3.0. MATERIALS AND METHODS

3.1. Work plan

The current study focused on the evaluation of the role of traditional Indian Medicines on the process of wound healing in normal and diabetic rat models. The following work plan (Figure 3.1) was adopted to achieve the defined objectives.

![Figure 3.1: Work Plan for the study](image)

3.2. List of chemicals/reagents/kits

1X PCR buffer: Thermo Scientific, USA

4-Hydroxy-L-proline: HiMedia Laboratories Pvt. Ltd., Mumbai, India

5,5’-Dithiobis(2-nitrobenzoic acid (DTNB): SISCO research laboratories, Mumbai, India

Accu-Chek Glucometer with Glucose oxidase-peroxide Reactive Strips: Roche Diagnostic India Pvt. Ltd, Mumbai, India

Acetic anhydride: Sigma Aldrich-Merck, USA
Acetone: Merck, India

Acid fuchsin: Sisco Research Laboratories, Mumbai, India

Agarose: Hi Media Laboratories, India

Anti-alpha smooth muscle Actin antibody: ab5694, Abcam, UK

Anti-IL1 beta antibody: ab9722, Abcam, UK

Bovine Serum Albumin (BSA): Sigma Aldrich-Merck, USA

Butylated hydroxytoluene (BHT): SISCO research laboratories, Mumbai, India

Celestine blue: Sigma Aldrich-Merck, USA

Chloroform: Sisco Research Laboratories, Mumbai, India

Citric acid: Sisco Research Laboratories, Mumbai, India

Collagenase/Dispase: Sigma Aldrich-Merck, USA

Copper Sulphate (CuSO\textsubscript{4}): Sisco Research Laboratories, Mumbai, India

Dibutyl Phthalate Xylene (DPX): Sigma Aldrich-Merck, USA

Dimethyl sulfoxide (DMSO): HIMEDIA, India

DNA polymerase: Thermo Scientific, USA

dNTP mix: Applied Biosystems, USA

Ehrlich’s Hematoxylin: British Drug Houses (BDH), London

Eosin Y: Sigma Aldrich-Merck, USA

Ethylenediamine tetraacetic acid (EDTA): Sisco Research Laboratories, Mumbai, India

Ethyl alcohol (C\textsubscript{2}H\textsubscript{6}O): Hayman Specialty Products, UK

Ferric chloride FeCl\textsubscript{3}: Merck, India

Fetal bovine serum: Gibco, Oklahoma, USA

Formaldehyde (CH\textsubscript{2}O): Sisco Research Laboratories, Mumbai, India
Forward and reverse primers: Bioserve, India
Glacial acetic Acid: Merck, India
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): Sigma Aldrich-Merck, USA
Goat anti-rabbit secondary antibody: ab6721, Abcam, UK
Hydrochloric acid (HCl): Merck, India
Hydrogen peroxide (\(\text{H}_2\text{O}_2\)): British Drug Houses (BDH), London
ImPACT NovaRED Peroxidase (HRP) Substrate: SK-4805, Vector Laboratories, USA
Iodine: Sisco Research Laboratories, Mumbai, India
Iscove's modified Dulbecco's medium: Life Technologies, Inc., HIMEDIA, India
Light Green SF Yellowish: Sigma Aldrich-Merck, USA
Mayer’s Hematoxylin: Sigma Aldrich-Merck, USA
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide): Sigma Aldrich-Merck, USA
Ninhydrin: Sigma Aldrich-Merck, USA
Normal goat serum: HIMEDIA, India
n-Propanol: Sisco Research Laboratories, Mumbai, India
Paradimethyl amino-benzaldehyde (4-Dimethylaminobenzaldehyde): Sigma Aldrich-Merck, USA
Paraffin wax: Sisco Research Laboratories, Mumbai, India
Penicillin-streptomycin: HIMEDIA, India
PeqSTAR Thermal cycler: PeqLab, Germany
Phosphomolybdic acid: Sigma Aldrich-Merck, USA
Picric acid: Sisco Research Laboratories, Mumbai, India
Poly-L-lysine: Sigma Aldrich-Merck, USA
Ponceau 2R: Sigma Aldrich-Merck, USA

Potassium chloride (KCl): SISCO research laboratories, Mumbai, India

Potassium Dihydrogen Orthophosphate (KH$_2$PO$_4$): Sisco Research Laboratories, Mumbai, India

Potassium iodide: Sisco Research Laboratories, Mumbai, India

PureLink RNA isolation kit: Invitrogen, USA

SOD Assay Kit: Sigma Aldrich-Merck, USA

Sodium hydroxide (NaOH): Sisco Research Laboratories, Mumbai, India

Sodium iodate: Sisco Research Laboratories, Mumbai, India

Sodium Phosphate Dibasic Dihydrate (Na$_2$HPO$_4$.2H$_2$O): Sisco Research Laboratories, Mumbai, India

Sodium Phosphate Monobasic (NaH$_2$PO$_4$): SISCO research laboratories, Mumbai, India

Sodium thiosulphate: British Drug Houses (BDH), London

Streptozotocin: Sigma Aldrich-Merck, USA

Sulphuric acid (H$_2$SO$_4$): Merck, India

SuperScript III First strand synthesis kit: Invitrogen, USA

Thiobarbituric acid (TBA): Sigma Aldrich-Merck, USA

Trichloroacetic acid (TCA): SISCO research laboratories, Mumbai, India

Tris base: Sigma Aldrich-Merck, USA

Tris Buffered Saline (TBS): Sigma Aldrich-Merck, USA

Tris-Borate-EDTA buffer: HIMEDIA, India

Trisodium citrate dihydrate: Sigma Aldrich-Merck, USA

Triton™ X-100: Sigma Aldrich-Merck, USA

Trypsin: HIMEDIA, India
TWEEN® 20: Sigma Aldrich-Merck, USA

Vecta Mount mounting medium: H-5000, Vector Laboratories, USA

Vector® Haematoxylin QS: H-3404, Vector Laboratories, USA

Xylene: Sisco Research Laboratories, Mumbai, India

α-naphthol: Sisco Research Laboratories, Mumbai, India
3.3. Test materials

3.3.1. Procurement of the test materials

Unprocessed Honey in its raw form, Cow’s Ghee, and roots of GG were procured from Sri Dharmasthala Manjunatheshwara (SDM) Ayurvedic pharmacy, Udyavara, Udupi. Honey and Ghee were subjected to microbiological examination to rule out any bacterial contamination. NI was collected in December from its natural habitat in Udupi and was identified and authenticated by Dr. K. Gopalakrishna Bhat, Professor of Botany (Rtd), Poornaprajna College, Udupi, Karnataka, India. A voucher specimen is preserved in the Dept. of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal (PP no. 603).

3.3.2. Preparation of aqueous extract of GG and NI

The roots of GG and leaves of NI after procurement were shade dried for seven days and then powdered. Aqueous extraction was carried out by hot maceration method (Davis, 1956). The powdered plant materials (200g each) were dissolved in 1500 ml of distilled water, and the decoction was prepared at 75-80 degrees Celsius. In the final stages (after boiling) of extract preparation, all possible sterility was maintained. The decoction was then cooled and filtered. The filtrate was finally evaporated to dryness using a lyophilizer. Before application on the wound, these test materials were membrane filtered to ensure that they were devoid of contamination.

3.3.3. Preliminary Phytochemical investigation of GG and NI

The aqueous extracts of GG and NI were exposed to preliminary phytochemical screening to detect the presence or absence of active constituents using the standard protocol (Kokate, 2008; Ugochukwu et al., 2013).
Test for Alkaloids (Wagner’s reagent)

3-5 drops of Wagner’s reagent, i.e., 1.27 grams of iodine and 2 grams of potassium iodide in 100 ml of water was added to a fraction of the extracts. The presence of alkaloids was indicated by the formation of a reddish-brown precipitate.

Test for Glycosides (Keller Kelliani’s test)

5 ml of the extracts were added to 2 ml of glacial acetic acid in separate test tubes. A drop of the solution of ferric chloride (FeCl\textsubscript{3}) was added to each of them. It was then underlaid with one ml concentrated sulphuric acid (conc. H\textsubscript{2}SO\textsubscript{4}). Presence of glycosides was indicated by the formation of a brown ring at the interface.

Test for Saponins (Foam test)

6 ml of water was added to 2 ml of the extracts in separate test tubes. The mixture was shaken vigorously. Development of an insistent foam confirmed the existence of saponins.

Test for Resin

Extracts were added with acetone and water. Turbidity specified the incidence of resin.

Test for Tannins (Braymer’s test)

2 ml of the extracts were added to 10% alcoholic FeCl\textsubscript{3} solution and perceived for the development of greenish/blue colored solution.
Test for Phlobatannins (Precipitate test)
Formation of a red precipitate when 2ml of the extracts were boiled with 1ml of 1% aqueous hydrochloric acid (HCl) indicated the existence of phlobatannins.

Test for Quinones
A small amount of the extracts were added to conc. HCl. Formation of yellow precipitate/coloration confirmed the incidence of quinones.

Test for Gums
A minor amount of the extracts were added to 25 ml each of absolute alcohol with continuous stirring and later filtered. The precipitate formed was then air dried and observed for swelling. The existence of swelling affirmed the presence of gums.

Test for Oxalate
A few drops of glacial ethanoic acid were added to 3ml of each extract. Formation of a greenish black coloration indicated the existence of oxalates.

Test for Proteins (1% ninhydrin solution in acetone)
2-5 drops of ninhydrin solution were added to 2ml of each filtrate in a boiling water bath for 1-2 minutes. Formation of a purple color indicated the presence of proteins.

Test for Carbohydrates (Molisch’s test)
Few drops of the Molisch’s reagent were added to 2ml of the extracts. It was then added with 2ml of conc. H₂SO₄ slowly along the side of the test tubes. The mixture was then allowed to stand for two/three minutes. The presence of carbohydrate was
confirmed by the production of a red/dull violet color at the interphase of the two layers.

**Test for Phenols (FeCl₃ test)**

A small portion of the extracts was added to aqueous 5% FeCl₃ and examined for the development of dark blue/black color.

**Test for Flavonoids (Alkaline reagent test)**

2ml of the extracts were treated with 2-3 drops of 20% solution of sodium hydroxide (NaOH). The presence of flavonoids was indicated by the development of intense yellow color, which turned colorless when dilute HCl was added.

**Test for Steroids (Liebermann-Burchard test)**

One ml of the extracts were treated with chloroform, acetic anhydride, and conc. H₂SO₄ and perceived for the development of dark pink/red color.

**Test for Terpenoids (Salkowki’s test)**

One ml of chloroform was added to 2ml of the extracts. To this mixture, a few drops of conc. H₂SO₄ was then added. A reddish brown precipitate formed affirms the existence of terpenoids.

**Test for Coumarins**

After dissolving the extracts in alcohol, they were added with 2-3 drops of 2 N solution of NaOH. Development of a dark yellow color indicated the presence of coumarins.
3.4. Experimental animals

Healthy adult Wistar Albino rats (150-200 g) of either sex were utilized for the studies. They were housed under standard environmental conditions of temperature and humidity (25±0.5 °C) and 12 hour light/dark cycle. The rats were fed a standard diet of pellets and water ad libitum. The experimental study was conducted in the central animal house of Kasturba Medical College, Manipal, Karnataka, India. The animal studies have been carried out after approval by the Institutional Animal Ethics Committee (Ref. No: IAEC/KMC/49/2013).

The rats were grouped into two main sets, i.e., normal and diabetic.

3.5. Induction of diabetes

The animals ordained to belong to the diabetic group were induced with diabetes using Streptozotocin (STZ) (Maritim et al., 2003).

Procedure:

Citrate buffer preparation (pH 4.5)

44.5 ml of 0.1 M citric acid and 55.5 ml of 0.1 M trisodium citrate dehydrate solutions were mixed to prepare the citrate buffer.

Preparation of 0.1M trisodium citrate dihydrate solution

2.94 g of trisodium citrate was added to 100 ml of distilled water to make 0.1M trisodium citrate dihydrate solution.
0.1M citric acid solution preparation
1.92g of citric acid was added to 100 ml of distilled water for the preparation of the 0.1M citric acid solution.

STZ dose preparation
A weighed quantity of STZ was dissolved in 0.1M freshly prepared ice cold citrate buffer solution just before the induction of diabetes. After seven days of acclimatization, Wistar rats were used for the induction of diabetes. The rats were injected with a single intraperitoneal dose of STZ (35 mg/kg) after fasting overnight. Drinking water was added with glucose (5%) to overcome the STZ induced hyperglycemia. Fasting blood glucose levels were measured using Accu-Chek glucometer with glucose oxidase-peroxide reactive strips (Roche Diagnostic India Pvt. Ltd, Mumbai). The animals with fasting blood glucose levels >250 mg/dl were considered for the diabetic models.

3.6. Wound model and treatment groups
Wistar rats (normal and diabetic) were used for incision and excision wounds. The animals were further classified into seven groups, i.e., untreated and treated groups (6 animals in each group): Control (untreated), Honey, Ghee, Honey+Ghee (H+G), GG, NI and combination of all test materials (Tot) (Figure 3.1).

3.7. Creation of wound and treatment plan
3.7.1. Incision wound: The animals were anesthetized with ketamine, before inflicting the wounds. The anesthetized animals were shaved off their dorsal fur. Two 6 cm long paravertebral incisions were made through the whole skin on the back. The
parted skin was then sutured intermittently, one cm apart using a curved needle and surgical thread. The wounds were left undressed (Figure 3.2).

**Figure 3.2:** Incision wound inflicted in the rat model

3.7.1.1. **Treatment plan:** 250 mg of the test materials were topically applied in the form of paste on the entire surface of the wound once a day for 11 days. The dosage was calculated based on the amount of test material required to cover the entire area of the wound uniformly. The nature of wound healing was regularly observed, and photographic evidence was obtained. At two different time points of the treatment plan (6th and 11th day), the skin with the incision was collected for histological and biochemical study. At the end of the treatment, the incision wound models were subjected to biomechanical analysis to measure the wound breaking strength.

3.7.2 **Excision wound:** The rats were anesthetized using ketamine, before inflicting the excision wounds. The animals were shaved off their dorsal fur and the wound area to be created was marked on the animals’ back with methylene blue using a circular stainless steel stencil. A full skin thickness excision wound with a width of 2.5 cm
(circular area = 4.90 cm²) and depth of 0.2 cm was made along the marking with the help of a surgical blade, toothed forceps, and pointed scissors. The wound was left undressed (Figure 3.3).

![Image](image_url)

**Figure 3.3**: Excision wound inflicted in the rat model

3.7.2.1. **Treatment plan**: The open wounds (covering the entire wound area and the adjacent area) were topically applied with reference dose of the test materials, i.e., 500mg once a day. Two subclasses of animals with seven groups each were created and followed up to study the nature of healing for 8 and 16 days respectively.

3.8. **Biomechanical analysis**

3.8.1. **Breaking strength**: The biomechanical strength was assessed in the incision wound model by the measurement of the breaking strength using Lee’s Constant Water flow technique (Lee, 1968). The breaking strength of the skin was measured on the 11th day after anesthetizing the animal. In each of the incised area, three points were selected one centimeter apart to measure the breaking strength (Figure 3.4). The findings were tabulated.
**Procedure:** In the constant water flow technique, the anesthetized animal was secured to the operation table. The ally’s forceps were firmly fixed on the lines facing each other. The forceps on one side is hooked to a metal rod to keep it in position while the other forceps were connected to a polythene reservoir by a string run over a pulley. The water was allowed to flow at a constant rate into the polythene reservoir, and then the pulling force necessary to disrupt the wound was gradually built under controlled condition. The flow of water was regulated with the help of an occlusion clamp on polythene tubing which was connected to the reservoir that was raised to a suitable height. As soon as the gaping of the wound was found, the water flow was cut off. The pulling force of the wound was immediately released by lifting up the polythene reservoir to avoid further opening of the wound. The volume of the water accumulated in the polythene reservoir was measured and was converted to corresponding weight by considering the density of water to be equal to one. The tensile strength was expressed as minimum weight of water necessary to bring about the gaping of the wound. Three such reading was recorded for a given incision wound, and the procedure was repeated on the other side, thus obtaining a total of 6 readings for each animal. The mean tensile strength of each animal was calculated by taking the average of six readings.
3.8.2. Epithelialization period: The number of days needed for fall of the remnants of the dead tissue (eschar/scab) without any persistent raw wound was considered as the time required for epithelialization in the excision wound.

3.8.3. Measurement of the Rate of wound closure/healing: The area of the wound in the excision model was measured every alternate day, following the initial wound (i.e., day 0) using a permanent marker and transparent paper. The wound marks recorded were scanned, and the area was measured using the image analyzer software- Image J. The percentage of wound closure was calculated using the following formula (Lundeberg et al., 1992):

\[
\text{Percentage area of wound not closed} = \frac{\text{Wound area on day 0} - \text{wound area on day } n \times 100}{\text{Wound area on day 0}}
\]

Wherein ‘n’= the number of days, i.e., day 2, 4, 6, etc.
Also, photographs of the wound with fixed distance and magnification were captured every alternate day until it was completely closed, and the rate of wound closure was compared with various groups of the study.

3.9. Tissue procurement

During the treatment plan, the skin tissue of the wounds were collected on day 6 and 11 for incision models for further histological and biochemical studies. In the excision models, the tissues were harvested on day 8 and 16 for further histological, biochemical and molecular studies.

3.10. Histological evaluation

The skin samples procured were washed and fixed in 10% formalin. The samples were then dehydrated using graded alcohol (50%, 70%, 90% and 100%), cleared in xylene and embedded in paraffin wax. Serial sections of 5μm thickness were cut using the rotary microtome. The sections were mounted on the slides. The slides were stained with hematoxylin and eosin (H & E) to identify the rate of wound healing and epithelialization. Further special stains like Masson’s trichrome and Verhoeff’s Van-Geison’s were used for assessing the area of healing, vascularity, inflammatory responses, fiber composition and distribution and tissue remodeling.

3.10.1. Hematoxylin & Eosin (H&E) staining

**Deparaffinization:** Sections were deparaffinized by two changes of xylene each lasting for two minutes to dissolve the paraffin.

**Hydration:** Sections were then hydrated by passing them for two minutes each in descending grades of alcohol (100%, 90%, 70% and 50%) and then in distilled water.
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Staining procedure: The slides were stained with Ehrlich’s hematoxylin solution for three minutes and then placed for five minutes in running tap water. The slides were then stained with 1% aqueous eosin for two minutes. After staining, the slides were rinsed in 90% alcohol for 10-15 seconds. Then, the sections were dehydrated in two changes of absolute alcohol, 15-30 seconds. After dehydration, sections were cleared in two changes of xylene for two minutes. Finally, the sections were mounted in DPX (Dibutyl Phthalate Xylene). The mounted slides were cleaned and made ready for light microscopic study.

3.10.2. Masson’s trichrome (MT) staining
Deparaffinization and Hydration: Same as above (i.e., H&E staining)
Staining procedure: The hydrated sections were treated with Celestine blue for five minutes. They were then rinsed with distilled water and later stained for five minutes with Mayer’s hematoxylin. After washing for five minutes in running tap water, the sections were treated with ponceau fuchsin for five minutes. After rinsing in distilled water, they were treated with phosphomolybdic acid for ten minutes. The slides were drained and covered with light green stain for ten minutes. After draining, the slides were treated with 1% acetic acid for 30 seconds. They were then rinsed in two changes of 5% alcohol. The sections were later dehydrated in two changes of absolute alcohol, cleared in xylol and mounted in DPX.

3.10.3. Verhoeff’s Van-Geison’s (VVG) staining
Deparaffinization and Hydration: Same as above (i.e., H&E staining)
Staining procedure: The hydrated sections were placed for ten minutes in Verhoeff’s staining solution. They were later placed under running tap water and washed in two
changes of distilled water. The sections were differentiated in 2% FeCl$_3$ for 15 seconds and later washed in running tap water to remove the FeCl$_3$. Sections were then examined under light microscope for correct differentiation. The elastic fibers appeared black on the gray background. The slides were then washed in running tap water and treated with 5% sodium thiosulfate for one minute. The solution was discarded, and the slides were washed in running tap water for five minutes. The sections were then counterstained with Van Gieson's solution for 3-5 minutes. They were later rinsed in 90% alcohol for 10-15 seconds and dehydrated in two changes of absolute alcohol, 15-30 seconds each. They were then cleared in two changes of xylene, two minutes each or until sections were clear. The sections were finally mounted in DPX.

3.10.4. Image Analysis and Quantification

The stained slides were analyzed and documented using Olympus microscope (BX43), Q Imaging camera and ImagePro Premier 9.1 software (Media Cybernetics, Rockville, USA). Parameters like the area of wound healing, the width of the healing wound at different levels in the dermis, rate of epithelization, cell population at the wound site, i.e., the cell count of lymphocytes, macrophages, and fibroblasts was quantified using Image-Pro Premier software.

Further, the vascularity in the wound (i.e., the number of blood vessels) was also quantified using Tissue Quant Software (Prasad et al., 2012).
3.11. Immunohistochemistry

Immunohistochemistry-Horseradish Peroxidase Method (IHC-HRP) was used to assess the following parameters:

1) The inflammatory activity at the wound site by measuring the expression of interleukins (IL-1). Primary antibody used: Anti-IL1 beta antibody

2) The activity of myofibroblasts at the wound site. Primary antibody used: Anti-alpha smooth muscle Actin antibody

3.11.1. IHC-HRP Procedure

Formalin-fixed tissue sections (5 m in thickness) from each group of excision wounds were cut and attached to the slides pretreated with poly-L-lysine (Sigma-Aldrich). The sections were deparaffinized in xylene and rehydrated with graded ethanol (100 to 50%). The retrieval of antigens was done by heating the slides in Tris-EDTA Buffer (i.e., a mixture of 10mM Tris Base, 1mM EDTA Solution, and 0.05% Tween 20) (pH 9.0) (0.01 M) for 30 minutes. The slides were then treated with 0.3% (v/v) H$_2$O$_2$ in water for five minutes to minimize the activity of endogenous peroxidase. The sections were later washed with Tris Buffered Saline (TBS) with 0.025% Triton X-100 and then blocked with 10% normal goat serum (HIMEDIA, India) and 1% Bovine Serum Albumin (BSA) in TBS for one hour at room temperature (RT). The sections were then incubated for 12 hours at 4 °C with Anti-IL1 beta antibody (ab9722, Abcam, UK, 1:1000 dilution) or Anti-alpha smooth muscle Actin antibody (ab5694, Abcam, UK, 1:100 dilution) diluted in TBS with 1% BSA. After washing with TBS with 0.025% Triton, the sections were incubated with the HRP conjugated goat anti-rabbit secondary antibody (ab6721, Abcam, UK, 1:1000 dilutions) for one hour at RT. It was followed by rinsing in TBS and
incubation with ImPACT NovaRED Peroxidase (HRP) Substrate (SK-4805, Vector Laboratories, U.S.A) as per the manufacturer’s guidelines. The slides were then counterstained with hematoxylin (Vector-Haematoxylin QS, H-3404, Vector Laboratories, U.S.A), dehydrated, cleared and mounted in Vecta Mount mounting medium (H-5000, Vector Laboratories, U.S.A).

3.11.2. Image Analysis and Quantification

The stained slides were analyzed and documented using Olympus microscope (BX43), Q-Imaging camera and ImagePro Premier 9.1 software (Media Cybernetics, Rockville, USA). Counting was performed manually in a fixed area at 40x magnification, and then the ratio of the cell count/mm² granulation tissue was calculated. Color markers were used in the software to avoid confusion.

3.12. Biochemical analysis

3.12.1. Estimation of Hydroxyproline

**Tissue Preparation:** The wet weight of the tissues were recorded, and later dried for 24 hours at 60°C. The dry weight of the tissues was then noted. The dried tissues were treated with 10mL 6N HCl and placed at 110°C for 24 hours. The acid hydrolysates that were neutralized were then utilized for the estimation of hydroxyproline content (Neuman and Logan, 1950).

**Estimation:** In each test tube, 0.3mL each of hydrolysate, 2.5N NaOH, 0.01M CuSO4, and 6%H2O2 were taken. The contents of the tubes were shaken vigorously and placed immediately in a hot water bath at 80°C. After fifteen minutes; tubes were removed and cooled for five minutes in cold water. 0.6mL of freshly prepared 5%
paradimethylaminobenzaldehyde solution in n-Propanol and 1.2mL of 3N H$_2$SO$_4$ was then added. The test tubes were placed in the hot water bath for 15 minutes at 75°C and then cooled for five minutes under running tap water. The intensity of the color was read at 540 nm against the blank. The amount of hydroxyproline was then measured as per the standard curve prepared with the standard 4-Hydroxy-L-proline (HiMedia Laboratories Pvt. Ltd., Mumbai, India), from 75 to 900 $\mu$g/0.3mL using 3mg/mL working solution (Neuman and Logan, 1950). The findings were expressed in $\mu$g/mg dry tissue weight.

3.12.2. Estimation of Antioxidants

3.12.2.1. Preparation of Tissue Homogenate: The skin tissues procured from the excision wounds were washed in ice-cold saline. They were then blotted dry and immediately weighed. A 10% skin homogenate was then prepared in ice-cold KCl (150Mm) using Teflon – glass homogenizer (Yamato LSG LH-21, Japan).

The homogenate was subjected to centrifugation at 10,000 rpm for 10 minutes, and the pellets were cast off. The supernatant collected was centrifuged again at 20,000 rpm for one hour at 4°C. The resultant supernatant was used in the estimation.

3.12.2.2. Catalase assay method

Catalase is present in abundance in the peroxisomes of the aerobic organisms. H$_2$O$_2$ being a by-product of the metabolic activity in the cells is toxic to the body. Catalase speeds up the reaction, i.e., it catalyzes the conversion of H$_2$O$_2$ to water and oxygen. During excessive oxidative stress, the level of catalase enzyme activity decreases due
to excessive stress caused due to ROS resulting in accumulation of H$_2$O$_2$ which is toxic to the body causing cell death. (Nilsson, 2010).

**Reagents:**

1. Phosphate buffer 50 mM (pH 7.0): 6.81 g of KH$_2$PO$_4$ (solution A) and 8.90 g Na$_2$HPO$_4$.2H$_2$O (solution B) were dissolved separately in distilled water and made up to 1000mL. The Solutions A and B were then mixed in the proportion 1:1.5 (v/v).

2. PBS- H$_2$O$_2$ solution (0.17 mM): 0.16 mL of (30% w/v) H$_2$O$_2$ was diluted with phosphate buffer and brought to 100 ml.

**Procedure:** One ml of H$_2$O$_2$- phosphate buffer solution and 0.015 mL of the tissue homogenate supernatant were mixed in a cuvette, and the absorbance was recorded at 240 nm for one minute. The difference in the absorbance per unit time indicated the amount of the catalase activity (Aebi, 1984).

**3.12.2.3. Estimation of GSH**

Reduced glutathione (GSH) located in almost every cell of the body is a non-protein thiol, a water-soluble tripeptide consisting of glutamine, cysteine, and glycine. GSH is one of the vital antioxidant systems present in high concentrations in the body. It is a potent reducing agent, an electrophile which is responsible for attenuation of free radical injury to the cells. GSH plays a vital role in cellular oxidative stress (Townsend et al., 2003).

**Reagents:**

5% TCA (Trichloroacetic acid): 5g in 100 mL of distilled water

PBS (0.2 M) (pH- 8.0): 0.218g NaH$_2$PO$_4$ and 2.641g of Na$_2$HPO$_4$ in 100 mL distilled water
DTNB (0.6 mM): 20 mg in 50 mL of phosphate buffer

**Procedure:** The skin homogenates were precipitated in 5% TCA. The solution was then centrifuged to collect the supernatant. One mL of the supernatant was then mixed with six mL 0.2M PBS and one mL of 0.6 mM DTNB and incubated for 10 min at RT. The samples’ absorbance was recorded against a blank at 412 nm using a UV-Visible spectrophotometer (Shimadzu UV-260, Shimadzu corp., Japan) (Sedlak and Lindsay, 1968).

### 3.12.2.4. Estimation of SOD

SOD is an active antioxidant enzyme located in mitochondria and peroxisomes. It scavenges superoxide free radicals generated during oxidative stress. It catalyzes the dismutase reaction forming a by-product of $\text{H}_2\text{O}_2$ (Naso et al., 2011).

**Procedure:** Estimation of SOD was carried out using the SOD assay kit method (Sigma Aldrich-Merck, USA) as per the manufacturer’s guidelines. The change in the absorbance was recorded at 480 nm ($A_0$-$A_{60}$). Change in absorbance was extrapolated to standard plot to find out the SOD activity, which was expressed in units/mg of protein (Kakkar, 1984).

### 3.12.2.5. Estimation of Lipid peroxidation

Lipid peroxidation is a process by which ROS, nitrite and superoxide radicals formed during the oxidative stress attack polyunsaturated fatty acids (PUFAs) of the cell membrane, undergo hydrogen abstraction and the addition of oxygen radical. This process leads to the formation of proxy radical which initiates a propagating chain reaction leading to the formation of hydroperoxides (LOOH). Also along with this, at the termination of lipid peroxidation, reactive aldehydes as end products such as
malondialdehyde and 4-hydroxynonenal are formed which disrupt the biological membrane rigidity.

Malondialdehyde (MDA), resulting from the breakdown of PUFAs, acts as an index for the estimation of the amount of peroxidation. MDA reacts with thiobarbituric acid (TBA) to produce TBARS that imparts a red color (Ayala et al., 2014).

**Reagents:**

TBA–TCA-BHT (Butylated hydroxytoluene) solution: TCA-15%, TBA-0.375%, BHT-0.05%

**Procedure:** 0.5mL of tissue homogenate and 2.5 ml (TBA-TCA-BHT solution) were mixed and incubated at 30°C for 5 minutes. It was then heated at 80°C for 10 minutes and centrifuged at 2000 rpm for 20 minutes. The absorbance was read at 532 nm by adjusting the blank with distilled water (Ohkawa et al., 1979).

### 3.13. Molecular analysis

#### 3.13.1. RNA isolation and Reverse transcription

Total RNA was obtained from the excision wound samples of the different experimental groups using PureLink RNA isolation kit (Invitrogen, USA). The cDNA was then made with the help of SuperScript III First strand synthesis kit (Invitrogen, USA) as per the manufacturer’s guidelines.

#### 3.13.2. Polymerase Chain Reaction (PCR)

Semi-quantitative PCR was done using primers specifically designed for mRNA templates of the growth factors TGFβ, FGF2, PDGF and VEGF which could distinguish between genomic DNA and cDNA. Only cDNA template was amplified in all primer sets. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal control and was used to normalize the
gene expression level. PCR was carried out in the 25µl reaction mixture in PeqSTAR Thermal cycler (PeqLab, Germany). The reaction mixture contained 1X PCR buffer (Thermo scientific, USA), 1µg of forward and reverse primers (Bioserve, India), 0.25mM dNTP mix (Applied Biosystems, USA) and two units of DNA polymerase (Thermo scientific, USA). After initial denaturation at 94°C for three minutes, the samples were subjected to 35 cycles of amplification comprising of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, elongation at 72°C for 45 seconds and a final extension at 72°C for ten minutes.

The PCR products were then examined by electrophoresis on 2% agarose gel, stained with ethidium bromide and photographed on a UV light transilluminator. The sequences of the primers are as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>CTAGAGACAGCCGCATC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Reverse</td>
<td>GGGTAGAGTCATACTGGAAC</td>
</tr>
<tr>
<td>TGF-b1</td>
<td>Forward</td>
<td>CTGGAAAGGGCTCAACACCT</td>
</tr>
<tr>
<td>TGF-b1</td>
<td>Reverse</td>
<td>GGCCCCCAGATGGGCTT</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Forward</td>
<td>GCCGAACGGGACAGATTCTT</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Reverse</td>
<td>TTCGCACACACTCCCTTGAT</td>
</tr>
<tr>
<td>PDGF</td>
<td>Forward</td>
<td>GTGTGAGACAGTAGTGACCC</td>
</tr>
<tr>
<td>PDGF</td>
<td>Reverse</td>
<td>ACGGACGAGGGAAACACATT</td>
</tr>
<tr>
<td>VEGF</td>
<td>Forward</td>
<td>CGTCCAACCTCTGGGCTCTTT</td>
</tr>
<tr>
<td>VEGF</td>
<td>Reverse</td>
<td>GCTTTCTGCTCCCCTCTGT</td>
</tr>
</tbody>
</table>
The intensity of the bands obtained was then measured using ImageJ software. The intensity of the control group was considered as 100%, and the intensity for the remaining groups was calculated and documented.

3.14. Cellular analysis- Cell Culture (In Vitro study)

3.14.1. Isolation of Rat Dermal Fibroblasts (RDFs) & Procurement of Keratinocytes (HaCaT Cells)

3.14.1.1. Isolation of Rat Dermal Fibroblasts (RDFs): The rat dermal fibroblasts (RDFs) were isolated from the skin samples of healthy adult Wistar rats following the standard protocol (Seluanov et al., 2010). After digestion of skin pieces with 0.05% trypsin and 0.1% collagenase at 37°C for 30 min, the dermis was separated and homogenized with continued pipetting and vertex. The cell suspension was cultured in Iscove's modified Dulbecco's medium (Life Technologies, Inc., HIMEDIA, India) with 10% fetal bovine serum (FBS) (Gibco, Oklahoma, USA) and 1% penicillin-streptomycin (Himedia) (Figure 3.5A).

3.14.1.2. Procurement of Keratinocytes (HaCaT Cells): HaCaT cell line (NCCS, Pune) was used instead of Rat keratinocytes. It was hard to isolate the keratinocytes from the rat skin due to recurrent contamination and was therefore not included in the study. Also, the HaCat cells have a greater ability to differentiate and proliferate in vitro (Schürer et al., 1993) compared to rat keratinocytes. Therefore, HaCaT cells were used in the study (Figure 3.5B).
3.14.2. RDF/ HaCaT Viability and Proliferation in vitro


A day before the assay, RDF/HaCaT cells were seeded in 96-well plates at an optimum concentration of $5 \times 10^3$ cells per 100 µL medium per well. The cell suspensions were then incubated at 37ºC to enable cell attachment. After 24 hours, the cells were treated with 100 µL of each concentration of serially diluted medicinal preparations. The concentrations considered for Honey, GG, NI, H+G, and Tot were 1000, 750, 500, 250 & 100 µg/ml. Ghee was administered in the concentrations of 10, 7.5, 5, 2.5 & 1 µL/ml. All concentrations were in triplicates and cells were treated for 24, 48 and 72 hours. The assay with NI was carried out further at lower concentrations in RDFs, i.e., 50, 25, 10 & 5 µg/ml owing to its cytotoxic effect. At the end of the stipulated time frame, 20µl MTT (5mg /mL) in PBS was added to the wells and incubated for 4 hours at 37ºC. After incubation, the media was discarded and formazan crystals formed were dissolved in 100 µl of DMSO and incubated for one hour at RT. The optical density (OD) was read at 570nm with a reference wavelength of 650nm using ELISA plate Reader (Bio-Rad Laboratories, Orlando, FL, USA).
USA, model 3550). The findings obtained were normalized to percent of control (wells without having any drugs) and plotted against drug concentration. The formula used for its calculation is represented below:

\[
\text{Value of the treatment group/ Mean value of control X 100}
\]

Wherein the treatment groups were: Control, Honey, Ghee, GG, NI, H+G, and Tot.

### 3.14.2.2. Wound Scratch Assay using RDF and HaCaT cells

RDFs and HaCaT cells were plated separately into 12 well plate at a concentration of \(1 \times 10^5\) cells. After 24 hours, the monolayer of cells was disturbed by scratching a line using micro-tips (Li et al., 2004). The detached cells were gently washed out using PBS. Monolayer cells were then treated with the different groups of medicinal preparations 100 \(\mu\)g/ml in case of Honey, GG, NI, H+G & Tot; 10 \(\mu\)L/ml in the event of Ghee respectively). Soon after adding the drugs, the plates were observed under a microscope to locate and mark the scratch in monolayer, and the images were captured. These pictures were documented as 0th-hour images. Cells were further incubated and regularly monitored. At regular intervals, i.e., 24, 48 and 72 hours, the fresh media was added to the cells with the same amount of test materials. Images were also captured at these intervals. The captured images were then analyzed using T Scratch software (CSE Lab). The area of the scratch in each image was estimated. Scratch area on the 0th hour was considered 100% and the reduction in area was calculated for each set in 24, 48 and 72 hours and plotted on a graph.

### 3.14.2.3. RNA isolation and Reverse transcription using RDFs

The RDFs were cultured in 6 well plates and treated with the best concentrations of the test materials (Honey- 1000 \(\mu\)g/ml, Ghee- 100 \(\mu\)l/ml, GG, NI & Tot- 100\(\mu\)g/ml).
The following concentrations were calculated based on the results of cell proliferation experiments (MTT assay). Total RNA was obtained from the different treatment groups using PureLink RNA isolation kit (Invitrogen, USA). The cDNA was then made with the help of SuperScript III First strand synthesis kit (Invitrogen, USA) as per the manufacturer’s guidelines.

**Polymerase Chain Reaction (PCR):** Semi-quantitative PCR was done using primers specifically designed for mRNA templates of FGF2 which could distinguish between genomic DNA and cDNA. Only cDNA template was amplified in all primer sets. GAPDH served as an internal control and was used to normalize FGF2 expression level. PCR was carried out in the 25µl reaction mixture in PeqSTAR Thermal cycler (PeqLab, Germany). The reaction mixture contained 1X PCR buffer (Thermo scientific, USA), 1µg of forward and reverse primers (Bioserve, India), 0.25mM dNTP mix (Applied Biosystems, USA) and two units of DNA polymerase (Thermo scientific, USA). After initial denaturation at 94\(^\circ\)C for 3 minutes, the samples were exposed to 35 cycles of amplification comprising of denaturation at 94\(^\circ\)C for 30 seconds, annealing at 50\(^\circ\)C for 30 seconds, elongation at 72\(^\circ\)C for 45 seconds and a final extension at 72\(^\circ\)C for 10 minutes.

The products of PCR were then examined by electrophoresis on 2% agarose gel, stained with ethidium bromide and photographed on a UV light transilluminator. The sequences of the primers are as follows:

GAPDH Forward-CTAGAGACAGCCGACATC
GAPDH Reverse-GGGTAGAGTCATACTGGAAC
FGF-2 Forward-GCCGAACGGGACAGATTCTT
FGF-2 Reverse-TTCGCACACACTCCCTTGAT
The intensity of the bands obtained was then measured using ImageJ software. The intensity of the control group was considered as 100%, and the intensity for the remaining groups was calculated and documented.

### 3.15. Statistical Analysis

Results were expressed in mean ± SEM (standard errors of the mean). The data were analyzed using Graph Pad Prism software (Microsoft, San Diego, CA, USA). ‘One-way ANOVA’ followed by Dunnett’s posthoc test was employed to compare between the treated groups and control (untreated). P value ≤0.05 was considered statistically significant.