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## Evaluation of In-vitro Antimicrobial and Wound Healing Activity of Polyherbal Formulation In Albino Wistar Rats

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### ABSTRACT

In Ayurveda, individual herbs are not sufficient to get the therapeutic effect in a desired way. Toxicity is reduced and therapeutic effect is produced in a better way when the composition with multiple herbs is formulated in a particular ratio. For developing such an intervention, ethanolic extract of *Momordica charantia*, *Nyctanthes arboristis*, *Azadirachta indica* were used to prepare the polyherbal extract. The zone of inhibition of each bacterial strains (against *Bacillus subtilis* and *Pseudomonas aeruginosa*) were measured in triplicate. Three Mixtures of extracts with three herbs in different ratio were used for evaluation of antimicrobial effect. Polyherbal mixture 1 *Bacillus subtilis* ( $14.8 \pm 1.17$ ) *Pseudomonas aeruginosa* ( $12 \pm 1.58$ ) Polyherbal mixture 2 *Bacillus subtilis* ( $18 \pm 1.19$ ) *Pseudomonas aeruginosa* ( $14.2 \pm 1.16$ ). Polyherbal mixture 3 *Bacillus subtilis* ( $25.8 \pm 1.11$ ) *Pseudomonas aeruginosa* ( $22 \pm 1.09$ ). Three polyherbal ointments Polyherbal ointment 1, Polyherbal ointment 2, Polyherbal ointment 3 were prepared by fusion method. Wound closure of PHO3 have increased ( $2.83 \pm 1.16$ ) when compared to PHO 1 ( $13.68 \pm 1.50$ ) and PHO2 ( $5.88 \pm 2.40$ ), the PHO promoted the wound healing process through accelerated remodelling process of damaged tissue. Polyherbal ointment where the ethanolic extract was prepared by maceration method and the wound healing is evaluated by excision where it was assessed by rate of wound contraction. Free formulation developed from the combination of ethanolic extracts of *Azadirachta indica*, *Momordica charantia*, *Nyctanthes arboristis* showed antimicrobial and wound healing effects on various stages on healing cascades. In neem, mainly nimbidin shows antibacterial activity where in bitter melon essential oil obtained from the seed of bitter melon was tested for antibacterial activity. In case of wound healing mainly D-mannitol and flavanoid involved. Neem contains active ingredients like nimbidin, nimbin, nimbidole, with antibacterial, anti-inflammatory that mainly helps in acceleration the wound healing process. Here in Bitter melon, the plant contains cucurbitane, triterpenoids, folic acids, glycolipids helpful for wound healing activities. In case of Night flowering Jasmine D-mannitol flavanol glycosides, tannic acid, ascorbic acid chemical constituents play a role in antibacterial and anti-inflammatory activity.

**Keywords:** *Momordica charantia*, *Nyctanthes arboristis*, *Azadirachta indica*, Polyherbal mixture, Polyherbal ointment, Wound closure

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## INTRODUCTION

Many reasons are there for increased use of the herbal medicines(1).90% of traditional medicine remedies contain medicinal plants that have preventive measures in disease control strategies.(2) Wound is a reversible or irreversible outcome of injury in which the part effected is torn, cut or punctured. This may be due to trauma, surgery or health disorders. The wounds are generally classified according to the depth of tissue loss .The classification is as follows, wounds with tissue loss and wounds without tissue loss . The wounds with tissue loss include burn wound (second and third degree burn wounds), diabetic foot ulcer, etc and wounds without tissue loss include laceration, first degree burn wound etc. Microbes are the major reason for infection and prevalence of the same is high in and around us. The major infection causing bacteria were *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. Epidermidis*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Escherichia coli* (*E. coli*). Once it gains entry into the body, these microbes grow immediately and start to form colonies. The microbes can easily enter the body through the wounds and can reach into deeper portions of the tissue. Furthermore, it can lead to internal infection. For the topical application to intact or broken skin or to mucous membranes ,number of medicaments have been used in the form of various semisolid consistency in the form of ointment creams pastes etc.(3) For the treatment and cure for number of diseases, the herbal origin medicines are the basis.(4). For wound healing inflammation snake bit ,leprosy ,diarrhea like therapeutic purposes many Indian folk medicines consisting of numerous prescriptions.(5) For the treatment of various skin diseases 80% of world's population depends on medicines of traditional origin.(6). For encouraging the suitable moist environment establishment for natural healing process is the main advantage occurring with herbal medicines in wound management(7).From the past time immemorial especially for the treatment of various ailments of skin and disorders of dermatology such as cuts, wounds ,burns.(8). The most common causative agents of wound infections are *Escherichia coli*, *Staphylococcus aureus* that is the rationale behind their selection. bacteremia, septicemia, and wound infection in hospitalized patients, especially patients who had the impaired immune system is caused by *Bacillus subtilis*.

## MATERIALS AND METHOD

### **Plant collection, authentication and extraction**

The leaves of plants (Neem, Bitter gourd, Night flowering jasmine) were collected from the local area of Visakhapatnam. Leaves of the plant were collected thoroughly with distilled water and shade dried for 10 days.

Dried leaves were ground into powder form where 23g powder was imbibed with 350 ml of 90% ethanol for 3hrs and transferred to percolator with additional of 150ml of 90% ethanol for maceration for 7 days with occasional stirring.

#### **Preparation of polyherbal ethanolic extract:**

(N,BJ,BJN,JNB) in proportion of 1:2:3,2:3:1,3:1:2 ratio respectively were taken on the basis of their presently reported individual anti-microbial efficacy and previously reported antimicrobial efficacy. (9) By using mortar and pestle each weighed extract mixed properly in a clockwise direction to attain a uniform consistency and obtained final mixture was stored at 4 °C for in vitro and in-vivo study.

#### **In-vitro antimicrobial activity of PHEE:**

##### **Agar well diffusion method:**

**Test microbial strains.** All the test extracts were tested for their effect on gram positive strains (*Bacillus subtilis*; and gram negative strains *Pseudomonas aeruginosa*; by using cup plate method.

##### **Preparation of agar plates and sampling of the test drugs:**

Agar plates were inoculated by streaking the swab of bacterial strains over the entire sterile agar surface for 2–3 times by rotating the agar plate at 60° for uniform distribution of the inoculum. The plates were dried at room temperature under aseptic condition following by boring of 9 mm diameter wells in them.

Test drugs (NEE, BGEE and NFJEE) of concentration viz. 30, 60,120, and 240mg/ml and standard drug Ofloxacin (10µg/ml) were prepared using dimethyl sulphoxide (DMSO) as solvent. The standard and test drugs (100µl) were added in wells by using sterile micropipette. The plates were then incubated at bio-Oxygen Demand (BOD) incubator at 37°C for 24 h. The zone of inhibition of each bacterial strains were measured in triplicate by using calibrated digital Vernier caliper.

##### **Determination of minimum inhibitory concentration (MIC) by broth dilution method:**

To each test tube,100µl of 105CFU/ml of tests train (*Bacillus subtilis*, and *Pseudomonas aeruginosa*) were inoculated with an equal volume of NBM. The tubes were incubated aerobically at 37 °C for 24–48 h. Three control tubes were also maintained for each strain (media control, organism control and extract control). The lowest concentration of the extract that produced no visible growth (no turbidity). in the 24 h when compared with the control tubes was considered as MIC . The MIC was determined against all four selected microorganism separately.

#### **Evaluation of Polyherbal Ointment for Wound Healing Activity in Wistar Rats**

#### **MATERIALS AND METHOD**

Collection of plant materials: Leaves of plants were collected from local area of Vizag.

**Animal Used:**

Healthy adult albino Wistar rats strain weighing 180-250gram were used for the study. The animals were obtained from animal house. On arrival, the animals were placed randomly and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at Temp of 24 and relative humidity of 30-70%. 12:12 light: day cycle was followed; all the animals were allowed to free access to water and fed with standard commercial pelleted rat chaw.

**Preparation of extract**

Leaves of Neem, bitter gourd, and night blooming jasmine were collected and washed thoroughly with the distilled water and shade dried for 10 days. Dried leaves were grind into powder form, 20gm powder was imbibed with 350ml of 90% ethanol for 3 hours and transferred to percolator with addition of 150ml of 90% ethanol for maceration for 7days with occasional stirring. Finally ethanol extract was collected and concentrated to get blackish green residue. The extract was stored in the airtight container at cool and dry place.

**Evaluation of Ointment Formulation:****a. Color and Odour: -**

Color and Odour of all ointments was examined by visual examination. (11)

**b. pH:-**

The pH of ointment formulation was determined by using digital pH meter. 1gm of ointment was dissolved in 100 ml of distilled water and stored for two hours. The measurement of pH of each formulation was done in triplicate and average values were depicted. (12)

**c. Spreadability Test: -**

Spreadability is expressed in terms of time in seconds taken by two slides. To flip out or from cream when placed in between the slides under the direction of certain load. Lesser the time taken for separation of two slides, better the spread ability. It is calculated by using the formula.  $S = M \times L/T$ , where, M = Weight tied to upper slide, S = Spread ability of formulation, L = Length of Glass Slides, T = Time taken to separate the slides. (12)

**Evaluation:**

**Colour and odour:** Physical parameters like colour and odour were examined by visual examination.

**Consistency:** Smooth and no greediness is observed.

**Spreadability:** The spreadability was determined by placing excess of sample in between two slides which was compressed to uniform thickness by placing a definite weight for definite time. The time required to separate the two slides was measured as spreadability.

**Procedure for preparation of herbal ointment:**

a) Initially ointment base was prepared by weighing accurately grated hard paraffin which was placed in evaporating dish on water bath. After melting of hard paraffin remaining ingredients were added and stirred gently to aid melting and mixing homogeneously followed by cooling of ointment base.

b) Herbal ointment was prepared by mixing accurately weighed neem, bitter gourd and night blooming jasmine leaf extracts to the ointment base by fusion method to prepare a smooth paste with 2 or 3 times its weight of base, gradually incorporating more bases until to form homogenous ointment, finally transferred in a suitable container.

**Induction of Excision wound:**

In the present investigation an excision wound was inflicted by cutting away a 500mm full thickness of skin from a traced area and left undressed to the open environment. Wound contraction was measured as percent contraction in each 2 days after wound formation. The wounds were left undressed to the open environment and observed daily. The treatments were applied topically twice a day, starting from the wound induction until complete healing to cover all wounds. Small skin samples were collected on 14th day.

The wound margins were traced on a transparent paper using permanent marker pen and then measured on a mm<sup>2</sup> graph paper (Planimetric method). The reduction in the wound area was expressed as % of the original wound size.

**% of wound closure =  $\frac{\text{healed area (mm)} \times 100}{\text{Original area (mm)}}$**

**Original area (mm)**

Healed area = initial wound area - final wound area

**PLAN OF WORK**

Procurement of albino winter rats (200-250gm body weight). Grouping of animals into 5 groups each contain 2 rats.

**Group 1 Disease control****Group 2 Standard****Group 3 PHO1****Group 4 PHO2****Group 5 PHO3**

All animals are subjected to anesthesia except group 1. The dorsal view side of the rat will be shaved to induce wound. An area of 500mm<sup>2</sup> wound was induced by excision method on 0th day. The wound area was measured on 0th day. All the animals are treated with respective drug

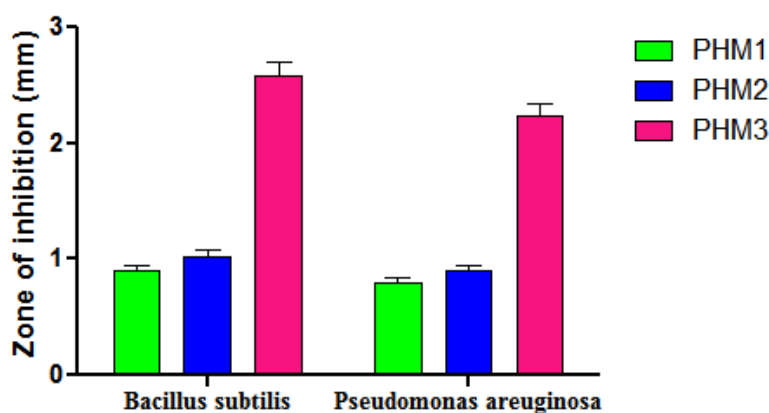
(ointment) twice daily for 14 days. The wound area was measured on 14th day and % wound contraction was calculated

## RESULTS AND DISCUSSION

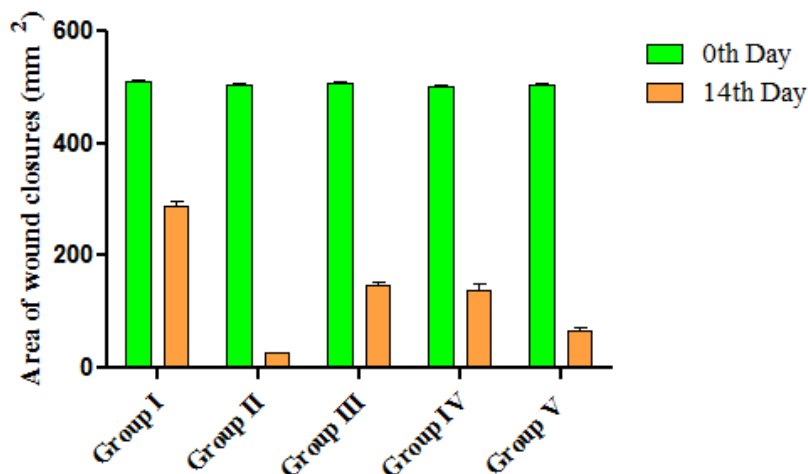
The table No.5 represents the determination of wound area (mm<sup>2</sup>) on 0th day in albino Wistar rats. Treatment with different groups shows dose dependent effect. PHO3 have decreased the wound area better when compared to PHO1 and PHO2 and disease control which is presented in Table No.8, Similarly percentage of Wound closure of PHO3 have increased ( $2.83 \pm 1.16$ ) when compared to PHO1 ( $13.68 \pm 1.50$ ) and PHO2 ( $5.88 \pm 2.40$ ). The Standard framycetin group showed decreased wound area as well as increased % of wound closure among all the groups. The % of wound closures of different groups is depicted in figure 3.



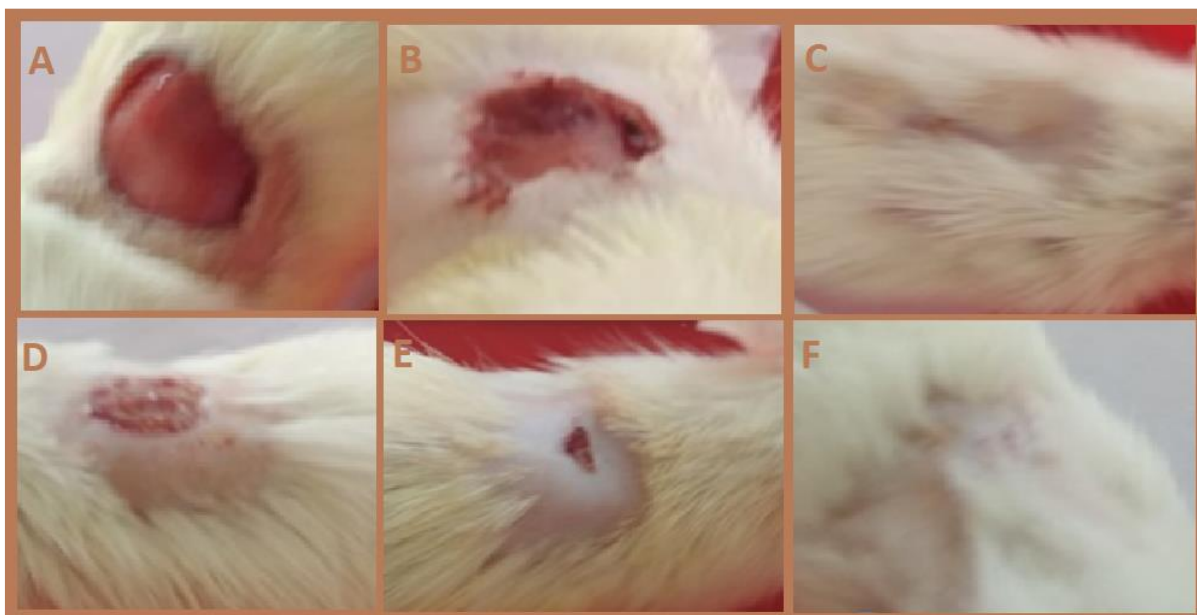
**Figure 1: Zone of inhibition of Polyherbal mixture 1,2,3 against gram +ve & gram -ve Bacterial strains**



**Figure 2: Anti-microbial efficacy of Polyherbal ethanolic extract against Bacillus subtilis and P. aeruginosa at different concentrations**



**Figure 3: The effect of different groups on physical appearance (histology) of wound on 14th day**



**Figure 4: Pictorial representation of excision wound of 0th and 14th day of treatment**

**A –Wound Area Of 0th Day: B-Wound Area of (Disease control) on14th Day: C-Wound Area ofstandardon14th Day D-Wound Area ofPHO1 on 14th Day: E-Wound Area of PHO2 14th Day: F-Wound Area of PHO3 14th Day.**

**Table 1: Determination of Zone of inhibition (mm) of poly herbal mixture Against Bacillus subtilis and Pseudomonas aeruginosa**

	Bacillus Subtilis		Pseudomonas Aeruginosa	
PHM1	0.9	0.04	0.8	0.04
PHM2	1.025	0.06	0.9	0.04
PHM3	2.575	0.13	2.23	0.11

**Table 2: Formulation of Herbal ointment PHO1**

S.NO	Name of Ingredient	Quantity to be taken
1.	Prepared extract of sample 1	0.1gm
2.	Prepared extract of sample 2	0.2gm
3.	Prepared extract of sample 3	0.3gm
4.	Ointment base	10gm

**Table 3: Formulation of herbal ointment: PHO2**

S.NO	Name of Ingredient	Quantity to be taken
1.	Prepared extract of sample 2	0.2gm
2.	Prepared extract of sample 3	0.3gm
3.	Prepared extract of sample 1	0.1gm
4.	Ointment base	10gm

**Table 4: Formulation of herbal ointment: PHO3**

S.NO	Name of Ingredient	Quantity to be taken
1.	Prepared extract of sample 3	0.3gm
2.	Prepared extract of sample 1	0.1gm
3.	Prepared extract of sample 2	0.2gm
4.	Ointment base	10gm

**Table 5: Determination of wound area (mm<sup>2</sup>) on 0th day in albino Wistar rats**

Groups	R1	R2	R3	R4	R5	R6	Mean±SEM
Group I Diseased control	510	512	489	522	498	520	508.50±5.23
Group II Standard	510	498	506	495	516	504	504.83±3.15
Group III PHO1	524	513	508	496	492	502	505.83±4.79
Group IV PHO2	488	502	492	496	508	513	499.83±3.92
Group V PHO3	502	488	492	518	506	510	502.67±4.58

**Table 6: Determination of wound area (mm<sup>2</sup>) on 14th day in albino Wistar rats**

Groups	R1	R2	R3	R4	R5	R6	Mean±SEM
Group I Diseased control	280	325	298	242	302	274	286.83±11.59
Group II Standard	20	32	24	21	34	24	25.83±2.37
Group III NBPH0	132	146	152	178	130	138	146.00±7.25
Group IV PHO2	152	178	138	146	110	105	138.17±11.15
Group V PHO3	84	56	76	61	67	47	65.17±5.50

**Table 7: Determination of % wound closure on 14th in albino Wistar rats**

Group	R1	R2	R3	R4	R5	R6	Mean±SEM
Group I Diseased Control	45.1	36.5	39.05	53.6	39.3	47.3	43.50±2.62



Group II Standard	96.0	93.7	95.2	95.7	92.6	95.2	94.89±0.46
Group III PHO1	72.9	70.9	69.1	64.11	74.40	72.50	71.10±1.55
Group IV PHO2	68.85	64.54	69.10	70.56	78.34	79.53	72.30±2.34
Group V PHO3	83.26	88.52	84.55	88.86	86.75	90.78	87.02±1.13

**Table 8: Determination of Mean  $\pm$  sem of area and %of wound closure on 0th day and 14thday in albino Wistar rats**

Groups	Wound closure on 0th day	Wound closure on 14th day Area	Wound closure on 14th day Percentage
Group I Diseased control	12.80±5.23	28.40±11.59	6.41±2.62
Group II Standard	7.70±3.15	5.81±2.37	1.31±0.54
Group III PHO1	11.74±4.79	17.75±7.25	3.68±1.50
Group IV PHO2	9.60±3.92	27.32±11.15	5.88±2.40
Group V PHO3	11.22±4.58	13.47±5.50	2.83±1.16

## CONCLUSION

Polyherbal ointment where the ethanolic extract was prepared by maceration method where the information were evaluated for following parameters like spreadability, skin irritation etc and the wound healing is evaluated by excision and incision wound models where it was assessed by rate of wound contraction etc. Our result indicated that free formulation developed from the combination of ethanolic extracts of *Azardica indica*, *Momodic acharantia*, *Nyctanthes asbortestis* showed anti microbial and wound healing effects on various stages on healing cascades.

In Neem, mainly nimbolide shows anti-bacterial activity where in bitter gourd essential obtained from the seed of bitter gourd was tested for anti bacterial activity. In NFJ both ethyl acetate and chloroform and extract shows significant anti-bacterial activity. In case of wound healing mainly D-mannitol and flavanoid involved Neem contains active ingredients like nimbi din, nimbin, nimbidole, with anti bacterial, anti- inflammatory that ma helps in acceleration the wound healing process. Here in BG, whole plant where it contains most essential antacids the plant contain cucurbitane, triterpinoids, folic acids glycolipids etc helpful for wound healing activities. In case of NFJ D-manitol flavanol glycosides, tannic acid, ascorbic acid etc chemical constituents are involved in wound healing where they play role in anti-bacterial and anti- inflammatory activity.

The **PHO3** have decreased the wound area better when compared to PHO1 and PHO2.

**PHO3** shows better wound healing activity.

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