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Evaluation of Anti-Microbial, Anti-Fungal and Wound Healing Activity of Ointment Formulations of Methanolic Extracts of Acalypha Indica and Piper Betel Leafs in Rats

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KEYWORDS

antimicrobial activity, anti-fungal activity, wound healing, *Acalypha Indica*, *Piper Betel leaf*, Hemostasis, Inflammation, Proliferation and Re-modeling.

ABSTRACT:

Plants have immense potential activity to treat and prevent different types of aliments. Herbs have been in use for thousands of years in Ayurveda and only a few have been scientifically validated for their molecular mechanisms. Wound healing is an important research aspect where rapid healing is desirable to balance the physical performance and health of the subjects. From ancient times plants were being used traditionally to treat several aliments including different types of wounds. Wound refers to an injury that damages the normal integrity of the skin. In wound healing a complex process involved like Hemostasis, Inflammation, Proliferation and Re-modeling phases. Certain plants are known to poses potential wound healing activities and many of them were not scientifically evaluated. The wound healing activity of plant extracts maybe due to specific type of phytoconstituents present in them. In the present study methanolic extracts of *Acalypha Indica* (10% w/w & 20% w/w) and *Piper Betel leaf* (10% w/w & 20% w/w) ointment studied against anti-bacterial, anti-fungal, incision wound and excision wound healing activities. The selected plant extracts showed increase in cell proliferation, faster contraction of wound, increase tensile strength and shortening the duration of healing period. The results were compared with the standard drug (Betadine) ointment. The selected plant extract ointment formulations showed dose dependent effect on wound healing between the two extracts, *Piper Betel leaf* (20%w/w) showed better activity than the *Acalypha Indica*. The polyherbal extract formulation showed very good activity and comparable with that of the standard (Betadine 5%) ointment.

INTRODUCTION:

Plants are having potential agents for prevention and treatment of various disorders and modern science has yielded many life-saving drugs. The biochemical events that are generating at the molecular level in the cell radiate the energy needed to fulfill biological life and its sustainability. Any desires taking place in the biological cycles resulted in cell insult and became a factor in the genesis of various forms of disease [1].

Injuries are linked to major health issues that come at a huge expense to individuals, healthcare systems and communities. Treatment for chronic wounds can cost several thousand to tens of thousands, depending on the type of wound and is expected to see yearly 8.2 million people's visits to hospitals globally. As a result of the limitations of current wound healing approaches, researchers have turned to natural remedies such as medicinal plants to explore their potential in wound management. One of the key advantages of using plant-based wound management strategies is their efficacy in wound treatment with minimal or no side effects.

Wounds generally occur on the surface of the skin which has three main compartments: Epidermis, Dermis and Subcutaneous tissue2. The skin's unique structures, functions and its pathophysiology are crucial to understanding wound healing. The process involved in

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wound healing is mainly by different distinct phases like Homeostasis, Inflammation, Proliferation and Re-modelling [3].

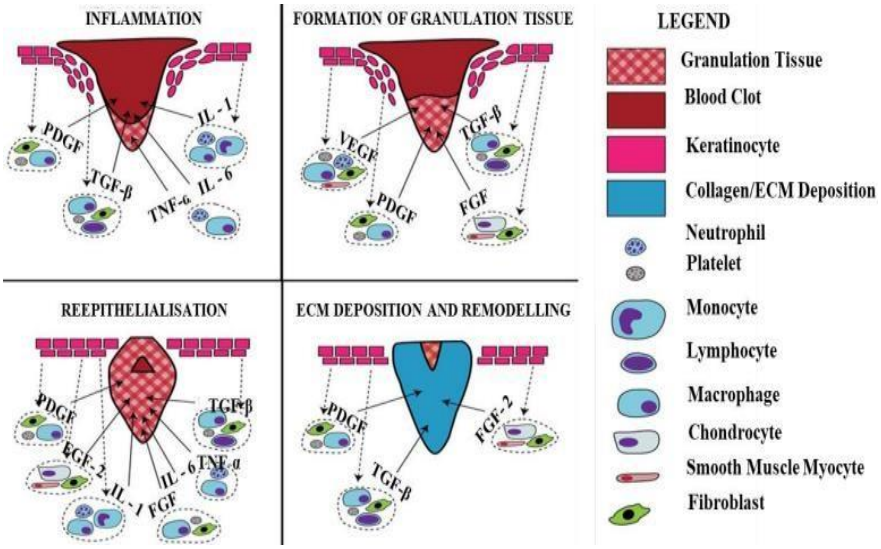

Fig. 1: Factors that contribute to better wound healing and tissue remodeling

MATERIALS AND METODS

Chemicals:

All the chemicals were analytical grade obtained from Sigma chemicals, Loba, Fisher fine chemicals and Qualigens. The plants methanolic extract of *Acalypha Indica* and *Piper Betel leaf* was gifted by Laila Neutraceuticals, Vijayawada, Andhra Pradesh, India.

Preparation of extract and ointment:

The plants were collected, cleaned neatly with water to remove the dirt and sand, shade dried and powdered. The dried powders were extracted by maceration method for five days with 95% methanol, 100 gm of plant powder was weighed and added with 500 ml of 95% methanol solution (1:5 ratio) 4. The extracts were concentrated and dried under vacuum. The dried extracts were used to perform further studies. The two extracts were formulated in an ointment base (Wool fat, hard paraffin, cetylalcohol & yellow soft paraffin) in single and in combination. The formulations were made as 10% w/w and 20% w/w with selected plant extracts.

Phytochemical Testing:

After complete the total evaporation of methanolic extract of plant materials of *Acalypha indica* and *Piper Betel leaf* were used for preliminary phytochemical testing to check for the presence of secondary metabolites like flavonoids, alkaloids, tannins, phenolic compounds, saponins, fixed oils, and fats [5].

Antimicrobial Activity:

Determination of Antibacterial Activity:

Media preparation:

Suspend nutrient agar powder of 11.5 g in distilled water of 500ml. To fully dissolve all components heat this mixture while stirring. Sterilize the medium by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow it to cool but not solidify, once the nutrient agar has been autoclaved. Nutrient agar was poured into each and every plate and plates were left on the sterile surface until the agar has solidified.

Test bacterial strains:

The clinical bacterial isolates such as Bacterial strain of Gram positive type *S. Aureus, B. Subtilis* and *S. Werneri.* Gram negative type *E. Coli, P. Aurgenosa* and *P. Putida.*

Determination of Zone of Inhibition by Agar Diffusion Method:

Agar well diffusion method was used to test the Antibacterial activity of plant extract. For this, agar plates were prepared separately and overnight culture of test bacterial strains were seeded individually over the surface of agar plates. Sterile cork borer was used to puncture 6mm diameter wells over the agar plates. The extracts were prepared by dissolving in DMSO. 50µL of extract were then filled in wells. Gentamycin (25µg/ml) was used as positive control. Incubation of

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the plates was done at 37°c for 24 hrs in triplicate. The extracts antibacterial activity was determined by measuring the diameter of zone of inhibition around the well filled with the extracts [6].

Determination of Antifungal activity:

Media preparation:

Suspend dextrose sabouraud agar medium (5.5 grams) in distilled water of 200 ml. later boiling was done to dissolve the medium completely. By autoclaving at 15 lbs pressure (121°C) for 15 minutes sterilization should be done.

Test fungal strain:

The clinical fungal isolates such as *Candida albicans*

Determination of Zone of Inhibition by Agar Well Diffusion Method:

Antifungal activity of the extracts was carried out using agar well diffusion method. sabouraud dextrose agar plates (SDA) media was prepared to determine the antifungal activity. 72 hrs culture of fungal strain were seeded over the surface of SDA plates with sterile cotton swab. Over the agar plates using sterile cork borer, approximately 6 mm diameter wells were punctured. The extracts were prepared by dissolving in DMSO. The wells were then filled with 50µL of extracts. Flucanozole (25µg/ml) was used as standard drug. The incubation of the plates was carried at 37°c for 48 hrs. The assay was carried out in triplicate. The extracts antifungal activity was determined by measuring zone of inhibition diameter around the well filled with extracts.

ANIMALS AND GROUPING:

Rats of either sex weighing about 150 – 200 gm were used for the study. Five animals were used in each group. All the animals are properly caged and maintained under standard diet and water ad libitum, placed in a properly air conditioned room with 12hrs light and 12hrs dark.

Group-1 was treated without ointment base, Group-2 was treated with ointment base, Group-3 was treated with standard (Betadine 5%), Group-4 was treated with *Acalypha Indica* 10% w/w, Group-5 was treated with *Acalypha Indica* 20% w/w, Group-6 was treated with *Piper Betel leaf* 10% w/w, Group-7 was treated with *Piper Betel leaf* 20% w/w, Group-8 was treated with *Acalypha Indica* 10% w/w & *Piper Betel leaf* 10% w/w and Group-9 was treated with *Acalypha Indica* 20% w/w & *Piper Betel leaf* 20% w/w ointment preparations were locally applied [7].

Incision wound model:

Animals were selected and shaved neatly the hair one day before to perform the incision process, made one parallel 6 cm paravertebral incisions through the full thickness of the skin, 1cm lateral to the mid line of the vertebral column after giving anesthesia 8. Closure of the wound was carried out with interrupted sutures 1cm apart with the help of suturing needles (curved needle) and using the silk thread for stitching. After the stitches the animals are placed individual cages. Completion of the 10th day of the process removal of the sutures was done. On the post wounding day in an anesthetized rats wound breaking strength (WBS) was measured. Standard weights were put slowly and steadily into the S-shaped hook. A gradual increase in weight was transmitted to the wound side hook apart the wound edges. The weight was stopped and recorded as and when the wound was just opened up. The procedure was carried out and three readings were recorded and the repetition of entire procedure was done. The mean reading of the group was taken as an individual value of breaking strength. Average value indicates the breaking strength for respective group.

Excision Wound Model:

Rats were fasted overnight before the process, anaesthetized with Lignocaine and a wound was made in area about 400 mm2. Full thickness of the marked skin was cut carefully and removed the skin. The measurement are taken on 1 mm2 graph paper, initially on the first day of wounding and up to complete wound was completely healed at a gap period of 3 days. Changes in the wound area were periodically measured and the wound contraction rate was calculated by the following formula given below

Percentage Wound Concentration = 100 – [Final diameter (cm)/Initial diameter (cm) X 100]

Significance in test treated groups wound healing was obtained by comparing the area of the wound healed on respective days with the negative control groups. The epithelisation period was recorded [9].

STATISTICAL ANALYSIS:

Statistical analysis was carried out by using either one-way analysis of variance (ANOVA) or unpaired t-test

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and then followed by post tests like Dunnett's test for multiple comparisons.

RESULTS:

In vitro evaluation

Table 1: In vitro evaluation of plants methanolic extracts ointment formulations for topical application

Assessed Parameters	*Acalypha Indica*		*Piper Betel leaf*	
	10%w/w	**20%w/w**	**10%w/w**	**20%w/w**
Colour	Light green	Light green	Dark green	Dark green
Solubility	Water, ethanol and Methanol	Water, Ethanol and Methanol	Methanol, ethanol and Warm Water	Ethanol, Methanol and Warm Water
Odour	Aromatic	Aromatic	Aromatic	Aromatic
Homogenecity	Homogenous in nature	Homogenous in nature	Homogenous in nature	Homogenous in nature
Wash ability	Washable with Water	Washable with Water	Washable with Water	Washable with Water
Texture	Smooth	Smooth	Smooth	Smooth
Stability at 4ºC, 25ºC & 37°C	Stable	Stable	Stable	Stable
pH	5.4	5.4	5.7	5.4

Antimicrobial activity:

Table 2: Antimicrobial and antifungal activity of Methanolic extract of *Acalypha Indica* and *Piper Betel leaf*

Bacteria	**Microorganism**	**Control (DMSO)**	***Acalypha Indica***		***Piper Betel leaf***		**Gentamycin (25 ug/ml)**
			100 mg/ ml	200 mg/ ml	100 mg/ ml	200 mg/ ml	
Gram positive bacteria	*Staphylococcus aureus*	7.21 ± 0.45	11.50 ± 0.55	13.36 ± 0.55	14.03 ± 0.87	18.01 ± 0.15*	15.21 ± 0.12
	Bacillus subtilis	6.23 ± 0.36	13.56 ± 0.41	15.53 ± 0.40	14.32 ± 0.58	18.04 ± 0.15*	19.23 ± 0.25
	Staphylococcus werneri	7.26 ± 0.45	9.26 ± 0.25	12.90 ± 0.85	13.40 ± 0.68	16.13 ± 0.28*	21.36 ± 0.55
Gram negative bacteria	*Escherichia coli*	8.10 ± 0.35	12.43 ± 0.51	14.56 ± 0.49	16.02 ± 0.23	20.10 ± 0.43*	22.13 ± 0.15
	Pseudomonas aeruginosa	6.45 ± 0.54	8.26 ± 0.31	12.53 ± 0.41	13.04 ± 0.65	15.23 ± 0.77*	15.11 ± 0.10
	Pseudomonas putida	8.22 ± 0.42	12.56 ± 0.62	16.31 ± 0.44	13.31 ± 0.33	17.26 ± 0.57*	17.43 ± 0.41
Fungal strain	*Candida albicans*	7.54 ± 0.63	11.40 ± 0.15	14.20 ± 0.56	11.82 ± 0.15	15.80 ± 0.42*	17.40 ± 0.21

Values are expressed as mean ± SEM; n=3; * $p < 0.05$.

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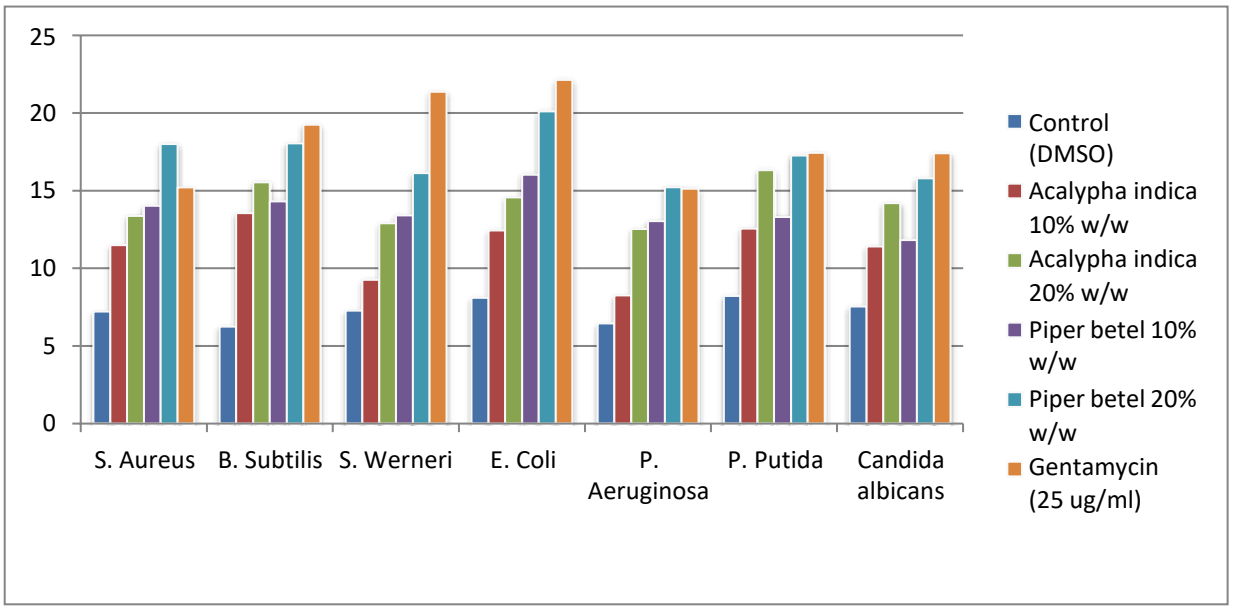

Fig. 2: Antimicrobial and antifungal activity of Methanolic extract of *Acalypha Indica* and *Piper Betel leaf*

Incision wound healing Model:

Table 3: Effects of Methanolic extracts ointment of *Acalypha Indica* and *Piper Betel* leaf on Incision wound healing model

Groups	**Period of Epithelialisation (mean time in days)**	**Tensile strength (gm)**
Group – 1	23.08 ± 0.16	190.25 ± 0.31
Group – 2	21.58 ± 0.16	200.25 ± 0.11
Group – 3	09.56 ± 0.15*	350.35 ± 0.05*
Group – 4	21.21 ± 2.22	229.45 ± 1.20
Group – 5	19.45 ± 1.19	239.69 ± 1.45
Group – 6	18.45 ± 0.19	250.25 ± 1.20
Group – 7	15.80 ± 0.76	255.65 ± 1.65
Group – 8	15.35 ± 0.98	272.25 ± 2.80
Group – 9	12.21 ± 0.16	288.69 ± 1.43

Values are expressed in terms of mean ± SEM; n=5, * $p < 0.05$.

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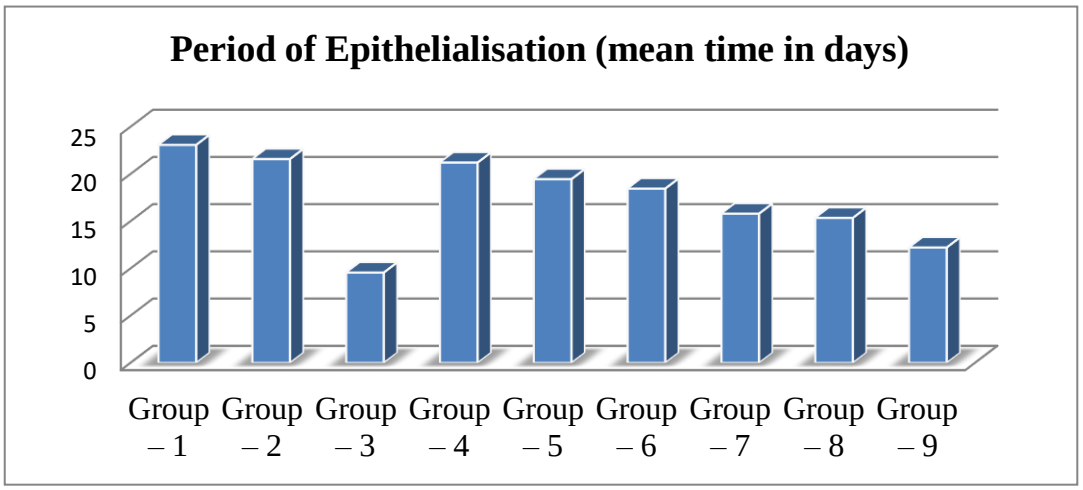

Fig. 3: Epithelialization period of Methanolic extracts ointment of *Acalypha Indica* and *Piper Betel leaf* on Incision wound healing model

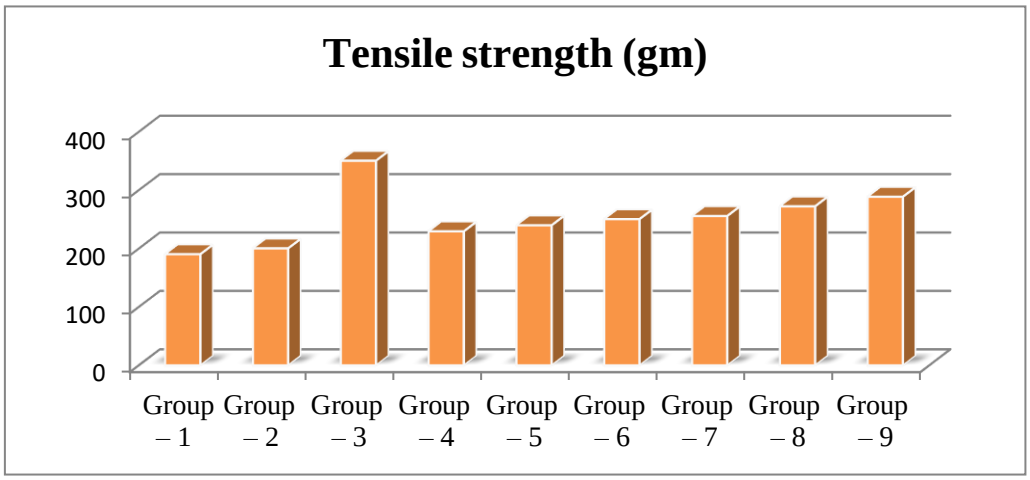

Fig. 4: Tensile strength of Methanolic extracts ointment of *Acalypha Indica* and *Piper Betel leaf* on Incision wound healing model

Excision wound healing Model:

Table 4: Effect of Methanolic extracts ointment of *Acalypha Indica* and *Piper Betel leaf* on Excision wound healing model

Groups	Period of epithelialization (in days)
Group – 1	24.16 ± 0.41
Group – 2	23.06 ± 0.04
Group – 3	13.06 ± 0.60*
Group – 4	20.05 ± 0.34
Group – 5	19.08 ± 0.72
Group – 6	19.01 ± 0.58

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Group – 7	18.34 ± 0.64
Group – 8	18.05 ± 0.89
Group – 9	15.72 ± 0.90

Values are expressed in terms of mean ± SEM; n=5, * $p < 0.05$

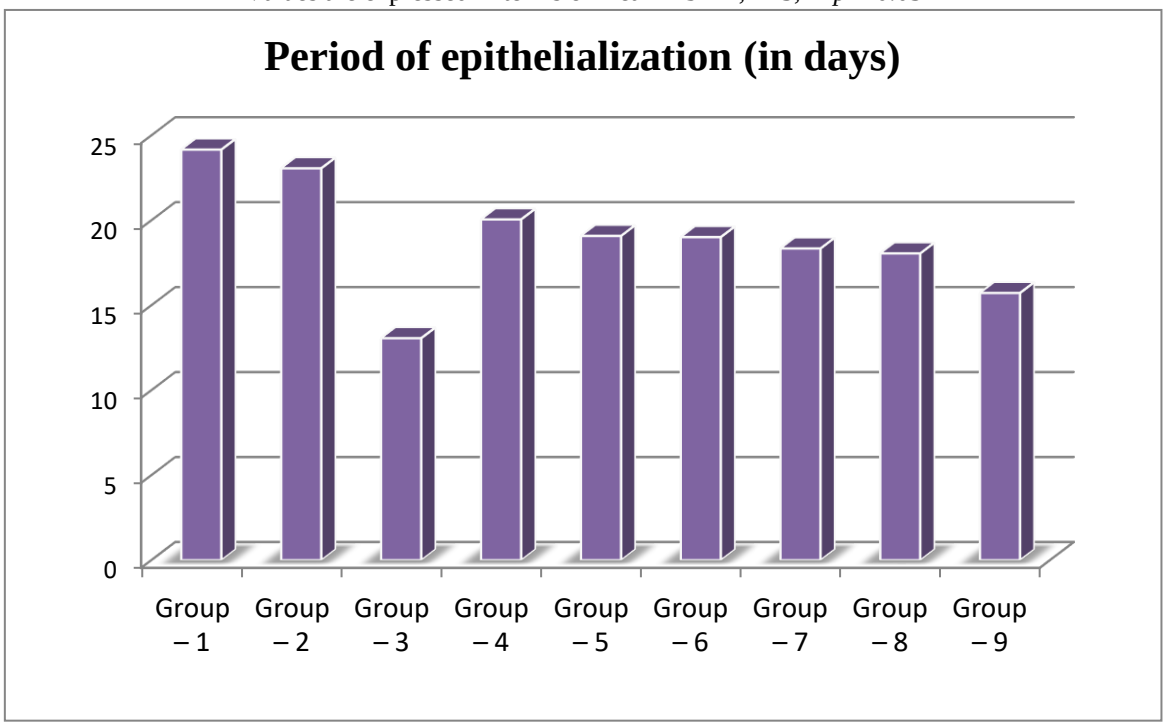

Fig. 5: Epithelialization period of Methanolic extracts ointment of *Acalypha Indica* and *Piper Betel leaf* on Excision wound healing model

DISCUSSION & CONCLUSION:

In vitro evaluation

Hence, in the current study, different formulations were prepared using methanolic extracts of *Acalypha Indica* and *Piper Betel leaf*. The extracts were assessed for their homogeneity (Table.1), like color- light and dark green, odour- aromatic, Solubility- soluble in water, ethanol, and methanol, Texture: having smoothness, Stability: stable at 4oC, 25oC and 37oC; pH: 5.4 to 5.7, which is closely related to skin pH and wash ability: washable with water [10].

Antimicrobial activity:

In the present study, the antimicrobial activity of methanolic extracts of *Piper Betel leaf* and *Acalypha Indica* was assessed at different doses of 100 mg/ml and 200 mg/ml against gram-positive bacteria, such as Bacillus subtilis, S. aureus, and S. werneri, and gram-negative bacteria, such as P. aeruginosa, E. coli, and P. putida, as well as fungi, such as Candida albicans, the typical medication for comparison against the standard drug (Gentamycin) (Table.2). When these extracts were evaluated for antibacterial activity, the methanolic extract of *Acalypha indica* were shown to be less effective against bacteria than the extract of *Piper Betel leaf* [11].

Incision Wound Model:

In incision model without the application of ointment base the wound healing took place 23.08 ± 0.16 days. The tensile strength was also low (190.25 ± 0.31 gm). In simple ointment base group-2 the wound healing took place 1.5 days early. The results clearly indicated ointment base alone showed no marked effect on wound healing and which ruled out significant wound healing

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effect on ointment base (table-3). The standard ointment Betadine (5%) showed wound healing effect on 9.56 ± 0.15 days, indicate rapid epithelialization and collagenization. Increase tensile strength indicating increase collagen, strength and facilitating wound healing. The low (10%) concentration of *Acalypha indica* and *Piper Betel leaf* extract ointment formulation treatment showed very mild improvement in wound healing effect and where as 20% extract ointment treatment produce more effect on rapid epithelialization and wound healing effect.

The *Piper Betel* extract ointment showed good wound healing effect than the *Acalypha indica* formulation. The results clearly indicated that the influence of extracts on wound healing mechanism and rapid repair process of the wound. Further, the dose dependent effect on wound healing activity maybe due to the high concentration of phytochemical present in the formulation.

The two extracts combination formulation also showed dose dependent wound healing activity (Group 8 and 9). Both the groups (10% w/w and 20% w/w ointment, table: 3) showed good wound healing activity than the single plant extract formulations. 20% w/w concentration of *Acalypha indica* and *Piper Betel leaf* extract formulation showed good healing activity and its period of epithelialization is close with that of the standard drug effect. The tensile strength also increased. The results clearly indicated positive activation of all phases of healing processes, rapid epithelialization, collagenization and increase in collagen facilitating wound healing [12].

Excision Wound Model:

In excision wound healing model, ointment base applied to group-2 animal showed mild healing activity when compared to without ointment base applied group-1 animals and shown by 1.2 days advancement in wound healing. Individual plant extract formulations are shown less activity in wound healing processes when compared with the standard drug treatment (Betadine 5%) group of animals (13.06 ± 0.60 days). Here also the formulation showed dose dependent effect on wound healing in days (table. 4).

The combination of *Acalypha indica* and *Piper Betel leaf* extract ointment formulations clearly has shown the dose dependent wound healing effects. The improvement and effects on wound healing maybe due to granulation, fibroblasts, macrophages13, collagen synthesis, neutrophils, and leukocytes14, new extra cellular matrix formation. During the wound development period it induces the extra cellular matrix migration, growth factors15 (Transforming Growth Factor, Interleukin-6) and granulation tissue formation which are involved in the formation of new epithelial cells, blood vessels and connective [16].

Over all the study revealed wound healing properties of plant extracts of *Acalypha indica* and *Piper Betel.* Further the study indicated polyherbal formulations may give better wound treatment effect. Further research is in progress with other potential plant extracts.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICAL APPROVALS

This study includes the animal study for which approval was obtained from Institutional Animal Ethics Committee (013/IAEC/NCPA/PHD/2021-22).

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