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In vivo Study of Modified Okra Mucilage/Acrylic Acid Hydrogels Loaded with Ethanolic Extracts of Calendula Officinalis for Wound Healing Application

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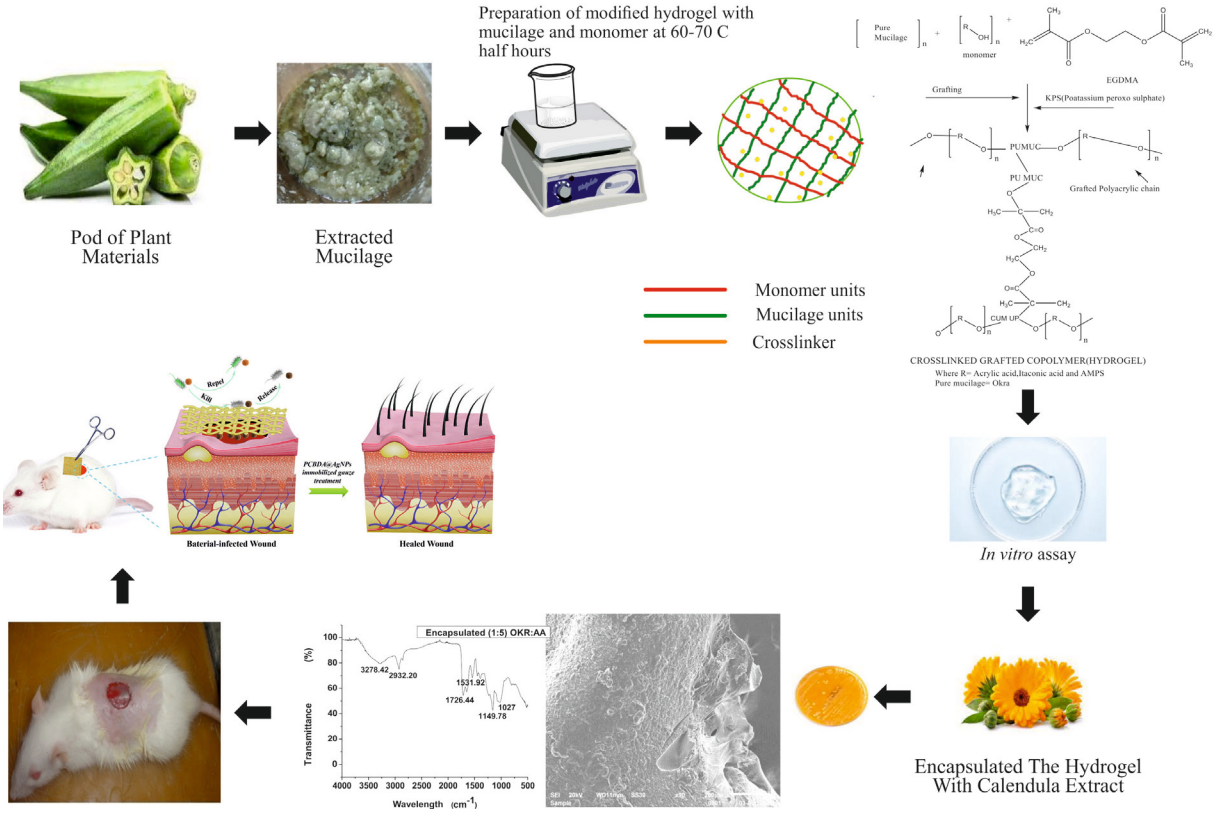
Abstract **Purpose:** The study aimed to evaluate the wound-healing efficacy of an ethanolic extract of Calendula officinalis flower petals, traditionally used by tribal communities. This extract was encapsulated in hydrogels made from modified okra mucilage and acrylic acid to enhance its therapeutic effects. **Methods:** Mature flower petals of Calendula officinalis were crushed to prepare an ethanolic extract. This extract was encapsulated in hydrogels and applied to excision wounds on animal models. A comparative analysis was conducted on encapsulated and unencapsulated hydrogels. Epithelization period, contraction area, hydroxyproline content, total protein, antioxidant activity, wound index and histopathological evaluations were among the key parameters analyzed. **Results:** The study revealed that animals treated with encapsulated hydrogels demonstrated significantly improved wound healing outcomes compared to those treated with unencapsulated hydrogels and control groups. Specifically, the encapsulated hydrogels led to greater wound contraction, a shorter epithelization period, and a lower wound index. Additionally, higher levels of hydroxyproline, total protein, and DNA concentrations were observed in healing tissue, indicating enhanced collagen synthesis and tissue regeneration. Histopathological evaluations supported these findings with more organized and mature tissue formation. **Conclusion:** The encapsulated hydrogel of Calendula officinalis ethanolic extract proved to be more effective in promoting wound healing than the unencapsulated versions. This novel hydrogel formulation shows promise as a potent pharmaceutical candidate for acute cutaneous wound care.

Graphical Abstract



Keywords Hydrogels, Wound Healing, Encapsulation, *Calendula officinalis*, Okra Mucilage

1. Introduction

A wound is an injury to the healthy skin, and its consequence may lead to a global health problem. Cosmetic products play an important role in rapid treatment and prevention. The pharmaceutical sector faces problems producing medical equipment and addressing consumer market demands. Using herbal ointments for wound healing or cosmetic items might give a choice to diverse customers. Therefore, it has attracted greater attention in recent years. This method is an ecologically sound strategy for maintaining a wet environment, reducing curing time, and minimizing (or largely preventing) disease. Wound healing may be greatly influenced by the bioactive compounds found in natural ingredients like plant juices, mainly because of their anti-inflammatory, antioxidant, and antimicrobial effects. Nature has been giving solutions for different medical illnesses for decades. Mucilages are plant-based polysaccharides that are highly hydrated, form gel, and often contain biologically active compounds. The large capacity and increasing demand for mucus create the need to understand the properties of natural materials for better use. Natural materials play a prominent role in producing products for external use and contain phytochemicals like flavonoids, phenols, alkaloids and terpenoids. These compounds have practical uses in medicine, skin procedures, and developing anti-inflammatory drugs while supporting fibroblast proliferation. For example, flavonoids exert anti-inflammatory effects by inhibiting cyclooxygenase and lipoxygenase, reducing reactive oxygen species and improving wound healing. Different precursor peptides in mucus can stimulate collagen synthesis, and aids in the wound healing process [1, 2]. Wound healing comprises complex phases, which include hemostasis, inflammation, migration, and remodeling. In the last few years, biomaterials for wound healing and medication release have shown significant progress in delivering more efficient patient treatment. To improve cutaneous wound care, scientists have developed several types of wound dressings, such as conventional, interactive, bioactive, etc. [3].

The desire for wound-healing materials has prompted developing novel materials, including natural biopolymers, semi-synthetic polymers, and synthetic polymers. In wound management, biopolymers have gained importance, as they possess versatile properties, such as biodegradability, biocompatibility, and durability, and are like the macromolecules recognized by the human body. To facilitate wound healing, one can consider adopting a few approaches, including the addition of in situ drugs,

drug encapsulation, wound site filling, anti-infection, anti-oxidation, blood clotting, and poor adhesion [4]. Current advances in wound dressing provide a better understanding of wound healing mechanisms, such as drug release mechanisms, polymer degradation kinetics, and new fabrication and development methods to produce a more efficient treatment method for individual patients. Hydrogels have emerged as promising options for medication delivery and wound care treatment for decades. Newer materials with better properties are a challenge for the development of hydrogels. The wound-healing efficiency of quince seed mucilage was studied in rabbits using wound closure model [5]. A study by scientists investigated the wound healing activity of composite sponges containing with mucilage and lipoidal matter derived from Hibiscus species [6]. The zinc oxide nanoparticles synthesized via biological route and produced from psyllium mucilage have demonstrated increased antibacterial and wound healing ability. According to scientific studies, psyllium mucilage has been found to enhance the bactericidal and wound-healing potential of biosynthesized zinc oxide nanoparticles [7]. Chitosan/PEO nanofibers and Calendula officinalis extract have been tailored and characterized for wound healing applications [8].

In our previous study, modified okra mucilage with acrylic was synthesized, optimized, and standardized in different ratios. The optimized ratio achieved was 1:5, i.e., mucilage: acrylic acid. These hydrogels were glucose-sensitive and pH-sensitive smart polymers with biocompatibility and hemocompatibility [9]. Calendula has antioxidant and antibacterial properties and has been encapsulated in the hydrogels. Release studies have followed non-Fickian release patterns and have shown that the polymer releases the encapsulated calendula extract. As a result, these hydrogels may be used in various biological processes.

The present study explores the antibacterial, anti-inflammatory, antiviral, collagen synthesis, and antioxidant properties of modified and encapsulated okra mucilage with Calendula officinalis extracts for wound healing. The polyphenols and flavonoids analysis have been studied to evaluate the calendula extract. This paper presents the comparative studies of encapsulated and unencapsulated samples. The investigation focused on several factors associated with wound healing, such as epithelization duration, wound index analysis, hydroxyproline content, total protein content, antioxidant activity, and histological assessments. The results showed significant differences in wound healing for different samples.

2. Experiments

2.1. Materials and Methods

Biochemical assays of hydroxyproline were performed using amino acid kits. Bovine serum albumin (BSA), hydrogen peroxide, sodium potassium tartrate, copper sulfate, and sodium carbonate were procured from Central Drug House (P) Ltd. Folin-Ciocalteu reagent, and Ehlrich reagent used for biomarker analysis were procured from SRL Laboratories Pvt. Ltd.

2.2. Mucilage Extraction from Okra and Phytochemicals from Petals of Calendula Officinalis

2.2.1. Okra Mucilage Procurement

Okra was purchased from the local market in May and July. Calendula officinalis flower petals were collected from the IFTM University Botanical Garden in Moradabad, India, in February and March 2019. Dr. Ashok Kumar, Assistant Professor, Department of Botany, IFTM University Moradabad (U.P.), India, performed the authentication. A voucher specimen (Ref. 2018/SOS/BOT /69.) was submitted to the department.

2.2.2. Extraction

The fruits of the okra plant were meticulously cleaned, diced, and then soaked in distilled water for an entire day. After centrifuging the crude material for five minutes at 3000 rpm and drying it, the mucilage was precipitated using acetone.

Calendula flower petals were collected, dried, and extracted using the Soxhlet apparatus with ethanol. The extract containing the phytochemical was further distilled and used after dilution to encapsulate the polymer. The stock solution was made using the residue. The concentration was 1mg of extract in 10 ml of ethanol, i.e., 1mg/10ml or 100 μg/ml. Vandna et al. [9] have reported the detailed procedure.

2.3. Preparation of Hydrogels

Hydrogels were synthesized using extracted okra mucilage (OKR) and acrylic acid(AA) monomers in various ratios of 1:5, 1:2, and 1:1 (OKR: AA) by free radical polymerization. The initator selected for polymersiation was potassium persulphate ($K_2S_2O_8$), and ethylene glycol dimethacrylate (EGDMA) was used as a cross-linker. The extracted mucilage, monomer, and initiator were thoroughly mixed till they form a homogenised solution, on a magnetic stirrer for 30 minutes.

The cross-linker was added after 30 minutes of mixing and then heated for another 45 minutes, at 80^0C, to obtain the hydrogels. The conditions of polymerization were standardized and optimized by varying the concentration of the polymer (OKR): monomer (AA), cross-linker (EGDMA) and initiator. The free radical polymerization mechanism has already been reported by Vandna et al. [9] and is given in Figure 1.

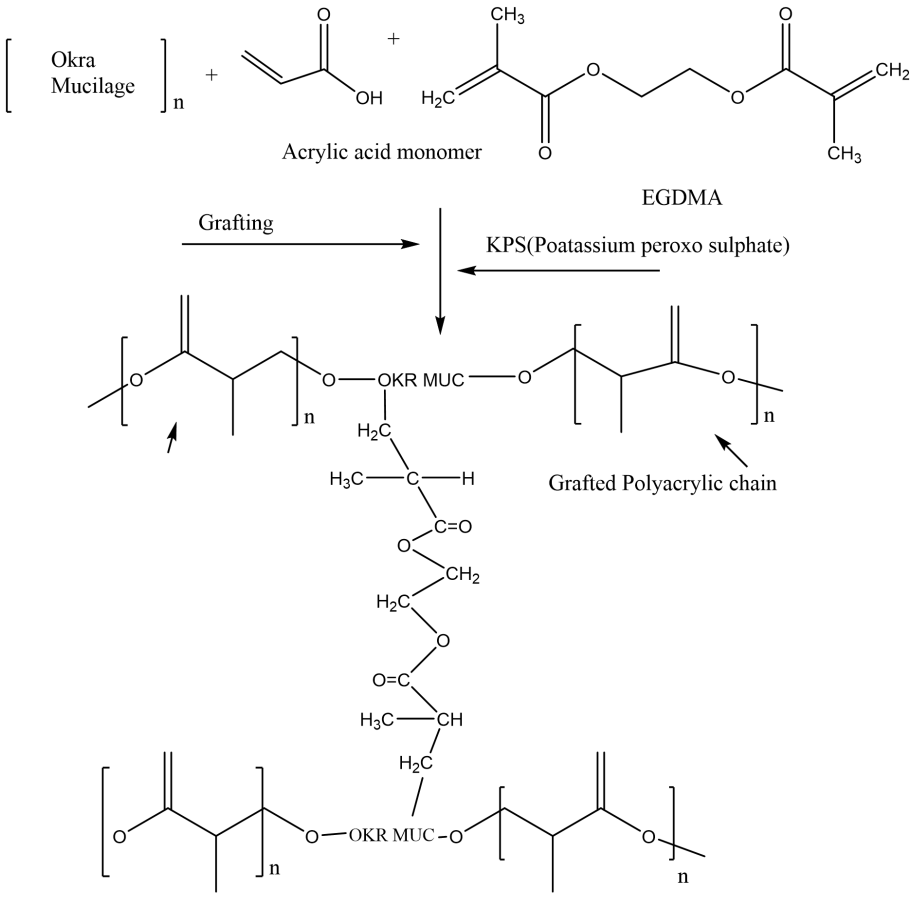


Figure 1. Reaction Mechanism for polymerization

2.4. Quantitative Estimation of the Phenolic, Flavonoid, and Antioxidants in the Ethanolic Extract of Calendula Officinalis (CE)

2.4.1. Phenol Estimation w.r.t. Gallic Acid

Ten milligrams of calendula extract with 10 milliliters of ethanol prepared 1 mg/mL of the extract. 1 ml solution was diluted further to 10 ml. In a 50 mL beaker, 1 mL of an ethanolic extract solution was combined with 9 mL of distilled water, 150 µl of Folin-Ciocalteu phenol reagent was added. After 5 mins, the resulting sample was mixed with 0.7% of 500 µl sodium carbonate solution. Various quantities of gallic acid ranging from 50 to 500 µg/ml were prepared using a stock solution, including distilled water; the standard curve was produced by recording the absorbance at 760 nm. A blue product, shown by the chemical reduction of a mixture of tungsten and molybdenum oxides derived from FC reagents independent of the structure of phenolic compounds, allows quantification of phenolic content [8-10].

2.4.2. Flavonoid Estimation w.r.t. Quercetin Content

Spectrophotometric analysis was used to determine flavonoid content. The flavonoid concentrations were determined by plotting a standard curve of quercetin standard solution of concentration varying from 2 to 20 µg/ml. To measure the concentration of the aqueous extract, 1 mL was combined with 0.3 mL of 10% Al_2Cl_3, 0.3 mL of 5% sodium nitrite solution, and 2 mL of 0.04 µg/mL. After 30 minutes, the samples were analyzed on a UV spectrophotometer (model UV-1800 Shimadzu) at 310 nm [8], [10].

2.4.3. Anti-Oxidant Activity Using 2, 2'-Dipheny-l-Picrylhdrazyl Radical (DPPH) of the Extract

DPPH assay was used to study the antioxidant activity of Calendula extract (CE). The structure of DPPH consists of a stable free radical of nitrogen. The compounds have the capability of removing free radicals or hydrogen. Equation 1 given below computed the DPPH free radical scavenging activity [11]. The details are given in **Supplementary S1** (https://docs.google.com/document/d/1_aA3opq5dtwPY6CZ4XiJmfXPEcg3J1fP/edit?usp=drive_link&ouid=106014090175249682283&rtpof=true&sd=true).

$$\text{DPPH (\%)} = \frac{\text{Abs control} - \text{Abs sample x100}}{\text{Abs control}} \quad (1)$$

2.5. Antimicrobial Activity

Antimicrobial activity was evaluated against the Staphylococcus aureus and Escherichia coli pathogens.

The agar disk diffusion method was followed [9]. This method is commonly used to study antimicrobial activity by diffusion of the test compounds through aqueous agar plates. The material's shape, polarity, and size govern the diffusion process. To study antimicrobial activity, sterilized nutrient agar medium (2.8 g nutrient agar in 100 ml of water) and nutrient broth (1.3 g in 100 ml of water) were prepared. The nutrient medium was used for bacterial growth. The nutrient medium was put in a petri dish, and one loop of each strain was streaked in the medium for 24 hours to form a single colony. The plate had two wells: one for the positive control, or ciprofloxacin, at a concentration of 0.8 ppm (C+), and the other for the negative control, water (C-). After incubation, we checked the plates the next day for clear zones around the wells, referred to as inhibition zones. They measured the diameters of these zones and recorded the measurements in mm. After adding bacterial cultures, we treated and incubated the tubes at 27 °C for 24-48 hours [9].

2.6. Animals

Healthy adult (either sex) Wistar rats (150-250 g) were used for the study procured from the IFTM University animal house. The group of animals used for the study is four, and the total number of rats in each group is four, i.e., 16 rats were used. They were kept at a temperature of 28± 2°C in polypropylene cages. The cages contained a relative humidity (RH) of 60-70%, followed by a dark/light cycle of 12 hrs. The animals were provided unrestricted access to standard pellet feed and mineral water throughout the research.

The protocols for pharmacological studies and acute toxicity were cleared by the Institutional Animal Ethics Committee (IAEC) of IFTM University of Moradabad, with reference no. CPCSEA was performed according to the ethical approval (876/PO/ReBiBt/S/04/CPCSEA) approved by OCED guidelines.

2.7. Study on Acute Dermal Toxicity

The skin irritation test was conducted on Wistar rats weighing 180-200 grams. The animals were fed conventional feed and given access to mineral-enriched water. Three groups of rats were used for the study, with three in each group (9 rats were used). Details are given in **Supplementary S2**. The animals were maintained under well-established laboratory conditions, and the extract was applied as an ointment with Vaseline as a base. OECD Guidelines detailed in No. 402 were used as a study protocol. The rats were divided into four treatment groups under the 1987 OECD Guidelines [12].

2.8. Excision Wound Model

Wistar rats weighing 150–250 g were anesthetized with ketamine HCl at 80 mg/kg of body weight (i. p.). The rats' dorsal hairs were shaved using shaving cream and cleaned with ethanol. An incision was made on the dorsal thoracic region of the rats using a surgical blade and scissors to remove two circular areas, each measuring 300 mm. Wound depth was not over 2 mm, and all animals were kept in individual polypropylene cages. After creating the wound, it was covered with polymeric material. [13] The dried polymeric material is encapsulated with (CE) Calendula ethanol extract (CE^+ OKRA: AA) and without encapsulation (CE^-OKRA: AA) applied.

2.8.1. Assessment of Wound Area

A full HD camera was used to monitor the wound region on specific days, including the 0th, 4th, 8th, 12th, and 16th, until it healed fully. Measurements were recorded on drafting paper, and the areas were calculated using mm scale graph paper [14]. The percentage reduction in the initial wound size was evaluated.

The formula used for wound contraction is given in Equation 2 below:

$$wound\ \% = \frac{Wo - Wn}{Wn} \times 100 \quad (2)$$

Where W_0 = Wound area on the Zeroth day

W_n = Wound area on nth (0^{th}, 4^{th}, 8^{th}, 12^{th}, and 16^{th}) day

2.8.2. Wound Index Measurement

The wound index was evaluated according to the parameters in Table 1, which covers Wachtel *et al.*'s randomized scoring system [15].

Table 1. Gross change in wound index by the scoring system

S.No.	Changes observed	Rating
1.	Pus and necrosis	4
2.	The environment is healthy. Healing has not been initiated	3
3.	Healing healthy but delayed	2
4.	Healthy healing but Incomplete	1
5.	Healing completed	0
6.	Score	**10**

2.8.3. Epithelization Period

The development of new skin over a wound is classified as either a healed wound or complete epithelization. The epithelization period is the number of days it takes for a wound to heal [16].

2.9. Estimation of Bio-Chemical Markers

The wound was created in the dorsal region. New tissues were raised and collected on the 16th day for the biochemical assay. The size of new tissue collected from the dorsal region is about 150mg. The granulated tissues were estimated for their protein and hydroxyproline

content. Free radical scavenging was used to evaluate the antioxidant levels in tissue.

2.9.1. Hydrolysate Preparation

150 mg of moist, dried tissue was placed in an airtight glass container containing 3 ml of 6N HCl. The hydrolysate container was autoclaved for 15 to 20 minutes at 121°C and then cooled to room temperature. The surplus acid was balanced out by adding 10N NaOH solution. The prepared hydrolysate was used to determine the tissue's collagen (hydroxyproline), total protein, and antioxidant levels by studying the free radical scavenging activity [17].

2.9.1.1. Hydroxyproline

The prepared protein hydrolysate sample tubes were labeled as "sample," "blank," and "standard." To the tube labeled "sample," 1 ml of the test sample was added. In the "blank" tube, 1 ml of double-distilled water was added, while the "standard" tube received 1 ml of standard hydroxyproline solution. Each tube was then supplemented with 1 ml of freshly prepared 0.01M copper sulfate solution, 1 ml of 2.5N sodium hydroxide, and 1 ml of 6% hydrogen peroxide. The mixtures were thoroughly mixed and heated in a water bath at 80°C for five minutes. After rapid cooling in an ice bath, 4 ml of 3N sulfuric acid was added to each tube. Next, 2 ml of Ehrlich's reagent was added, and the solutions were heated at 70°C for 15 minutes. The optical density at 540 nm was then measured using a digital colorimeter [17].

2.9.1.2. Estimation of Protein Content

Protein concentration was estimated using the Lowry et al. method. Standard Bovine Serum Albumin (BSA) was taken as a standard [18]. Varying concentrations of BSA, ranging from 30 to 150 mg/ml, were prepared. From these varying concentrations, 0.2 ml of the sample solution was taken to prepare the standard solution. Then, 4.5 ml of the solution containing three reagents was added: 2% sodium carbonate in 0.1N sodium hydroxide as reagent A. Reagent B consisted of 1% sodium potassium tartrate (7 mM), and 0.5 ml of 0.5% copper sulfate was prepared from a 50 ml stock solution. Subsequently, 0.5 ml of a 1:1 mixture of Folin phenol reagent and water was added. Absorbance was measured at 650 nm using a UV spectrophotometer, with readings taken in triplicate for both the blank and sample solutions. The absorbance was noted at 650 nm on a UV spectrophotometer. Absorbance readings were taken in triplicate, including a blank and sample solutions [18].

2.9.1.3. Estimation of Anti-Oxidant Activity

The Ruch et al. (1989) method evaluated the tissues' ability to remove hydrogen peroxide. A solution of hydrogen peroxide was prepared at 40 mM concentration using 30% hydrogen peroxide, a phosphate buffer of pH 7.4. 0.4ml of hydrolysate sample was mixed with 0.6ml of 40mM H_2O_2 and further diluted to make it 2ml. Then, 50mM sodium phosphate buffer was added and incubated for 40 mins at 30ºC. Following ten minutes, the mixture's absorbance was measured at 240 nm concerning the phosphate buffer, which served as a blank.

Equation 3 below was used to calculate the percentage of hydrogen peroxide in the sample [19].

$$\% \text{ Scavenged } [H_2O_2] = [(A_C - A_S)/A_C] \times 100 \quad (3)$$

Where A_C is the absorbance of the control, and A_S is the absorbance in the presence of the sample.

2.10. Histopathological Examination

On the 16th day, the new skin formed on the healed wound was collected from the rats of each group to study the histopathological alterations. The samples were fixed in a formalin solution (i.p. 2007), and a 5 µm thin slice was stained with hematoxylin and eosin. Samples were taken and photomicrographed at a 100X magnification.

2.11. Statistical Analysis

ANOVA was used to statistically compare the groups with the control group. The GraphPad Prism software designed by Insightful Science was used for all statistical analyses [14].

3. Result and Discussion

3.1. Estimation of Phenols, Flavonoid, and Antioxidant Activity

Table 2 presents the results of the total phenols and flavonoids and total antioxidant content in Calendula extract.

The calendula officinalis (CE) ethanol extract containing phenols and flavonoids has high anti-oxidant activity, as these antioxidants are highly effective at capturing and neutralizing free radicals. **Supplementary Data S1** explains the graphical representation of flavonoid content. The reagent DPPH was used to evaluate the antioxidative potential of the extract. The purple DPPH form is converted into its non-radical yellow form of 2, 2'-diphenyl-1-picrylhydrazine (DPPH). The reaction involved is discussed below (Figure 2). The increasing concentration of the sample increases the activity of the samples, which is indicated by the low IC50 values. This suggests better protective action and an increase in radical scavenging activity.

3.2. Antimicrobial Activity

The inhibitory zone for the ethanolic extract of Calendula (CE) was investigated for two microbial strains, S. aureus, and E. coli, at 30, 60, and 90 µg/ml concentrations. The results were compared to a positive

control group of Ciprofloxacin and a negative control of water. The study in Table 3 and Figure 3 shows that the extract demonstrated better effectiveness against E. coli than S. aureus at 30 µg/ml and 60 µg/ml concentrations. However, at 90 µg/ml, S. aureus exhibited the highest zone of inhibition.

Table 2. Estimation of the phenols, flavonoid, and antioxidant activity

Plant extracts (CE)	Total Phenol contents (GAE) (µg/ml)	Total Flavanoid content QAE (µg/ml)	Total antioxidant DPPH content (µg/ml)
Calendula ethanol extract	81.66	46.42	246
Line of Regression	y=0008x+0.1811 R^2=0.9898	y=0.0009x+0.0476 R^2=0.9862	Y=0.1766+6.7302 R^2= 0.987

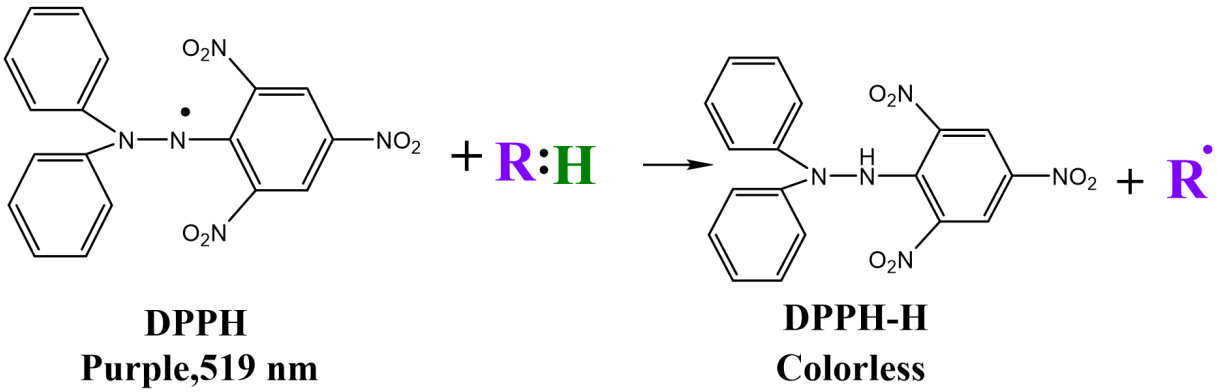


Figure 2. Reaction of DPPH free radical

Table 3. Inhibitory Zone (mm)

	Microbial strains	**Inhibitory zone (mm)**	**Microbial strains**	**Inhibitory zone (mm)**
CE-01 (30µg/ml)	*Staphylococcus aureus*	Nil	*E. coli*	21
CE-02 (60µg/ml)		Nil		22
CE-03 (90µg/ml)		29		24
Ciprofloxin (+ ve)		42		30
Water (-ve)		Nil		Nil

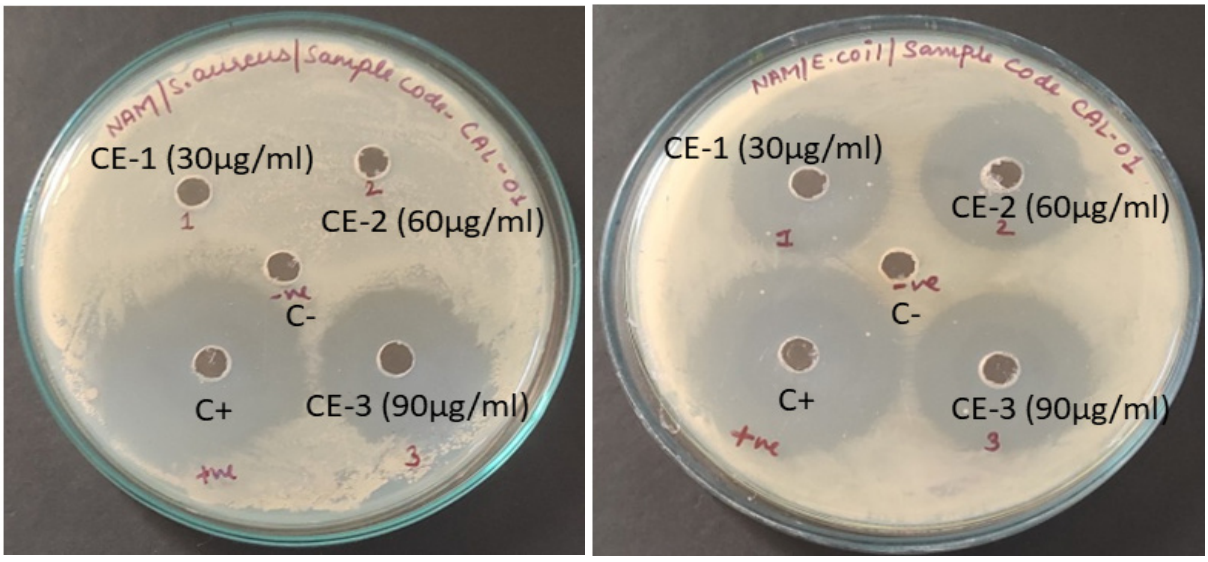


Figure 3. Antimicrobial activity of the sample (CE) against S. aureus and E. coli respectively

3.3. *In vivo* Analysis

3.3.1. *In vivo* Analysis of Modified Okra Mucilage

The selection of modified Okra mucilages in a 1:5 ratio of mucilage monomer was used for in vivo studies [8]. The polymer in a 1:5 ratio was encapsulated with ethanolic calendula extract (CE^{+}OKR: AA). Two samples were used for the in vivo studies: one encapsulated with ethanolic calendula extract (CE^{+}OKR: AA) and one unencapsulated (CE^{-}OKR: AA). The studies used four sets of animals, as outlined in Table 4.

Table 4. Animal classification

Group-1	(Control) untreated wounds
Group-2	Okra polymeric material (CE-)
Group-3	Okra -encapsulated polymeric material (CE^{+})
Group-4	Soframycin (standard)

3.3.2. Study on Acute Dermal Toxicity

Three sets of rats were used to assess the dermal toxicity of the extract. Three samples used were the control, paraffin, and ethanolic extracts of Calendula officinalis (CE). The OECD Guidelines No. 404 (OCED, 1981) were followed to perform this test on rats. Toxicity refers to the capacity of a substance to elicit a reaction in an animal upon contact with the skin. The extent to which toxic substances are absorbed through the skin is determined by their chemical composition and solubility. All the studied cases showed no observable effect. Thus, all the studied cases show no observable effect, proving that we have observed no acute dermal toxicity. The Calendula officinalis extract (CE) has shown no toxic effect on the liver and kidneys, as shown by the results presented in **Supplementary Data S2**.

3.3.3. Wound Closure

The closure of the wound shows its effect on wound healing. The wound area was assessed in each group on the 4th, 8th, 12th, and 16th days. These are the control, standard (Soframycin® skin cream), and both polymeric samples with (CE^{+}OKR: AA) and without encapsulation (CE-OKR: AA) with the extract of Calendula officinalis.The wound closure in the case of an encapsulated sample (CE^{+}OKR: AA) better than the wound closure observed in the case of an unencapsulated polymer (CE-OKR: AA), as shown in Figure 4 and Table 5.

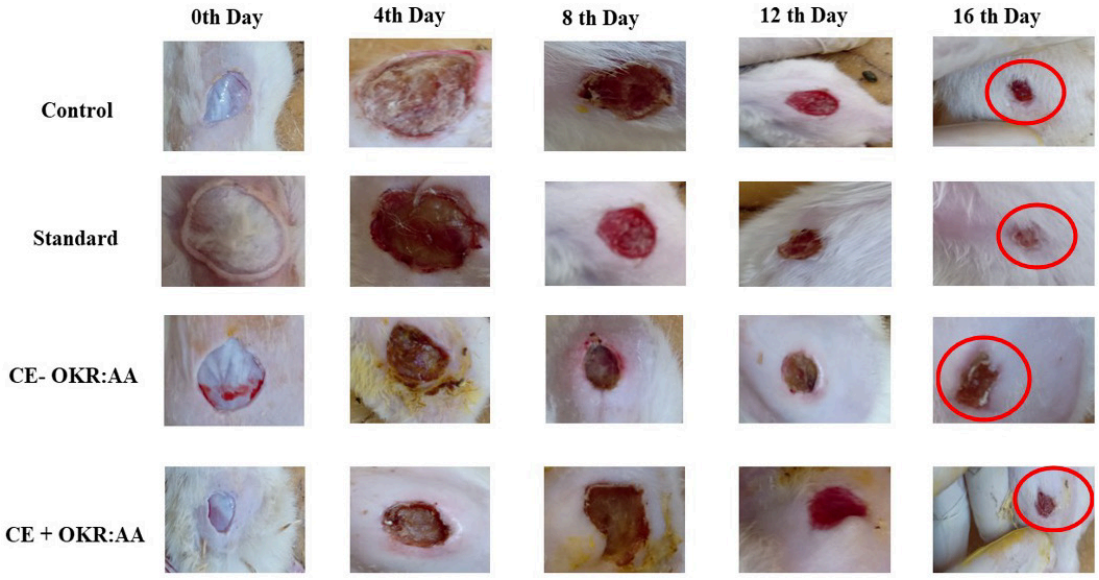


Figure 4. Wound closure of the Okra Polymer (n=4) with acrylic acid

Table 5. Observed Wound contraction

Groups	**Wound contraction (%)**			
Treatment Group	**4th Day**	**8th Day**	**12th Day**	**16th Day**
Control (Blank)	9.1±0.5	*46.0 ± 1.4	60.0 ± 1.4	84.5 ± 1.6
Soframycin Cream (w/w)	10.0 ± 0.5	*62.1 ± 1.5	** 76.3 ± 2.1	*** 99.0 ± 0.7
CE-OKRA:AA (w/w)	9.6 ± 0.8	57.0 ± 1.5	71.5 ± 5.3	95.5 ± 2.0
CE+OKRA:AA (w/w)	10.0 ± 0.7	59.7 ± 2.3	76.0 ± 2.6	**96.1 ± 0.9

Data are presented as mean ± S.E.M from four rats and analyzed using ANOVA followed by Tukey tests.

*P < 0.05, **P < 0.01, and ***P < 0.001 indicate significance compared to the control group.

The encapsulation of synthesized polymer samples, derived from modifying okra mucilage with acrylic acid, was conducted using calendula extract (CE+OKR: AA) to investigate its effect on wound healing. The wound healing efficacy of encapsulated (CE+OKR: AA) and unencapsulated samples (CE-OKR: AA) was compared. The encapsulated okra samples showed a wound closure of 10.0 ± 0.7 on the 4th day, while the unencapsulated sample exhibited a closure of 9.6 ± 0.8, compared to the control sample, which had a closure of 9.1 ± 0.5. Data analysis revealed that the encapsulated okra samples showed a percentage wound closure of 59.7 ± 2.3% on the 8th day, whereas the unencapsulated hydrogel (CE-OKR: AA) demonstrated a closure of 56.98 ± 1.52%. By the 12th day, the encapsulated sample exhibited a wound contraction of 76.0 ± 2.6%, significantly different from the control. However, the encapsulated okra-based polymer samples showed a contraction of 96.1 ± 0.9% on the 16th day, in contrast to the control, which showed 84.5 ± 1.6%.

3.3.4. Effect of Calendula Extract (CE) on Wound Index

Table 6 provides the wound index for healing based on the method by Wachtel et al. The encapsulated sample (CE^{+}OKRA: AA) demonstrated a significantly lower wound index value of 2.7 ± 0.0, differing from the control group. The unencapsulated okra sample (CE-OKRA: AA) showed a wound index value of 2.1 ± 0.0, indicating a good response. The encapsulated sample (CE^{+}OKRA: AA) has exhibited the least wound index value compared to the control and unencapsulated samples, highlighting its application for wound healing (Table 7).

3.3.5. Effect of Polymers on Biochemical Markers

a) Estimation of Hydroxyproline content

Hydroxyproline is a biomarker for collagen determination that helps in wound healing. The encapsulated okra sample (CE+OKRA: AA) showed higher levels of hydroxyproline (255.7 ± 0.8 µg/mg) than the unencapsulated samples (CE-OKRA: AA) and control. Similarly, in the absence of encapsulation, CE-OKRA: AA showed a higher hydroxyproline content (215.1 ± 2.7) than the control group.

Encapsulated polymer samples (CE-OKRA: AA) had the highest hydroxyproline concentration and showed better wound healing, as shown in Table 7 and Figure 5.

b) Estimation of total protein

Protein content is another biochemical marker carried out after healing the wound. Proteins play a crucial role in wound healing by regulating the formation of RNA and DNA, collagen and elastic tissue, immune system function, epidermal growth, and keratinization. The protein content was evaluated for encapsulated and unencapsulated samples in the wound sample, which provided insights into the healing process. Compared to the control sample, the okra (CE^{+}OKR: AA) encapsulated sample exhibited a higher protein content of 157 ± 3 µg/mg. There was a difference of 119.6 ± 2 µg/mg in protein content between the control group and the sample without encapsulation (CE-OKR: AA), with the control group showing lower levels. The polymer samples without encapsulation (CE-OKR: AA) have a lower protein content than the encapsulated sample (CE^{+}OKR: AA). The control sample has a lower protein content. Therefore, the study proves that the modified mucilage samples would help treat wounds (Table 8 and Figure 4). However, compared with standard Soframycin samples, the wound healing parameters display higher values. We observed relatively better results for encapsulated and non-encapsulated samples when compared with controls.

Table 6. Effect of polymer samples on wound index

S.No.	Groups	Wound index	Gross Change	Score
1.	Control (Blank)	4	Formation of necrosis	4
2.	Soframycin cream (w/w)	1.9	Incomplete but healthy healing	1
3.	Without encapsulation (CE-OKRA: AA)	2.1	healthy healing but delayed	2
4.	Encapsulated (CE+OKRA: AA)	2.7	Delayed but healthy healing	2

The mean ± S.E.M. of four rats' data is shown, and one-way ANOVA is used for analysis before Turkey tests are run.
*P < 0.05, **P < 0.01, ***P < 0.001 vs to the animal in the control group.

Table 7. Hydroxyproline content (µg/mg) of treated samples

S.No.	**Group**	**Hydroxyproline content (µg/mg)**
1.	Control	168.40 ± 2.5
2.	Standard (Soframycin Cream (w/w))	**262.1 ± 2.8
3.	Without encapsulation (CE-OKR: AA)	215.1± 2.7
4.	Encapsulated (CE+OKR: AA)	**255.7 ± 0.8

The mean ± S.E.M. of four rats' worth of data are shown, and one-way ANOVA is used for analysis before Turkey tests are run.
*P < 0.05, **P < 0.01, ***P < 0.001 vs to the animal in the control group.

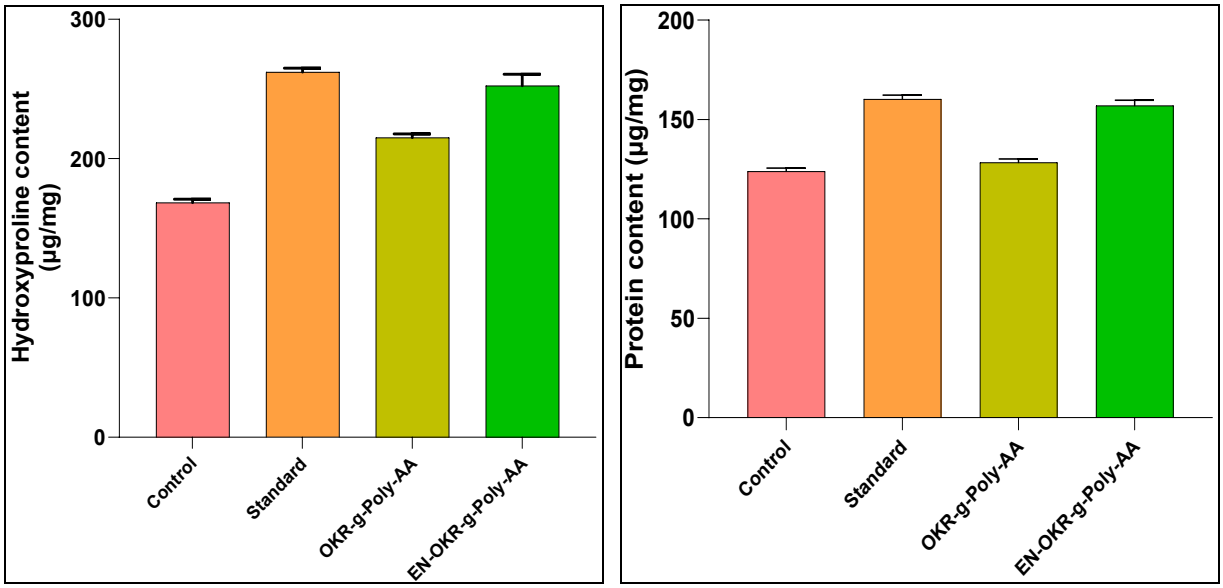


Figure 5. Graphical representation of hydroxyproline and protein contents of okra mucilage and acrylic acid sample

Table 8. Protein content (µg/mg) of the treated sample

S.No.	Group	Protein content (µg/mg)
1.	Control	128.4 ± 1.8
2.	Standard (Soframycin Cream (w/w))	**160.3 ± 1.9
3.	Without encapsulation (CE-OKR: AA)	119.6 ± 2.0
4.	Encapsulated (CE+OKR: AA)	**157.0 ± 3.0

Data are expressed as mean ± S.E.M from four rats and analyzed by one-way ANOVA followed by Tukey tests.

Significance levels are indicated as *P < 0.05, **P < 0.01, and ***P < 0.001 compared to the control group.

3.3.6. Anti-Oxidant Activity

Oxygen plays a vital role in the wound healing process, which affects the killing of bacteria, collagen synthesis, epithelialization, and angiogenesis. It breaks down quickly into oxygen and water, producing hydroxyl radicals that cause DNA damage and lipid peroxidation. The presence of phenolic groups provides electrons to hydrogen peroxide and neutralizes it in water. The Calendula officinalis extract can effectively scavenge hydrogen peroxide. This is thus beneficial for wound healing. Calendula officinalis ethanol extract has shown a concentration-dependent scavenging action on hydrogen peroxide (50–500 µg/ml). The inhibition percentage is better for the encapsulated sample (CE+OKR: AA). The encapsulated polymer sample with an ethanolic extract of Calendula officinalis (IC50 =396.1 µg/ml) exhibits stronger hydrogen peroxide scavenging activity compared to the material without encapsulation (CE-OKR: AA)(IC50 = 419.6 µg/ml). The difference in the percentage inhibition of hydrogen peroxide extracts is statistically significant ($P < 0.05$).

3.3.7. Histopathological Examination

The wound healing process comprises complex and highly programmed processes, including inflammation. Hematoxylin and Eosin help stain and identify different cells and tissues and provide important information about the structure and shape of cells in the tissue. The standard group arrow shows the formation of collagen in the Figure 5. Without encapsulation of okra mucilage and acrylic acid, inflammatory cells in the dermis characterized the formation of collagen dermatitis. In contrast, encapsulated EN-OKR-g-Poly-AA showed no change in the dermis. Our results show the role of encapsulation; without encapsulation, it may help heal the wound (Figure 6).

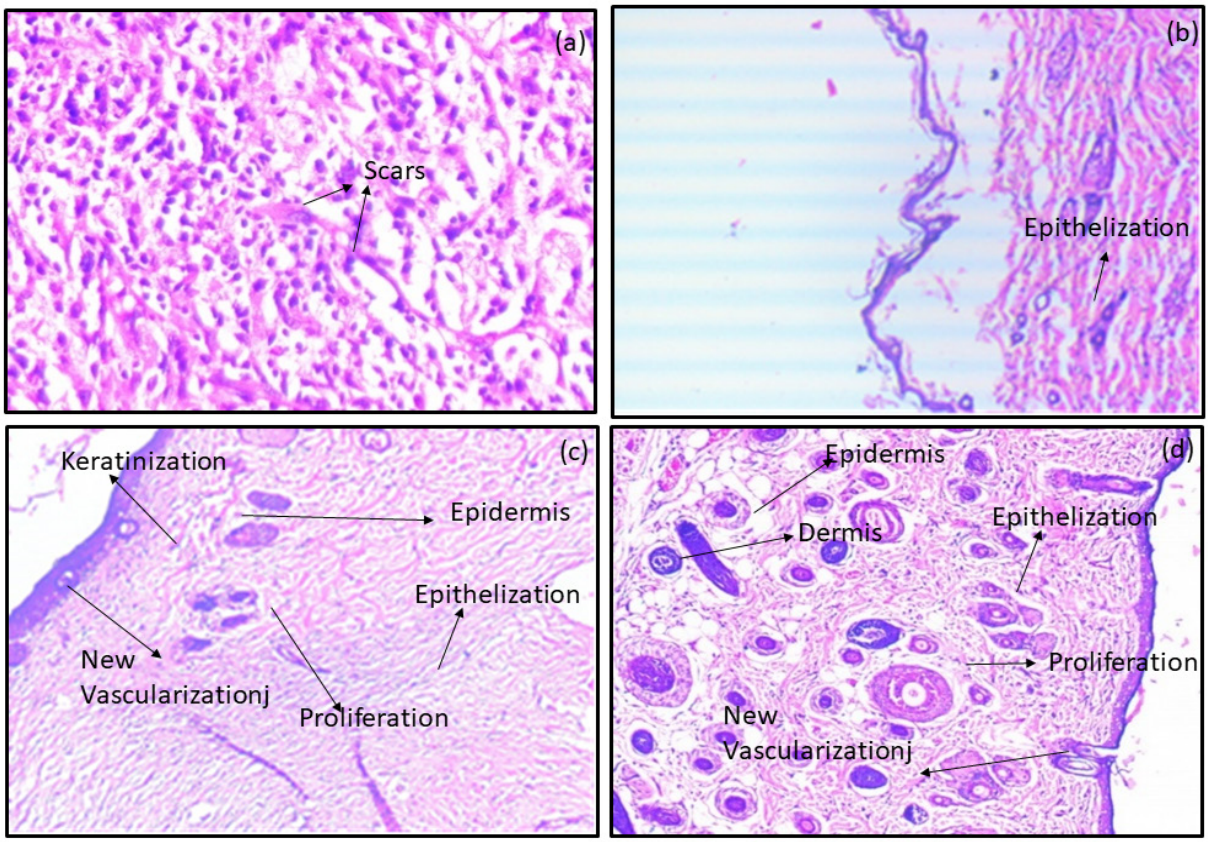


Figure 6. (a) Control (b) Standard (c) Unencapsulated (CE-OKR: AA) (d) Encapsulated (CE+OKR: AA)

4. Conclusions

This research aimed to explore the synthesis of a potentially safe, sustainable, and biocompatible hydrogel based on Okra mucilage, modified with acrylic acid, and incorporating an ethanolic extract of *Calendula officinalis* flowers. Preliminary findings suggest that the wound bed expressed healing markers, leading to notable wound contraction after two weeks of applying a Calendula-loaded hydrogel on a rat skin as studied by the excision wound model. Both encapsulated and non-encapsulated hydrogels appeared to promote efficient wound closure, likely enhancing the formation of collagen organization while minimizing secondary defects and scar formation.

The study indicated that the hydrogel film, used as a wound healing patch, significantly improves epidermal regeneration and collagen formation and reduces the inflammatory response. However, further research is necessary to fully confirm the long-term effects and safety of the hydrogel, as well as to assess its potential for clinical applications. Future studies should focus on optimizing the hydrogel formulation, exploring its performance across different wound types, and determining the scalability of its production. If these aspects are successfully addressed, the hydrogel with polymers in a dressing film could serve as an effective delivery system for *Calendula officinalis*, enhancing wound healing outcomes.

Ethical Consideration

Ethical considerations are taken care of. The Institutional Animal Ethics Committee approves the protocols for animal-based experiments.

Financial Disclosure

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Declaration of Competing Interest

No competing interests.

Data Availability Statement

The data generated is with the author and can be provided on request.

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