10^6 cells/2 ml and incubated in a CO_2 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 x 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.

3.4.5. *In vivo* studies

3.4.5.1. 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.

3.4.5.2. 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

3.4.5.3. Parameters studied

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

3.4.6. Statistical analysis

All numerical data is presented as the mean value ± 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_{50}) was determined by plotting a nonlinear regression graph between % call inhibition and Log_{10} 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.