Chapter 3

Materials & Methods

3.1 Plant materials and sample preparation

Plant materials of Certain xerophytic plants undertaken for research are *Securinega leucopyrus* (Willd.) Müll.Arg. [84]{NTP/15/07/2020-/03} *Vitex negundo L.* [89] {NTP/15/07/2020-/04}, *Senegalia catechu* (L.f.) P.J.H. Hurter & Mabb. [98] {NTP/15/07/2020-/01}and *Azadirachta indica A. Juss.* [100] {NTP/15/07/2020-/02}, collected from Saurashtra region, and their botanical identities were confirmed by a certified botanist Dr. Neha Patel, Atmiya University. The plant samples were washed, air-dried under shade at 27 °C-30 °C for 5 days, and then ground into a fine powder using a mechanical grinder. The powdered samples were stored in air-tight containers until further use.

3.2 Preparation of the Ayurvedic polyherbal formulation

The Ayurvedic polyherbal formulation was prepared through decoction, a traditional method of extracting bioactive compounds from plant materials. A specific ratio of the powdered plant materials was mixed and boiled in distilled water until the volume reduced to One-fourth. The decoction was then filtered and concentrated using a rotary evaporator to obtain the final extract [77].

3.3 Physiological Properties

The various physicochemical parameters that were determined as per The Unani Pharmacopoeia of India- viz. Acid value, Saponification value, pH, Viscosity, Specific gravity, Refractive index, Organoleptic property and Relative density.

3.3.1 Acid value

10mL of polyherbal formulation was added on with 25 mL of ethanol and ether simultaneously. Phenolphthalein as indicator was used and performs titrimetric method with 0.1M potassium

$$Acid\ value = \frac{5.61n}{w}$$

where,

n= number of mL of 0.1M KOH

w= weight of polyherbal formulation

3.3.2 Saponification value

2g of polyherbal formulation with 250mL iodine was correctly weighted. Then add 25mL of 0.5M potassium hydroxide and homogenized in water bath for 30 minutes. Phenolphthalein as indicator was used and perform titrimetric method with 0.5M HCL. Similarly blank was prepared without the sample.

$$Saponification\ value = \frac{28.05(b-a)}{w}$$

where,

b= blank,

a=sample

w= weight in grams of solution.

3.3.3 pH determination

pH of polyherbal formulation was measured using pH meter

3.3.4 Viscosity

Viscosity of polyherbal formulation was measured using Brookfield viscometer.

3.3.5 Specific gravity

Specific gravity of polyherbal formulation was determined by specific gravity bottle.

$$Specific\ gravity = \frac{Weight\ of\ distilled\ water - Weight\ of\ sample}{10ml}$$

3.3.6 Refractive index

Refractive index of polyherbal formulation was measured using Refractometer.

3.3.7 Organoleptic property

Color, odor, Sensitivity test / sensory properties were determined, respectively. Polyherbal formulation was apply gently on backside of hand and exposed under sunlight for 5 minutes to check irritation on skin.

3.3.8 Relative density

Relative density of polyherbal formulation was determined by gravity bottle.

$$Relative\ density = \frac{Oil\ with\ gravity\ bottle}{Weight\ with\ gravity\ bottle\ of\ distilled\ water\ as\ reference}$$

3.4 Phytochemical analysis

The qualitative phytochemical analysis of the polyherbal formulation was conducted using standard methods to identify the presence of various phytoconstituents, such as alkaloids, flavonoids, tannins, saponins, and terpenoids. Quantitative phytochemical analysis was performed to determine the total phenolic content, total flavonoid content, and total tannin content of the polyherbal formulation using spectrophotometric methods and standard calibration curves. Also, the Gas chromatography-mass spectrometry (GC-MS) analysis was employed to further elucidate the volatile compounds present in the polyherbal formulation [41]. The analysis was conducted using a gas chromatograph equipped with a mass selective detector. The resulting data were used to identify and characterize the volatile compounds based on their retention time, mass spectra, and comparison with reference libraries.

3.4.1 Qualitative analysis of polyherbal formulation:

The study was conducted performing qualitative analysis of polyherbal formulation to check the presence of secondary metabolites or phytochemicals such as flavonoids, saponins, alkaloids, tannins, terpenoids etc. The given below tests were performed.

3.4.1.1 Fehling's test for reducing sugar:

Preparation of Fehling's solution:

Fehling's A: 34.66 g of copper sulphate was taken and distilled water was added to make the total volume 100mL.

Fehling's B: 173g of potassium sodium tartarate was taken with 50g NaOH and distilled water was added to make the total volume 100mL.

Fehling's Test: 1mL each of Fehling's solution A and B was taken with 1 mL of polyherbal solution and boiled in water bath. formation of red precipitates will indicate the presence of reducing sugar.

3.4.1.2 Kellar-Killani test for glycosides:

1 mL of polyherbal formulation was added with the 1.5 mL of glacial acetic acid in a test tube, a drop of 5% ferric chloride was added with pouring few drops of sulfuric acid along the side of the test tube. formation of a blue coloured solution in acetic acid layer will indicate the presence of glycosides.

3.4.1.3 Millon'stest for proteins and amino acids:

A few drops of Millon's reagent were added into the 2mL of polyherbal formulation solution. formation of white precipitate will indicate the presence of proteins and amino acids.

3.4.1.4 Alkaline reagent test for flavonoids:

1mL of polyherbal formulation was taken with 2mL of 2% sodium hydroxide and few drops of diluted hydrochloric acid. An intense yellow colour which disappears on addition of acid will indicate the presence of flavonoids.

3.4.1.5 Gelatine test for phenolic compounds and tannins

Polyherbal solution is dissolved in 5mL distilled water and 1% gelatine solution, 10% sodium chloride was added. formation of white precipitate will indicate the presence of phenolic compounds and tannins.

3.4.1.6 Frothing test for saponins:

Polyherbal formulation was mixed with distilled water in a ratio of 1:3, it was shaken vigorously for about 5 minutes, and it was allowed to stand for 30 minutes. Froth having a structure like a hOneycomb will indicate the presence of saponins.

3.4.1.7 Test for terpenoids:

2mL of chloroform was added to 5mL polyherbal formulation and was evaporated through water bath, then 3mL concentrated sulphuric was added. Then again, the solution was treated with water bath. A grey coloured solution will indicate the presence of terpenoid.

3.5 Extraction of phytochemicals

Phytochemical extracts were prepared for obtaining the bioactive compounds from the powdered mixture. for extraction of different bioactive compounds, different 6 solvents like two polar solvents methanol & water, two semi- polar solvents chloroform & hexane and two non- polar solvents ethanol & petroleum ether. Solvent extraction is performed in a Soxhlet apparatus to extract the bioactive compounds from the powdered mixture. The Soxhlet apparatus consists of a glass extractor, fitted in between a round bottom flask at the bottom and a bulb condenser at the top. Within the glass thimble holder, pouch made of filter paper containing the powdered mixture is placed inside thimble. The bottomed distillation flask initially contained an extracting solvent and it's heated up by electro thermal heating mantle having 100°C maximum temperature, 500mL remains the maximum capacity. as the solvent vapor goes up to the condenser, it condenses and accumulates within the extractor. Here, the solvent comes in contact with the powdered mixture and starts dissolving bioactive compounds from powder into solvent. Major amount of dissolution from the powder to solvent happens once the accumulated solvent moves up purely due to the hydrostatic pressure head. After passing the pressure head condensate pours down to the round bottom flask. This cycle is repeated for several times until it gets concentrated. for extraction of different bioactive compounds different solvents were used. These extracts would be stored for further characterisation studies aimed to isolate the bioactive compound/ s.

3.6 Quantitative analysis of plant extract:

Based on qualitative analysis of phytochemicals, quantitative analysis was also dOne on major phytochemicals that were the terpenoids and flavonoids. Terpenoids being One of the foremost bioactive compounds in plants. Likewise, Flavonoids in recent studies, considered as an essential compOnent in a variety of therapeutics, pharmaceutical, medicinal and cosmetic applications. Therefore, Quantitative analysis for the terpenoid and flavonoid was performed with the mixture of plant extract of Neem *(Azadirachta indica)*, Nirgundi *(Vitex negundo)*, Katupila *(Securinega leucopyrus)*, and Khadir *(Acacia catechu)*prepared in ratio of 4:1:1:1.

3.6.1 Quantification of Terpenoid

Dried plant extract 100mg (initial weight = wi) was taken and soaked in 9mL of ethanol for 24 hours. The extract after filtration, was extracted with 10mL of petroleum ether using a separating funnel. The ether extract was separated and waited for its complete drying (final weight = wf). Ether was evaporated till extract got dried and the yield (%) of total terpenoids contents was measured by the formula.

$$\% \text{ Terpenoid content} = \frac{wi - wf}{wi} \times 100$$

Where, wi= initial weight of extract

wf = final weight of the dried extract after ether treatment.

3.6.2 Quantification of Flavonoid

1g of plant extract was measured and 10 mL of 80% aqueous methanol was added repeatedly. After that, filtration of the solution was performed with the use of Whatman filter. Later on, filtered solution was vaporised and dried out under water bath until it become dried completely and weighed to obtain constant weight comparing to the initial weight. and it is compared using the below given formula.

$$\% \text{ Flavanoid content} = \frac{wf}{wi} \times 100$$

Where, wi= initial weight of extract

wf = final weight of the dried extract after methanol treatment.

3.7 GCMS Analysis

Gas chromatography-mass spectrometry (GCMS) analysis was employed to further elucidate the volatile compounds present in the polyherbal formulation. The analysis was conducted using a gas chromatograph equipped with a mass selective detector. The resulting data were used to identify and characterize the volatile compounds based on their retention time, mass spectra, and comparison with reference libraries.

3.8 *In vitro* Antioxidant Activity

The formulation has been made of different concentrations and various radical scavenging assays viz. DPPH assay, Hydrogen peroxide scavenging assay, reducing power assay, and antioxidant activity by phosphomolybdenum assay were performed.

3.8.1 DPPH radical scavenging assay

The free radical scavenging prospective of various extracts were measured as stated by Kulisic with some variations. An aliquot of 50µL of polyherbal formulations of various concentration were mixed with 0.3mM DPPH in methanolic solution and kept at room temperature for 30 minutes. The absorbance of the reaction mixture was recorded at 518 nm using UV – Visible spectrophotometer. ascorbic acid as a standard and DPPH in methanolic solution was set as blank [62].

DPPH Scavenging activity (%) = $\left(\frac{Absorbance\ of\ standard - Absorbance\ of\ sample}{Absorbance\ of\ standard}\right) \times 100$

3.8.2 Hydrogen peroxide scavenging assay

The potential of the extracts to scavenge H_2O_2 was measured according to the method of Ruch et al. Aliquot of 0.1 mL of polyherbal formulations of various concentration (500,1000,1500,2000 and 2500 µg/mL) was take into test tube and add 0.4 mL with 50mM phosphate buffer (pH7.4) followed by the incorporation of 0.6mL of 2mM H_2O_2 solution. The reaction mixture was vortexed and after 10 minutes of incubation time, absorbance was measured at 230 nm. ascorbic acid as a positive control and blank was determined without polyherbal formulation [112].

H_2O_2 scavenging activity (%) = $\left(\frac{Absorbance\ of\ standard - Absorbance\ of\ sample}{Absorbance\ of\ standard}\right) \times 100$

3.8.3 Reducing power assay

The reducing power was measured The reducing power was determined according to the Oyaizu et al. method with some modifications. Aliquot of 0.2 mL of various concentrations of the extracts were mixed separately with0.5 mL of phosphate buffer (0.2 M, pH 6.6) and 0.5 mL of 1% potassium ferricyanide. The mixture was incubated in a water bath at 50°C for 20 min. After cooling at room temperature, 0.5 mL of 10% trichloroacetic acid was added to it followed by centrifugation a 3,000 rpm for 10 min. Supernatant (0.5 mL) was collected and mixed with 0.5 mL of distilled water. Ferric chloride (0.1 mL of 0.1%) was added to it and the mixture was left at room temperature for 10 min. The absorbance was measured at 700 nm. Ascorbic acid was used as positive control and blank was determined without polyherbal formulation [64].

3.8.4 Anti-oxidant activity by phosphomolydenum assay

The total antioxidant activity was conduct by phosphomolybdenum assay, the method of Prieto et al. was followed. An aliquot of 0.1 mL of sample solution of different concentrations treated with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were incubated at 95°C in a water bath for 90 min. The polyherbal formulation cooled to room temperature and their absorbance was recorded at 765 nm. Ascorbic acid was used as the positive control [62]. Antioxidant capacity was estimated by using following equation:

$$Antioxidant\ activity\ (\%) = \left(\frac{Absorbance\ of\ standard - Absorbance\ of\ sample}{Absorbance\ of\ standard}\right) \times 100$$

3.9 Molecular Biology assay

The mouse skin fibroblast cell line m5S (4×10^4 cells per mL) were cultured using medium α MEM supplemented with fetal bovine serum (10%) and antibacterial cocktail (1%) at room temperature (37 °C), with a continuous supply of CO_2 and with maintenance of 95% humidity. The cell lines were made hyperglycaemic and various assays were performed viz. Cell Proliferation assay by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), Cell Proliferation by Flow Cytometer, Quantification of intracellular reactive oxygen species (ROS) assay, Phagocytosis assay, *in vitro* cell migration assay, Live Cell Migration assay.

3.9.1 Cell Proliferation assay by MTT

This was used for study of cell proliferation. Untreated cells were taken as Control. Cells were seeded in 96 well plates at a density of 1×10^4cells were treated with different doses of formulation for normal cell, for hyperglycaemic cell 0.5µl/mL, 1µl/mL in oil, 150µl/mL. Treated cells were washed with phosphate buffered solution (PBS). 100µl MTT solution was added into each well, and cells were incubated at 370C for 4 hr. The resulting intracellular purple formazon was quantified with a spectrophotometer at an absorbance of 570nm (Multiskan FC, Thermo Fischer Scientific, inc., Pittsburgh, PA, USA)

3.9.2 Quantification of intracellular reactive oxygen species (ROS) assay

Intracellular ROS was quantified by using Oxiselect^{TM} intracellular ROS assay kit (DoJindo, inc, Washington, DC, USA). Cells were seeded on coverslips in 6-well plates at a density of 3×10^5 cells/well. Cells were washed with PBS solution, 25µM DCFH-DA was added to cells 1 hr prior to treatment and incubated at 37°C. After incubation formulation was added on the basis of Cell Proliferation assay (dose dependent) 30µl for normal cell and for hyperglycaemic cell formulation doses were 0.5µl, 1µl and then incubated at 37°C for 2 and 4 hr. After treatment coverslip was removed in separate well washed with PBS solution 1-2 times. The cells were fixed on coverslip with 4%PFA or 70% Slides were mounted and images were captured using a confocal and fluorescence microscope (BZ-9000, Keyence, Osaka, Japan) [112].

3.9.3 Cell Proliferation by Flow Cytometer

Cell Apoptosis was quantified by an Annexin V-FITC apoptosis detection kit (Nacalai Tesque, Tokyo, Japan). Normal and hyperglycaemic cells were seeded in 6-well plate density at – cells/well in the semi confluent phase were treated with varied concentration of formulation for 2 and 4 hr. Cells were washed with PBS solution. Trypsin-EDTA was to for detach the cell from surface. Normal and hyperglycaemic medium was added in normal and hyperglycaemic cell respectively. Annexin binding buffer was added in the cell suspension, and then incubated with Annexin conjugate at room temperature for 15 min in the dark. Stained cells were added to PI (Propidium Iodide) solution before analyzed by Flow cytometry machine (BD Biosciences 7000).

3.9.4 Phagocytosis assay

Phagocytosis was determined using FITC (fluorescein-isothiocyanate) phagocytosis kit (Cayman chemical, Ann Arbor, MI, USA). Hyperglycaemic cells seeded in 12-well plates at a 1×10^4 density of cells/well were treated with formulation and for 2hr and 4hr and LPS was added for 4hr. After LPS treatment, cells were grown in hyperglycaemic DMEM medium for 24hr. After 24 hr, the cells were washed with PBS, and incubated with rabbit I_gG-FITC conjugates latex beads for 3 hr. Nuclei were counterstained with DAPI and fluorescence images were obtained using confocal laser scanning microscope TCS SP8 (Leica, Germany).

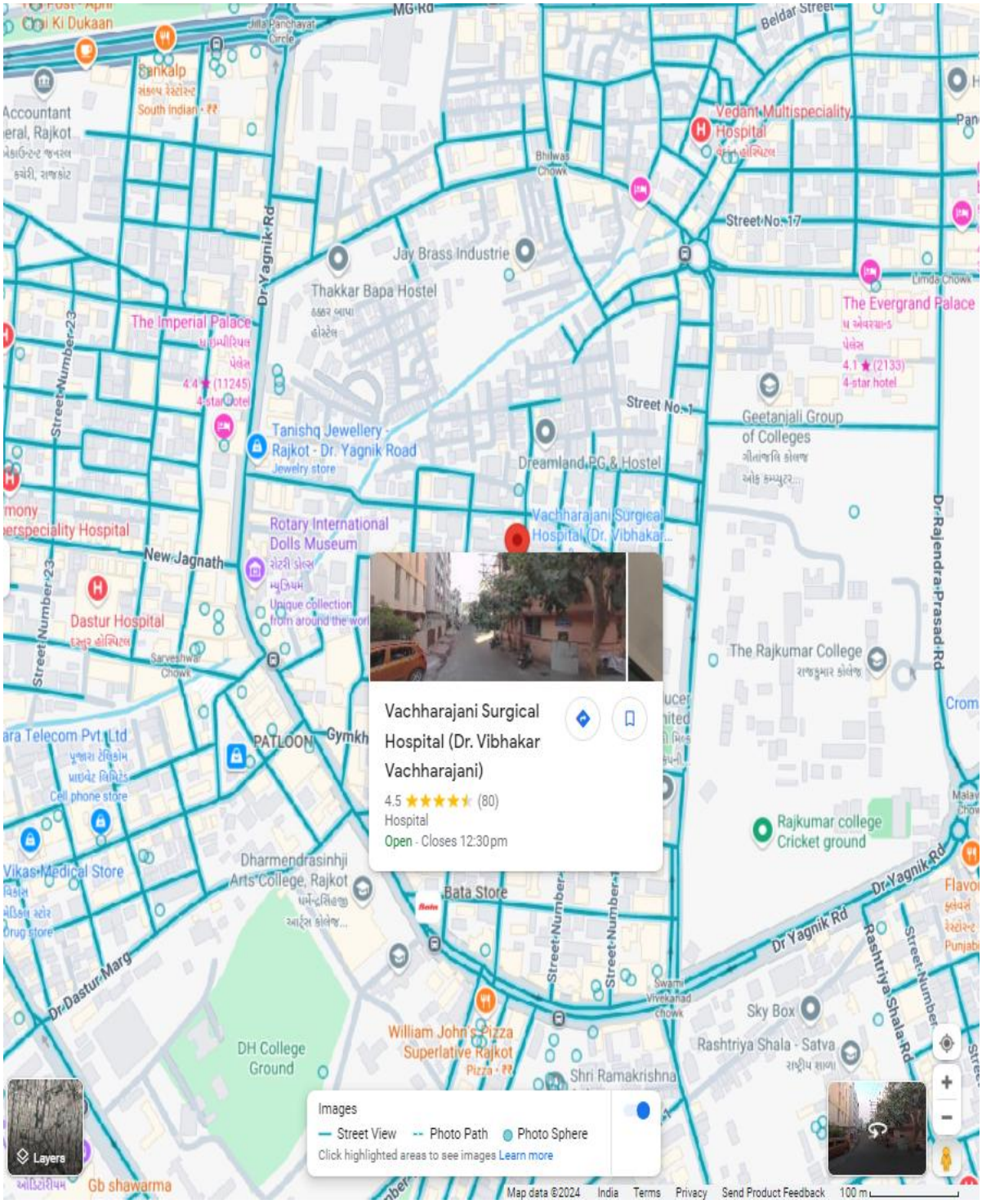
3.9.5 *In vitro* cell migration assay

in vitro wound healing assay determined by using µ-well culture inserts (Ibidi Suppliers, Lochhamer, Grafelfing, Germany). Skin fibroblast cells (M5s) were culture in µ-well with normal medium and glucose medium culture insert to fully confluent stage. Cells were treated with different concentration of formulation and for 2 hr and 4 hr incubated at 37°C. After 2 hr and 4 hr insert were slowly removed without disturbing the edge and cells were fixed with 100% Methanol for 10 min in room temperature. Fixed cells were washed with PBS and stained with 0.5% crystal violet cell staining dye for 5 min. Excess stain was removed by washing continuously with distilled water. Air dry the µ-well in aseptic condition. Photographs taken in (IX71, OLYMPUS, Japan).

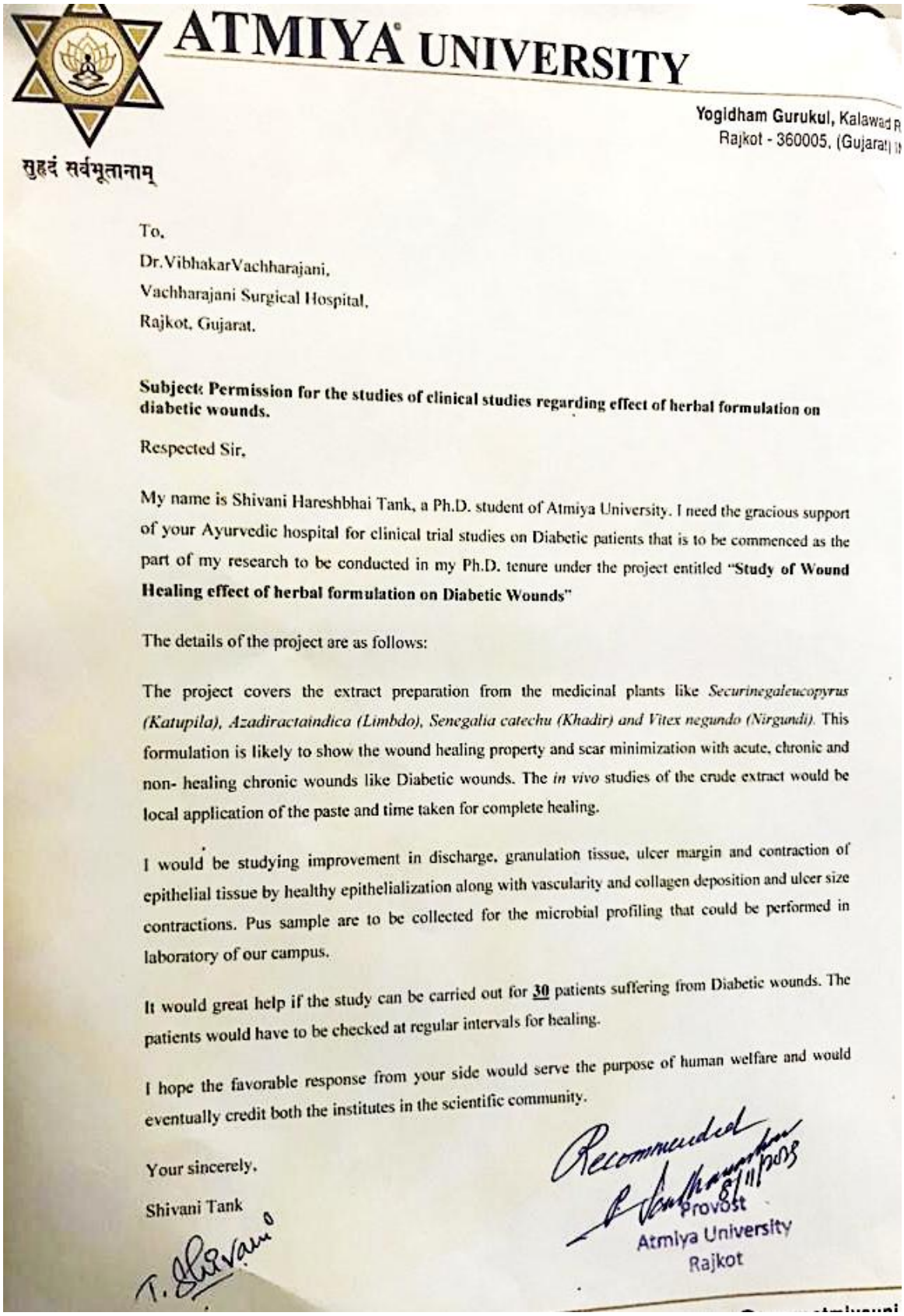
3.10 Clinical Trials

The *in vivo* studies included the clinical trials on Diabetes mellitus patients of *Dr. Vibhakar Vachhrajani Surgical Hospital, behind. Kathiawar Gymkhana, Hemu Gadhvi Marg, Jagnath Plot, Rajkot, Gujarat 360001*. Here, the patients were treated especially with Diabetic Foot Ulcers (DFUs) of various age groups and different diabetes status. However, the medical officers treating the patients applied the polyherbal formulation as dressings. The collaboration was of 30 patients, out of which the complete data of 24 patients were monitored. The official collaboration letter & Ethical Committee certification has been attached in the supplementary data. Consent forms & Clinical Research forms were collected and are stored with the hospital authorities for all of these patients. The patient's population included the gradation of wounds from Grade 0 to Grade 3.

3.10.1 Location:



3.10.2 Collaboration Letter:



ATMIYA UNIVERSITY

Yogidham Gurukul, Kalawad R
Rajkot - 360005. (Gujarat) I

सुहृदं सर्वभूतानाम्

To,
Dr.VibhakarVachharajani,
Vachharajani Surgical Hospital,
Rajkot, Gujarat.

Subject: Permission for the studies of clinical studies regarding effect of herbal formulation on diabetic wounds.

Respected Sir,

My name is Shivani Hareshbhai Tank, a Ph.D. student of Atmiya University. I need the gracious support of your Ayurvedic hospital for clinical trial studies on Diabetic patients that is to be commenced as the part of my research to be conducted in my Ph.D. tenure under the project entitled "**Study of Wound Healing effect of herbal formulation on Diabetic Wounds**"

The details of the project are as follows:

The project covers the extract preparation from the medicinal plants like *Securinegaleucopyrus (Katupila), Azadiractaindica (Limbdo), Senegalia catechu (Khadir) and Vitex negundo (Nirgundi).* This formulation is likely to show the wound healing property and scar minimization with acute, chronic and non- healing chronic wounds like Diabetic wounds. The *in vivo* studies of the crude extract would be local application of the paste and time taken for complete healing.

I would be studying improvement in discharge, granulation tissue, ulcer margin and contraction of epithelial tissue by healthy epithelialization along with vascularity and collagen deposition and ulcer size contractions. Pus sample are to be collected for the microbial profiling that could be performed in laboratory of our campus.

It would great help if the study can be carried out for **30** patients suffering from Diabetic wounds. The patients would have to be checked at regular intervals for healing.

I hope the favorable response from your side would serve the purpose of human welfare and would eventually credit both the institutes in the scientific community.

Your sincerely,

Shivani Tank

Recommended
8/11/2018
Provost
Atmiya University
Rajkot

3.10.3 Clinical Research form:

CLINICAL RESEARCH FORM

"Study on effects of crude extracts of Polyherbal formulation in wound healing"

Principal Investigator: Shivani Tank

Name of the patients:	Age : ☐ years
Address :	Sex : Male ☐ Female ☐
	OPD No: ☐ IPD No: ☐
Tel:	Blood Group:
Religion: H ☐ M ☐ Ch ☐ J ☐	Any Important Information
Marital Status: M ☐ UM ☐ D ☐ W ☐	
Education : Literate ☐ Illiterate ☐	Date of Sample Collection:
	Date of Admission:
Socio-eco. Status : Poor ☐ Middle class ☐ Rich ☐	

CHIEF COMPLAINTS:

1) **VRANA (Wound)**

Avadhi (Duration)	Days :	Month:	Years:
Prarambha (Onset)	Traumatic:	Recurrent:	Chronic"
Vrana Sankhya	Single:	Multiple No :	
Any Other:			

2) **SRAVA (Discharge)** Present: Absent:

Varna (colour):	Salilpraksha:		Peetavbhasa:
	Sarpipraksha:		Rakta:
	Puya:		Shleshma:
Consistency	Ghana:		Thanu:
Pramana	Mild	Moderate	Profuse

3) **VEDANA (Pain)** Present: Absent:

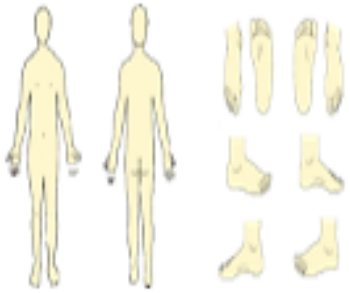
	Character	Toda	Daha	
	Periodicity	Continuous	Intermittent	Occasional
	Intensity	Mild	Moderate	Severe

4) **GANDHA (Odour)** Present Absent

5) **KANDU (Itching sensation)** Present Absent

6) **JVARA (Fever)** Present Absent

Location of Wound:



GENERAL EXAMINATION:

Pulse	Regular	Irregular
B.P	...../.....mmHg	
Weight	.....kg	
Height	.....cm	

□

LOCAL EXAMINATION

Site of wound -
Shape & size -
Edge -
Base -
Floor -
Margins -
Swelling -
Discharge -
Odour -
Colour of surrounding skin
Pain -

HISTORY OF PAST ILLNESS:

History of similar complaints	:Yes	□	No	□
Diabetes	:Yes	□	No	□
Thyroid disease	:Yes	□	No	□
Hypertension	:Yes	□	No	□
History of any surgery	:Yes	□	No	□

FAMILY HISTORY:

History of similar complaints	Yes □	No □
Tuberculosis	Yes □	No □
Diabetes	Yes □	No □
Thyroid disease	Yes □	No □
Hypertension	Yes □	No □

TREATMET HISTORY: Yes □ No □

SYSTEMIC EXAMINATION:

Cardio Vascular System .....

Respiratory System.....

Nervous System.....

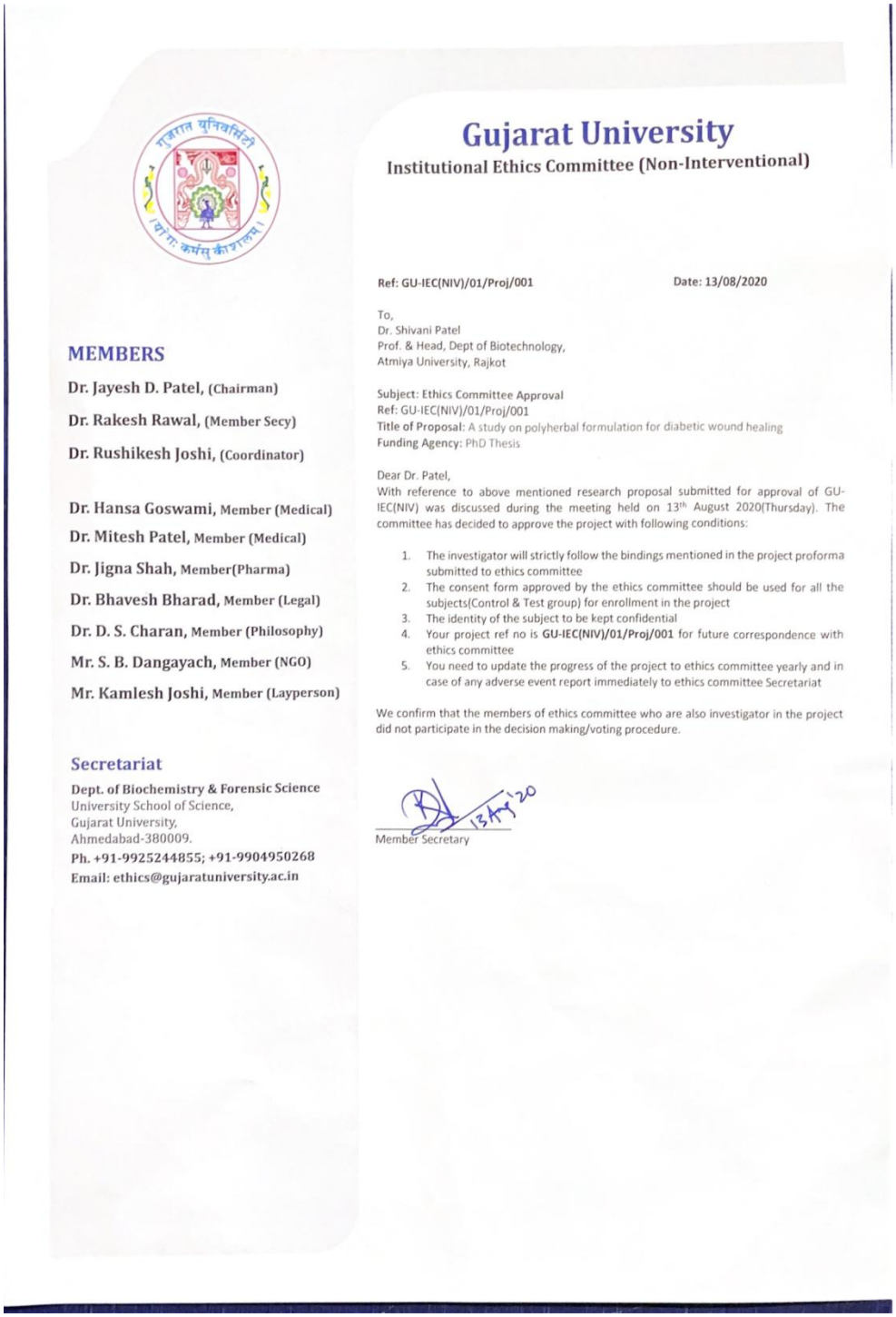
Gastro Intestinal System.....

Urinary System.....

Gradations of Assessment of result:
VAS score

Criteria	Gradations			
	0	1	2	3
Colour of surrounding Skin (Varna)	NO Marginal induration & Normal Pigmentation surrounding skin	Mild Marginal induration & Normal Pigmentation surrounding skin	Moderate Marginal induration & Moderate Pigmentation surrounding skin	Severe Marginal induration & Severe Pigmentation surrounding skin
Discharge (Srava)	NO discharge	Serous (Yellow/Clear) discharge	Sero-purulent discharge	Purulent (Yellow/Green/Brown)
Pain (vedana)	No Pain	Pain on dressing change	Intermittent pain	Continuous pain
Odour (Gandha)	No odour	Mal odour	----	----
Swelling (Shotha)	Swelling absent	Swelling present	----	----
Itching (Kandu)	Itching absent	Itching present	----	----
Fever (Jwara)	Afebrile	Febrile	----	----

3.10.4 Ethical Committee Certificate:



Gujarat University

Institutional Ethics Committee (Non-Interventional)

MEMBERS

Dr. Jayesh D. Patel, (Chairman)

Dr. Rakesh Rawal, (Member Secy)

Dr. Rushikesh Joshi, (Coordinator)

Dr. Hansa Goswami, Member (Medical)

Dr. Mitesh Patel, Member (Medical)

Dr. Jigna Shah, Member(Pharma)

Dr. Bhavesh Bharad, Member (Legal)

Dr. D. S. Charan, Member (Philosophy)

Mr. S. B. Dangayach, Member (NGO)

Mr. Kamlesh Joshi, Member (Layperson)

Secretariat

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Ph. +91-9925244855; +91-9904950268
Email: ethics@gujaratuniversity.ac.in

Ref: GU-IEC(NIV)/01/Proj/001 **Date: 13/08/2020**

To,
Dr. Shivani Patel
Prof. & Head, Dept of Biotechnology,
Atmiya University, Rajkot

Subject: Ethics Committee Approval
Ref: GU-IEC(NIV)/01/Proj/001
Title of Proposal: A study on polyherbal formulation for diabetic wound healing
Funding Agency: PhD Thesis

Dear Dr. Patel,
With reference to above mentioned research proposal submitted for approval of GU-IEC(NIV) was discussed during the meeting held on 13th August 2020(Thursday). The committee has decided to approve the project with following conditions:

1. The investigator will strictly follow the bindings mentioned in the project proforma submitted to ethics committee
2. The consent form approved by the ethics committee should be used for all the subjects(Control & Test group) for enrollment in the project
3. The identity of the subject to be kept confidential
4. Your project ref no is **GU-IEC(NIV)/01/Proj/001** for future correspondence with ethics committee
5. You need to update the progress of the project to ethics committee yearly and in case of any adverse event report immediately to ethics committee Secretariat

We confirm that the members of ethics committee who are also investigator in the project did not participate in the decision making/voting procedure.

13/8/'20
Member Secretary

3.11 Microbial Profiling from the purulent samples

The microbial profiling was obtained by swirling with Sterile Cotton Swab in screw capped polypropylene tube, Cotton bud with polypropylene stick (size 75 mm, packed in 12 mm diameter tube) onto the purulent wounds of Diabetic patients. The swab samples were streaked onto the Nutrient Agar, Luria Bertani agar, Mac-Conkey agar and Blood agar plates and incubated for 24- 48hrs period. The preliminary biochemical analysis might help the microbes to grow preferentially on selective and differential media. The colony characterization and biochemical characterization were carried out with the standard protocols and Bergey's Manual was taken as reference for the preliminary identification of the genera of the 38 isolates and their preservation was carried out.

3.11.1 Gram's staining and Pigmentation

The Gram's staining was performed by standard method. The loopful culture was heat fixed on a clean slide. Then 2-3 drops of crystal violet was poured, followed by flooding the slide with the gram's iodine. Washed with ethanol until decolourization was achieved. Washed under running water. Air dried. Counter stained with safranine for 1 min and washed under running water. A small drop of paraffin oil was added and observed under 100X lens. Out of 38 isolates, 3 were pigmented and rest 35 showed cream to whitish colonies.

3.11.2 Biochemical Characterisation

The preliminary analysis was followed certain biochemical characterisation that included IMViC test, sugar utilisation tests, Triple sugar iron test, Catalase test, 1% peptOne etc. Bergey's manual was referred for preliminary identification.

3.11.2.1 Indole Test

This test determines the ability of bacteria to produce indole from tryptophan using the enzyme tryptophanase. Bacteria are incubated in a tryptophan broth, and after 24 hours, Kovac's reagent is added. A red ring indicates a positive result (Kovac, 1928).

3.11.2.2. Methyl Red Test

Developed by Clark and Lubs in 1915, the methyl red test identifies bacteria that produce stable acid end-products from glucose fermentation. After 48 hours of incubation in MR-VP broth, methyl red indicator is added. A red color indicates a positive result, showing stable acid production.

3.11.2.3 Voges-Proskauer Test

Created by Voges and Proskauer in 1898, this test detects acetoin, a neutral fermentation product. Bacteria are grown in MR-VP broth, and Barritt's reagent (α-naphthol and KOH) is added. A red color indicates a positive result.

3.11.2.4 Citrate Utilization Test

Developed by Simmons in 1926, this test identifies bacteria that can use citrate as a carbon source. Bacteria are grown on Simmons citrate agar; a color change from green to blue indicates a positive result, signifying citrate utilization.

3.11.2.5 Catalase Test

This test, introduced by Loew in 1901, detects the presence of catalase, an enzyme that decomposes hydrogen peroxide into water and oxygen. A positive test results in the production of bubbles when hydrogen peroxide is added to the bacterial colony.

3.11.2.6 *1%* PeptOne Water

PeptOne water is a nutrient broth with 1% peptOne, used to grow bacteria for various biochemical tests. It serves as a basal medium to observe microbial growth.

3.11.2.7 Triple Sugar Iron Test (TSI)

Described by Sulkin and Willett in 1940, TSI tests the ability of bacteria to ferment sugars (glucose, lactose, sucrose and mannitol) and produce hydrogen sulfide. A color change in the slant and butt of the medium indicates sugar fermentation and gas or H_2S production.

3.11.2.8 Sugar Utilization Tests

These tests evaluate the ability of bacteria to ferment specific sugar, producing acid and/or gas. Phenol red broth is commonly used, with a color change indicating acid production (Durham, 1891).

3.12 Determination of Minimum inhibitory Concentration (MIC)

Minimum inhibitory Concentration (MIC) refers to the lowest concentration of an antimicrobial agent that inhibits visible growth of a microorganism after a specified period of incubation. It is a critical parameter used in microbiology to evaluate the effectiveness of antibiotics, antifungals, or other antimicrobial agents against bacteria, fungi, or other pathogens. The **Broth Dilution Method** is One of the most widely used techniques for determining MIC. It involves diluting an antimicrobial agent in a liquid growth medium (broth) and then inoculating it with a standardized concentration of the test organism. The isolates were inoculated in a nutrient broth and incubate overnight at 35-37°C. The turbidity of the bacterial suspensions was adjusted to match the 0.5 McFarland standards (approximately 1-2 x 10^8 CFU/mL). A serial two-fold dilutions of the antimicrobial agent was prepared in Mueller-Hinton broth. The final concentrations would cover a range that includes expected MIC values (e.g., 1 mg/mL to 0.001 mg/mL). for microdilution, 100 µl of each antimicrobial dilution was added to the corresponding wells of a 96-well plate. The 5 µl of the bacterial suspension was added to each well, achieving a final bacterial concentration of approximately 5 x 10^5 CFU/mL. The plate was incubated at 35-37°C for 18-24 hours. After incubation, visually the wells were monitored for bacterial growth (turbidity). The lowest concentration of the antimicrobial agent that completely inhibits visible growth is considered the MIC. To ensure, spectrophotometer was used to measure the optical density (OD) at 600 nm so as to confirm the absence of growth in wells with inhibited bacterial activity. Positive and negative controls were kept and the standard antibiotic viz. Ampicillin and Gentamicin were employed in concentration range of 1 mg/mL to 0.001 mg/mL.

3.13 Anti- Biofilm assay

One of the commonly used method is Crystal violet staining employing micro-titre plate, for qualitative and quantitative methods to study biofilm formation and efficacy studies of test samples. Amidst the microbial research community, crystal violet remains the best known primary stain from Gram's staining to biofilm research as it is cost effective and easy to handle. Researchers rely on the use of CV staining to quantify adherent biofilm biomass within the wells of a microtiter plate. The biofilm inhibition procedure uses solely the readout of CV staining, whereas the biofilm eradication procedure builds on the CV staining of biofilms by incorporating a metabolic stain into the protocol to monitor metabolic activity of biofilms in the presence of antibiotics, host defence peptides (HDPs) or other potential antibiofilm agents. Biofilm inhibition is by adding a compound of interest at the same time as a freshly prepared bacterial suspension and then incubating at a predetermined time followed by quantification of adhered biomass by CV staining. The biofilm inhibition capacity of a peptide or antimicrobial can then be assessed by examining the dose–response curve of CV staining as a function of concentration. Biofilm eradication is more nuanced and requires companion assays to help elucidate the manner of 'eradication' (i.e., dispersal or killing). The eradication assay begins by preestablishing consistent biofilms in the wells of a 96-well plate and incubating the plates overnight. The following day, the adhered biofilms are rinsed then treated with the compound of interest.

Chapter 4

Results & Discussion