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ARTICLE

# ANTIMICROBIAL, ANTIOXIDANT AND ANTICANCER ACTIVITY OF KEFIRAN EXTRACTED FROM *PEDIOCOCCUS PENTOSACEUS* STRAIN TNAR03

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## ABSTRACT

***Background:*** *Kefir is a microbial symbiont mixture that produces slimy grains with collection of bacteria and yeast. The polysaccharide extracts (kefiran) from kefir have antimicrobial, antioxidative and anticancer activity.* ***Methods:*** *Extraction of kefiran in laboratory environment was performed by batch culture of Pediococcus pentosaceus strain TNAR03. The supernatant was used to evaluate the antibacterial, anticancer and antioxidative activities of kefiran. Antimicrobial activity was estimated by Disc Plate Method, Minimum Inhibitory Concentration and Minimum Bactericidal Concentration. Antioxidative activity, including radical-scavenging effects was analysed by DPPH method. Anticancer activity by MTT assay were investigated therein.* ***Results:*** *The intestinal pathogens from MTCC showed excellent decline in the growth when the kefiran extract were added to it. The antioxidant-scavenging properties by DPPH showed an enhanced antioxidant effect in the extract of kefiran. Vero and HepG2 cell were exposed to serial concentrations of kefiran to evaluate its cytotoxic activities. Results showed that kefiran significantly affected the viability of both tested cancer cell lines in a dose-dependent manner with $IC_{50}$ values of 298.8 ± 1.71 and 371.2 ± 1.32 µg/ml for Vero and HepG2 cells, respectively.* ***Conclusion:*** *These findings have demonstrated that kefirs possess antioxidant activity, thereby suggesting that kefirs are potential applicants for the role of useful natural antioxidant enhancements for the human diet.*

**KEY WORDS**
*Kefiran, Antimicrobial, Antioxidant, Anticancer activity, EPS*

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## INTRODUCTION

Probiotic bacteria are microorganism that can be used as a nutrition for the benefit for health. The kefir grains initiating the fermentation are a combination of lactic acid bacteria (LAB) and yeasts in a matrix of proteins, lipids, and sugars. This symbiotic culture of bacteria and yeast forms "grains"[1]. Kefir is a microbial symbiont mixture that produces jelly like grains as it grows, that contain both lactic acid bacteria (*Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Lactococcus*, etc.) and yeasts (*Candida*, *Saccharomyces* sp. etc.). Both bacteria and yeasts are surrounded by a polysaccharide matrix, named kefiran, a water-soluble branched glucogalactan, which has been reported to have antibacterial and anticancer activity [2,3].

Kefir is needed to act against the pathogenic genera and to have anti-inflammatory activities. The main properties of kefir might be of use as an alternative medication for patients infected with a single or multi-resistant strains of bacteria [4].

The importance of probiotics in food industry is growing nowadays and further research between different microorganism and their interactions can result in curing and preventing human diseases (Irritable bowel syndrome, Baterial vafinosis, traveler's diarrhea, small interstinal bacterial overgrowth) and other disorders[5]. Antibiotic use became widespread that resulted in obstinately developed resistance in bacteria. Because of this, efforts have been made to develop and study new compounds outside conventional antibiotic therapy [6]

This work focuses on the production, characterization of kefiran and its antimicrobial, anticancer and antioxidative analysis.

## MATERIALS AND METHODS

### Microorganisms used

The microorganisms used were *Bacillus subtilis* MTCC 441, *Bacillus cereus* MTCC 1272, *Staphylococcus aureus* MTCC 1144, *Pseudomonas aeruginosa* MTCC 741, *Escherichia coli MTCC* 739, *Klebsiella aerogenes* MTCC 39 *Vibrio cholerae* MTCC 3906. All strains were purchased from MTCC and revived by using appropriate media following the standard instruction provided [7].

### Kefir

Starter grain was purchased and maintained under appropriate condition. After 24 hours, the grains appeared to settle down at the bottom of the flask. The sour taste and the turbid appearance of the kefir drink indicated that it has been fermented. The medium was changed at 24h intervals and the grains washed with sterile water [7]. The organisms from kefir were isolated, identified by biochemical method and molecular 16S rRNA identification method and the sequence was submitted to NCBI using BANKIT Tool. Suspensions and kefir grains contained significant number of *Pediococcus* sp, and *Leuconostoc* sp.

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## Extraction of kefiran

Kefir was extracted for kefiran production for use in the antibacterial experiments. The polysaccharide matrix (kefiran) also used was isolated from kefir grains using the method described by Micheli et al. [8]. The stirred grains were washed with boiled distilled water for 1 hour (1/100 v/v).The mixture was then cooled and centrifuged (Remi R-4C) at 10000 rpm for 15 min. The procedure was repeated with the sediment. The polysaccharide dissolved in the combined supernatants was precipitated by the addition of an equal volume of cold ethanol at 4°C overnight. The precipitate was re-dissolved in hot water (1:100) for 1 h at 70°C with stirring and the precipitation procedure was repeated twice. The precipitate was finally dissolved in 100mL distilled water, and subjected to dialysis in distilled water.

## Susceptibility studies

Antibiotic susceptibility was studied by modified Kriby -Bauer method. The wells were made using sterile agar gel borer and loaded with different concentration of kefiran extract on pre-swabbed plate with organism in it [9].

Antimicrobial susceptibility was analyzed and interpreted using the guidelines for reference broth microdilution method as described by the Clinical & Laboratory Standards Institute (CLSI) [10].

The Minimum Inhibitory Concentration (MIC) was defined as the lowest antimicrobial concentration able to completely inhibit bacterial growth up to 24h. MIC parameters were determined in triplicates using 0.1mL of bacterial suspensions ($5\times10^8$ CFU/mL) in tubes containing 10mL of minimal media and the same amounts of kefiran as described above. Tubes were mixed by vortexing and incubated at 37°C for 24 h. Minimal bactericidal values were obtained based on the results for MIC values. Plates containing 25mL of Brain Heart Infusion (BHI) agar medium were inoculated with 0.1mL of the tubes showing no growth and incubated for 24 and 48h at 37°C. Controls were analysed similarly using the antimicrobial agents listed above [11].

## Scavenging effect upon DPPH radicals

The effect of kefiran upon DPPH radicals was measured according to the method [12]. Various concentrations of kefiran (0.8 ml, 0-4 mg/mL) were separately mixed with 0.2 mL of a methanolic solution containing DPPH radicals to give a final concentration of 0.2mM DPPH. The mixture was shaken vigorously and left to stand for 30min in the dark, and its absorbance was than measured at 517 nm. The capability to scavenge DPPH radicals was calculated as

DPPH radical-scavenging assay%
= 1- (absorbance of sample at 517 nm) / (absorbance of control at 517 nm) ×100

The percent DPPH decolonization of the sample was calculated. L-Ascorbic acid was used as a positive control [13].

## Anticancer activity of kefiran

Vero and HepG2 cell lines were grown on RPMI-1640 medium supplemented with 10% inactivated foetal calf serum and 50µg/mL of antibiotic. The cells were preserved at 37°C in a humidified environment with 5% $CO_2$ and were subcultured two to three times a week. Potential cytotoxicity of the compounds was evaluated on tumour cells using the method of Bertram [14]. Working dilutions were freshly prepared on the day of testing. After 72h incubation, the cell growth rate was evaluated by performing the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which detects dehydrogenase activity in viable cells. Each test was performed in quadruplicate in three individual experiments. The results are expressed as $IC_{50}$, which is the concentration necessary for 50% of inhibition. The $IC_{50}$ values for each compound are calculated from concentration–response curves using linear regression analysis by fitting the test concentrations that give values above and below the reference value (i.e.,50%). If however, for a given cell line all of the tested concentrations exceeding the respective reference level of effect, then the highest tested concentration is assigned as the default value, which is preceded by a '>' sign. Each result is a mean value from three separate experiments.

$$1-\frac{OD_t}{OD_C}\times100$$

Where, $OD_t$ is the mean optical density of wells treated with the tested sample and
$OD_c$ is the mean optical density of untreated cells.

## Statistical analysis

All values were expressed as mean ± S.D. Antimicrobial activity data from diffusion experiments were evaluated using the least squares method adjusted to the data and by one-way ANOVA using SPSS[15].

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## RESULTS

### Isolation

The organism isolated from milk kefir was further screened and identified as *Pediococcus* sp. Confirmation of the organism was done using 16S rRNA method and the sequence was submitted in NCBI. The accession number was obtained as KY817786 [Fig. 1]. Further the organism was enhanced to produce kefiran using batch culture

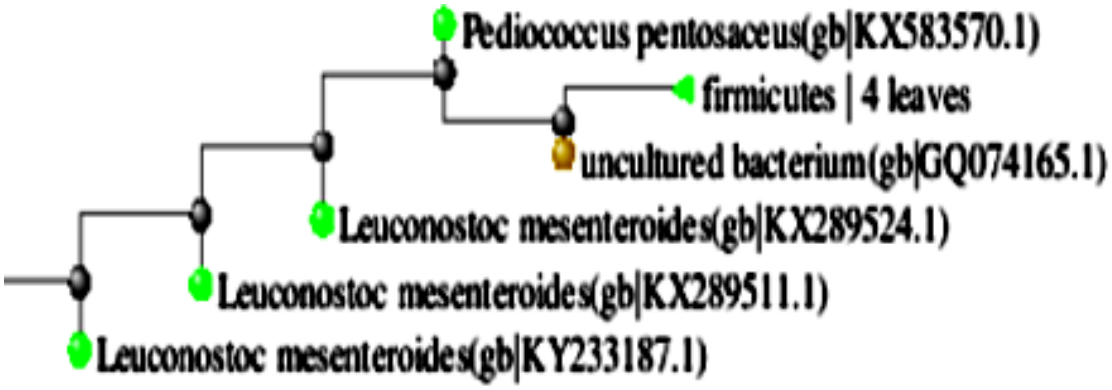


**Fig. 1:** Phylogenetic analysis of organism

### Sequencing results of *Pediococcus pentosaceus*

GCTAGGGAACCGGTAGTTGGTTTCCCCTGGATTTTGATCGTGGTCAGGCATCGAACAGGTAACCGTAGAGGATAAAAAA
AGTTGGTAGGCGCATTGTACGAATAYTGTTACGACTTCCTGAGCCAGGGTCAAACTCTAGGTTCCTTGTTAGATCTTGTTA
CAACTTCCTGACTAGGGTCAAACTCTATAGGTTCCTTGTTACAACATCTTGATACACCTTCCTGATCTATGGTCACACTCTA
TGGATTCCCTGTTTCGACATCCTYCCCAACTTAAAAACCCATGATCAACCTTATCGGAACCTAGAACATTCCTGACAATAAG
GGGCATGATGATCTGACGTCGTCCCCGCCTTCCTCCGGTTTGTCCCGGCGTCTCGCTAGAGTGCCCATCTGAATGCTGG
CAACTAACAATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACACCATGCACACC
TGTCACTTTGTCTCCGAAACACTTCTATCTCTAAAAGCTTCAAAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTC
GAATTAAACCACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGTACTCCCCAG
GCGGAACACTTAATGCGTTAGCTTCGGCACTAAGAGGCGGAAACCTCCTAACACCTAGTGTTCATCGTTTACGGTGTGG
ACTACCAGGGTATCTAATCCTGTTTGCTACCCACACTTTCGAGCCTCAACGTCAGTTGCAGTCCAGTAAGCCGCCTTCGC
CACTGGTGTTCTTCCATATATCTACGCATTCCACCGCTACACATGAGTTCCACTTACCTCTACTGCACTCAAGTTACCAGTT
CSATGCCATTCCGGAGTTGAGCT

### Susceptibility tests

Inhibition ratios of kefiran against the pathogenic strains were determined from minimum least squares applied to different concentration at 5, 20 and 50. The results show *Pseudomonas aeruginosa* to be the most sensitive microorganism to kefiran, followed by *Bacillus subtilis* and *Bacillus cereus*. *Vibrio cholerae* and *Staphylococcus aureus* were less sensitive to kefiran [Table -1] and *Klebsiella aerogenes* and *E. coli* the least sensitive. Minimum Inhibitory concentration (MIC) and Minimum Bactericidal Concentration (MBC) values for kefiran against all strains tested, ranged from 256 (MIC) to 128 mg/L (MBC) showing only a small increase in concentration to achieve a killing effect

**Table 1:** Antibiotic sensitivity pattern of various antibiotics on different organisms

| S.No | Name of the Antibiotics | Zone of inhibition in mm | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | SA | BS | BC | PA | EC | KA | VC |
| 1 | Amoxicillin | 16.5 | 21.2 | 27.2 | 24.5 | 22.1 | 11.9 | 23.3 |
| 2 | Amikacin | 17.6 | 29.4 | 12.3 | 34.6 | 11.2 | 12.5 | 25.5 |
| 3 | Cefotaxime | 25.1 | 28.1 | 23.4 | 35.2 | 28.5 | 22.1 | 28.1 |
| 4 | Chloramphenicol | 22.4 | 24.1 | 28.6 | 13.4 | 7.3 | 9.5 | 19.2 |
| 5 | Erythromycin | 15.5 | 11.5 | 46.7 | 23.6 | 12.7 | 16.4 | 18.4 |
| 6 | Gentamycin | 17.7 | 42.2 | 49.5 | 46.1 | 11.9 | 8.7 | 10.9 |
| 7 | Streptomycin | 19.8 | 28.9 | 24.4 | 43.3 | 8.4 | 12.9 | 7.7 |
| 8 | Ciprofloxacin | 18.1 | 32.8 | 46.6 | 19.6 | 19.6 | 12.6 | 16.2 |
| 9 | Tetracycline | 16.8 | 26.3 | 6.4 | 3.2 | 6.4 | 8.3 | 6.4 |
| 10 | Kefiran | 29.2 | 30.1 | 53.2 | 42.5 | 30.9 | 28.5 | 31.4 |

SA-*Staphylococcus aureus* MTCC 1144; BS - *Bacillus subtilis* MTCC 441; BC- *Bacillus cereus* MTCC 1272; PA - *Pseudomonas aeruginosa* MTCC 741; EC - *Escherichia coli* MTCC 739; KA- *Klebsiella aerogenes* MTCC 39;VC -

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*Vibrio cholerae* MTCC 3906;

The results represent the mean zone diameters (in mm) using the agar diffusion method. MIC/MBC values of kefir and kefiran showed within a narrow range.

### Scavenging effect upon DPPH radicals

DPPH is a compound that possesses free radical and used to determine its radical scavenging action. DPPH exhibits a characteristic absorption at 517 nm and its purple color fades when it encounters radical scavengers. At a dosage of 4.0 mg/ml, kefiran showed a significantly greater level of scavenging activity of DPPH radicals. The antioxidant activity for the milk based products were reported in different literature [5,8,10]. Hence the compound synthesized by the microorganism may have a similar property.

### Evaluation of *in vitro* cytotoxicity of kefiran polysaccharides

The kefiran polysaccharides produced by *Pediococcus pentosaceus* strain TNAR03 were evaluated for its *in vitro* cytotoxic properties against Vero and HepG2 cells using standard MTT assay. [Fig. 2] shows the cytotoxic effect of kefiran, which was type-dependent. The $IC_{50}$ values for Vero and HepG2 cells were 298.8 ± 1.71 and 371.2 ± 1.32 µg/mL, respectively. In increasing concentration of Kefiran extract, the cytotoxicity was prominent in the Vero cell line, compared with that of Vero.

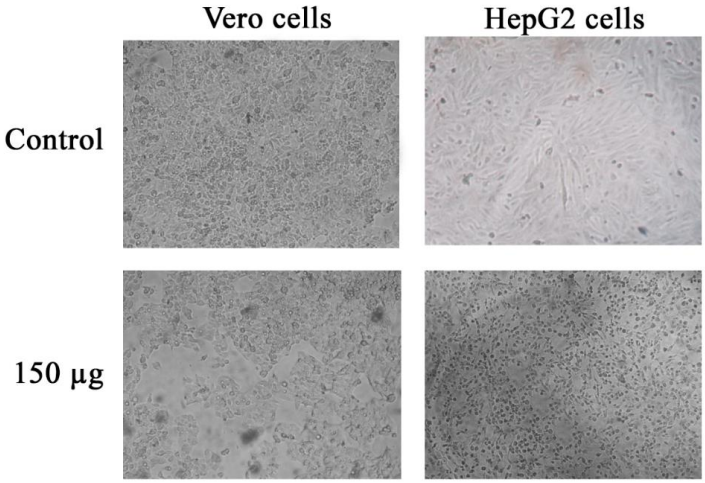


**Fig. 2:** Effect of kefiran on morphological characteristics of Vero and HepG2 cells after 24 h. Images were captured using inverted contrast microscope at 10x magnification.

## CONCLUSION

Kefir is a symbiont organism mixture that produces slimy grains with collection of bacteria and yeast. The polysaccharide extracts (kefiran) from kefir have antimicrobial, antioxidative and anticancer activity. Therefore, kefirs are potential candidates for the role of useful and natural antioxidant supplements in the human diet.

### CONFLICT OF INTEREST

Authors declare no conflict of interest

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None

### FINANCIAL DISCLOSURE

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