Alternative Preservatives of a "Nisin A" with Silver Nanoparticles for Bacteria Isolation from the Local Food Markets of Baghdad City

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Abstract

Today technology using nanoparticle when treatment pathogentic microorganism and we focused on this here. It was found that the species of *Staphylococcus epidermidis* used in present study were sensitive to Levofloxacin. The aim of report effect (Nisin-Silver Nanoparticles) on ability of complete healing injury comparing using Nisin only . In vivo study revealed that silver nanopasrticles treatment of *S. epidermidis* contaminated injured skin showed good healing process contain complete regeneration of the epithelial cells of the epidermis and good prognosis and increase of cellulartiy of the dermal content compared with untreated group. In conclusion, treatment of skin infected with *S.epidermidis* using silver nanoparticles at different concentration may limited the skin damage, localized the lesion to the incision site and enhance the healing process.

Keywords: *Staphylococcus epidermidis, Nisin A, silver nano particles.*

Introduction

The process of contamination of foods leading to loss of color, texture and their nutritive value and permits growth of pathogenic microbes, which deteriorates the quality of the product and makes it. Food contamination can occur with its exposure to the environment while slaughtering, food processing and packaging.(1)

Conventional food packaging aims at shelf life extension, maintenance of quality and assurance of safety of the food product. However, now a days foods ecurity is a big issue and, therefore, antimicrobial packaging system is specifically designed to control the microorganisms that adversely affect the above three goals.(2)

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Antimicrobial agents have different activities on different pathogenic microorganisms due to their various diverse physiologies.(3) Silver nanoparticles (AgNPs), have strong permeability and effective broad-spectrum antibacterial properties and it used to produce a range of antibacterial therapeutic products, such as, toothpaste, gynecologic suppository and wound dressing.(4)

Thus, there is a need for "green chemistry" that comprises a clean, nontoxic and environmentally-friendly manner of nanoparticles synthesis, an alternative to conventional techniques, biological method are considered ecologically sound for the nanomaterials fabrication and safe.(5)(6)

Nisin A is a bacteriocin formed by Lactobacillus lactis. It is the most bacteriocin studied and assort as the generally recognized as safe (GRAS) in 1988 by the FDA according to low toxicity to human body (US Food and Drug Administration, 1988). It is embedded to lantibiotic class kind a liner with small amino acid peptide chain (34 amino acid residues).(7)

Bacteriocins are antimicrobial peptidesproduce

by certain bacterial strains, which are immune to them, to compete by preventing the growth of other bacteria existing in their environment. Nisin is a 35-mer bacteriocin of the lantibiotics group, whose use as a food preservative was approved by the Joint Food and Agriculture Organization[8]

Many reportslantibiotic sextensively subclass of bacteriocins, which includes (staphylococcin C55 and NisinA) amongst others, Several type highly activity anti- clinically relevant and food-borne pathogens [9]

Material and Method

Media Preparation

Ready to Use Media: "The media were prepared according to instructions of the manufacturer. They were brought to boil in water to dissolve all constituents completely, then the pH was adjusted to 7.2 and sterilized by autoclaving at 121˚C (15Ib/In^2) for 15min. They were incubated at 37˚C for (18- 24) hrs to sterilization."[10]

Samples Collection: A total of 40 food samples were purchased from local markets Baghdad/Iraq Octuber 2018 to january starting from 2019 as illustrated in (type of samples are: Cake without cream, Cake with cream; Arab Cheese; Kemar Arab; Raw milk (no. of samples 8 per each type to give total samples are 40)). The samples were taken after recording the labeling information (commercial name of product,), then putting it in the ice box and transport to the microbiology laboratory to isolate pathogen bacteria on the same day

Cultivation of Bacteria: Each samples put 10g or 10 ml according to type of sample then diluted in 90ml sterile enrichment peptone water and incubated at 37°C for (18- 24)hrs. Then loopful of incubated culture was cultured on were cultured first on Mannitol salt agar and MacConkey agar plates to differentiated between Gve + and Gve- bacteria.(Forbes *et al.,* 2007).

Biochemical Analysis: The morphology and biochemical tests were conducted according to the method described by Bergey's manual of determinative bacteriology.[11]

Antimicrobial susceptibility testing: Bacteria gram positive and negativ were detected by antibiotic disk in DDM. This method was implemented subsequently the instructions of the clinical and Laboratory Standards Institute. [12]

Molecular assay:

DNA extraction: Genomic DNA was extracted from the detected bacterial isolates according to the protocol of Wizard Genomic DNA Purification Kit, Promega. Quantus Florometer was used to detect the concentration of extracted DNA.[13]

Well Diffusion Method: (WDA) Well diffusion method Jérôme *etal*., (2014) then100 µL of the suspension was spread on the test plate (Muller Hinton Agar). And put (10 µL) of the essential oils and placed on well in agar. Plates were incubated at 37^0 C for 24 hours and the zone of inhibition was measured. The experiment was performed in triplicate and the average diameter of inhibition was calculated.[14]

Preparation of Nisin A stock solution: Nisin A Figure (3-4) (Sigma Nisaplin 2/5%) was prepared by dissolving with 100mg from Nisin A powder in 10 ml HCl (0.02 N) to provide10^4 IU/ml (40 IU=1 g) concentration. after that the solution was passed through 0.45 filters for sterilization and was maintained at –20°C.[15]

Preparation of Bio Extract: Fresh peel 20 gm of Lemon leaves were washed with tap water and then washed with distilled water, air dried a little and then they were finely cut and soaked in 100 ml boiling distilled water for 5-10 minutes and filtered through Whatman filter paper no. 42. This extract was used for generating Silver Nano particles.

Preparation of 1mM Silver Nitrate: Silver nitrate was brought from Lobachemie. Weigh 0.0169gm of silver nitrate and dissolve in 100mL of distilled water in amber coloured bottle.

Synthesis of Silver Nanoparticles: 3mL of prepared extract was added to 40mL of silver nitrate solution in 100mL conical flaskcontain lemon peel extraction Incubate at room temperature for 2- 3 hours. Control is made containing only 40 mL of silver nitrate solution. Show Figure (1)

Scanning Electron Microscope (SEM Analysis): The morphological features of synthesized silver nanoparticles from neem plant extract were studied by Scanning Electron Microscope (JSM-6480 LV). After 24Hrs. of the addition of AgNO3 the SEM slides were prepared by making a smear of the solutions on slides. A thin layer of platinum was coated to make the samples conductive. Then the samples were characterized in the SEM at an accelerating voltage of 20 KV

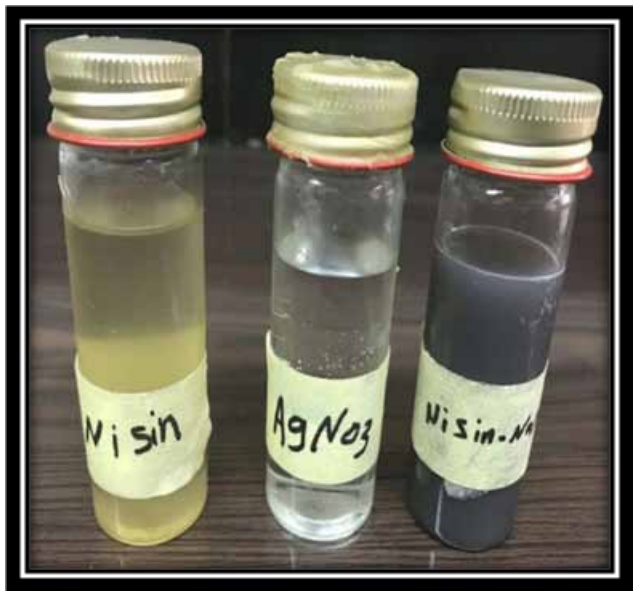

Figure (1): Formation of Brown colour solution

Antibiotic Sensitivity Test: For inoculums, standard homogenized *Staphylococcus epidermidis* was prepared in normal saline and the suspending was diluted to 0.5× 10^8 CFU ml compared with McFarland tubes.(10)

Antibiotic sensitivity test for *S. epidermidis* was done by Kirby-Bauer disk diffusion method againstOxacillinOX (1), Erythromycin E (15), Levofloxacin LEV (5), Cloxacillin, CX (1), Cefepime, FEP (30). The zone of inhibitionwere measured (mm) and compared with pretivechart a documented standard,the zone of inhibition (in mm) Clinical and Laboratory StandardsInstitute(16).

Primers Selection: The set of primers 27F (AGAGTTTGATCTTGGCTCAG) and 1492R(TACGGTTACCTTGTTACGACTT) was used for amplification of 16s rRNA for identification of bacteria at gene level.(17)

Preparation of Silver Nitrate: Silver nitrate was brought from Lobachemie. Weigh 0.0169gm of silver nitrate and dissolve in 100 ml of distilled water to obtain three concentration (25, 50 and 75mM) in ambercolored bottle.

Characterization of Silver Nanoparticles: Then synthesis of silver nanoparticles was checked in UV-Visible spectroscopy at the wavelength of 300 - 700nm.

Synthesis and optimization of Nisin-Silver nanoparticles compounds: Fifty mM of AgNO3 solution was prepared and different concentrations of Nisin Making double serial dilutions (16,32 and 64 µg/ml) in collection beaker (10 ml) to 50 mM silver nitrate solution after that was mixed with continuous stirring slowly .

Synthesis of $AgNO^3$ at Room temperature: The solution above mentioned are incubated at 37C (Room temperature) for 27hrs and observed the change color and checking every one day. UV-V is spectra using for the solution to monitored and measured then the flask was incubation at room temperature for another two days until the completion of the reaction.

Bacteriocin Nisin A: Bacterocin was preparation from a commercial (Nisin A obtained from Cayman Chemical Company) asaccording to(18) at concentration (31.25, 62.50 and 125µg\ml).

Agar Well Diffusion Assay (AWD): Efficiency different concentration of Nisin A measurement activity was carried out by serial two-fold dilutions method by AWD .

Result and Discussion

Isolation Bacteria: The symptomatic cases are shown in Table (1), were out of 40 food samples 35(60%) isolates were identified as *S. aureus* gave positive and *E. coli* isolates reached 22. The highest percentage of isolates was present in raw milk then in cheese and cake with cream reached to respectively.

Table (1): Prevalence of Bacterial isolates from food samples.

Sample	No. Sample	*S. aureus* %	*E. coli* %	*Klebsiella*spp %	Enterobacter %	Staphylococcus % epidermidi
Raw milk	8	35 (43)	22(32)	16(27.5)	12(31.5)	9(30)
Kemar Arab	8	15(18.5)	19 (27.9)	16(27.5)	9(23.6)	8(26.6)
Arab Cheese	8	17(20.9)	15 (22)	11(18.9)	10(26)	7(23)
Cake without cream	8	8 (9.8)	9(13)	7(12)	4(10.5)	4(13)
Cake with cream	8	6 (9.8)	3(4.4)	8(13.7)	3(7.8)	2(6.6)
Total	40	81 (81)	68(68)	58(58)	38(38)	30(30)
Chi-Square (P-value)	---	9.935 ** (0.0001)	9.173 ** (0.0003)	6.004 ** (0.0092)	8.469 ** (0.0004)	8.931 ** (0.0002)
** (P<0.01).						

Various levels susceptibilities to different antibiotics among isolates that were observed by Disk diffusion method isolates was multi-resistance for antibiotics with a high level against, Oxacillin, Cefoxitin, Erythromycin. But sensitivity to Levofloxacinthis result was similar to that acquired by.[19]

Staphylococcus spp. count in the samples was low and all the strains were considered as coagulase-negative strains on coagulase-test. *S. aureus* is considered as the most important foodborne pathogen of this group and it is considered as coagulase-positive specie. Based on such results according to.[20]

Isolation and identification of Bacteria:

Isolation and Identification of Gram positive: (*Staphylococcus. epidermidis* and *Staphylococcus. aureus*)

The macroscopic examination of isolates on Mannitol Salt Agar have an ability to ferment mannitol and turn the color of medium from red to yellow were classified as a presumptive *S. aureus* and *S. epidermidis* isolates

The isolates on Blood agar showed yellow-gray colonies are (4-3) mm in diameter on the zones of β-hemolysis

Isolationand Identification of Gram Negative: The macroscopic examination of presumptive (*E. coli,P. aeruginosa, Klebsiella*spp and *Enterobacter* pp). (Figure 2).

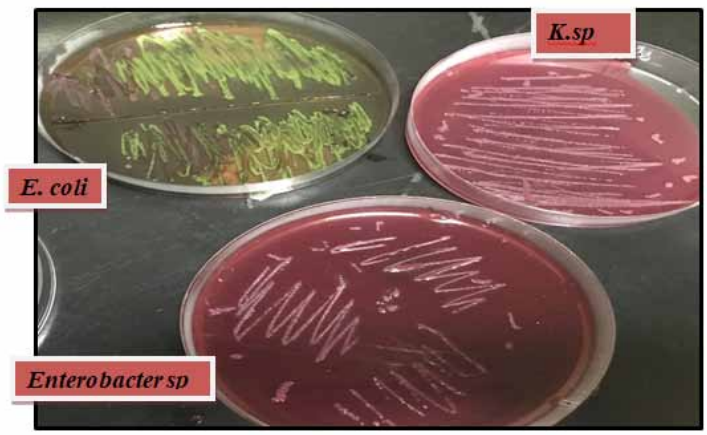

Figure (2): Bacterial isolation from Food sample on MacConkey and Eosin methicillin blue agar mediums at 24hrs at 27°C.

Were found to be (*E. coli*, *P.aeruginosa*, *Enterococcus faecalis* and *K. pneumoniae*) resistant to between (92.7-96.5 %) but moderately resistant to other between (70.5-67.2 %). Show the *E. coli* isolates β-lactam resistance is probably due to the production of (TEM β-lactamases), which may be the chromosome or on a plasmid genetically localized on. this result was similar to that obtained by[21]. Showes most isolates of *Staphylococcus aureus* were multi-resistance for antibiotics with a high level against Methicillin, Penicillin G, Cefoxitin, Erythromycin, Oxacillin, Chloramphenicol and Tetracyclin. But sensitivity to Azitromycin.[22]

In this study, the genomic DNA of bacterial isolate was successfully extracted and the extracted DNA has an appropriate quality to perform PCR (10 ng/µl). 16S rDNA (Fig. 3).

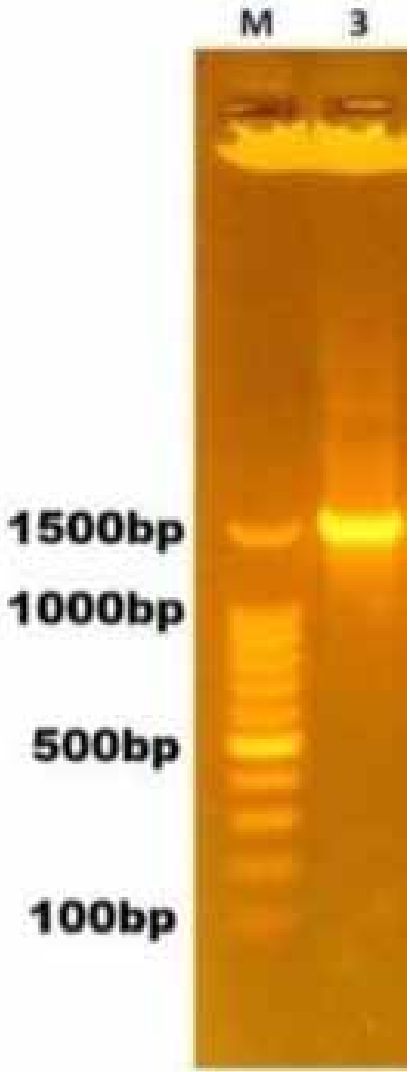

Figure (3): PCR product the band size 1250 bp. DNA ladder (100). *Enterobacter sp*

Study of Nisin-Silver Nanoparticles compounds characterization:

- **Spectral Properties of the Nisin-Silver Nanoparticles**

A strong surface plasmon centered around 340 nm.

Atomic Force Electron Microscopy (AFM): The AFM micrograph obtained for the Nisin-silver nanoparticles Figure (4) For the sample the roughness value was 56 nm and the section analysis of the sample's grain size value was 42 nm.

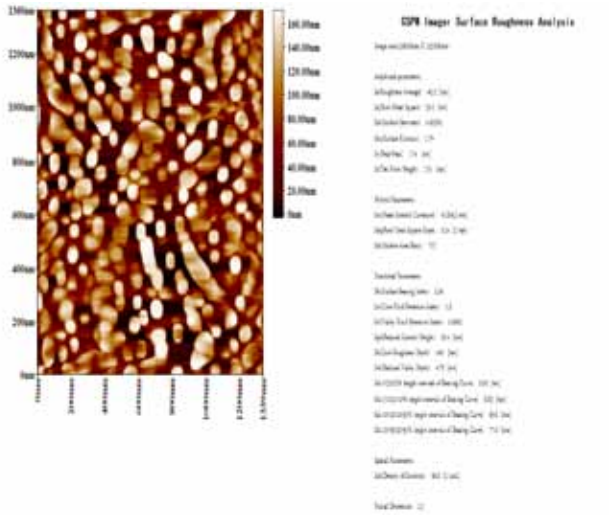

Figure (4). AFM for nisin-silver nanoparticles

Transmission Electron Microscopy analysis (TEM) of silver nanoparticles:

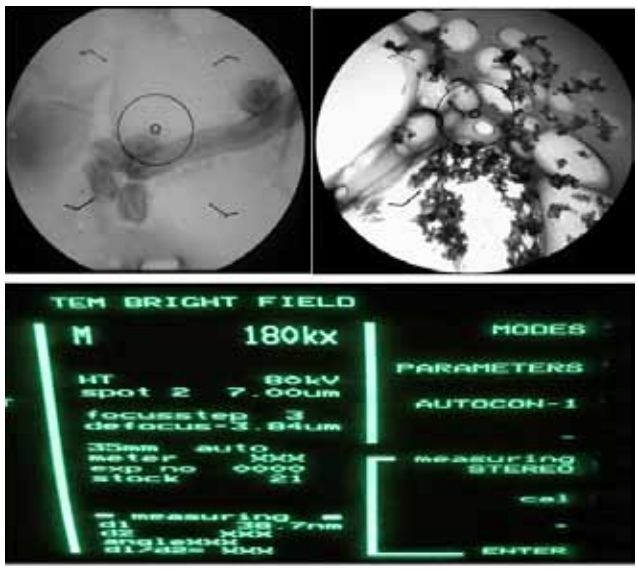

Figure 5: TEM of A) *S. epidermidis* before treatment Nisin-Silver Nanoparticles B) *Enterobacter sp* after treatment Nisin-Silver Nanoparticles

The well diffusion agar method (WDA) was used to detection *S epidermidis* sensitivity to word Nisin-Silver Nanoparticles. The concentration (31, 62 and 124) µg\ml was appeared the inhibition zone size is (11 and 9) mm recpectively while at 125µg\ml not any inhibition,the result agree with[23]. The previous study by[23] showed that Nisin A was active against MRSA and *Staphylococcusepidermidis* less than other strain in MIC range 2mg/l at 24 hrs. Many studies have regarded the activity of Nisin for use as antimicrobial therapeutic[24].

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: Non

Funding: Self-funding

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