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EVALUATION OF ANTI-MICROBIAL ACTIVITY AND WOUND HEALING OF KEFIR

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ABSTRACT: Objective: *Pseudomonas aeruginosa* has emerged as an important cause of serious wound infection and death in burn patients. There are a limited numbers of antimicrobial agents affecting the bacterium due to its inherent resistance. Kefir is a natural probiotic which contains diverse spectrum of bacteria, yeasts and their metabolites with potential antimicrobial activity. Current study was designed to investigate the antimicrobial activity of Kefir extract against *P. aeruginosa* (ATCC 27853) as well as burn clinical isolates. **Materials and Methods:** In this descriptive study, the Kefir fermented in MRS Broth medium for 24 hours (hrs), 48 hrs and 96 hrs and their extract were used for evaluation of antibacterial effect. The antibacterial effect of different kefir extract were compared based on measurement of diameter of inhibition zone in diffusion plate method, and assessment of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The density of bacteria and percentage of organic acids (lactic and acetic acids) were also tested. Kefir gels were prepared by mixing of equal portions of gel base and 96 hours supernatant of kefir grain cultivated in MRS Broth medium. Antibacterial effects of kefir grain supernantants taken at 24, 48, 72 and 96 hours times intervals were evaluated. Burn injuries were created on dorsal skin surface of 32 rats. After 24 h wounds were infected with *Pseudomonas aeruginosa.* The infected rats were divided in to 4 groups of 8 rats each. Burn wounds area were measured before treatment as well as one and two weeks after. Animals in all groups were sacrificed and whole skin wound areas were removed and the percentages of epithelization, scar formation, inflammation and angiogenesis were assessed. **Results:** After two weeks treatment the 96 h kefir gel showed lower percentage of inflammation and also better epithelization compared to silver sulfadiazine 1%, base gel and untreated groups. **Conclusion:** Kefir extract showed a promising anti-pseudomonal effect and was able to significantly improve wound healing. However further experiments and clinical trials needs to be conducted before it could be used in human.

INTRODUCTION: *Pseudomonas aeruginosa* (*P. aeruginosa)* is one of the leading gram-negative organisms involved in nosocomial infections [1] and has emerged as the most important life-threatening nosocomial pathogen in patients with burn-wound infections and remains a serious cause of death in burn patients [2, 3].

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This organism is intrinsically resistant to a large number of antimicrobial agents and has a remarkable capacity for acquiring new resistance mechanisms which results in increasing the therapeutic problems [4, 5]. So, searching new compounds for ideal therapy of burns infected with this pathogen is going on [6].

Kefir is a probiotic mixture of diverse spectrum of yeasts, lactic acid and acetic acid bacteria [7] which has been reported to have antibacterial and immunostimulating activities [8, 9]. Moreover recent studies have identified that Kefir displays notable anti-inflammatory properties and speeds wound

healing process following accumulation of lymphocytes, macrophages and poly morphonuclear cells in site of injury [11, 12].

In this regard, we have recently reported the wound healing activity and antimicrobial effects of Kefir in experimental burn wounds infected with *P. aeruginosa* [13]. In another study, Kefir and Kefiran (insoluble polysaccharide of Kefir) have shown the antimicrobial and cicatrizing activities on rats [14]. However, the present study was designed in order to evaluate the antimicrobial effects of different Kefir extracts against *P. aeruginosa* samples taken from patients with burns.

MATERIALS AND METHODS:

This investigation was a descriptive cross-sectional study.

Preparation of Kefir extracts

Kefir grains (50 g) were continuously cultured in 100 g/l of MRS (Man, Rogosa, Sharpe) Broth medium at 35 °C for 24, 48 and 96 hours in a CO2 incubator. The supernatants of culture fermentation were centrifuged at 6,000 rpm for 20 minutes at about 15 °C. Then they were filtered through a 0.22-micron Millipore filter and named as Kefir 24 h, Kefir 48 h and Kefir 96 hours. In order to ensure the sterility of Kefir extracts, it was necessary to culture them on MHA (Mueller-Hinton Agar) at 37 °C for 24 hours [7, 15]. Moreover, the pH of supernatants was measured by digital pH meter.

Measurement of organic acids

The percentages of citric and acetic acids in different Kefir extracts were measured by the reverse phase High Performance Liquid Chromatography (HPLC) assay using C18 column, UV detection wavelength: 254 nm, mobile phase of deionized water, flow: 1; then Compared to the 1%, 5% and 10% lactic acid and acetic acid.

Identification and differential diagnosis of pathogenic bacteria The *P. aeruginosa* bacteria (Standard 27853 ATCC) and sample from patients with burns was cultured on MHA medium and incubated at 37 °C for 24 h. Then differential diagnosis of *P. aeruginosa* bacteria was made using Simon's citrate agar, oxidase and carbohydrate fermentation tests. After isolating the *P. aeruginosa*, a pure culture of these bacteria was made on MHA medium based on McFarland 0.5 solution, then antibiotic disks Selected and transferred to the culture medium and incubated at 37 °C for 24 hours.

Determination of antimicrobial activity of Kefir

Antibiotic activity of Kefir extracts were evaluated using the disk plate and well test methods. These methods measure the potency of antimicrobial substances by diffusion in to the agar medium, The inhibition zone appears around the antimicrobial compound.

In disk plate method, 100 ml of Kefir extracts obtained from fermentation after 24 hrs, 48 hrs and 72 hrs And 96 hrs inoculated on the disks with diameter of 6mm (Blank paper) and then prepared disks transferred to the MHA medium that already inoculated with 100 microliters of *P. aeruginosa* suspensions obtained from patients and standard (ATCC27853) and were diluted in 200 µL of saline to create 0.5 McFarland unit suspensions and one-tenth of a milliliter of each suspension was cultured on MHA and incubated at 37 ◦C for 24 h. In Well test method, small wells made with sterile Pasteur pipette with diameter of 6 mm on MHA medium wich previously cultured with *P. aeruginosa* and various Kefir extracts spilled in separate wells and incubated at 37 ° C for 24 h.

Turbidity assay:

Turbidity were determined by using 0.1mL of suspensions of P. aeruginosa bacteria (Standard 27853 ATCC) and sample from patients with burns (3×108 CFU/mL) in tubes containing 10mL of BHI solution and the same amounts of Kefir. Tubes were mixed using a vortex for 60 s and incubated at 37 ◦C for 24 hours, then all prepared broth media was read at 530 nm wavelength, at this wavelength reduction of light absorption (optical density under 0.65) indicates the antimicrobial activity of Kefir extracts on *P.aeruginosa*.

Susceptibility studies

The antimicrobial susceptibility was evaluated by the broth micro dilution method as previously described by the NCCLS [14, 16]. The MIC was defined as the lowest antimicrobial concentration able to completely inhibit bacterial growth up to 24

h. MIC parameters were determined in triplicates using 0.1mL of suspensions of P. aeruginosa bacteria (Standard 27853 ATCC) and sample from patients with burns (3×108 CFU/mL) in tubes containing 10mLof BHI solution and the same amount of Kefir. Tubes were mixed using a Vortex for 60 s and incubated at 37 ◦C for 24 h. MBC values were obtained based on the results for MIC values. Plates containing 25mL of BHI agar medium were inoculated with 0.1mL of the tubes showing no growth and incubated for 24 and 48 h at 37 ◦C.

Preparation of kefir extracts and kefir gels:
Kefir grains (50 g) were continuously cultured in 100 g/l of MRS Broth medium for 96 h. The supernatants of culture fermentation were centrifuged, filtered and named as kefir 96 h. kefir gel product was prepared from above extract named as kefir kefir 96 h gel [18, 19]. The kefir gels were formulated by addition of 100 ml of extract 100 g to 100 g gel base.

Experimental protocol
Animals
32 male Wistar rats, aged six months old weighting 200 ± 10 g were purchased from Pastor Institute Karaj city, I.R. Iran. The rats were caged under controlled conditions of light, room temperature and humidity for a week prior to study. This study was approved by the ethical committee of Islamic Azad University, Tehran, Iran.

Burn wounds induction
The 3rd degree burn wounds were induced on shaved area of dorsal skin of the rats under anesthesia (intraperitoneal injection of 100/5 mg/kg ketamin/xaylazin) using hot plate sized 3± 1cm cm at temperature of 156 8F or 69 8C for 3 s [21].

The rats were placed in an isolated cage to inhibit transmission of infection. The wounds were examined after 24 h and in case of necrotic tissue, the same was removed. Debridement procedure under the standard way was done for all the animals.

Burn wounds treatment Twenty-four hours after rats were caged individually and divided into 4 groups of 8 rats each as follows:

- Untreated group: the burn wounds received no medication.
- Base gel group: the base gel was applied on burn wounds.
- Silver sulfadiazine group: the silver sulfadiazine 1% was applied on burn wounds.
- Kefir 96 h gel group: the kefir 96 h gel was applied on burn wounds.

The gels and silver sulfadiazine thin layer were applied on burn wounds twice a day.

Burn wounds histological assessment
After 2 weeks the animals were sacrificed by spinal cord injury under anesthesia and 3.5 cm ±1.2 cm wound skin tissue in its full thickness were removed and paraffin embedded sections were prepared. The sections were cut with a microtome 2 mm thick, cutting perpendiculars to the thickness of skin surface.The sections were stained with Haematoxylin & eosin. The percentage of epithelization, scar formation, inflammation and angiogenesis were evaluated in all specimens [24].

RESULT:
Identification and differential diagnosis of *P. aeruginosa*
The results obtained from the samples taken of patients with burns incubation on MHA for 24 hours indicated the round and smooth colonies with a green fluorescent and the production of gas bubbles in oxidase test represent the presence of *P.aeruginosa* and also the gram stain of these bacteria showed gram-negative, rod-shaped bacteria. Moreover, in the samples taken from patients with burns plated on Simmons' citrate agar, After 24 h at 37°C, citrate-positive colonies were identified by the color change of the agar from green to blue.

P. aeruginosa strains isolated from burn patients were respectively resistant to Erythromycin, Kanamycin, penicillin, gentamicin, ceftizoxim and moderately susceptible to ceftriaxone, ceftazidime and amikacin.

Percentage of organic acids and pH
The results showed that by increasing the time of fermentation, concentration of lactic and acetic acids in orders of Kefir 96 hrs > Kefir 48 hrs >

Kefir 24 hrs increased, but acetic acid was not produced in Kefir extract 24h **(Table 1) (Fig 1)**.

TABLE 1: PH KEFIR EXTRACT FERMENTATION AT DIFFERENT TIMES

pH	Fermented Kefir extracts from different time
4.54 ± 0.15 c	Kefir Extract from 24 h fermented
4.84 ± 0.22 b	Kefir Extract from 48 h fermented
5.18 ± 0.10 a	Kefir Extract from 72 h fermented
4.25 ±0.6 d	Kefir Extract from 96 h fermented

Columns with non-common letters are significantly different (p <0.05).

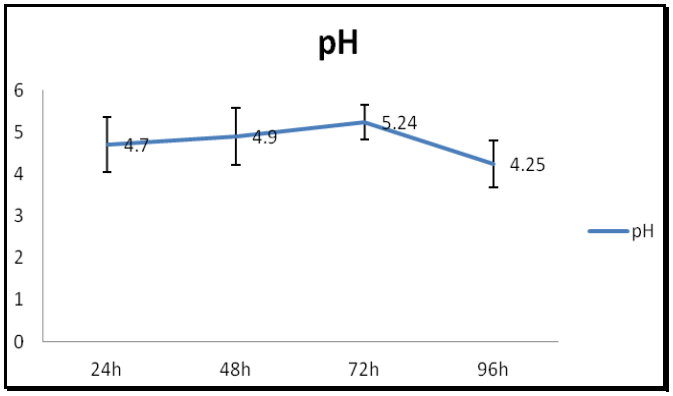


FIGURE 1: pH KEFIR EXTRACTS AT DIFFERENT TIMES OF FERMENTATION

Further, Acid-producing activity of this product as revealed by pH attained after 24, 48 and 96 h in different Kefir extracts. It was indicated that the lowest pH was related to the 96 h fermented Kefir extract and this extract produced the highest amount of lactic and acetic acid indicated by HPLC **(Fig 2-3)(Graph 1-2)**.

Disk plate and well test

The results indicated that by increasing the time of fermentation the diameter of inhibition zone increased and the maximum diameter of inhibition zone in disk plate and well test were seen in 96 h (p <0.05 and p<0.001 respectively) **(Table 2-3).**

TABLE 2: THE MEAN INHIBITION ZONE DIAMETER IN MILLIMETERS OF KEFIR EXTRACTS THE DISK METHOD

Inhibition zone diameter in millimeters		
P. aeruginosa in standared sample (ATCC 27853)	Samples taken from patients with burns	Fermented Kefir extracts from different time
11.32 ± 0.47 b	11.5 ± 0.4 b	Kefir Extract from 24 h fermented
12.17 ± 0.23 b	13.65 ± 1.88 b	Kefir Extract from 48 h fermented
16.17 ± 2.77 a	18.32 ± 2.62 a	Kefir Extract from 96 h fermented

Columns with non-common letters are significantly different (p <0.05).

TABLE 3: THE MEAN INHIBITION ZONE DIAMETER IN MILLIMETERS OF KEFIR EXTRACTS THE WELL TEST

Inhibition zone diameter in millimeters		
P. aeruginosa in standared sample (ATCC 27853)	samples taken from patients with burns	Fermented Kefir extracts from different time
12.5 ± 0.4 b	13 ± 0 b	Kefir Extract from 24 h fermented
14 ± 1.36 C	13.5 ± 1.22 B	Kefir Extract from 48 h fermented
16.75 ± 0.2 a	15.75 ± 0.2 a	Kefir Extract from 96 h fermented

Columns with non-common letters are significantly different (p <0.05).

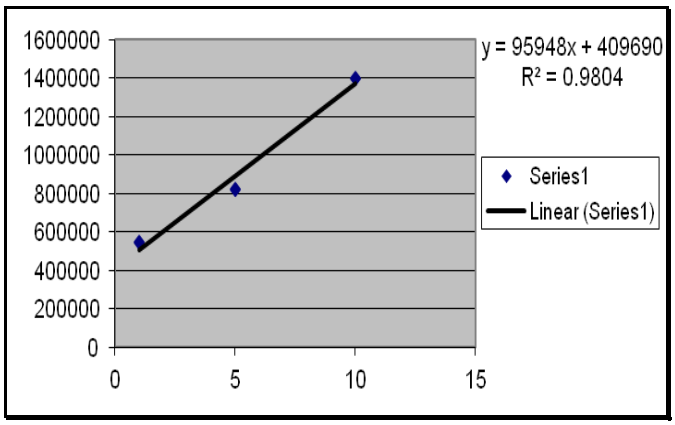


FIGURE 2: DIAGRAM OF THE THREE STANDARD LINEAR ACETIC ACID

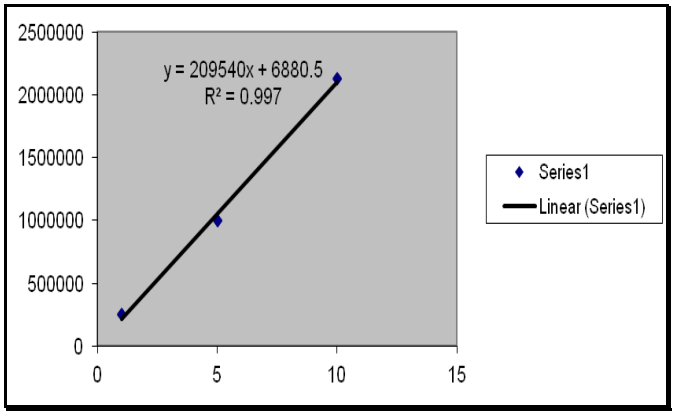
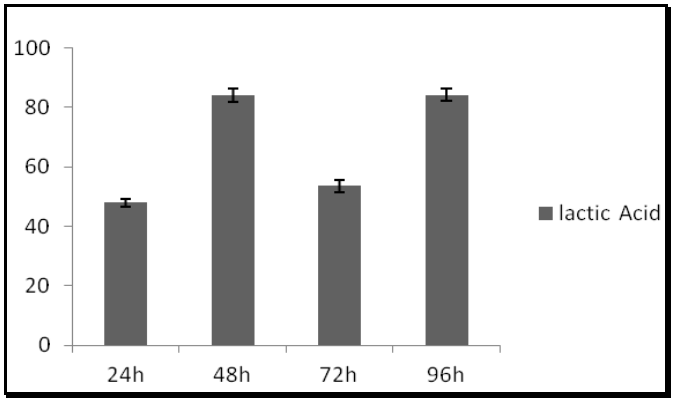
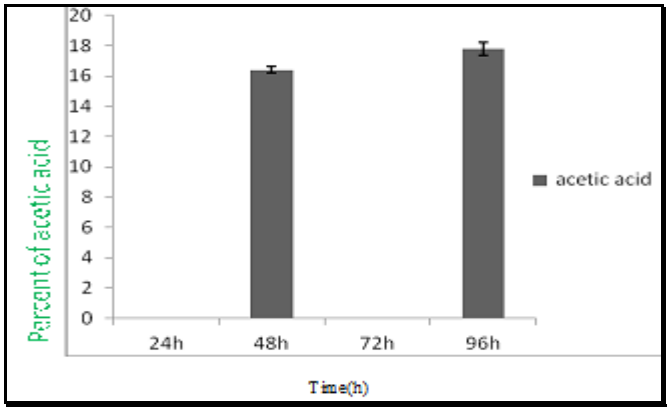


FIGURE 3: DIAGRAM OF THE THREE STANDARD LINEAR LACTIC ACID



GRAPH 1: THE PERCENTAGE LACTIC ACID PRODUCED BY FERMENTING THE KEFIR AT DIFFERENT TIMES FERMENTATION



GRAPH 2: THE PERCENTAGE OF ACETIC ACID PRODUCED BY FERMENTING THE KEFIR AT DIFFERENT TIMES FERMENTATION

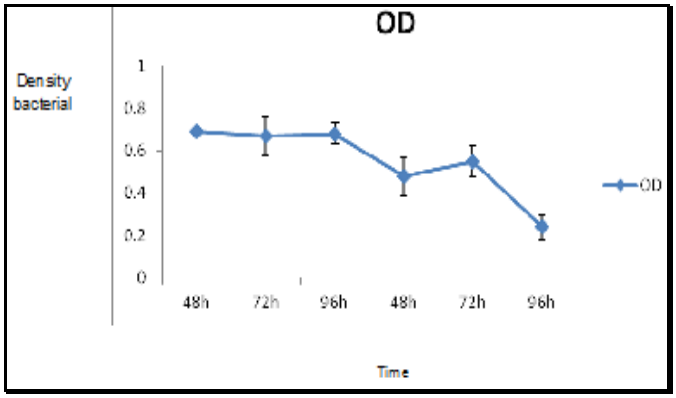


FIGURE 4: BACTERIAL DENSITY OF *PSEUDOMONAS AERUGINOSA*, DIFFERENT TIMES AFTER TREATMENT WITH THE EXTRACT OF FERMENTED KEFIR AT WAVELENGTH OF 530nm USING A TURBIDITY MEASUREMENT

Turbidity assay:

Extracts from the Kefir ferment for 96 hours had the highest antimicrobial effect because it showed the lowest light absorption that indicates the highest antimicrobial effect and 72hrs ferments showed lowest antimicrobial effect.

Respectively: 48hrs>24 hrs>72hrs ferments **(Figure 4)** The results of measurements of MIC and MBC in the several concentrations of kefir extracts showed antibacterial effect 24 h, 48 h and 96 h fermented kefir extracts at only in concentration of 250 mg/ml was antimicrobial effect on *P. aeruginosa*.

Histological examination:

The percentage of inflammation, angiogenesis, epithelization and scar formation at the end of 2nd week in all groups is summarized in Result showed that at the end of the 2nd weeks the percentage of epithelization and scar formation were significantly higher in kefir 96 h gel ($p < 0.001$), where as the percentage of inflammation were significantly lower in kefir 96 h gel ($p < 0.001$) as compared to silver sulfadiazine 1%, base gel and untreated groups. Angiogenesis were not significantly different between the groups. The data are summarized in **(Table 4)**.

TABLE 4: PERCENTAGE OF INFLAMMATION, ANGIOGENESIS, EPITHELIZATION, AND SCAR FORMATION AT 14TH DAYS OF TREATMENT IN 4 GROUPS OF 8 RATS EACH (MEAN±SD).

Treated groups	Scar formation	Epithelization	Angiogeneses	Inflammation
Kefir gel	61.0 ± 5.6	72.5 ± 6.5	97.5 ± 3.5	11.4 ± 2.8
Untreated	0 ± 0	2.5 ± 1.1	97.5 ± 3.5	97.5 ± 3.5
Silversulfadiazine1%	0 ± 0	5.0 ± 1.0	97.5 ± 3.5	95.0 ± 7.0
Base gel	0 ± 0	2.0 ± 0 .1	95.0 ± 7.0	97.5 ± 3.5

DISCUSSIONS: One possible mechanism underlying the antibacterial activity of Kefir is production of acetic and lactic acids. The results in the present study showed that Kefir extract as a probiotic has the ability to inhibit the activity of *P. aeruginosa* in samples taken from patients with burns and standard sample.

It was found that, the diameter of inhibition zones have been increased linearly with increasing the time of fermentation. So, continuously cultured Kefir grains in MRS Broth medium up to 96 h increases these properties of extract.

These results are parallel to our previous findings showing the wound healing activity and antimicrobial effects of Kefir gels in burn wounds infected with *P. aeruginosa* in rats. Those data indicated that there was a great reduction in percentage of wound size in Kefir grains 96 h gel and also wound healing time was significantly shorter in Kefir 96 h gel among different groups of fermentation [13]. Furthermore, in several in vivo and in vitro studies, Kefir possesses antimicrobial activity against a wide variety of Gram-positive, Gram-negative bacteria and some yeasts [14, 17, 18]. Besides its antibiotic and cicatrizing properties, previous researches have also demonstrated that

microorganisms of Kefir have immunomodulating effects [22]. Kefir extracts inhibit microbial growth through various mechanisms, maybe in part by the antagonistic action of various microorganisms present in Kefir, which are also capable to prevent the normal action of pathogenic microorganisms [23, 24]. Recent studies have explained the role of lactobacilli in the prevention and treatment of gastrointestinal disorders [28, 29].

Kefir extracts contained several natural antimicrobials and inhibitory substances such as organic acids (lactic acid and acetic acid), hydrogen peroxide, bacteriocins, reuterin, and reutericyclin that may effect on pathogens [25, 26].

Lactic acid penetrates the microbial cell membrane, causing the acidification of the cytoplasm and the enzyme activity inhibition and at a higher intracellular pH the acids dissociate to produce hydrogen ions, which interfere with important metabolic functions such as oxidative phosphorylation, a possible explanation for the inhibition of aerobic species [24]. Additionally, in another study by Garrote et al [27], it was found that lactic and acetic acid have an excellent synergistic inhibitory effect when produced together in Kefir extract and this effect is associated to the potentiation of acetic acid at the lower pH produced by lactic acid.

Although the mechanism of pathogen inhibition by lactic acid bacteria (LAB) is not completely understood, it is known that these microorganisms inhibit the adherence, multiplication and pathogenic action of invasive microorganisms [29]. The antagonistic mechanisms of LAB may include the activity of organic acids, hydrogen peroxide diacetyl, bacteriocins and other compounds [30]. Yuksekgag et al. demonstrated that microorganisms isolated from the Kefir grains inhibited the growth of S. *aureus* and *Pseudomonas aeruginosa*. These authors suggested that organic acids, hydrogen peroxide and other substances were responsible for the inhibition. [28]

In this study, by increasing the time of fermentation, concentration of lactic and acetic acids increased in orders of Kefir 96 hrs > Kefir 48 hrs > Kefir 24 hrs and this resulted in continuous increasing of the diameter of inhibition zone and decreasing the density of bacteria following inhibition of *P. aeruginosa* growth.

In present study the wound healing activity and antimicrobial effects of kefir gels were tested in experimental. The antimicrobial activity of kefir 96 h gel was similar to silver sulfadiazine 1% ointment but wound healing time were lower in kefir 96 h gel as compared to silver sulfadiazine ointment. Furthermore the process of burn wound healing took place within 14 days for kefir gel 96 h in our study, but it was for 24 days for silver sulfadiazine 1% reported in previous study [22]. These data indicated that, continuously cultured kefir grains in MRS Broth medium up to 96 h increases the wound healing properties of extract.

The antimicrobial properties of kefir were reported on several microorganisms in laboratories as well as in human diarrheas disease and urinary tract infection [31–18]. Several mechanisms were reported for antimicrobial effects of kefir grains. Farnworth[32] reported that the antimicrobial effects of kefir grains are due to lactic acid and antibiotics produced by microorganisms. Kumthavee [33] proposed that, bacteriocin and lactic acids from *lactobacillus rhamnosus* isolated from kefir grains are responsible for such antimicrobial effects.

However several other mechanisms such as production of organic acids, ethanol, bacteriocines and hydrogenperoxyde, in fermented process were proposed for antimicrobial activity kefir extracts [33–34]. The anti-inflammatory property is also influence process of wound healing[12]. Medeiros et al. [35] reported that the positive effects of hyaluronic acid on burn injuries are due to its anti-inflammatory effects. The anti-inflammatory properties of polysaccharide present in kefir extract may also influence in process of wound healing [37, 36]. However the lactic acid, acetic acid, polysaccharide and other chemicals present in kefir preparation are important factors for antimicrobial, anti-inflammatory and wound healing properties observe in present study.

However in future studies we try to standardize kefir gel product by determination of lactic acid, acetic acid and polysaccharide concentration along

with its burn wounds healing properties in animal studies.

CONCLUSIONS: In conclusion, as the processes time of the Kefir fermentation increased from 24 hrs to 96 hrs, the lactic and acetic acid concentrations and accordingly the antimicrobial activity of Kefir extract against *P. aeruginosa* were increased. the kefir gel therapy especially kefir 96 h gel with longer culture fermentation time strongly improves clinical outcomes after thermal injury as compared to conventional silver sulfadiazine treatment.

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