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Skin healing effects of an innovative polymer-based oil *Nigella sativa*: a rabbit model experimental study

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Abstract. – OBJECTIVE: Natural wound dressings composed of gelatin (GEL) and chitosan (CH) impregnated with bioactive compounds (*Nigella sativa* oil) were prepared and characterized to evaluate their potential application.

MATERIALS AND METHODS: The formulated composite was subjected to γ-irradiation. *In vitro*, the ferric-reducing antioxidant power (FRAP) assay and antibiofilm activities were evaluated. *In vivo*, the tissue wound-healing process was studied by applying GEL-CH-*Nigella* in dorsal skin rabbit tissue. On days 7 and 14, the biochemical biomarker and histological analysis were determined.

RESULTS: At 10 kGy, FRAP assays exhibited the highest antioxidant activity (380 mmol/kg). A significant inhibition of anti-biofilm activity was observed against *Staphylococcus aureus (S. aureus)* and *Escherichia coli (E. coli)* ($p<0.01$). Fourteen days post-surgery, a significant reduction in thiobarbituric acid-reactive compounds (TBARs) was observed compared to the GEL-CH group. Concerning oxidative stress status, GEL-CH-*Nigella* significantly improved superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities. A histological analysis revealed that GEL-CH-*Nigella* accelerated wound closure and improved collagenisation and enhanced epidermal tissue thickness.

CONCLUSIONS: These results indicate that GEL-CH-Nigella wound dressing is a promising biomaterial for engineered tissue.

Key Words:
Nigella sativa, Gelatin, Chitosan, Wound dressing, γ-irradiation, Anti-biofilm inhibition, Oxidative stress, Rabbit model.

Introduction

Many advantages of chitosan (CH) on skin injuries have been reported[1] including attractive pharmacological actions such as biocompatibility, antibacterial, anti-inflammatory, hemostatic and cell skin regenerative behavior. The amino and hydroxyl groups of these molecular chains allow the grafting of other groups and chemical components to enhance certain biological functions.

Despite its favorable biological properties, the weak mechanical strength of CH has limited the scope of its application[2]. Many physicomechanical properties are needed in an ideal wound dressing material, such as high elongation and good tensile strength[3]. Gelatin (GEL), a collagen-derived substance, exhibits attractive mechanical properties that associate with desired biocompatible properties[4].

GEL does not trigger any immune response in the human body[5]. It is made of proline, hydroxyproline and glycine which help cells join togeth-

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er[6]. Human tissue has the ability to metabolize GEL, which is one of the reasons for its popularity as a pharmaceutical ingredient. In the case of wound healing phenomenon, the mechanisms that occur are hemostasis, coagulation, inflammation, angiogenesis, granulation tissue formation, and re-epithelialization.

The moist, nutrient-rich environment of a wound is ideal for bacterial development[7,8]. When the host immune system fails to eliminate all invading germs, bacterial infections ensue. As such, wound dressings' antibacterial qualities must be carefully evaluated. While GEL was proved to be a non-antibacterial material, *Nigella sativa* oil contains active substance such as thymoquinone, dithymoquinone, thymohydroquinone and thymol, which exhibit a strong bacterial inhibitory effect[9].

It has been reported[10] that thymoquinone delivery caused a high collagen deposition in the healing tissue. Another study[11] concludes that *Nigella sativa* promotes better wound healing *via* its anti-inflammatory capabilities. In fact, it has exhibited wound healing potential by regulation of inflammatory expression (NF-B, TNF-α, and ILs), and up regulation of growth factor expression (VEGF and TGF-β), thus modulating collagen-1 expression to induce angiogenesis[12,13]. The quinine constituent is found as the most potent and pharmacologically active compound in *Nigella sativa,* which exhibits useful influence on infected skin and joints[14].

Like any biomedical product, wound dressing requires sterilization, but polymers exhibit different changes in physical properties[15]. Radiation can cause chain scission in some polymers, while others remain largely unaffected. The effect of radiation on the polymers themselves must be considered and requires evaluation and characterization. Hence, the aim of the present research was to develop a bioactive wound dressing system based on polymers and *Nigella sativa* oil, as well as to determine the minimum dosage required to completely sterilize the product without weakening the biomaterial itself. In addition, this study evaluated the potential use of GEL-CH-*Nigella* as a wound dressing in a skin rabbit model.

Materials and Methods

Preparation of GEL-CH-Nigella Wound Healing Dressing

The GEL solutions with concentration of 5.0 weight % were prepared by dissolving 5 g GEL powder (Merck KGaA, Darmstadt, Germany) in 100 ml distilled water for 30 min, which was then heated at 50°C for 30 min under continuous stirring. Chitosan (CH) (90% DD, degree of deacetylation) was purchased from (Sigma Aldrich, St. Louis, MO, USA). The oil of *Nigella sativa* 10% was added. All mixtures were warmed and stirred at 50°C for 30 min to obtain a homogenous solution. Finally, the mixture was prepared through repetitive freeze-thawing for four cycles[16].

In Vitro Study

Antioxidant activity: ferric reducing antioxidant power (FRAP) assay

A modified version of the procedure published by Benzie and Strain[17] was used to carry out the FRAP assay. Briefly, 1.5 mL of FRAP reagent was combined with 200 µl of adequately diluted composite (0.1 g/mL). The reaction mixture was incubated at 37°C for 4 minutes. After that, the absorbance was measured at 593 nm in comparison to a distilled water-prepared blank. The FRAP reagent was freshly made by combining 10 volumes of 300 mM/L acetate buffer (Sigma Aldrich, St. Louis, MO, USA) (pH 3.6), 1 volume of 10 mmol 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) solution in 40 mM/L HCl (Sigma Aldrich, St. Louis, MO, USA), and 1 volume of 20 mM ferric chloride (Sigma Aldrich, St. Louis, MO, USA). The mixture was then pre-warmed to 37°C ($FeCl_3.6H_2O$). An aqueous solution of ferrous sulfate ($FeSO_4.7H_2O$) (Sigma Aldrich, St. Louis, MO, USA) at different concentrations was used to create a calibration curve.

Cristal violet biofilm assay

A static biofilm formation assay was performed in 96-well polystyrene plates (Greiner Bio-One, Frickenhausen, Germany). The bacteria were grown overnight in the LB and diluted into fresh LB to an OD_{600} of 0.02. Then, 200 µl of the bacterial suspensions were mixed with 200 µl of various samples and incubated for 24 h (stationary phase) without shaking at 37°C. The LB medium was discarded the following day, and the plates were washed three times with a PBS buffer. Then, bacterial biofilms were visualized by staining with 125 µl of 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at room temperature. The plates were then washed, and the amount of biofilm quantified, after dissolving in 100 µl of 95% ethanol, by measuring the OD at 570 nm.

In Vivo Study

Surgical and postoperative protocols

New Zealand white rabbits (3 kg), bred in the central animal house for 10 days were used for the experiments in this study. The animals were fed on a pellet diet (Micco, Tunisia) and water *ad libitum*. All the animals were kept under climate-controlled conditions (25°C; 55% humidity; 12 h of light alternating with 12 h of darkness). All rabbits were randomly divided into 3 groups (five rabbits per group): the first group (I) was used as negative control (CT). Groups II and III received GEL-CH and GEL-CH-*Nigella* biomaterial, respectively. Local anesthesia was applied using 4 mg/kg carprofen (Rimadyl, Pfizer, Paris, France). For the last two groups, each rabbit's dorsal hair was clipped off and then cleaned with 70% ethanol and betadine. Using a template, two full-thickness wounds measuring 4 cm^2 in diameter were marked. Using dissecting scissors and forceps, the tissue was removed to the level of the panniculus carnosus. All the rabbits were sacrificed then the regenerated cutaneous tissue was recovered, and specimens were harvested for biological evaluation after 7 and 14 days. The rabbits were checked daily for clinical lameness or other complications. The study followed the guidelines for the care and use of laboratory animals outlined by the National Centre for Sciences and Nuclear Technologies (Tunisia), which refers to Guide for the Care and Use for Laboratory Animals (Protocol code: LFNT/1529/2019). The experiment was approved by the local Ethical Committee of Energy and Matter Research Laboratory (LR16CNSTN02).

Tissue Preparation and Stress Oxidative

Thiobarbituric acid-reactive substance measurements

The skin tissues were removed and rapidly frozen by dry ice. The tissues from each group were chopped, homogenized (100 mg/ml), and centrifuged at 3,000 g for 15 min at 48°C in a 0.1 mol/l Tris-HCl buffer pH 7.4. By testing thiobarbituric acid reactive substances (TBARS), which represent the end result of lipid peroxidation, the amount of lipid peroxidation in the tissue homogenate was calculated[18].

Antioxidant enzyme studies

Superoxide dismutase (SOD) activity was measured using a spectrophotometric technique[19]. The technique used to evaluate the activity of the glutathione peroxidase (GPx) was taken from Pagila and Valentine[20]. The calorimetric measurement of catalase (CAT) activity at 240 nm was quantified as the number of moles of H_2O_2 used per minute per milligram of protein[21]. Bovine serum albumin (Merck KGaA, Darmstadt, Germany) was used to measure the total protein level, as described by Lowry et al[22].

Histological Analysis

To conduct a histological study of the skin tissue, the samples were fixed in 10% formalin and embedded in paraffin. Next, 4-5 mm paraffin sections were stained with hematoxylin-eosin and subjected to microscopic examination.

Measurement of Biochemical Biomarker in Serum

Venous blood draws were performed to acquire the serum, which was then utilized to quantify the levels of IL-1, IL-6, TNF-α, and VEGF by ELISA (Sigma Aldrich, St. Louis, MO, USA) in accordance with the manufacturer's recommendations.

Statistical Analysis

All experimental results were then presented as means (n=3) ± standard deviations (SD). Multiple comparisons were performed using the analysis of variance (ANOVA) followed by Tukey's range test. The probability value of $p<0.05$ was considered significant.

Results

Ferric Reducing Antioxidant Power (FRAP) Assay

The γ-irradiation significantly increased the antioxidant activities of the GEL-CH-*Nigella* wound dressing material observed in doses of 5 up to 25 kGy (Figure 1). Gamma rays improved the ferric reducing power of all composites compared to corresponding un-irradiated samples. Notably, the data showed that all the experimented composites treated with 10 kGy had the most significant antioxidant properties as compared to the non-treated sample (26.33±1.09%) for concentration corresponding to 2 mg/ml ($p<0.01$).

Anti-Biofilm Activities

Due to the ability of many resistant bacteria to form biofilm, the treatment against their infections is usually very appealing. Interesting-

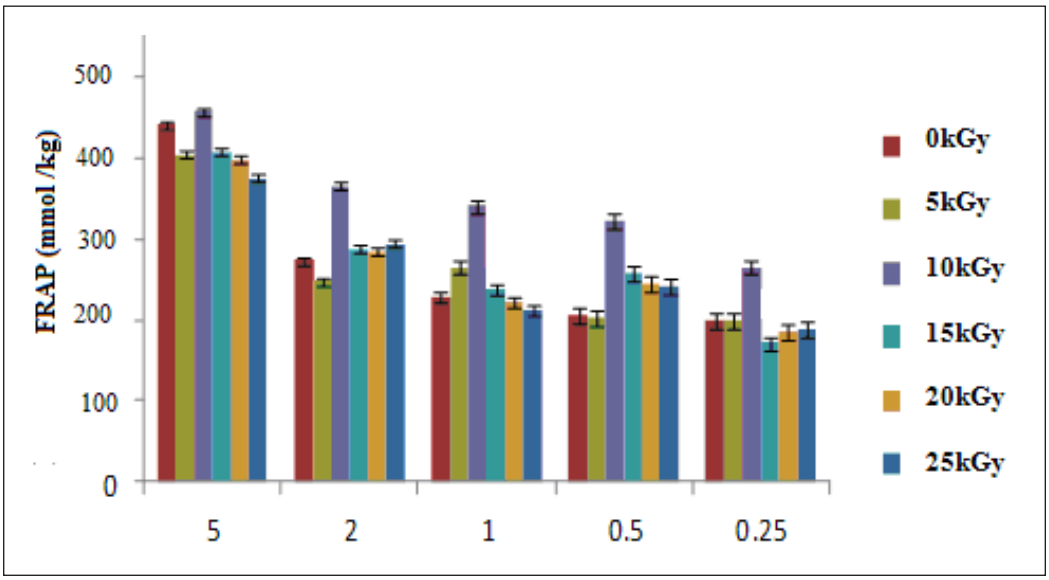

Figure 1. Determination of the ferric reducing antioxidant power (FRAP) of the GEL-CH-*Nigella* composite after different γ-radiation doses 5, 10, 15, 20 and 25 kGy compared to the control samples (0 kGy).

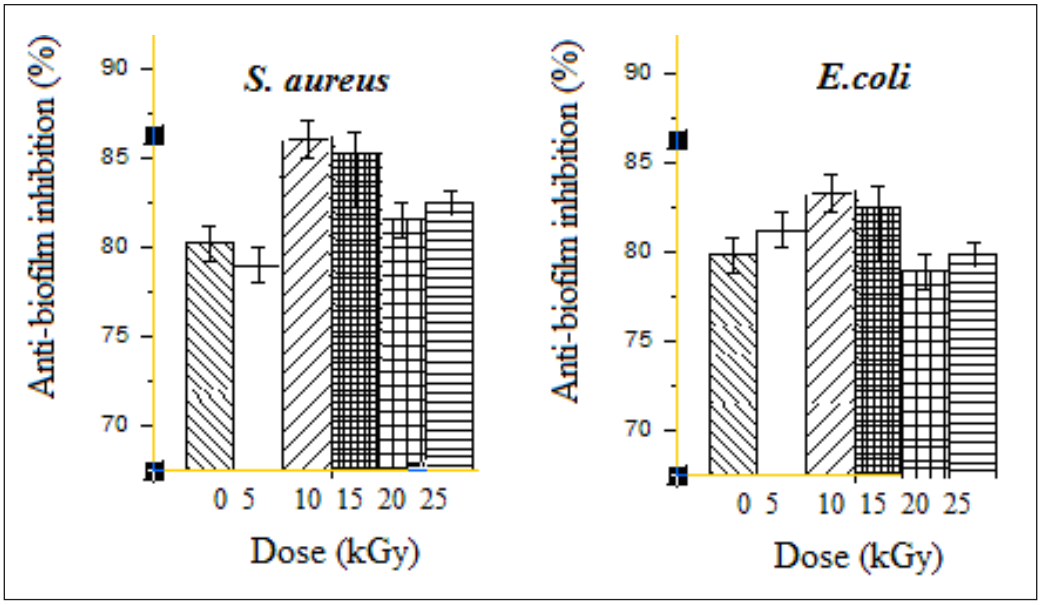

Figure 2. Determination of anti-biofilm activities effects of GEL-CH-*Nigella* composite against *S. aureus* and *E. coli* strain with different γ-radiation doses 5, 10, 15, 20 and 25 kGy in comparison with control samples (0 kGy).

ly, GEL-CH-*Nigella* dressing showed promising biofilm inhibition results, as shown in Figure 2. At 10 kGy, the dressing showed the highest anti-biofilm activity of *S. aureus* at 86.84±1.50% and 83.56±0.60% for *E. coli*. The anti-biofilm activities showed a low inhibition at 15 and 20 kGy for both strains. The anti-biofilm activity was dose-independent manner with the most important inhibition detected at 10 kGy for both *S. aureus* and *E. coli*. The dose rate of gamma ray plays an important role in the anti-biofilm activities. In fact, the GEL-CH-*Nigella* dressing showed different behaviors and effects against bacteria biofilm.

Table I. Effects of GEL-CH-Nigella wound dressing on MDA levels, CAT, SOD, and GPx activities in cutaneous tissue levels after 7 and 14 days.

BOS	MDA (mmol/L.pro)		SOD (mmol/L.pro)		CAT (mmol/L.pro)		GPx (mmol/L.pro)	
Groups	7 d	14 d	7 d	14 d	7 d	14 d	7 d	14 d
CT	1.5± 0.01	1.59±0.02	60± 1.92	53± 1.62	0.66±0.01	0.71±0.02	1.4± 0.02	1.51±0.04
GEL-CH	4.1± 0.03*	3.88±0.04*	20±0.025*	36±0.02*	0.33±0.01*	0.55±0.03*	1.1±0.01*	1.3±0.02*
GEL-CH-*Nigella*	4.3 ±0.03*	1.68±0.025	26±0.025*	47±0.025	0.29±1.92	0.69±1.92	0.98±0.03*	1.55±0.09

BOS: biomarkers of oxidative stress; d: days; *: significant difference between the indicated group and control group (CT) (p<0.05). Values are expressed as mean±SD of five rabbits.

Table II. Levels of IL-1β, IL-6, TNF-α, and VEGF factors in the GEL-CH-*Nigella*, GEL-CH and CT groups after 7 and 14 days post-surgery.

BBS (ng/l)	IL-1β		IL-6		TNF-α		VEGF	
Groups	7 d	14 d	7 d	14 d	7 d	14 d	7 d	14 d
CT	48±1.80	49±1.77	23±1.96	22.9±1.08	58.2±1.66	59.1±1.55	27.5±1.25	26.9±1.05
GEL-CH	70±2.10	72±2.90	47±1.33	45±1.56	77±2.30	76.2±3.23	57.5±1.40	56.9±2.20
GEL-CH-*Nigella*	71±2.30	56±1.90*	41±1.88	32±1.12*	67±2.32*	60.1±2.33*	47.4±1.66	36.8±1.32*

BBS: biochemical biomarker in serum, IL: interleukin; TNF-α: Tumor necrosis factor; VEGF: vascular endothelial growth factor; d: days; *: significant difference between the indicated group and CT group (p<0.01). Values are expressed as mean±SD of five rabbits.

Oxidative Damage in the Cutaneous Tissue

The evaluation of the oxidative stress biomarker, as well as the antioxidant enzyme activity after the GEL-CH-*Nigella* application in the cutaneous tissue are shown in Table I. Malondialdehyde (MDA) level in the GEL-CH-*Nigella* tissue following 7 days of graft were significantly different when compared with that of control CT (p<0.05). CAT, SOD, and GPx activities in the same rabbit group tissues exhibited a highly significant decline when compared with those of CT rabbit tissues (p<0.05). However, after 14 days, there were significantly enhanced enzyme activities in the regenerated tissue when compared with that of GEL-CH rabbit group (p<0.01).

Measurement of Biochemical Biomarker in Serum

The serum level measurements in the different treated rabbits are shown in Table II. The levels of IL-1β, IL-6, TNF-α and VEGF in the group that received GEL-CH-*Nigella* composite were 56.9±1.9, 32±1.12, 60.1±2.33 and 36.8±1.32 ng/l, respectively. These values were decreased in the rabbit group that received GEL-CH-*Nigella* composite with statistically significant differences as compared with that of GEL-CH group (p<0.01).

Skin Histological Observation

As presented in Figure 3, the histological comparison with the control group (Figure 3A-C) demonstrated an incomplete re-epithelialization of the epidermis on day 14 post wounding in the GEL-CH group. Tissue regeneration was limited to the extremity region of the wound (Figure 3D). Particularly, abnormal collagen remodeling, and reorganization was observed (Figure 3E). The analysis indicated that application of GEL-CH did not efficiently improve the re-epithelialization of the epidermis. In GEL-CH-*Nigella* group a proliferative phase of cells that become closely packed together and exhibited mitosis was clearly observed (Figure 3F). An enhanced and full re-epithelialized wound surface was observed on the 14 day of post wounding period (Figure 3G). The GEL-CH-*Nigella* group showed an enhanced vascularization. Also, a new blood vessels development (angiogenesis) was detected. Moreover, a well-collagenized fibrous scar presenting very little inflammatory cells and thinly distributed blood vessels was observed. The coating epithelium was shown to be thickened and presented numerous bulbous buds and showed central keratinization (Figure 3H) which is compatible with basic cutaneous attachment (hair follicles).

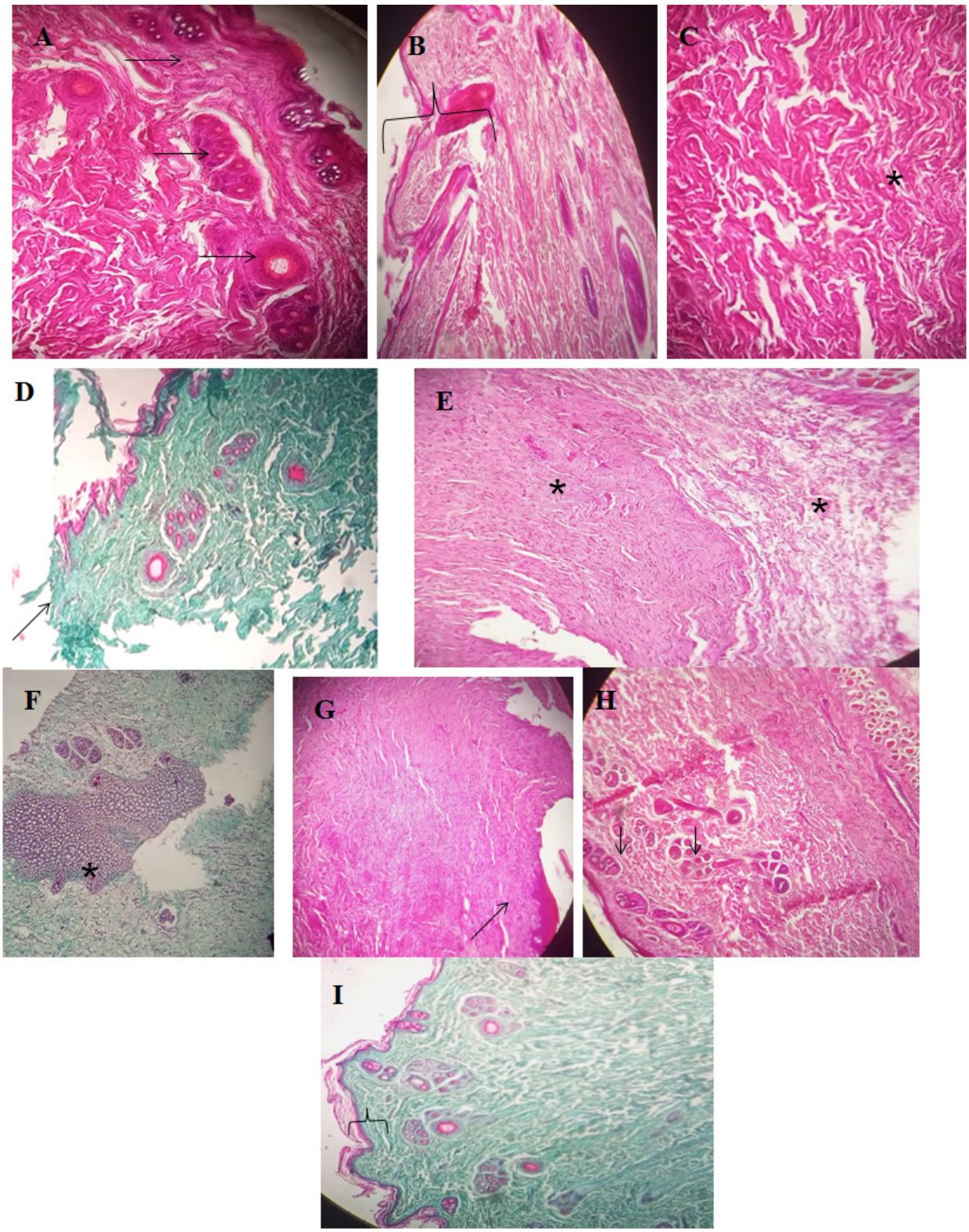

Figure 3. CT group: (**A**) arrow: sebaceous gland, (**B**) hair root: hair shaft and epithelialized tissue, (**C**) normal collagen deposition. GEL-CH application: **(D)** Arrow: marginal re-epithelisation. Normal collagen face to abnormal collagen deposition. GEL-CH-*Nigella* application: (**E-F**) cell mitosis (*), (**G**); full re-epithelization, (**H**) sebaceous gland, (**I**) central keratinization compatible with hair follicles attachment. Hematoxylin and eosin and Papanicolaou Stain (**A-C**, **E** x200; **D**, **F-H** x200).

Discussion

In the present research, a novel bioactive dressing containing a nature-derived compounds (GEL and CH) and the bio-functional agents *Nigella sativa* oil was prepared. Herein, the CH is designed to prevent bacterial penetration and to fight oxidative stress. The GEL was designed to absorb wound exudates, smoothly adhere to the wet wound bed, and accommodate newly formed tissue. Its exclusive physical properties have aroused particular interest in their release of bioactive substance from *Nigella sativa* oil.

Gamma irradiation has been widely used for sterilizing. However, γ-irradiation has been known to cleave chemical bonds of polymers including the peptide bonds of proteins. As such, the polymers were characterized after irradiation, because one of the major problems with polymers, such as GEL and CH, is to maintain the native structural integrity of the material.

Concerning anti-biofilm activities, GEL has no antibacterial effects, however, CH is considered as a strong antibacterial. One proposed mechanism suggests 1) the binding of CH with microbial DNA[23]. 2) The CH molecules are assumed to be able to pass through the bacterial cell wall[24]. 3) The chelation of metals, suppression of spore elements and binding to essential nutrients to microbial growth[25].

One of the suggested mechanisms of *Nigella sativa* oil bactericidal ability observed is the weakening effect on the integrity of bacterial membranes[26]. The ability of the GEL-CH-*Nigella* wound dressing to inhibit microbial biofilm growth, can be explained by the synergetic effect of CH and *Nigella sativa* oil. In this regard, with the dangerous evolution of antibiotic resistant bacteria, antibiotic delivery systems are urgently needed. However, increases of antioxidative potential may related, in part, to the increases of the phenol content.

After γ-rays exposition, the total phenolic at about 4 kGy on almond skin showed increased phenolic content[27]. For cumin seeds, it was found a non-significant increase in the phenolic (thymohydroquinone + thymol) content in the irradiated cumin[28]. The differences in effects were attributed to the different phenolic compounds present in the various plants. The release of phenolic compounds from a glycosidic component may be due to the breakdown of bigger phenolic compounds into smaller ones by γ-radiation[29].

The synergetic biological activities might have shortened the acute phase of inflammation. On day 14, the remodeling phase of wound skin healing was achieved, and no substantial inflammatory reaction was found. Cutaneous defects after skin surgery can be subjected to increased oxidative stress, which triggers the development of metabolic disorders such as the increased production of reactive oxygen species (ROS).

Lipid peroxidation was evaluated by TBARS. The results demonstrated that the group that performed a surgical defect presented the highest level as a result of the increased production of free radicals. These elements attack various components in the cell, which leads to biochemical changes and macromolecule modifications[30,31]. The present study demonstrated that harmful oxidative molecules such as malondialdehyde (MDA) are produced in the rabbit tissue.

ROS have a harmful effect on the cellular antioxidant defense mechanisms by falling the level of antioxidant enzymes in cutaneous tissue, especially catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). The results are a consequent failure of the body to be protected against free radicals. The formulated biomaterials contain natural antioxidants derived from *Nigella sativa* that were applied to detoxify the increased levels of the free radicals. In fact, after the GEL-CH-*Nigella* graft, the antioxidant mechanisms of the body were enhanced as shown by elevated skin GP_X, SOD and CAT activities accompanied with the reduction of MDA cutaneous level. These results might be attributed to the reaction between the free radicals and the free residual amino groups to form ammonium groups from CH.

Various phenolic compounds such as thymoquinone, gallic acid, kaempferol, rutin, apigenin, naringenin and quercetin, were found[32] to be anti-oxidative compound. The role of antioxidants is to lower or terminate these chain reactions by removing free radicals or inhibiting other oxidation reactions by being oxidized themselves[33]. The observed wound dressing is promising because of its widely favorable biological properties and the possibility of integration into engineered structures.

Generally, the dose requested for this type of biomaterial can go up to 25 kGy. However, in the present study, γ-irradiation at this dose has a limited effect on the biological activity. Maximum results were obtained at 10 kGy. Unfortunately, this dosage is insufficient to destroy or inhibit all types of bacteria growth.

Conclusions

The bioactive dressings were prepared with the aim of delivering active substances to the wound environment to enhance the process of wound healing. *In vitro,* an improve in the antioxidant performance without altering anti-biofilm properties of GEL-CH composite against *E. coli* and *S. aureus* was observed. *In vivo,* modulate antioxidant enzyme activity, such as that of SOD, GPx, and CAT was approved. The serum levels of proinflammatory cytokines during 14 post-operative days were significantly decreased in the GEL-CH-*Nigella* group. This indicate that the prepared GEL-CH-*Nigella* wound dressing may provide new biomaterials to promote healing while protecting the affected area from infection.

Data Availability

The data used to support the findings of this study are available from the corresponding author (Ghada Ben Salah) upon request.

Conflict of Interest

The authors declare there are no competing interests.

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Ethics Approval

The animal study protocol was approved by the local Ethical Committee of Laboratory of Energy and Matter Research Laboratory (LR16CNSTN02) of the National Center for Sciences and Nuclear Technologies (Tunisia) (protocol code: LFNT/1529/2019).

Informed Consent

Not applicable.

Authors' Contributions

Conceptualization, J.S.; Methodology, J.S., S.B, and H.K.; Software, J.S. and G.B.S; Validation, G.B.S, and M.A.A; Formal analysis, J.S., N.M. and G.B.S.; Resources, G.B.S.; Data curation, S.J and G.B.S.; Writing-original draft preparation, S.J., G.B.S., and M.A.; Writing-review and editing, G.B.S.; Visualization, G.B.S; Supervision, J.S., and G.B.S; Project administration, J.S.; Funding acquisition, J.S. All authors have read and agreed to the published version of the manuscript.

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