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Short communication

Biochemical properties of *Hemigraphis alternata* incorporated chitosan hydrogel scaffold

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ABSTRACT

In this work, *Hemigraphis alternata* extract incorporated chitosan scaffold was synthesized and characterized for wound healing. The antibacterial activity of Hemigraphis incorporated chitosan scaffold (HIC) against *Escherichia coli* and *Staphylococcus aureus* was evaluated which showed a reduction in total colony forming units by 45-folds toward *E. coli* and 25-fold against *S. aureus* respectively. Cell viability studies using Human Dermal Fibroblast cells (HDF) showed 90% viability even at 48 h when compared to the chitosan control. The herbal scaffold made from chitosan was highly haemostatic and antibacterial. The obtained results were in support that the herbal scaffold can be effectively applied for infectious wounds.

1. Introduction

Chitosan is used as a haemostatic wound dressing due to its biodegradable and biocompatible nature. *N*-Acetyl-glucosamine is the degradation product of chitosan. It is an initiator of proliferation phase (Jayakumar, Prabaharan, Sudheesh Kumar, Nair, & Tamura, 2011) in wound healing. An infectious wound needs an active antimicrobial agent for its recovery which is not effectively met by chitosan alone. *Hemigraphis alternata* (*H. colorata*) is a well known folk medicine. The herb is a perennial, belonging to the family Apocyanacea. The foliage carries attractive violet color, growing well in the presence of sun light. The use of crude leaf extract for anti-inflammatory activity (Subramoniam, Evans, Rajasekharan, & Sreekandan Nair, 2001) has already been studied. The phytochemical screening of *Hemigraphis* leaves and their antibacterial activity has already been worked on (Anitha, Marimuthu, Antonisamy, & Jeeva, 2011). In the present work some of the properties of chitosan scaffold have been compared with the herbal scaffold in vitro.

2. Materials and methods

2.1. Materials

H. alternata was collected from Kakkanad, Kerala. Chitosan (molecular weight – 100 kDa and degree of deacetylation – 80%) was obtained from Koyo Chemical Co. Ltd., Japan. Bacterial samples (ATCC), *Escherichia coli*-25922 and *Staphylococcus aureus*-25923 were obtained from the microbiology department of Amrita Hospital.

2.2. Preparation of chitosan scaffold and HIC

Chitosan was dissolved in 1% acetic acid by constant stirring. The solution was neutralized by the addition of NaOH solution. To obtain hydrogel, the solution was centrifuged at 10,000 rpm for 10 min. The obtained hydrogel yield was 1250%. The pellet obtained was freeze dried to obtain the scaffold. Fresh crude extract was collected from *Hemigraphis* leaves. The extract was lyophilized and added to the hydrogel, stirred for uniform mixing and freeze dried to obtain HIC (Fig. 1).

2.3. Characterization

Surface morphology was done using SEM (JEOL, JFC-1600, Japan). Thermogravimetric analysis was done at a temperature

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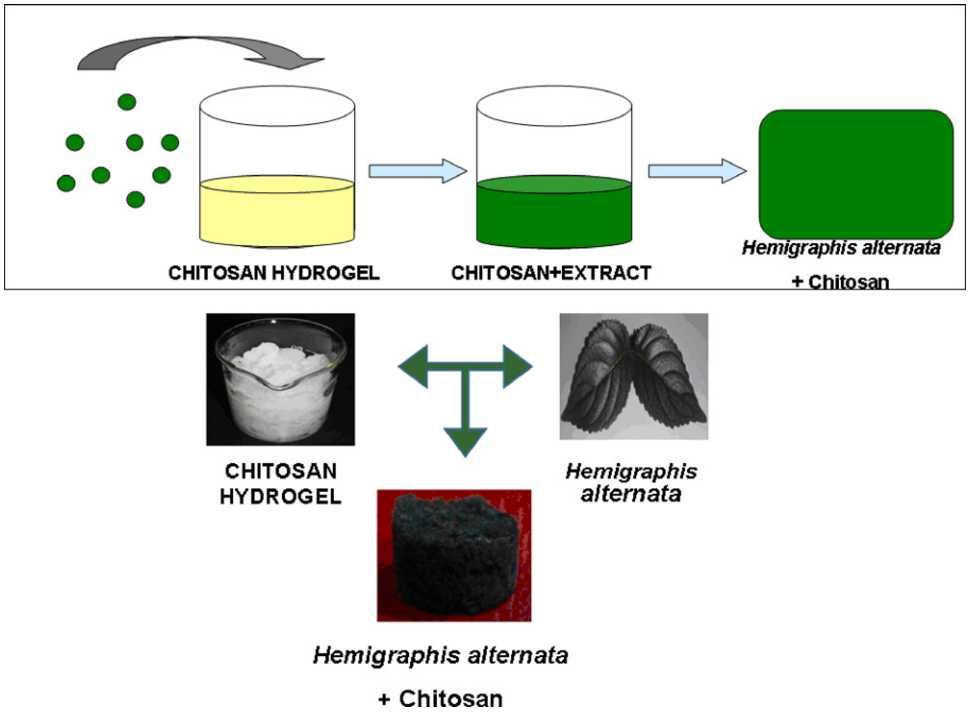

Fig. 1. Schematic representation of the preparation of *Hemigraphis alternata* incorporated chitosan scaffold.

range between 0 and 500 °C using TGA instrument (SII TG-DTA6200). Analysis using FTIR spectroscopy (Perkin Elmer Spectrum RX1) was done within a range of 400–4000 cm^{-1}.

2.4. Porosity, water uptake and swelling studies

Scaffolds of equal size were immersed in 95% ethanol for 24 h until saturation. The final weights of scaffolds were noted. Porosity can be calculated as follows

$$\text{Porosity} = \frac{\text{Wetwt} - \text{drywt}}{\text{Density} \times \text{volume}} \times 100$$

To determine the water uptake and swelling ratio, equally weighed scaffolds were immersed in water and PBS respectively followed by incubation at 37 °C for 14 days. The weights were noted on 1st, 7th and 14th days. The ratio can be calculated as

$$\text{DS/WU} = \frac{\text{Wtf} - \text{Wti}}{\text{Wti}}$$

where WU is water uptake, DS is degree of swelling, Wtf is the final weight and Wti is the initial weight.

2.5. In vitro biodegradation study

The scaffolds were equally weighed and immersed in PBS (pH 7.4) containing lysozyme followed by incubation at 37 °C. The final weights were noted after freeze drying the scaffolds. The percentage of biodegradation is calculated as

$$\% = \frac{\text{Wtf} - \text{Wti}}{\text{Wti}} \times 100$$

2.6. Platelet activation study and whole blood clotting assay

Plasma collected from fresh human (O+) blood samples were added to the scaffolds and incubated. The samples were fixed with gluteraldehyde, dried using ethanol and analyzed using SEM. The blood clotting property of HIC was compared with kaltostat and chitosan control. The samples for blood clotting studies were incubated with blood samples at 37 °C. Water was added slowly through the sides of the plate and free hemoglobin release at 540 nm was plotted.

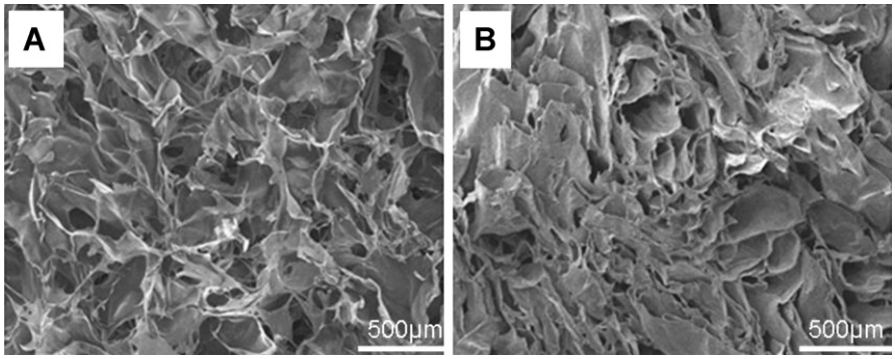

Fig. 2. (A) SEM image of chitosan control and (B) *Hemigraphis alternata* + chitosan.

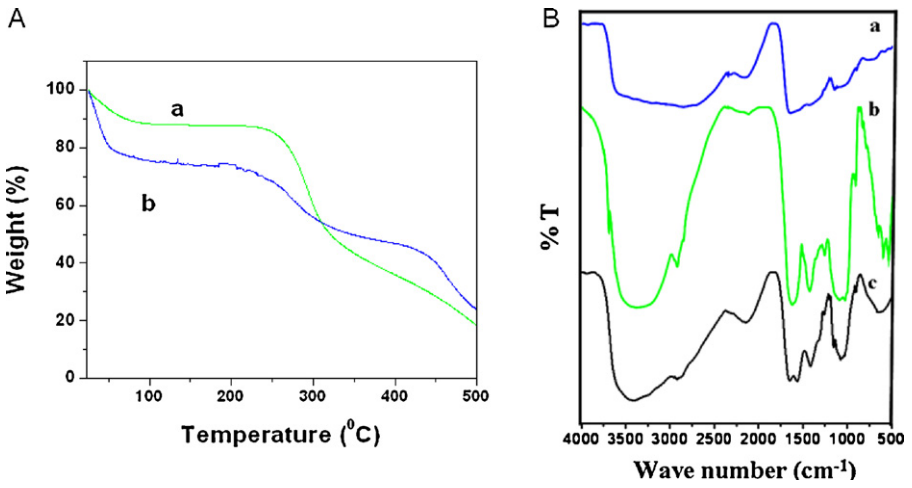

Fig. 3. (A) TGA of (a) chitosan and (b) *Hemigraphis alternata* incorporated chitosan scaffold (B) FTIR of (a) *Hemigraphis* extract (b) *Hemigraphis alternata* + chitosan and (c) chitosan scaffold.

2.7. Antibacterial activity

To test the antibacterial activity, *E. coli* and *S. aureus* (ATCC 25922 and 25923 respectively) were selected. The bacterial cultures were added equally to different test tubes after standardizing it with Mc Farland at 595 nm. Samples were serially diluted in normal saline after incubation for 24 h with samples of equal weight followed by plating on LB Agar. The colonies formed were counted and plotted.

2.8. Cell viability and proliferation

10,000 HDF cells were seeded per well on sterile scaffolds, followed by incubation for 24 and 48 h respectively. After each time interval, the media was replaced with 10% Alamar blue and absorbance was measured (Biotek Power Wave XS) at 570 nm with 620 nm as reference wavelength. For DAPI staining, the samples were fixed with 4% formaldehyde and were observed under fluorescence microscopy (Olympus-BX-51). The sample for SEM analysis was subjected to gluteraldehyde fixation followed by ethanol drying.

3. Results and discussion

3.1. Preparation and characterization of HIC

The minimal concentration of HIC that showed good antibacterial and mechanical properties was 16.6%. Hence this particular concentration was only subjected to further studies. Pore sizes of the range 200–300 μm was obtained with chitosan control and HIC respectively (Fig. 2). The addition of extract made the scaffold softer with a lamellar morphology.

TGA profile as shown in Fig. 3A shows a sudden steep decrease in weight at 0–50 °C for HIC compared to chitosan control. A uniform weight of about 95% was maintained until 250 °C while the weight of control decreases by 78% up to 200 °C. Further decrease is prolonged for HIC until 450 °C after which there is a fall in the peak indicating the stable weight loss for HIC for every 100 °C. Chitosan

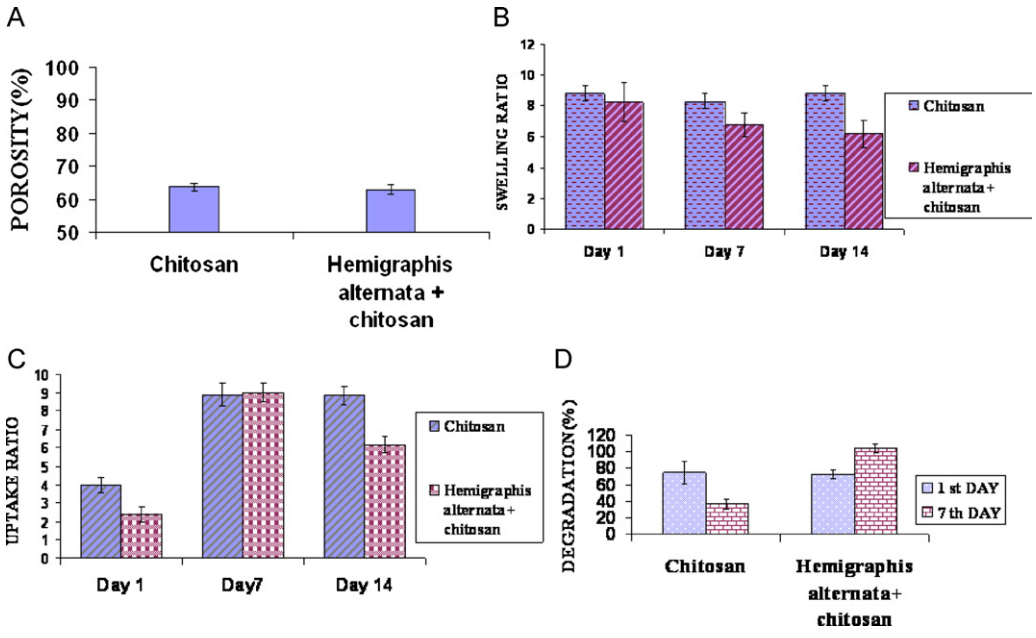

Fig. 4. (A) Porosity, (B) water uptake, (C) swelling ratio and (D) degradation studies of the prepared scaffolds.

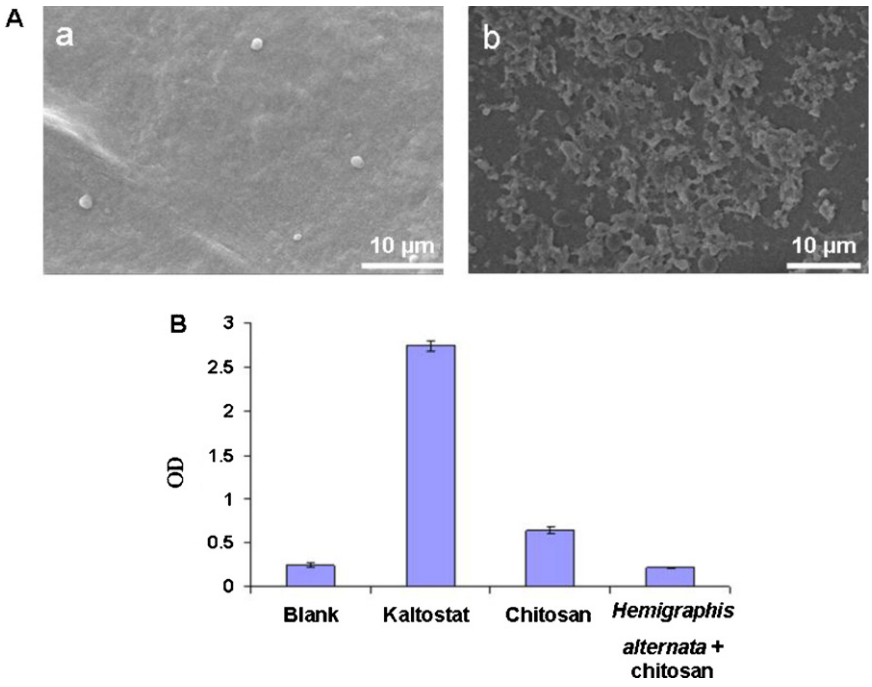

Fig. 5. (A) SEM images of platelet activation on (a) chitosan and (b) *Hemigraphis alternata* + chitosan scaffold and (B) graph showing whole blood clotting study of the scaffolds.

on the other hand, loses its half weight at 300 °C. The HIC showed higher decomposition than chitosan control.

FTIR spectra of leaf extract (Fig. 3B) shows prominent peaks that corresponds to O—H stretch of alcohol at 3627 cm^{-1} and that of carboxylic acid at 3122 cm^{-1} which is characteristic of organic acids such as chlorogenate, cinnamic acids and tannins (Anitha et al., 2011) N—H bend of amine, C—O stretch of alcohol and aromatic C—H bend at 670 cm^{-1} were also observed which further confirms the presence of phenolic acids. The spectra of HIC reveals the N—H bend of amine at 1557 cm^{-1} and C—O stretch of alcohol at 1024 cm^{-1}. The shift in peak may be due to stretching of the functional groups on binding to chitosan. The additional peaks in HIC correspond to the major secondary metabolite, cinnamate in *Hemigraphis*.

3.2. Porosity, water uptake and swelling studies

The porosity of both HIC and control (Fig. 4A) was almost 63%. The higher porosity enhances gaseous and vapor exchange at the wound interface (Petrulyte, 2008). The water uptake ability (Fig. 4C) was lower for HIC when compared to the control on 1st day. However, it increases and is similar to that of control on day 7 followed by further decrease on day 14. Therefore, slow and controlled water uptake of HIC attains saturation by 7th day.

The swelling rate (Fig. 4B) was almost same for the control on the observed days. Whereas, it decreases steadily with time for HIC. The maximum swelling capacity for HIC was up to 24 h thereafter the decrease was observed. The decrease in weight can be attributed to the conversion of scaffold material to mobile phase (Brandon, Shahana, Omar, Ali, & Nicholas, 2009).

3.3. In vitro biodegradation

HIC showed lesser degradation on day 1 (Fig. 4D) followed by a maximum degradation of 100% on 7th day whereas chitosan showed maximum degradation initially followed by lesser degradation after a week. Higher rate of degradation of scaffolds would help in faster release of its byproducts.

3.4. Platelet activation and whole blood clotting assay

More number of platelet aggregations is visible as shown by SEM image (Fig. 5) in the HIC when compared to the Chitosan control. The morphology of platelets showed more aggregation and spreading nature with characteristic pseudopods. The greater number of proteins adsorbed to chitosan scaffold would have enhanced

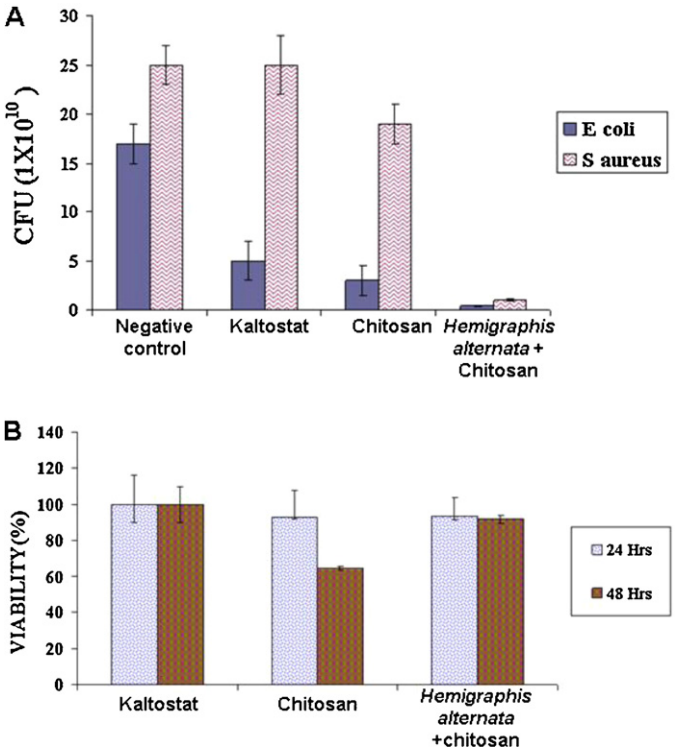

Fig. 6. (A) Antibacterial activity of *Hemigraphis alternata* incorporated chitosan scaffold against *E. coli* and *S. aureus* and (B) cell viability on HDF at 24 and 48 h respectively.

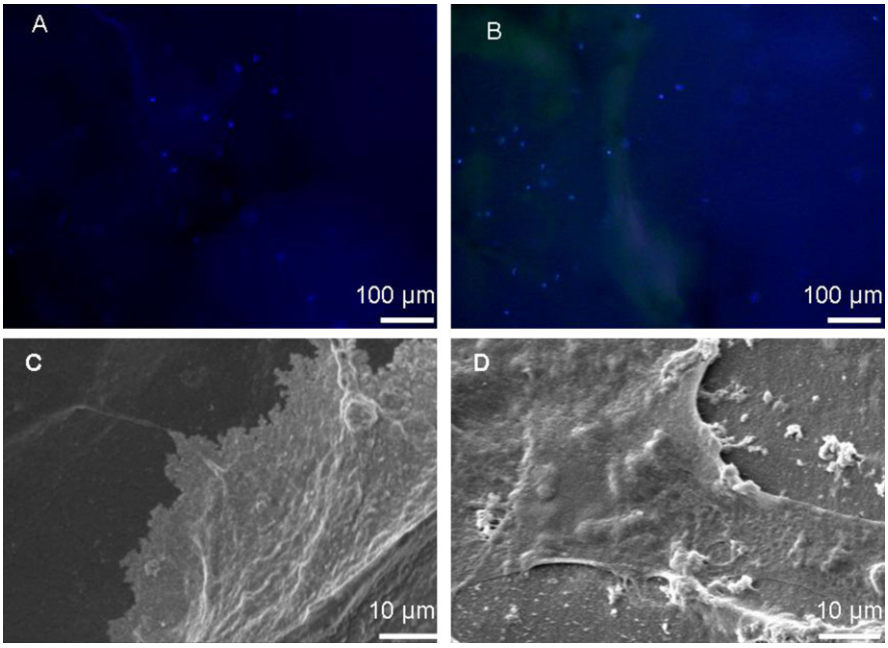

Fig. 7. DAPI stained images of HDF showing proliferation on (A) chitosan and (B) *Hemigraphis alternata* + chitosan scaffold and SEM image showing cell attachment on (C) chitosan and (D) HIC at 48 h.

the platelet aggregation (Megan, Bill, McCarthy, Moon Sun, & John, 2011). The haemostatic property of chitosan and HIC were compared with kaltostat (Fig. 5). A lower OD value at 540 nm indicates the higher blood clotting ability of the scaffolds. Of all the scaffolds, HIC showed least OD value suggesting the higher clotting ability than other materials.

3.5. Antibacterial activity

Antibacterial activity of HIC was higher than kaltostat and chitosan (Fig. 6). All the scaffolds showed significant reduction in *E. coli* colonies. The kaltostat and the chitosan control showed lesser activity toward *S. aureus* when compared to HIC. The presence of secondary metabolites such as phenolics, flavanoids and coumarin in the *Hemigraphis* might be responsible for higher activity than HIC (Rivière et al., 2009). Cinnamate, one of the major phenolic acid in hemigraphis is a potent antibacterial agent. Therefore, when the chitosan hydrogel is being incorporated to the extract, there is synergistic effect against Gram-positive and Gram-negative bacteria (Anitha et al., 2011).

3.6. Cell viability and proliferation

Cell viability (Fig. 6B) toward HDF showed a viability of almost 100% with kaltostat and HIC at 24 and 48 h, whereas the chitosan control showed slight decrease in viability of about 64% after 48 h. Fig. 7A shows more even distribution of cells on HIC scaffolds than on control at 48 h. In the SEM image (Fig. 7B) increased numbers of cells are seen attached on the surface of HIC than the control.

4. Conclusions

The HIC was prepared and characterized using SEM, FTIR and TGA. The HIC showed controlled porosity, swelling and biodegradation. The HIC showed enhanced platelet activation, blood clotting property and antibacterial activity. The scaffold also showed enhanced HDF cell attachment and proliferation and proved its non-toxic nature. Hence HIC can be used for wound healing.

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