Research Protocol
“Comparative Histological Study of Wound Healing Potential of *Ehretia Laevis Roxb.* plant leaves on Animal Model Against Standard”

Dr. Rushikesh Thakre, Dr. Arvinda Bhave, Dr. Pradip Tekade
Mrs. Ketaki Harne, Dr. Preeti Sujit Borkar

1. Assistant Professor , Department of Samhita Siddhant, MGACH & RC, Datta Meghe Institute Medical Sciences (DU) Wardha (MS) India
2. Professor Department of Pathology, JNMC, Datta Meghe Institute Medical Sciences (DU) Wardha (MS) India
3. Associate Professor Department of Chemistry Bajaj College of Science (Formerly known as Jankidevi Bajaj College of Science), Wardha (MS), India
4. PhD scholar (Chemistry), Rashtra Santa Tukadoji Maharaj Nagpur University Nagpur (MS), India
5. Professor & Head, Department of Samhita & Siddhant MGACH & RC, Datta Meghe Institute Medical Sciences (DU) Wardha (MS) India.

Abstract: Background: Ayurveda is considered as ancient science for healing. Many medicinal plants are mentioned in Ayurvedic literature for wound healing. Folklore medicines for various medical conditions including wound healing are being practiced in India. Herbal plants contain many phytochemical useful for many medical conditions and prevent the progression of diseases. All these traditional practices need to be evaluated on scientific grounds, to pave the path for further clinical trials and new drug developments. This effort will avoid the excessive use of antibiotics and to avoid side effects of modern medicine.

*Ehretia Laevis Roxb.* called as Ajan Vruksha and Khandu Chakka. They are traditionally being used in wounds, body ache & minor fractures in the form of local application by traditional peoples in the state of Maharashtra India. The scope of the study is to know the wound healing efficacy of *Ehretia Laevis Roxb.* on the histological parameters in animal model. Topical Phenyltoin ointment as a control will be used.

*Ehretia Laevis Roxb.* contains many chemicals useful in promotion of healing & repair of wounds. These chemical compounds used full in anti-inflammatory, fungal infection, viral infection, bacterial infection, parasite infection, clotting of blood plasma, analgesic, anti-apoptotic, anti-oxidant, improves immune responses, promotes collagen synthesis & regeneration of tissue. These properties are important in wound healing.

Methodology: Assessment of biopsy from wound site will be done by Epithelialization, Vascularity, Fibroblastic cell proliferation, Leucocytic infiltration, Appearance of granulation tissue. Histological parameters for calculating healing score will be granulation tissue quantity, inflammatory infiltrate, orientation of collagen fiber, collagen pattern, early collagen quantity, quantity of mature collagen.
Result: - In this study we will evaluate quantification of granulation tissue, gradation of inflammatory infiltrates, quantification & pattern of collagen after local paste application of Ehretia Laevis Roxb. compared to topical Phenytoin. Ehretia Laevis Roxb. leaves paste application promotes the growth & proliferation of cell of healing & repair epithelial and stromal at the site of mechanical wounds.

Conclusion: Conclusion will be drawn with reference to discussion

Background:

Plants introduction: - Ehretia laevis Roxb.is known as: ovate-leaved ivory wood, In Gujarati: Vadhavaradi , In Hindi: bhairi and chamror, In Konkani: kalo gamdo, In Malayalam: Caranti, Marathi: Datrangi( It colors teeth in red, Ajanvruksa (Sant Dnyaneshwar Maharaj from Alandi Maharashtra India took the Sanjivam Samadhi at the base of this tree).

In Ayurveda, It is mentioned for Prameha ( In Diabetics) and Vishaghna (As a anti-venom) (Su.Chi 11/10, Su.Kal.5/46, and Cha.Chi.23/66).

Taxonomical classification:-

**Botanical name**–Ehertia Laevis Roxb.

**Classifications**

Plantae $\Rightarrow$ Tracheophyta $\Rightarrow$ Magnoliopsida $\Rightarrow$ Boraginales

Boraginaceae $\Rightarrow$ Ehretia $\Rightarrow$ Ehretia Laevis (Roxb.)

**Habit**: It is a small tree.

**Leaves**: Its leaves are very small alternate, elliptic-oblong, entire, glabrous and nearly so.

**Flowers**: Its flowers are white in colour and up to 8-10 mm across.

**Flowering and Fruiting time**: It is from January to April

**Fruits**: Its fruits are small drupe, at first red and then black.

**Chemical Composition of various parts of Ehretal Laevis Roxb. plants are as follows**-

Naphthoquinone derivative lewisone, n-octatricontane, baurenol acetate, baurenol, ursolic acid (1). Proteins, Amino acids, Lipids Minerals such as Ca, Mg, Na, Fe, NH3, Mn, P, K, Zn, Cu and Si, Gallic acid, tannins, Vit C, rutin , decanoic acids, acontanes ,phthalic acid, phenylephrine, $\alpha$ and $\beta$ amyrin, phytol, piperazine, (2). Benzoquinones: - 1,4naphthoquinone lewisone, Bauerenol acetate, Bauerenol , $\alpha$-amyrin, Lupeol, Betulin, Betulinic acid, $\beta$-sitosterol(3). Dodecanol, Tetradecane , Tridecane , n Octylcyclohexane, Hexadecane , Tridecanol Decyl cyclohexane , Nonadecane , Heptadecane , Tetradecanone (4). Di – n octyl phthalate (5). Amino acid- Butyric acid, Ornithine, Cysteine, Arginine, Serine, Histidine, Lysine, Glutamic acid, Proline, Hydroxy proline, Tryptamine having various therapeutic properties (6).

3,7,11,15-Tetramethyl-2-hexadecen-1-ol ; Hexadecanoic acid, 15-methyl-, methyl ester ;Tridecanolic acid ; Propionic acid,3-(1-hydroxy-2-isopropyl-5* methylcyclohexyl)Methyl 2-hydroxy-octadeca-9,12,15-trienoate; 1,2,15,16-Diepoxyhexadecane ; 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl ; 8,11-Octadecadienoic acid, methyl
ester; Methyl 8,11,14-heptadecatrienoate; 5-Chloro-3beta-hydroxy-6beta-nitro-5alpha-androstan-17-one; 12,15-Octadecadienoic acid, methyl ester

Propane, 1,2-dichloro-2-methyl, 1-Chloro-2-ethoxy-2-methoxy-propane, 4-Chloro-2,4-dimethylhexane, dodecamethyl, Cyclohexasiloxane, Cycloheptasiloxane, tetradecamethyl, Phenol, 2,4-bis(1,1-dimethylethyl), Cyclooctasiloxane, hexadecamethyl; 1,1,3,3,5,5,7,7,9,9,11,13,15,15-hexadecamethylloctasiloxane; 3,7,11,15-Tetramethyl-2-hexadecen-1-ol; 9-Eicosyne; 1,2-Benzenedicarboxylic acid, butyloctyl ester; isobutyl octadecyl ester; Oxirane, Phthalic acid, hexadecyl-; Methyl 6,10-octadecadienoate; Phthalic acid, octyl 2-propylpentyl ester; 9,10-Dimethyltricyclo[4.2.1.1(2,5)]decane-9,10-diol; 9,12,15-Octadecatrienoic acid, (Z,Z,Z)-

Z,Z-4,16-Octadecadien-1-ol acetate; Silane(pregn-5-ene-3a,11a,17,20a-tetrayltetraoxy)tetrakis[trimethyl-(5a)Pregnane-3,20a-diol; 4-(Dimethylaminomethyl-5-hydroxybenzofuran-3-y1)(4-methoxyphenyl)methanone; Benzoic acid, 2,6-bis [(trimethylsilyl)oxy]-trimethylsilyl ester Chromone, 5-hydroxy-6,7,8-trimethoxy-2,3-dimethyl-; 6,7-Epoxy pregn-4-ene-9,11,18-triol-3,20-dione; 2-Trimethylsiloxy-6-hexadecenoic acid, methyl ester; 5,8,11,14-Eicosatetraynoic acid, trimethylsilyl ester; 2,7-Diphenyl-1,6-dioxopyridazine[4,5'-2',3']pyrrol o[4',5'-d]pyridazine; 2,15-Heptadecadiene, 9-(ethoxyethyl); 3,9-Epoxy pregn-16-ene-14-18-diol-20-one, 7,11-diacetoxy-3-methoxy-Ethyl iso-allocholate; 4-Methoxyphenoxoyformamide N-methyl-N-[4-(1-pyrrolidinyl)-2-butenyl]; Phthalic acid, butyl oct-3-yl ester; Hexadecanoic acid, 14-methyl-, methyl ester Methyl 9,12-epithio-9,11-octadecanoate; 2,7-Diphenyl-1,6-dioxopyridazino[4,5'-2',3'] pyrrolo [4',5'-d] pyridazine. Methyl 4,7,10,13,16,19-docosahexaenoate; (8)

Other specified therapeutic advantages

1. Decoction of the fresh root is used in syphilis & decoction of bark of stem used in diphtheria. Tender leaves paste is used in eczema. Crude powder of flowers mixed with milk is used as an aphrodisiac. Leaves paste is used in ulcers and in head ache. Fruit are used as, antihelmentic, diuretic, demulcent, expectorant and used in urine infection, It is also used in diseases of spleen & lungs. Seeds are used as antihelmintic. (8)

2. Phenytoin:- Topical Phenytion promotes healing of wound by fibroblast proliferation stimulation, collagen deposition facilitation, antagonism of glucocorticoid, and antimicrobial activity. Also Phenytoin enhances healing of necrotizing soft tissue wound which was unresponsive to conventional treatment. (9)

The first use of Phenytoin was in 1937 for disease of convulsive disorders.

The one of the side effect of phenytoin is fibrous overgrowth of gingiva. This side effect of on connective tissue may be used in wound healing.

The healing process contains inflammatory responses, regeneration of the epidermis, wound shrinkage, connective remodelling & tissue formation. On 4th day new granulation tissue forming starts with new capillaries. Fibroblast migrates into the wounds and begins the synthesis of the extracellular matrix.

The use of phenytoin in treatment of wound is still not confirmed. Also the mechanism of wound healing by phenytoin is not clear. Some trials shows topical phenytoin enhances the
healing process: fibroblastic proliferation stimulation, enhancement of granulation tissue, decreasing collagenase activity, promoting of collagen deposition with other connective tissue components, antimicrobial activity and reducing wound exudate. Wound biopsies treated by phenytoin show neovascularization and collagenization. Also it has anti microbial activity, Local pain relief action by membrane-stabilizing.

The above findings taken into consideration for application of phenytoin, for promotion of healing process in wound in the designed study in a control group.

**Antimicrobial activity of Ehretia Laevis Roxb.**

This Plant has antimicrobial activity on Staphylococcus aureus gram-positive & Escherichia coli gram-negative. \(^{(11)}\), Staphylococcus aureus and Pseudomonas aeruginosa gram-negative \(^{(12)}\)

Acetone extracts of E. leavis have higher antimicrobial activity than M. pubescens extracts at lower concentrations against human salivary microflora. \(^{(13)}\)

Methanol, chloroform and Aqueous extract of Ehretia Laevis Roxb. also shows significance antimicrobial activity on Escherichia coli gram-negative, Staphylococcus aureus gram-positive, Pseudomonas aeruginosa gram-negative, Bacillus subtilis gram-positive up to 50 (µg/ml) \(^{(14)}\)

- Stages of wound healing

1. Clotting phase:- At beginning of wound healing clot forms to stop bleeding. Then it is followed by neutrophil invasion.
2. Inflammation phase:- Phagocytic & macrophages cells kill bacteria. Debridement of damaged tissue. Growth hormones releases causes enhancement of fibroblasts, epithelial & endothelial cells to form new blood vessels.
3. Proliferative phase:- Fibroblast produces large amount of type III collagen, this causes filling of defect. Granulation tissue shifts from the border of wound to centre of wound.
4. Maturation phase:- Extra vessels formed in granulation tissue are eliminated by apoptosis process. Collagen of type III is replaced by type I.

One traditional plant species is being practised wound ulcer, arthritis pain and minor fractures by local tribes in Maharashtra India, which is now identified as Ehretia Laevis Roxb and in ayurveda it is known as Charma Vruksha. \(^{(15-21)}\) Local tribes of Wardha District use the freshly crushed leaves paste of Ehretia laevis Roxb.(Khandu Chakka) from several years for controlling the joint pain, body ache, fractures and wound healing. \(^{(8)}\) Several studies has been done so far on this plant species related to pain, wound healing and anti microbial activities. Histological study of wound tissue for wound healing potential of Ehretia Laevis Roxb. plant in burn is still not done. Hence it very necessary to know about histological changes in wound tissue by application of Ehretia Laevis Roxb. leaves paste in burn wound against standard.

The main aim of this exersie is to study the comparative wound healing potential & histological changes by Ehretia Laevis Roxb. and Phenytoin in burn wound, which will be helpful to provide the cheap convenient and alternative medicine for wound healing.
Rationale:

Its folklore claim of wound healing property has been confirmed on scientific basis. Histological study of wound tissue for wound healing potential of *Ehretia Laevis Roxb* plant is still not done. Hence it very necessary to know about histological changes in wound tissue by application of *Ehretia Laevis Roxb*. leaves paste on wound against control. It has many chemical compound which shows anti oxidant, protective effect on blood vasculature, wound healing, inhibit cellular senescence, angiogenic, promotes collagen which maintains the structure and strength of connective tissue.

**Burns** are one of the major health problems. The majority of deaths occur in low and middle-socio-economical countries. World health organization estimates about 2,65,000 deaths in each year from fires.

In India every year, around 70,000/- people get burn injuries. And 1, 40,000 deaths and 2, 40,000 peoples affected by disability.

Deaths due to burn are less in developed countries. Burn is 11th leading cause of death in 1-9 yrs age groups. Infants have the highest death rates by burns. Burns injuries are also a leading cause of morbidity.

Many expensive antibiotics are in use to treat the burn wound which specifically not affordable by rural tribes. In addition, development of resistance from broad-spectrum antibiotics have also increased the risk. There is only single study on wound healing in human being, but this drug not tested in burn wound for healing and histological study of wound healing in animal model. It will be helpful to provide the cheap convenient and alternative medicine for wound healing. This plant contains may chemicals having many medicinal uses in various medical conditions.

**Aim:**

To assess the comparative histological changes for wound healing potential of *Ehretia Laevis Roxb*. plant laeves paste on animal model against standard

**Objective:**

1. To assess the histological changes for wound healing potential of *Ehretia Laevis Roxb*.plant leaves on animal model
2. To assess the histological changes for wound healing potential of phenytoin ointment on animal model
3. To compare outcome of both the studies.

**Hypothesis:** *Ehretia Laevis Roxb*. leaves paste application promotes the growth & proliferation of cell of healing & repair( epithelial and stromal at the site of mechanical wounds).

**Methodology:**

**Study Material:**

- Healthy 10 Rabbits will be selected from DMIMS animal house. Then they will be divided into two groups of 5 in each group.
Leaves paste will be prepared under all aseptic precautions. Leaves paste will be applied on wound in first group and considered as Group A.

Phenytoin ointment as a control will be used in second group and considered as Group B.

Animals will be anesthetized before creating wound.

The skin will be shaved, disinfected with 70% spirit and then injected with 1 ml of Lignocaine HCl (2%, 100 mg/5 ml) for local anaesthesia.

Approval from institutional animal ethical committee will be taken.

External wound protocols

- Physically external wound will be made by burn injury of 2X2cm. The edges of the wound will be sampled by forcep biopsy.
- Daily leaves paste will be applied in Group A and Local Phenytoin ointment in group B. Biopsies for assessment of healing & repair will be taken on 0, 7th and on 15th day.

Place of study:

1. Mahatma Gandhi Ayurved College Hospital and Research Centre, Salod (Hi) Wardha (MS) under the ambit of Datta Meghe Institute of Medical Sciences (Deemed to be University) Wardha (MS).

2. Animal house of Datta Meghe Institute of Medical Sciences (Deemed to be University) Wardha (MS).

3. Department of pathology Jawaharlal Nehru Medical College under the ambit of Datta Meghe Institute of Medical Sciences (Deemed to be University) Wardha (MS).

Assessment of biopsy from wound site was done by following histological features.

- Epithelialization
- Leucocytic infiltration
- Vascularity
- Fibroblastic cell proliferation
- Appearance of granulation tissue

Histological Parameter

The following histological parameters will be used to calculate healing score.

1. Granulation tissue (profound – grade- 1, moderate – grade- 2, scanty- grade -3, absent- grade- 4)
2. Inflammatory infiltrate (plenty – grade- 1, moderate – grade -2, a few- grade- 3 )
3. Collagen fiber orientation (vertical - grade -1, mixed - grade -2, horizontal- grade -3)
4. Pattern of collagen (reticular – grade- 1, mixed- grade- 2, fascicle- grade -3)
5. Early collagen (profound- grade- 1, moderate – grade- 2, minimal- grade- 3, absent- grade -4)
6. Mature collagen (profound- grade- 1, moderate – grade- 2, minimal- grade -3)

Histological assessments of wound were Semi Quantitative and Quantitative methods as described below.
**Semi-quantitative method:** - Wound reepithelialisation, Migration of keratinocytes, Bridging of cells, keratinisation, Inflammatory cells: absence/ Presence: (mild/ moderate/ marked), Fibroblasts: absence/ presence(mild/ moderate/ marked). New vessels: absence/ presence(mild/ moderate/ marked), Collagen: absence/ presence(mild/ moderate/ marked)

**Quantitative method:** - Polymorpho nuclear leucocytes/ tissue macrophages ratio, reepithelialisation percentage, granulation tissue area

### Study Design

**Expected Results:**

*Ehretia Laevis Roxb.* leaves paste application promotes the growth & proliferation of cell of healing & repair (epithelial and stromal at the site of mechanical wounds).

**Discussion:**

A number of articles in modern medicine related to wound healing are reported [31,32,33]. Kambale et al conducted a comparative study on wound healing using placentrex, collagen, vitamin c and insulin [34]. Dasari and Dhaniwala reported studies on management of traumatic wounds of hand and foot [35]. Dhaniwala reported platelet-rich plasma as an ‘elixir’ for wound healing [36]. Kable et al reported on effect of oral vitamin d supplements on wound healing in
patient with diabetic foot ulcer\footnote{37}(38). Discussion will be done on the basis observations and results & number of articles related to topic.

**Conclusion:** Conclusion will be drawn with reference to discussion.

**References:**


[16] http://envis.frlht.org/plantdetails/e0561ae6874616e01764bf1f46709e3c0/1ca7a00459bf9 a5451ddd6fdfe856acd ( cited on 2.6.2020)

