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EVALUATION OF ANTIOXIDANT AND WOUND HEALING POTENTIAL OF POMEGRANATE PEEL GEL FORMULATION

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Punica granatum, Extract, Gel formulation, Antioxidant activity, Antibacterial activity, Excision model

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ABSTRACT: The objective of the study was to formulate and evaluate the antioxidant activity and the wound healing effect of the *Punica granatum* peel extracts formulation. Tannins, alkaloids, flavonoids, proteins, and phenols were present in the ethanolic extract, which is further formulated in a gel formulation. Seven herbal gel formulations were prepared using 1.5% of the gelling agent's carbopol 940 (F1-F7). Formulations were evaluated for physical appearance, viscosity, extrudability, pH, and spreadability. The antioxidant study was evaluated *in-vitro*, using 2, 2-diphenylpicrylhydrazyl (DPPH), and reducing power assays. It was found that all the gel formulation has good power to inhibit DPPH and good reducing power. The antimicrobial properties were evaluated by agar well diffusion method. The result indicated that F5 and F7 exhibited good antimicrobial properties. The highest potential was observed in the F7 against *E. coli* and *B. subtilis*. Wound healing was studied using excision wounds on rat models. Treatment of wound with a gel containing 2% and 10% (w/w) ethanolic extract exhibited better-wound healing activity than the positive control. This study illustrated an excellent potential of the *Punica granatum* peel extracts formulation therapy on dermal wound healing, with a tentative mechanism of action related to improved collagen deposition and reduced inflammatory reaction.

INTRODUCTION: Traditional medicine uses medical aspects of traditional knowledge that developed over generations within various societies before the era of modern medicine. WHO defines traditional medicine as "the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness."

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Traditionally, medicinal plants are often used to obtain preparations beneficial for wound healing purposes [1]. The wound has been defined as the disruption of anatomic or functional continuity of living tissue. Wound infection is one of the most common diseases in developing countries due to poor hygienic conditions [2].

Wounds are the physical injuries that end in a gap or breaking of the skin. The applicable methodology for the healing of wounds is important for the restoration of discontinuous anatomical continuity [3]. The wound healing method holds many steps that involve natural process, inflammation, the formation of granulation tissue, matrix formation, remodeling of connective tissue, and acquisition of wound strength [4]. In the Ayurvedic system, the peels of *P. granatum* fruits are used in the treatment of diarrhea, dysentery [5].

The most abundant phytochemical constituents in pomegranate juice are polyphenols, including the hydrolyzable tannins or ellagitannins, the main components are ellagic acid or gallic acid [6]. These are bound with a carbohydrate known as punicalagin. Many researchers have reported the antioxidant properties of Pomegranate [7]. The ethanolic extract of pomegranate peel has an ameliorative effect against chlorpyrifos-ethyl-induced oxidative stress in rats. *P. granatum* peel used in the treatment of the severe condition of a bed sore, is studied against open wounds in male Albino Wistar rats [8].

The purpose of this study was to investigate the effect of *P. granatum* peel gel on wound healing and its antioxidant effect. The gel is formulated with dried pomegranate peel extract and mixed with Zinc oxide in % w/w concentration. Zinc is an essential micronutrient, present at less than 50 mg/kg, in the human body. It is important for human health and disease due to its critical roles in growth and development of bone metabolism, the central nervous system, immune function, and in wound healing [9]. Wound closure was significantly shortened when peel extract gel was applied to the wounds of diabetic rats. The present study was performed to assess the synergistic wound healing potential of the aforementioned herb extract formulation blend in excision wound models in rats when applied topically in comparison to a marketed formulation [10]. Surveying the literature revealed that the synergistic wound healing activity of such blend had not been previously examined. In addition, the study also aimed to evaluate the antioxidant and antimicrobial activities of the different formulation [11].

EXPERIMENTAL:

Materials: Carbopol 940 & propylene glycol was obtained as a gift sample from LOBA Chemie Pvt. Ltd, Mumbai, India. Propyl glycol and triethanolamine were purchased from Merck Pvt. Ltd, Mumbai, India. Povidon-Iodine ointment USP (manufactured by Nanz Med Science Pharma Pvt. Ltd.). All other chemicals used were of analytical grade and obtained commercially.

Plant Materials and Extract Preparation: *Punica granatum* (Family: Punicaceae) were collected from Indapur, Maharashtra and authenticated at the Regional Ayurveda Institute for Fundamental Research, Nehru Garden Kothrud, Pune. Peel was manually removed and cut into small pieces. Sun-dried peel of pomegranate was powered using a mixer grinder. This coarse powder was extracted using a Soxhlet extractor with n-hexane to remove fatty matter, then 100 gm of powder mix in 300 ml of ethanol in Soxhlet extractor for 24 h. The mixture was evaporated to dryness in a rotary flash evaporator and stored in the refrigerator. The condensed extracts were used for preliminary screening of phytochemicals and used for the formulation of the gel.

Animals: Male Albino Wistar rats weighing 150-200 g of either sex in the animal house of BVDU Poona College of Pharmacy, Pune, was selected for wound healing evaluation. They were housed under controlled conditions of room temperature (25 ºC), humidity (50 ± 5), and 10-14 h light and dark cycles. The animals were housed individually in polypropylene cages containing sterile paddy husk bedding and free access to food and water. The experiments were designed and conducted in accordance with ethical norms approved by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPSCEA) and Institutional Animal Ethical Committee (KMCRET/DRDO/01/2011, dt.16/07 /2011).

Preparation of Peel Extract Gel: Gels of pomegranate peel extract (PPE) and (Zinc Oxide) ZnO were prepared with carbopol 940, propyl glycol, triethyl amine and water according to the following formula:

F1= 1% PPE + 1% ZnO

F2= 2% PPE + 1% ZnO

F3= 5% PPE + 1% ZnO

F4= 1% PPE + 2% ZnO

F5= 2% PPE + 2% ZnO

F6= 5% PPE + 2% ZnO

F7= 10% PPE +1% ZnO

Wound Healing Activity: The rats were shaved and circular wounds of approximately 2 cm were produced on the dorsal thoracic region using sharp scissors after anesthesia with thiopental sodium [12].

The wounds were measured the next day of wound creation, and the reading served as initial reading. The wound area was recorded on a measurement scale. After this, each animal was placed in a separate cage for full recovery from anesthesia before being returned to holding rooms. Group arranged as blank; Standard treated with povidone-iodine. The prepared formulations gel was applied to different groups. The application was done on alternate days and recorded. The animals were monitored daily for their health and behavior. From this, wound areas were read, and the percentage of wound contraction was calculated by the given formula.

% Wound Contraction = Initial wound size – Final wound size × 100 / Initial wound size

Free-radical Scavenging Activity: The free-radical scavenging activities of different formulations were measured by a decrease in the absorbance of the methanol solution of DPPH. A stock solution of DPPH was prepared in methanol, which gave initial absorbance of 0.92, and 5 ml of this stock solution was added to the formulation extract solution at different concentrations. After 30 min, absorbance was measured at 517 nm. The antiradical activity was calculated as % inhibition from the given formula [13].

% Anti-radical Activity = Control Abs – Sample Abs × 100 / Control Abs

Reducing Power Assay: The reducing power of different formulation was determined as per the reported method by Oyaizu M. Different concentrations of formulations in 1 ml of ethanol were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferrocyanide (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power [14].

Anti-microbial Activity: The cup plate agar diffusion method was used to evaluate the antimicrobial activity of Pomegranate peel extract gel formulations. Twenty-five ml of the molten agar medium was poured onto sterile Petri dishes (90 mm in diameter) to provide a depth of 4 ± 0.5 mm. The agar was left to solidify. The following standard strains were used to evaluate the antibacterial and antifungal activities of the topical formulations: *Escherichia coli* ATCC 25218, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 29213, Methicillin-Resistant *S. aureus* ATCC 29213, *Bacillus subtilis* ATCC 10400. The agar plates were inoculated with the above stains. The inoculated plates were then left to dry at room temperature. The dried inoculated plates were used for the agar well diffusion assay. A sterile cork borer was used to make four wells per plate by perforating holes on the inoculated plate. Each well was 7 mm in diameter, and the cut pieces of the agar were removed using a sterile needle. Then, 100 mg of each formulation was placed into each well. The inoculated agar plates were kept at 37 ºC for 18 h. The detected diameters of the inhibition zones were measured using a ruler to the nearest millimeter [15].

Stability Studies of Topical Herbal Gel Formulation: The main objective of the stability testing is to provide evidence on how the quality of the drug product varies with time under the influence of temperature and humidity. The stability study for the topical herbal gel formulation was done as per ICH guidelines in a stability chamber for a period of 6 months. Samples were withdrawn at an initial, first, second, third, and sixth months and evaluated for change in color, odor, homogeneity, and pH.

RESULTS AND DISCUSSION: *Punica granatumis* one of the widely used drugs in various Ayurvedic and herbal formulations. Certain plants show antioxidant activity because of their phenolic constituents. Flavonoids are a broad class of low-molecular-weight, secondary metabolites widely distributed in plants [16]. The beneficial effects of flavonoids are attributed to their antioxidant and chelating abilities. Large amounts of polyphenols are found in pomegranate peel, for example, ellagic tannins, ellagic acid, and gallic acid. Because of polyphenols and flavanoids, its peel formulation exhibit good antioxidant, wound healing and antimicrobial activity [17].

The biological process of wound healing consists of a series of complex interactions between cells, cytokines, and the extracellular matrix. It seems that interventions in different steps, from the onset of injury up to scar formation, can accelerate the healing process [18]. Wound healing has different phases, including inflammation, proliferation, and reconstruction. Each phase interferes with the others and thus is not fully distinguishable from other phases. Various methods, especially in the form of topically applied materials, have been proposed to improve the wound-healing process by preventing the proliferation of keratinocytes, fibroblasts, granulation tissue maturation, and regular collagen accumulation [19]. Epithelialization has an important role in the healing of full-thickness wounds. The results of this study give scientific proof for the use of pomegranate peel gel in treating the wound. In the present study, zinc oxide is used because zinc is a necessary micronutrient, present at less than 50 mg/kg, in the human body. It is necessary for human health and disease because of its important roles in the growth and development of bone metabolism, CNS, immune function, and wound healing [20]. The formulation can be more subjected to stability studies and changed to suit the native population. The *in-vitro* pharmacological studies like anti-oxidant and anti-microbial studies are done on the ethanolic extract of *P. granatum* peel, and its shows antibacterial activity.

Evaluation of Topical Herbal Gel Formulation: Seven different gel formulations (F1 to F7) were prepared using different concentrations (1%, 2%, 5% w/w) of *Punica granatum* peel extract, with Zinc oxide respectively. Carbopol 940 was used as gelling agent in the formulation as they are biodegradable, bioadhesive, biocompatible, irritation-free and not absorbed into the body. The percentage of the polymer was optimized after preparing the gel with various concentrations from 1%, 2%, 5% where the 1.5% of carbopol 940 containing gels was found to be compatible with the requirements of gel formulations.

From the quality control test, as a 1.5% of the gelling agent was found to be superior to the gel formulations prepared with Carbopol 940 (F1 to F7) and spreadability parameters with Carbopol 940 were found to be good. The *in-vitro* release and stability studies were carried out for the best herbal gel formulation F3, F5, F7. Propylene glycols are reported to be the two best permeation enhancers in the preparation of the gel formulation. Triethanolamine was used in the formulation in order to adjust the pH of the formulation.

The pH values of all the formulations were in the close range of neutral pH (6.5-7.8). Hence, it caused no skin irritation, which is also supported by skin irritation study.

TABLE 1: EVALUATION PARAMETERS FOR TOPICAL HERBAL GEL FORMULATION

Code	pH	Viscosity (Poise)	Spreadability	Net content %w/w	Physical appearance
F1	6.8	462	462	99.7	Brownish-yellow, smooth gel
F2	7.5	850	850	90	Brownish yellow, smooth gel
F3	7.1	250	250	90	Yellowish-brown, smooth gel
F4	6.9	365	365	90	Yellowish-brown, smooth gel
F5	7.3	255	255	92	Brown, smooth gel
F6	6.5	347	347	95	Brown smooth gel
F7	7.0	300	300	101	Off White smooth gel

TABLE 2: ANTIOXIDANT ACTIVITY OF *PUNICA GRANATUM* PEEL EXTRACT FORMULATION

S. no.	Formulation	DPPH (IC_{50} ug/ml)	Reducing power (mg equivalent Ascorbic acid/g of formulation)
1	F1	235.71 ± 1.26	87.89 ± 0.37
2	F2	185.18 ± 0.07	156.47 ± 0.47
3	F3	155.18 ± 0.07	133.04 ± 0.77
4	F4	86.45 ± 0.93	83.29 ± 0.37
5	F5	25.5 ± 0.81	253.29 ± 0.41
6	F6	50.6 ± 0.53	203.54 ± 0.62
7	F7	8.62 ± 0.07	289.91 ± 0.97
8	Ascorbic Acid	2.17 ± 0.01	---

Antioxidant Activity: Antioxidant activity was performed by DPPH radical scavenging activity and reducing power assay. Oxygen radicals are toxic waste products that produce oxidative stress during the inflammatory phase of wound healing. Scavenger's application to the injury site has been reported to be effective in inflammatory conditions and wound healing. The antioxidant reacts with DPPH radical (purple color) and converts it into a colorless α-α-diphenyl-β-picrylhydrazyl. The amount of DPPH reduced could be quantified by measuring a decrease in absorbance at 517 nm. A different formulation of *P. granatum* peel extract shows different inhibition % of DPPH radical. The IC_{50} value of DPPH for F7 was found to be 8.6 µg/ml; the results are in **Table 2**.

The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron. In this assay, the ability of the formulation to reduce Fe^{3+} to Fe^{2+} was determined. The presence of antioxidants in the formulations resulted in a reduction of the ferric cyanide complex (Fe^{3+}) to the ferrous cyanide form (Fe^{2+}). In reducing power assay, antioxidants cause the reduction of the Fe^{3+} into Fe^{2+}, thereby changing the solution into various shades from green to blue, depending on the reducing power of the compounds. Strong reducing agents, however, formed Perl's Prussian blue color and absorbed at 700 nm. **Table 2** data showed the reducing activities of various formulations in comparison with ascorbic acid as standard. The higher the absorbance of the reaction mixture, the higher would be the reducing power. All formulations showed some degree of electron donation. Reducing the power of different formulation increased with the concentration. F1 and F3 showed less degree of Fe^{3+} reduction than the F5 and F7. The reducing power of the reference compound (Ascorbic acid) was found to be higher than all the tested formulation. It has been reported that the reducing power of substances is probably because of their hydrogen-donating ability. Ethanolic extracts of peel might, therefore, contain high amount of reductones, which showed good activity.

Antimicrobial Activity: Antimicrobial activity of *P. granatum* peel extract formulations was performed on the agar disc diffusion method, and the results are shown in **Table 3**. The results revealed that all of the tested formulations have excellent antibacterial activities against gram-negative bacteria, such as *E. coli* ATCC25218, Gram-positive bacteria such as *S. aureus* ATCC 29213, MRSA ATCC29213 and *B. subtilis* ATCC10400. No formulations showed any activity against Gram-negative *P. aeruginosa* ATCC15442. The result showed that a maximum inhibition zone of 25 mm was obtained against *E. coli* for formulation F7 and *S. aureus* 17 mm for F5.

TABLE 3: EVALUATION OF ANTIMICROBIAL ACTIVITIES OF POMEGRANATE PEELS EXTRACT FORMULATION BY CUP PLATE DIFFUSION METHOD. CUP PLATE DIFFUSION METHOD BLANK AS WATER AND CONTROL ONLY PLACEBO WITHOUT PPE AND ZnO

Formulation	*E. coli*	*P. aeruginosa*	*S. aureus*	MRSA	*B. subtilis*
Blank	No	No	No	No	No
Control	No	No	No	No	No
F1	22	8	12	21	18
F2	18	1	12	12	13
F3	17	3	15	15	16
F4	21	5	11	12	11
F5	14	4	17	20	19
F6	16	1	12	13	10
F7	25	4	14	17	18

Wound Healing Activity: The main processes that are involved in wound healing are epithelisation, contraction, and connective tissue deposition. Healing processes are governed by the biosynthesis and deposition of new collagen at the site of the wound [21]. The wound healing activity of *Punica granatum* peel extracts was investigated by formulating it into gel formulations. The study was performed as an in-depth investigation of the synergistic wound healing activities of the peel extract formulation blend in excision and dead space wound models in rats when applied topically on wounds in comparison to a marketed formulation.

Initial investigation studies showed that formulation F5 and F7 exhibited promising results for antioxidants, viscosity, spreadability, and anti-microbial studies. It had been discovered that formulation F5 showed a shorter amount of epithelisation and a larger rate of wound contraction. The optimized formulation was compared with the marketed product. It had been concluded that the *Punica granatum* peel extracts gel using Carbopol 940 base has higher wound healing properties [22].

TABLE 4: WOUND HEALING STUDY FOR DIFFERENT POMEGRANATE PEEL GEL FORMULATION

Formulation	Wound Size				
	0 day	5 day	10 day	15 day	21 day
F1	2 cm	1.8 cm	0.9 cm	0.7 cm	0.7 cm
F2	2 cm	1.9 cm	1.0 cm	0.7 cm	0.6 cm
F3	2 cm	1.8 cm	1.3 cm	0.9 cm	0.5 cm
F4	2 cm	1.9 cm	1.2 cm	0.8 cm	0.7 cm
F5	2 cm	1.0 cm	0.6 cm	0.2 cm	0.1 cm
F6	2 cm	1.7 cm	1.2 cm	0.7 cm	0.7 cm
F7	2 cm	1.0 cm	0.7 cm	0.3 cm	0.2 cm
Control	2 cm	1.8 cm	1.5 cm	1 cm	0.7 cm
Standard	2 cm	1.3 cm	1 cm	0.5 cm	0.2 cm

CONCLUSION: On the basis of the results exhibited by this study, it can be concluded that *Punica granatum* peel extract gel formulation has significant anti-microbial and antioxidant property as evident from the result of the anti-radicular assay. This also demonstrated the wound healing effect by accelerating wound closure and epithelialization. This effect may be due to the antioxidant potential of the extract and may be attributed, at least partly, on its improvement of collagen deposition. The wound-healing property and antioxidant activity co-exist in many plant species from a variety of families. However, to understand the process of wound healing fully, it is essential to study the basic cell biology, immunology and biochemistry involved in the processes of inflammation and collagen metabolism, and how these pathways are regulated.

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