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Evaluation of Wound Healing Activity of Aqueous Extract of Polyherbal Preparation

Pasupula Rajeswari*, Venkata Naga Jyothi, Sree Teja K, Jaha Sultana, Prsannakumari T

Department of Pharmaceutics, College of Pharmacy, Guntur, India

***Corresponding author:** Pasupula Rajeswari, Department of Pharmaceutics, College of Pharmacy, Guntur, India, E-mail: rajeswaripasupula9@gmail.com*

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ABSTRACT

Background: *A breakdown in the protective function of skin with a loss of framework of epithelium is defined as wound. A wound can range from simple to complex pathological process and remained as clinical challenge. Complications of wound cause morbidity and mortality.*

Materials and Methods: *The present study was carried out using Aqueous Polyherbal extract Preparation (APHP) (250 mg/kg and 500 mg/kg) of* Indigofera zollingeriana, Dillenia indica, Strobilanthes barbatus, Mimusops elengi. *Povidone iodine was taken as standard. Wound healing activity was assessed using excision and incision models. Antioxidant assays were performed for SOD and catalase. Western blotting for MMP-2 and MMP-9 and histopathology were performed.*

Results: *APHP had showed a significant dose dependent effect on surface area of wound, duration of epitheliazation, hydroxyproline content, dry weight of tissue and tensile strength ($p<0.05$). APHP in a dose dependent manner had significantly increased the levels of SOD and CAT ($p<0.05$).MMP-2 and MMP-9 expressions were significantly increased in a dose dependent manner ($p<0.05$). Histopathological studies had also revealed that APHP is having wound healing potential in a dose dependent manner.*

Keywords: MMP-2, Western blot, APHP, Histopathology

INTRODUCTION

Wound is defined as a breakdown in the protective function of the skin; the loss of continuity of the epithelium, with or without loss of underlying connective tissue *viz*. muscle, bone, nerves. It is due to trauma, pathological process, burns or surgical procedure. Depending on the cause, site and depth, a wound can range from simple to complex that endangers the life [1]. In everyday pathology, wounds remain a challenging clinical problem, with early and late complications presenting a frequent cause of morbidity and mortality [2]. Due to the complications that accompany acute wounds, when their repair does not progress in timely and orderly manner, they can also get transformed into chronic wounds which are more difficult to manage [3]. In the present investigation Aqueous extract of Polyherbal Preparation (APHP) prepared from *Indigofera zollingeriana, Dillenia indica, Strobilanthes barbatus, Mimusops elengi* was used to evaluate wound healing activity. It was reported that this APHP has no signs

of acute and sub-chronic toxicity [4,5]. According to Ayurveda, individual herbs are insufficient to attain a desired therapeutic effect. When it is optimized as multiple herbs composition in a particular ratio it will give a therapeutic effect in a preferable way with reduced toxicity. In order to develop such intervention, the present study was helps to develop a polyherbal drug from methanolic extracts of *Plumbago zeylanica Linn*, *Datura stramonium Linn* and *Argemone mexicana Linn*. The study also aimed to evaluate the effect of polyherbalism on antimicrobial and antioxidant effect, there after the ratio of individual plant extracts was optimized consequently to treat the wound. The poyherbal drug was put on preclinical trial to approach the anti-inflammatory and wound healing activity as 2% and 5% polyherbal carbopol-940 gels. The antimicrobial activity was evaluated by agar well diffusion and broth dilution process while wound healing activity was evaluated by excision and incision wound models. Topical anti-inflammatory effect was assessed by carrageenan induced paw oedema. This study revealed the synergistic antimicrobial potential of Polyherbal drug against gram-positive and negative strains. Polyherbal carbopol- 940 gels (2% and 5% w/w) promoted the wound healing and anti-inflammatory effect. The high rate of wound contraction (<0.0001), early epithelialization period (<0.0001) and increased wound breaking strength (<0.0001) were noted 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 implement the wound healing process through accelerated remodelling of damaged tissue.

MATERIALS AND METHODS

In Indian society, Ayurveda is also known as “Goddess of All Healing” and is considered as one of the most accurate traditional system of medicine with many curing and healing properties. Most of the plant extracts and their phytoconstituents are known as a promising alternative for wound healing agents due to the occurence of diverse active components, ease of access and minimal side effects. The Ayurvedic literature “Sarangdhar Samhita” has also special attention for the concept of polyherbalism in which products with combined extracts of plants are considered more effective rather than individual ones. The active phytoconstituents of individual plants have been identified but are generally present in a small quantities, which is insufficient to produce the desired therapeutic effect for curing wounds. Medicinal plants with antimicrobial, antioxidant and anti-inflammatory properties have alleviated the wound healing process. Polyherbalism results in cheaper medication by reducing the duration of therapy or individual cost for anti-inflammatory and antimicrobial medications. The incidences of new and relapsing infectious disease and antibiotic resistance has widely increased the susceptibility of delayed healing.

Group I (C): Animals with wound and left untreated.
Group II (PC): Animals with wound and treated with Povidone ointment (5% w/w).
Group III (APHP 250 mg/Kg): Animals with wound and treated with 250 mg/kg APHP, p.o.
Group IV (APHP 500 mg/Kg): Animals with wound and treated with 500 mg/kg APHP, p.o

Albino wistar rats were anaesthetised with an intramuscular injection of ketamine-xylazine mixture (90 mg/kg ketamine, 9 mg/kg xylazine) and their anterior cervical region was shaved and depilated with a commercial cream (Veet®, Reckitt Benckiser, UK). Intramuscular Ibuprofen (Carpofen-5 mg/kg) was used as analgesic. The skin was sprayed with 70% (v/v) ethanol and left to dry prior to wound creation. Circular shaped wounds were created in the skin of the cervical region using a scalpel after depilation [6].

Excision model

Measurement of wound area: The wound healing progress was evaluated by measuring wound areas using a transparency sheet and a permanent marker [7]. The evaluated surface area was used to calculate the percentage of wound contraction, taking initial size of the wound (300 mm^2) as 100 % [8].

Epithelialization period: Epithelialization time was measured from the initial day to the day when the scab fell off from the wound surface exclusive of leaving a raw wound behind [9].

Percentage wound contraction: The percentage of wound closure was calculated as followed by using the initial and final area drawn on glass slides during the experiments. Percentage wound contraction was measured on day 0, 7, 14 and 21st day [10].

Estimation of collagen: Hydroxyproline was measured as marker for collagen [11]. The excised granulation tissues were air dried in hot air oven and digested with acid to release the tissues bound hydroxyproline; released hydroxyproline was neutralized and oxidized with chloramin-T [12]. The oxidized hydroxyproline gives the colored complex with Eherlich reagent [13]. The colour intensity was measured at 557 nm spectrophotometrically [14].

Antioxidant assays: Antioxidant assays of SOD and Catalase were performed using standard assay procedures.

Incision wound model

Two para vertebral straight incisions of 6 cm length each were made through the entire thickness of the skin, on either side of the vertebral column with the help of a sharp scalpel. After complete hemostasis the wound was closed by means of interrupted sutures placed at equipoints about 1cm apart. Allis forceps were firmly applied on either side of incision wound 3 mm away from wound margin on adjacent normal skin .The forceps on one side was hooked to affixed metal rod while the other forceps was attached to a thread suspended by weights running over a pulley. As soon as gapping of the wound occurred addition of weights were lifted so as to avoid opening of the wound The weights required to produce gapping of the wound were noted. Average of six readings was taken from each animal a group [15].

RESULTS AND DISCUSSION

The selected herbs for this study aimed different pharmacological targets involved in the wound healing like suppression of the production of inflammatory cytokines and inflammatory transduction cascades, which reduce oxidative factors, and enhance anti-oxidative enzymes and prevention of the microbial growth at wound site. The development of formulation for the wound healing having antimicrobial, antioxidant and anti-inflammatory properties is very important now days. Healthy adult Swiss albino mice of either gender weighing 20 g to 30 g were used for this study. Animals were kept under 12 h. light: 12 h. dark cycle in animal house. The animals were acclimatized to laboratory condition for one week prior to the experiments and were fed with standard pellet diet and water. The animals were randomly sorted into four groups (n=6 per group). The treatment regimen was performed by administering the APHP as mentioned below:

Protein extraction

Wound tissue was homogenized on ice in protein lysis buffer and centrifuged to remove tissue debris. Wound fluid protein was extracted by incubating the filter paper for 2 h at 37°C in a lysis buffer (Tables 1 and 2) (Figure 1).

Table 1: Effect of APHP on wound surface area

Treated group	Day 3	Day 7	Day 11	Day 14	Day 21
Control (vehicle)	269.13 ± 6.56	173.18 ± 7.19*	84.50 ± 11.76*	31.94 ± 3.19*	5.76 ± 0.83*
PC	236.19 ± 14.97**	140.32 ± 7.98**	20.68 ± 3.76**	4.5 ± 0.001**	-
APHPI	241.92 ± 18.27**	137.25 ± 13.35**	18.61 ± 2.77**	4.75 ± 0.001**	-
APHPII	236.87 ± 19.65**	132.02 ± 23.32*	16.01 ± 16.01**	4.49 ± 0.001**	-

Note: C=Control, PC= Povidone Control, APHP-I=APHP 250 mg/kg, APHP-II, APHP 500 mg/kg. Values are expressed in mean ± S.D. *p<0.05 is considered as significant, **p<0.01 is considered as most significant calculated by one way ANOVA followed by Dunnett's test.

Figure 1: Wound healing progress by using a transparency sheet and a permanent marker

Table 2: Effect of APHP on duration of Epithelialization (Initial wound surface area=300 mm^2) percentage of wound contraction and duration of epithelialization

Treated group	7th Day	14th Day	21st Day	Duration
Vehicle(control)	42.27 ± 1.76	89.35 ± 3.53*	98.08 ± 2.43**	21 ± 0.36
Standard	53.22 ± 1.13	98.35 ± 3.43*	-	16 ± 0.27
APHP250 mg/kg	54.25 ± 1.54	98.45 ± 3.42*	-	17 ± 0.40
APHP500 mg/kg	55.99 ± 1.24	98.50 ± 3.80*	-	16 ± 0.36

Note: C=Control, PC= Povidone Control, APHP-I=APHP 250 mg/kg, APHP-II, APHP 500 mg/kg. Values are expressed in mean ± S.D. *p<0.05 is considered as significant, **p<0.001 is considered as most significant calculated by one way ANOVA followed by Dunnett's test.

Western blotting (MMP-2 and MMP-9)

Western blot analysis was used to identify specific proteins from complex tissue homogenate cells. The proteins were separated based on the size/charge and transferred into a solid support (PVDF/ Nitrocellulose membranes) [12]. Following transfer, the protein of interest can be detected by incubation of the membrane with antibodies (primary) specific to the target protein followed by detection with an enzymatically labeled secondary antibody. Secondary antibody is then detected by adding an appropriate substrate for enzyme conjugated to the secondary antibody (Tables 3-5).

Table 3: Effect of APHP on Hydroxyproline content and dry weight of tissue

Treated group	Hydroxyproline	Dry weight of tissue
Vehicle (control)	84.55 ± 2.81	81.8 ± 3.9
Standard	104.47 ± 4.05*	106.8 ± 3.1*
APHP-250 mg/kg	102.69 ± 3.19*	100.4 ± 2.9*
APHP-500 mg/kg	101.27 ± 2.65*	104.2 ± 3.9*
Note: C=Control, PC= Povidone Control, hydroxyproline expressed in mg/g tissue. Dry weight of tissue expressed in mg/100g .Values are expressed in mean ± S.D. *p<0.05 is considered as significant, **p<0.01 is considered as most significant calculated by one way ANOVA followed by Dunnett's test.		

Table 4: Effect of APHP on SOD and catalase

Treated group	SOD	Catalase
Vehicle (control)	0.52 ± 0.01	55.1 ± 4.53
Povidone iodine (5%)	0.32 ± 0.01*	37.19 ± 5.45*
APHP 250mg/kg	0.55 ± 0.01*	58.67 ± 6.43*
APHP500mg/kg	0.65 ± 0.01*	76.34 ± 6.4*
Note: SOD and Catalase expressed in U/mg protein and Values are expressed in mean ± S.D. *p<0.05 is considered as significant, **p<0.01 is considered as most significant calculated by one way ANOVA followed by Dunnett's test.		

Table 5: Effect of APHP on MMP Expression (Initial Wound surface area=300 mm^2).

Expression	Vehicle	Standard (5%)	APHP-250 mg/kg	APHP-500 mg/kg
MMP-2	1.58 ± 0.03	1.93 ± 0.05*	1.63 ± 0.03*	1.91 ± 0.06*
MMP-9	1.61 ± 0.17	2.56 ± 0.22*	1.99 ± 0.27*	2.77 ± 0.29*
Note: G-I-control; G-II-Standard; G-III-APHP250 mg/kg; G-IV-APHP500 mg/kg; Values are expressed in mean ± S.D. *p<0.05 is considered as significant,**p<0.01 is considered as most significant calculated by one way ANOVA followed by Dunnett's test.				

Histopathology: Wound tissues were surgically excised and immediately fixed in 10% (v/v) formalin fixative and embedded in paraffin wax. Five micron thick sections were cut under microtome, spread on glass slides and cleared with xylene. The sections were rehydrated with series of ethanol, rinsed with water then stained using hematoxylin and washed with running water (20 min). The slides were counterstained with Eosin and dehydrated with series of ethanol [13]. Finally slides were then mounted in DPX, viewed under microscope and photographed (Figure 2).

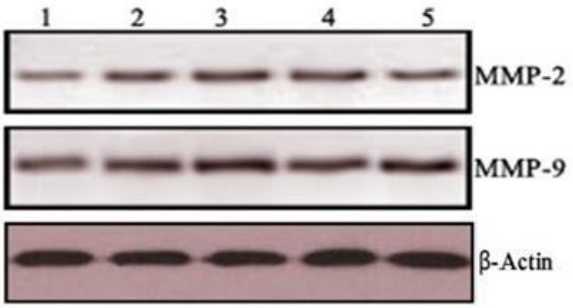

Figure 2: Shows the expression pattern of MMP-2 and MMP-9 in control and experimental groups

The MMP-2 and 9 levels in wound induced group (C) was significantly increased (P<0.05) when compared to control group. Whereas, treatment with APHP significantly reduced (P<0.05) the level of MMP-2 and 9 than that of control and PC treated groups (Table 6 and Figure 3).

Table 6: Effect of APHP on Tensile Strength in wound healing study

Treated group	Tensile strength in gram	% Tensile strength
Vehicle	204.116 ± 14.39	-
Standard (5%)	384.1333 ± 11.24 **	56.00%
APHP250 mg/kg	393.7733 ± 12.87 **	59.12%
APHP500 mg/kg	371.6450 ± 10.39 **	50.90%
Note: Values are expressed in mean ± S.D. *p<0.05 is considered as significant, **p<0.001 is considered as most significant calculated by one way ANOVA followed by Dunnett's test.		

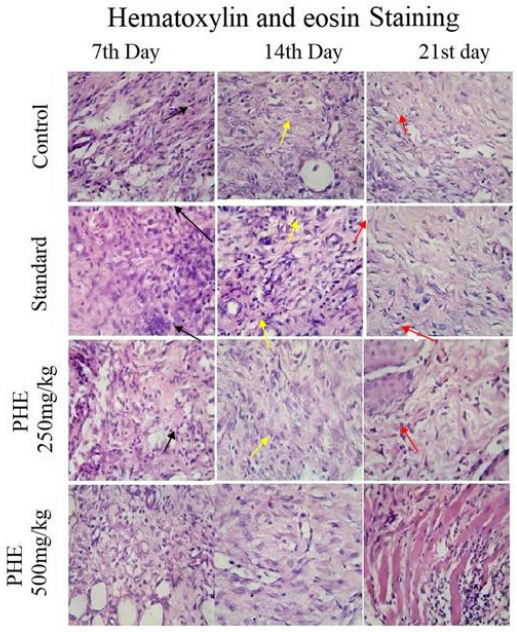

Figure 3: Blue colour indicates infiltration of neutrophils; Pink colur indicates fibro collagen. Orange Arrow indicates highly vascularised granular tissue. Yellow arrow indicates pro collagen and Black arrow indicates infiltration of neutrophils

CONCLUSION

Western blot analysis was used to identify specific proteins from complex tissue homogenate cells. The proteins were separated based on the size/charge and transferred into a solid support (PVDF/ Nitrocellulose membranes). Following transfer, the protein of interest can be detected by incubation of the membrane with antibodies (primary) specific to the target protein followed by detection with an enzymatically labeled secondary antibody. Secondary antibody is then detected by adding an appropriate substrate for enzyme conjugated to the secondary antibody.

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