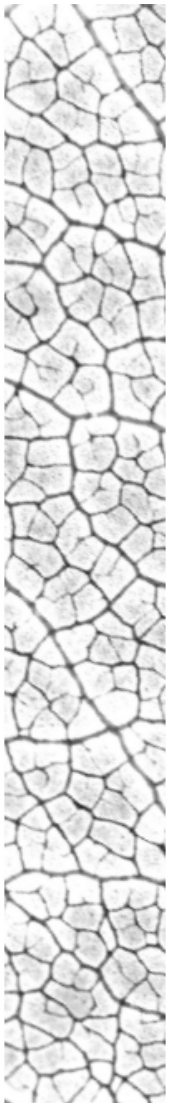

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Artemisia dracunculus in combination with chitosan nanoparticle biofilm improves wound healing in MRSA infected excisional wounds: An animal model study

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Abstract
The objective of the present study was to assess effect of Artemisia dracunculus in combination with chitosan nanoparticle biofilm on MRSA infected excisional wounds. Thirty rats were randomized into five groups of six rats each. Group I: Animals with uninfected wounds treated with 0.9% saline solution. Group II: Animals with infected wounds treated with saline. Group III: Animals with infected wounds were dressed with chitosan nanoparticle biofilm. Group IV: Animals with infected wounds were treated topically with Artemisia dracunculus and Group V: Animals with infected wounds were treated topically with Artemisia dracunculus and dressed with chitosan nanoparticle biofilm. Wound size was measured on 6, 9, 12, 15, 18 and 21days after surgery. Microbiology, reduction in wound area and hydroxyproline contents indicated that there was significant difference ($P < 0.05$) between group V and other groups. Quantitative histological studies and mean rank of the qualitative studies demonstrated that there was significant difference ($P < 0.05$) between group V and other groups. It was concluded that the Artemisia dracunculus with chitosan nanoparticle biofilm had a reproducible wound healing potential and hereby justified its use in practice.

Keywords: Artemisia dracunculus, chitosan nanoparticle biofilm, MRSA, wound, rat

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INTRODUCTION

Wound healing is a complex process by which heal through the same process (Cotran et al. 1999). Dysfunctional immune system and presence of an infection which may be the normal flora or other opportunistic microbes that are in the environment (Ataee et al. 2012). The most common infections in the wounds are caused by *Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus species and Escherichia coli* (Ranjbar et al. 2016, 2017, Rajan 2012). Different course of antibiotic resistance, especially in methicillin resistant *Staphylococcus aureus* (MRSA) demand a new approach to management of innovative treatments (Maghsoudi et al. 2017, Zetola et al. 2005). To manage medical treatment of infected wounds, lots of agents and protocols have been proposed in the literature (Ranjbar and Ashrafzadeh-Takhtfooladi 2016a, 2016b, Ranjbar et al. 2016).

Artemisia dracunculus is a member of the Asteraceae that grow in dry or semi-dry habitats (Meepagala et al. 2002). Plants in this family are rich sources of various types of biologically active compounds like Phototoxic and antibiotic activities and antioxidant features (Lopez-lutz et al. 2008). Many Artemisia species possess allelopathic and antifungal chemicals (Gholami et al. 2012). Artemisia species are also used as spices and in folk remedies as antiseptics. Powdered leaves of Artemisia species have been applied externally in salves and washes by North American native people for treating sores and wounds and internally to treat chest infections. The aromatic leaves of Artemisia dracunculus (tarragon) have been also used as spice and to preserve meat. Artemisia dracunculus ethanolic extract significantly reduced hyperglycemia in mice with chemically induced insulin deficiency and diabetes. The positive analgesic effect of tarragon in diabetic neuropathy have been approved. The artemisinin from Artemisia annua is now being developed as a potential drug for the treatment of malaria. Artemisia dracunculus, has a traditional Persian history of use as a natural cleanser of the blood and for the treatment of headaches and dizziness (Gholami et al. 2012, Kordali et al. 2005, Schmidt et al. 2007, Yazdanparast and Saee 1999).

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Chitosan is a non-toxic cationic biopolymer usually obtained by alkaline deacetylation from chitin, which is the principal component of crustacean exoskeletons (Jonaidi Jafari et al. 2018, Sinha et al. 2004). Chitosan presents with biocompatibility, chelating capacity and also antimicrobial effects against a broad range of gram positive and gram-negative bacteria as well as fungi (Kishen et al. 2008, No et al. 2002). Previous in vitro studies have demonstrated the significant biofilm efficacy of chitosan nanoparticles (CNPs) (Calamari et al. 2011, Silva et al. 2013).

To the best knowledge of the authors the literature is poor regarding potentiation effects of Artemisia dracunculus in combination with chitosan nanoparticle biofilm on wound healing in full thickness infected wounds with antibiotic resistant gram positive bacteria. Therefore, the present study aimed to study effects of combination of Artemisia dracunculus loaded chitosan nanoparticle biofilm on wound healing in full thickness infected wounds with methicillin resistant Staphylococcus aureus.

MATERIALS AND METHODS

The study was approved by the institutional animal research ethics committee and 3R's principle were strictly followed. Thirty adult healthy male Wistar rats weighting 200– 250g were used and housed in individual cages under constant temperature (22ºC) and humidity with 12-h light/dark cycle, and had *ad libitum* access to chow and water throughout the study.

Preparation of MP (Carrier)

To prepare 100 g of Macrogol ointment, 40 g of polyethylene glycol 3350 (Ineos Manufacturing, Deutschland GmbH, Germany) was mixed with 60 g of polyethylene glycol 400 (DOW Chemical Company, USA). The two ingredients were heated in water bath at 65°C until complete melting and then allowed to cool down to room temperature while stirring until the mixture was congealed.

Formation of the Artemisia Dracunculus Paste

The Artemisia dracunculus herb was harvested as the total herb above the root mass. The harvested herb was stored frozen at -20°C. For extraction, 4 kg of frozen herb was heated with 80% ethanol (v/v) to 80 $^{\circ}$C for 2 h and allowed to continue to extract for 10 additional hours at 20 $^{\circ}$C. The extract was then filtered through cheesecloth to remove particulates (Sayyah et al. 2004).

Preparation of Chitosan Nanoparticle Biofilm

The chitosan nanoparticles were prepared based on a procedure described by others (Rampinoa et al. 2013). A 2.5 mg/mL chitosan solution was prepared by dissolving LMW or VLMW chitosan in a 0.05% (v/v) acetic acid solution and leaving it under stirring for 24 h. The pH was adjusted to 5.5with a 0.5 M sodium hydroxide solution and diluted in deionized water to the final desired concentrations. The tripolyphosphate (TPP) was dissolved in deionized water to a final concentration of 0.25 mg/mL. TPP and chitosan solutions were filtered through a 0.45 µm membrane (Millipore). Then, the TPP solution was added to the chitosan solution drop wise (0.3 mL/min) at different TPP: chitosan ratios under vigorous magnetic stirring at room temperature. The resulting suspension was dissolved in 100 mL of 1% acetic acid and stirred for 24 h at room temperature. The obtained solution was then filtered through G4 sand filter in order to remove the impurities and undissolved particles. The prepared plain polysulfone (PSf/TiO2) membrane (100 cm^2) was pasted on the glass plate separately using tape with thickness of 1 mm. The stuck membrane was washed with distilled water and wiped with smooth tissue paper. A thin film of saturated polyvinyl alcohol solution was brush coated on the substrate. Chitosan (30 mL) was slowly poured in the center of the substrate and spread evenly throughout the substrate. Further, the thin film was dried at 60 8C for 4 h in a hot air oven. After drying, the membrane was allowed to reach room temperature, and was then washed with 1% NaOH to remove excess acetic acid. Finally, the membrane was washed with distilled water until the washed water reached neutral ph. The same was repeated for bare PSf membranes (Nayak et al. 2015). The obtained membranes were used to dress the wounds.

Study Design

The rats were randomly selected and allocated into five groups of six rats each. A power calculation based on earlier studies suggested that 6 animals in each group would be sufficient to detect a statistically significant difference in bacterial count, which was the primary outcome in this study. Group I: Animals with uninfected wounds treated with 0.9% saline solution. Group II: Animals with infected wounds treated with saline. Group III: Animals with infected wounds were dressed with chitosan nanoparticle biofilm. Group IV: Animals with infected wounds were treated topically with Artemisia dracunculus and Group V: Animals with infected wounds were treated topically with Artemisia dracunculus and dressed with chitosan nanoparticle biofilm.

The Procedures for Wound Creation and Infection

Rats were anesthetized by an intraperitoneal injection of ketamine (70 mg/kg of b. w.) and xylazine (5mg/kg of b. w.), the hair on their back was shaved and the skin cleansed with 70% alcohol solution. Following shaving and aseptic preparation, a circular excision wound was made by cutting away approximately 300 mm^2 full thickness of predetermined area on the anterior-dorsal side of each rat. Small gauze was placed over each wound and then inoculated with 5×10^7 CFU of *Staphylococcus aureus* ATCC 43300, The methicillin-

resistant *S. aureus* ATCC 43300 strain was commercially available. The pocket was closed by means of 4-0 nylon sutures and this procedure resulted in a local abscess after 24 h. The rats were returned to individual cages and they were examined daily. After 24 h, the wounds were opened, the gauze removed for quantitative bacterial cultures and treatment started. Postoperative pain was controlled using meperidine (Hameln, Germany); 10 mg/kg were injected subcutaneously once daily for three days.

Animal Grouping

In group I, the sterile saline 0.9% solution was added to the uninfected wounds. In group II, the animals with infected wound were only treated with saline solution. In group III, animals with infected wounds were dressed with chitosan nanoparticle biofilm. In group IV, the animals with infected wounds had topical Artemisia dracunculus paste application once daily at a concentration of 200 mg/kg on the created wound. In group V, the animals with infected wounds had topical Artemisia dracunculus paste application once daily at a concentration of 200 mg/kg on the created wound and were dressed with chitosan nanoparticle biofilm. All the test formulations were applied for 7 days starting from the day of wounding.

Microbiological Examination

At the end of 6th day of treatment, a sample tissue was taken from each wound, homogenized, weighed and 1:4 wt/vol dilutions were made with sterile 0.9% saline. Quantization of viable bacteria was performed by culturing ten-fold dilutions of each sample. 0.1 ml of the bacterial suspension from each group was put in sterile blood agar flat bottom plates. All plates were incubated at 37°C for 48 h and evaluated for the presence of the *Staphylococcal* strain. The number of colony-forming units/g (CFUs/g) of tissue homogenate was used to express the colonization.

Excision Wound Model and Planimetric Studies

Wound-healing property was evaluated by wound contraction percentage and wound closure time. Photographs were taken immediately after wounding and on days 6, 9, 12, 15, 18 and 21 post-operation by a digital camera while a ruler was placed near the wounds. The wound areas were analyzed by Measuring Tool of Adobe Acrobat 9 Pro Extended software (Adobe Systems Inc, San Jose, CA, USA) and wound contraction percentage was calculated using the following formula: Percentage of wound contraction = $(A_0 – A_t) / A_0 \times 100$

Where A_0 is the original wound area and A_t is the wound area at the time of imaging. The animals were left in separate cages for four days at room conditions for acclimatization. Animal houses were in standard environmental conditions of temperature (22 ±3°C), humidity (60 ± 5%), and a 12h light/dark cycle. The animals were maintained on standard pellet diet and tap water. All rats were closely observed for any infection and if they showed signs of infection were separated, excluded from the study and replaced.

Determination of Hydroxyproline Levels

On the day 21 after surgery, a piece of skin from the healed wound area was collected and analyzed for hydroxyproline content. As a major part of collagen, hydroxyproline has an essential role in collagen stability. The collagen is the major component of extracellular tissue, which gives support and strength. The hydroxyproline contents were estimated using a method described by others (Qiu and Kwon 2007). Briefly, tissues were dried in a hot air oven at 60–70 ◦C to constant weight and were hydrolyzed in 6N HCl at 130 ◦C for 4 h in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4M perchloric acid and color was developed with the help of Ehrlich reagent at 60 ºC and measured at 557 nm using UV-visible spectrophotometer (CamSpec M330, Cambridge CB2 4BG, UK).

Histological Preparation and Quantitative Morphometric Studies

The tissue samples were taken on 7, 14, 21 days after surgery from periphery of the wound along with normal skin and fixed in 10% buffered formalin, dehydrated and embedded in paraffin wax, sectioned at 5 µm and stained with hematoxylin and eosin (H&E) and Masson's trichrome stains. Photomicrographs were obtained under light microscope to assess the predominant stage of wound healing. Three parallel sections were obtained from each specimen. Cellular infiltration including the number of mononuclear cells, poly morphonuclear cells and fibroblastic aggregation were quantitatively evaluated. Acute hemorrhage, congestion, vascularization, epithelialization, collagen production and density were also evaluated qualitatively. Morphological findings were scored using image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). The histological parameters were classified according to the intensity of occurrence in five levels (- absence; + discrete; ++ moderate; +++ intense; ++++ very intense) (Rampinoa et al. 2013).

Statistical Analysis

Differences among groups in excisional model, hydroxyproline level test were evaluated by Kruskal–Wallis variance analysis. When the P-value from the Kruskal–Wallis test statistics was statistically significant, multiple comparison tests were used to know differences. Student's t-test was used for evaluation of mechanical test results. Comparison among days was assessed by Mann–Whitney U-test. The Bonferroni correction was applied for all possible multiple

Table 1. Wound bacterial count in experimental groups

Groups	Wound bacterial count (CFU/g)
I	0.00 ± 0.00
II	1449.77 ± 752.15
III	274.11 ± 28.67
IV	243.12 ± 63.43
V	164.46 ± 51.57*

CFU: Colony-forming units. *P<0.05 *vs* other experimental groups

Table 2. Effect of Artemisia dracunculus and/or chitosan nanoparticle biofilm on circular excision wound contraction area (mm2). Values are given as mean ± SEM

	Wound area in days (mm²)					
Groups	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21
I	251.71 ±0.91	102.43 ±1.54	88.73 ±0.65	45.77 ±1.35	22.20 ±1.10	7.88 ±0.38
II	257.12 ±0.69	200.82 ±1.19	189.15 ±0.25	147.77 ±1.94	95.67 ±1.19	75.15 ±1.25
III	205.25 ±1.15	133.53 ±1.73	105.05 ±0.06	87.57 ±0.99	54.15 ±1.02	25.67 ±1.35
IV	215.15 ±1.27	169.49 ±1.74	127.59 ±1.34	106.68 ±0.20	68.17 ±1.10	30.11 ±1.35
V	157.58 ±1.35*	117.20 ±0.30*	77.54 ±0.11*	37.76 ±0.19*	14.35 ±0.57*	4.56 ±0.34*

The treated groups are compared by Student t test with other groups. *: The mean difference is significant at the.05 level *vs* other experimental groups

comparisons. SPSS 11.5 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A P-value was set at 0·05.

RESULTS

Microbiological Examination

No animals died due to infection or anesthetics. The culture at 24 h after wounds inoculation of groups II and IV rats with MRSA showed CFU/g count > 1.000. On the 6th day, the uninfected wounds treated with saline had no CFU/g of *S. aureus* count. In animals of group II whose infected wounds were treated with saline, the counts of *S. aureus* cultured in the wound tissues were significantly higher than in the infected wounds of groups IV and V (P<0.05,). In animals of group V whose infected wounds were treated with both Artemisia dracunculus paste and chitosan nanoparticle biofilm , the counts of *S. aureus* cultured in the wound tissues were significantly lower than in the infected wounds of group IV (P<0.05) (**Table 1**).

Reduction in Wound Area

Wound contraction percentage in different groups during the course of study is shown in **Table 2**. The healing rate of wounds in group V was significantly different compared to the control group (P< 0.05).

Hydroxyproline Content of Wound

Proline is hydroxylated to form hydroxyproline after protein synthesis. Hydroxyproline contents in the groups I to V were found to be 46.13±3.57, 58.63±2.89, 68.71±1.57, 75.33±3.56 and 85.17±3.11 mg g^{-1}, respectively. Hydroxyproline contents were increased significantly in the group V which implies more collagen deposition compared to other experimental groups (P <0.05).

Table 3. Intensity of histological parameters assessed in experimental animals

		Histological parameters				
Groups	Days	Acute Hemorrhage	Congestion	Vascularization	Epithelialization	Collagen
I	7	++	+++	+	-	+
	14	++	+	++	+	++
	21	-	-	++	++	++
II	7	+++	+++	-	-	-
	14	+++	+++	+	+	+
	21	++	++	+	+	+
III	7	++	++	++	+	++
	14	+	-	++	++	++
	21	-	-	+++	++	+++
IV	7	++	++	++	-	+
	14	+	-	++	+	++
	21	-	-	+++	++	++
V	7	+*	+*	++*	++*	++*
	14	-	-	+++*	++*	++*
	21	-	-	+++*	+++*	+++*

Classification of histological parameters according to the intensity of occurrence: - absence; + discrete ;++ moderate; +++ intense; ++++ very intense. Histopatological damages were assessed as explained under material and methods on days, 7, 14 and 21 of lesion. *p<0.05 vs other experimental groups

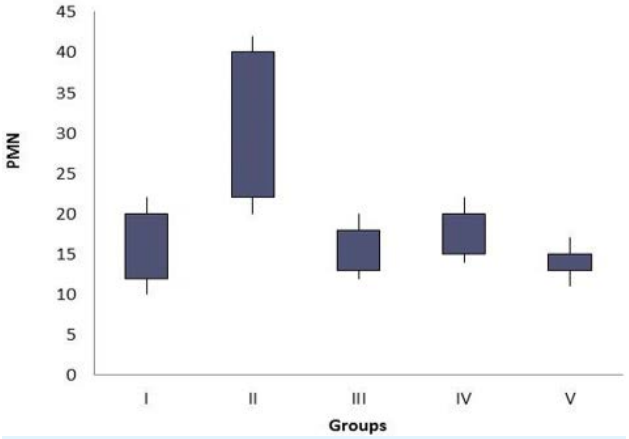

Fig. 1. Box-and-whisker plots of number of polymorphnuclear cells (PMN) in excisional model of the rat's skin in experimental groups. Results were expressed as mean ± SEM

Histological and Morphometric Findings

There were significant differences in comparisons of group V and other groups, particularly in terms of cellular infiltration, acute hemorrhage, congestion, edema, collagen production and density, reepithelialisation and neovascularization. During the study period, scores for reepithelialisation and neovascularisation were significantly higher in group V rats than other groups (P <0.05). Polymorphonuclear (PMN) and mononuclear (MNC) cell count, fibroblast cell proliferation and also Mean Rank of the qualitative study of acute hemorrhage, edema and collagen production score in group V were significantly higher than those of other experimental groups (P <0.05) (**Table 3**) (**Fig. 1**-**4**).

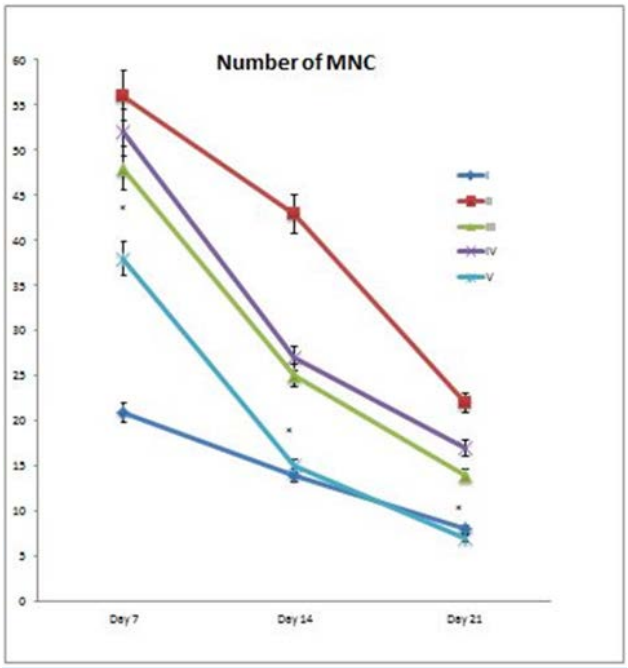

Fig. 2. Line graph indicating number of mononuclear cells (MNC) in excisional model of the rat's skin in experimental groups. Results were expressed as mean ± SEM. * P < 0.05 vs other experimental groups

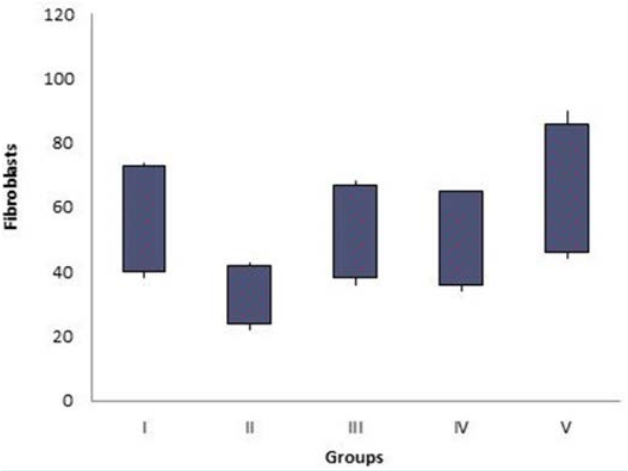

Fig. 3. Box-and-whisker plots of number of fibroblasts in excisional model of the rat's skin in experimental groups. Results were expressed as mean ± SEM

DISCUSSION

Inflammation, proliferation and tissue remodeling are three phases of healing process which occur following tissue damages as closely as possible to its natural state. The healing process is activated when platelets come into contact with exposed collagen leading to platelet aggregation and the release of clotting factors resulting in the deposition of a fibrin clot at the site of injury. The fibrin clot serves as a provisional matrix and sets the stage for the subsequent events of healing. Inflammatory cells also arrive along with the platelets at the injury site providing key signals known as growth factors. The fibroblast is the connective tissue cell responsible for collagen deposition required to repair the tissue injury. The collagen is the main constituent of extra cellular tissue, which is responsible for support and strength (Martin et al. 2010).

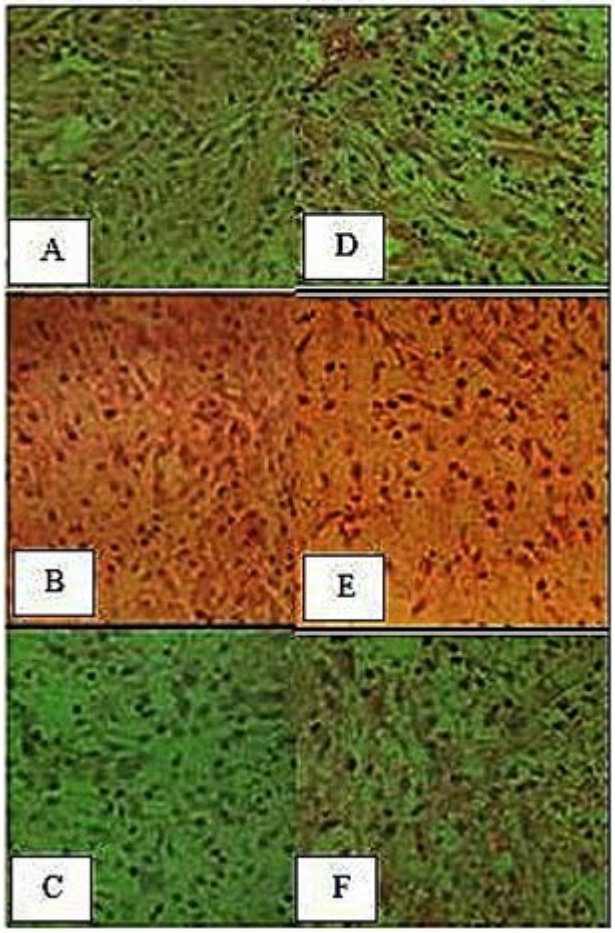

Fig. 4. Histological characteristics of rat skin on the 7th (A-C) and 14th day (D-F) after wound creation in excisional wound model. A and D: III, B and E: IV, C and F: V. Wounds with surrounding skin were prepared for histological microscopic evaluation by Masson trichrome staining. (×400)

Nanoparticles have become significant in the regenerative medicine field in the last two decades (McLaughlin et al. 2016). Many biological processes happen at through mechanisms that fundamentally act at the nanometer scale. Thus, materials such as NPs can be used as unique tools for drug delivery, imaging, sensing, and probing biological processes (Wang and Wang 2016). In the context of wound healing, the special properties of NPs like electric conductivity, antimicrobial activity, and high surface to volume ratio, swelling, and contraction make NPs versatile resources.

Several reports have demonstrated that there is a beneficial effect of chitosan as a biologically active dressing in wound management. It has been reported

that the application of chitosan to the open wounds in dogs induced exudate, which has a high growth factor activity, and induced infiltration by inflammatory cells and granulation tissue formation accompanied by angiogenesis (Mizuno et al. 2003, Okamoto et al. 1995). Chitosan-membrane-based wound products have been investigated both in laboratory animals and humans, however, are still at the early stages of development. Since 1980, chitosan and its derivatives have been used in skin and wound management products in Japan. Beschitin W, an artificial skin prepared from chitin threads, has been developed for human use and is on the market (Azad et al. 2004, Koji 1992). Chitosan microspheres have been demonstrated to bear robust antimicrobial activity against S. aurous (Seetharaman et al. 2011).

We selected chitosan as a dressing material due to its biocompatibility, biodegradability, haemostatic activity, anti-inflectional activity and property to accelerate wound healing (Archana et al. 2013). The N-acetyl glucosamine (NAG) present in chitin and chitosan is a major component of dermal tissue which is essential for repair of scar tissue. Its positive surface charge enables it to effectively support cell growth and promotes surface induced thrombosis and blood coagulation. Free amino groups which are present on the chitosan membrane surface may form polyelectrolyte complexes with acidic groups of the cellular elements of blood (Seetharaman et al. 2011). It has several advantages over other type of disinfectants because it possesses a higher antimicrobial activity, a broader spectrum of activity, a higher killing rate and a lower toxicity toward mammalian cells. However, synthetic polymers are available at a lower price than biopolymer chitosan, substitution of chitosan by these synthetic polymers could reduce the price of chitosan-based films with safe effect on their functionality (Archana et al. 2013).

Antimicrobial properties of Artemisia dracunculus are historically mentioned. Some studies have highlighted the role of Artemisia dracunculus as the solvent employed for the extraction of Artemisia that may influence the potency of its antimicrobial activity (Kordali et al. 2005, Lopez-lutz et al. 2008). Artemisia dracunculus also shows anti-inflammatory and analgesic effects (Roghani et al. 2006). It is approved that Artemisia dracunculus also have antifungal, antioxidant properties (Lopez-lutz et al. 2008, Meepagala et al. 2002).

In excisional wound model there was a significant decrease in wound area in Artemisia dracunculus and/or chitosan treated animals. This indicated improved collagen maturation by increased cross linking. The balance between synthesis and breakdown and so deposition of collagen is important in wound healing and development of wound strength (Toreti et al. 2013). Hydroxyproline is a major component of the collagen that permits the sharp twisting of the collagen helix. It helps on providing stability to the triple-helical structure of collagen by forming hydrogen bonds. Hydroxyproline is found in few proteins other than collagen. For this reason, hydroxyproline content has been used as an indicator to determine collagen content (Dogan et al. 2009). Increase in hydroxyproline content in group V indicated increased collagen content, since hydroxyproline is the direct estimate of collagen synthesis. Mechanical testing is sensitive to changes that occur during the progression of wound healing, and can be used as a tool to measure the quality of healing.

Biomaterials derived from natural products can provide materials with greater complexity and composition. In order to mimic the extracellular matrix (ECM) conditions of the wound and to provide a scaffold for the fibroblasts for collagen deposition, ECM-based therapies have gained popularity (Martin et al. 2010). A phase I clinical trial using fibroin to enhance wound healing is currently underway. Finally, there have been numerous marine polysaccharide hydropastes like marine collagen from *Stomolophus nomurai meleagris*, *Oncorhynchus keta*, *Lates calcarifer*, *Stichopus japonicas*, and *Salmo salar*, alginate from *Macrocystis pyrifera*, chitosan from crabs and shrimps, which are bioactive and increase wound healing rates in mice (Chandika et al. 2015, Das and Baker 2016).

In the present study, histopathological examination and scoring revealed that there was a significant difference by means of wound healing scores in group V compared to other experimental groups. Artemisia dracunculus with chitosan nanoparticle biofilm decreased the maturation time of granulation tissue and wound contraction which means that it enhanced reepithelialisation with significant effect on inflammatory infiltration and number of fibroblasts in time-dependent activity.

Artemisia dracunculus with chitosan nanoparticle biofilm resulted in significant improvement of full thickness wound healing. Thus, from this study we concluded that the Artemisia dracunculus with chitosan nanoparticle biofilm have a reproducible wound healing potential and hereby justifies its use in practice.

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