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Wound Healing Potential of Polyherbal Formulation Containing Pereskia Aculeate, Cinnamomum Verum, Sphagneticola Trilobata, Glycyrrhiza Glabra Extract

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ABSTRACT:

The use of herbal plant extracts in wound healing is known through decades, but it is necessary to provide scientific data through reverse pharmacology. The selected plants (Pereskia Aculeate, Cinnamomum Verum, Sphagneticola Trilobata, Glycyrrhiza Glabra) were studied individually for many medicinal activities and good results were obtained. This work is carried out in the combinations of extracts in different ratios and then formation of polyherbal ointment using polyherbal extracts and evaluate for wound healing activity. The formulation was found to contain high amounts of flavonoids, tannins and phenols which facilitate wound healing. Polyherbal formulation prepared from the plant extracts accelerates wound healing process by proliferation and mobilization of fibroblast and keratinocytes, and angiogenesis at the site of injury. It also shows fast contraction of wound with its beneficial improvement in tissue biochemical and antioxidant parameters.

Introduction

Wound healing is a process of reconstruction of injured skin, coordinated by interaction of various epithelial and mesenchymal cells with cytokines, chemokines and growth factors. Keratinocyte growth factor is a paracrine growth factor synthesized by fibroblasts, endothelial cells, smooth muscle cells and dendritic epidermal T-cells [1]. KGF also known to induce mitogen activated protein activation and directly acts as angiogenic factor in vitro. Wounds have affected humans since prehistoric times and the treatment and healing of wounds is an art as ol as humanity. Due to the increasing life expectancy coupled with a more modern way of life, wounds and particularly chronic wounds increasingly affect a growing number of elderly patients and seriously reduce their quality of life. Current estimates indicate that nearly 6 million people suffer from chronic wounds causing great physiological and mental trauma. In the United States, chronic wounds cost the nation $20 billion to $25 billion a year, and acute or traumatic wounds add another $7 billion to $10 billion annually. Research on wound healing drugs is a rapidly developing area of modern biomedical sciences [2-3].

The progress in this field has allowed the synthesis of large numbers of molecules associated with wound repair process. Delivery of exogenous growth factors in order to mimic the natural microenvironments of tissue formation and repair is believed to be therapeutically effective. The most important among the growth factors are recombinant human platelet-derived growth factor-BB and granulocyte colony-stimulating factor. Despite finding new methods of stimulation of the wound repair process, wound care has returned to the roots of medicine and is embracing some of the remedies used millennia ago [4].

Natural plant products play major role in proliferation of fibroblasts and keratinocytes. Plant products were reported to contain growth factors, cell signaling molecules and cell adhesion molecules. Plant-derived natural products are significant as sources of medicinal agents and models for the design of new remedies [5]. Such pharmaceuticals as morphine isolated from the opium poppy, salicylic acid from the bark of the willow tree or ephedrine from the Chinese herb mahuang are but a few examples of the many medicinally important substances. Nowadays plant-derived compounds play an important role in drug development as exemplified by taxol and camptothecin, artemisinin (the Chinese antimalarial drug), and forskolin (the East Indian Ayurvedic drug). As plants are a source of many bioactive compounds and many plant ingredients are traditionally used to accelerate healing, scientists go back to traditional folk medicines as they are generally characterized by high acceptability and good toleration [6-7].

Pereskia aculeata, also known as Barbados gooseberry or Ora-pro-nobis, has leaves that are edible and packed with nutrients. They are a good source of protein, minerals, vitamins, and dietary fiber. The leaves are also known for their mucilage content, which has been explored for its potential in food processing and wound healing. Leaves are typically simple, entire, and deciduous in the dry season. They are 4-10 cm long and 1.5-3.5 cm wide, and can be described as elliptic to obovate, obtuse to acuminate, with an obtuse to cuneate base. The leaves are also shortly petiolate, meaning they have a short stalk. In terms of color, the leaves are green or sometimes purplish on the lower surface. They are also known to have a smooth surface [8].

Cinnamomum Verum (also known as Ceylon cinnamon or true cinnamon) bark is typically semi-tubular or tubular, with pieces ranging from 0.1 to 0.5 cm in thickness, and 5 to 50 cm in length. The bark's outer surface is dark red-brown, while the inner surface is red-brown and smooth. It is brittle and has a slightly fibrous, red-brown fractured surface. The bark is usually between 0.1 and 0.5 cm thick. Typical pieces are 5 to 50 cm long. The bark curls into rolls (quills) when dried. The outer surface is dark reddish-brown, and the inner surface is red-brown and smooth. The bark is brittle and the fractured surface is slightly fibrous [9].

Sphagneticola trilobata, commonly known as Singapore daisy or wedelia, has opposite, fleshy, and hairy leaves. The leaves are typically obovate to elliptic, with serrated or irregularly toothed margins and can be dark green above and lighter green below. They are often described as having pairs of lateral lobes. The leaves are generally obovate (egg-shaped) to elliptic, meaning they are wider at the base and taper towards the tip. They are arranged oppositely on the stem, meaning two leaves emerge from the same node on opposite sides. The leaves are fleshy and can be described as hairy or glabrous. gins: The edges of the leaves are serrated (like a saw) or irregularly toothed, sometimes with distinct lateral lobes. The leaves are typically dark green on the upper surface and a lighter shade of green on the underside. The leaves are usually 4-9 cm long and 2-5 cm wide. The leaves can root at the nodes, and they are often described as being part of a dense ground cover [10-13].

The selected plants (Pereskia Aculeate, Cinnamomum Verum, Sphagneticola Trilobata, Glycyrrhiza Glabra) were studied individually for many medicinal activities and good results were obtained. This work is carried out in the combinations of extracts in different ratios and then formation of polyherbal ointment using polyherbal extracts and evaluate for wound healing activity.

Materials and Methods

Collection and identification of plant material

Pereskia Aculeate, Cinnamomum Verum, Sphagneticola Trilobata, Glycyrrhiza Glabra were purchased or collected from local area of Bhopal. Plant material had been recognized and authenticated. The plant substances had been dried in colour, powdered fairly and bypass via sieve No. 10.

Extraction of plant material

The powdered plant materials (250 gm) have been extracted in a soxhlet equipment with ethanol. After extraction take a look at changed into carried out to look whether the drug were absolutely exhausted or no longer. The of completion of extract changed into confirmed via evaporating some drops of the extract on the watch glass and ensuring that no residue remained after evaporating the solvent. The liquid extracts were collected in a tare conical flask. The solvent removed by using distillation method. The ultimate lines of solvent being eliminated below vacuum. The extract changed to a consistent weight and percent w/w yield changed into calculated

Preparation of poly herbal ointment formulation: Polyherbal ointment formulation was prepared with extract of herbal drug extract coding from PO1 to PO5 and evaluated. The required amount of hard paraffin was taken in a china dish and melt it in hot plate with low

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temperature. After that the required quantity of cetostearyl alcohol, wool fat, and white soft paraffin was weighed, added to the china dish one by one. After the preparation of base, the extracted herbal extract were incorporated into the ointment by levigation method using a slab and knife. After 2 h, the semiliquid solution containing the drug was neutralized by the addition of the alkali triethanolamine act as tickening agents was added slowly with maximum viscosity. The polyherbal ointment was finally transferred in aluminium collapsible tube and labelled. Different composition in preparing poly herbal ointment is mentioned in Table 1.

Table 1: Formulae of polyherbal ointment

Formulation	PO1	PO2	PO3	PO4	PO5
Pereskia Aculeate extract (PA) (g)	0.5	1	1	1	1.5
Cinnamomum Verum extract (CV) (g)	1	1.5	1	0.5	1
Sphagneticola Trilobata extract (ST) (g)	1.5	1	1	1	0.5
Glycyrrhiza Glabra extract (GG) (g)	1	0.5	1	1.5	1
White soft paraffin (g)	0.5	0.5	0.5	0.5	0.5
Cetostearyl Alcohol (g)	0.5	0.5	0.5	0.5	0.5
Hard paraffin (g)	0.5	0.5	0.5	0.5	0.5
Wool fat (g)	0.5	0.5	0.5	0.5	0.5
Glycerine (ml)	5	5	5	5	5
PEG (ml)	5	5	5	5	5
Triethanolamine (ml)	1	1	1	1	1

Wound healing activity:

Group of animals: The excision, incision and dead space wound models were used to evaluate the wound-healing activity of formulated polyherbal ointment (PO1-PO5). The wistar rats were divided into various groups, each group containing six animals, for excision and incision wound models. Formulated polyherbal ointment (PO1-PO5) were applied topically to each animal once a day.

Group I: The animals of received ointment base (control)

Group II: Treated group with a marketed povidone formulation

Group III: Polyherbal ointment PO1

Group IV: Polyherbal ointment PO2

Group V: Polyherbal ointment PO3

Group VI: Polyherbal ointment PO4

Group VII: Polyherbal ointment PO5

Excision wound model: The animals were divided into groups with six in each were anaesthetized by open mask method with anesthetic ether before wound creation. The particular skin area was shaved 1 day prior to the experiment. An excision wound was inflicted by cutting away a 300 mm^2 full thickness of skin from a predetermined shaved area. The wounds were left undressed to the open environment. The base ointment, standard drug ointment (povidone) and prepared polyherbal ointment (PO1-PO5) was applied topically to the control group, standard group and treated group respectively, till the wound was completely healed. In this model, wound contraction and epithelialization period was monitored. Wound contraction was measured as percent contraction in each 2 days after wound formation. From the healed wound, a specimen sample of tissue was collected from each rat for histopathological examination.

Incision wound model: In incision wound model, all the animals of each group were anaesthetized under light

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ether anesthesia. Two full thickness paravertebral long incisions were made through the skin at the distance of about 1 cm from midline on each side of the depilated back of rat. After the incision was made the both edges of skin kept together and stitched with black silk surgical thread (no. 000) and a curved needle (no. 11) was used for stitching. The continuous threads on both wound edges were tightened for good closure of the wound. After stitching, wound was left undressed then ointment base, standard ointment and extracts ointment were applied daily up to 10 days; when wounds were cured thoroughly the sutures were removed on the day 10 and tensile strength of cured wound skin was measured using tensiometer**.**

Dead Space Wounds: Animals were anaesthetized by light ether and wound was made by implantation of two polypropylene tubes (2.0×0.5), one on either side, in the lumber region on the dorsal surface in each animal. On the eighth post wounding day, granuloma tissue formed on an implanted tube was excised carefully. Granuloma tissue from one tube was dried (60◦C) and stored in 10% formalin for the biochemical parameters and histopathological study, while the other part of granuloma tissue was used for determination of tensile strength. Tensile strength was measured with the help of tensiometer**.**

Wound healing evaluation parameters

A. Measurement of wound contraction: An excision wound margin was traced by following the progressive changes in wound area planimetrically, excluding the day of wounding. The size of wounds was traced on a transparent paper in every 2 days, throughout the monitoring period. The tracing was then shifted to graph paper, from which the wound surface area was evaluated**.** The evaluated surface area was then employed to calculate the percentage of wound contraction, taking initial size of wound, 300 mm2, as 100%, by using the following formula as

% wound contraction = initial wound size − specific day wound size initial wound size × 100

B. Epithelialization period: It was evaluated by noting the number of days required for the Escher to fall off from the wound surface exclusive of leaving a raw wound behind.

C. Measurement of tensile strength: The force required to open the healing action is known as tensile strength. It is used to measure the completeness of healing. It also indicates how much the repaired tissue resists to breaking under tension and may indicate in part the quality of repaired tissue. The sutures were removed on the 9th day after wounding and the tensile strength was measured on 10th day. For this purpose, the newly formed tissue including scar was excised and tensile strength was measured with the help of tensiometer. In this method, wound-breaking strength was measured as the weight of water at the time of wound breaking per area of the specimen.

D. Hydroxyproline estimation: Hydroxyproline is an uncommon amino acid present in the collagen fibres of granulation tissues. Its estimation helps clinically to understand progress rate at which the healing process is going on in the connective tissue of the wound. For the determination of hydroxyproline content, the wound tissues were excised and dried in a hot air oven at 60–70 ◦C to constant weight and were hydrolysed in 6N HCl at 130 ◦C for 4 h in sealed glass tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to Chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4 M perchloric acid and color was developed with the help of Ehrlich reagent at 60 ◦C. The absorbance was measured at 557 nm using a uv spectrophotometer (Shimadzu, Japan). The amount of hydroxyproline in the samples was calculated using a standard curve prepared with pure l-hydroxyproline**.**

RESULTS AND DISCUSSION

Polyherbal ointment formulation was prepared with extract of herbal drug extract coding from PO1 to PO5 and evaluated. The physical evaluation as the colour of prepared herbal gels was pale green in colour and appearance of polyherbal ointment was translucent in nature and smooth on application at skin. The individual properties such as consistency of formulations were good and texture of prepared polyherbal ointment was found to be smooth. The pH value of the prepared gel formulation was observed at room temperature and valued range at 7.2 to 7.8. The literature informed that from epidermis to dermis, pH of the skin increases and attained the neutral value. The results of gel formulation having pH range 7.2 to 7.6 are desirable to skin pH, since they do not interfere with the physiology of skin.

Journal of Chemical Health Risks
www.jchr.org
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Table 2: Evaluation parameters of polyherbal ointment formulation

Parameters	Formulations				
	PO1	**PO2**	**PO3**	**PO4**	**PO5**
Colours	Pale green colour	Pale green colour	Pale green colour	Pale green colour	Pale green colour
Appearance	Translucent	Translucent	Translucent	Translucent	Translucent
Odour	Pleasant odour	Pleasant odour	Pleasant odour	Pleasant odour	Pleasant odour
Spreadability (g.cm/sec)	10.7	10.4	10.1	10.3	10.4
Homogeneity	Good	Good	Good	Good	Good
Feel of application	Smooth	Smooth	Smooth	Smooth	Smooth
Consistency	Good	Good	Good	Good	Good
pH	7.8	7.6	7.2	7.7	7.3
Viscosity (cps)	0.97	0.99	1.15	1.09	0.96
Extrudibility	Excellent	Excellent	Excellent	Excellent	Excellent
Stability	Stable	Stable	Stable	Stable	Stable

The prepared polyherbal ointment formulations were evaluated or stored to accelerated stability testing. The physical parameters were evaluated during study period. The result of the study indicates that the preparation is physically stable at all temperatures during storage period.

Wound healing activity:

Wound contraction: A better healing pattern with complete wound closure was observed in standard and treated group within 10 and 12 days respectively while it was about 24 days in control rats. An excision wound was inflicted by cutting away a 300 mm2 full thickness of skin from a predetermined shaved area. The wounds were left undressed to the open environment. The base gel, standard drug ointment (povidone) and herbal gel PO1- PO5 was applied topically to the control group, standard group and treated group respectively, till the wound was completely healed. In this model, wound contraction and epithelialization period was monitored. Wound contraction was measured as percent contraction in each 2 days after wound formation.

Group V animal which received herbal ointment PO3 showed percent wound contraction in 16 days. herbal ointment PO1 showed percent wound contraction in 21 days. herbal ointment PO3 treated animal group showed best results among all formulation.

Table 3: Effect of polyherbal ointment formulation on % of wound contraction of excision wound models in rats

Group	**0 Days**	**4 Days**	**8 Days**	**12 Days**	**14 Days**	**16 Days**	**20 Days**	**24 Days**
Group I	0	25.12	41.28	51.59	72.15	78.43	91.76	100
Group II	0	51.24	73.32	97.15	100			
Group III	0	22.12	42.21	62.11	72.02	88.02	100	
Group IV	0	20.14	48.02	69.24	76.17	93.24	100	

Journal of Chemical Health Risks
www.jchr.org
JCHR (2025) 15(6), 2855-2864 | ISSN:2251-6727

Group V	0	46.68	62.61	82.41	98.82	100		
Group VI	0	34.21	54.24	72.61	88.45	97.32	100	
Group VII	0	29.11	50.02	67.31	81.415	92.25	100	

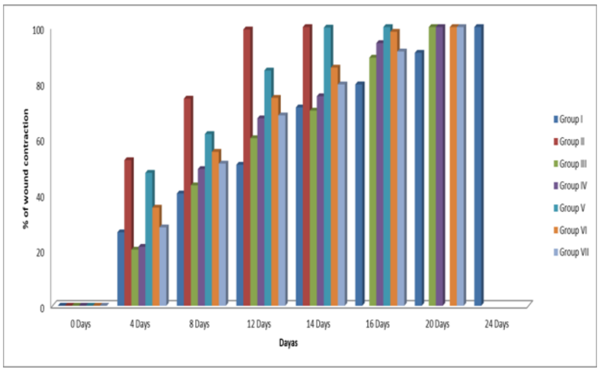

Figure 1: Effect of polyherbal ointment formulation on percent (%) of wound contraction of excision wound models in rats

Epithelialization period: The epithelialization time was measured from the first day. The epithelialization time was found to be significantly ($P < 0.01$) reduced in Groups II and IV. Group V animal which received polyherbal gel PO3 showed Epithelialization time 13 days. Whereas Group III animal received PO1 showed percent wound contraction in 17 days. Polyherbal ointment PO3 treated animal group showed significant recovery among all formulation.

Table 3: Effect of herbal gel formulation on Epithelialization time of excision wound models in rats

Group	Epithelialization time (Days)
Group I	22
Group II	12
Group III	17
Group IV	15
Group V	13
Group VI	15
Group VII	16

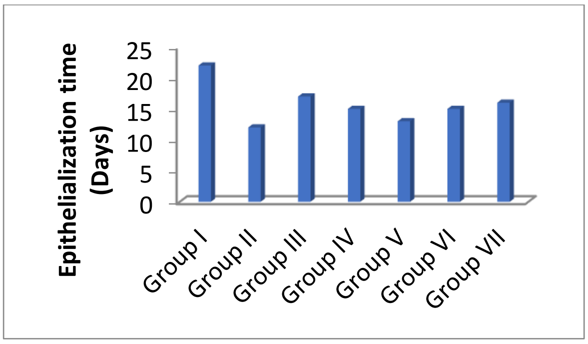

Figure 2: Effect of polyherbal ointment formulation on epithelialization time of excision wound models in rats

6.8.3. Hydroxyproline: Hydroxyproline is a major component in the ground substance of granulation tissue. herbal gel PO4 treated group animal showed hydroxyproline content 58.13 ± 0.01 mg/g tissue which is significant comparable to standard treated group 70.22 ± 0.04. Whereas Group III animal received PO1 showed hydroxyproline content 44.02 ± 0.02. Polyherbal ointment PO3 treated animal group showed significant high level of hydroxyproline among all formulation.

Table 4: Effect of polyherbal ointment formulation on hydroxyproline on incision wound models

Group	Hydroxyproline (mg/g tissue)
Group I	28.11 ± 0.05
Group II	70.22 ± 0.04
Group III	44.02 ± 0.02
Group IV	53.13 ± 0.01
Group V	68.02 ± 0.02
Group VI	58.13 ± 0.01
Group VII	55.04 ± 0.02

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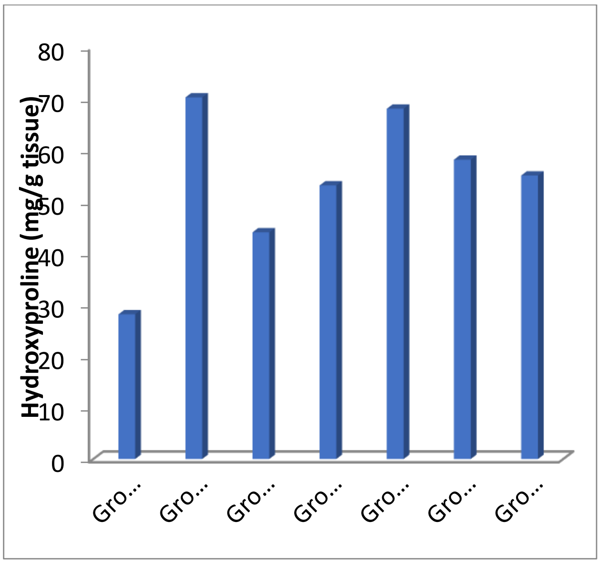

Figure 3: Effect of polyherbal ointment formulation on hydroxyproline on incision wound models

6.8.4. Tensile strength: The tensile strength of a wound is a measurement of its load capacity per unit area. The bursting strength of a wound is the force required to break a wound regardless of its dimension. Bursting strength varies with skin thickness. Polyherbal ointment PO3 treated group animal showed tensile strength 589.34 ± 2.89 g/mm2 which is significant comparable to standard treated group 590.04 ± 4.01 g/mm2. Whereas Group III animal received PO1 showed tensile strength 479.31 ± 3.01 g/mm2. Polyherbal ointment PO3 treated animal group showed significant tensile strength among all formulation.

Table 5: Effect of polyherbal ointment formulation on tensile strength on incision wound models in rats

Group	Tensile strength (g/mm2)
Group I	391.21 ± 2.11
Group II	596.04 ± 3.08
Group III	479.31 ± 2.95
Group IV	511.21 ± 2.18
Group V	589.34 ± 2.89
Group VI	537.32 ± 2.54
Group VII	533.02 ± 2.13

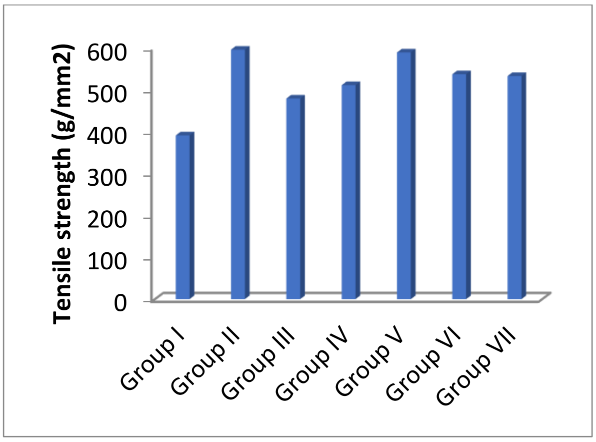

Figure 4: Effect of Polyherbal ointment formulation on tensile strength on incision wound models

6.8.5. Hydroxyproline content: Treated group showed significant increase in hydroxyproline content when compared to control group ($P < 0.01$).

In healing phase collagen content of the granulation can be measured by monitoring the concentration of hydroxyproline, which is a marker of collagen biosynthesis. Higher concentration of hydroxyproline indicates faster rate of wound–healing, which reflects increased cellular proliferation and thereby increased collagen synthesis. Lower concentration of hydroxyproline indicates poor wound healing. Group V treated with PO3 polyherbal ointment increased the level of hydroxyproline content in wound granulation tissue.

Table 6: Effect of herbal gel formulation on Hydroxyproline on dead space wound models

Group	Hydroxyproline (mg/g tissue)
Group I	27.15 ± 0.012
Group II	73.26 ± 0.011
Group III	48.12 ± 0.012
Group IV	58.23 ± 0.015
Group V	73.02 ± 0.011
Group VI	63.13 ± 0.013
Group VII	58.03 ± 0.011

Journal of Chemical Health Risks
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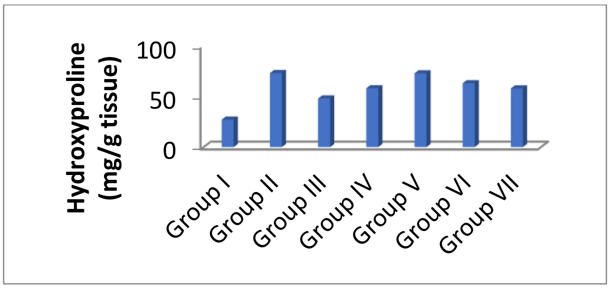

Figure 5: Effect of herbal gel formulation on Hydroxyproline on dead space wound models

Tensile strength: Tensile strength for the treated group on 10th day was found to be significant ($P < 0.01$) than control group. Polyherbal ointment PO3 treated group V animal showed tensile strength 593.14 ± 2.25 g/mm2 which is significant comparable to standard treated group 596.04 ± 1.01g/mm2. Whereas Group III animal received PO1 showed tensile strength 474.31 ± 3.11 g/mm2. Polyherbal ointment PO3 treated animal group showed significant tensile strength on dead space wound models in rats among all formulation.

Table 7: Effect of polyherbal ointment formulation on tensile strength on dead space wound models

Group	Tensile strength (g/mm2)
Group I	397.14 ± 3.11
Group II	596.04 ± 1.01
Group III	474.31 ± 3.11
Group IV	537.11 ± 2.18
Group V	593.14 ± 2.25
Group VI	575.32 ± 2.31
Group VII	567.11 ± 2.05

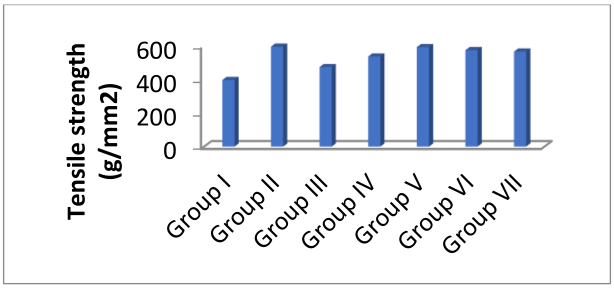

Figure 6: Effect of polyherbal ointment formulation on tensile strength on dead space wound models

Granuloma weight content: Granuloma weight content on dead space wound models estimated at Group V result was showed 456.1 ± 5.7 (mg/100 g), which was best result other extract and significant with treated group. The result concluded that PO3 showed best effect than other extract and significant with treated group.

Table 8: Effect of polyherbal ointment formulation on granuloma weight on dead space wound models in rats

Group	Granuloma weight mg/100 g
Group I	215.8 ± 6.2
Group II	455.1 ± 5.4
Group III	401.5 ± 4.2
Group IV	409.2 ± 9.3
Group V	456.1 ± 5.7
Group VI	431.7 ± 8.1
Group VII	423.1 ±6.

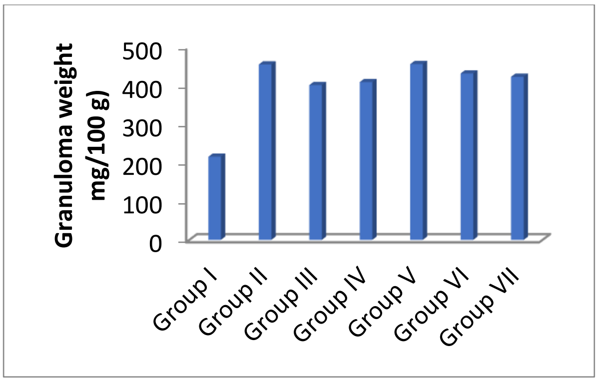

Figure 7: Effect of polyherbal ointment formulation on granuloma weight on dead space wound models

6.8.8. Protein content: Protein content on dead space anemic wound models estimated at Group V result was showed 77.3± 1.2 (mg/g wet tissue), which was best result other polyherbal and significant with treated group. The result concluded that PO3 showed best effect than other polyherbal ointment and significant with treated group.

Journal of Chemical Health Risks

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JCHR (2025) 15(6), 2855-2864 | ISSN:2251-6727

Table 9: Effect of polyherbal ointment formulation on Protein content on dead space wound models

Group	Protein content mg/g wet tissue
Group I	53.1 ± 1.4
Group II	83.5 ± 1.1
Group III	52.2± 1.3
Group IV	63.1± 2.1
Group V	77.3± 1.2
Group VI	66.1± 1.5
Group VII	62.5± 1.3

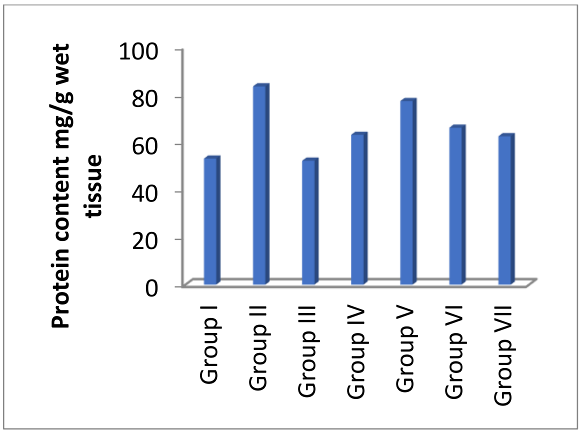

Figure 8: Effect of polyherbal ointment formulation on protein content on dead space wound models in rats

Conclusion

A process of wound healing comprises of three phases i.e. inflammation, angiogenesis and collagen deposition. An angiogenesis refers to the formation of new capillaries formed as band like structures from pre-existing vessels adjacent to the wound. Phytochemical analysis showed presence of flavonoids, phenols and tannins in the formulation. Tannins are phenolic compounds that typically act as astringent and are found in a variety of plant products used in wound healing. The astringent property is responsible for wound contraction and accelerates rate of epithelialization at the granulation formation and scar remodeling phases. Therapeutic potential of a tannic acid cross-linked collagen scaffolds is reported earlier and demonstrated significant effect in wound closure and wound healing rate. The present formulation showed significant wound contraction as compared to control untreated group at the end of 15 days. For healing of wounds, collagen is an important constituent of extra cellular matrix. Collagen synthesis is always directly proportional to hydroxyproline.

In the present study, hydroxyproline levels in newly formed tissue were found to be significantly increased in polyherbal formulation treated animals as compared to control group. Synthesis of collagen formation accelerated in newly formed tissue indicating increased collagen turnover after treatment with formulation. The conventional assays to determine efficacy of plant products for wound healing comprises of painful invasive procedures in animal models. Thus minimum essential parameters of wound healing properties of any herbal preparation could be screened using in vitro assays described in the present study, and unnecessary usage of experimental animals could be avoided. Group V animal which received herbal ointment PO3 showed percent wound contraction in 16 days. herbal ointment PO1 showed percent wound contraction in 21 days. herbal ointment PO3 treated animal group showed best results among all formulation. Polyherbal formulation prepared from the plant extracts (Pereskia Aculeate, Cinnamomum Verum, Sphagneticola Trilobata, Glycyrrhiza Glabra) accelerates wound healing process by proliferation and mobilization of fibroblast and keratinocytes, and promotes angiogenesis at the site of injury.

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