DEVELOPMENT AND EVALUATION OF POLY HERBAL OINTMENT FOR WOUND HEALING ACTIVITY

A Dissertation Submitted to

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSITY

CHENNAI- 600032

In partial fulfillment of the requirements for the award of the Degree of

MASTER OF PHARMACY

IN

PHARMACOLOGY

Submitted by

L. KOUSALYA

(REG NO: 261725808)

Under the guidance of

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Head, Department of Pharmacognosy

KMCH COLLEGE OF PHARMACY

KOVAI ESTATE, KALAPPATTI ROAD,

COIMBATORE-641 048

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CERTIFICATE

This is to certify that the dissertation work entitled "**DEVELOPMENT AND EVALUATION OF POLY HERBAL OINTMENT FOR WOUND HEALING ACTIVITY"** was carried out by **Ms. L. KOUSALYA** (**Reg No: 261725808).** The work mentioned in the dissertation was carried out at the **Department of Pharmacology**, **KMCH College of Pharmacy, Coimbatore, Tamil Nadu,** under the guidance of **Dr. N. ADHIRAJAN, M.Pharm., Ph.D.,** for the partial fulfilment for the Degree of Master of Pharmacy during the academic year 2018-2019.

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Date: **Dr. N. ADHIRAJAN, M. Pharm., Ph.D.,**

Place: Coimbatore

DECLARATION

I hereby declare that the dissertation work entitled **"DEVELOPMENT AND EVALUATION OF POLY HERBAL OINTMENT FOR WOUND HEALING ACTIVITY"** submitted to The Tamil Nadu Dr. M.G.R. Medical University, Chennai, in partial fulfillment for the Degree of **Master of Pharmacy** in **pharmacology** was carried out under the guidance of **Dr. N. ADHIRAJAN, M.Pharm., Ph.D.,** at the **Department of Pharmacology**, KMCH College of Pharmacy, Coimbatore, Tamil Nadu during the academic year 2018-2019.

This research work either in part or full does not constitute any of other thesis / dissertation.

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EVALUATION CERTIFICATE

This is to certify that the research work entitled "**DEVELOPMENT AND EVALUATION OF POLY HERBAL OINTMENT FOR WOUND HEALING ACTIVITY**" submitted by **Ms. L. KOUSALYA (Reg.No: 261725808)** to the Tamil Nadu Dr. M.G.R. Medical University, Chennai, in the partial fulfilment for the Degree of **Master of Pharmacy** at the **Department of pharmacology**, is a bonafide work carried out by the candidate at KMCH College of Pharmacy, Coimbatore, Tamil Nadu during the academic year 2018-2019 and the same was evaluated.

Examination Centre: KMCH College of Pharmacy, Coimbatore

Date:

Internal Examiner **External Examiner**

Convener of Examination

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L. KOUSALYA
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Dedicated to Almighty,

My Beloved parents,

friends, Teaching and Non

Teaching Staffs…

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L. KOUSALYA
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ABBREVIATIONS

ABBREVIATIONS	FULL FORM
WHO	World Health Organization
IFN-γ	Interferon Gamma
NSAIDs	Non Steroid Anti-Inflammatory Drugs
DPPH	1, 1-diphenyl-2-picrylhydrazyl
GAE	Gallic acid Equivalent
ANOVA	Analysis of Variance
TGF	Transforming Growth Factor
VEGF	Vascular Endothelial Growth Factor
PDGF	Platelet Derived Growth Factor
MEPHF	Methanoloic Extract of Poly Herbal Formulation
μg	Microgram
μl	Microliter
HPTLC	High Performance Thin Layer Chromatography
mm	Millimeter
Rf	Retention Factor

ABSTRACT

Wound is a physical trauma where the skin is torn, cut or punctured. Several drugs obtained from plant sources are known to increase the healing of different types of wounds. The present study was aimed to develop a herbal formulation using herbs which has been proved to enhance the cell proliferation, cell migration and alleviate the inflammation and infection thus hasten the healing process. The selected plants for the present study were the leaves of *Tridax procumbens, Azadirachta indica and Aloe vera, rhizomes of Curcuma longa.* The plants were collected, authenticated, dried and powdered. The powders of each crude drugs were mixed in equal parts and subjected to soxhlet extraction using methanol as solvent. The obtained extract MEPH was evaluated for their *in vitro* antioxidant, antimicrobial and total phenol and flavonoid content. Poly herbal ointment was prepared using MEPH in different ratio with simple ointment base. The formulations were evaluated for its physiochemical properties and the selected poly herbal formulation was subjected to skin irritation and in vivo excision wound healing activity in albino wistar rats. The wound healing activity was compared with standard herbal ointment available in the market. The Phytochemical analysis revealed the total phenolic content was 74.7mg/g of extract calculated as Gallic acid equivalent and total flavonoid content was 40.1 mg/g of extract calculated as Quercetin equivalent. The in vivo wound healing activity showed that the % wound closure was comparable with standard. The result may be attributed to the phytoconstituents such as flavonoids and phenolics present in it which may be due to their enhanced wound healing and provided scientific evidence to the ethno medicinal futures of poly herbal formulations. These findings could justify the inclusion of this formulation in the management of wound healing.

Keywords: Wound healing, Poly herbal formulation, Aloe vera, Tridox procumbens, Curcuma longa, Azadirachta indica

1. INTRODUCTION

1.1 Herbal Medicines

Herbal medicines are plant based medicines made from differing combinations of plant parts. E.g. leaves, flowers and roots. Each part can have different medicinal uses and the many types of chemical constituents require different extraction methods. Both fresh and dried plant matter are used depending upon the herb. Herbal medicines which formed the basis of health care throughout the world since the earliest days of mankind are still widely used, and have considerable importance in international trade. Recognition of their clinical, pharmaceutical and economic value is still growing, although this varies broadly between countries.

Plant is an important source of medicine and plays a key role in world health. Medicinal herbs or plants have been known to be an important potential source of therapeutics or curative aids. The use of medicinal plants has attained a commanding role in health system all over the world. Many countries in the world, that is, two-third of the world's population depends on herbal medicine for primary health care. The reasons for this is because of their better cultural acceptability, better compatibility and adaptability with the human body and pose lesser side effects.[1]

Medicinal plants may be defined as those plants that are commonly used in treating and preventing specific ailments and diseases and that are generally considered to be harmful to humans. Medicinal plants have provided mankind a large variety of potent drugs to alleviate or eradicate infections and suffering from diseases in spite of advancement in synthetic drugs, some of the plant-derived drugs still retained their importance and relevance. The use of plant-based drugs all over world is increasing.

Through recent researches on herbal plants or medicine, there have been great developments in the pharmacological evaluation of various plants used in traditional systems of medicine. Consequently, plants can be described as a major source of medicines, not only as isolated active principles to be dispensed in standardized dosage form but also as crude drugs for the population.[2]

India is a birth place of indigenous medicine such as Siddha, Ayurveda and Unani where many herbs have been used for treatment of human ailments. According to the World

Health Organization (WHO) about 80% of developing countries depend on traditional medicines for their primary health care needs.

The most common reasons for using traditional medicine are that it is more affordable, more closely corresponds to the patient's ideology, allays concerns about the adverse effects of chemical (synthetic) medicines, satisfies a desire for more personalized health care, and allows greater public access to health information. The major use of herbal medicines is for health promotion and therapy for chronic, as opposed to life-threatening, conditions.[3]

However, usage of traditional remedies increases when conventional medicine is ineffective in the treatment of disease. Currently, herbs are applied to the treatment of chronic and acute conditions and various ailments and problems such as cardiovascular disease, prostate problems, depression, inflammation, and to boost the immune system.

Medicinal plants play an important role in the development of potent therapeutic agents. Herbal drugs referred as plants materials or herbals, involves the use of whole plants or parts of plants, to treat injuries or illnesses. Herbal drugs are use of therapeutic herbs to prevent and treat diseases and ailments or to support health and healing. Herbal drugs are the oldest form of health care known to mankind. World Health Organization (WHO) has distinct herbal drugs as complete, labeled medicinal products that have vigorous ingredients, aerial or secretive parts of the plant or other plant material or combinations.

World Health Organization has set precise guidelines for the evaluation of the safety, efficacy, and quality of herbal medicines. Herbal drug is a chief constituent in traditional medicine and a common constituent in ayurvedic, homeopathic, naturopathic and other medicine systems. Herbs are usually considered as safe since they belong to natural sources. The use of herbal drugs due to toxicity and side effects of allopathic medicines, has led to rapid increase in the number of herbal drug manufacturers.[4]

1.1.1 Advantages of Herbal Drugs

- High low/minimum cost
- Complete accessibility
- Enhanced tolerance
- More protection
- Fewer side effects
- Potency and efficacy is very high

1.1.2 Disadvantages of Herbal Drugs

- Not able to cure rapid sickness and accidents
- Risk with self-dosing
- Complexity in standardizations.

1.2 Inflammation

Inflammation is the immune system's response to harmful stimuli, such as pathogens, damaged cells, toxic compounds or irradiation and acts by removing injurious stimuli and initiating the healing process.

The purpose is to localize and eliminate the injurious agent and to remove damaged tissue components so that the body can begin to heal. The response consists of changes in blood flow, an increase in permeability of blood vessels, and the migration of fluid, proteins, and white blood cells (leukocytes) from the circulation to the site of tissue damage.[5]

The classical signs of acute inflammation are pain, heat, redness, swelling, and loss of function. Which result from local immune, vascular and inflammatory cell responses to infection or injury. Inflammation is a generic response, and therefore it is considered as a mechanism of innate immunity, as compared to adaptive immunity, which is specific for each pathogen.

Inflammation is tightly regulated by the body. Too little inflammation could lead to progressive tissue destruction by the harmful stimulus (e.g.bacteria) and compromise the survival of the organism.[6]

In contrast, chronic inflammation may lead to a host of diseases, such hay fever, periodontitis, atherosclerosis, rheumatoid arthritis, and even cancer (e.g., gallbladder carcinoma). Inflammation is therefore normally closely regulated by the body.

Inflammation can be classified as either acute or chronic.

Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. Inflammatory response that lasts only a few days is called acute inflammation.

While a response of longer duration is referred to as chronic inflammation. It leads to a progressive shift in the type of cells present at the site of inflammation, such as mononuclear cells, and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process. Inflammation is not a synonym for infection. Infection describes the interaction between the action of microbial invasion and the reaction of the body's inflammatory defensive response.

Table: 1 Comparison between Acute and Chronic Inflammation

Factors	Acute Inflammation	Chronic Inflammation
Causative agent	Bacterial pathogens, injured tissues	Persistent acute inflammation due to non-degradable pathogens, viral infection, persistent foreign bodies, or autoimmune reactions.
Major cells involved	Neutrophils (primarily), basophils (inflammatory response) and eosinophil's (response to helminth worms and parasites)	Mononuclear cells (monocytes, macrophages, lymphocytes, plasma cells), fibroblasts
Primary mediators	Vasoactive amines, Eicosanoids.	IFN-γ and other cytokines, growth factors, reactive oxygen species, hydrolytic enzymes
Onset	Immediate	Delayed

Duration	Few days	Up to many months, or years
Outcomes	Resolution, chronic inflammation	Tissue destruction, fibrosis, necrosis

Acute inflammation is a short-term process, usually appearing within a few minutes or hours and begins to cease upon the removal of the injurious stimulus. In a normal healthy response, it becomes activated, clears the pathogen and begins a repair process and then ceases. It is characterized by five cardinal signs.

The key symptoms is "PRISH", for pain, redness, immobility (loss of function), swelling and heat.

The traditional names for signs of inflammation come from Latin:

- Dolor (pain)
- Calor (heat)
- Rubor (redness)
- Tumor (swelling)
- Functiolaesa (loss of function)

Redness and heat are due to increased blood flow at body core temperature to the inflamed site. Swelling is caused by accumulation of fluid. Pain is due to the release of chemicals such as bradykinin and histamine that stimulate nerve endings. Loss of function has multiple causes.[7]

1.2.1 Inflammatory disorders

Inflammatory abnormalities are a large group of disorders that underlie a vast variety of human diseases. The immune system is often involved with inflammatory disorders.[8]

Examples of disorders associated with inflammation include:

- Acne vulgaris
- Asthma
- Auto immune diseases
- Auto inflammatory diseases
- Celiac disease
- Chronic prostatitis
- Colitis
- Diverticulitis
- Glomerulonephritis
- Hidradenitis suppurativa
- Hypersensitivities
- Inflammatory bowel diseases
- Interstitial cystitis

1.3 Wound Healing Activity

Wound is a physical trauma where the skin is torn, cut or punctured. On exposure to air, microorganisms enter the wound which leads to wound contamination and finally development of infection. Wound healing is a complex multiphase process that involves a chain of well orchestrated biochemical and cellular events.[9] The phases of normal wound healing include hemostasis, inflammation, proliferation and remodeling. Each phase of wound healing is distinct, although the wound healing process is continuous, with each phase overlapping the next and characterized by migration and proliferation of fibroblasts, epithelial cells, deposition of connective tissue, angiogenesis, re-epithelization, and finally contraction of wound.[10]

1.3.1 Classification of Wounds

Wounds are classified as open wounds and closed wounds on the basis of underlying cause of wound creation and as acute and chronic wounds on the basis of physiology of wound healing.

(a) Open wound:

Though the open wound blood escapes the body and bleeding is clearly visible.

Open wound is further classified as:

- Incised Wounds:

  It is an injury with no tissue loss and minimal tissue damage. It is caused by a sharp object such as knife. Bleeding in such cases can be profuse, so immediate action should be taken.

- Abrasions or superficial Wounds:

  It is caused by sliding fall on to a rough surface. During abrasion the topmost layer of the skin i.e. epidermis is scraped off that exposes nerve ending resulting in a painful injury. Blood loss similar to a burn can result from serious abrasions.

- Laceration wound or tears Wounds:

  This is a nonsurgical injury in conjunction with some type of trauma, resulting in tissue injury and damage.

- Puncture Wounds:

  They are caused by some object puncturing the skin, such as needle or nail. Chances of injection in them are common because dirt can enter into the depth of wound.

- Gunshot Wounds:

  They are caused by a bullet or similar driving into or through the body

- Penetration Wounds:

  Penetration wounds are caused by an object such as knife entering and coming out from the skin.

(a) Closed wounds:

In closed wounds blood escapes the circulating system but remain in the body. It includes contusion or bruises, heamatomas or blood tumor, crush injury etc.

- Contusions or bruises:

  Bruises are caused by a blunt force trauma that damage tissue under the skin.

- Hematomas or blood tumor:

  They are caused by damage to a blood vessel that consequently causes blood to collect under the skin.

- Crush injury:

  Crush injury is caused when great or extreme amount of force is applied on the skin over long period of time.[11]

- Acute Wounds:

  An acute wound is a tissue injury that normally proceeds through an orderly and timely reparative process that results in sustained restoration of anatomic and functional integrity. Acute wounds are usually caused by cuts or surgical incisions and complete the wound healing process within the expected time frame. Acute wounds include burns and chemical injuries.

- Chronic wounds:

  Chronic wounds are wounds that have failed to progress through the normal stages of healing and therefore enter a state of pathologic inflammation. Local infection, hypoxia, trauma, foreign bodies and systemic disorders such as diabetes mellitus, malnutrition, immunodeficiency or medications are the most frequent causes of chronic wound. Chronic wounds may result from various causes, including naturopathic, pressure, arterial and venous insufficiency, burns and vasculitis.[12]

1.3.2 Factors Affecting Wound Healing

Wound healing is affected by various factors which can be categorized as local and systemic factors.[13]

Table: 2 Factors Affecting Wound Healing

Local factors	Systemic factors
Oxygenation	Diseases: Diabetes, Jaundice, Obesity
Infection	Medications: Glucocorticoid steroids, NSAIDS, chemotherapy
Poor blood supply	Alcoholism and Smoking
Wound infection	Nutrition
Poor wound hygiene	Immune suppression

1.3.3 Phases Involved in Wound Healing

Normal wound healing involves there are 4 overlapping phases. They are,

- ❖ Hemostasis phase
- ❖ Inflammatory phase
- ❖ Proliferative phase
- ❖ Remodeling phase

1.3.3.1 Hemostasis Phase:

It is the first phase, Bleeding usually occurs when the skin is injured and serves to flush out bacteria and antigens from the wound. Platelets are the cells that are deputed for sealing off the damaged blood vessels. They secrete vasoconstrictors that stimulate the constriction of broken blood vessels and thereby help to reduce blood loss. Within the first few minutes of injury, platelets in the blood begin to stick to the injured site. They change into an amorphous shape, more suitable for clotting, and they release chemical signals to promote clotting. This results in the activation of fibrin, which forms a mesh and acts as “glue” to bind platelets to each other. This makes a clot that serves to plug the break in the blood vessel, slowing/preventing further bleeding.[14]

1.3.3.2 Inflammatory Phase:

The inflammatory phase starts immediately after the injury that usually last between 24 and 48 h and may persist for up to 2 weeks. This phase is characterized by vasodilatation and phagocytosis to produce inflammation at the wound site. The inflammatory cells migrate to the injury site to scavenge bacteria, and prepare the injury site for healing. Neutrophils is the first cells to appear at the injury site, cleanse debris and bacteria to provide a good environment for wound healing. In the following, macrophages accumulate and facilitate phagocytosis of bacteria damage tissue and apoptotic neutrophils then leads to removal of chemokines from the area of inflammation, preventing further leukocyte influx. Several cytokines and growth factors are known to be secreted by macrophages. Such growth factors include TGF-β, TGF-α, basic FGF (bFGF), VEGF and PDGF. These growth factors activate and attract local endothelial cells, fibroblasts and keratinocytes, and enable wound healing by causing cell proliferation.[15]

1.3.3.3 Proliferative Phase:

Proliferative phase (2 days to 3 weeks) includes:

- Granulation stage: Fibroblasts lay bed of collagen matrix and produces new capillaries.
- Contraction stage: Wound edges pull together to reduce defect.
- Epithelialization stage: keratinocytes divides and migrate about 3cm from point of origin in all directions.

1.3.3.4 Remodeling Phase:

This phase last for 3 weeks to 2 years. New collagen is formed in this phase. Tissue tensile strength is increased due to intermolecular cross-linking of collagen via vitamin-C dependent hydroxylation. The scar flattens and scar tissues become 80% as strong as the original. During maturation and remodeling, collagen is realigned along tension lines, and cells that are no longer needed are removed by programmed cell death, or apoptosis.[16]

1.3.4 Role of Plants in Wound Healing

The wound healing activities of plants have since been explored in folklore. Many ayurvedic herbal plants have a very important role in the process of wound healing. Extensive research has been carried out in the area of wound healing management through medicinal plants. Many herbal plants have been proved to have significant role in the process of wound healing and they promote the repair mechanisms in the natural way.[17] Herbal medicines in the wound management involve disinfection, debridement and providing a moist environment to encourage the establishment of the suitable environment for natural healing process. Medicinal plants heal wound healing process by promoting blood clotting, fighting against infection and accelerating wound healing.[18]

Herbal medicines with the following properties are beneficial in the wound management of both acute and chronic type wound. They are,

(a) Antioxidant Effect:

The production of free radicals at or around the wound may contribute to delay in wound healing through the destruction of lipids, proteins, collagen, proteoglycan and hyaluronic acid.

Agents that demonstrate a significant antioxidant activity may, therefore, preserve viable tissue and facilitate wound healing.

(b) Antimicrobial Activity:

Wound healing can also be delayed when microorganisms are present in large enough numbers. Therefore, reducing the bacterial load of a wound may be necessary to facilitate wound healing as well as to reduce local inflammation and tissue destruction. An ideal agent for the prevention and control of wound infection would therefore be one that directly destroys the pathogens while also stimulate immune activity.

(c) Anti-inflammatory activity:

Wounds in persistent inflammatory phase may delay the healing process. Preventing prolonged inflammatory phase hasten the healing process.

(d) Cell proliferation and migration:

Plants or plant products enhances cell proliferation and cell migration hasten the natural healing process.

2. LITERATURE REVIEW

1. **khatoon safina et al.,** has reported the *T. Procumbens* is a plant with its all parts having noble pharmacological activities. Especially antimicrobial action of *T. Procumbens* are quiet significant as in the present era continued increase in antibiotic resistance has fuelled the need for development of new antibiotics.[19]
2. **Kumar *et al.*,** The *T. procumbens* is reported for identification, phytochemical, pharmacogonostic study and its pharmacological activities like hepatoprotective activity, antimicrobial activity, immunomodulating property, defluoridation activity, hypotensive action, anti viral action, anti oxidant action, antiurolithiatic action and anti inflammatory action.[20]
3. The ethyl acetate extract of rhizomes of *C. longa* has high antibacterial activity than the methanol extract or water extract as per **Kim *et al.*,** further he claimed that The *C. longa* may have antibacterial activity and the potential to restore the effectiveness of β-lactams against MRSA, and inhibit the MRSA invasion. Ethyl acetate extract of *C. longa* markedly lowered the MICs of ampicillin and oxacillin against MRSA. [21]
4. **Teplicki *et al.*,** Has demonstrated that the *Aloe vera* accelerates wound healing by promoting fibroblast and keratinocyte proliferation and moderately stimulating cell migration. *Aloe vera* also shows protective effects against preservative-induced death of keratinocytes.[22]
5. **Peng-Hui *et al.,*** Has reported that wound healing is an important physiological process to maintain the integrity of skin after trauma. The normal wound healing involves three successive but overlapping phases, including hemostasis/inflammatory phase, proliferative phase, and remodeling phase. (i) wound healing; (ii) wound healing in fetus and adult; (iii) prostaglandins and wound healing; (iv) the pathogenesis of excessive wound healing; (v) the epidemiology of excessive wound healing; (vi)in vitro and in vivo studies for excessive wound healing; (vii) stem cell therapy for excessive wound healing; and (viii) the prevention strategy for excessive wound healing.[23]
6. The hydroalcoholic extract of poly herbal formulations such as *Tridax procumbens, Aloe vera, Euphorbia hirta* are exhibited significant wound healing activity in excision, incision, burn and dead space wound model, which is comparable to the marketed *Aloe vera* gel formulation. This finding by **Muqeem Nasir *et al.*,** provides an insight into the usage of the

poly herbal formulation in traditional treatment of wounds or burns associated with bacterial infections**.**[24]

7. **Olamide adebiyi *et al.,*** Has reported the total phenolic content was 19.08 ± 1.21 mg gallic acid equivalent (GAE)/g extract and 14.85 ± 1.09 mg GAE/g extract for the leaf and stem respectively while the flavonoid content was 9.00 ± 0.13 and 13.22 ± 1.53 mg quercetin/g extract. The antioxidant activity of *Grewia carpinifolia* extract may be due to the high level of flavonoids and phenols in the plant.[25]
8. **Suresh Kumar Dev *et al.,*** The high rate of wound contraction (< 0.0001), early epithelialization period (< 0.0001) and increased wound breaking strength (< 0.0001) were observed in 2% and 5% polyherbal gel treated group when compared to the normal control and negative control group. The antimicrobial and anti-inflammatory effect of Polyherbal drug provoked and promoted the wound healing process through accelerated remodelling of damaged tissue.[26]
9. **Victoria N *et al.,*** Has reported the leaf extract of *F.exasperata* in the treatment of cutaneous wounds more than any of its fractions. However, *F.exasperata* may not be safe at higher doses, especially for the management of chronic disease conditions like hypertension and diabetes, as could be observed in the sub chronic toxicity and histopathology findings.[27]
10. **Demilew W *et al.,*** Has results demonstrate that the crude extract of *A. polystachyus* leaves possesses wound healing activities. This justifies the traditional claimed use of the plant for treating uninfected and infected wounds caused by S. aureus.[28]
11. **Nagesh HN *et al.,*** The ethanolic extract of *A. indica* leaves showed highly significant pro-healing effect almost equivalent to standard drug, which may be partly due to the anti-inflammatory activity, proliferation of fibrocollagenous tissue and angiogenesis properties.[29]
12. **Shirin Fahimi *et al.,*** HPTLC fingerprinting of PHO demonstrated the presence of several phenolic constituents corresponding to the plant extracts. Regarding to the role of phenolic compounds in wound healing process, PHO could be an appropriate candidate for burn healing with respect to its traditional use in ITM. Moreover, HPTLC fingerprinting could be utilized as an applicable method for quality control of the prepared formulation.[30]
13. **Yogesh PT *et al.,*** Polyherbal formulation prepared from the plant extracts (*V. negundo, E. officinalis* and *T. procumbens*) accelerates wound healing process by proliferation and

mobilization of fibroblast and keratinocytes, and promotes angiogenesis at the site of injury.[31]

14. **Yaduvanshi B *et al.,*** *T.Procumbens* (1 mg/g) was found to be effective in healing dermal wound and its pharmacodynamic effect was comparable to VEGF (1 µg/g) treatment. *T.Procumbens* (1 mg/g) acted by stimulating collagen synthesis, which has been reported to be an essential step in faster healing of wound.[32]
15. **Chandra Pratap Singh *et al.,*** Has demonstrated design, formulate and evaluate the polyherbal Tridax procumbens based cream comprising *of Aloe vera, Marigold, Henna, Papaya* and *Neem.* The cream was evaluated for pharmaceutical parameters & wound healing activity. That the topical application of cream increases the percentage of wound contraction and decreases epithelization time in treatment group as compared to other groups, which may be due to additive activity of the phytoconstituents present in the extract and hence, may be used as a potential herbal formulation for wound healing.[33]
16. **Shilpa S *et al.,*** The Herbal ointment containing hydrochloric extracts of plants *Psorolea corylifalia, Achryanthes aspera* ,was formulated as ointment and the hydro alcoholic extract was prepared by maceration method. These Formulations were evaluated for the following parameters: pH, Spread ability, grittiness, skin irritation study, stability. The wound healing activity is assessed by the rate of wound contraction, Period of epithelisation and skin breaking strength.[34]
17. **Heiko Sorg *et al.,*** The integrity of healthy skin plays an important role in maintaining physiological homeostasis of the human body. Many instances are described which lead to insufficient healing necessitating further intervention. Although wound healing mechanisms and specific cell functions in wound repair have been delineated in part, many underlying pathophysiological processes are still unknown and we are only able to design new and effective wound healing therapies if we better understand this complex interplay.[35]

3. AIM AND OBJECTIVES

Aim

To develop and evaluate poly herbal ointment for wound healing activity.

Objectives

- Collection and authentication of the selected plants.
- Preparation of herbal extracts.
- Phytochemical analysis for the prepared extracts.
- Development of herbal formulations for the prepared extracts.
- Characterization of the developed formulations.
- *In vitro* and *in vivo* pharmacological evaluation of the developed formulation.

4. PLAN OF WORK

1. Review of Literature
2. Collection and authentication of the selected plants
3. Preparation of herbal extracts
4. Phytochemical analysis for the prepared extracts
5. Quantification of total Phenol and Flavonoid content.
6. Development of herbal formulations for the prepared extract
7. Evaluation of the developed formulations for
    - *In vitro* antioxidant study
        - DPPH radical scavenging assay.
    - *In vitro* antimicrobial activity.
        - Antibacterial.
    - *In vivo* wound healing study using Excision wound model.
8. Histopathological study
9. Statistical analysis.

5. PLANTS SELECTED FOR THE PRESENT STUDY

In present study the following plants which have been demonstrated for wound healing activity are selected and were used for the development of herbal formulations.

5.1 *Tridax procumbens*

Tridax procumbens Linn is a common plant found in tropical areas of all countries, growing primarily during rainy season. It is commonly known as 'Coat button' in English. It belongs to the family **asteraceae**.[36]

Figure: 1 *Tridax procumbens*

Medicinal uses: wound healing, antimicrobial, antidiabetic, anti-inflammatory.

5.1.1 Scientific Classification:

Kingdom : plantae

Subkingdom: Tracheobionta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Asteridae

Order : Asterales

Family : Asteraceae

Genus : Tridax

5.1.2 Vernacular Names:

Botanical name: *Tridax procumbens L.*

English name : Coat Buttons

Tamil : Vettukkaaya Thalai

Hindi : Khal Muriya

Telugu : Gaddichamanthi

Kannada : Sanna Gida

Malayalam : Kurikootticheera

5.2 *Azadirachta indica*

Azadirachta indica, commonly known as neem or nimtree. It belongs to the family **meliaceae**. It is typically grown in tropical and semi-tropical regions.[37]

Figure: 2 *Azadirachta indica*

Medicinal uses: wound healing, anti-inflammatory, antibacterial. antiseptic, antifungal.

5.2.1 Scientific Classification:

Kingdom : Plantae

Subkingdom: Tracheobionta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Rosidae

Order : Sapindales

Family : Meliaceae

Genus : Azadirachta

Species : Indica

5.2.2 Vernacular Names:

Botanical name**:** *Azadirachta indica A. Juss*

English **:** Neem tree

Tamil **:** Vembu

Hindi **:** Neem

Malayalam **:** Ariyaveppu

5.3 *Curcuma longa*

Curcuma longa is a flowering plant of the ginger family **zingiberaceae.** It requires temperature between 20 to 30 degree Celsius. It is native to the Indian subcontinent and southeast Asia.[38]

Figure: 3 *curcuma longa*

Medicinal uses: wound healing, antioxidant, Alzheimer's disease, anticancer.

5.3.1 Scientific Classification:

Kingdom : Plantae

Subkingdom: Tracheobionta

Division : Magnoliophyta

Class : Liliopsida

Subclass : Zingiberidae

Order : Zingiberales

Family : Zingiberaceae

Genus : Curcuma L

Species : *C. longa L*

5.3.2 Vernacular Names:

Botanical name: *Curcuma longa L.*

English : Turmeric

Hindi : Haldi

Malayalam : Manjal

Telugu : Haridra

Tamil : Manjal

5.4 *Aloe vera*

Aloe vera is a succulent plant species of genus aloe. An evergreen perennial, it is originated from Arabian peninsula but grows wild in tropical climates around the world and is cultivated for agriculture and medicinal uses.[39]

Figure: 4 *Aloe vera*

Medicinal uses: Wound healing, Heartburn relief, Lower blood sugar.

5.4.1 Scientific Classification:

Kingdom : Plantae

Subkingdom: Tracheobionta

Division : Magnoliophyta

Class : Liliopsida

Subclass : Liliidae

Family : Aloaceae

Genus : Aloe L

Order : Liliales

Species : Aloe vera (L)

5.4.2 Vernacular Names:

Botanical name: *Aloe barbadensis*

English : Aloe vera

Tamil : Kattrazhai

Malayalam : Kattarvala

Telugu : kalabanda

Hindi : Ghikumari

6. METHODOLOGY

6.1 Collection and Authentication of Plants

The leaves of *Aloe vera* collected from the surrounding area of Coimbatore district and authenticated by Botanical survey of India (BSI) southern circle, Coimbatore, Tamilnadu. The authentication certificate number is No.BSI/SRC/5/23/2018/Tech/3033. Soon after collection the leaves were cleaned, gel was collected using sharp knife and dried in hot air oven at 40^0C, powdered to coarse using mortar and pestle. The powder was stored in an air tight container, until further use.

The leaves of *Tridax procumbens* collected from the surrounding area of Coimbatore district and authenticated by Botanical survey of India (BSI) southern circle, Coimbatore, Tamilnadu. The authentication certificate number is No.BSI/SRC/5/23/2018/Tech/3034. Soon after collection the leaves were cleaned, dried in shade and crushed to a coarse powder, stored in an air tight plastic container, until further use.

The leaves of *Azadirachta indica* collected from the surrounding area of Coimbatore district and authenticated by Botanical survey of India (BSI) southern circle, Coimbatore, Tamilnadu. The authentication certificate number is No.BSI/SRC/5/23/2018/Tech/3035. Soon after collection the leaves were cleaned, dried in shade and crushed to a coarse powder, stored in an air tight plastic container, until further use.

The rhizomes of *Curcuma longa* collected from the surrounding area of salem district and authenticated by Botanical survey of India (BSI) southern circle, Coimbatore, Tamilnadu. The authentication certificate number is No.BSI/SRC/5/23/2018/Tech/3036. Soon after collection the rhizomes were cleaned, dried in shade and crushed to a coarse powder, stored in an air tight plastic container, until further use.

6.2 Extraction of the Plant Materials

6.2.1 Soxhlet Extraction

6.2.1.1 Principle:

Soxhlet extraction is a continuous solid / liquid extraction. A solid which contains the material to be extracted is placed in what is called a thimble. A thimble is made out of a material which will contain the solid but allow liquids to pass through. The thimble containing the material is placed in the Soxhlet extractor. An organic solvent is then heated at reflux. As it boils its vapors rise up and are condensed by a condenser. The condensed solvent then fills up the thimble. After it fills with enough solvent it automatically back down into the container of organic solvent.[36]

This process takes place over and over again until all the material to be extracted from the solid in the thimble is now extracted into the organic solvent. Typically, a Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. The solvent vapor travels up a distillation arm, and floods into the chamber housing the thimble of solid. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask.

This cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled.[37]

6.2.1.2 Procedure

The collected *Neem* leaf, *Tridax* leaf, *Aloe* gel and *Turmeric* rhizome were dried, powdered, mixed and placed inside a thimble made from thick filter paper, which was loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor was placed onto a flask containing the extraction solvent. The Soxhlet was then equipped with a condenser.

The solvent were heated to reflux. The solvent vapor travels up a distillation arm, and floods into the chamber housing the thimble of solid. The condenser ensures that any solvent vapor cools, and drips back down into the chamber housing the solid material. The chamber containing the solid material slowly fills with warm solvent. Some of the desired compound was then dissolved in the warm solvent.

When the Soxhlet chamber was almost full, the chamber were automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times, over hours or days. During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. After extraction the solvent is removed, and the non-soluble portion of the extracted solid remains in the thimble, and is discarded.[38]

6.2.2 Simple Distillation

6.2.2.1 Principle

Simple distillation can be used when the liquids to be separated have boiling points that are quite different Distillation is a separation process that involves heating a liquid to its boiling point, transferring the vapor to a different portion of the apparatus, then condensing the vapour and collecting the condensate in another container. This technique is one of the most useful for separating a mixture of liquids when the components have different boiling points. Industrially, distillation is the basis for the separation of crude oil into the various, more useful hydrocarbon fractions. Chemically, distillation is the principal method for purifying liquids (e.g. samples, or solvents for performing reactions). Successful distillation depends on several factors, including the difference in boiling points of the materials in the mixture, and therefore the difference in their vapor pressures, the type of apparatus used, and the care exercised by the experimentalist.[39]

6.2.2.2 Procedure

A pure liquid has a constant boiling point as long as liquid and vapor are in equilibrium. In a simple distillation of a pure substance, as the temperature rises, the vapor pressure increases. As the vapor expands, it passes out of the heated portion of the apparatus until it comes into

contact with the cold surface of the water-cooled condenser. When the vapor is cooled, it condenses and passes down the condenser into the receiver.[40]

6.3 Qualitative Phytochemical Analysis of MEPHF

6.3.1 Test for Carbohydrates

A small quantity of the extract was dissolved in 5ml of distilled water and filtered. The filtrate was tested to detect the presence of various phytochemical constituents in the sample.[41]

- **Molisch's test**

Few drops of Molisch's reagent was added to 2-3ml of filtrate, followed by addition of concentrated sulphuric acid along the sides of the test tube. Formation of violet color ring at the junction of two liquids indicates the presence of carbohydrates.

- **Fehling's test**

1ml Fehling's-A (copper sulphate in distilled water) was added to 1ml of Fehling's-B (potassium tartarate and sodium hydroxide in distilled water) solution, boiled for one minute. To this added 1ml of filtrate and heated gently. Formation of brick red precipitate indicates the presence of reducing sugars.

- **Benedict's test**

Few ml of filtrate was mixed with equal volume of Benedict's reagent (alkaline solution containing cupric citrate complex) and heated in boiling water bath for 5min. Formation of reddish brown precipitate infers the presence of reducing sugars.

6.3.2 Test for Alkaloids

Small amount of extract mixed with few ml of dilute hydrochloric acid. Shaken well and filtered. Following tests were performed with the obtained filtrate.[42]

- **Dragendorff's test**

A few drops of Dragendorff's reagent (potassium bismuth iodide solution) was added to 2-3ml of filtrate. Orange red precipitate indicates the presence of alkaloids.

- **Mayer's test**

A few drops of Mayer's reagent (potassium mercuric iodide solution) was added to 2-3ml of filtrate. Cream (dull white) precipitate was formed.

- **Wagner's test**

A few drops of Wagner's reagent (solution of iodine in potassium iodide) was added to 2-3ml of filtrate. Reddish brown precipitate was obtained.

- **Hager's test**

A few drops of Hager's reagent (Picric acid) was added to 2-3ml of filtrate. Yellow precipitate was obtained.

6.3.3 Detection of Steroids and Terpenoids:

- **Salkowski Test**

About 50mg of the extract, 2ml of chloroform and 2ml of concentrated sulphuric acid were added and shaken well. Then observed the coloration of chloroform and acid layers. Appearance of chloroform layer as red in color and acid layer as greenish yellow fluorescence indicates the presence of steroids.

- **Liebermann -Burchard Test**

About 50 mg of the extract was dissolved in 2ml of acetic anhydride in a test tube, added 2ml chloroform, heated to boiling and cooled. Then 1ml of concentrated sulphuric acid was added along the sides of the test tube and observed for the formation of color at the junction. Formation of red, pink or violet color at the junction of the liquids indicates the presence of steroidal terpenoids.[43]

6.3.4 Test for Glycosides

- **Legal's test**

1ml of pyridine and 1ml of sodium nitroprusside was added to 1ml of extract. Pink to red color indicates the presence of glycosides.

- **Keller-Killiani test**

Glacial acetic acid was added to 2ml extract, followed by the addition of trace quantity of ferric chloride and 2 to 3drops of concentrated sulphuric acid. Reddish brown color appears at the junction of two liquid indicates the presence of cardiac glycosides.

6.3.5 Detection of Phenolic Compounds and Tannins

- **Ferric chloride test**

1ml of the alcoholic solution of the extract was added to 2ml of distilled water followed by few drops of 10% ferric chloride. Formation of blue or green color indicates the presence of phenols.

- **Lead acetate test**

A few drop of lead acetate was added to 5ml of aqueous extract. Formation of yellow or red color precipitate indicates the presence of tannins.

6.3.6 Test for Saponins

- **Foam Test:**

1ml of test sample was diluted with 20ml of distilled water and shaken it in a graduated cylinder for 3minutes. Foam of 1cm after 10min indicates the presence of saponins.

- **Froth test:**

5ml of test sample was added to sodium bicarbonate solution. After vigorous shaking the mixture, kept it for 3minutes. A honey comb like froth formation indicates the presence of saponins.[44]

6.3.7 Test for Flavonoids

- **Alkaline reagent test**

A few drop of sodium hydroxide solution was added to the extract. Formation of an intense yellow color, which turns to colorless on addition of few drops of dilute hydrochloric acid, indicates the presence of flavonoids.

- **Shinodas test[Magnesium hydrochloride reduction test]**

Alcoholic solution of extract was treated with a small piece of magnesium ribbon and a few drops of concentrated HCl was added and heated. Appearance of crimson red or occasionally green to blue color infers the presence of flavonoid.

6.3.8 Test for Proteins and Amino Acids

- **Biuret test**

3ml of test solution was added to 4% sodium hydroxide and few drops of 1% copper sulphate solution. Formation of violet color indicates the presence of proteins.

- **Ninhydrin test**

A mixture of 3ml test solution and 3drops of 5% Ninhydrin solution was heated in a boiling water bath for 10min. Formation of purple or bluish color indicates the presence of free amino acids.

6.4 Quantification of Total Phenolics and Flavonoids

6.4.1 Estimation of Total Phenolics content

6.4.1.1 Reagents

- Folin-Ciocalteu's reagent
- Gallic acid (1 mg/ml)
- 20% sodium carbonate

6.4.1.2 Preparation of standard

Standard solution of Gallic acid was prepared by adding 10 mg of accurately weighed Gallic acid in 10 ml of distilled water.

6.4.1.3 Preparation of sample

10 mg of the accurately weighed MEPHF was dissolved in 10 ml methanol and used for the estimation.

6.4.1.4 Procedure

The total phenolic content of the MEPHF was determined by Folin-Ciocalteau assay method. To an aliquot 100µl of MEPHF (1mg/ml) or standard solution of Gallic acid (10, 20, 40, 60, 80, 100 µg/ml) added 50µl of Folin-ciocalteau reagent followed by 860µl of distilled water and the mixture was incubated for 5min at room temperature. 100µl of 20% sodium carbonate and 890µl of distilled water were added to make the final solution to 2ml. It was incubated for 30 min in dark to complete the reaction after that absorbance of the mixture was measured at 725 nm against blank. Distilled water was used as reagent blank. The tests were performed in triplicate to get the mean values. The total phenolic content was found out from the calibration curve of Gallic acid, and it was expressed as milligrams of Gallic Acid Equivalents (GAE) per gram of extract.[45]

6.4.2 Estimation of Total Flavonoid Content

6.4.2.1 Reagents

- 10% aluminium chloride
- 1M Potassium acetate

6.4.2.2 Preparation of standard

Standard solution of Quercetin was prepared by adding 10 mg of accurately weighed Quercetin in 10 ml of methanol.

6.4.2.3 Preparation of sample

10 mg of the accurately weighed MEPHF was dissolved in 10 ml methanol and used for the estimation.

6.4.2.4 Procedure

The total flavonoid content of the MEPHF was determined by using Aluminium chloride by colorimetric method. To an aliquot of 1 ml of extract (1 mg/ml) or standard solutions of Quercetin (10, 20, 40, 60, 80, 100µg/ml) methanol was added separately to make up the solution upto 2 ml. The resulting mixture was treated with 0.1 ml of 10% aluminium chloride, 0.1ml of 1M potassium acetate and 2.8 ml of distilled water. Shaken well and incubated at room temperature for 30 min. The absorbance was measured at 415nm against blank, where a solution of 2ml ethanol, 0.1 ml potassium acetate, 2.8 ml distilled water and 0.1 ml of aluminium chloride serve as blank solution. The total flavonoid content was determined from the standard Quercetin calibration curve. And it was expressed as milligrams of Quercetin equivalents per gram of extract.[46]

6.5 HPTLC Method for Estimation of Biomarkers in MEPHF

The basic principle of HPTLC is adsorption. Where the mobile phase used is non-polar and the stationary phase is polar. Chemical or active constituents present in the plant extract will move through the plate according to the relative solubility of the constituents in the two phases and will be separated. The non- polar compound will be eluted first and the more polar later. The compounds can be identified based on the Rf value.[47]

6.5.1 Experimental condition

Stationary phase : Aluminium plates precoated with silica gel 60F254(10x10cm)

Mobile phase : Toluene:Ethyl acetate:Formic acid:Methanol (3;6:1.6:0.4)

Sample application : CAMAG Linomat 5

Chamber type : Twin Trough Chamber 10x10cm

Chamber saturation : 5min

Development time : 30min

Detection : Camag scanner 3

Development distance : 7cm

Data system : Win CATS planar chromatography manager.

6.5.2 Instrumental Parameters

Number of track : 9

Band length : 6.0mm

Application position : 10mm

Solvent front position : 80.0mm

Solvent volume : 10ml

Position of first track : 10mm

Distance between tracks : 10mm

Scan start position Y : 5.0mm

Scan end position : 75.0mm

Slit dimension : 6.00 × 0.45mm, Micro

Optimized optical system : light

Scanning speed : 20mm/sec

Data resolution : 100µM/ step

6.5.3 Measurement table

Wavelength : 254nm

Lamp : D2&W

Measurement : Remission

Measurement mode : Absorption

Optical filter : Second order

Detector mode : Automatic

6.5.4 Preparation of standard

Stock solutions of Standard compounds were prepared by dissolving accurately weighed 100mg of Gallic acid, Rutin and Quercetin in 100ml of methanol (HPTLC grade), filtered by using Whatmann No.1 filter paper and stored in amber colored container at 40C.

6.5.5 Preparation of Sample

Accurately weighed 100mg of MEPHF was dissolved in 100ml of methanol, filtered by using Whatmann No.1 filter paper and stored in amber colored container at 40C.

6.5.6 Procedure

5 µl of Gallic acid, Rutin, Quercetin and MEPHF were spotted in form of bands with a Camag microlite syringe on pre-coated Silica Gel glass plate 60F254 (10×10 cm with 0.2 mm thickness) using a Camag Linomat 5 applicator. The plates were prewashed with methanol and activated at 600C for 10 min prior to chromatography. The sample loaded plate was kept in TLC twin trough developing chamber after chamber saturation with respective mobile phase. The optimized chamber saturation time for mobile phase was 5 min at room temperature. Linear ascending development was carried out and the plate was developed in the respective mobile phase up to 7 cm. The developed plate was then dried by hot air to evaporate solvents from plate and also for the development of bands. The dried plate was observed under UV light at 254 nm and 366 nm and photo documentation was done. The percentage of active constituents present in the extracts were compared with that of standard.

6.6 Anti-Microbial Activity of MEPHF

6.6.1 Antibacterial study

6.6.1.1 Preparation of Inoculums

The inoculums for the experiment were prepared in fresh nutrient broth from the preserved slant culture. The turbidity of the culture can be adjusted by the addition of broth or sterile saline (if it is excessive) or by further incubation to get the required turbidity, and the newly prepared inoculums were standardized by adjusting the turbidity of the culture to that of McFarland standards.

6.6.1.2 Preparation of Sterile Swabs

Cotton wool swab on wooden applicator or plastics were prepared and sterilized by autoclaving or by dry heat (only for the wooden swabs). It was sterilized by packing the swabs in culture tubes, papers or tins etc

6.6.1.3 Sterilization of Forceps

Forceps can be sterilized by dipping in alcohol and burning off the alcohol.

6.6.1.4 Experiment

The standardized inoculums is inoculated in the sterilized plates prepared earlier (aseptically) by dipping a sterile in the inoculums removing the excess of inoculums by passing and rotating the swab firmly against the side of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3times rotating the plate through an angle of 60º after each application. Finally pass the swab round the edge of the agar surface. Leave the inoculums to dry at room temperature with the lid closed. The sterile discs are soaked overnight in sample solutions. Each petri dish is divided into 3 parts. First compartment were loaded with sample disc as sample (100µg) and second compartment were loaded with sample disc as sample (50µg) third compartment were loaded with standard ciprofloxacin (5µg) with the help of sterile forceps. After that petri dishes are placed in the refrigerator at 4º C or at room temperature for 1hour for diffusion. Incubate at 37ºC for 24hours. Observe the zone of inhibition produced by different samples. Measure it using a scale and record the average of two diameters of each zone of inhibition.[48]

6.7 In Vitro Antioxidant Study of MEPHF

6.7.1 DPPH Free Radical Scavenging Assay

6.7.1.1 Principle

The DPPH [1, 1-diphenyl-2-picrylhydrazyl (α, α-diphenyl-β-picrylhydrazyl)] assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH with an odd electron gives a maximum absorption at 517nm (purple color). When Antioxidants react with DPPH, which is a stable free radical becomes paired off in the presence of a hydrogen donor (e.g., a free radical scavenging antioxidant) and is reduced to the DPPHH and as

consequence the absorbance's decreased from the DPPH. DPPH radical is a stable radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerize, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet color, characterized by an absorption band in methanol solution centred at about 517nm Radical to the DPPH form, results in decolorization (yellow color) with respect to the number of electrons captured. More the decolorization more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of any new drug. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Diphenyl picryl hydrazine; non radical) with the loss of this violet color (although there would be expected to be a residual pale yellow color from the picryl group still present).

6.7.1.2 Procedure

The effect of the extracts on DPPH radical was estimated using micro plate method. Gallic acid standard solutions (5, 10, 20, 40, 80µl) were run each time in all plates in the linear range of concentrations prepared from 0.3mM stock solution.10µg/ml concentration of sample extract (10, 20, 40µl) pipeted followed by 100 µl DPPH reagent into each well and allowed to react for 30 min. Plates were sealed with clear adhesive seals to protect samples from external oxidation. The seals were removed before measurement. The absorbance was measured at 517 nm in the spectrophotometer. A control reaction was also carried out without the test sample. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples. Free radical scavenging activity was expressed as percentage inhibition (I%) and calculated using the following equation:

$$\textbf{Percentage inhibition (I\%)} = (Abs_{control} - Abs_{sample} / Abs_{control}) \times 100$$

Different sample concentrations were used in order to obtain calibration curves and to calculate the IC_{50} values. (IC_{50} - concentration required to obtain a 50% radical scavenging activity).[49]

6.8 Development of Poly Herbal Formulation

6.8.1 Preparation of Simple Ointment Base

6.8.1.1 Ingredients:

Wool fat 5.0g

Hard paraffin 5.0g

Cetostearyl alcohol 5.0g

White soft paraffin 85.0g

6.8.1.2 Type of preparation: Absorption ointment base

6.8.1.3 Procedure:

Hard paraffin and cetostearyl alcohol taken in a china dish kept on water-bath at 70^0C. Wool fat and white soft paraffin are added to this mixture and stirred until all the ingredients are melted. If required decanted or strained and stirred until cold and packed in suitable container.

6.8.2 Formula for the ointment

Table 3: The extract was incorporated into the ointment base at different proportion

S. No	INGREDIENTS	F1	F2	F3	F4
1	Extracts (MEPHF)	0.5g	1g	2g	4g
2	Wool fat	5g	5g	5g	5g
3	Hard paraffin	5g	5g	4g	3g
4	Cetostearyl alcohol	4.5g	4g	4g	3g
5	White soft paraffin	85g	85g	85g	85g

6.8.2.1 Preparation of poly herbal ointment

6.8.2.2 Procedure:

The Hard paraffin and cetostearyl alcohol taken in a china dish kept on water-bath at 70^0C. Wool fat and white soft paraffin are added to this mixture and stirred until all the ingredients are melted and then added the extracts with continuous stirring. ointment was prepared by using the

extracts of *Azadirachta indica*, *Tridax procumbens*, *Curcuma longa*, *Aloe barbadensis* and taking different proportions of extracts.

6.8.3 Physiochemical Characterization of the Formulation

6.8.3.1 pH

The pH meter was calibrated using standard buffer solution. About 0.5g of the ointment was weighed and dissolved in 50.0 ml of distilled water and set aside for 2 h and then the pH was measured.

6.8.3.2 Centrifugation:

It is believed to be an unique tool for the evaluation of accelerated deterioration of ointments. It was determined by using Remi centrifuge in 10 ml-graduated cylinder at 10,000 rpm for 10 min.

6.8.3.3 Spreadability:

The spreadability was determined by placing excess of sample (1g) in between two slides which was compressed to uniform thickness by placing a definite weight (50g) for definite time (5 min). The time required to separate the two slides was measured as spreadability.

Spreadability was calculated by following formula

$$S = M \times L / T$$

Where, S= Spreadability

M= Weight tide to the upper slide

L= Length of glass slide

T= Time taken to separate the slides

6.8.3.4 Viscosity:

Viscosity of the formulation was determined by Brookfield Viscometer at 50rpm, using spindle no.E64.[50]

6.9 Pharmacological Evaluation of MEPHF

6.9.1 Animals and Management

Female Wistar albino rats of 6-8 weeks old and 150-200g body weight were procured from the Biogen laboratory, Bangalore. All rats were kept at room temperature and allowed to accommodate in standard conditions at 12 h light and 12 h dark cycle in the animal house. Animals were fed with modified diet and water ad libitum freely throughout the study. The animals were left for 15 days for acclimatization prior to the beginning of the experiment. The

experimental procedure was approved by IAEC (Institutional animal ethical committee) governed by CPCSEA, Government of India.

6.9.2 Skin irritation study:

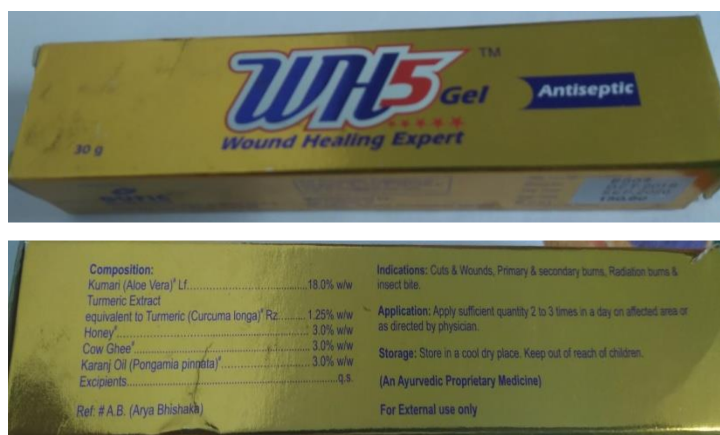
Skin irritation study was conducted on albino rats as per OECD guide lines number 404. The back of the animals was shaved free of fur with an electric clipper 24 hours before application of the sample. This was carefully done to avoid skin injury which could alter its permeability. Ointment were applied on the skin patches of albino rats, and the site of application in terms of erythema and edema was examined at 24, 48, and 72 h for changes in any dermal reactions. Scores corresponding to the skin reactions were attributed according to the scoring systems described by Draize test.[51]

Table 4: Grading of Skin Reactions

ERYTHEMA	SCORE	OEDEMA	SCORE
No erythema	0	No oedema	0
Very slight erythema	1	Very slight oedema	1
Well defined erythema	2	Slight oedema	2
Moderate tom severe erythema	3	Moderate oedema	3
Severe erythema to eschar formation	4	Severe oedema	4

6.9.3 DRUG

Commercially available wound healing herbal ointment were used as standard drug. The ointment were applied topically over the wound area.



6.9.4 PREPARATION OF TEST DRUG

MEPH ointment was formulated using (4%) in simple base ointment.

6.9.5 EXPERIMENTAL MODEL

6.9.6 EVALUATION OF WOUND HEALING EFFECT OF MEPHO IN EXCISION WOUND MODEL

Rats were divided into 4 groups each containing 6 animals as follows.

Table 5: Experimental Design for Excision Wound Model

S.NO	GROUP	SAMPLE SIZE	GROUP SPECIFICATION	TREATMENT
1	Group I	6	Control	Untreated
2	Group II	6	Ointment base	Only with simple ointment base
3	Group III	6	Standard	Poly herbal ointment from market
4	Group IV	6	Treated	MEPH ointment (4%)

6.9.6.1 INDUCTION OF WOUND

The rats were anaesthetized prior to creation of the wounds, by ether anaesthesia. The dorsal area were shaved and disinfected using 70% alcohol. The dorsal fur of the animal was shaved with an electric clipper. A full thickness of the excision wound of 1 cm were created along the markings using toothed forceps, a surgical blade and pointed scissors. After 24h of wound creation, the ointments was applied gently to cover the wounded area once daily until complete healing Wound area and wound contraction content were monitored.[52]

6.9.7 ESTIMATION OF PARAMETERS

6.9.7.1 Measurement of wound closure

The progression of wound healing was judged by the periodic assessment of the contraction of excision wounds. Wound contraction was monitored by tracing the outline of the wound on tracing sheet and then using graph sheet to calculate the area of the wound size. All

animals in each group were monitored until complete healing of wounds occurred and the day at which each wound healed was recorded. Mean of all healed wounds was determined.[53]

$$\text{Percent wound contraction} = \frac{\text{Healed area}}{\text{Total area}} \times 100$$

6.10 HISTOLOGICAL ASSESSMENT

Histological studies of wounded tissues provide accurate diagnosis of level of healing of the wound. Histopathology is the microscopical study of tissues for pathological alterations. When wound was completely healed, fibroblastic proliferation and vascular proliferation covered with granulation tissue was also observed in test and standard drug treated animals.[54]

6.10.1 Fixation:

Kept the tissue in fixative for 24-48hours at room temperature

The fixation was useful in the following ways:

a) Serves to harden the tissues by coagulating the cell protein,
b) Prevents autolysis,
c) Preserves the structure of the tissue, and
d) Prevents shrinkage

Common Fixatives: 10% Formalin

6.11 STATISTICAL ANALYSIS

Data's of all the parameters were analyzed using the Graph pad software. Analysis of Variance (ANOVA); one way ANOVA followed by Dunnet's comparison test was performed. The values were expressed as Mean ± SEM.

7. RESULTS

7.1. Preparation of Extracts

Methanolic extracts of the poly herbs such as *Neem* leaf, *Tridax* leaf, *Aloe* gel and *Turmeric* rhizome was obtained by soxhlet extraction followed by distillation.

The % yield of the extract was found to be 15 %w/w.

The appearance of the extract showed that the color was green with semisolid greasy consistency.

7.2 Preliminary Phytochemical Screening

The extract was studied for its phytochemical analysis by qualitative chemical test. The presence of major phytoconstituents were presented in Table 6.

Table 6: Preliminary Phytochemical Screening of Extract

S.NO	PHYTOCHEMICAL CONSTITUENTS	TEST	RESULT
1.	Carbohydrates	Molisch's test Fehling's test Benedict's test	+ + +
2.	Alkaloids	Dragendorff's test Mayer's test Wagner's test Hager's test	+ + + +
3.	Steroids and Terpenoids	Salkowski Test Liebermann -Burchard Test	+ +
4.	Glycosides	Legal's test Keller-Killiani test	+ +
5.	Phenolic Compounds and Tannins	Ferric chloride test Lead acetate test	+ +

6.	Saponins	Foam Test Froth test	+ +
7.	Flavonoids	Alkaline reagent test Shinodas test	+ +
8.	Proteins and Amino Acids	Biuret test Ninhydrin test	+ +

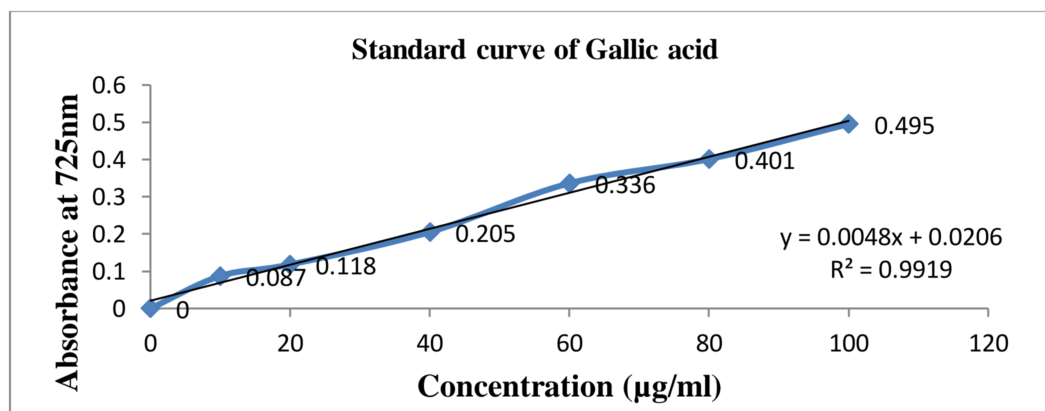
7.3 Quantification of Total Phenol and Flavonoids

7.3.1 Estimation of Total Phenol of MEPHF

Table 7: Estimation of Total Phenolic Content of MEPHF

Sample	Concentration (µg/ml)	Absorbance
Standard (Gallic acid) 1mg/ml	10	0.087
	20	0.118
	40	0.205
	60	0.336
	80	0.401
	100	0.495
MEPHF	100	0.3185

Figure 5: Estimation of Total Phenolic Content of MEPHF



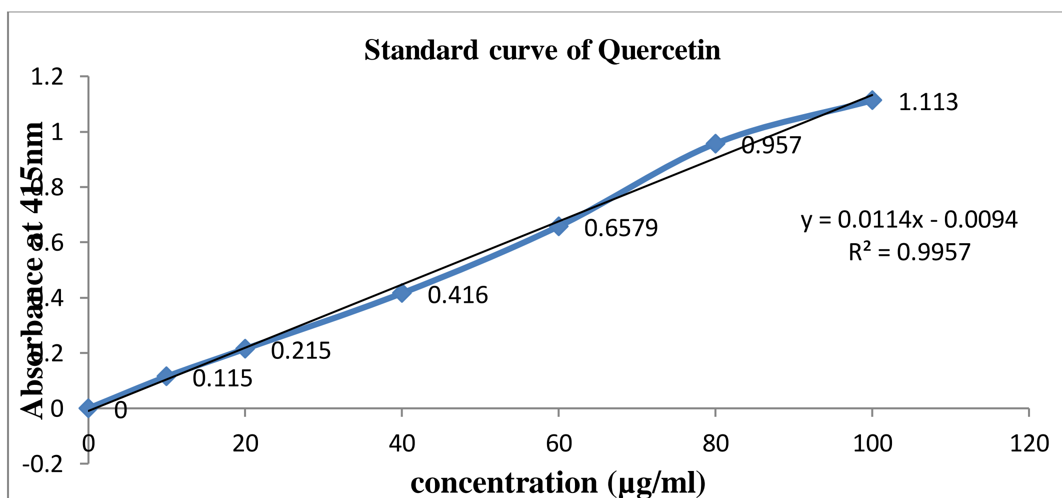
The total phenolic content in MEPHF were found to be 74.7mg/gm of extract calculated as Gallic acid equivalent

7.3.2 Estimation of Total Flavonoid Content of MEPHF

Table 8: Estimation of Total Flavonoid Content of MEPHF

Sample	Concentration (µg/ml)	Absorbance
Standard (Quercetin) 1mg/ml	10	0.1150
	20	0.2150
	40	0.4160
	60	0.6579
	80	0.957
	100	1.113
MEPHF	100	0.4321

Figure 6: Estimation of Total Flavonoid Content of MEPHF



The total flavonoid content in MEPHF were found to be 40.1 mg/gm of extract calculated as Quercetin equivalent.

7.4 *In Vitro* Antioxidant Activity of MEPHF

7.4.1 DPPH Radical Scavenging Activity

Table 9: Percentage inhibition and IC_{50} values of DPPH radical by Gallic acid

Treatment	Concentration (µg/ml)	% Inhibition	IC_{50} (µg/ml)
Standard (Gallic acid)	0.05	5.24	0.407
	0.1	14.25	
	0.2	29.76	
	0.4	59.74	
	0.8	89.09	

Figure 7: DPPH radical scavenging activity of Gallic acid

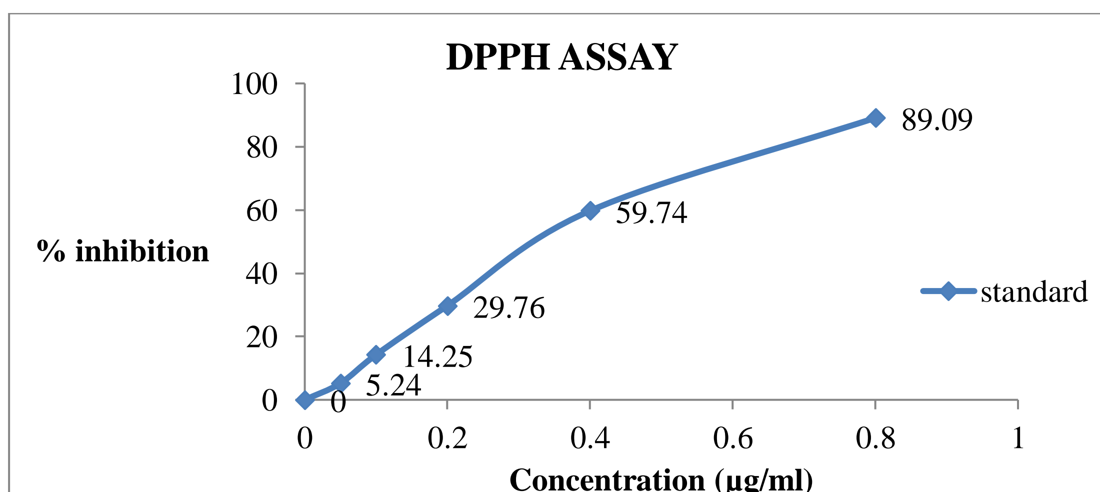
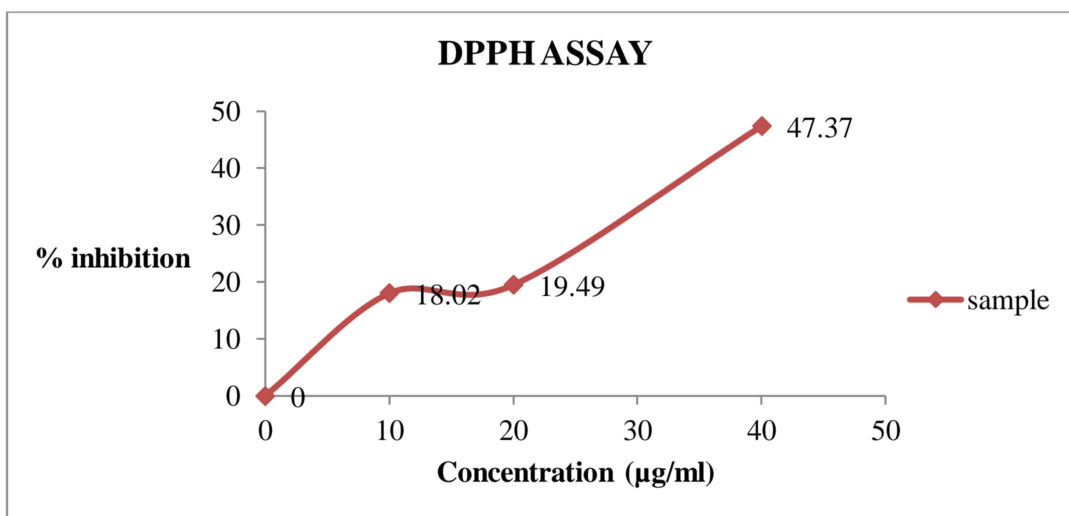


Table 10: Percentage inhibition and IC_{50} values of DPPH radical by MEPHF

Treatment	Concentration (µg/ml)	% Inhibition	IC_{50} (µg/ml)
Sample (MEPHF)	10	18.02	43.22
	20	19.49	
	40	47.37	

Figure 8: DPPH radical scavenging activity of MEPHF



7.5 High Performance Thin Layer Chromatography:

7.5.1 Phytochemical Screening of the Formulation by HPTLC Technique:

HPTLC was performed to screen the number of phytoconstituents present in the formulation.

Figure 9: Photographic representation of HPTLC

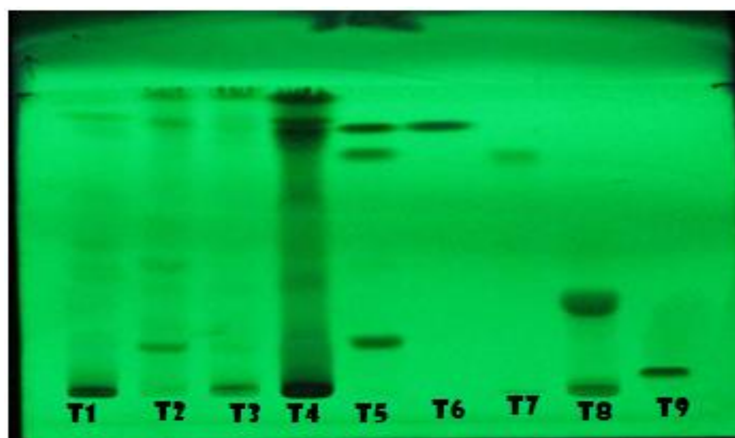


Figure 10: Track 1: Aloe vera

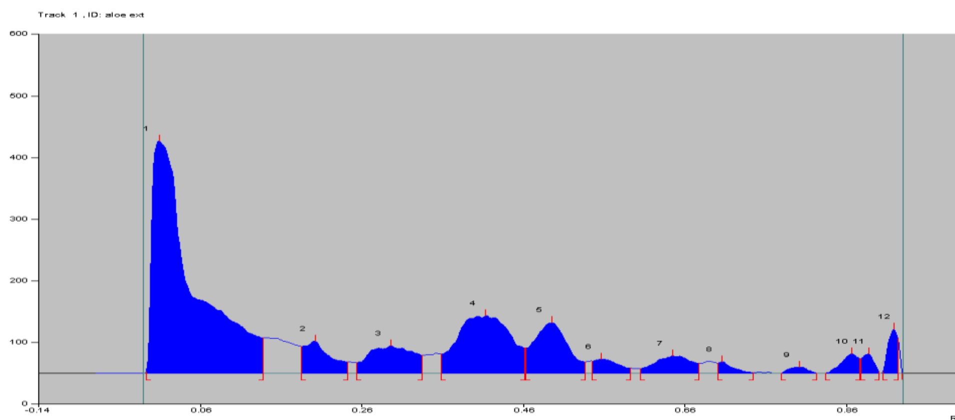


Figure 11: Track: 2 Neem sample

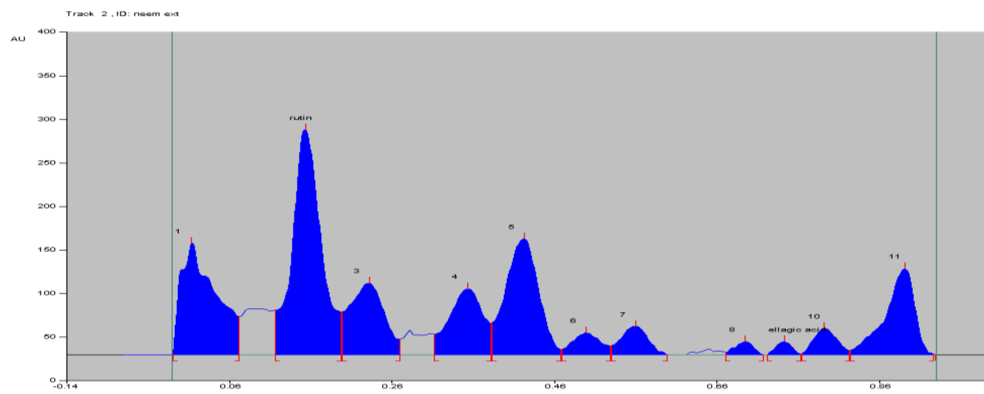


Figure 12: Track: 3 Tridax sample

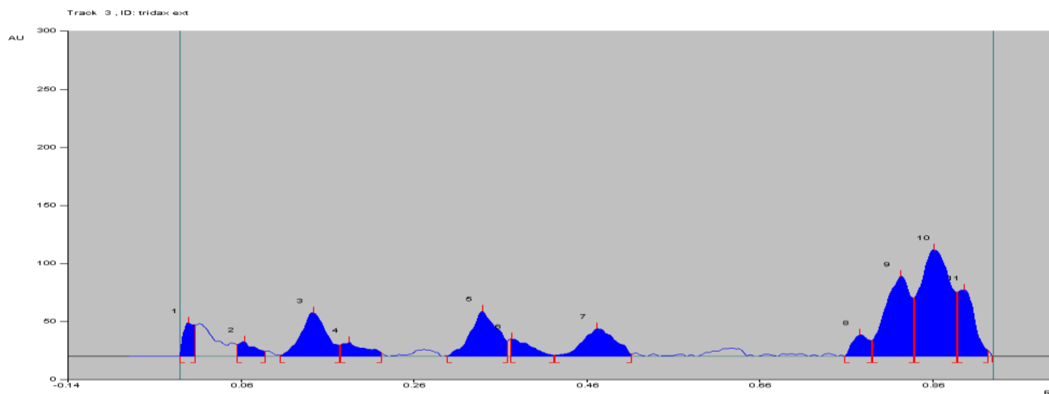


Figure 13: Track: 4 MEPHF

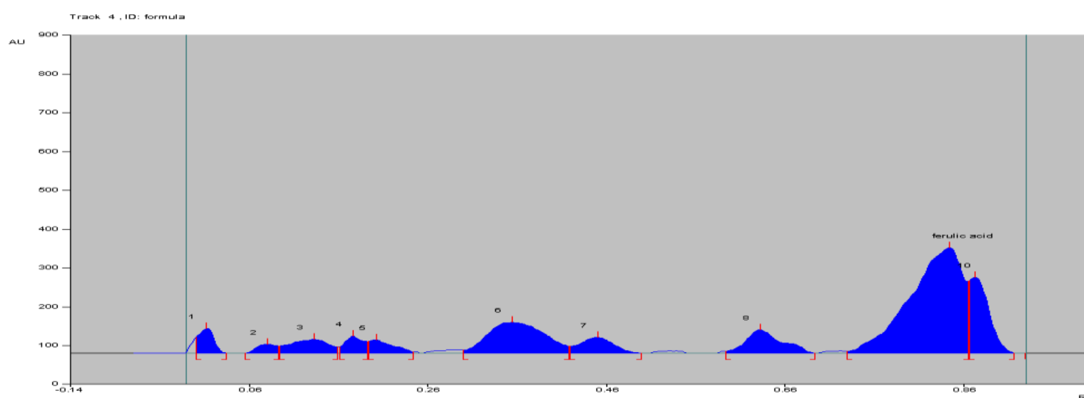


Figure 14: Track: 5 STD (Rutin, Gallic acid, Quercetin)

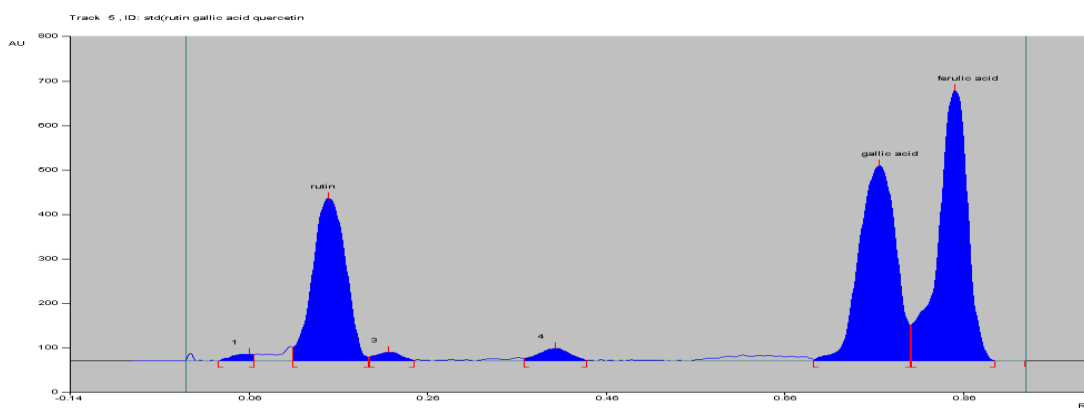


Figure 15: Track: 6 Ferulic acid

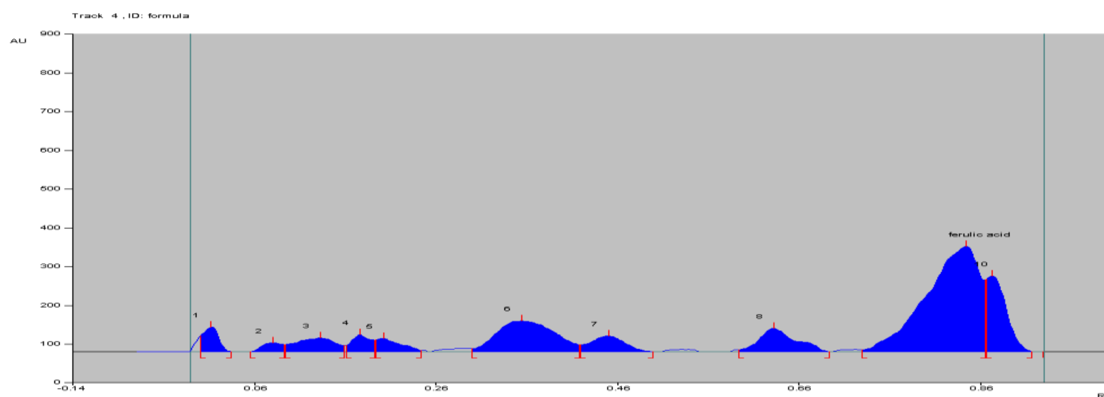


Figure 16: Track: 7 Elagic acid

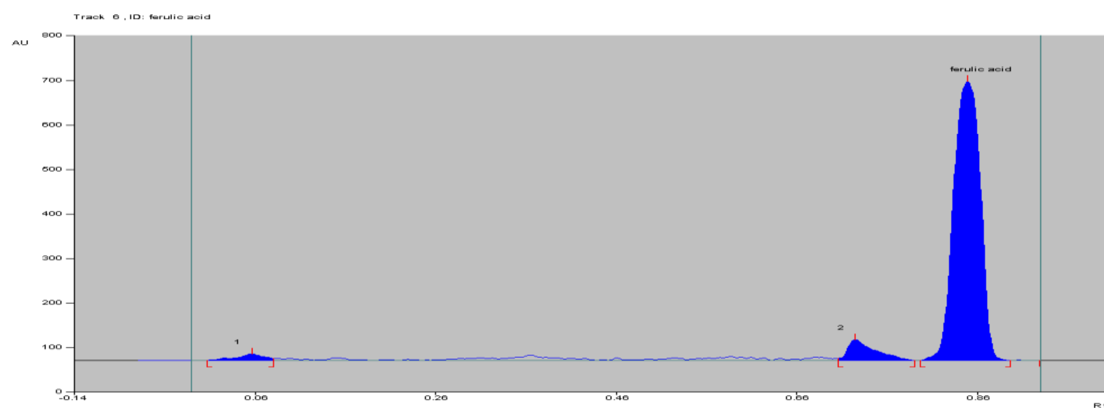


Figure 17: Track: 8 Mangiferin

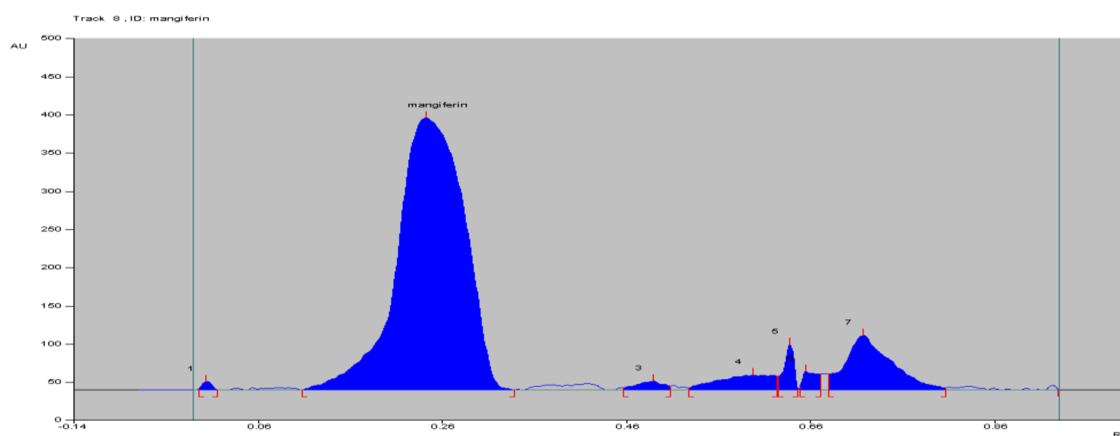


Figure 18: Track: 9 Trigonilline

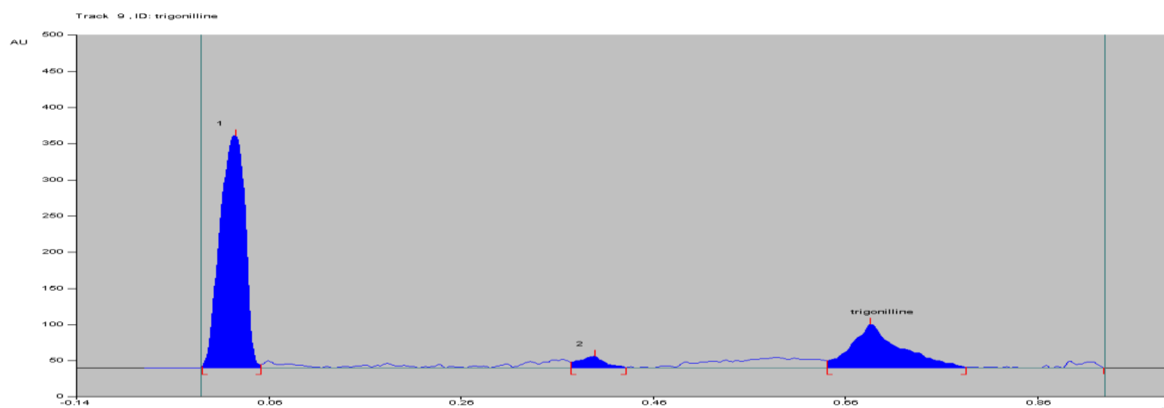


Table 11: Phytoconstituents Present in the Extracts Analyzed by HPTLC

Track	Sample	Volume	% Rutin Content	% GA Content	% QC content	% EA content	% TG content
Track1	Aloe	10µl	-	-	+	-	+
Track2	Neem	10µl	+	+	-	+	-
Track3	Tridax	10µl	+	+	+	-	-
Track4	MEPHF	10µl	+	-	+	-	-

(+) PRESENT (-) ABSENT

7.6 Anti-Microbial Activity

Antimicrobial activity was carried out using agar disc diffusion method by using various microorganisms and the zone of inhibition was determined.

7.6.1 Anti-Bacterial Activity of Extract:

7.6.1.1 Anti-bacterial activity against gram +ve organisms:

The *in-vitro*anti-bacterial activity of selected polyherbal extract against gram +ve organisms were studied and zone of inhibition was reported in the following table 11.

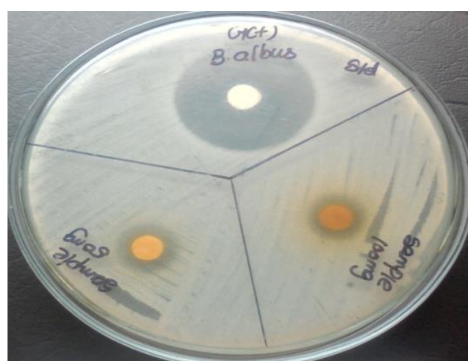
Table 12: Zone of inhibition for Gram +ve organisms

SI NO	GRAM +VE ORGANISMS	Zone of inhibition (mm)	
		STD- Ciprofloxacin	MEPH
1	*Staphylococcus aureus*	27	8
2	*Bacillus subtilis*	28	7
3	*Micrococcus luteus*	30	8
4	*Bacillus albus*	30	9

The maximum zone of inhibition (9mm) was found in methanolic extract of poly herbal formulations against *Bacillus albus* for the Gram +ve organism.

Figure 19: Zone of inhibition for Gram +ve organisms

s.aureus | *Bacillus subtilis*



Bacillus albus

Micrococcus luteus

7.6.1.2. Anti-bacterial Activity against Gram Negative Organisms:

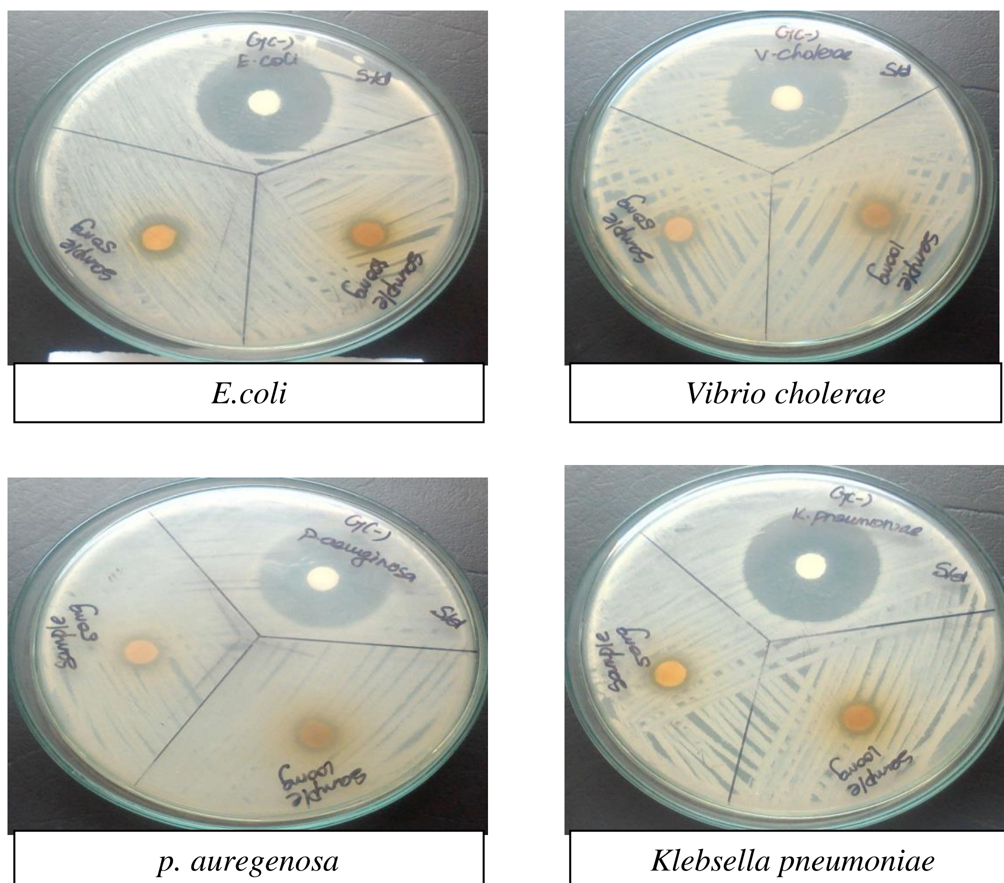
The in-*vitro* anti-bacterial activity of polyherbal extract against selected gram -ve organisms were studied and zone of inhibition was presented in Table 12.

Table 13: Zone of inhibition for Gram –ve organisms

SI NO	GRAM -VE ORGANISMS	Zone of inhibition (mm)	
		STD-Ciprofloxacin	MEPH
1	*E.Coli*	29	9
2	*Klebsiella pneumoniae*	30	8
3	*Vibrio cholerae*	28	8
4	*Pseudomonas auregenosa*	28	10

The maximum zone of inhibition (10mm) was found in MEPHF against *Pseudomonas auregenosa* for the Gram -ve organism.

Figure 20: Zone of inhibition for Gram –ve organisms



E.coli | *Vibrio cholerae*

p. auregenosa | *Klebsella pneumoniae*

7.7 Evaluation of Prepared Poly herbal Ointments

The ointments prepared (F1, F2, F3 and F4) were evaluated as per I.P for physiochemical characters. The character of ointments such as irritancy test, spreadability, viscosity, pH, centrifugation were studied and the results were presented.

Table 14: physical evaluation parameters of the formulations

S.NO	FORMULATIONS	P^H	CENTRIFUGATION	SPREADABILITY	VISCOSITY
1	F1	6.3	No phase separation	40 mm	1258 cps

2	F2	6.4	No phase separation	56 mm	1125 cps
3	F3	6.7	No phase separation	58 mm	970 cps
4	F4	6.9	No phase separation	58 mm	950 cps

7.8 Pharmacological Studies

7.8.1 Skin Irritation Study

Table 15: Grading of Erythema and Eschar Formation at Different Time Intervals

GROUP	GRADING AND TIME INTERVALS		
	24 h	**48 h**	**72 h**
I	0	0	0
II	0	0	0
III	0	0	0

It shows that there is no sign of dermal toxicity as no erythema or eschar of any kind was observed. The ointment did not cause moderate or severe erythema even after 72 h of observation.

Table 16: Grading of Edema Formation at Different Time Intervals

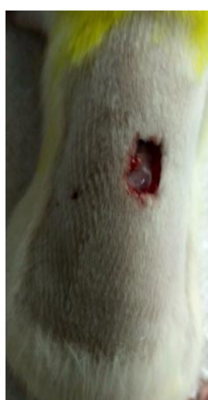
GROUP	GRADING AND TIME INTERVALS		
	24 h	**48 h**	**72 h**
I	0	0	0
II	0	0	0
III	0	0	0

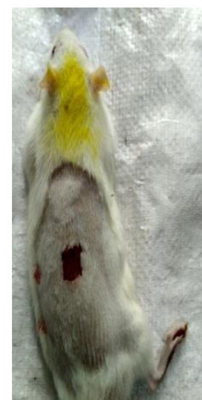
The result shows that there was no oedema observed on the skin of the rats after topical application of the ointments

7.8.2 Evaluation of Wound Healing Effect of MEPHF In Excision Wound Model

Figure 21: Percentage wound contraction on excision wound model

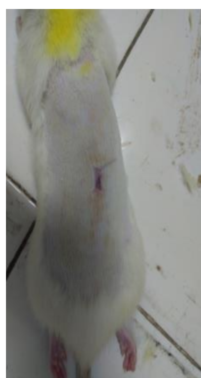
On 0th day





Control | Simple Ointment Base | Standard | Treated

On 15th day



Control | Simple Ointment Base | Standard | Treated

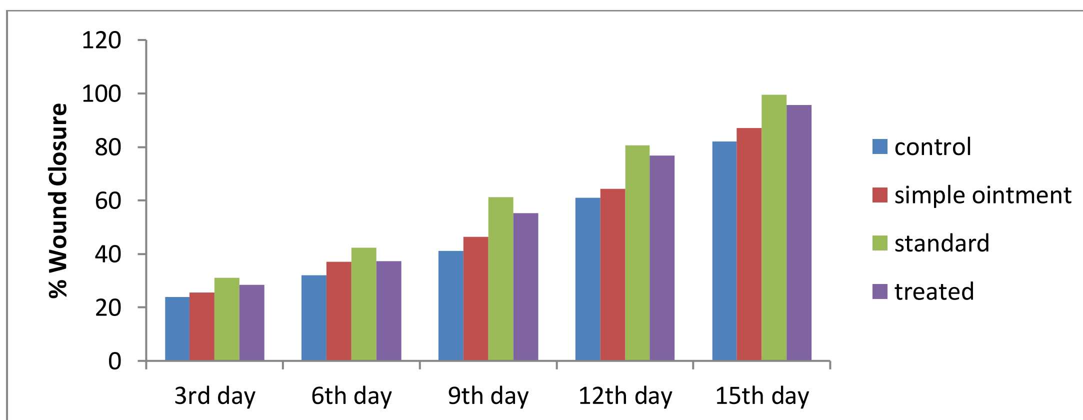
7.9. Estimation of Parameters

7.9.1. Percentage Wound Contraction

Table 17: Percentage wound contraction in excision wound model

COMPOUNDS	% OF WOUND CLOSURE					
	0th Day	3rd Day	6th Day	9th Day	12th Day	15th Day
Control	0	24±1.06	32.1±0.94	41±0.73	63±1.06	83.3±1.06
Simple ointment base	0	25.6±1.14	37.1±0.94	46.3±1.14	65.3±0.82	86.2±1.35
Standard	0	31.1±1.07	42.3±1.22	61.1±1.07	85.5±0.76	99.5±1.12
MEPH ointment	0	28.3±0.98	37.3±1.29	55.4±0.94	76.8±1.07	95.6±0.76

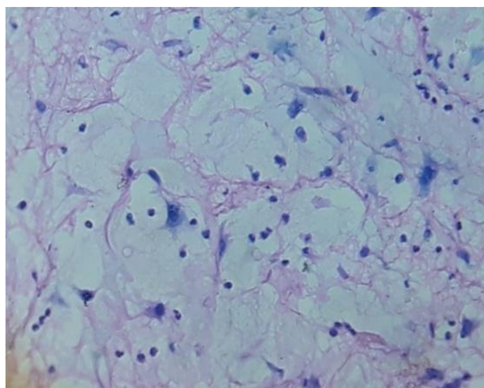
Figure 22: % wound contraction in excision wound model



7.10. Histopathological Study

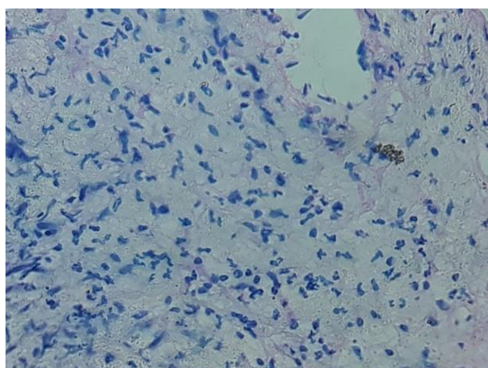
Histopathological evaluation of excision wound model.

Figure 23: Group 1- Control



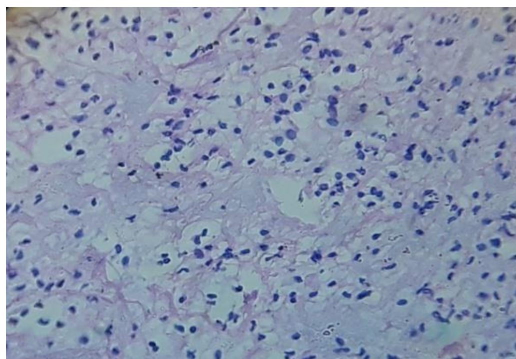
The section of the skin and epidermis shows incomplete healing with less epithelialization. It indicated the incomplete wound healing.

Figure 24: Group 2- Simple ointment base



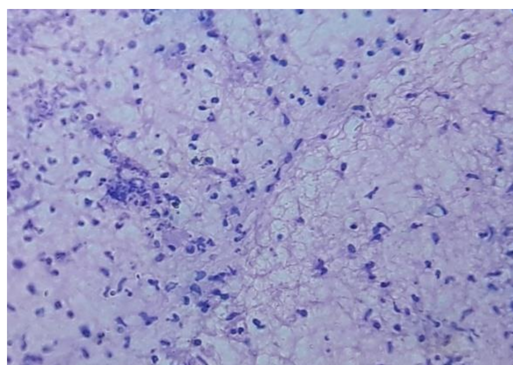
The section of the skin and epidermis shows incomplete healing. Section of the skin and epidermis shows proliferation of fibroblasts.

Figure 25: Group 3- Standard



Section of the epidermis shows Re-epithelialization with the proliferation of fibroblasts.

Figure 26: Group 4 - MEPHO treated



Section of the epidermis shows Re-epithelialization with the proliferation of fibroblasts, of dense fibrous tissue and blood capillaries was observed.

8. DISCUSSION

Wound is a clinical entity and is as old as mankind, often considered as major problem in clinical practice. Each year, millions of people experience burns, suffer from chronic wounds, or have acute wounds that become complicated by infection, dehiscence or problematic scarring. Wounds are normally resolved in a few days, but chronic wounds represent a major burden because of economic and social factors. There by, the search for new agents is ongoing and natural products become a great target. Individual factors such as stress or diabetes can cause delays in the healing process or increase the risk of infection in the wound. Due to unawareness of the society this may lead to chronic diseases which may damage the other organs. Impaired wound healing can result into severe morbidity leading to long hospitalization of patients. States (US) population, which results in a significant economic burden estimated to be nearly 2–4% of the health budgets.

The *Tridax procumbens, Azadirachta indica, Curcuma longa, Aloe vera* plant has a long history in herbal medicine in various countries. The leaves and rhizomes of these plants is well known for its different types of pharmacological properties such as antidiabetic, hepatoprotective, anti-inflammatory, Alzheimer's disease, anticancer, anti bacterial, anti fungal and wound healing. The present study was aimed to evaluate the wound healing potential of methanolic extract of poly herbal ointments for excision wound model of rats.

Phytochemical screening of MEPHF were performed and the results revealed the presence of carbohydrates, alkaloids, Steroids and Terpenoids, Glycosides, flavonoids, tannins, phenols, saponins, proteins and amino acids. The main attraction of the phytochemical was the presence of phenols and flavonoids which was concluded by the colorimetric estimation of these constituents in the extract.

Flavonoids are coming under the category of polyphenols, where its action is mainly attributed to anti-inflammatory action and it provides a symptomatic relief in wound. Phytopharmaceuticals are gaining importance in modern medicine as well as in traditional system of medicine owing to their therapeutic effect due to the presence of phytochemicals such as polyphenols, flavonoids, terpenoids etc.

Flavonoids are wide spread plant secondary metabolites that have shown free radical scavenging activity and protection against oxidative stress. Studies were revealed that flavonoids are also known to promote the wound healing process mainly due to their astringent and antimicrobial properties which appear to be responsible for the wound healing and increased rate of epithelialization. Results obtained in the present study revealed that the levels of these phytoconstituents were considerable and the total phenol and flavonoid content was found to be MEPHF- 74.7mg/gm and MEPHF- 40.1mg/g respectively.

HPTLC was used to assess the phytochemical constituents in the extract. The solvent system Toluene:Ethyl acetate:Formic acid:Methanol (3;6:1.6:0.4) ratio showed good separation and demonstrated 9 prominent constituents. HPTLC also confirms the presence of quercetin,rutin, gallic acid, mangiferin,elagic acid,trigonilline in the extract.

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. They are radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ageing, dementia, cancer etc. Therefore in the present study, the potential of the MEPHF to serve as antioxidants was assayed. Free-radicals play an important role in the oxidative damage of biological systems. Several complementary methods have been adopted to trap free radicals through antioxidant activity, among which DPPH assay is the most common.

The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability. The free radical scavenging activity of the extract was estimated by comparing the %inhibition of MEPHF with standard Gallic acid. IC_{50} was also calculated to determine the amount of extract needed to quench 50% of radicals. Methanolic extract of poly herbal formulations exhibited a dose dependent scavenging activity with IC_{50} values of 43.22µg/ml respectively for MEPHF. Where the IC_{50} for standard Gallic acid was found to be 0.407µg/ml. Thus from the result obtained it could be concluded that MEPHF shows a good antioxidant activity which might be attributed to the presence of phytochemicals such as polyphenols and flavonoids.

The standard ciprofloxacin was found to have significant antimicrobial activity against bacteria respectively. The zone of inhibition was observed from both Gram +ve and Gram –ve

bacteria strains .The maximum zone of inhibition (9mm) was found in methanolic extract of poly herbal formulations against *Bacillus albus* for the Gram +ve organism. And for Gram –ve organisms the maximum zone of inhibition (10mm) was found in MEPHF against *Pseudomonas auregenosa.*

The extract was formulated in to poly herbal ointment using four different concentration of 0.5%, 1%, 2% and 4%w/w of extract was incorporated into ointment base and the resulted formulations were named as F1, F2, F3 and F4 respectively. The prepared formulations were evaluated for its physiochemical characters and revealed that all the formulations demonstrated ideal characteristic of herbal ointment. Formulations complied with the physical evaluation parameters like pH, centrigugation,viscosity,spreadability were found to be acceptable which were notified in (table).

As the skin irritation studies on the animals didn't show any significant effects like erythema, edema, itching etc., it was stated to be safer in clinical practice.

Methanolic extract of poly herbal formulations demonstrated wound healing properties comparable with standard ointment. Animals in the untreated group showed some degree of healing. As earlier suggested, healing in this untreated group may be due to self-immunity. It is important to throughout the period of wound treatment, the extracts did not cause irritation or pain to the animals. In this investigation one group were used to assess the effect of MEPHO applied topically.

Extract treated excision wounds showed an increased rate of wound contraction, leading to faster healing as confirmed by the increased healed area when compared to the control untreated group. The MEPHO was recorded similar effectiveness when compared to the group treated with a commercial wound healing herbal ointment but the activity was lesser than standard.

9. CONCLUSION

Wound healing is a complex and continuous process that begins immediately after injury, followed by homeostasis, blood clotting, inflammation, proliferation and remodeling phases. All these phases can promote or prolong healing by influencing external or internal factors including infection sex hormones and nutrition. Delay in healing process increases the possibility of getting infected, improper recovery, and formation of unpleasant scar.

The study thus demonstrated the wound healing activity of methanolic extract of poly herbal ointment were found to be effective in the functional recovery of the wound. The extracts promote wound closure of excision wound as compared to control group. The result may be attributed to the phytoconstituents such as flavonoids and phenolics present in it which may be due to their enhanced wound healing and provided scientific evidence to the ethno medicinal futures of poly herbal formulations.

The study concludes that the poly herbal extract obtained by the dried powders of *Neem* leaves, *Tridax* leaves, *Aloe* gel and *Turmeric* rhizome posses antimicrobial activity. Incorporation of the poly herbal extract into ointment base at 4% (F4) demonstrated ideal physiochemical characteristics. These findings could justify the inclusion of this formulations in the management of wound healing.

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