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Antibacterial Activity and Molecular Detection of Plantaricin

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Abstract: *Escherichia coli* O157:H7 is the cause of more people and animals getting sick with diseases like hemolytic uremic syndrome (HUS) and enterohemorrhagic diseases (EHEC).This study was conducted for molecular detection and evaluate the antibacterial activity and mechanism of action of plantaricin against *E. coli* O157:H7 in comparison with ciprofloxacin. This experiment was carried out through obtaining plantaricin from *Lactobacillus plantarum* and study the sensitivity of the *E.coli* O157H7 by MIC and MBC and identify its mechanism of action by Fe-Scanning electron microscope (Fe-SEM). The plantaricin showed pronounced concentration dependent antibacterial activity. The results of the presence study activity suggest plantaricin may have the perfect to be choice in clinical control. The result of inhibition of plantaricin was (25.72 mm to 30.83 mm) while the result of MIC was (0. 625mg/ ml) and MBC was (1.25 mg/ ml) , while for ciprofloxacin, the result was(16.75 mm to 22.04 mm) , at (20 and 40µg/ ml) respectively . I was concluded The Fe-SEM identification indicated that DNA leaked from cells due to the cell lysis of *E.coli* O 157:H7 and plantaricin interacts with the target cell membrane.

Keywords: Plantaricin, *E. coli* O157:H7, MIC, MBC and Fe-SEM.

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Introduction

Escherichia coli O157:H7 is the cause of more people and animals getting sick with diseases like hemolytic uremic syndrome (HUS) and enterohemorrhagic diseases (EHEC). Most of these cases are attributed to an infection with the bacteria that produce Shiga toxin (STEC). While O157 remains the most common serotype of Shiga toxin-producing *Escherichia coli* (STEC) (1, 2). Infection caused by *Escherichia coli* that produces Shiga toxin is linked to dysentery and the hemolytic uremic syndrome, characterized by the presence of microangiopathic hemolytic anemia (HA), rapid kidney damage, and thrombocytopeni (3).*Escherichia coli (E. coli)* has a disadvantage in that an insignificant recombination of genes event may result in the emergence of a highly pathogenic variant. This variant is responsible for a broad range of bacterial diseases that are prevalent globally, including sepsis, newborn meningitis, pneumonia, bacteremia, and traveler's diarrhea (4). The main sources of *E. coli* O157:H7 include undercooked vegetables, beef ground up, milk, and livestock. Hemolytic uremic syndrome (HUS), which can have major health, and financial repercussions, and diarrhea are only two examples of disorders that can be brought on by very low infectious doses in people. (5, 6). Every strain of *Escherichia coli* has specific somatic (O) and flagellar (H) antigens as well as

distinctive virulence traits. *Escherichia coli* O157:H7 is the prototypical strain (7, 8, 9). The use of antibiotics is crucial for treating pathogenic bacterial infections, but it has grown increasingly difficult in recent years due to rising resistance to commonly used medicines (10). *Lactobacillus plantarum* produces the new bacteriocin known as plantaricin which usually included in both class I and II. Class I includes bacteriocins which named plantaricins (11, 12). Plantricin has the potential to be useful as an effective substitute to standard antibiotics for the purpose of preventing and treating diseases that are infectious (13 14, 15). This study aimed to evaluate antibacterial activity of plantaricin against *E. coli* O157:H7.

Materials and methods

Source of *E.coli* O 157:H7

E. coli O157: H7 bacteria were obtained from AL-Karama hospital in Wasit Governorate, obtained from female suffering acute UTIs. Morphological colonies characterization was recorded on the media by using MacConkey agar, eosin methylene blue (EMB) agar, sorbitol MacConkey agar and chromogenic agar while , Biochemical test identification of *E.coli* O 157 H7 include indole, catalase test, coagulase test, oxidase test, motility test, methyl red test and voges-proskauer (16) in addition, Vetik II system , PCR and Lattex agglutination.

Extraction of plantaricin from *Lactobacillus plantarum*

Plantaricin was produced from *lactobacillus plantarum* that was isolated from local samples (sourdough samples) by using a growth medium called deMan, Rogosa, and Sharpe (MRS) broth according to (17). The plantaricin gene identified by PCR according to (18).

Antibacterial activity of the plantaricin

The agar well diffusion technique was used for testing the antibacterial activity of plantaricin **(**19) , 500 ml of sterile Mueller Hinton agar were combined with 5 ml of standardized *E. coli* O157:H7 bacterial stock solution (1.5 x10^8 cfu/ml), and a volume of 25 ml of MHA was dropped onto each sterile petri plate. Following a 10-minute settling duration, four wells with a diameter of 6 mm each were generated using a sterilized glass pipette on the agar. After that, wells were filled with 100 microliters containing different concentrations of plantaricin, and last filled with distilled water, it was provided with two hours to disperse at ambient temperature, its concentration were performed on the plates over the course of a 24-hour incubation period at 37°C. By using a specialized ruler known as a "caliper ", the diameter of the inhibition zone surrounding each well was measured to evaluate the antibacterial effect (20).

Determination of minimum inhibitory concentration (MIC)

A stock solution of plantaricin were prepared in Mueller-Hinton broth, then make series dilution in different concentration (5, 2.5, 1.25, 0.625, 0312 and 0.156 mg/ml) of plantaricin and put in 96 well micro-titer plate that have shape as a U bottom, each well was infected with 100 µl of a bacterial suspension containing 10^6 colony-forming units per milliliter of *Escherichia coli* O157:H7. The samples were then incubated at a of 37 ºC for 24 hours (21). To establish a control group, a volume of 200 µl of Muller Hinton broth was introduced into the designated wells that were intentionally devoid of microorganisms. For colorimetric identification of bacterial growth, adding 20µl of alamar indicator (0.125% w/v) to

each well of the test and re-incubated for two hours (22).

Field emission -scanning electron microscope (Fe-SEM)

The Fe-SEM were used to observe the morphological changes according to (23 ,24). The bacterial sample was prepared for scanning electron microscope by prepare four tubes contained on 100 µl of the bacterial suspensions (1×10^8 CFU/ml) that inoculated onto Mueller–Hinton broth (MHB) tubes containing different concentration of plantaricin. The tubes were incubated at a temperature of 37°C for 4 hours.

Following incubation, a volume of 600 µl from each tube was dispensed onto glass cover slides measuring 1x1 cm. After treatments coverlids were washed with 0.1M of phosphate buffer saline. After dehydration, the samples were dried in a silica desiccator for a 72 hrs before being analyzed by a scanning electron microscope **(**25).

Statistical analysis

Statistical Analysis System- SAS (26) program was utilized. In this study, a significant comparison of means was made using the difference that was least significant (LSD) test (ANOVA).

Results and discussion

Biochemical identification of *E. coli* O157:H7 Results of biochemical tests are shown in table 1, and these results were consistent with the findings reported in reference (27, 1).

Table (1): Biochemical tests for identification of *Escherichia*.coli O157:H7

No.	Biochemical tests	Results
1	Catalase	+
2	Citrate utilization	-
3	Gelatinase	-
4	Indole	+
5	KIA	**A/A**
6	Methyl red	+
7	Motility	+
8	Oxides	-
9	Ureas	-
10	Voges-Proskauer	-

(+) positive result, (-) negative result, (KIA) Kliger Iron Agar test, (A/A) Acid slant/ Acid bottom

Cultural characterization of *E. coli* O157:H7

The result showed in (Figures1,2). MacConkey agar showed a pink discoloration, as indicated by the colony of positive *E. coli* O157H7 isolates. In eosin methylene blue (EMB) was applied for selection and differentiation and was seen as a rapid means of distinguishing *E. coli* from other gram-negative bacteria, the colonies appeared as green metallic sheen and that indicated a vigorous fermentation of lactose, and acid production which precipitated the green metallic pigment that agree with (28,29). The use of particular compounds identified by -D-galactosidase and -D-glucuronidase, H7 may be differentiated from *E. coli* non-O157. All strains of *E. coli* produce B-D-Galactosidase, whereas all strains of *E. coli* produce -D-glucuronidase with the exception of STEC O157:H7, which does not ferment sorbitol which agree with (30), The isolates showed various colonies on selective culture media grown at 37°C. The colonies of *E.coli* O157: H7 showed a tiny, round morphology on the medium, with a colorless or amber-like appearance. The findings of this study suggested that the use of chrome agar

assisted in the diagnosis of *E. coli* O157: H7. Specifically, the chromogenic substrates present in the agar resulted in the formation of colonies with a mauve color. By employing chrome agar, it became possible to assume the presence of E. coli O157: H7 on the primary isolation plate and differentiate it from other species (31,32). A comparable outcome by (33,1) the chromogenic techniques were apparently offered recently for the identification of Shiga toxin-producing *E. coli* (STEC) O157:H7 in people, food, and animals. The media being used comprised a special blend of synthetic chromogenic conjugates made up of a chromophore and a substrate for an enzyme specific to *E. coli* (34,32).

Figure (1): (A) Pink Colonies of *E. coli* O157:H7 when grown on MacConkey agar at 37°C for 24 hrs.; (B) Colonies of *E. coli* O157:H7 (green metallic sheen) grew on EMB agar at 37°C for 24 hrs (C) The pallid-brown colonies of *E. Coli* O157:H7, because it was a non-sorbitol fermenter, when grown on Sorbitol MacConkey agar at 37°C. For 24 hrs.

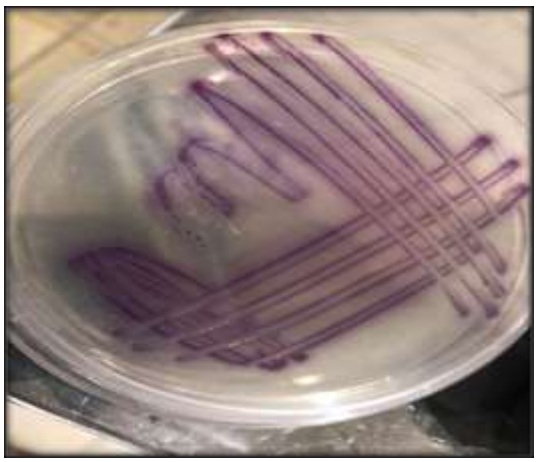
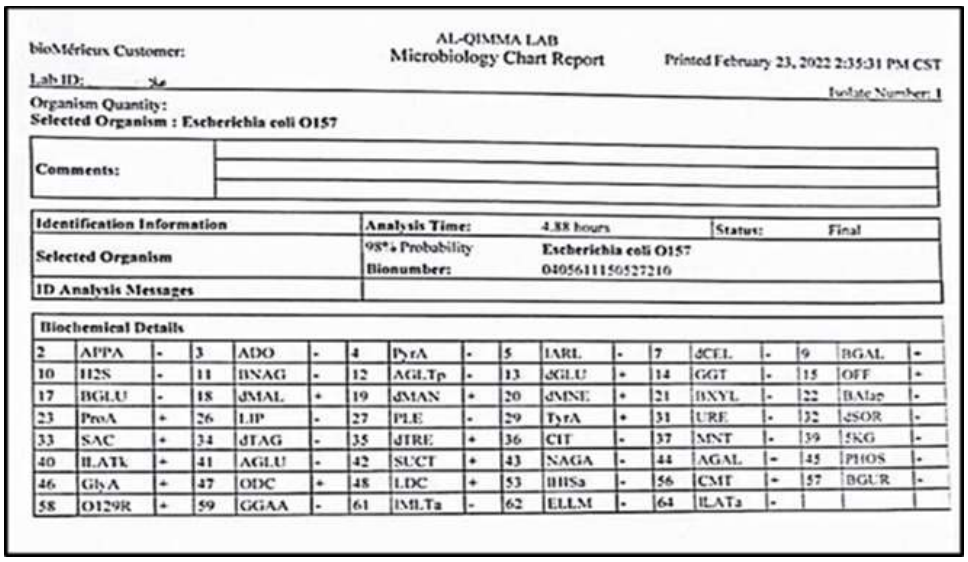


Figure (2): Characteristics of the culture *E. coli* O157:H7 colonies are pinkish or purple on chromogenic coliform agar

VITEK II System

Confirmation of *E. Coli* O157:H7 of identification was performed with the automated VITEK II system by using GN-ID cards which contain many biochemical tests, The isolated have shown a high degree of identification accuracy, reaching a probability of 98% as indicated by the technical datasheet provided by the manufacturer (Figure 3). This method is distinguished by its rapid bacterial detection capabilities, reducing the need for extensive culture medium and minimizing the occurrence of culture contamination (36). The use of automated identification of bacteria techniques in medical laboratory settings offers a fast and dependable means of diagnosing a wide range of pathogens associated with infectious disorders while

maintaining a notable degree of accuracy in identification (35). On the other hand, the VITEK II system is beneficial for comparing the biochemical characteristics of *E. coli* O157:H7 (37).



bioMérieux Customer: AL-QIMMA LAB
Microbiology Chart Report
Printed February 23, 2022 2:35:31 PM CST
Lab ID: Isolate Number: 1
Organism Quantity:
Selected Organism : Escherichia coli O157

Comments:	

Identification Information	Analysis Time:	4.88 hours	Status:	Final
Selected Organism	98% Probability	Escherichia coli O157		
	Bionumber:	0405611150527216		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	+	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	+	47	ODC	+	48	LDC	+	53	BHSa	-	56	CMT	-	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	-	62	ELLM	-	64	ILATa	-			

Figure (3): **Identification of *E. coli* O157:H7 by VITEK II system.**

Serotyping test (Wellcolex *E.coli* O157:H7, Remel) latex agglutination test

The O157 and H7 antigens of *E. coli* colonies were identified using the wellcolex *E. coli* O157:H7, remel. Isolates that responded positively to the O157 antigen were subcultured overnight on blood agar to detect the flagellar antigen (H7). Red color agglutination showed a positive result for the O antigen in comparison to the clear red color of the control, while blue color agglutination showed a positive result for the H antigen, also known as the flagler antigen., as in (Figure 4).

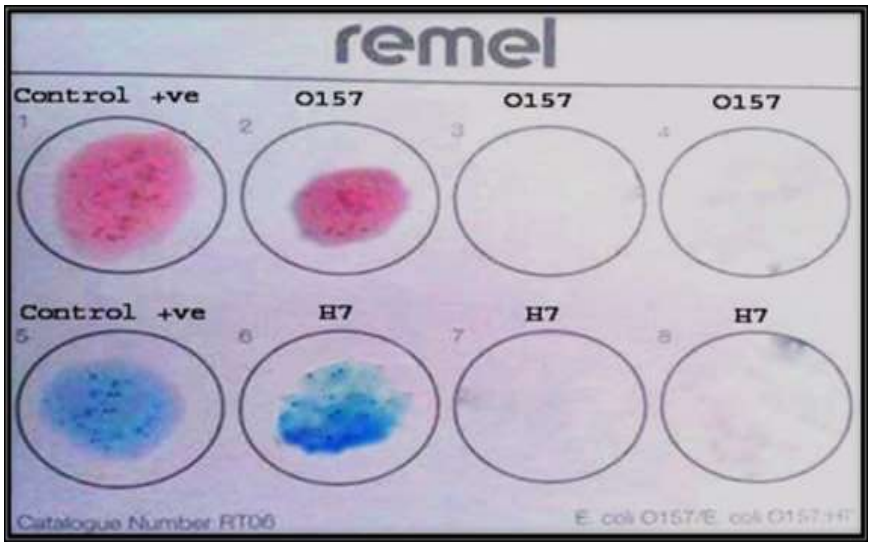


Figure (4): The latex agglutination test for *E. coli* O157:H7 has shown positive findings for the detection of O157 and H7 antigens.

This result was in acquiescence with (38, 33, 27) that demonstrated the use of the latex agglutination test for the serotyping of *E. coli* O157:H7 isolates and demonstrated how quick, simple, and

easy it was to perform the test and understand the results.

Extraction of plantaricin from *Lactobacillus plantarum*

The 37 ◦C was found to be the optimum temperature for growth while 30 ◦C and 37 ◦C were better for plantaricin production which was in agreement with (39, 18) and the identification of plantaricin was found to be as following:

FASTA sequence for plantaricin gene

AGTGCTTAAACTTGATGGCTTGAA CTATCCGTGGATGAATCCTCGGAC AGCGCTAATGAC 60 Sbjct 474 ..... 533 Query61 CCAATCGGCAGGCCCAACAGCACT TTTATAATTATTCCGAGCGCCACG CGCGCTATAGGC 120 534 .....**A**..... 593. Query 121ATGGAAAACGCCACCTGAAAT AGCATTTAATTCACGGTCACGCAA AACTAGAAAATTTTT 180 594 .....**T**..... 653 Query 181 CATAATTGTTGATCTCCCCCAAGA AAATTAACGAATACTTTTCAAAAT ACCACGAATGCC

Based on molecular weight, the discovered protein was anticipated to be plantaricin that agrees with (40, 41) .

Antibacterial activity of Plantaricin against *E. coli* O157:H7 well diffusion method

The results of the study revealed that *E. coli* O157:H7 showed variable degrees of resistance to various doses of antimicrobial drugs. The sensitivity of the *E. coli* O157:H7 isolate to plantaricin was assessed at various doses, including 5, 2.5, 1.25, 0.625, 0.312, and 0.156 µg/ml. The result of antimicrobial activity was summarized in tables (2, 3). It was seen that 4 concentrations (5,2.5,1.25and 0.625mg/ml) of plantaricin had antibacterial activity against *E. coli* O157:H7. (Figure 5.A), this isolate was resistant to the other concentrations of plantaricin (0.312 and 0.156 mg/ml). While *E.coli* O157:H7 was resistant to other concentrations of ciprofloxacin also showed intermediate resistance at the concentration (20 µg//ml) but it was sensitive only to (80 and 40 µg/ml) ciprofloxacin. (Figure 5.B).

Table (2): **Antibacterial effect of plantaricin against *E.coli* O157:H7**

Plantaricin concentration (mg/ml)	Zone of inhibition(mm) M ± SE		
	Plantaricin	D.W	LSD value
2.5	30.56 ±2.18 A a	0 ±0 B a	5.19 *
5	30.83 ±1.89 A a	0 ±0 B a	4.78 *
1.25	26.70 ±1.52 A a	0 ±0 B a	4.03 *
0.625	25.72 ±1.35 A a	0 ±0 A c	4.17 *
0.312	0 ±0 A b	0 ±0 A c	0.00 NS
0.156	0 ±0 A b	0 ±0 A c	0.00 NS
LSD value	6.722 *	0.00 NS	---

This means that there is a substantial difference between the lowercase characters within the same column and the lowercase letters within the same row, shown by * ($P \leq 0.05$).

Table (3): Antibacterial effect of ciprofloxacin against *E.coliO157:H7*

Ciprofloxacin concentration (µg/ml)	Zone of inhibition(mm) M ± SE		
	Ciprofloxacin	D.W	LSD value
80	22.04 ±1.37 A a	0 ±0 A c	3.47 *
40	20.23 ±1.08 A a	0 ±0 A c	3.07 *
20	20.23 ±1.08 A a	0 ±0 A c	2.88 *
10	0 ±0 A c	0 ±0 A c	0.00 NS
5	0 ±0 A c	0 ±0 A c	0.00 NS
2.5	0 ±0 A c	0 ±0 A c	0.00 NS
LSD value	3.28 *	0.00 NS	---
This means that there is a substantial difference between the lowercase characters within the same column and the lowercase letters within the same row, shown by * (P≤0.05).			

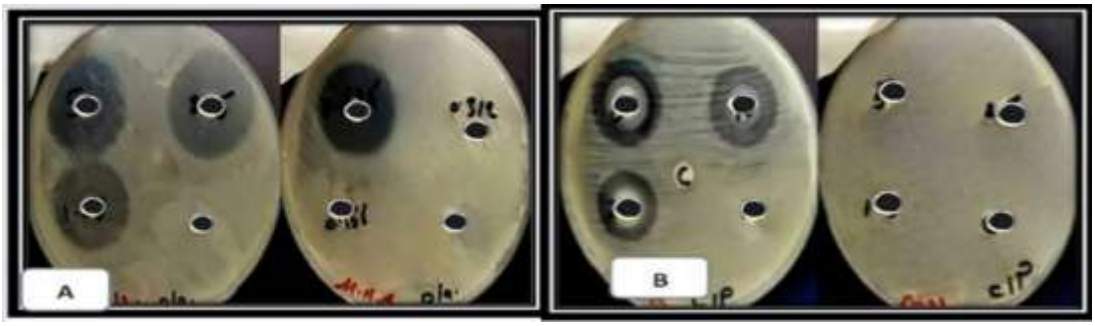


Figure 5 : (A) Senstivity of *E. coli* O157 :H7 to different concentrations of plantarcin. (B) Senstivity of *E. coli* O157 :H7 to different concentrations of ciprofloxacin
Note*((C)) means: the solvent as control is distilled water for ciprofloxacin while phosphate buffer saline for plantaricin

Results of antimicrobial activity was in agreement with (42) who demonstrated that plantaricin has antibacterial action versus *E. coli* by testing plantaricin against indicator bacteria. A susceptibly test result of this study was in agreement with**. (**43,44, 54**)** who attributed that plantaricin, consider a potential source of antibacterial compounds and is reported to be effective to inhibit the growth of different bacterial strains especially those which have acquired resistance to antibiotics. In otherhand (45, 46,47,18) reported that the plantaricin inhibits the growth of *E. coli* that cause disease by peptides which have efficacy against bacteria.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of plantaricin against *E.coli* O157:H7

To establish the minimal inhibitor y concentration, the H7 isolate was tested againstvarious concentrations of plantaricin and ciprofloxacin. MIC values were (0.625 mg/ml) for plantaricin and (20 µg/ml) for ciprofloxacin while the MBC values were (1.25 mg/ml) and (40 µg/ml) respectively (Figure 6).

Figure (6): Broth microdilution method to determination MIC and MBC of plantarcin and ciprofloxacin aganst *E. coli* O157:H7. Wells with blue color had no growth, but, Wells with red color with growth.

Depending on visual readings(22) conducted by observing the transformation of blue, which comes from alamar, into formazan (pink or purple), or not. When determining the MIC and MBC, the lowest concentration was taken into consideration and no color development took place, corroborating the experiment's findings that's in agreement with (48,30,49). The micro-dilution test has been approved by both the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) as the predominant and precise technique for measuring the minimum inhibitory concentration (MIC). Plantaricin has notable inhibitory efficacy against many harmful microorganisms, such as *Escherichia coli*. (50, 51).

Scanning Electron Microscope (SEM)

The use of Field Emission Scanning Electron Microscopy (Fe-SEM) assessment was used in order to describe the dimensions, design, and morphology of the bacteria afterward to treatment with antibacterial agents. Additionally, Scanning Electron Microscopy (SEM) analysis was utilized to identify alterations in the surface morphology and integrity of the cell membrane of *E. coli* O157:H7 cells following exposure to various antibacterial agents. The results of Fe-SEM showed plantaricin action and damage at the level of the cell wall or membrane. In this study plantaricin caused damage in the cell surfaces and blebs to form all over the indentation cell membrane, giving treated cells a rougher look than normal cells. Additionally, the manipulated cells were only seen in sparse clusters of linked cells (Figure 7). Greatest destruction was seen in plantaricin-treated cells, which also had a few fully lysed cells and surface blebbing (intracellular vesicles), indentations, convolutions, and well-defined wrinkling of the cell wall. In a few instances, the injured cell also had intracellular material flowing out of it **(**Figure 8).

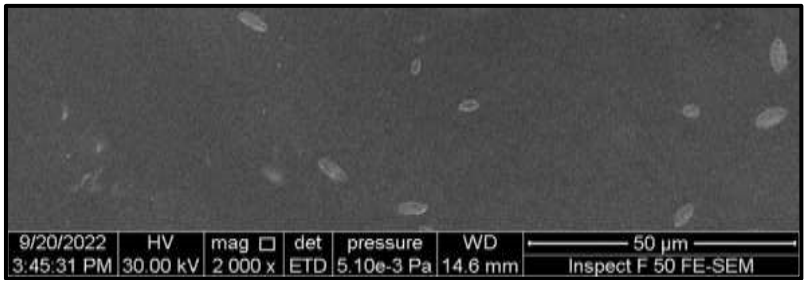


Figure (7): Fe scanning electron microscope photograph show effect of plantaricin on *E.coli* O157 H7 which lead to form intracellular vacuoles and ruptures

These findings showed that plantaracin has negative impacts on cell surface layers and hence entire structure. The bacterial cell wall, following treatment to a concentration of plantaricin equal to one times the minimum inhibitory concentration (MIC), exhibited the presence of vacuoles inside the cells and the release of internal contents by hole development, resulting in the lysis of *E. coli* O157:H7 cells (Figure 8).There have been some hypothesized models for antimicrobial peptide membrane lysis (52). A particular peptide may have various processes in various membrane settings depending on the diversity of the microbial membrane ultrastructure. Multiple experiments have demonstrated that antimicrobial peptides cause barrier holes to develop, ions and intercellular molecules to seep out, metabolic alterations, and death of cells (52). As a consequence of the data, it is clear that plantaricins' inhibitory action is markedly boosted (53). The cellular surface exhibited distinct pitting and rupture, as seen in *Figure 8*. These results are suggestive of the susceptibility of *E. coli* O157:H7 to plantaricin, which aligns with the findings reported by reference 54. The observed action may be related to the chelating properties of plantaricin, as well as its capacity to disrupt the lipid layer of bacterial cells, resulting in cell membrane rupture, cellular depression, and inhibition of the growth of *E. coli* O157:H7.

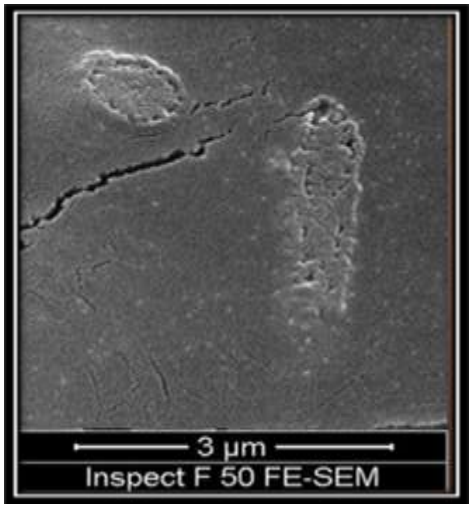


Figure (8) :Fe-scanning-electron microscope photograph shows the effect of Plantaricin on *E.coli* O157 H7 which leads to distraction of cell wall rupture and accumulation of cell debris

Conclusions

I was concluded The Fe-SEM identification indicated that DNA leaked from cells due to the cell lysis of *E.coli* O 157:H7 and plantaricin interacts with the target cell membrane.

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