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Bacteriological Evaluation of *Aloe vera L.* Fresh Gel on Experimental Infected Full-Thickness Open Wounds Induced with *Staphylococcus aureus* in Dogs

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Abstract

Objective - to evaluate both in-vivo and in-vitro antibacterial property of *Aloe vera.L* fresh gel**.**
Design - experimental study.
Animals - five adult male mixed breed dogs aged 2-4 years.
Procedures - for in-vivo experiment, under general anesthesia and sterile condition 8 symmetrical full-thickness wounds measured 2 cm×2 cm were surgically created on the back of each dogs on 0, 7, 13 and 21 days of study. After wound creation, 1 ml fluid contains 105 CFU *Staphylococcus aureus* was inoculated on each wound. Right wounds in all dogs were covered with 1 ml *Aloe vera* fresh gel whereas the left wounds were not received any treatment. Antibacterial activity of *Aloe vera* gel was evaluated on days 7, 15, 21 and 28 by wound biopsies. Antimicrobial susceptibility test was applied for in-vitro evaluation. For this purpose, measurement of inhibition zone of *Aloe vera* gel was compared to some commercially available antibiotic discs.
Results - no significant differences were seen by counting viable bacteria between the treatment and control wounds at the particular days of the period study ($P > 0.05$). The gel inhibited the growth of *S.aureus* by appearance of 12 mm inhibitory zone.

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Conclusions and Clinical Relevance - *Aloe vera* has antibacterial property against *S.areus*.
Key Words: *Aloe vera*, Infected full-thickness wounds, *Staphylococcus aureus*

Introduction

In spite of advances in controlling the infection of surgical wounds, bacterial wound contamination is still remains the most common post-operative complication. Presence of infection in wound, in addition to interference with healing process can be resulted in increasing the duration of wound repair, therapeutic period, costs and even morbidity and mortality rate.[1]Various bacterial species isolated from wounds, but *Staphylococcus aureus* is the most frequent organism responsible for wound infection.[1,2] Due to resistance against common antibiotics and its high prevalence, it seems that this bacterium is the best indicator for evaluation of the prevention and treatment infection in wounds.[1,3] Although mechanisms of *S.aureus* on delayed wound healing is not well identified but the extracellur adherent protein (EAP) is often responsible for inhibiting wound healing. Inflammation and neovascularization are two phases of healing process that would be affected.[4]
Nowadays, excessive and inappropriate use of antimicrobial drugs have developed the resistant bacteria and difficulty in management of infected wounds, so consideration to new antibacterial agents and least adverse effects seems necessary.[5-7] Numerous plants are known for use related pharmaceutical activities. *Aloe vera* (*Aloe vera L., Aloe barbadensis*) is a well-known medical plant with historical records that in recent decades is used for its unique properties such as anti-inflammatory, Anti-oxidant, wound healing promoting, immunomodulatory and anti-microbial activities.[6-9] Antibacterial properties of *Aloe vera* was evaluated in various in-vitro experiments against many species bacteria involved like *S.aureus, Pseudomonas aeroginosa, Escherichia coli* and *Helicobacter pylori* but only a few in-vivo studies exist to investigate its antibacterial properties.[9] The present study was designed to assess the in-vitro and in-vivo antibacterial potential of *Aloe vera* fresh gel against *S.aureus*.

Materials and Methods

Experimental animals

Five healthy male mixed breed dogs aged 2-4 years-old weighing 30±5 kg (Mean±SD) were used. The animals were kept in individual cages having free access to water and fed by standard feed. All the procedures in this study were approved by the animal ethics committee for studies on laboratory animals of the department of clinical sciences review Board at Faculty of Veterinary Medicine of Ferdowsi University of Mashhad in accordance to the Iranian community guidelines for laboratory animals and the principles of laboratory animal care (NIH publication NO. 86-23, revised 1985).

Wounds Creation

The animals were anesthetized with intramuscular injection of xylazine hydrochloride 2% (Alfazyne®, Alfasan, Woerden Holland) at the dose of 1 mg/kg body weight and ketamine hydrochloride 10% (Alfamine®, Alfasan, Woerden Holland) at the dose of 15 mg/kg. First pair of full-thickness wounds measured 20 mm×20 mm were surgically created on the

shoulder region of each dog with approximate 50 mm distance from mid line. In order to provide different-age wounds, further 3 pairs of wounds with the same size were caudally created on days 7, 13 and 21 of the study at a distance of 100 mm to each other. So that at the end of the study each animal has 8 symmetrical wounds aged 7, 15, 21 and 28 days-old. Immediately after wound creation, 1 ml fluid containing 10^5 CFU (ATCC 25923) *Staphylococcus aureus* was inoculated on each wound. Next, the wounds were covered with sterile vaseline gauzes (Paraffin gauze, Latif bandage & gauze Co, Iran) (day 0). After 72 hours, (day 3) to confirm the presence of bacteria and infection in the wounds, sterile swab sampling were performed from exudates of wounds for gram staining and inoculation on culture media.

Preparation of Aloe vera gel

Before performing study, the species of selected *Aloe vera* (*Aloe vera L.*) plant was approved by Herbaceous Sciences Research Center of Ferdowsi University of Mashhad. Anti-bacterial activity of the *Aloe vera L.* leaf gel was examined and confirmed against *S. aureus* in-vitro by Reference Bacteriology Labratory of Veterinary Faculty of Ferdowsi University of Mashhad. A fresh mature *Aloe vera* leaf was cut from the plant on each of the treatment days. Leaf was cleaned and disinfected by chlorine solution. The cuticle layer of the leaf was removed to expose the fillet layer (gel). This layer was homogenized in a sterile blender and then pasteurized under indirect heat at 65°C for 15 minutes.[10] The preparation method was used throughout the study period.

Treatment protocol

The wounds in all animals were divided into 2 groups: right wounds as a treatment group and left wounds as a control group. The wounds in the treatment group topically were received 1 ml of the prepared *Aloe vera* gel once daily according to treatment protocol and were covered with sterile vaseline gauzes whereas the control group did not receive any treatment except for changing of sterile gauze wound dressing on the same days. The first treatment was done on day 3 after bacteriological sampling. Treatments were continued at the days of the study period according to below protocol:
28 days-old wounds: days 3, 4, 5, 6, 7, 8, 9, 11, 13, 15, 18, 21 and 24 of study period.
21 days-old wounds: days 10, 11, 12, 13, 14, 15, 16, 18, 20, 23 and 25 of study period.
15 days-old wounds: days 16, 17, 18, 19, 20, 21, 22, 24 and 26 of study period.
7 days-old wounds: days of study 24, 25, 26 and 27 of study period.
To avoid self trauma, animal bodies were covered with special designed clothes.

Bacteriological evaluations
In-vivo

Under general anesthesia, the wound samples were collected by punch biopsy technique at the end of study period (day 28) from both treatment and control groups. These samples were placed in Eppendorf tube containing 1 ml sterile distilled water and transferred to the laboratory. Immediately after transportation the tissue sample collection were vortex very well, then the final suspension were used for counting viable bacteria. For this purpose, the spread method was used. 6 various dilutions were prepared from the tissue suspension and 0.1 mm of each dilution were streaked onto the surface of the Kant agar medium plates. These

cultured plates were incubated at 37˚C. Determination of viable bacteria was performed by counting of black *Staphylococcus aureus* colonies after 24 hours of incubation period in plates with colonies among 30 to 300. To calculate the number of viable bacteria in tissue samples with 1 ml volume, the following formula was used.
The number of counted colonies × inoculation rate × 1/dilution = CFU/ml
Since the inoculum rate was 0.1 ml in all samples, for calculate of CFU in 1 ml in this formula, 10 was considered as an inoculation rate.

Statistical analysis

The mean of obtained numbers were used for statistical analysis. Repeated Measure ANOVA test was used to comparing the differences between treatment and control during the study period. A P value less than 0.05 was considered significant. All data were analyzed with SPSS 16.0 (SPSS Inc, Chicago, IL, USA).

In-vitro

For in vitro evaluation of antibacterial activities of *Aloe vera L.*fresh gel, 3 various techniques were considered: disc diffusion, well diffusion agar and spots culture methods. The gel was prepared according to the specified protocol for treatment of in-vivo experiment. In disc diffusion method, the matched bacterial suspension with 0.5 standard Mac Farland index was inoculated onto the entire of Muller-Hinton agar plates. The sterile blank disc were smeared by *Aloe vera* gel and the commercially antibiotic discs involved trimethoprim-sulfamethoxazole, gentamicin, lincomycin, oxytetracycline, ampicillin, novabiocin, penicillin, streptomycin and cephalothin as positive controls and the blank containing water without pyrogenic agents as a negative agent were placed on the surface of these plates. Then plates were incubated at 37˚C for 24 hours. After this time, the presence or absence of inhibition zone for *Aloe vera* fresh gel was measured and compared with other antibiotic discs. In well diffusion agar method, suspension of isolated *S. aureus*from animals, equivalent to 0.5 McFarland standard were inoculated on Mueller-Hinton agar, then the *Aloe vera* fresh gel was filled into its wells. The plate was incubated for 24 hours at 37˚C. In the spots method, a piece of *Aloe vera* intact gel was placed on the surface of plate containing Mueller-Hinton that *S. aureus* was cultured by spots plate technique then this plate like another plates was incubated on same condition.

Results

In-vitro investigation was shown that *Aloe vera* with formation of inhibition zone (12 mm) has anti-bacterial activity in agar gel diffusion (Figure 1).The inhibition zones were 16 mm, 20 mm and 30 mm for penicillin, ampicillin and trimethoprim-sulfamethoxazole respectively (Table 1). In well diffusion agar and spots culture techniques, *Aloe vera* formed inhibition zone around *S.aureus* and inhibited its growth but in the in-vivo evaluation, there was no significant difference in the wound bacterial count between the treatment and control wounds during the period study ($P > 0.05$) (Figure 2).

Table 1. Inhibition zones diameter of *Aloe vera* and some other antibiotics by disc diffusion technique.

Antibacterial agents	*Aloe vera* gel	Penicillin	Ampicillin	Trimethoprim-Sulfamethoxazole
Zone of inhibition(mm)	12	16	20	30

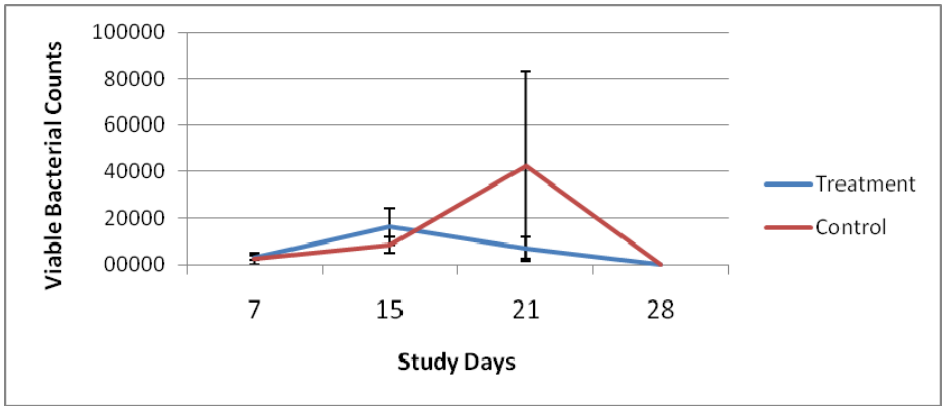

Figure 1. Zone of inhibition of *S.areus* with *Aloe vera* gel after 24 h 0f incubation at 37˚C in disc and well diffusion agar techniques (Right and left arrows respectively).

Figure 2. Bacterial count viable of treatment and control groups. There was no significant difference between treatment and control groups ($P > 0.05$).

Discussions and conclusions

Increase of bacterial number more than 10^6 organisms per gram of tissue or per milliliter of exudate in wound site defines as infection. Replication of bacteria due to inducing a host defense mechanism can result in tissue damage.[11] Although use of systemic chemical antibiotics for prevention of infection is common but appearance of drug resistance beside their side effects, are serious problems that cause inclination to application of natural topical

antibacterial agents.[12] In the present study, the antibacterial potential of *Aloe vera* fresh gel was evaluated both in-vivo and in-vitro condition. In-vitro experiment was stabilized that *Aloe vera* fresh gel has anti-*S.aureus* activity with appearance of inhibitory zone in all 3 tests. The zone of inhibition was 12 mm in agar gel diffusion technique. Lawrence *et al* (2004) by using of well diffusion agar technique showed that various components of *Aloe vera* inhibit growth of *S.areus*.[13] Anti-*S.areus* activity was confirmed by Bashir *et al* (2011) in isolated bacteria from skin infections in well diffusion agar test.[14] This inhibitory effect of aloe vera against *S.areus* was also emphasized by Agarry *et al* (2005), Ilaiyaraja *et al* (2010) and Mehrotra *et al* (2013).[12,14-16] But although these studies confirmed antibacterial effects of *Aloe vera* gel against a broad range of bacteria including *S.areus*, Cock *et al* (2000) showed this bacterium is resistance to *Aloe vera*.[7]

Parallel to in-vitro study, *Aloe vera* fresh gel was applied clinically for investigation its antibacterial property in open wounds induced with *S.aureus*. The data obtained during the period of investigation reveal no significant differences in bacterial load attenuation between treatment and control groups during the study. These findings are comparable with Kumari *et al* (2010). They revealed no significant decline in bacteria number after seven days by application autoclaved *Aloe vera* gel for treatment of *K. pneumonia* induced burn wounds.[17] In contrast these reports, numerous studies emphasize significant decrease in bacterial count. Yun *et al* (2009) reported that CFU of bacteria significantly decreased after use of *Aloe vera* in induced experimentally sepsis and expressed that *Aloe vera* exerts its effect via reducing over production of pro-inflammatory cytokines.[18] Cuttle *et al* (2008) demonstrated that the colonization chance of some microflora such as *Staphylococcus* species decrease in experimental full-thickness burns in Aloe vera treatment group due to its antibacterial property.[19] Parallel to this bacteriological evaluation, we histopathologically investigated the effect of *Aloe vera* on healing process in these experimental infectious wounds and we reported that *Aloe vera* enhances skin wound healing process,[9] although in bacteriological study there was no significant diminution in bacterial colonies formation. *S.areus* is opportunistic bacterium with a range of manifestations from mild to severe infections. Several various virulence factors have been identified for its pathogenicity. Among of them, the EAP plays an important role. The pathogen exerts its damaging effects through the binding of EAP to plasma and extracellular components including prothromobin, fibrinogen, fibronectin, collagen, elastin, fibroblasts, epithelial and endothelial cells.[4,9,20-22] EAP as an anti-inflammatory agent can exert its main negative effect via inhibition the infiltration of leukocytes, significantly neutrophils and macrophages, from bloodstream and their recruitment into the infection site at the first time. So it is able to delay wound healing process by anti-inflammatory mechanism.[4,20-22] On the other hand some studies reveal that *Aloe vera* by reducing of leukocytes adhesion has an anti-inflammatory role.[23,24] These contradictory results obtained from various studies and even variation in composition of *Aloe vera*, makes it complicate to identify its antibacterial properties. There was no evidence on antibacterial activity of *Aloe vera* in this study, probably due to synergistic effects between anti-inflammatory property of EAP as a virulence factor of *S.areus* and anti-inflammatory mechanism of *Aloe vera* in reduction of leukocytes-endothelium cells interaction. However, histopathologically shown improvement of wound healing process in *Aloe vera* treatment group compared with control group and formation of inhibitory zone in-vitro against *S.areus* in recent study revealed that *Aloe vera* via effect on proliferative phase of healing and antibacterial property can improve infected wound healing processes. Future investigation of antimicrobial activity of *Aloe vera* and its effect on infected wound healing is recommended.

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چکیده

بررسی باکتریولوژیک اثر درمانی ژل تازه *صبر زرد* (آلوئه‌ورا ال) بر زخم‌های پوستی تمام ضخامت باز عفونی شده با استافیلوکوکوس اورئوس در سگ

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هدف- این مطالعه به منظور ارزیابی خواص ضد باکتریایی ژل تازه گیاه *صبر زرد* در زخم‌های عفونی در موجود زنده و در شرایط آزمایشگاهی انجام شد.

نوع مطالعه- مطالعه تجربی بر روی حیوان زنده

حیوانات- پنج قلاده سگ نر نژاد مخلوط با محدوده سنی ۲ تا ۴ سال

روش کار- برای این منظور ۴ جفت زخم تمام ضخامت پوستی متقارن در شرایط جراحی در پشت حیوانات در روزهای مختلف مطالعه ایجاد شد. سپس این زخم‌ها به وسیله تلقیح ۱ میلی‌لیتر مایع حاوی 10^5 CFU/*استافیلوکوکوس اورئوس* آلوده شدند. زخم‌های گروه درمان در طی دوره مطالعه با ژل تازه *صبر زرد* درمان شدند در حالی‌که زخم‌های گروه کنترل هیچ درمانی دریافت نکردند. پس از اتمام دوره مطالعه، فعالیت ضد باکتریایی ژل تازه *صبرزرد* با استفاده از شمارش پرگنه‌های سیاه رنگ *استافیلوکوکوس اورئوس* حاصل از تلقیح سوسپانسیون نمونه‌های اخذ شده بافتی روی محیط کشت در روزهای ۷، ۱۵، ۲۱ و ۲۸ در دو گروه درمان و کنترل بررسی شد. علاوه بر این، فعالیت ضد باکتریایی ژل تازه این گیاه نیز در شرایط آزمایشگاهی با برخی از آنتی‌بیوتیک‌ها و با بررسی تشکیل هاله ممانعت از رشد انجام گرفت.

نتایج- در شمارش باکتری‌های زنده تفاوت معنی‌داری بین گروه‌های درمان وکنترل وجود نداشت. ضمن این‌که ژل تازه صبرزرد با تشکیل هاله ۱۲ میلی‌متری مانع رشد باکتری *استافیلوکوکوس اورئوس* شده بود.

نتیجه‌گیری و کاربرد بالینی- ژل *صبر زرد* قادر به مهار رشد باکتری *استافیلوکوکوس اورئوس* می‌باشد.

کلید واژگان- صبرزرد، زخم‌های باز تمام ضخامت عفونی، *استافیلوکوکوس اورئوس*.