3

Materials and Methods

3.1 Selection of Medicinal plant

The medicinal plant, *Kaempferia galanga* Linn was selected on the basis of its use in the traditional system of medicine of Meghalaya and its therapeutic significance. It is widely used as folk medicine for the treatment of indigestion, cold and dry cough, pectoral and abdominal pain, fever, headaches, rheumatism, inflammations, diuretic, and several other therapeutic indications.

3.1.1 Classification of *Kaempferia galanga* Linn

Kingdom: Plantae
Phylum: Tracheophyta
Class: Liliopsoda
Order: Zingiberales
Family: Zingiberaceae
Genus: *Kaempferia*
Species: *galanga* Linn

It is a perennial aromatic herb bearing strong fragrance; leaves are two or more, spreading flat on the ground, oval, thin and usually deep green; petioles very short; flowers are white in colour with purplish spots in the axillary fascicles; fruits are oblong, 3-celled and 3- valved capsules and the seeds arillate. The undergroundrhizome is characterized by vertically oriented tuberous root and other small secondarytubers and roots whose tips are tuberous in nature as shown in Fig. 3.1.

A. *Kaempferia galanga* Linn leaves

B. Rhizome of *Kaempferia galanga* Linn

Fig. 3.1-Pictorial representation of *Kaempferia galanga* Linn leaves and rhizome

It is also known by various names as follows:

Common name- Aromatic Ginger, Sand ginger

Hindi- Chandramula, Sidhoul

Assamese- Chandramula

Sanskrit- Chandramauli, Sathi

Meghalaya- Sying Khmoh

The plant is a native of Meghalaya and has been naturalised in parts of China [121].

3.2 Collection of plant material

The plant material was collected from Ri-Bhoi district, Nongpoh, Meghalaya in December 2020. Fig. 3.2 shows the geographical location of the cultivation of *K. galanga* L. The plant was chosen based on their uses among the local people for different medicinal purpose. The rhizome of the plant was dug out, washed and dried. Once dried, it was packed in poly-bags and transported to the laboratory. In the laboratory, the plant rhizomes (3 kgs) were again washed and the skin peeled off. The rhizome was sliced into small pieces and allowed to dry prior to which the pieces were grounded into fine powder (528 g) and packed in sterile bags and kept at 4°C until use.

Fig. 3.2 Location of Ri-Bhoi district from where *Kaempferia galanga* Linn was collected: 2448 sq.km and lies between E 91°20'30" and E 92°17'00" Longitude and N 25°40' to N 26°20' Latitude.

3.3 Isolation of Essential oil

Isolation of essential oil was carried out using Soxhlet apparatus (JSGW). Extraction was carried out with slight modifications [212], where the grounded air-dried rhizome (500g) was subjected to Soxhlet extraction for 6 h using hexane as a solvent. The final solvent mixed with the essential oil was evaporated using Rotatory evaporator. The oil was stored in sealed vials at 4°C until further assays. The yield of essential oil obtained after isolation was calculated using the formula:

$$\text{Yield of essential oil } (\%) = \left\{\frac{Amount\ of\ E.O.obtained\ (g)}{Aount\ of\ raw\ material\ used\ (g)}\right\} \times 100 \qquad \textbf{(Eq.4.1)}$$

3.4 GC-MS analysis and identification of the compounds

The essential oil of *Kaempferia galanga* L. was analyzed for the presence of possible bioactive compounds through GC-MS analysis which was performed at Biotech-Park, IIT Guwahati, in a *Perkin Elmer Clarus 680 GC/600C MS*, bearing the acquisition parameters: Oven: Initial temp 40°C for 2 min, ramp 10°C/min to 140°C, hold 2 min, ramp 7°C/ min to 300°C, hold 5 min, Inj Auto=280°C, Volume= 10µL, Split= 10:1, Carrier gas= He, Solvent Delay= 8.00 min, Transfer Temp= 160°C, Source Temp= 150°C, Scan: 50 to 600 Da, Column 60.0m× 250µm.. Compound identification was done by comparing the NIST library data of the peaks with those reported in literature [221].

3.5 Biochemical Analysis of essential oil

The essential oil was further evaluated for their biochemical constituents.

3.5.1 Total phenol content (TPC)

The total phenol content of *Kaempferia galanga* L. essential oil was determined by Folin-Ciocalteu reagent with slight modification [216], using gallic acid as a standard. 1ml of essential oil was taken and mixed with 0.5 ml of 1N Folin-Ciocalteu reagent (1:1 diluted with distilled water). After 5 minutes, 1 ml of 20% sodium carbonate was added to the mixture. The samples were incubated for 10 minutes at room temperatureand then the absorbance was measured at 750 nm. The calibration curve of gallic acid was constructed and linearity was obtained in the range of 10- 50 µg/mL. The final concentration of the phenolics was calculated as gallic acid equivalent.

3.5.2 Total flavonoid content (TFC)

The total flavonoid content of *Kaempferia galanga* L. essential oil was determined by aluminium chloride method with slight modification [216], using quercetin as a standard. 1ml of essential oil was taken and treated with 5% sodium nitrite. After 5 minutes of incubation, 10% aluminium chloride and 2.8 ml of distilled water was added. The solution was incubated at room temperature for 30 min. After incubation, the absorbance was measured at 510 nm. The calibration curve of quercetin was constructed and linearity was obtained in the range of 25- 150µg/mL. The final concentration of the flavonoid was calculated as quercetin equivalent.

3.6 *In-vitro* free radical scavenging assay

3.6.1 2,2-diphenyl-1- picrylhydrazyl (DPPH) assay

Antioxidant activity was determined by the 2,2-diphenyl-1- picrylhydrazyl (DPPH) assay with slight modification [217]. 200 µL of five different concentrations (20, 40, 60, 80, 100 µg/mL) of the essential oil from *Kaempferia galanga* L. in methanol were added to an aliquot to which 2.8 mL of 0.1 mM methanolic solution of DPPH was added, for each concentration. The mixture was shaken vigorously and left to stand at the room temperature for 30 min in dark. When DPPH was reduced, the change of colour from deep violet to light yellow was observed and the absorbance was determined by UV spectrophotometry at 517 nm. The radical scavenging activity was then calculated using the following formula:

$$\text{DPPH scavenging activity } \% = \left\{\frac{Ab - Aa}{Ab}\right\} \times 100 \qquad \textbf{(Eq.4.3)}$$

in which Ab is absorption of the control and Aa is absorption of the sample.

In this study, butylated hydroxytoluene was taken as the standard. All the tests were carried out in triplicates. The IC_{50} value was also determined by plotting a graph of % inhibition against concentration of essential oil.

3.6.2 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) radical cation-based assay

ABTS scavenging assay was carried out with slight modifications [218]. The ABTS radical solution was prepared mixing 7.4 mmol/L ABTS and 2.6 mmol/L potassium persulfate. 100µL of essential oil with different concentration (20-100µg/ml) were subsequently mixed with 1900µL ABTS radical solution (prepared 16 hours and kept in dark prior use), and the absorbance of the resulting mixtures was measured after 20 min at 737 nm using UV spectrophotometer. BHT was used as a standard. The free radical-scavenging capacity was calculated by the following equation:

$$\text{Radical scavenging } (\%) = \left\{\frac{Acontrol - Asample}{Acontrol}\right\} \times 100 \qquad \textbf{(Eq.4.4)}$$

Where A_{sample} is the absorbance of the ABTS mixed with the sample, A_{control} is the absorbance of the ABTS mixed with deionized water.

3.6.3. Total Antioxidant Activity (TAC) assay (Phosphomolybdenum assay)

The total antioxidant capacity of the essential oil was evaluated [219] with slight modifications. An aliquot of 0.5 mL of the sample's solution combined with 4.5 mL of

reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was taken. The tubes were then incubated in a boiling water bath at 95°C for 90 min. The samples were then cooled at room temperature and the absorbance was measured at 695 nm. BHT was taken as standard, and values were expressed as BHT equivalent (mg BHT/g). The higher absorbance value indicated higher antioxidant activity.

3.7 Haemolysis assay

Cytotoxicity was performed on red blood cells by centrifuging at 1000 rpm for 10 min at 4°C. The supernatant was removed and the pellet containing the red blood cells (RBCs) was washed five times with Phosphate Buffer Saline (PBS) at pH-7.4 and then re-suspended in PBS to obtain blood suspension. This was followed by the addition of 180μL blood suspension into different microcentrifuge tubes containing 20μL of 2-fold serial dilutions of essential oil prepared in 1% DMSO, ranging from 0.02 to 0.1 mg/ml. The final suspensions were then incubated for 60 min at 37°C. After incubation, the tubes were then placed on ice for 5 min to stop the reaction. The samples were then centrifuged for 5 min at 1000 rpm and the supernatants were transferred to 96-well plates and the haemoglobin release was measured by absorbance at 540 nm. Triton X-100 and PBS were used as positive and negative controls respectively [220]. Experiments were carried out in triplicate. The degree of haemolysis was calculated using the following formula:

$$\text{Haemolysis (\%)} = \left\{\frac{OD540\ of\ sample - OD540\ of\ negative\ control}{OD540\ of\ positve\ control - OD540\ of\ negative\ control}\right\} \times 100 \quad \textbf{(Eq.4.5)}$$

3.8 Screening of medicinal plants for antibacterial activity

3.8.1 Test microorganism

Staphylococcus aureus ATCC 6538, was used during the study. The bacteria was cultured in nutrient broth (Hi-media, M002) at 37°C and was sub-cultured once a month and the isolated cultures were stored at 4°C for further utilization.

3.8.2 Antibacterial assay by Agar well diffusion method

Antibacterial activity of essential oil was determined by agar well diffusion method using Muller-Hinton agar medium with slight modifications [213]. Wells were punched on solidified Muller-Hinton agar (M173) and the inoculum bacteria was spread onto the solidified medium surface. Then, 50μl of essential oil was loaded into the respective

well. The plates were allowed to diffuse at room temperature for 1 hour and kept for incubation in upright position at 37ºC for 24 h. The activity was measured as zone of inhibition in mm and interpreted with standard antibiotic chloramphenicol which served as positive reference against the bacteria. All experiments were carried in triplicates.

3.8.3 Minimum inhibitory concentration (MIC) of the essential oil

The minimum inhibitory concentration (MIC) of the essential oil was carried out by using the broth microdilution test with slight modification [214]. DMSO (dimethyl sulfoxide) solution was used in the preparation of 1% stock solution of the essential oil. 200 µl of the prepared stock solutions were initially pipetted into the first well of the first row of a 96-well plate. 100 µl of the microbiological media-Luria Bertini Miller broth (M1245) was also pipetted into each of the remaining wells. After that, serial dilutions were then carried out so that each of the following wells contained 100 µl of the stock solution. Now, to each of these wells, 100 µl of *S. aureus* microbial cultures was added. The positive and negative controls used in the study were chloramphenicol (25mg/ml) and 1% DMSO respectively. The 96 well micro plates were then incubated at 37ºC for 12h. Once the incubation period was over, the MIC was investigated by adding 40 µl (0.5mg/ml) of 3-(4,5-Dimethyl- thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent into each well, followed by aerobic incubation at 37°C for 45 min. Appearance of clear/yellowish colour was indicative of cidal activity while dark blue colour indicated bacterial growth.

3.8.4 Antibiofilm assay of the essential oil

Biofilm inhibition assay was performed using the process of staining with crystal violet with slight modifications [215]. Test tubes consisting of essential oil at different concentrations of 0.25, 0.50, 0.75, and 1.0 mg/ml were taken. To the test tubes 50µl culture of *S. aureus* ATCC 6538 at 0.4 OD_{600} *was* added followed by adding Muller Hinton Broth (LQ182V) making the final volume to 5 ml. Now the test tubes containing the bacterial culture were incubated at 37°C for 48 h in a static condition. Once the incubation period was completed, the glass tubes were washed twice using Phosphate-Buffered saline (PBS) of pH 7.2 to remove any media and other planktonic bacteria. 0.1% Crystal violet was now used to stain the attached bacterial cells on the glass tubes and then incubated for further 30 min. The tubes were washed again with PBS to remove unbound stain and subsequently air dried. The quantification of the biofilm was carried out after solubilizing the bound crystal violet in methanol at optical densities of 570 nm. The positive and negative control used in the study were methanol and

gentamicin (2mg/ml). The formula used for calculation of percentage biofilm inhibition is:

$$\% \text{ Biofilm inhibition} = \{\frac{ODcontrol - ODtest}{ODcontrol}\} \times 100 \qquad \textbf{(Eq.4.2)}$$

Where, the OD_{test} and $OD_{control}$ represents the absorbance of treated culture and untreated control respectively.

3.9 *In-silico* investigation

3.9.1 Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) analysis

All eleven compounds identified using GCMS were screened for their ADMET properties using pkCSM software as per the previous protocols [222]. Various parameters of ADMET were investigated. For carrying out the analysis, the compounds were retrieved from PubChem and canonical smiles were taken.

3.9.2 Molecular Docking

The molecular docking analysis was carried out to determine the best binding compounds against two of the selected proteins of *Staphylococcus aureus*. The target proteins were selected for their role in biofilm synthesis and formation. In our study, we used CrtM and SarA proteins of *S. aureus*. CrtM is involved in the first step of staphyloxanthin biosynthesis in *S. aureus* and SarA is a virulence factor responsible in adhesion and tissue spread thereby enhancing biofilm formation. The crystal structure of CrtM (PDB ID: 2ZCO) and SarA protein (PDB ID: 2FNP) were retrieved from Research Collaboratory for Structural Bioinformatics (RSCB) Protein Data Bank database [223]. Eleven compounds from essential oil of *Kaempferia galanga* were considered for the molecular docking study against the two target proteins. The compounds were converted into .pdb file using Online SMILES Translator and Structure File Generator of National Cancer Institute (NCI) of National Institute of Health (NIH) [224]. The molecular docking was performed by using Autodock Vina wizard of PyRx [225-227]. To perform docking study, the grid box centre and dimensions were set accordingly to cover the substrate-binding sites of the protein. For CrtM, the grid box centre was reserved at $X = 54.8562$, $Y = 11.8491$, and $Z = 49.9251$,

and the dimension was $X = 52.7908$, $Y = 38.7607$, and $Z = 43.4988$. In case of SarA, the grid box centre was secured at $X = 5.8651$, $Y = 3.7167$, and $Z = 11.706$ and the dimension was $X = 30.2410$, $Y = 34.877$, and $Z = 38.935$. In both the cases, ciprofloxacin was used as standard drug to compare the docking results [228]. The best docked poses of ligand-protein complexes were visualized in BIOVIA Discovery Studio (Version 4.5) [229].

3.9.3 Molecular dynamics (MD) simulation

The Desmond 2020.1 from Schrödinger, LLC was used in the MD simulation experiments for 2FNP-Caryophyllene and 2ZCO-γ-elemene. The independent simulations were carried out at 37°C. The OPLS-2005 force field [230-232] and the explicit solvent model with the SPC water molecules were used in this system [233] in period boundary salvation box of 10 Å x 10 Å x 10 Å dimensions. Na^{+} ions were added to neutralize the charge and 0.15 M NaCl solutions were added to the system to simulate the physiological environment. NVT ensemble was used initially to equilibrate the system for 10 ns to retrain over the protein ligand complexes. As a follow up of the previous step, a short run of equilibration and minimization was done using NPT ensemble for 12 ns. The Nose-Hoover chain coupling scheme [234] was used for the ensembling of the NPT with relaxation time of 1.0 ps, varying temperature and a pressure of 1 bar was maintained in all the simulations carried out for the present study including a time step of 2fs. The chain coupling scheme barostat method of Martyna-Tuckerman–Klein [235] was used for control of pressure which included a relaxation time of 2 ps. The long-range electrostatic interactions were calculated by using the particle mesh Ewald method [236]. Radius for coulomb interactions was kept at 9Å. The RESPA integrator was used in the study for a time step of 2 fs for each of the trajectories so as to find out the bonded forces. The final production run was carried out at 100 ns each. The root means square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (Rg), and Solvent accessible surface area (SASA) were calculated to monitor the stability of the MD simulations.

3.9.4 Binding free energy analysis

The study of the binding free energies of the ligand protein complexes were carried out using the molecular mechanics merged with generalized Born surface area (MM-GBSA) approach [237]. The Python script thermal mmgbsa.py was used for calculation of the prime MM-GBSA binding free energy. It was used in the simulation trajectory

for last 50 frames with a 1-step sampling size. The principle of additivity was used for estimation of the binding free energy of Prime MM-GBSA (kcal/mol). Here individual energy modules such as columbic, hydrogen bond, van der Waals, covalent, self-contact, solvation of protein, lipophilic and ligand were added collectively. The equation used to calculate ΔG_{bind} is as follows:

$$\Delta G_{bind} = \Delta G_{MM} + \Delta G_{solv} - \Delta G_{SA} \qquad \textbf{(Eq.4.6)}$$

Where,

- ΔG_{bind} represents the binding free energy,
- ΔG_{MM} denotes the difference between the free energies of ligand-protein complexes and total energies of protein and ligand in their isolated form,
- ΔG_{Solv} represents difference in the GSA solvation energies of the ligand-receptor complex and sum of the solvation energies of the receptor and ligand in the unbound state,
- ΔG_{SA} denotes the difference in the surface area energies for the protein and the ligand.

3.10 Ointment formulation

Four different essential oil concentrations (w/w) (2%, 4%, 6%, and 10%) were taken for preparation of the topical ointment formulations by fusion method. The oil was incorporated into the soft mass of the different dermatological bases whose composition is tabulated below in Table 3.1.

The ointment was prepared as the following:

1. White petroleum was melted at 70°C in a hot plate.
2. Stearyl alcohol was then simultaneously added maintaining the temperature of 70°C with constant stirring.
3. The temperature was then reduced to 45°C until the mixture was in a liquid homogeneous state.
4. After the mixture was cooled at 45°C, propylene glycol was added to the melted homogenous liquid.
5. Sodium lauryl sulfate, prepared in water at temperature 25°C, was added to the homogeneous mixture.

6. Lastly, different concentrations of essential oil were added into the prepared homogenous ointment base.

Table 3.1 Hydrophilic ointment base composition

Components	Percentage (%)
White petrolatum ($C_{10}H_{15}N_5O_3$)	25
1-octadecanol ($CH_3(CH_2)_{16}CH_2OH$) (Stearyl alcohol)	25
Propane-1,2-diol ($C_3H_8O_2$) (Propylene glycol)	12
Sodium lauryl sulfate ($NaC_{12}H_{25}SO_4$)	1
Water	37

3.10.1 Physicochemical Evaluation of the Topical Formulation

The ointment after formulation was investigated for its various physiochemical properties such as organoleptic examination, pH, spreadibility, stability, viscosity, phase separation and grittiness with some modifications [238-240] described in detail as the following:

1. **Organoleptic Examination**- The formulations were examined vividly for their physical appearance, texture, color, and homogeneity. Appearance and color were examined visually; while homogeneity and texture characteristics were evaluated by pressing small quantity of the formulated product between thumb and index finger, which also determined the presence of coarse particles in the formulation.

2. **Creaming and Coalescence**- 10g of each formulation was stored at room temperature in a beaker for 3 months and subsequently their physical stability was examined after one week and after one- and three-month storage.
3. **Determination of pH**- The pH of the ointment was determined by using digital pH meter where, 1g of ointment was dissolved in 50ml of distilled water and the pH was determined.
4. **Spreadibility**- Two sets of standard-sized glass slides were taken and the ointment was applied to one of the slides. The second slide was placed on top of the ointment such that the ointment was sandwiched between the two slides along with the spacing of approximately 7cm. On the upper slide, a hundred-gram weight of ointment was put such that the ointment between the two slides was uniformly compressed to produce a thin layer. The weight was subsequently eliminated, and the extra ointment adhered to the slides was removed. The two in-place slides were fastened to a stand without causing the least disruption and in such a way that only the upper slide could be dislodged by the weight linked to them. A 10g weight was carefully attached to the upper slide. Under the impact of the weight, the time required for the top slide to transverse the distance of 7.5cm and separate from the lower slide was recorded. The tests were performed in triplicates. Finally, the spreadibility was calculated by using the following formula:

   $$S=\frac{m\times l}{t} \qquad \textbf{(Eq.4.7)}$$

   Where,

   S= Spreadibility

   m= Weight tied to upper slide (10g)

   l= Length of the glass slide (7.5cm)

   t= Time taken in seconds
5. **Viscosity**- The measurement of viscosity of the prepared ointment was carried out with a Brookfield Viscometer. The ointments were rotated at 20 and 30 rpm using spindle no. 64. At each speed, the corresponding dial reading was noted.
6. **Phase separation**- A 10 g portion of each of the formulation was placed in a centrifuge tube (1 cm diameter) and centrifuged at 2000 rpm for 5, 15, 30, and 60 min. Then the phase separation and solid sedimentation of the samples were evaluated.

7. **Grittiness**- All the formulations were evaluated microscopically for the presence of particles if any.

3.11 Animal experimental model

3.11.1 Experimental rats

Thirty healthy, nine-week-old Wistar Albino rats weighing 100-250g were used in the present study, with equal number of male and female rats. The animals were housed in individual plastic cages with access to ambient temperature of 23±3°C, stable air humidity, and a natural day/night cycle with proper monitoring. They were all fed sterile rodent laboratory diets and water was given ad libitum. The animals were handled on a regular daily basis for 14 days prior to wound incision so as to acclimatize them with the testing area and experiments. All animal handling protocols were carried out in strict accordance with proper guidelines for the use of experimental animals. The present study was carried out after seeking ethical approval by the Institutional animal ethics committee (IAEC) of Girijananda Chaudhury Institute of Pharmaceutical Science, GIPS, Guwahati, Assam (Approval number GIPS/IAEC/PhD/PRO/03/2023).

3.11.2 Skin irritancy

Fifteen Wistar Albino rats weighing 100-250g were used for the skin irritation test following OECD guidelines. The animals were divided into three groups of five animals each comprising control and formulation groups Five rats were used as test rats (Tr) with 4% and 10% formulation each, whereas five other rats were used as control rats (Cr). Hair from the back of the rats was depilated using a sterile razor towards the lower mid-position to about 20 cm in diameter and caged respectively. The rats were left undisturbed for 24 hours, prior to which Mupirocin (control) and the essential oil topical ointment formulation (4% and 10%) was topically applied evenly on the shaved portion on the rats. Observation of the sites was done at 24 h after application for 72 hours to check upon for skin irritancy such as oedema and erythema and scored according to the Draize scoring system as tabulated below in Table 3.2. [241].

Table 3.2 Draize scoring system

Erythema Formation	Value	Oedema Formation	Value
No Erythema	0	No Oedema	0
Very slight erythema (barely perceptible)	1	Very slight Oedema (barely perceptible)	1
Well-defined erythema	2	Slight Oedema (edges of area well defined by definite raising)	2
Moderate to severe erythema	3	Moderate Oedema (raised approximately 1mm)	3

3.11.3 Acute dermal toxicity

This experiment was performed in accordance with the OECD guidelines Fifteen Wistar Albino rats weighing between 100-250 g were used for this test. About 10% of the body surface area on the dorsal positions of the rats were shaved with a razor blade. They were then left undisturbed for 24 h in their respective clean cages. The rats were grouped into three groups of five rats each (test groups and a control group). The essential oil-based ointment formulation (4% and 10%) was applied for 14 days in both test and control groups with a daily observation for clinical changes of rats was done. Oedema and erythema scores were evaluated based on the Draize dermal irritation scoring system as shown in Table 3.2 [242].

3.11.4 Animal Usage

In the present study, total 30 rats were used, 6 in each group which were divided into 5 groups as follows and listed as follows:

Group 1: (Normal group): This group received normal diet+ water ad libitum (untreated skin without wound or healthy skin) **(6 rats**)

Group 2: Rats with untreated wound skin (Negative control) (**6 rats)**

Group 3: Wounded skin of the rats was treated with standard marketed wound healing ointment, 0.5g Mupirocin ointment (Positive control) **(6 rats)**

Group 4: Test group with 4% formulated ointment **(6 rats)**

Group 5: Test group with 10% formulated ointment **(6 rats)**

3.11.5 Wound infection and dressing

Before creation of the wounds, the rats were first anesthetized with isoflurane, a gaseous anaesthesia and the back hairs were depilated by shaving. A circular wound model was created on the dorsal inter-scapular region of each animal by excising the skin with a 5 mm biopsy punch and the wounds were left open. Immediately after wounding, an aliquot of 25×10^7 *Staphylococcus aureus* (ATCC 6538) suspended in 50-µL PBS were applied on the wound. After twenty-four hours, the ointments were topically applied once a day till the wound was completely healed and no longer visible in the wound area. The animals in each subgroup were examined for 14 days post-injury and tissue samples from the wound site were collected for histopathological analysis on the 14th day. Furthermore, the wound area contraction was also calculated. The animals were rehabilitated post study as per the standard guidelines [243,245].

3.11.6 Rate of wound healing

Wound contraction rate was determined [244]. Wound closure percentage was calculated using the following formula:

Percentage of wound closure (%) $=\left\{\frac{\text{Wound area on day 0} - \text{Wound area on day } x}{\text{Wound area on day 0}}\right\} \times 10$ **(Eq.4.8)**

"X" = days post injury

3.11.7 Histological analysis

The tissues were excised from the wounded area on the 14th day post-injury and immediately fixed in 10% buffered neutral formalin. This was followed by processing the tissue in an automatic tissue processor and programmed to run a series of treatment where they were immersed in acetone, xylene, and paraffin. The processed tissues were then cut into 4µm sections using a Leica microtome and subsequently stained with haematoxylin and eosin (H&E) stain after removing the paraffin. The tissues were then examined with light microscope and simultaneously photomicrographs were taken at 100x magnification. Tissues were observed for the degree of cell repair, re-epithelialization, collagen content, and granular tissue formation [241].

Statistical analysis

OriginLab, Version 8 (Northampton, Massachusetts, USA.) was used for analysis and preparation of all graphs, in which all values are reported as the mean ± SD. The tests were performed in triplicates. The statistical significance was evaluated using One-Way Analysis of Variance (ANOVA), one sample t-test and Wilcoxon test using GraphPad(Version 9) and SPSS software. Differences were considered significant at $p=0.005$.