

Topical delivery of Eugenol and Resveratrol loaded Carbopol based hydrogels for cutaneous wound healing in Wistar strain albino rats

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Abstract

Wound healing remains a significant clinical challenge, highlighting the need for effective bioactive agents with antimicrobial, antioxidant, anti-inflammatory and biocompatible properties. Eugenol and Resveratrol, both plant-derived compounds, have demonstrated potential in reducing oxidative stress and supporting tissue repair. The objective of the study was to assess and compare the wound contraction rates and epithelialization periods of topically administered Eugenol and Resveratrol in an experimental Wistar strain albino rat model. The animals were divided into seven (7) distinct groups for the individual treatments including control. Incision wounds were created on the dorsal skin of each animal for topical application of prepared Carbopol 940 based hydrogel formulations loaded with Eugenol and Resveratrol individually. Measurement of wound contraction was studied at fixed intervals on day 5, 10 and 15, till complete epithelialization. Eugenol 0.5% and Resveratrol 1% demonstrated the optimum wound contraction, indicating their potential to enhance cutaneous wound healing. The group treated with Gel *per se* did not show significant change in wound contraction rates which indicates that Carbopol 940 has no significance in wound healing but acts as an excellent delivery system. Overall, the findings suggest that Eugenol 0.5% and Resveratrol 1% may be promising candidates for promoting wound repair, with further studies warranted to elucidate their mechanisms of action and therapeutic potential.

Key words: Wound healing, Eugenol, Resveratrol, Carbopol 940, Wistar albino rats.

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Introduction

Disruption of live tissue architecture may be regarded as a wound. Skin is the largest organ of the human body, and its important functions are to hydrate and protect internal organs from chemicals and pathogens, activate Vitamin D secretion, excretion and homeostasis [1,2]. Wound healing is a multifaceted, finely tuned process that is critical to maintaining these functions of skin. Wound healing takes place in three overlapping synchronised phases: 1. Haemostasis/inflammation phase, 2. Proliferation phase, and 3. Remodelling phase [3]. Any alterations in any of these phases can trigger chronic wound development and may hinder or delay wound healing [4].

Haemostasis/inflammatory phase

The wound exposes the sub-endothelium, and the collagen and tissue factors activate the aggregation of platelets at the site of injury, which results in degranulation and release of chemokines and growth factors (GFs) to form the fibrin clot achieving successful haemostasis [5,6]. Neutrophils and macrophages accumulate at the wound area and phagocytise bacteria and the damaged tissue providing a proper environment for wound healing [6,7,8]. The haemostasis/inflammatory phase often takes 72 h to accomplish.

Proliferative phase

The proliferative phase is characterized with an accumulation of cells and excessive connective tissue, the wound now comprises of fibroblasts, keratinocytes, and endothelial cells. A granular tissue composed of extracellular matrix (ECM),

including proteoglycans, hyaluronic acid, collagen, and elastin is formed to replace the original clot. Cytokines, growth factors, interleukin and angiogenesis factors (Vascular epidermal growth factor) participate in this phase of wound healing, which continues for days and week [9, 10].

Remodelling phase

During the remodelling phase, new cells replace the existing cells by apoptosis. The immature type III collagen is transformed to mature type I collagen, along with gradual degradation of profuse ECM. Any aberration in this phase may lead to excessive wound healing or chronic wound [10].

Chronic wounds fail to heal and remain in the inflammatory phase for a prolonged period which are often destructive [11]. These wounds are influenced by local factors (oxygenation, foreign bodies and infection) and systemic conditions (age, nutrition, diabetes, obesity, lifestyle etc.,) or a combination of both [12]. Apart from these factors and conditions biofilms play a significant role in non-healing wounds causing infections. The bacteria that make up biofilms form a rigid polysaccharide membrane that cannot be penetrated by antibiotics, prolonging inflammation and breaking down the extracellular matrix resulting in formation of keloids. These keloids are made up of hyperactive fibroblast, type III collagen, irregular epidermal cells and macrophages [11]. Early diagnosis and professional wound care improve the prognosis of chronic wounds [12].

Hydrogels

Hydrogels are also called hydrophilic gels are cross-linked materials that absorb large quantities of water without dissolving. They are unique materials because of their softness, smartness, and capacity to store water. The hydrogel's ability to absorb water is because of hydrophilic functional groups connected to the polymer's backbone, while its resistance to dissolution is because of cross-links between network chains [13]. Topical treatment of skin wounds using hydrogels have shown to have greater advantage. They can be directly applied on wound site which avoids the dilution of gel if taken orally by fluids in hepatic and gastrointestinal system. The concentration of the drug is highest at the surface of the wound site up to 4000-fold in contrast to systemic administration of oral and intravenous administration. Direct application avoids vascular damage which evades adverse systemic effects [14]. Other advantages of hydrogel include easy to change because the moist interface between the gel and the wound bed prevents dressing adherence; their semi-transparent nature permits visual observation of the wound bed without removing the wound dressing [15]. The above-mentioned advantages and physical properties make hydrogels a promising candidate for applied medicine topical drug delivery system. Hydrogels come with some disadvantages, the most important is their low mechanical strength when swollen, heterogeneity of hydrophobic drugs, faster release of drug, vulnerability to bacterial growth, and semi-permeability to gases and water vapour [16]. Polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyacrylic acid (PAA), chitosan, alginate, hyaluronic acid and gelatine are commonly used hydrogels for drug delivery.

Carbopol 940

Carbopol 940 is one such promising hydrogel for drug delivery. They are synthetic high molecular weight three-dimensional acrylic cross-linked polymer (polyacrylic acid- PAA) networks with a high moisture content [17]. They provide a moist, thermally insulating environment for dry wounds, aiding in autolytic debridement [18].

Eugenol

Eugenol, an aromatic phenolic compound (phytochemical) found in plants such as cinnamon, basil, nutmeg and mainly in clove. It has shown diverse pharmacological effects, including analgesic, antioxidant, anti-inflammatory, antibacterial, antiviral and antifungal activity, with synergistic effects with antibiotics and numerous other potential therapeutic applications. The antioxidant property is due to its phenolic structure, enabling free radical scavenging by reducing lipid peroxidation and oxidative stress [19,20]. It disrupts quorum sensing, inhibits virulence factors and prevents biofilm formation [21]. Eugenol has generally regarded as safe (GRAS) status by FDA and EFSA at low concentrations, and its typical

usage range is between 0.1-1% in food and up to 100mg/kg in pharmaceuticals [22].

Resveratrol

Resveratrol is a well-known bioactive stilbene (phytoestrogen) which is mainly found in fruits such as berries, grapes, peanuts, cocoa and Japanese knotweed [23]. It is synthesized in response to ionising radiations and infections. *Vitis vinifera* synthesize these stilbenes in higher concentrations in response to fungal infections. It exists in trans and cis forms, the trans isomer being the active form is sold as a nutraceutical as it possesses a broad spectrum of properties beneficial to human health [24]. It exhibits antimicrobial property against *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* by inhibiting the electron transport chain. Resveratrol modulates the wound healing pathway such as NFκB and p53 and inhibits tumour growth, suppresses cytokines thereby reduces inflammation, augments endothelial function and controls blood pressure [23]. Studies has shown it induces apoptosis by suppressing fibroblast proliferation inhibiting fibro genesis in keloids which further reduced the production of pro-inflammatory factors elevating the level of SIRT1 in severe burn [25].

Considering the multi-faceted properties and pharmacological effects of Eugenol and Resveratrol, the present work was hypothesised exploring the wound healing potential of these bioactive phyto compounds by synthesising Carbopol 940 based hydrogels for *in vivo* wound healing studies in rat models.

Materials and methods

The Carbopol 940 and Eugenol used for the preparation of the formula was purchased from Himedia, and were of analytical grade, and Resveratrol (HPLC grade) from Merck, Germany.

Gel preparation: Two different concentrations of eugenol and resveratrol were loaded into Carbopol 940 to prepare the hydrogel formulations. 1.5% Carbopol 940 was mixed with double distilled water till a homogenous mixture was obtained. The solution was kept overnight at room temperature allowing complete swelling of the polymer. About 3-5ml of glycerine was added to increase the appearance of the hydrogel and the pH was adjusted with triethanolamine. Tri-ethanolamine is a common and safe stabilizer, fatty acid neutralizer, and pH adjuster in skincare products [26]. The solution was made up to 100 mL (Carbopol 940, 1.5%, w/v) by adding double distilled water and stirred until the transparent hydrogels were formed [27,28]. Different concentrations of drugs (Eugenol-0.25%, 0.5% and Resveratrol- 0.5%, 1%) were afterwards mixed in the hydrogels and stirred again until homogeneous. Similarly blank gel is prepared. The gels were stored at 4-8°C till further evaluation. The composition of the formulations of the gels are as shown in Table 1.

Formula	Concentration of Carbopol 940	Drug concentrations
F1	1.5%	Carbopol (Blank)
F2		0.25% Eugenol
F3		0.5% Eugenol
F4		0.5% Resveratrol
F5		1% Resveratrol

Table 1 showing the different compositions prepared with Carbopol 940

Evaluation

Physical appearance: All the hydrogels after preparation was evaluated for colour, odour, clarity, appearance and texture.

Physical characterisation & homogeneity: The gels were characterized and studied based on their washability, pH, spreadability and stability.

Determination of pH: The pH of hydrogels was determined by digital pH meter. One gram of gel was dissolved in 25 ml of distilled water, and the electrode was dipped in the gel for 30 min until constant reading was obtained and noted. The measurement of pH of each formulation was done in triplicate and average values were calculated [28].

Washability: Formulations were applied on the skin and their ease and extent of washing with water were checked manually.

Spreadability: Two glass slides of standard dimensions (6×2) were selected. The hydrogel formulation whose spreadability had to be determined was placed over one of the slides.

$S = m \times l / t$ where S = Spreadability (gcm/s), m = weight tied to the upper slide (20 grams), l = length of glass slide (6cms), t = time taken in seconds [29].

Stability Studies: It assesses changes in physical appearance, pH, and drug content on storage. The hydrogels were stored in a closed container at 40°C at 75% humidity for a period of 90 days. The gel samples were drawn on day 30, 60 and 90 and evaluated [30].

Evaluation of cutaneous wound repair in Wistar strain Albino rats

The study was conducted at Vaarunya Biolabs, Bengaluru and was approved by Institutional Animal Ethics Committee (IAEC) and the approval number for the project is VBPL/IAEC/68. The animals were divided into seven groups (n=6) as shown in Table 2 and were studied for a duration of 15 days.

Group 1	Control
Group 2	Gel <i>perse</i> (F1)
Group 3	5% Povidone Iodine
Group 4	Eugenol 0.25% (F2)
Group 5	Eugenol 0.5%(F3)

Group 6	Resveratrol 0.5%(F4)
Group 7	Resveratrol 1%(F5)

Table 2. The number and designation of the rat groups used in the study (F-Formulation)

The animals obtained weighed approximately 200 to 225grams and were maintained in an adequately air-conditioned area with 12 hours of light and dark periods. All the animals were caged, fed with a standard meal, and given unlimited access to water. An intramuscular injection of 30mg/kg ketamine was given to each rat to induce anaesthesia. A linear incision wound measuring 1cm (10mm) size was created on the dorsal skin of the animal using a surgical blade, cleaned and the prepared formulation was applied twice daily and dressed using a surgical gauze [31].

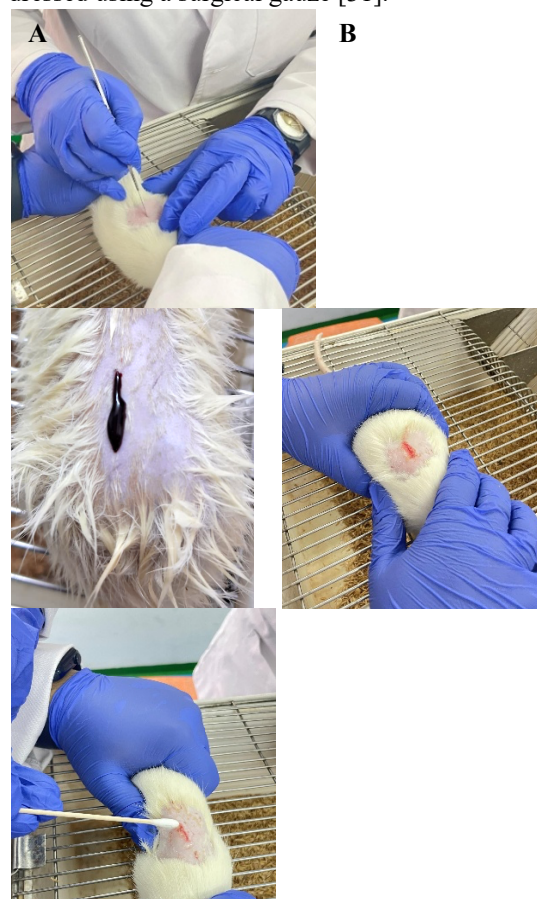


Figure 1: A & B - induction of incision wound, C - incision wound, D-topical gel application.

Gel application was repeated for consecutive days up to wound closure and scar formation(epithelialization). The changes in the wound size were recorded. The animals were euthanized by carbon dioxide asphyxiation after the study was completed. The wound healing potential of the formulations was determined by comparing the wound area healed on day 5,10 and 15 with the negative control groups. The duration of epithelialization was noted [29].

The percentage of wound contraction was calculated using the formula $(A_0 - A_t) / A_0 \times 100$ where A_0 is the initial wound size on day1 and A_t is the wound size on day 15. It was calculated to track the healing progress, compare the treatments in different groups and to assess the healing efficiency of the formulations [32,33].

Results and Discussion

Preparation Carbopol 940 based hydrogels

Physical appearance:

All the 5 formulas were examined macroscopically for the existence of structures that could be observed physically. The formulas were homogenous, lump-free, and without any roughness. The prepared formulas were clear and transparent. The physical appearance of the 5 formulas was as shown in the table 3.

Physical appearance	Result
Colour	Eugenol loaded: Colourless Resveratrol loaded: orange brown Gel <i>per se</i> : Colourless
Odour	Eugenol loaded: aromatic Resveratrol loaded: Odourless
Taste	Mucilaginous
Appearance	Viscous
Texture	Fine

Table 3 represents the physical appearance of the hydrogels

Physical characteristics

pH of the formulations: The pH of the formulations was as shown in table 4. The pH was observed to be compatible with that of the skin (5.5–6.0) to avoid irritation. The pH plays a very significant role in wound healing, Roberts *et.al*[34] and Hoffman [35] and his co-workers had demonstrated that high alkaline pH wounds had a lower rate of healing compared with wounds with a pH closer to neutral, they also reported that within alkaline conditions, wound healing progression decreased. Therefore, a pH neutral formulation helps in acceleration of wound closure by maintaining the pH of the wound environment.

Washability: All the formulations showed excellent washability with water. It did not require any soap or detergent to be washed because Carbopol being hydrophilic polymer expands by absorbing water and forms a structure which makes it non-greasy and water soluble.

Spreadability: Spreadability acutely influences the ease of application, and as an optimal value ensures

uniform distribution and enhanced therapeutic effect of the drug across the target site. The spreadability of the final gel formulation observed was as in table 4. The values indicate good spreadability, easy application, and effective topical delivery. The values of spreadability were further supported by the gel's pseudoplastic behaviour, which explains the gel's mechanical strength, ease of application and higher retention time on skin [15].

Formula number	pH	Spreadability in gcm/sec
1	5.8	12.9 ± 0.04
2	5.7	13.7 ± 0.05
3	6	15.75 ± 0.03
4	5.8	15.4 ± 0.06
5	5.9	13.12 ± 0.02

Table 4 showing the pH and spreadability values of the formulations

Stability studies: The hydrogels showed good stability on storage for a period of 90 days with no significant changes in pH, physical appearance, spreadability, drug content and washability.

Assessment of Wound size and Wound contraction

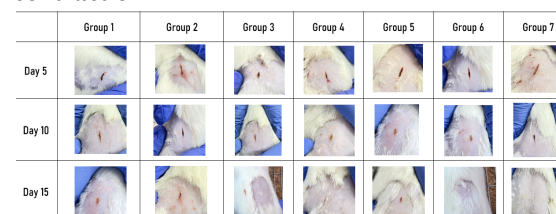


Figure 2 showing the progress in wound healing from the day of wound induction to day 15 in different treated groups. Group 1: control, Group 2: gel *per se*, Group 3: 5% Povidone Iodine, Group 4: 0.25% Eugenol, Group 5: 0.5% Eugenol, Group 6: 0.5% Resveratrol, Group 7: 1% Resveratrol.

The wound size was assessed (measured) on day 5, 10 and 15 after induction of incision wound. All the values are expressed as mean ± SD (n=6). Statistical analysis was done by Student's *t-test*, and the values were statistically significant.

Group	Wound contraction in %
Control	58.4 ± 4.7
Gel <i>per se</i>	58.8 ± 5
Standard (5% Povidone iodine)	81.3 ± 5.7
Eugenol 0.25%	83.4 ± 7.1
Eugenol 0.5%	86.7 ± 7.8
Resveratrol 0.5%	83.4 ± 7.2
Resveratrol 1%	88.4 ± 7.9

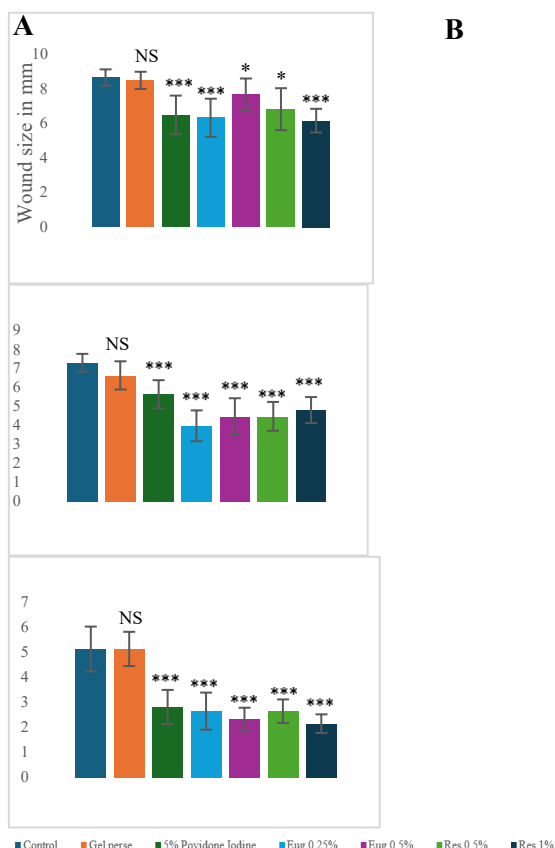


Figure 3. The effect of different concentrations of eugenol and resveratrol in comparison with control and standard (5% Povidone iodine) were evaluated. Figure A depicts wound size on day 5 of observation, figure B on day 10 and figure C on day 15. At these time intervals the wound sizes were consistently decreased in all groups treated with hydrogels.

($p < 0.001^{***}$, $p < 0.01^{**}$, $p < 0.05^{*}$, NS-Non-significant).

Significant decrease in wound size in groups treated with Eugenol (0.25% and 0.5%) and Resveratrol (0.5 and 1% concentrations) was observed on day 5 compared to group treated with 5% Povidone Iodine (Standard). No significant wound closure was seen in control and group treated with Gel *perse* which reflects that Carbopol has no role in wound healing and acts as a carrier polymer. The same trend was observed on day 10 whereas, on day 15 the groups treated with the formulations showed more than 80% wound closure and epithelialisation had commenced. 1% Resveratrol treated group displayed the highest wound closure on day 15 followed by Eugenol 0.5%, Resveratrol 0.5%, Eugenol 0.25%, 5% Povidone Iodine (Standard) and least closure in gel *perse* and untreated control groups.

The percentage of wound contraction was calculated to track the healing progress, compare the treatments in different groups and assess the healing efficiency of the formulations.

Table 5 shows the percentage of wound contraction/healing from day 1 to day 15. All the values were expressed as mean $\pm$ S.D (n=6)

A dose dependent increase in the healing of the wound was noticed in both Eugenol and Resveratrol treated groups. Significant difference in wound contraction was observed between standard and hydrogel treated groups. The highest percentage of wound contractions was observed in Resveratrol 1% followed by Eugenol 0.5% treated groups and a difference of 1.7% can be noted. Whereas in the Eugenol 0.25% and Resveratrol 0.5% groups displayed similar percentage of contraction.

Studies have shown that Eugenol has anti-inflammatory properties, promotes angiogenesis by enhanced blood vessel formation, increasing expression of VEGF and FGF growth factors. It also enhances proliferation of fibroblast and endothelial cells, hence improves cell migration. It supports re-epithelialization and collagen synthesis, hence promotes tissue regeneration [10].

Resveratrol has a positive impact on wound healing as it reduces inflammation by cytokine inhibition and suppression of NF- κ B. In vivo assays have shown it has strong antioxidant properties which protects the cells from reactive oxygen species, promotes angiogenesis by stimulating formation of new blood vessels, proliferation of fibroblasts and wound scar formation. Previous works have shown prevention of excessive collagen deposition by effectively reducing the risk of thick hypertrophic scars on treatment with Resveratrol. Studies in mouse models with full thickness wounds resulted in less scar formation when injected with Resveratrol trans-dermally. The wounds were better organised with thinner collagen fibres [37]. Resveratrol loaded hydrogel dressings have also shown similar results with less scar formation due to better organisation of collagen on treatment with resveratrol hydrogels [37]. Since Resveratrol has a low bioavailability and breaks down quickly in the body, topical application is the most desirable method of drug delivery by loading it to Carbopol 940 hydrogels and applying it directly to wounds for sustained release and localised wound closure [38].

Conclusion:

Eugenol is water insoluble because of its non-polar molecular structure whereas Resveratrol is poorly water insoluble. Both the compounds show less bioavailability due to their poor solubility in body fluids and are rapidly metabolized in liver and intestines before reaching systemic circulation. Resveratrol is enzymatically converted rapidly to its metabolic products making very less free form available for circulation. The oral and intravenous route of administration of these compounds is not feasible for treating any kind of diagnosis. Eugenol at higher concentrations is cytotoxic and by increasing the dosage the bioavailability cannot be increased, Resveratrol due to its rapid breakdown is excreted in urine within a short duration of administration. By synthesizing Eugenol and Resveratrol loaded hydrogels for drug delivery on

the wound site by topical application these limitations can be bypassed, and the drug can be delivered efficiently without the loss of the drug. The Carbopol 940 hydrogels used in the present study have excellent biocompatibility showing promising results for wound care by topical application in a period of two weeks with minimal scar formation. The weights of all animals remained constant throughout the study duration signifying the gel formulations did not interfere with the growth and metabolism of the animals and it worked as an excellent topical delivery system for wound treatment.

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