



Characterization and evaluation for wound healing of chitosan extracted from the exoskeleton of freshwater crab *Potamonautes niloticus*

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ABSTRACT

The freshwater crab *Potamonautes niloticus* (Potamonautidae: Decapoda: Crustacea) is common in River Nile and its tributaries but has no commercial value as edible crab for human beings. Therefore, the present study aims to evaluate the role of chitosan extracted from its exoskeleton for wound healing process as a medical application. The structure of chitosan has been physically investigated by using fourier transformer infrared spectroscopy to reveal the vibrational frequencies of different structural functional groups, such as primary amine groups, hydroxyl groups and amide groups. The efficiency of the extracted chitosan was assessed by morphological and histological examination of wound healing in albino rats. Sections of isolated wounds were stained with hematoxylin and eosin to evaluate the histological changes during the wound healing process. Chitosan was yielded 30.64% from the crab shells and the results of wound healing assessments showed that the chitosan treated group (Ch 1%) was more efficient and faster than other groups (Ch 2, 3%) versus control groups. Also, the total leucocyte counts showed shortened inflammatory phase in chitosan-treated groups. The present results showed the efficiency of extracted chitosan in wound healing process and can be used in medical applications.

INTRODUCTION

The two Afrotropical species of freshwater crabs, *Potamonautes berardi* (Audouin, 1826) and *Potamonautes niloticus* (H. Milne Edwards, 1837) belong to Family Potamonautidae are well known in the River Nile and its tributaries. These species are in close to the Palaearctic species, *Potamon potamios* Olivier, 1804 (Family: Potamidae), an eastern Mediterranean taxon whose range extends into the Sinai Peninsula (Brandis *et al.*, 2000). *Potamonautes niloticus* and *P. berardi* are common and widespread throughout the River Nile basin from the delta in Egypt to Lake Victoria and its tributaries in equatorial Africa. The most common species in the northern limit is *P. niloticus* which has a very wide distribution; from Egypt, Sudan, Ethiopia, Uganda, Kenya and Rwanda. Fishermen have the crabs caught in their fishing nets

while targeting mainly crayfish and then they discard the undesired caught crabs because these populations of crabs are to the best of my knowledge not yet commercially evaluated.

In fact, freshwater crabs were considered as an important source of chitin as the other crustaceans (Bolat *et al.*, 2010). Chitosan is traditionally obtained from crustacean shells (crabs, shrimps and crayfish) with natural cationic polysaccharide composed of 2-acetamido-2-deoxy-b-D-glucopyranose and 2-amino-2-deoxy-b-D-glucopyranose (De Oliveira *et al.*, 2014).

Depending on the species and season, crustacean shells or exoskeletons are composed of 30%–40% protein, 30%–50% calcium carbonate and 20%–30% chitin. A rate of 1010–1011 tons per year is the amount of chitin produced from crustaceans, insect exoskeletons and cell wall of fungi, which are unusable wastes (Gopalan and Dufresne, 2003). It was determined that the shrimp (*Pandalus borealis*) contains about 17%–32.2% chitin (Shahidi and Synowiecki, 1991) while the chitin isolated from the crab, *Chionoecetes opilio*, contains 26.6% chitin (Hong and Mun, 1995).

Because of the outstanding biological features of chitosan, including its biodegradability, biocompatibility and

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nontoxicity; it is used in the food, agriculture, textile, water treatment, cosmetics and other industries with or without other natural polymers (alginates, starch and gelatin) additives (Kammoun *et al.*, 2013). Since chitosan was an applicable compound to use in medical and commercial industries, it considers as biodegradable, non-toxic, anti-fungal and antibacterial biomaterial compound (Ahmed and Sastry, 2011; Baldrick, 2010; Daniela and Camelia, 2008; Gokarneshan, 2017; Shi *et al.*, 2009).

The effect of chitin and its derivatives on fibroblasts and production of cytokines were reported by Mori *et al.* (1997); while Usami *et al.* (1994a, 1994b) found that chitin and chitosan have enhanced the activation of bovine and canine polymorph nuclear cells. Moreover, chitosan is of biomedical importance, for example, wound healing processes (Shakeel and Ikram, 2016). Moreover, Patrulea *et al.* (2015) concluded from their review that the chitosan is an efficient compound in wound healing application; also, it is a good accelerator material for wound healing (Yilmaz, 2004).

Wound repair or healing in which tissue repair itself after injury (Cordoso *et al.*, 2010; Nilani *et al.*, 2011) one of the applications that can be used to test the effect of chitosan on various tissues. The healing process begins with blood clotting and can be generally classified to: inflammation, granular tissue formation, re-epithelialization, matrix production and remodeling (Janis *et al.*, 2010). While Barbul and Regan (1993) classified this process to three phases comprised: inflammation, proliferation and maturation. Archana *et al.* (2013) stated that the wound healing is a series of complex steps starting with inflammation followed by cell migration, angiogenesis provisional matrix synthesis, collagen deposition and re-epithelization.

Generally, there are two models to test wound healing: incisional and excisional (Dorsett-Martin, 2004), but excisional model is more appropriate to determine histological and morphological changes during the healing process. Azuma *et al.* (2015) studied the excisional model on rat's wounds and found that wounds healed in group treated with chitosan are 99% higher than control groups 82%.

Hence, the present study aims to test an applicable material (chitosan) extracted from the non-economic freshwater crab *P. niloticus* on wound healing process caused by circular excision of skin in normal healthy rats.

MATERIALS AND METHODS

Extraction of chitosan

Forty six individuals of freshwater crab *P. niloticus* were collected from River Nile Tributaries, then transported to the laboratory, Faculty of Science, Al-Azhar University during August/2018. Crab exoskeletons were separated after dissecting all individuals in pre-iced jars. All crab exoskeleton wastes were washed with tap water then boiled and dried in room temperature for 24 hours. The extraction of chitosan was carried out according to Hossain and Iqbal (2014) with modification in demineralization step, using glacial acetic acid instead of hydrochloric acid. 1) Demineralization, by glacial acetic acid 5% for 1 hour at room temp. 2) Deproteinization by NaOH 4% for 2 hours at 65°C and 3) Deacetylation by NaOH 50% for 20 hours at 65°C.

Physical characterization of chitosan

Chitosan was characterized by using fourier transformer infrared spectroscopy (FTIR spectra) in a range of 400 to 3,600 cm^{-1} .

Wound healing experiment

A total of 25 male albino rats were obtained from Vacsera Farm, Helwan branch by delivery mediator and transported to the Animal House, Faculty of Science, to perform this experiment during October/2018. The animals were acclimated for 2 weeks before the experiment, all cages containing the experimental animals were cleaned, sterilized and changed the bedding (sawdust) every 2 days. Animals were separated into five groups (five in each) comprised: (1) -ve CG (negative control group without any treatment), (2) +ve CG (positive control group treated with commercial ointment of 25% w/w β -sitosterol), (3) Ch 1% (chitosan film 1% treated group), (4) Ch 2% (chitosan film 2% treated group) and (5) Ch 3% (chitosan film 3% treated group). Chitosan concentrations were determined based on studies carried out by Bujang *et al.* (2013) and No *et al.* (2002). Diethyl ether was used as anesthetic before wound injury. A small sterilized puncher used for performing two round wounds, ~ 1 cm in diameter, on the back of each rat (Fig. 1).

Morphological evaluation

Wound closure was measured by Vernier Caliper at each: 1, 4, 8, 12 and 16 day's intervals after surgery (Fig. 1).

Histological evaluation

It was performed by excision the rounded wounds of sacrificed rats (sacrificed by diethyl ether inhalation before excision). Then, the excised wounds preserved in formalin 10% and followed the basic histological preparation (fixation, dehydration, embedding and cutting sections). Sections were stained with hematoxylin-eosin to assess the phases of wound healing.

Total leucocytes count was estimated by automated Complete Blood Count veterinary Counter.

Morphometric measurements

The stained sections were examined under light microscope (Leica DM -LB2) provided with a digital camera (Sanyo vcc-6580PE) for photography. For each section, prepared samples from each rat was subjected to the following measurements: i) Thickness of epidermal layer (μm), ii) Thickness

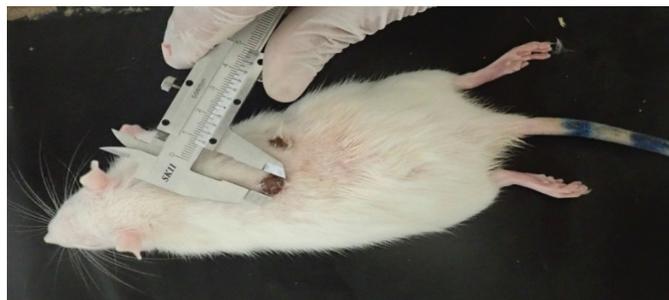


Figure 1. Measuring of wound closure using Vernier Caliper during healing process after treatment.

of dermal layer (μm), iii) Number of hair follicles and Sebaceous glands /field.

Statistical analyses

Statistical analyses were carried out using Microsoft Office 365 Excel program, ANOVA (Single factor).

RESULTS AND DISCUSSION

Chitosan yield

A total of 57.6 g chitosan was extracted from 46 individual's exoskeleton of the freshwater crab *P. niloticus*. This amount represents 30.64% out of 188 g of dried crab exoskeleton after final step of deacetylation, which in the range of those reported in some studied crustaceans (Cho *et al.*, 1999) and being higher than that determined in crab *C. opilio* and shrimp *P. borealis* (Hong and Mun, 1995; Shahidi and Synowiecki, 1991).

Physical characterization

Fig. 2 represents FTIR transmittance spectra of chitosan powder isolated and prepared from *P. niloticus*. Stretching vibrations of primary and secondary hydroxyl groups are represented by the

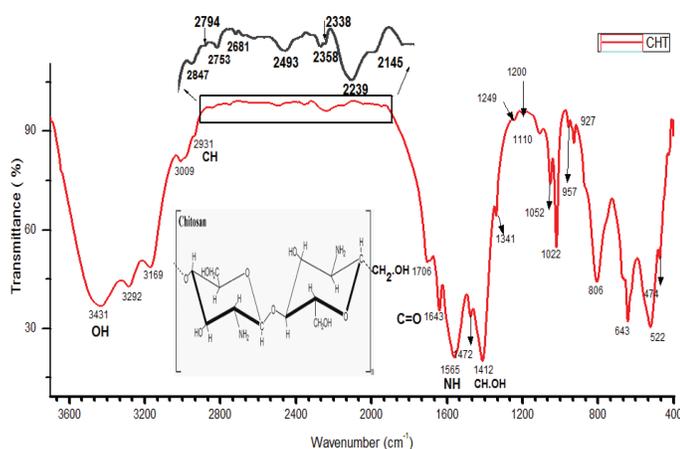


Figure 2. FTIR transmittance spectra of chitosan powder isolated from *P. niloticus* (the graph range between 2,850 and 2,000 has been enlarged due to lower intensity of peak existence).

principal spectral broad band between 3,431 cm^{-1} to 3,009 cm^{-1} . The stretching vibrations of C=O and C-O of primary alcohols were located at 1,413 and 1,052 cm^{-1} , respectively. The band at 1,338 cm^{-1} was assigned to amide III and the spectral band at 1,243 cm^{-1} was attributed to complex vibration of NHCO group. The C-O-C glycoside linkage vibration was observed at 1,198 cm^{-1} . The bands at 1,052 and 1,022 were ascribed to stretching of C-O bonds of the primary alcohols. An angular deformation of C-H bonds of methyl group were observed at the band of 1,475 cm^{-1} . The existence of a band at 3,282 cm^{-1} is attributed to the hydrogen bonded NH groups of the secondary amide. The ring vibrations in C-C were represented by several bands at 677, 806 and 957 cm^{-1} . The presence of spectral peak at 1,563 corresponds to the protonation of NH_3^+ groups. The bands at 2,931, 2,847, 957 cm^{-1} were ascribed to C-H bonds stretching. The stretching vibrations in amide II group was located at 1,643 cm^{-1} . All bands that are observed in FTIR spectrum are detailed in Table 1.

Noteworthy, the band of 1,643 cm^{-1} , that is assigned to C=O of the primary amide group of the extracted chitosan shows similarity with the reported peak by Norhidayah *et al.* (2017) for commercial chitosan. The appearance of bands at 1,022 and 1,052 cm^{-1} is contributed by the stretching of C=O bonds of the primary alcohols. However, few bands of the chitosan prepared in the present study are absent in the commercial chitosan presented by Norhidayah *et al.* (2017).

Morphological and histological evaluation of wound healing

The uses of morphological and histological examination of healing rat wounds using the present extracted chitosan as a treatment are more appropriate methods to assess the wound healing factors as previously proposed (Gal *et al.*, 2008; Vidinsky *et al.*, 2006).

To evaluate the effectiveness of chitosan extracted from the crab exoskeleton waste as a wound dressing material, an *in vivo* macroscopic wound closure study was performed. During this study, the macroscopic observation of wound closure in control groups, including (-ve) untreated and (+ve) treated with commercial wound healing material versus treated groups with chitosan film (1%, 2% and 3%) was examined at 4, 8, 12 and 16 days after surgery (Fig. 3a and b).

In -ve control group, the closure of wound not completely healed at the end of the experiment at Day 16, but the +ve control

Table 1. Characterization of chitosan (present study) using IR in comparison with commercial chitosan.

Frequency wave number (cm^{-1})		Assignment
Chitosan present study	Commercial chitosan (Norhidayah <i>et al.</i> , 2017)	
3,431–3,009	3,435	Primary and secondary Hydroxyl groups (O-H) groups stretching
3,282	–	Hydrogen bonded NH groups of Amide II
2,931 & 2,847	2,921	C-H bonds stretching
1,563	–	Protonated NH_3^+ groups
1,643	1,643	C=O stretching of the amide II group
1,475	–	Angular deformations of C-H group of methyl group
1,413	1,430	CH ₂ bending and C=O stretching
1,338	–	Amide III [2013 reference no 29 says that its peak is at 1,320]
1,052 & 1,022	1,070	Stretching of C-O bonds of the primary alcohols.
1,243	1,262	Complex vibration of NHCO group
1,198	1,154	C-O-C glycoside linkage
677-806-957	699	Ring vibrations C-C

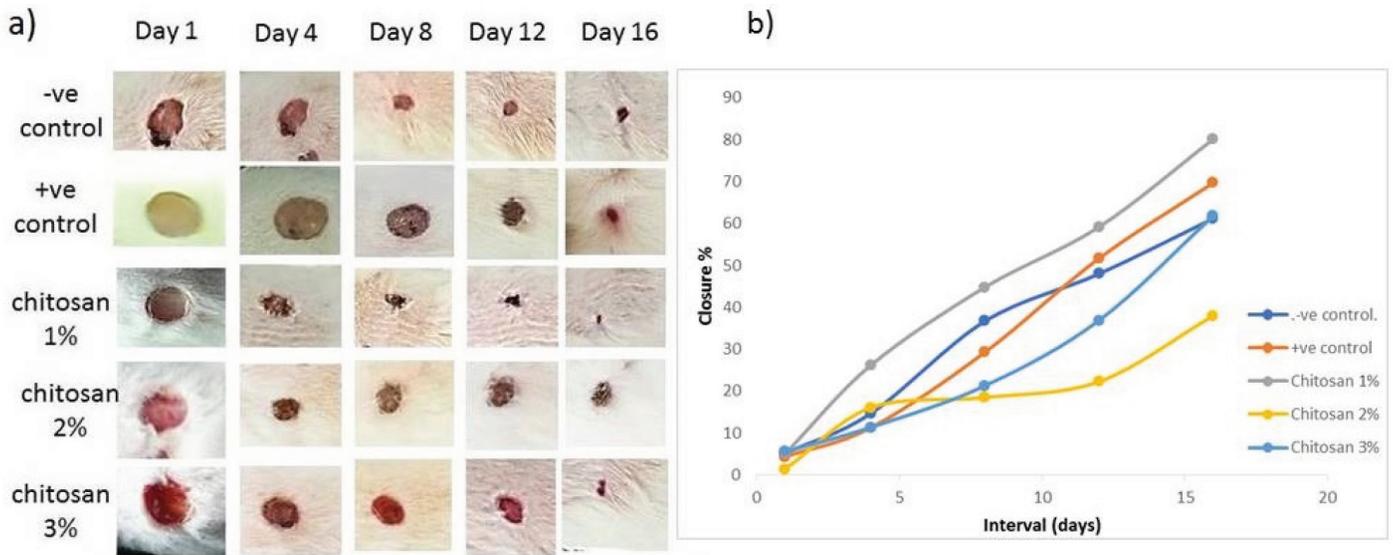


Figure 3. (a) Morphological assessment of wound healing closure treated with different doses of chitosan compared with control. (b) Closure rate of treated groups versus control groups.

showed more healing performance at Days 12 and 16. Moreover, scar formation weakly observed in both previous groups. On the other hand, the Ch 1% treated group showed the best performance of wound closure compared with control groups started at Day 4, in addition to the formation of scar at Days 4, 8, 12, with closure rate, 44.7%, 59.1% and 80%, respectively and almost completed healing at Day 16, this was less than reported complete healing range period of 3 weeks (Dreifke *et al.*, 2015). However, the macroscopic observation for treated groups, Ch 2% and 3% showed less effectiveness of wound closure during the period of the experiment despite of the formation of the scar, with lower closure rate. The present results showed that the most effective treatment on wound closure rate was chitosan treated group (1%) followed by positive control group (commercial product), indicating that the present prepared chitosan is more effective at concentration 1% comparable with commercial products and other concentrations and the statistical analyses confirmed the difference in closure rate, it was significant ($p < 0.05$) between groups.

Histological evaluation of wound healing

Microscopic examinations for control and treated groups sectioned samples of rat wounds stained with H&E at 4, 8 and 16 days intervals were carried out to evaluate the recovery of skin tissue (Fig. 4). After Day 4, the H&E stained sections showed no scar formation in -ve and +ve control groups but was clearly observed in Ch 1% treatment group and hardly indicated in the treatment groups of Ch 2% and 3%. On the other hand, re-epithelization was showed only in +ve CG and Ch 1% treatment groups. These results are in well agreement with Azuma *et al.* (2015) on the assessment of chitosan derivatives after 4 and 8 days and the re-epithelization was completed after 16 days indicating that chitosan prompts good re-epithelization with no scar formation which agree with No *et al.* (2002).

Moreover, sections of the treated groups were highly filled with newly formed blood vessels. In -ve and +ve control groups, new blood vessel formation delayed and observed at Day 8, while the granulation in the epidermis recorded first in the treatment groups at Day 8.

At Day 16, sebaceous glands observed in all groups except -ve CG, while hair follicles observed only in treatment groups; remarkably in Ch 1% and 2% groups. On the other hand, the thickness of the epidermis in Ch 1% & 2% groups were higher than Ch 3%, but the complete wound healing observed only in Ch 1% and 2% treatment groups at the end of the experiment.

By contrast, regeneration of tissues in wounds of treated groups were achieved at Days 4 and 8 but, delayed in control groups till Day 8. Moreover, the thickness of epidermis was increased significantly in Ch 1% and 2% treated groups at Days 4 and 8 comparable to control groups.

Total leucocyte count

The total averages of leucocyte count in the treatment groups versus control groups after 4, 8, 12, 16 days are shown in Table 2. Despite of there was no significance difference ($p > 0.05$) in the total leucocytes count between groups; the present results exhibit remarkable gradual decreasing in leucocyte number with an increasing period of treatment with chitosan. The maximum total leucocyte count estimated at Day 4 for all treated groups. The highest averaged values were 8.3, 6.1 and 11.6 for 1%, 2% and 3% chitosan treated groups, respectively. However, these values had decreased gradually at Days 8, 12 and reached the lowest values at Day 16 in the treated groups. On the other hand, control groups showed variable pattern at Days 4–16 with relatively lower values (Table 2). This indicates that chitosan promotes the inflammatory phase faster than control groups, as mentioned by both of Muzzarelli *et al.* (1990) and Seyfarth *et al.* (2008).

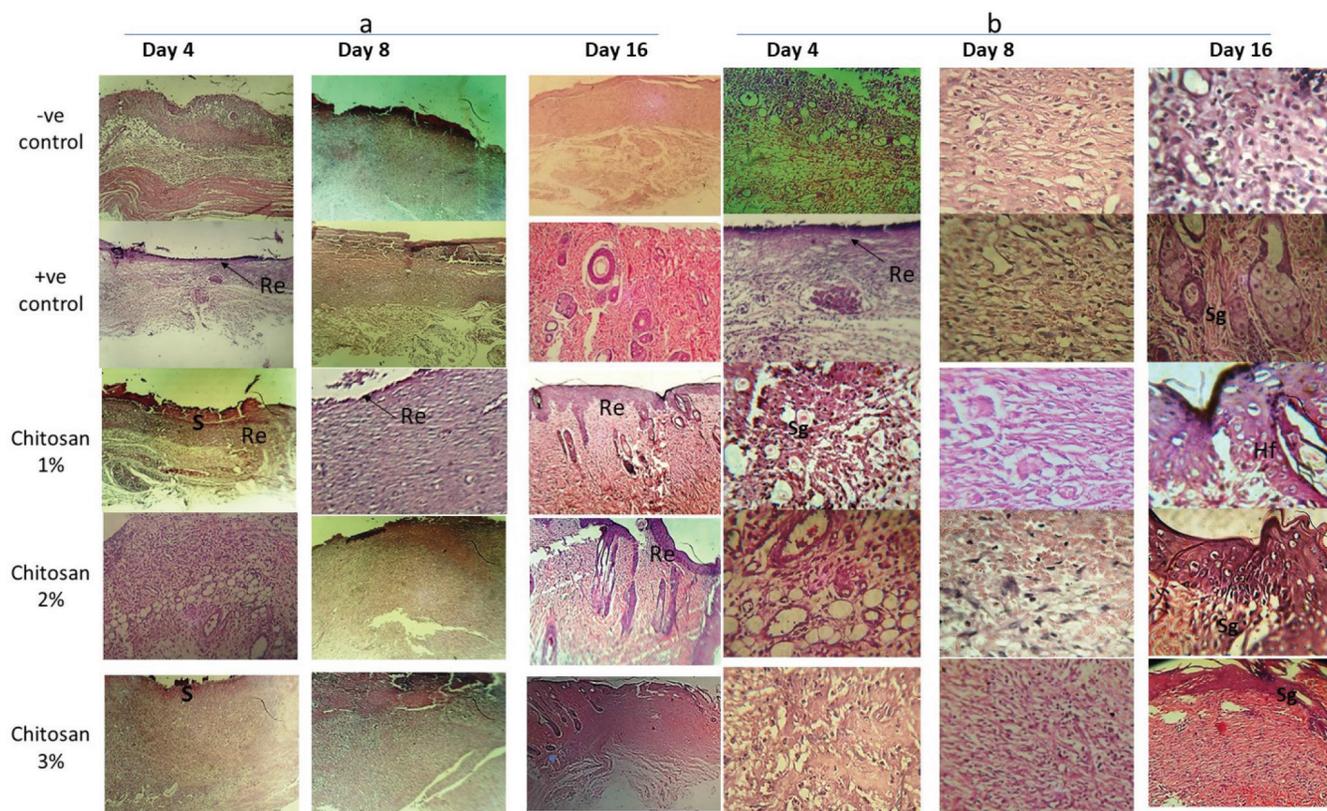


Figure 4. Histological examination of rat skin wounds H&E staining for control (-ve & +ve) versus treated groups (ch. 1%, 2% and 3%) after 4, 8 and 16 days of treatment; re-epithelization indicated by arrows, S (scar formation), Sg; sebaceous glands and Hf; hair follicle. (a) magnification 50×. (b) magnification 100×.

Table 2. Showing average \pm standard deviation total count of leucocytes in the chitosan treated groups versus control groups during this study.

Groups Period (post-surgery)	Groups				
	Ch 1%	Ch 2%	Ch 3%	-ve CG	+ve CG
Day 4	8.3 \pm 0.27	6.1 \pm 0.34	11.6 \pm 0.48	3.9 \pm .46	4.2 \pm 0.12
Day 8	5.4 \pm 0.43	4.7 \pm 0.39	7.7 \pm 0.27	2.8 \pm 0.34	7.3 \pm 0.32
Day 12	4.3 \pm 0.15	4.1 \pm 0.72	5.3 \pm 0.42	3.7 \pm 0.52	4.7 \pm 0.28
Day 16	3.5 \pm 0.22	4.1 \pm 0.23	3.1 \pm 0.47	2.8 \pm 0.31	3.9 \pm 0.37

CONCLUSION

The present study provides a commercial importance of freshwater crab *P. niloticus* which considers as a good source of chitosan extracted from its exoskeleton that evaluated in present study for wound healing application. The present results ensure the efficiency of chitosan in accelerating the wound healing process with less inflammatory phase.

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CONFLICT OF INTERESTS

The authors declare that there are no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The present studied animals were treated according to “Ethical guidelines for the care and use of animals in education & scientific Research” and approved through the Ethical Committee of Faculty of Pharmacy, Ain Shams University. (Reference Number: ENREC-ASU.2019-99)

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AUTHORS' CONTRIBUTIONS

M.A. collected the crab specimens and extracted the chitosan material; the wound healing experiment was investigated

also by M.A. E.A. involved in the physical characterization of the chitosan material. M.A. and E.A. contributed in the preparing and editing the whole manuscript. All authors have read and approved the manuscript.

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