SYNOPSIS
Of The Thesis Entitled

“SCREENING OF SELECTED MEDICINAL PLANTS FOR
WOUND HEALING AND ANTIAGING ACTIVITY”

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Research Guide

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INTRODUCTION

Optimum healing of a cutaneous wound requires a well-orchestrated integration of the complex biological and molecular events of cell migration and proliferation and of extracellular matrix deposition and remodeling. Wound infections are most common in developing countries because of poor hygienic conditions. *Staphylococcus aureus, Streptococcus pyogenes, Corynebacterium spp., Escherichia coli*, and *Pseudomonas aeruginosa* are some pathogenic organisms causing wound infection. Wound care and maintenance involves number of measures including dressing and administration of painkillers, use of anti-inflammatory agents, topical systemic antimicrobial agents, and healing promoting drugs. Wound healing studies mainly aim to detect various means and factors influencing healing process, so they could be either used or avoided in clinical practice to favorably alter the healing process.

Skin aging goes along with numerous functional and structural changes. Aging of the skin is the result of continuous “wear and tear” processes. Aging is driven by intrinsic and extrinsic factors. Recent advances in molecular biology and cellular immunology have allowed us a better understanding of the aging process. A number of morphologic changes, especially in collagen and elastin fibers have been demonstrated including depletion of dermal extracellular matrix components like collagen and hyaluronic acid (HA). Because collagen and elastin are responsible for the strength and resiliency of skin, their disarrangement with aging causes skin to appear aged.

Chronological skin aging is a universal and inevitable process while in contrast, photoaging results from the UV rays of sunlight, and the damage become apparent in sun-exposed skin.

Conventional synthetic drug therapies available for wound repair are effective but it may lose their effectiveness because of many adverse drug reactions. Instead of relying upon medical skin care products in general, these days, people are increasingly depending on herbs and herbal remedies. Current researches are devoted to validating their efficacy and to uncover the mechanisms responsible for this activity. Some of these plants owe their effects to direct effect on the wound healing and skin aging processes and some to their antioxidant, anti-inflammatory and anti-microbial effects.

Thus considering the above scenario medicinal plants with potential effect in wound healing and anti-aging were explored, evaluated and established.
In this context the medicinal plants proposed are-

*Rosa damascena* belonging to the Family Rosaceae, is a perennial shrub indigenous to Europe and Middle East countries, Iran, Turkey and is cultivated in India as a commercial crop. The damask rose oil plays an important role in high class perfumes and cosmetics. Its flowers are reported to have astringent, analgesic, anti-inflammatory, antidepressant, antibacterial, diuretic and anti-HIV activity and used in folk medicine as a mild laxative. Petals are the source of attar of roses and rose water used in flavouring drinks, sweets, baked goods, etc.\(^6\)

*Mimusops elengi* Linn. belongs to the Family Sapotaceae. It is an evergreen tree, 5-8 m tall and is cultivated throughout our country as an ornamental tree. The bark is used as a gargle for odontopathy. It has been used in the indigenous system of medicine for the treatment of various ailments. Several therapeutic uses as cardiotonic, stomachic, anthelmintic and astringent have been ascribed to the bark of *Mimusops elengi*. It has been reported as dantarogahara (treats and prevent tooth decay and tooth disease) in Ayurveda. A decoction of the bark is used as a gargle in salivation in weak and spongy gums, pyorrhea, stomatitis and ulcerated throat. Compound powder made of the bark is recommended as tooth powder in cases of spongy gums.\(^7\)

**RATIONALE OF THE PROJECT:**

Skin aging is a complex phenomenon and the most common amongst the visible signs of aging are wrinkles, pigmentation, dryness. As a consequence of aging, skin tissue cell regeneration capacity declines and the connective tissue (elastin, collagen, extra-cellular matrix) dwindles. The skin moisture levels, visco-elasticity and mechanical strength also decrease. Phenomena of aging provokes decline in defense, healing perception mechanisms and in thermoregulation of the skin tissue.

In excision and incision wound healing animal models, parameters studied are wound contraction, hydroxyproline (collagen) content and skin breaking strength which in turn is indicative of the aging parameters like tissue cell regeneration capacity, collagenation capacity and mechanical strength of skin. Hence incision and excision models can be used as a preliminary efficacy evaluation of anti-aging activity.\(^8\)
The screening of herbal extracts has been of great interest for the discovery of new effective drugs for wound and skin care.

So the rationale of the present study is to identify bioactive extracts of selected plants having the above mentioned effects in wound healing and antiaging.

AIMS AND OBJECTIVES

The research work aims at detailed and systematic phytochemical and pharmacological evaluation of various extracts of *Mimusops elengi* fruit and bark and *Rosa damascena* flower petals in Wound healing and Anti aging.

The objectives are as follows:

1. Screening of plant extracts for treatment in wound healing
2. Development of suitable dosage forms (topical formulations) containing bioactive extract/s.
3. Screening of topical formulations for treatment in wound healing
5. Evaluation of optimized topical formulation for the treatment in skin aging
6. Standardization of developed formulation/s containing bioactive extract/s using HPLC.

PLAN OF WORK

1. Literature survey and review of literature
2. Selection, Procurement and Authentication of plant materials and its standardization
3. Preparation of plant extracts
4. Phytochemical screening of plant extracts using qualitative tests
5. Screening of extracts by *in vitro* antimicrobial assays:
   a. Ditch Plate method
   b. Cup Plate method
6. Screening of extracts by *in vitro* antioxidant assays:
   a. DPPH free radical scavenging activity
   b. Nitric oxide reducing activity
7. Evaluation of *in vivo* wound healing activity of bioactive extracts by using following animal models:
   a. Excision wound model
   b. Incision wound model
   c. Dead space wound model

8. Phytochemical analysis of bioactive extracts
   a. Total phenolic content
   b. Total flavanoid content

9. Formulation and evaluation of suitable topical dosage formulation/s containing bioactive extract/s.

10. Primary skin irritation studies of topical formulation/s.

11. Evaluation of *in vivo* wound healing activity of the developed formulation/s by using following animal models:
   a. Excision wound model
   b. Incision wound model
   c. Dead space wound model

12. Evaluation of *in vivo* wound healing activity of the developed formulation/s in experimentally induced diabetic rats by using following animal models:
   a. Excision wound model
   b. Incision wound model
   c. Dead space wound model

13. Evaluation of *in-vitro* wound healing activity of optimized topical formulation by:
   a. Scratch assay

14. Evaluation of *in vitro* skin anti-aging activity of optimized formulation by using following methodology:
   a. Anti-Collagenase activity
   b. Anti-Tyrosinase activity.
   c. Anti-Hyaluronidase activity
   d. Anti-Elastase activity

15. Standardization of developed optimized formulation using bioactive marker/s.
RESEARCH METHODOLOGY

1. Literature survey and review of literature:

Extensive literature search on traditional plants used in wound healing and antiaging, extraction methods of plant materials, animal models of wound healing, phytochemical and analytical techniques and various formulation development techniques was done.

2. Selection, Procurement and Authentication of plant materials and its standardization

a. Selection, Procurement and Authentication of plant materials:

Based on the literature search, *Mimusops elengi* fruit and bark (Family-Sapotaceae) and *Rosa damascena* flower petals (family- Rosaceae) were chosen for the study. The authenticated plant materials were procured from Amsar Pvt Ltd. The samples were powdered and stored in air tight container and used for further study.

b. Standardization of plant materials:

Standardization of plant materials were carried out using following parameters:

1. Total ash, Acid insoluble and Water soluble ash values
2. Loss on drying

3. Preparation of plant extracts:

Extracts of the plant materials were prepared using various solvents such as methanol, water, ethyl acetate and petroleum ether (60-80).

Extraction of *Mimusops elengi* fruit and bark and *Rosa damascena* flower petals was carried out using refluxation and soxhlet extraction method.

4. Phytochemical screening of plant extracts using qualitative tests:

Qualitative evaluation of the plant extracts was carried out to determine the presence/absence of phytoconstituents present in the extracts using qualitative tests.

5. Screening of extracts by *in vitro* antimicrobial assays:

The plant extracts were screened for antimicrobial activity by following *in vitro* techniques:
a. Ditch plate method: Primary screening of extracts against various wound pathogens was carried out using ditch plate method. The test organisms were as follows;
Gram positive microorganisms: *Staphylococcus aureus*, *Streptococcus pyogens* and *Clostridium perfringens*
Gram negative microorganisms: *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia* and *Klebsiella aerogens*
Fungi: *Candida albicans* and *Aspergillus niger*

b. Cup plate method: Bioactive extracts obtained from ditch plate method were tested at various concentrations against the above mentioned microorganisms.¹⁰

6. Screening of extracts by *in vitro* antioxidant assays:
The plant extracts were screened for antioxidant activity by following *in vitro* assays:

a. DPPH (1, 1, diphenyl 2-picryl hydrazyl) assay:
The free radical scavenging activity of the extracts was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH spectrophotometrically at 517 nm. Decreasing the absorbance of the DPPH solution indicates an increase in DPPH radical scavenging activity.

b. Nitric oxide reducing activity assay:
The Nitric oxide radical scavenging activity of the extracts was measured in terms generation of nitric oxide from sodium nitroprusside which interacts with oxygen to produce nitrite ions that were estimated by use of Greiss reagent.¹¹

7. Evaluation of *in vivo* wound healing activity of bioactive extracts by using following animal models:
*In-vivo* wound healing activity is done by excision, incision and dead space model and the parameters associated with wound healing are measured. In excision model wound contraction and period of epithelization are studied. In Incision model, tensile strength of wounds is measured. In dead space model, formation of granulation tissue is determined.
i. **Excision Wound model:** Excision wound was made by excising the full thickness circular skin on the back of the animal. The bioactive extracts were applied on the open wound daily till complete epithelization. The parameters studied were wound contraction and period of epithelization.

ii. **Incision Wound model:** A longitudinal paravertebral incision was made through the skin on the back. The parted skin was sutured using a surgical thread and curved needle. Bioactive extracts were applied topically. The sutures will be removed on 8th post wounding day. Measurement of tensile strength and histopathological evaluation of the incised skin was done.

iii. **Dead space Wound model:** Dead space wounds were inflicted by implanting two sterilized cotton pellets (10 mg), one on either side of in the lumbar region on the ventral surface of each rat. On the 10th post wounding day, the granulation tissue formed on the implanted cotton pellet were assessed for hydroxyproline content, hexosamine content and weight of dry and wet granulation tissue.\(^\text{12}\)

8. **Phytochemical analysis of bioactive extracts\(^\text{13,14}\)**

Quantitative analysis of bioactive extracts were carried out using total phenolic and total flavanoid content method.

9. **Formulation and evaluation of suitable topical dosage formulation/s containing bioactive extract/s.**

   **Formulation I – Cream:** Topical cream containing methanol extracts of *Mimusops elengi* bark and *Rosa damascena* flower petals at varying concentrations was prepared. The formulations will be evaluated for their pH, viscosity, spreadabiltiy, drug content and stability studies as per ICH guidelines.

   **Formulation II – Gel**

Topical gel containing methanol extracts of *Mimusops elengi* bark and *Rosa damascena* flower petals at varying concentrations was prepared. The formulations were evaluated for their pH, viscosity, spreadabiltiy, drug content and stability studies as per ICH guidelines.
10. Primary skin irritation studies of topical formulation/s.

0.5gm of test and blank formulations will be applied on the dorsal side of shaved skin of albino rats and will be covered with cotton bandage. All animals should be examined for signs of erythema and oedema, and the responses scored at 60 minutes, and then at 24, 48 and 72 hours after patch removal. The reaction if any will be graded as: 0- No reaction, 1- Slightly, patchy erythema, 3- Slight but confluent or moderate but patchy erythema, 4- Moderate erythema, 5- Severe erythema (beef redness) to eschar formation.15

11. Evaluation of *in vivo* wound healing activity of developed formulation/s

The creams and gels prepared were then evaluated for wound healing activity by using following animal models:

a. Excision wound model
b. Incision wound model
c. Dead space wound model

12. Evaluation of *in vivo* wound healing activity of optimized cream and gel formulation/s on experimentally induced diabetic rats

Diabetic wounds are slow, non-healing wounds that can persist for weeks despite adequate and appropriate care. Such wounds are difficult to heal and tough to manage. Such conditions specially require the use of agents, which can facilitate healing. Very few synthetic agents are available for treatment of diabetic wounds and for other chronic wounds, but these are not very effective and have many side effects. Use of plant extracts can be the best alternative as they contain many phytoconstituents having diverse pharmacological activities which many times are complimentary to each other.

**Procedure:**

- Induction of Diabetes:
  Animals were fasted overnight. The animals were then injected with single dose of Alloxan monohydrate (120mg/kg) intraperitonealy (i.p) for induction of diabetes. The fasting blood glucose level was determined after 72 h of alloxan injection. The rats having serum blood glucose levels above 200 mg/dl were considered as diabetic and were selected for the study.
The wound healing activity is evaluated by using following animal models:
  a. Excision wound model
  b. Incision wound model
  c. Dead space wound model

From the results of \textit{in-vivo} wound healing activity, the optimized formulation was further evaluated for its \textit{in-vitro} wound healing and anti-aging potential.


\textbf{a. Scratch assay:} Skin fibroblast proliferation is very important in tissue repair as fibroblasts are involved in migration, proliferation, contractions and collagen production. The ability to stimulate fibroblast cell growth is a useful model for testing wound healing activity \textit{in vitro}.

Procedure: Using a sterile, Disposable scratch loop, three separate wounds were scratched through the cells. The test formulation was added to the scratch. Cell Migration in the induced wound was observed for 72 hours. The observations of the test formulation were compared with the PBS control on the basis of cell migration rate\textsuperscript{17}.

14. Evaluation of \textit{in-vitro} skin anti-aging activity of optimized topical (gel G2) formulation:

Connective tissues, particularly skin extra-cellular matrix (ECM), undergo significant alterations during aging. ECM is made up of fibrous matrix proteins like collagen, elastin, hyaluronic acid and tyrosine which are important for maintaining skin’s elasticity, resilience, moisture and melanin content. These connective tissue proteins are constantly attacked by by several enzymes like collagenases, elastases, tyrosinase and hyaluronidase which leads to decrease in thickness of skin and it becomes dry and wrinkled. The ability of to inhibit these enzymes is useful for testing anti-aging activity \textit{in-vitro}\textsuperscript{18}.

\textbf{a. Anti-Tyrosinase Activity:}

Test formulation (G2 gel) was incubated with enzyme (tyrosinase) and substrate (tyrosine) in phosphate buffer. The test tubes were placed in an ice bath to terminate the reaction.
amount of DOPA-quinone released was determined by measuring the absorbance at 475 nm. Higher the absorbance, more is the inhibitory effect of the test sample.

b. Anti-Elastase Activity:
The test sample was added to the buffer. The enzyme was added and the reaction was initiated by adding substrate. Each test reaction was incubated and the absorbance was read at 410 nm. Higher the absorbance, more is the inhibitory effect of the test sample.

c. Anti-Hyaluronidase Activity:
Bovine hyaluronidase was mixed with of various concentrations of test formulation G2. Hyaluronidase was activated by adding calcium chloride in reaction mixture and incubated. The Ca$^{2+}$ activated hyaluronidase was subjected to sodium hyaluronate and then incubated. Reaction mixture was allowed to cool to room temperature, p-Dimethyl amino benzaldehyde was added to the reaction mixture and it was then incubated. The absorbance was measured at 585 nm. Higher the absorbance, more is the inhibitory effect of the test sample.

d. Anti-Collagenase Activity:
Bovine collagen was mixed with TES buffer containing the test formulation (G2) and incubated. Start the reaction by adding 0.1 ml of enzyme dilution to appropriate tubes. The collagenase reaction was stopped by adding ninhydrin-citric acid mixture. The absorbance were read 600 nm. Higher the absorbance, more is the inhibitory effect of the test sample.

15. Analytical method development and validation:
A HPLC Method for detection of betulinic acid in methanol extract of *Mimusops elengi* bark, quercetin and kaempferol in methanol extract of *Rosa damascena* flower petals was developed. The same method was used for standardization of the formulation containing methanol extracts of *Mimusops elengi* bark and *Rosa damascena* flower petals.

ii. A simultaneous HPLC Method for detection of betulinic acid, quercetin and kaempferol was developed using above standard biomarkers. The same method was used for
standardization of the formulation containing methanol extract of both *Mimusops elengi* bark and *Rosa damascena* flower petals.

The developed HPLC methods were validated using various parameters like linearity, repeatability, accuracy, precision, robustness, limit of detection (LOD) and limit of quantification (LOQ) as per ICH guidelines.
REFERENCES:


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