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Acute dermal toxicity evaluation of eugenol-incorporated hydrogel in Sprague Dawley rats

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ABSTRACT

Background: Multidrug-resistant *Staphylococcus aureus* (MDRSA) infections significantly impair wound healing, often leading to chronic wounds and systemic complications. There is a need for wound dressings that are not only antibacterial but also biocompatible. Essential oils, such as eugenol, possess potent antibacterial properties, but their direct application is limited by volatility and instability. Incorporating eugenol into a polymeric hydrogel provides a stable delivery system, while ensuring safety prior to *in vivo* use is essential. **Objectives:** This study aimed to evaluate the dermal safety of eugenol-incorporated chitosan-PVA hydrogel (Eug-CH) in Sprague Dawley rats, following OECD Test Guideline 402. **Materials and Methods:** The inert chitosan-PVA hydrogel was synthesised via chemical crosslinking, followed by incorporation of eugenol as an emulsion. Acute dermal toxicity testing was conducted according to OECD 402, with topical application to the dorsal skin under semi-occlusive conditions. Sequential doses up to 2000 mg/kg were administered, with a total of five female rats used. Animals were monitored intensively for 24 h post-application and daily for 14 days, assessing dermal integrity, behaviour and systemic signs of toxicity. **Results:** Topical application of Eug-CH produced no mortality or toxicity at doses up to the tested limit dose of 2000 mg/kg. Observations over 14 days revealed no adverse effects, including erythema, edema, tremors, convulsions, or behavioural abnormalities. Gross necropsy showed no organ abnormalities and a normal increase in body weight was noted. These findings indicate that the Eug-CH formulation is safe for dermal application in rats even upto the limit dose level. **Conclusion:** The Eug-CH hydrogel showed no mortality, skin irritation or pathological changes at doses ranging from 200 to 2000 mg/kg, with normal body weight gain observed over 14 days. With an LD₅₀ exceeding 2000 mg/kg, the formulation is classified as GHS Category 5 (low hazard), confirming its safety for regulatory purposes. These results indicate that the eugenol incorporated hydrogel is highly biocompatible and suitable as an antibacterial wound dressing without risk of dermal toxicity.

Keywords: Acute Dermal Toxicity, Eugenol, Chitosan-Pva Hydrogel, Antibacterial.

INTRODUCTION

Wounds encompass any form of injury that compromises the continuity of the skin, mucosal surfaces, or internal organ tissues [1]. The process of wound healing stands as one of the human body's most elaborate biological mechanisms, orchestrating multiple cell types through temporally overlapping stages including hemostasis, inflammation, proliferation, re-epithelialisation and tissue remodelling to rebuild and reinstate the structural wholeness of injured skin [2]. Delayed or impaired wound healing can occur due to factors such as the size and depth of the wound, infections, age and general health [3]. Multidrug-resistant *S. aureus* (MDRSA) presents a significant therapeutic obstacle, typically characterised by non-susceptibility to at least one antibiotic within three or more distinct drug categories [4].

Although traditional dressings can absorb wound exudate, they are unable to drain exudate in time, often resulting in a poor feature with wound healing [5]. Characterised by a highly hydrated three-dimensional polymeric framework, hydrogels can sequester several times their dry weight in water, effectively sustaining a moist environment in the wound bed [6]. Chitosan-based hydrogels are gaining attention as viable substitutes for traditional antibiotics in infected wound therapy, attributed to their excellent biocompatibility, biodegradability, inherent antibacterial activity and their role in promoting wound healing and hemostasis [7]. Poly vinyl alcohol (PVA) bolsters chitosan's film - forming potential,

mechanical robustness and thermal gelation characteristics, allowing for the production of resilient, biocompatible hydrogel matrices through intermolecular physical forces that construct a sturdy three-dimensional architecture optimal for wound management [8].

Essential oils (EOs) are volatile aromatic blends from plants containing monoterpenes, sesquiterpenes, oxygenates and phenolics that are efficiently extracted via steam distillation and valued for antibacterial, antioxidant, anticancer, antiviral, antidiabetic and aromatherapeutic effects in novel therapeutic formulations [9]. Eugenol demonstrates strong antimicrobial effects against wound-related bacteria such as *S. aureus* through membrane damage and biofilm disruption, promoting faster recovery from infected injuries when incorporated into hydrogel matrices [10]. Chitosan-PVA hydrogels loaded with clove oil (rich in eugenol) significantly enhance antimicrobial activity against *S. aureus* and *P. aeruginosa* in diabetic foot ulcer microenvironments, combining chitosan's inherent bactericidal properties with PVA's mechanical flexibility and EOs' membrane-disrupting effects for superior infected wound management [11]. Hence, the present study explores the acute dermal toxicity of eugenol incorporated chitosan PVA hydrogel to ensure its safety in wound management applications.

MATERIAL AND METHODS

Animal

The study was carried out after obtaining approval from the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary and Animal Sciences, Mannuthy, Kerala Veterinary and Animal Sciences University (CVAS/MTY/IAEC/25/94). Five healthy female rats, all nulliparous and non-pregnant, were used. They were maintained under controlled conditions at approximately 22 °C with 30-70% relative humidity and a 12-hour light-dark cycle, along with standard housing, feeding, and care throughout the study.

Synthesis of Chitosan-PVA hydrogel

The inert chitosan -PVA hydrogel was synthesized via chemical crosslinking. Initially, a chitosan solution (1% w/v) was prepared by dissolving chitosan in 2% (v/v) acetic acid under magnetic stirring at 1250 rpm for 1 h until complete dissolution. Separately, PVA was dissolved in distilled water by constant manual stirring in a water bath maintained at 80 - 90 °C. The PVA solution was then added dropwise to the chitosan solution over a period of 3 h under continuous magnetic stirring. Subsequently, 1.5 mL of 37% (w/v) formaldehyde was added as a crosslinking agent, and the reaction mixture was maintained at 40 °C with stirring for 2 h. The resulting solution was cast into petri dishes and allowed to gel during incubation for 48 h, followed by drying in a hot air oven at 50 °C for 2 h. The formed inert hydrogel films were carefully peeled from the petri dishes and stored until further use [12].

Preparation of Eugenol-Incorporated Hydrogel (Eug-CH)

A 10% (v/v) eugenol formulation was initially prepared using Tween 80 as an emulsifying agent. The required volume of eugenol was mixed with distilled water and vortexed thoroughly to obtain a uniform emulsion. The prepared eugenol emulsion was incorporated into the preformed chitosan - PVA hydrogel by gradual dropwise addition onto the hydrogel surface, ensuring complete absorption into the polymeric matrix. The eugenol-loaded hydrogel was then air-dried at room temperature and stored under appropriate conditions until further use.

Acute Dermal Toxicity Assessment

The acute dermal toxicity of the Eug-CH was evaluated in compliance with OECD Test Guideline 402. Five healthy female rats weighing between 200 and 300 g were used for the study. Prior to application, the dorsal region of each animal was clipped free of hair

approximately 24 h in advance, ensuring that the skin remained intact and free from injury.

A dose range finding test was initially conducted by administering graded doses of 200, 1000, and 2000 mg/kg body weight to individual animals. Based on the absence of adverse effects, two additional animals were subsequently exposed to the limit test dose of 2000 mg/kg in the main study to confirm the safety of the formulation [13].

Application and Clinical Observation

Approximately 10% of the dorsal body surface area was exposed, and the test formulation was applied evenly and maintained in contact with the skin for 24 h under semi-occlusive conditions. During the exposure and observation period, animals were housed individually in separate cages. Clinical observations were carried out continuously during the first 30 min after application, followed by periodic monitoring at regular intervals for the next 6 h and thereafter once daily for a total duration of 14 days. Observations included evaluation of skin condition, fur texture, eyes and mucous membranes, respiratory and cardiovascular functions, autonomic and central nervous system responses, as well as motor activity and behavioural changes. Any signs of toxicity, including tremors, convulsions, salivation, diarrhoea, lethargy, sleep disturbances or coma were also recorded [13].

RESULTS

Observations of Range finding study and Main study

During the preliminary dose-range finding phase, topical administration of Eug-CH at doses of 200, 1000 and 2000 mg/kg to individual animals produced neither mortality nor treatment-related clinical signs throughout the day of exposure and the subsequent 14-day observation period. In the main study, application of Eug-CH at 2000 mg/kg to two additional animals likewise resulted in the absence of mortality or observable toxicity. Animals were closely observed at regular intervals during the first 24 h post-application and monitored daily thereafter. No adverse dermal or systemic effects, including erythema, edema, behavioral alterations, neuromuscular disturbances, ocular abnormalities or other signs of toxicity were noted. Terminal gross pathological examination conducted at the end of the study period revealed no treatment-related abnormalities, indicating that the Eug-CH formulation is well tolerated and non-toxic following topical exposure up to the limit dose of 2000 mg/kg in rats, indicating an LD50 \geq 2000 mg/kg in the dermal toxicity test.

Body weight

Throughout the observation period, all animals exhibited a gradual and consistent increase in body weight, with a net gain of approximately 10–15 g per animal, indicating normal growth patterns and the absence of treatment-related adverse effects.

Gross pathology

Gross pathological examination at necropsy revealed no visible abnormalities in any of the internal organs, indicating the absence of treatment-related pathological changes (Figure 1).

DISCUSSION

In this investigation, the acute dermal toxicity of the eugenol-loaded chitosan hydrogel (Eug-CH) was evaluated in accordance with OECD Test Guideline 402 to determine its preliminary safety profile (OECD, 2017). Topical exposure to Eug-CH at a dose of 2000 mg/kg did not produce any signs of dermal irritation or systemic toxicity throughout the 14-day post-application observation period. Based on these findings and the criteria outlined in OECD 402, the formulation may be categorized under Globally Harmonized System (GHS) Category 5, indicating a low level of acute dermal toxicity.

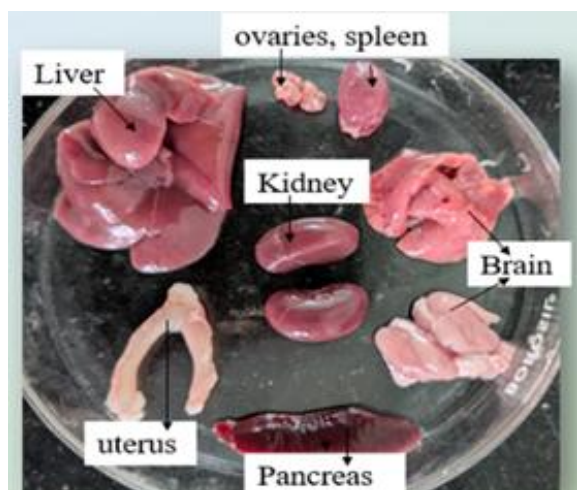


Figure 1: Gross pathological changes of internal organs

These observations are in agreement with earlier studies in which rats were exposed to eugenol aerosol at concentrations ranging from 0.77 to 2.58 mg/L with particle sizes under 1 μm for four hours. The animals exhibited only mild and temporary signs such as increased salivation, agitation, and short-term reductions in food and water intake, all of which resolved within 14 days. Examination of lung tissue revealed no treatment-related lesions, with findings similar to those seen in control animals. Importantly, the total systemic dose received through inhalation was relatively low (around 1–3 mg/kg), considerably less than the dermal limit dose tested in the current study, yet no significant adverse effects were observed. Encapsulation of eugenol in the chitosan-PVA hydrogel likely restricted its release and absorption, further enhancing safety. Overall, these results confirm the low acute toxicity of eugenol and support the safe use of Eug-CH for topical wound management [14].

Eugenol nanoparticles have been shown to exhibit excellent safety profiles at doses up to 2000 mg/kg in rats, with no significant effects on body weight, food and water intake, hematological and biochemical parameters, organ weights, histopathology or plasma cytokine levels (IL-1, IL-6, TNF- α). Similarly, in the present OECD 402 acute dermal toxicity study, the eugenol-incorporated chitosan hydrogel (Eug-CH) produced no clinical signs of toxicity, skin irritation, behavioral changes, or gross pathological alterations at the 2000 mg/kg limit dose. These findings indicate that Eug-CH is non-toxic upon topical application, with an LD₅₀ exceeding 2000 mg/kg, and can be categorized under GHS Category 5, highlighting its suitability for wound healing applications [15].

CONCLUSION

The acute dermal toxicity evaluation of the eugenol-incorporated chitosan hydrogel demonstrated no mortality, skin irritation, or gross pathological alterations throughout both the range-finding (200–2000 mg/kg) and main study (2000 mg/kg) phases, with normal body weight gains observed in all animals over 14 days. The formulation thus exhibited an LD₅₀ greater than 2000 mg/kg, placing it in GHS Category 5 and confirming its low hazard potential. These findings underscore the excellent biocompatibility of the hydrogel, supporting its safe use as an antibacterial wound dressing without risk of dermal toxicity.

Conflict of interest

The authors declared no conflict of interest.

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