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PREPARATION AND CHARACTERIZATION OF BLACK SEED OIL LOADED POLYMERIC BANDAGE FOR DERMAL WOUND HEALING

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Abstract

The right choice of biopolymer for bandages could enhance the healing of wound significantly compared to the conventional dressing materials. In order to analyze the preliminary effect of black seed oil in the healing area characterization of black seed oil was done. The prepared bandages were characterized physiochemical and its structural determination was done by FTIR. In addition, in-vitro swelling ability and in-vitro degradation of bandages were investigated to ensure the applicability of bandages as wound dressing material. Similarly, in-vitro drug release kinetics of the bandages were examined with UV spectrophotometer at 254nm that showed sustained release of drug in bandage with chitosan to alginate ratio of 1:1. Cytotoxicity test was done to ensure maximum cellular viability. Furthermore, in-vivo evaluation in Swiss albino rats revealed black seed oil loaded bandages enhanced wound healing rate with re-epithelization and better collagen orientation than that of void bandages and control. The obtained data strongly encourages the use of these bandages for topical wound healing in near future.

Keyword: anti-inflammatory, sustained release, biodegradable, Thymoquinone, re-epithelization

1.INTRODUCTION

Wound healing is a complicated multistage process that includes inflammation, cell proliferation, matrix deposition and remodeling phases. It is frequently connected with oxidative stress and subsequent prolonged inflammation, resulting in impaired wound healing. The longer it takes for spontaneous wound healing, the worse the outcome normally is, with improving probability of developing hypertrophic scaring and undesired alterations in pigmentation. Moreover, under unfavorable conditions, the self-sustaining inflammatory cascade may result in increasing tissue destruction and necrosis rather than healing.

Skin wounds are a public health issue that can influence the survival of injured people and decrease their quality of life. It attracts the attention of the medical community because it is expensive and, thus far, therapy has been unsuccessful.[1] The goal of clinical treatment is to have a fast wound recovery time to properly restore structure and function.

The chemical composition of Black seed (*Nigella sativa*) is very rich and diverse. It contains the phytochemicals thymoquinone and crystalline nigellone as well as anti-oxidants, amino acids, proteins, carbohydrates, fixed oils, volatile oils, alkaloids, saponin, and fiber, as well as minerals such as calcium, iron, sodium and potassium.[2] There are still many components in Black Seed that haven't been identified and research is going on around the world to help shed more light into this remarkable little seed.

Chitosan and alginate are well known for their healing properties. Chitosan is a cationic polymer obtained from chitin, is biologically renewable, biodegradable, biocompatible, and non-antigenic. It is able to accelerate the wound healing process enhancing functions of inflammatory cells, macrophages, and fibroblasts. Alginate is an anionic hydrophilic polymer, is also biocompatible and biodegradable under normal

physiological conditions. It is able to maintain a physiologically moist microenvironment that promotes the formation of granulation tissue.[3]–[5]

Recently, several groups have demonstrated that oleic acid (OA), an unsaturated fatty acid, may modulate the inflammation by downregulating COX2 expression and stimulating the production of cytokine-induced neutrophil chemoattractant in inflammation alpha/beta [6], [7]. This immunomodulation response of Oleic acid (OA) at the wound site is probable to enhance faster wound reparative processes. Additionally, its viscous properties could provide a platform for sustained release of the drug by slow drug diffusion in solubilized form which can enhance the therapeutic activity. Furthermore, it has an enhanced cell penetrating and inflammatory modulation property.[6], [7]

The only motive of our project is the formulation of wound healing technique in an unsophisticated manner. Various projects on wound healing done till date are found to be formulated using sophisticated technique. The mechanism by which the bandage will be prepared make the sustain release of required drug in the healing site in a very high amount that causes healing faster compared to any other technique introduced till now. The reason behind doing this project is due to the negligible toxicity and biocompatibility of the chemical used with our body. The preparation method is environmentally friendly as less organic solvent is required.

2.MATERIALS AND METHODS

2.1. Materials

Chitosan (degree of deacetylation=85%, molecular weight 650 kDa) derived from crab shell in the form of fibrillar flakes, sodium alginate (Molecular weight 150 kDa) and oleic acid were purchased from sunrise medical suppliers, Nepal. Black seed oil (100% pure) was obtained from Sara Worldwide Pvt. Ltd, Nepal. All other chemicals were purchased from Sunrise Medical suppliers, Nepal without further purification.

2.2. Preparation of black seed oil loaded and void polymeric bandages

Briefly, chitosan solution (0.5% w/v) was prepared by dissolving 0.1 gm chitosan powder in 20 ml of deionized water containing 0.2 mL of acetic acid at room temperature. Sodium alginate solution (0.5% w/v) was prepared by dissolving 0.1 gm of sodium alginate powder in 20 ml of deionized water. For the preparation of BOP bandages, 50 mg of black seed oil (BSO) was mixed with 1.75ml of OA with equal volume of ethanol. Chitosan solution was added to the suspension of OA and BSO and was stirred for 1 hour. The resultant solution was mixed with sodium alginate solution and was further stirred until an opaque aqueous solution was obtained. In this way, black seed oil loaded polymeric (BOP) bandage solution with different ratios of chitosan and alginate (1:1, 2:1, 3:1) were poured into 6-well plate and was lyophilized for five days at -50^{0}C. Similarly, Void polymeric (VOP) bandages were prepared following the above process without addition of BSO in it.

2.3. Fourier Transform Infrared Spectroscopy (FTIR)

The characteristic absorption of bandages was recorded at 500 to 4000 cm^{-1} on infrared spectrophotometer.

2.4. Moisture content

The moisture content of VOP bandages was determined by keeping the known weight of bandages in a desiccator containing fused calcium chloride and was left for 48 hours. The final weight was taken and percentage moisture content was calculated as follows:

$$\text{Moisture content } (\%) = \frac{(\text{Wi}-\text{Wf})}{\text{Wf}} \times 100\% \qquad (1)$$

Where, Wi and Wf denotes initial weight and final weight of bandages respectively. Each experiment was repeated six times and average value was taken.

2.5. Folding endurance

Bandages folded at the same place without breaking. The number of times it could be folded gives the folding endurance. It was repeated six times to calculate the mean value of folding endurance.

2.6. Porosity

Porosity of the lyophilized bandages was evaluated using an alcohol displacement method. Briefly, the bandages were immersed in absolute ethanol until saturated. The bandages were weighted before and after the immersion in alcohol. The porosity was calculated as per the protocol of Fan et al. [8]

2.7. In-vitro swelling ability study of bandages

The swelling ability of bandages were studied by immersing the bandages in phosphate buffer saline (PBS) (pH 7.4) at room temperature. The initial and final weights of bandages were taken. Final weights were taken at the pre-determined period of time by first blotting the bandages with filter paper to remove water from the surface of the bandages. Percentage swelling ability was calculated as follows.

$$\text{Swelling ability } (\%) = \frac{(\text{Wf}-\text{Wi})}{\text{Wi}} \times 100\% \qquad (2)$$

Where, Wi and Wf are the initial and final weight of bandages respectively.

2.8. In-vitro degradation test

For the determination of degradation of bandages, the bandages were incubated in PBS with pH 7.4 with lysozyme of minimum activity 30,000 U/mg in 6 well plate and was kept at 37 °C. At required period of time for up to 25 days, the bandages were taken out, washed and lyophilized. The weights of the bandages were weighed and percentage weight was calculated as:

$$\text{Weight loss } (\%) = \frac{(\text{Wi}-\text{Wf})}{\text{Wi}} \times 100\% \qquad (3)$$

Where, Wi and Wf are the initial and final weight of bandages respectively.

2.9. In-vitro drug release kinetics

Six different concentration of BSO i.e. 2 µg/ml, 4 µg/ml, 5 µg/ml, 6 µg/ml, 8 µg/ml, 10 µg/ml were taken to plot the calibration curve using UV spectroscopic analysis at 254nm. All the samples were triplicated. For the drug release profile, Different formulations of BOP bandage of size 5 cm × 5 cm containing 50 mg of black seed oil was suspended in 10 mL of PBS (0.01 M, pH 7.4) and was kept in a VDRL shaker at 37 °C, rotating at 150 rpm. At predetermined time intervals up to 7 days, the samples were collected and replaced with the same volume of fresh PBS (0.01 M, pH 7.4). The collected samples were then subjected to centrifugation at 13,800 rpm, 4 °C for 10 min to obtain the supernatant containing released black seed oil. The released black seed oil concentration was analyzed using UV Spectrometer at 254 nm.

2.10. Cytotoxicity testing of bandages

The cytotoxicity of the bandage solution was assessed using an MTT based on colorimetry where viable cells can reduce water-soluble MTT to a colored formazan product. Hela cells were cultured at a density of 1.0×10^4 cells/mL on 96-well plates (100 µl/well) in a CO2 (5%) incubator at 37 °C. After incubation for 24 h, the bandage solutions were added to 96-well plates (100 µL/well) in a CO_2 (5%) incubator at 37 °C. After incubation for 24 h, 72 h, and 120 h, 10 µL of MTT solution was added to each well, and the cultures were incubated at 37 °C for 4 h. The post incubation media containing MTT was removed, and the purple formazan crystals that formed were dissolved by incubating the bandage solution in 1.5 mL of dimethyl sulfoxide (DMSO) for 15 to 20 min at room temperature (RT) with constant shaking. The absorbance of the solution was measured at 595 nm. All the samples were triplicated.

2.11. Blood coagulation time

In-vitro blood coagulation time was evaluated by using two formulation of bandages. To this aim, 50 mg of dry bandages was portioned into different test tubes. 1 ml of fresh human blood was added to those test tubes. The same volume of blood was added to empty test tubes served as a control. Test tubes was then rotated for 1 min, and was set up vertically on lab bench. Tubes was inverted every minute till blood aggregate completely and the time was recorded.

2.12. In-vivo wound healing test

For this experiment, 30 Swiss albino male rats (150−170 g, 12 weeks) were used. All the rats were kept under 12-hrs light and 12-hrs dark cycle. In brief, the rats were divided into three groups, each group comprising 10 rats: group I, control; group II, VOP bandage treated group; group III, BOP bandage treated group. The animals were anesthetized intramuscularly by ketamine (100 mg/kg) and xylazine (10 mg/kg). The dorsal hair of the rats was removed. A full-thickness wound of 1×1 cm^2 was excised from the back of the rats. Each wound was covered with an equal size of BOP bandage, or a VOP bandage, or cotton gauze as a control for comparison. All the treated rats were kept in different compartments with single rat per compartment. Treated rats was observed and photographed on the 0th, 3rd, 6th and 9th days using a digital camera. The area of the

wound was calculated by measuring the length and breadth of the wound with digital slide calipers or ruler. Percentage of wound reduction was calculated by observing the wound area at the present time.

2.13. Histological analysis of wounded tissue

Wounded tissues from different groups of rats was excised after the 3rd, 6th and 9th days' post wounding and was fixed with paraformaldehyde (4% in PBS, 0.01 M, pH 7.4) for two days at 4 °C. Their cryosection was then taken and stained with hematoxylin and eosin to assess the predominant stages of healing.

2.14. Statistical analysis

All the experiments were carried out six times and the results are presented as mean ± SD. Statistical analysis was performed by using one-way ANOVA with Tukey's Multiple comparison for paired comparison for comparison of means. The statistical tool used for these analyses is GraphPad Prism 6. Values of $p<0.05^{*}$ were indicative of significant difference and $p<0.005^{*}$ were indicative of a very significant difference.

3.RESULTS AND DISCUSSION

3.1. Preparation of black seed oil loaded and void polymeric bandages

The chitosan alginate bandages with different blend ratios were prepared which is found to be very sufficiently flexible and is shown in Figure 1.

3.2. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was performed to examine the chemical structure of the bandages. Figure 2 shows the FTIR spectra of various bandages. It can be seen that the bond between the bandages were formed due to the interaction of positively charged chitosan and negatively charged alginate. Being more precise, the amide and amino group of chitosan interacts with the carboxyl group of alginates. The interaction of these groups in each bandage is different due to different blend ratio of polymers.

3.3. Moisture content

The values of moisture content of bandages are shown in Table 1. The appropriate moisture content in the bandage ensures sufficient supply of moisture to the wound.

3.4. Folding endurance

The value of folding endurance is shown in Table 1. The result indicates sufficient flexibility of the bandage so that it do not break in the general folding of skin. [9]

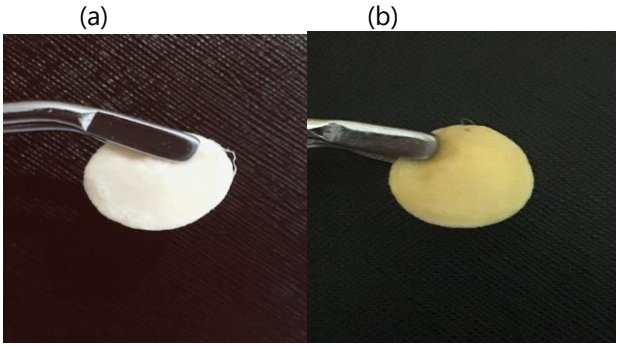

Figure 1 Photographs of VOP and BOP bandages (a) VOP bandage (b) BOP bandage

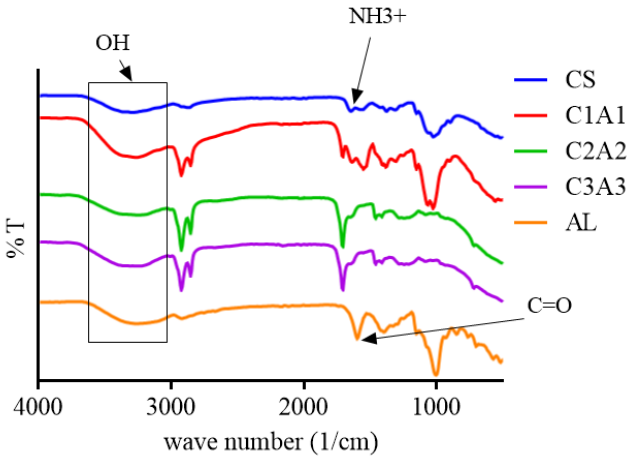

Figure 2 FTIR spectra of various bandages

Table 1. Table showing Moisture content (%), Folding endurance and porosity (%) of bandages

Bandage	Moisture content (%)	Folding endurance	Porosity (%)
C1A1	1.32±0.07	251.16±3.59	27.46±0.85
C2A2	1.52±0.09	254.33±3.16	34.49±3.37
C3A3	1.88±0.07	269.5±2.217	42.86±2.62

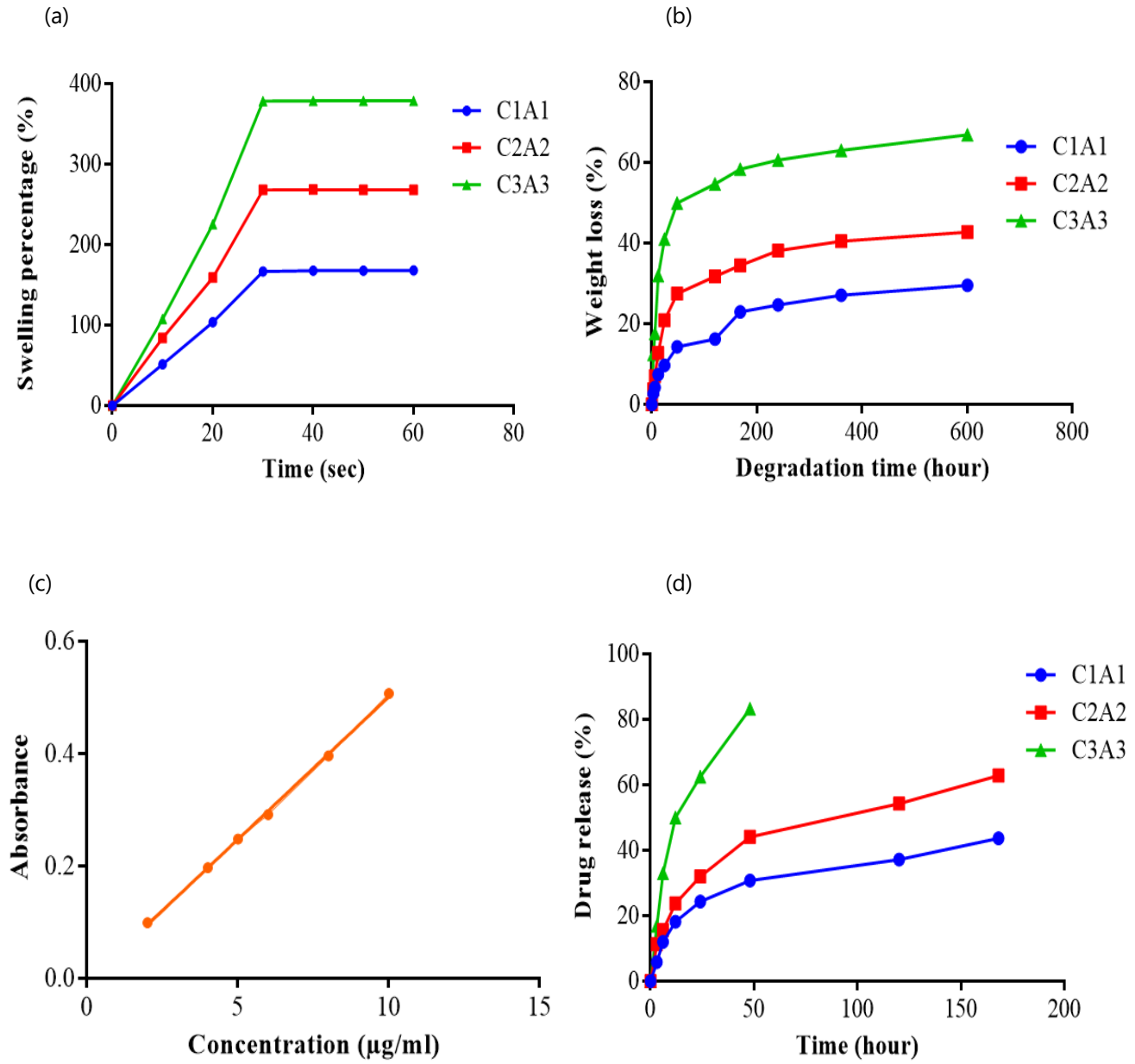

Figure 3 In-vitro test of different formulations of BOP bandages and VOP bandages. 3(a) In -vitro swelling ability of different formulations of VOP bandages with chitosan to alginate in different molar ratio, i.e., 1:1, 2:1 and 3:1 denoted by C1A1, C2A2 and C3A3 respectively. (b) In-vitro degradation of different formulations of VOP bandages (C1A1, C2A2 and C3A3) in PBS lysozyme solution. (c) The standard curve or linear regression of black seed oil using UV spectrophotometry at 254 nm. (d) The in-vitro release kinetics of black seed oil from BOP bandages (C1A1, C2A2 and C3A3).

3.5. Porosity

The mean value of porosity of the bandages is given in Table 1. The result of this test suggests the benefit it could give by absorbing exudates from the wound surface.[8] Furthermore, the pressure of large value of porosity is undesired as it can hinder the sustained release of drug.

3.6. In-vitro swelling ability study of bandages

Figure 3(a) shows the swelling ability of bandages. All the bandages absorbed significant amount of PBS achieving swelling equilibrium within 30 sec. The water uptake capacity ranges from 168% to 378% which revealed C1A1 bandages had minimum value of swelling percentage whereas C3A3 possessed the maximum value.

3.7. In-vitro degradation test

This test was performed to examined the stability of biodegradable bandages when in contact with wound. Figure 3(b) shows the percentage weight loss as a function of time. The value ranges form 20% to 65%. C3A3 showed 1.5-3 times higher weight than C1A1 and C2A2 bandages. The results suggests that C1A1 bandages was the most stable bandages which is due to the strong crosslinking of polymers in the bandage.

3.8. In-vitro drug release kinetics

Standard curve was plotted to obtain the linear equation as shown in Figure 3(c). The drug release profile indicates wheather the release is sustained or not. Figure 3(d) shows the drug release profile of BOP bandages. In 7 days, about 43% and 62% drug were released from C1A1 and C2A2 bandages respectively. While 78% of the drug from C3A3 bandage was released in 2 days. This result indicates that C1A1 showed sustained release of drug due to strong interaction of polymers.

3.9. Cytotoxicity testing of bandages

An ideal soft wound dressing should not release toxic products or produce adverse reactions, which can be evaluated through an *in-vitro* cytotoxicity test. Incubation of cells with two bandage solutions for one, three and five days resulted in significant differences as shown in Figure 4. It can be seen that VOP bandage inhibited the proliferation of Hela cells slightly on the first day, and the relative growth rate was 97.26%. BOP bandage showed better growth rate. Figure 5 shows the formation of formazan during the test.

3.10. Blood coagulation time

Coagulation time was evaluated for VOP and BOP bandages with chitosan to alginate ratio 1:1 along with control. The coagulation time of control, VOP and BOP was observed to be 8 minutes, 6 minutes and 3 minutes respectively. This result suggests that BOP bandage coagulates blood faster, thus shortening the wound healing phase.

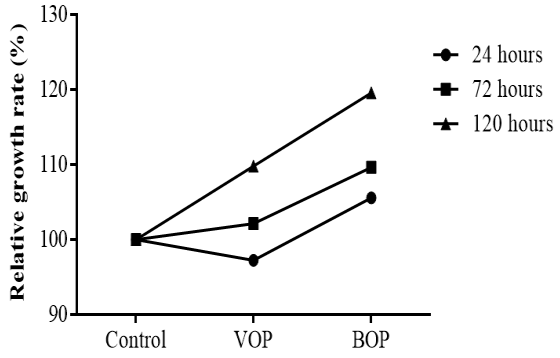

Figure 4 In-vitro cytotoxicity test showing Relative growth rate (%) of VOP and BOP bandage solution

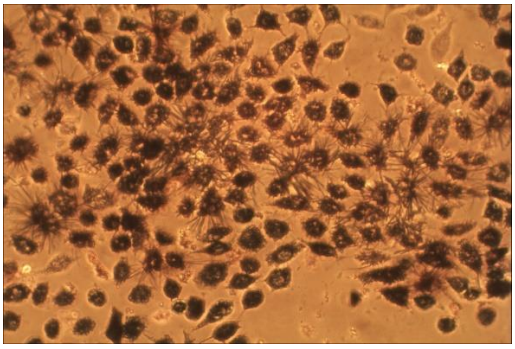

Figure 5 Formazan crystal formed by the living cells during cytotoxicity test (40X)

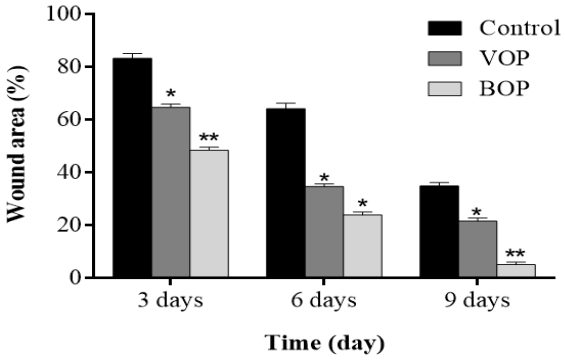

Figure 6 Total wound area of skin over time as a percentage of original wound size. Data as mean ± SD; $p < 0.05$ and $p < 0.005$**, VOP and BOP vs control.*

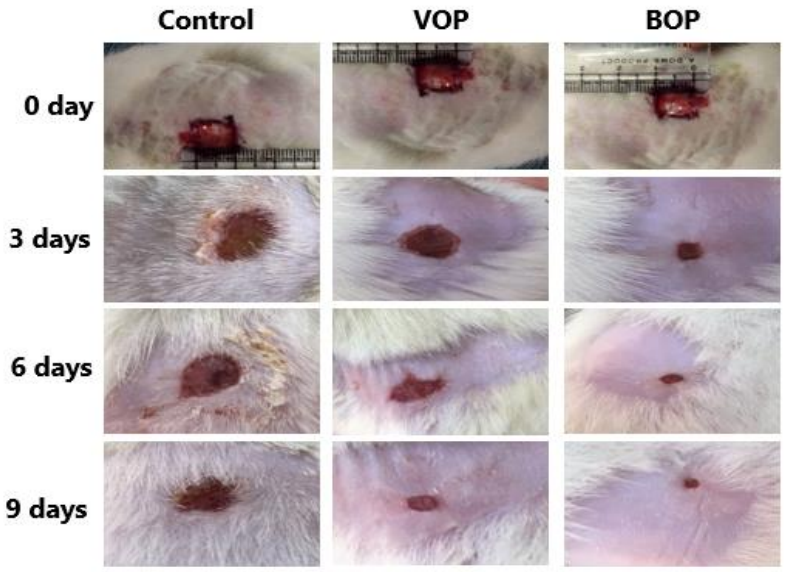

Figure 7 Photographs of macroscopic appearance of wound repair covered with cotton gauze (as control), VOP (1:1) and BOP (1:1) bandages at different post-wounding day.

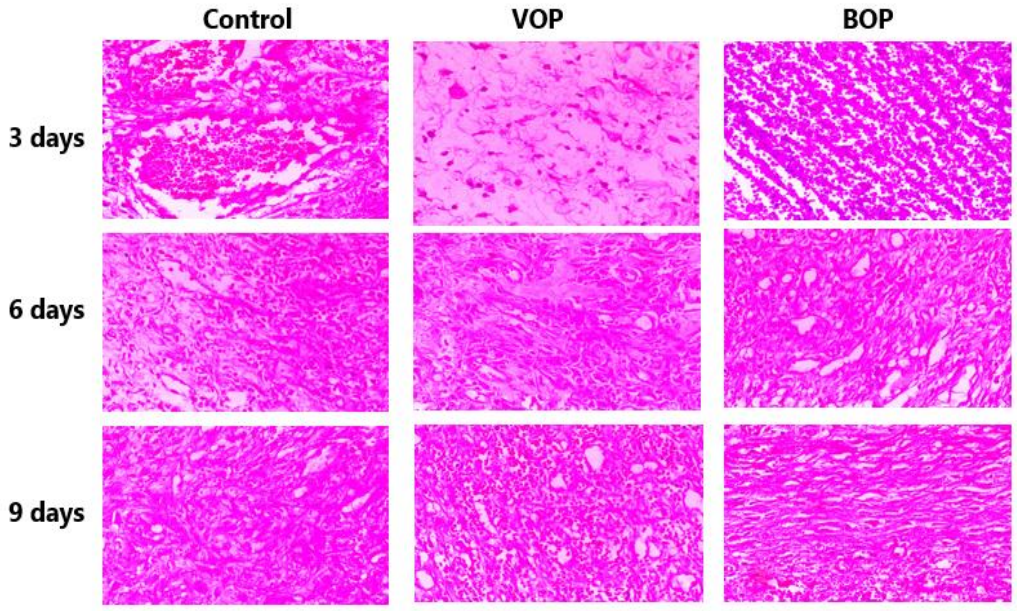

Figure 8 Histological study showing the effect of control, VOP and BOP bandage on wound healing in rats treated on the 3rd, 6th and 9th days post-wounding (hematoxylin–eosin (HE) stain) (40X).

3.11. In-vivo wound healing test

C1A1 bandages with molar ratios of chitosan to alginate 1:1 for both BOP and VOP bandages were used for in-vivo test. Initially, on 3rd post-operative day, VOP and BOP bandage absorbed the bleed and exudation at the wound site but cotton gauge adhered to surface and removal of it resulted in loss of tissue at wound surface. With reference to Figure 7, BOP bandage showed significantly better contraction of wound than VOP and gauge-treated wound over period of 9 days. Figure 6 shows the change in wound area in different treatment groups. Very little difference could be seen in the wound area of 3 groups on the 3rd postoperative day. By 6th day,

VOP and BOP treated wound healed faster than cotton gauge. Similarly, on 9th day the wound in control, VOP and BOP group contracted to 65%, 78% and 94% respectively. This indicates that polymeric bandage could be a better material to be used as wound dressing and black seed oil along with the polymeric bandage showed better wound healing property.

3.12. Histological analysis of wounded tissue

Figure 8 represents the result of histological examination of differently treated wound of Swiss albino rat. On the 3rd day, mild infiltration of polymorphonuclear leukocytes (PMNL) seen in case of control. While, the number of PMNL cells were increased in VOP and BOP treated wound. On the 6th day, the number of PMN cells in control increased whereas fibroblast with euchromatin nucleus was seen in VOP treated wound. BOP treated wound showed the formation of macrophages and fibroblast cells. Likewise, on the 9th day, wound remodeling was seen in BOP treated wound, with better orientation of connective tissue including collagen fiber. In VOP treated wound less amount of collagen fiber could be seen whereas in control, high number of PMNL cells were seen with absence of collagen fibers.

4.CONCLUSION

In summary, the present study represents the preparation of black seed oil loaded polymeric bandage in the presence of chitosan and alginate polymers and its therapeutic potential in dermal wound healing in a rat model have been investigated. The observed results demonstrated that dressings with black seed oil loaded polymeric bandage comparatively showed more healing response than control and void bandages. We assume that this may be due to anti-inflammatory, anti-oxidant and anti-microbial property found in the thymoquinone of the black seed oil. Based on the results of drug release of black seed oil, it was found that the C2A2 bandages have a sustained release behavior for up to 7 days. This shows that the C2A2 bandage could be a good drug support to be employed for sustained release. When this BOP bandage was used to cure dermal wound, a higher wound healing ratio in less time can be observed compared to VOP bandage and some reported dressings such as cotton gauze, and this result was further proved by histological analysis, demonstrating early collagen deposition and re-epithelialization for BOP treated wounds compared to control and VOP bandages where remodeling was under the process. However, further investigation is needed regarding the use of black seed oil in the dermal wound healing and further findings are necessary on how to make the bio polymeric bandage more absorbent to the essentials oils.

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