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EVALUATION OF THE SYNERGISTIC EFFECT OF A NOVEL LENTUSCIN-VANCOMYCIN ANTICANCER AGENT

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ABSTRACT

Vancomycin's declining efficacy against clinical Staphylococcus isolates has sparked worries about the limited availability of antibiotic therapy alternatives. Novel treatments, such as several promising antimicrobial peptides, have come into focus in response. Notably, several experimental investigations have demonstrated the strong biological activity of specific bacteriocins, indicating selective cytotoxicity by specifically targeting distinct cancer cell types in addition to their ability to combat bacterial pathogens. This study succinctly summarizes its objectives, methods, results, and conclusions. The alternative synergistic combination of vancomycin and lentuscin for treating the cancer cell line WRL68 decreased both the cell viability and the IC50 values of A375. The goal of this study was to compare the antimicrobial activity of bacteriocin from Staphylococcus lentus with that of a combination of vancomycin and lentuscin (bacteriocin produced from Staphylococcus lentus). Pathogens like S. lentus were isolated from clinical samples (e.g., mid-stream urine). It is essential to create a novel antimicrobial agent that is synergistic with the antibiotic vancomycin and that has been evaluated for mammalian cell cytotoxicity. The bacteriocin was produced in modified media supplemented with 1% glucose at 37°C for 72 hr with an inoculum size of 1.2 × 109 CFU/ml and a pH of 7. The important steps for the extraction of the bacteriocin were purification with 70% ammonium sulfate salt and purification by DEAE-cellulose chromatography. The alternative synergistic drug vancomycin, combined with lentuscin for treating the cancer cell line, Cell line derived from embryonic liver (WRL68), decreased the cell viability and IC50 values of A375. Conclusion This is the first report demonstrating the anticancer properties of lentuscin and vancomycin. Using these bacteriocins as single agents or in combination would be effective against drug-resistant cancer cells with specific cytotoxicity. This is the first report on the synergistic anticancer effect of lentuscin (a bacteriocin from S. lentus) with vancomycin.

Keywords: *S. lentus*, Primary Screening, Antimicrobial peptides, WRL68, vancomycin, mammalian cell cytotoxicity

INTRODUCTION

Prokaryotic antimicrobial peptides, also known by another name as bacteriocins, belong to a distinct taxonomic group. Bacteriocin is synthesized ribosomally as a protein with antibacterial activity against other bacteria **(Sugrue et al.,2020)**. The molecular weights of most active peptides decrease because of the cationic nature of the membrane, which results in pore formation, leading to cell death. Antimicrobial peptides contribute significantly to infection-related global mortality (**Norouzi *et al*., 2018**). Plasmid-encoded or chromosomal genes encode bacteriocins, including genes for the active protein, resistance mechanisms, and export systems (**Ahmed *et al*., 2023**). Bacteriocins are short polypeptides or proteins that are ribosomally synthesized, have antibacterial properties, and are closely associated with their producing strains. Bacteriocins work by engaging particular surface receptors on cells and eliminating them. Cells within a microbial community may be resistant to each bacteriocin, sensitive to it, or bacteriocinogenic (able to produce bacteriocin). Only a tiny portion of bacteriocinogenic cells will be stimulated to generate and release bacteriocin when all three cell types are present and compete for limited resources (**Ahmed *et al*., 2025**). According to the World Health Organization, cancer is the second leading cause of death globally. Compared with antibiotics, bacteriocins are smaller polypeptides encoded by genes that exhibit high activity (**Ahmed & Al-Awadi, 2024**). In bioengineering, in vitro cloning of biosynthetic bacteriocin genes is required for antimicrobial peptide production (**Abdulhasan *et al*., 2020**). Skin inflammation is caused by Staphylococcus spp bacteria. Thus, this study aimed to identify the species involved in staphylococcal acne and to determine how diverse it is. Using 16S rRNA gene amplification and sequencing as a basis (**Ďuračka *et al*., 2021**), Staphylococcus. Ventus is an important pathogen that causes a wide variety of diseases in humans, ranging from skin tissue infections to diseases (**Fawzi & Ahmed, 2024**). Peptide toxins called bacteriocins, which are produced by bacteria, have great promise as adjuncts or alternatives for existing therapeutic agents. Bactericidal peptides are not effective against producer strains of bacteria, but these poisonous peptides show considerable activity against some bacteria, particularly multidrug-resistant (MDR) species (**Karanam *et al*, 2020**). The complex process of cytotoxicity affects several variables and pathways. These characteristics represent the activities of cellular organelles that are triggered by drugs to undergo necrosis and apoptosis. Nuclear morphology, membrane permeability, and mitochondrial function—all of which result in the release of cytochrome C from mitochondria and a reduction in the mitochondrial membrane potential are excellent examples of cytotoxicity parameters (**Romi & Ahmed, 2024**). Even if traditional chemotherapy is still the mainstay of cancer treatment, its serious side effects and the rising problem of drug resistance make the hunt for more efficient and less harmful anticancer medications valuable. Bacteriocins are antimicrobial peptides of microbial origin that have attracted a lot of attention lately because of their targeted anticancer effect. Their cationic and amphiphilic properties allow them to selectively destroy tumor cells while sparing normal cells (**Wang, Y *et al*.,2024**) In the present study, S. lentus, which was isolated from a clinical sample, was used to produce, purify, and characterize synergistic antibiotic-bacteriocin compounds in a cytotoxic assay. antibiotic resistance (declining vancomycin sensitivity) and explores alternatives (bacteriocins) with dual antimicrobial/anticancer potential. The growing isolation of pathogens and opportunistic bacteria, such as S. lentus, from clinical samples underscores their rising clinical relevance in both hospitals and community-acquired infections. Nonetheless, some S. lentus strains are also capable of producing antimicrobial peptides, such as lentuscin, which could be a defensive mechanism in microbial environments. Using these bioactive substances presents a viable path toward the creation of new antimicrobial drugs, especially in light of the growing prevalence of multidrug resistance. The discovery and description of lentuscin from S. lentus highlight the therapeutic potential of bacteriocins, which, when paired with traditional medications like vancomycin, can contribute not only to infection control but also to cancer treatment.

MATERIALS AND METHODS

Seventy-five seminal fluid samples were obtained from genital tract infection patients. Clinical samples were obtained from patients receiving care at Baghdad Medical City and Al-Yarmook Teaching Hospital in Baghdad, Iraq. Clean cup and "Mid-stream urine samples were collected from patients to minimize contamination and improve the reliability of microbial cultures." were collected in

sterile containers, and within 30 minutes of collection, the samples were cultured on both blood and mannitol salt agar. If not, the urine samples were cultured after being kept at 4°C for a while (**Ahmed *et al*., 2021**). The patients' seminal fluid samples were collected and placed in a sterile container. After the patient had not had coitus for five to seven days, the semen was extracted. Three hours after collection, the semen was cultured on mannitol salt agar and blood agar according to the World Health Organization (WHO) standards. The indicator strains, which included both gram-positive and gram-negative bacteria obtained from the College of Science/Baghdad University, were identified by the Vitek 2 system).

Antibiotic Sensitivity Test

Using a caste containing 16 different antibiotics—benzylpenicillin, oxacillin, gentamicin, levofloxacin, moxifloxacin, erythromycin, clindamycin, linezolid, teicoplanin, tetracycline, vancomycin, tigecycline, nitrofurantoin, fusidic acid, rifampicin, and trimethoprim/sulfamethoxazole finally, identification by the Vitek 2-compact system was used to test the antibiotic sensitivity of all 60 isolates. Mueller Hinton broth was used to cultivate the bacteria, and the resulting suspension was then incubated for eighteen hours at 37°C (**Romi & Ahmed, 2024**).

Determination of the minimum inhibitory concentration (MIC) of vancomycin

Vancomycin's antibacterial activity towards both gram-positive and gram-negative isolates has been measured using the broth microdilution method (Mais and Sana, 2018). In 1 milliliter of Mueller-Hinton broth (MHB), each vancomycin solution (64 µg/ml stock solution) was serially diluted twice at concentrations varying from 4 to 64 µg/ml. 100 µl of vancomycin dilutions were injected in ten microplate wells that contained 10 µl of a bacterial solution with a turbidity of 0.5 McFarland standard. A bacterial suspension with any additions was used for the positive control, whereas MHB medium served as the negative control. The smallest concentration that prevents noticeable development is known as the MIC. According the guidelines provided by **CLSI (2023**), the minimum inhibitory concentration (MIC) of vancomycin for each isolate was established. For twenty-four hours, the microplate was incubated at 37°C. Following the measurement of the optical density at 450 nm using a microtiter plate reader, the growth of bacteria was examined in each well. The MIC was defined as the smallest concentration that prevented discernible growth. (**Mahdi *et al*, 2024**).

Detection of bacteriocin production

Primary Screening

Inoculum of bacteria S. lentus (1.5×108 CFU/ml) was inoculation with Muller Hinton Broth for two days at 37°C in an aerobic environment. The cultures were centrifuged for 15 minutes at 6000 rpm. On MHA agar, 0.1 ml of the activated test bacterial solution (108 cells/ml) was spread out. A sterile 6-millimeter cork borer was used to create wells in the pour plates, and 100 microliters of cell-free supernatant (CFS) were placed inside (**Mahdi, S. M *et al*, 2024)**. After two hours at room temperature, the plates were incubated for 18 to 24 hours at 37°C. Ultimately, the well-formed inhibitory zones were measured in millimeters and contrasted with those of the control group, which contained only MHB broth. Using the bacteriocin production isolate that was chosen from the screening section, multiple optimization experiments were carried out to determine the medium and culture conditions that promoted the maximal production of bacteriocin **(Ahmed & Seddiq, 2018).**

Bacteriocin activity assay

The bacteriocin-like inhibitory substances, BLIS or crude bacteriocin, were serially diluted two times with physiological saline solution to measure the bacteriocin activity. Using an agar well diffusion experiment, these dilutions were utilized to test the antibacterial activity of the bacteriocin against the indicator yeast (as previously described). The definition of bacteriocin activity, which was given as AU/ml, was the reciprocal of the maximum dilution that revealed a clear inhibitory zone of the indicator test (**Ahmed, M. E, 2018).**

Optimization of bacteriocin production

Effects of sugar sources: Three different sugars (glucose, sucrose, and esculin) were introduced to the media MHB. One percent of each of the sugars was added to the culture medium that supported the highest generation of bacteriocin without carbohydrates, and four different cultures were examined individually. One percent of the 18–24-hour-old bacterial isolate culture was added to each medium, and the mixture was then incubated for 24 hours at 37°C. The amount of protein and MRSA activity was measured. The best carbon source's ideal concentration was found by individually adding 1% of the carbon source to broth without glucose, inoculating the solution with 1% of the isolate for 18 hours of culture, and then incubating the mixture at 37°C for 18 to 24 hours (**Mais *et al*., 2022**).

Effects of temperature on growth and bacteriocin production

To investigate the suppressive impact of incubation temperature, the bacterial isolates were cultured in the best production media at various incubation temperatures (25, 30, 37, 40, and 45°C). A 10-milliliter inoculation of the producer strain (1.5 × 108 CFU/ml) was followed by a 24-hour aerobic incubation period (**Mun *et al.*,2012)**

Influence of pH

The impact of pH on the development and bacteriocin production of the isolates was investigated. Brain heart infusion broth was prepared in 10-milliliter tubes at various pH levels, as shown in numbers 5, 6, 7, 8, and 9, using HCl or one-nail NaOH after autoclaving. Then, the tubes were infected with specific isolates and incubated for 24 hours at 37°C in an aerobic environment. Following a pH shift, bacterial growth and bacteriocin production were measured**.**

Partial purification of the bacteriocin

The bacterial isolate that produced lentuscin was added to MHB broth, which was then incubated for 48 hours at 37°C. Centrifugation was performed for 15 minutes at 6000 rpm to harvest the cells. The CFS was heated to 80°C for ten minutes, cooled, and then centrifuged for fifteen minutes at 6000 rpm(**Cherukuri, P *et al*.,2019**). After the pH was neutralized with 1 N NaOH and solid ammonium sulfate was added at the saturation level (70%), 720 mL of CFS was precipitated. This was followed by a one-hour incubation period to confirm the presence of bacteriocin. Ammonium sulfate (209.52 g) was gradually added to the CFS at a 70% concentration, and the mixture was continuously mixed for one hour while stirring in an ice bath. Following an overnight period of constant mixing at 4°C.The mixture was centrifuged for 20 minutes at 4°C at 10,000 rpm. The production of bacteriocins was the reason for screening. Following the acquisition of the material in its raw form and the application of Lowry's method (**Lowry, O *et al*.,1951**) to measure the protein content, primary and secondary screening approaches were employed. The bacteriocin activity was expressed as AU/mL. AU was computed as (1000/100) × D, where D is the dilution factor, 1000 is the constant, and 100 is the supernatant volume in a well (µl). Figure 1 shows the steps of purification bacteriocin.

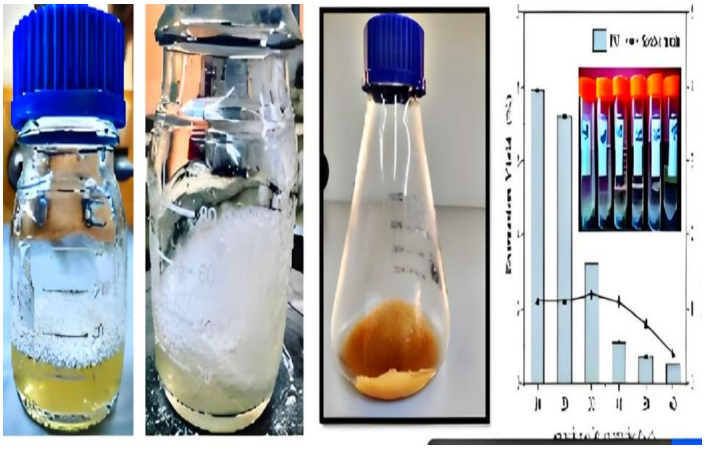


Figure 1 Partial purification of the bacteriocin

Dialysis

After being boiled for five minutes in a solution containing 0.05% EDTA (ethyl diamine tetraacetic acid) and 2% (w/v) sodium bicarbonate (NaHCO3), a sufficient-length dialysis membrane (SIGMA) was chilled and then boiled once again for ten minutes in sterile distilled water. The bottom of the dialysis membrane was sealed, and a precipitate that had been dissolved in 10 milliliters of pH 7 buffer was added through the other opening. The membrane was then once again tightly sealed. To allow for moderate swirling of the dialysis membrane, the membrane was placed in a beaker with one liter of the same buffer and placed on a magnetic stirrer. The dialysis process was conducted overnight at 4°C with numerous buffer changes to eliminate ammonium sulfate from the bacteriocin, which could impede subsequent purification tests(**Ahmed, M *et al*.,2018**). Figure 2: Steps of Partial purification.

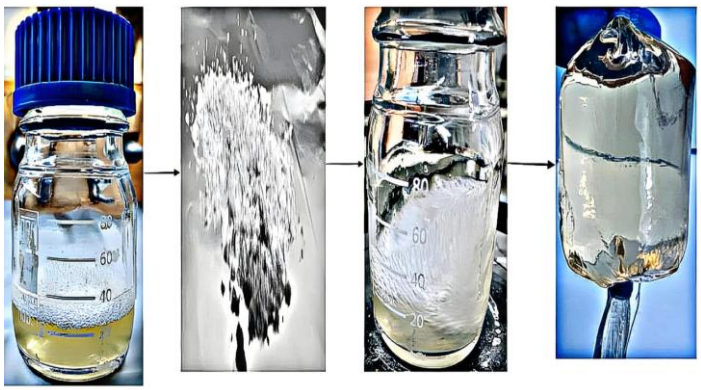


Figure 2 Stapes of Partial purification of lentuscin

Ion-exchange chromatography (DEAE-cellulose)

A total of 20 g of DEAE-cellulose powder was used for this purpose. The solution was then suspended in 1000 mL of DW and allowed to settle. This process was repeated multiple times until the supernatant became clear, after which the supernatant was discarded. After 30 minutes of activation with 0.25 N HCl, DEAE-cellulose was filtered through a Buchner funnel filled with Whatman No. 1 filter paper and rinsed three times with DW. Next, 0.25 N NaOH was used to activate the DEAE cellulose. There were two iterations of the filtering and washing procedures. After being equilibrated with 10 mM Tris buffer (pH 7.5), the activated DEAE-cellulose was packed in a column measuring 2 × 40 cm. The ion-exchanger column was filled with concentrated protein from the earlier stages. For washing, the flow rate was set at 0.8 milliliters per minute (**Stellwagen, E. 1990**): Fig. 3 Step of Ion-exchange chromatography (DEAE-cellulose)

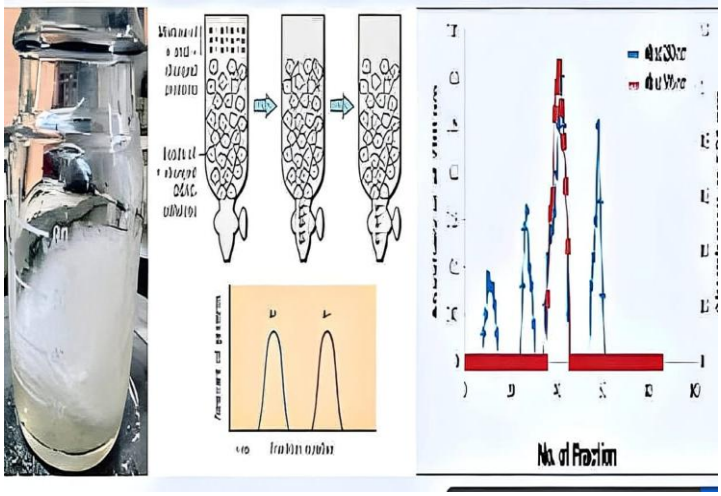


Figure 3 Ion-exchange chromatography (DEAE-cellulose)

Determination of molecular weight

The molecular weight of lentuscin was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to previous methods.

Cell line maintenance

After being suspended in complete Roswell Park Memorial Institute Medium (RPMI media), Cells derived from embryonic liver (WRL68). A375 cells were propagated in culture flasks for 24 hours at 37°C in a humidified environment with 5% CO_2. The growth medium was removed, and the sticky cells were rinsed twice with PBS once the cells reached 80% confluency. After adding two to three milliliters of trypsin/versene solution to the flask, the mixture was gently shaken to completely cover the monolayer. The flask was incubated for one to two minutes at 37°C or until the cells were separated from the flask surface.Using the trypan exclusion cell counting method, the necessary cell concentration was achieved by combining one volume of cell suspension with one volume of trypan blue stain. At three-minute intervals, the cells were counted under a microscope with a hemocytometer according to the following formula: Total Cell Count/ml = Cell count × Sample Volume × Dilution Factor × 10^4 In 96 flat-well microtiter plates, cells ($1x10^4$–$1x10^6$ cells/ml) were cultured in a final volume of 200 µl of complete culture media (RPMI-1640) in each well. Sterilized parafilm was added to the microplate, and the plate was carefully shaken. The plates were incubated for 24 hours at 37°C with 5% CO2 (**Zahraa *et al*., 2022**).

Cytotoxic Assay

Isolation of human lymphocytes

Lymphocytes were isolated from the plasma layer and transferred to a new 15 ml tube; the cells were then diluted with 10 ml of PBS and centrifuged at 1000 g to extract the supernatant. 4) and incubated for five minutes at 37°C. The lymphocytes were then suspended in erythrocyte lysis buffer (150 mM NH4Cl, 10 mM NaHCO3, 1 mM). The donor was a healthy nonsmoker at the time of blood collection (**Mais and Khadija, 2020)**

Ethical Statement

This research was approved by the Committee of Ethical Standards in the College of Science, University of Baghdad. The study protocol, the subject information, and the consent form were reviewed and approved by a local ethics committee according to document number CSEC/1022/0131 dated October 22, 2021.

Statistical analysis

GraphPad Prism 6 was used for statistical analysis. The study utilized the unpaired t-test. Conclusions have been displayed using the standard deviation as the mean of the three tests (SD).

RESULTS

Every sample was grown directly on blood agar and mannitol salt agar plates (Figure 4). Overall, only 42 of the samples tested positive for *S. lentus* (Table 1). The numbers for the isolates were L1–L42. Based on the morphology of the colonies on mannitol salt agar, blood agar, Gram stain, and the catalase test, identification was performed. These colonies were smaller than the characteristic yellow pinhead colonies of S. *lentus*. Clusters of gram-positive cocci were visible in the colonies on Gram's stain, and the catalase test yielded positive results, while S. *aureus* showed full mannitol sugar fermentation as colony the yellow verify the diagnosis of bacterial isolates extracted from clinical samples, the System Compact Vitek-2 was used. This system offers the 64 biochemical assays required for the diagnosis of bacterial isolates. *S. lentus* was confirmed as a chosen organism with a probability of 98–99% by the Vitek 2 method.

Table 1 Prevalence of *S. lentus* and *Staphylococcus* spp. Isolates in clinical samples

Source of Samples	No. of Samples	No of *Staphylococcus spp* (%)	No. of *S lentus* (%)
Seminal fluid	75	33(44%)	42(56%)
Total	75	33	42

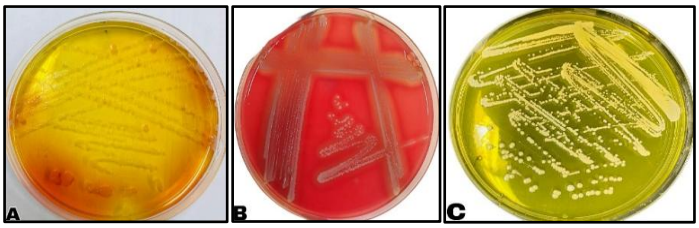


Figure 4 A) *S. lentus* on mannitol salt agar (SA) B) *S. aureus* on blood agar at 37°C for 24 hr

Antibiotic susceptibility test

According to the findings, all 42 (100%) isolates exhibited high levels of resistance to benzylpenicillin and oxacillin. Erythromycin (90%), fusidic acid (86.6%), and tetracycline (85%) were the next most resistant antibiotics. Among the isolates, 71.6% were resistant to both gentamicin and clindamycin. The isolates showed a moderate level of resistance to trimethoprim/sulfamethoxazole (43.3%) and loxifloxacin (58.3%). Teicoplanin (28.3%), levofloxacin, and rifampicin (26.6%) had the highest degree of resistance, followed by vancomycin (20%) and nitrofurantoin (3%). All (100%) of the isolates displayed a significant degree of sensitivity to TGC and linzolid. Figure (5)

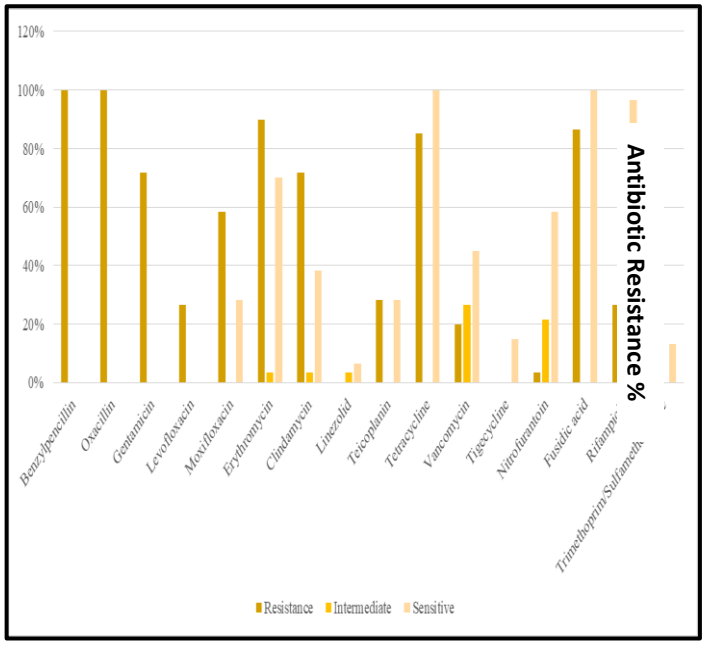


Figure 5 Percentage of antibiotic profiles for *S. lentus* isolates

Screening for the Production of Crude Bacteriocin

Based on their broadest inhibition zone, which measured 34 mm for the basic indicator *S. aureus,* the *S. lentus* L_6 isolate from seminal fluid, the bacteriocins produced by these strains had considerable antibacterial activity. Among all 8 isolates (L_1, L_2, L_3, L_4, L_5, L_6, L_7, and L_8), one was determined to be a good generator of crude lentiscus. The Agar well diffusion assay (AWD) was used to investigate the bacterial productivity of the bacteriocin against test isolation *S. aureus* Figure 6)

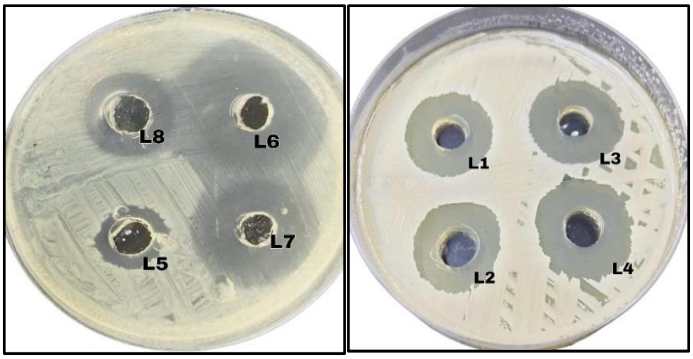


Figure 6 Screening of Crud lentuscin from *eight isolates of S. lentus* against *S. aureus* on MHA at 37°C for 72 hr via the WDA Method

Optimal Carbon Source

The various carbon sources of sugars are linked to the consumption of these sugars by bacteria during metabolic processes. The growth of *S. lentus* was observed in the presence of 1% glucose, and the optimal carbon source for isolation had the largest inhibition zone (39 mm) in comparison to other carbon sources and no source at all. When comparing the carbon sources (sucrose, ethanol, and glucose) without any carbon source, glucose was the most effective, whereas sucrose had the least impact. (Figure 7)

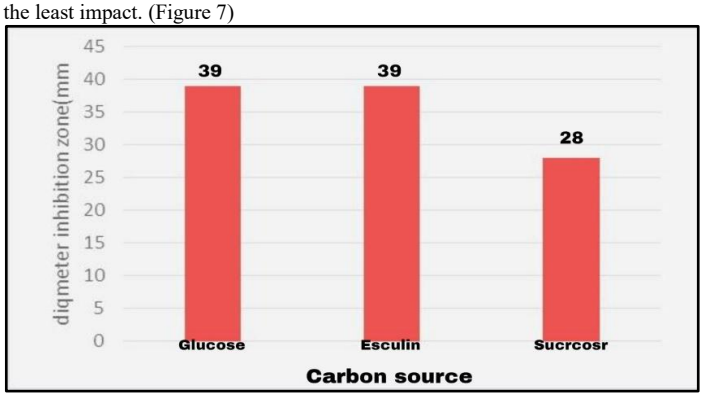


Figure 7 Effect of carbon source on lentuscin synthesis

Effect of the Incubation Temperature

Although it had little effect on the ultimate cell yield, temperature was shown to be the most significant element influencing the lag phase of isolation. Temperature also played a significant role in the proliferation of bacteria. Furthermore, throughout the exponential growth phase, the bacteriocin activity of the strains increased steadily, peaking at the end of the growth phase. Temperature is essential for both the development of bacteria and the synthesis of bacteriocin. The experiment revealed that the ideal temperature for the formation of bacteriocin was 37°C, with maximum production being reached at that temperature, and inhibitory zones against the studied bacteria measuring 32 mm. Figure 8.

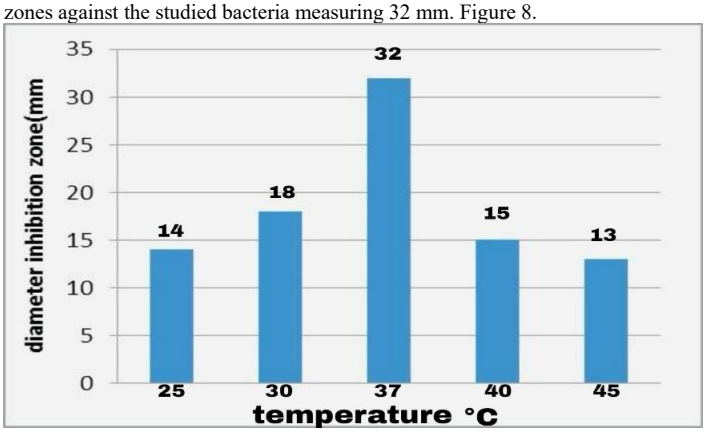


Figure 8 Effect of temperature on the effect of medium pH on lentiscus production

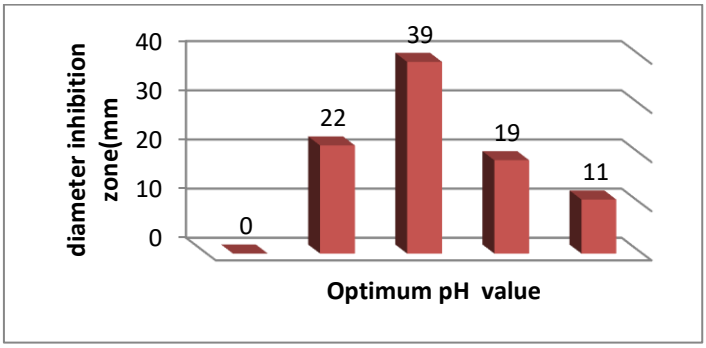


Figure 9 Effect of PH on lentuscin production

The optimal pH for the growth of *S. lentus* was determined. Since the normal pH of the stratum corneum is very high, a wide range of pH values was used to simulate the pH change in human fluid and to cover all the possible scenarios. The ideal pH for the growth of S. *lentus* was determined. Since the usual pH of the stratum is 4.1–7, a wide range of pH values was employed to simulate the pH change in human fluid and to cover all possible scenarios. Figure 9.

Purification of lentuscin

DEAE cellulose column chromatography was used for further purification. Crude bacterocin It was discovered that while the protein quantity declined at each stage, from the crude to the final active fraction, the antibacterial activity progressively increased. The specific activity increased from 1.30 to 1.20 mg/ml. Ion exchange chromatography (IEC) was applied to the concentrated bacteriocin on a DEAE-cellulose column in a sequential manner. The fractions from which the isolates were isolated (23, 42, and 63) showed the highest bacteriocin activity in the elution profile at an absorption wavelength of 280 nm, as shown in Figure 10.

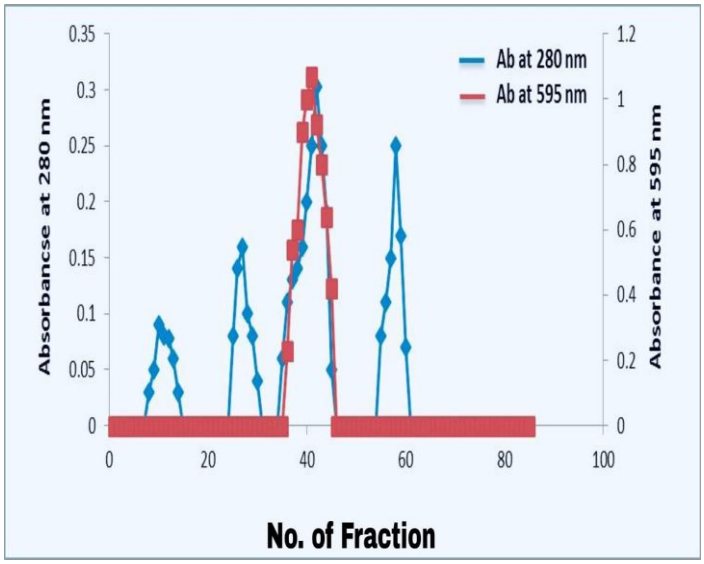


Figure 10 Purification of lentuscin by ion exchange chromatography using a DEAE-cellulose column (2×40 cm).

Molecular Mass Determination

Using Tricine-SDS–PAGE, purified bacteriocin samples obtained from various purification stages were examined. The resulting bacteriocin is a single active peptide in nature, as indicated by the appearance of a single target band in Figure 11. Upon determining the length of the gel and the sample migration distance relative to the protein standard (Biolabs, England; broad range: 10-250 kDa), the molecular mass of *S. lentus* was approximately 29 kDa. Purified bacteriocin samples from different purification stages were examined using Tricine-SDS-PAGE. A single significant band at about 29 kDa was found (Fig. 11), indicating that the bacteriocin is a single active peptide. By comparing the band's migration distance with that of a broad-range protein marker (10–250 kDa, Biolabs, England), the molecular mass was determined. Although the homogeneity of the purified peptide is supported by the presence of a single band, it is crucial to remember that the antibacterial and anticancer properties of this particular band were not explicitly verified. To verify that the biological effects reported are solely attributable to the 29 kDa peptide, no functional assays such as activity staining, in-gel bioautography, or band elution and testing were performed. Therefore, although the results suggest a link between this band and bioactivity, more validation is required to definitively attribute the antibacterial and anticancer properties of the 29 kDa protein.

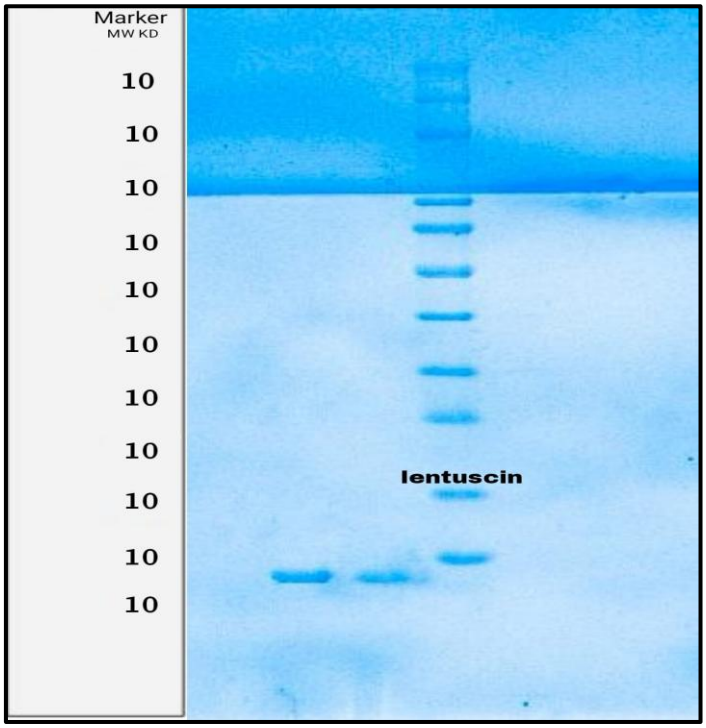


Figure 11 SDS–PAGE of Purified lentuscin determined Molecular weight marker

Even while the purified fractions' SDS-PAGE examination showed a noticeable protein band of about 29 kDa, this result by itself does not prove that the observed antibacterial and anticancer properties are caused by this particular protein. It is still possible that the bioactive component could be a distinct molecule, a co-purified chemical, or a post-translationally changed variation of the detected band in the absence of direct evidence connecting the band to biological activity. Additional research is necessary to confirm lentuscin's functional identification. To verify activity retention, these ought to involve the removal and elution of the 29 kDa band, followed by bioassays**.**

Minimum inhibitory concentration of vancomycin

Furthermore, earlier research has shown that exposure to beta-lactam antibiotics also increases vancomycin activity. One of the main mechanisms by which *Staphylococcus aureus* is resistant to vancomycin is a thicker cell wall. Furthermore, earlier research has shown that exposure to beta-lactam antibiotics also increases vancomycin activity. One of the main mechanisms by which *Staphylococcus aureus* is resistant to vancomycin is a thicker cell wall. Table 2 presents a comparative analysis of the inhibition zone diameter of every test bacterium following treatment with varying vancomycin concentrations (4, 8, 16, 32, and 64 µg/ml). The lowest concentration (4 µg/ml) and the lowest concentration of *S. aureus* strongly inhibited each other. Using the good diffusion assay (WDA), the inhibitory zone size of 14 mm was widened with increasing concentrations (32 µg/ml) of the tested *P. aeruginosa strains*.

Table 2 Mean (± SD) Zone of Bacterial Inhibition in mm with Different Concentrations (in µg/mL) of Vancomycin

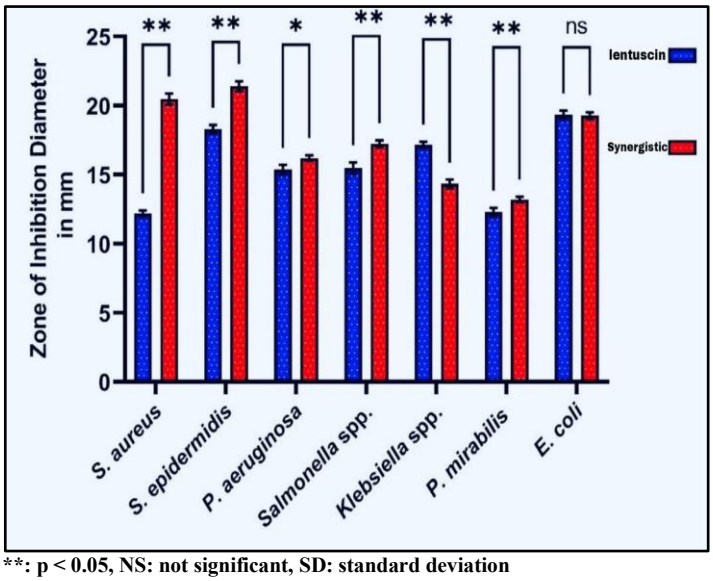
Bacterial Isolate	Mean ± SD Zone of Bacterial Inhibition in mm treated with vancomycin					*p* Value
	4	8	16	32	64	
S. aureus	5.2±0.2[a]	8.23±0.21[b]	10.23±0.3[c]	13.13±1.2[d]	15.2±0.8[e]	<0.0001 **
S. epidermidis	5.2±0.2	8.45±0.3[b]	9.3±0.3[b]	11.91±0.8[c]	12.17±0.21[c]	<0.0001 **
***Klebsiella* Sp.**	3.3±0.3[a]	6.3±0.25[b]	9.3±0.31[c]	11.27±0.4[d]	13.6±0.79[e]	<0.0001 **
P. aeruginosa	4.16±0.14[a]	7.13±0.16[b]	10.27±0.6[c]	14.3±0.4[d]	16.23±0.5[e]	<0.0001 **
E. coli	3.2±0.15[a]	4.7±0.31[b]	8.63±0.31[c]	10.28±0.67[d]	12.11±0.3[e]	<0.0001 **
P. mirabilis	3.17±0.19[a]	6.4±0.35[b]	10.2±0.21[c]	11.27±0.45[d]	12.2±0.2[e]	<0.0001 **

Different letters (a, b, c, d, e) indicate significant differences at $p < 0.05$ between groups in

Synergistic effect of the MICs of vancomycin and lentuscin

Vancomycin activity is therefore facilitated by a decrease in the cell wall thickness of beta-lactams. Additionally, it appears that beta-lactams chemically and morphologically modify the surface of *S. aureus*, enhancing target-specific vancomycin binding. The results of this study offer more proof of the advantages of utilizing beta-lactams in conjunction with vancomycin-lentuscin against various bacterial species, even though the combination of MIC values may not directly translate into therapeutic benefit. To ascertain the clinical value of this unique combination, more research is necessary. The antibacterial activity of lentuscin and antibiotics, together with their mechanisms of action at various bacterial species' cellular levels, were examined and described in the preceding section. Moreover, antibiotics and nanostructured materials can be used in tandem to strengthen their antibacterial properties. Figure. 12 shows that lentuscin and the synergistic combination of VAN and lentuscin are more effective against the gram-positive bacteria *S. aureus* and *S. epidermidis* than against other gram-negative bacteria. Vancomycin and β-lactams can facilitate the entry of vancomycin into bacterial cells by creating a route that could work in concert. Furthermore, identifying the

sensitivity of antimicrobial drugs to collateral effects may provide new insight into the synergistic effects of vancomycin and bacteriocin.



**: p < 0.05, NS: not significant, SD: standard deviation

Figure 12 Mean ± SD zone of bacterial inhibition in mm after treatment with the synergistic agent. Standard deviation, (n = 3).

Vancomycin and lentuscin work in concert to show encouraging antibacterial capabilities, especially against gram-positive bacteria like Staphylococcus aureus and Staphylococcus epidermidis. This synergy is most likely caused by beta-lactam-induced changes to the architecture of the bacterial cell wall, such as decreased thickness and surface modifications, which improve the binding efficacy of vancomycin. Without more testing, the encouraging synergy at the MIC level might not immediately translate into clinical efficacy. Furthermore, combining antibiotics like lentuscin with nanostructured materials may enhance their antibacterial properties. To completely determine the therapeutic benefit and molecular underpinnings of vancomycin–lentuscin synergy, future studies should investigate in vivo models and evaluate collateral sensitivity patterns.

Evidence of A Synergistic Effect on Tumor Cell Lines

In the A375 melanoma cell line, the combination of lentuscin and vancomycin dramatically reduced IC50 values and cell survival, suggesting a synergistic anticancer impact. Previously, however, the article confused these findings with effects on the non-tumorigenic liver cell line WRL68. Making a precise distinction between these two cell lines is crucial: The cancer model is represented by A375, and the reference for normal cell toxicity is WRL68. A degree of selectivity is supported by the lesser cytotoxicity seen in WRL68 as opposed to A375, indicating that lentuscin, either by itself or in conjunction with vancomycin, preferentially targets cancerous cells. Antimicrobial agents were assessed for their potential anticancer effects on skin and human melanoma cell lines using the MTT assay because of their increased cytotoxicity toward melanoma cancer cells. The greatest challenges to the therapeutic use of bacteriocins still involve systemic safety and long-term consequences on the body are currently defining their pharmacokinetic profiles to better comprehend how polypeptides act inside the body. Necrosis is caused by bacteriocin peptides interacting with the cell surface, which target and kill tumor-forming cells only while sparing healthy, normal cells. the synthesis of bacteriocins from *Staphylococcus* sp. bacteria, their interactions with cancer cell lines, and various additional applications. Necrosis is caused by bacteriocin peptides interacting with the cell surface, which target and kill tumor-forming cells only while sparing healthy, normal cells. A cell viability test (perhaps an MTT or a comparable assay) assessing the effects of a treatment on two cell lines is depicted in the image you provided: WRL68: Blue circles represent a normal liver cell line. Red squares represent the human melanoma cancer cell line A375. As the treatment concentration (log µg/ml) increases, the viability of both cell lines declines.

- Half-maximal inhibitory concentrations, or IC_{50}, are as follows:
- The IC_{50} for tumor cells A375 is 120.7 µg/ml.
- WRL68: IC_{50} = 152.4 µg/ml (normal cells)

Compared to normal cell viability, the therapy is more successful at reducing cancer cell viability.A375's lower IC_{50} value suggests that cancer cells are more sensitive to therapy. Partial selectivity is shown by the moderate discrepancy in IC_{50} values. Lentuscin-vancomycin combination's dose-dependent cytotoxicity against cancer cell lines (A375 melanoma, WRL68 hepatic embryonic), with A375's IC50 being lower (120.7 µg/mL) than WRL68's (152.4 µg/mL).The cytotoxic effects of the synergistic agent on the cell line were studied, as shown in Figure 13. The synergistic cytotoxic effect of lentuscin+ VAN was observed in the normal cell line A375 and the tumor cell line WRL68. The IC50 of A375 reached 120.7 µg/mL, while the IC50 of WRL68 (152.4 (µg/mL) increased with increasing synergistic concentration and displayed a dose-dependent sequence of progressive cytotoxicity beginning at lower concentrations, with 45% inhibition of A375 cells and 30% inhibition of WRL68 cells. The inhibition rate of WRL68 cells significantly ($p \leq 0.05$) increased with increasing synergistic concentration compared with that of normal A375 cells, especially at concentrations of 25, 50, and 100 (µg/mL). WRL68 is a non-tumorigenic human embryonic liver cell line that is frequently used as a normal control; it is wrong to classify it as a tumor cell line. WRL68's recognized role is contradicted if the study approaches it as a cancer model, which necessitates a compelling experimental or literature-based explanation. Furthermore, it defies expectations to find a greater IC50 in WRL68 compared to A375 (a verified melanoma line) because normal cells normally exhibit reduced, not higher, tolerance to anticancer drugs. Claims of selectivity are called into question by this disparity, which needs to be explained to prevent misunderstandings.

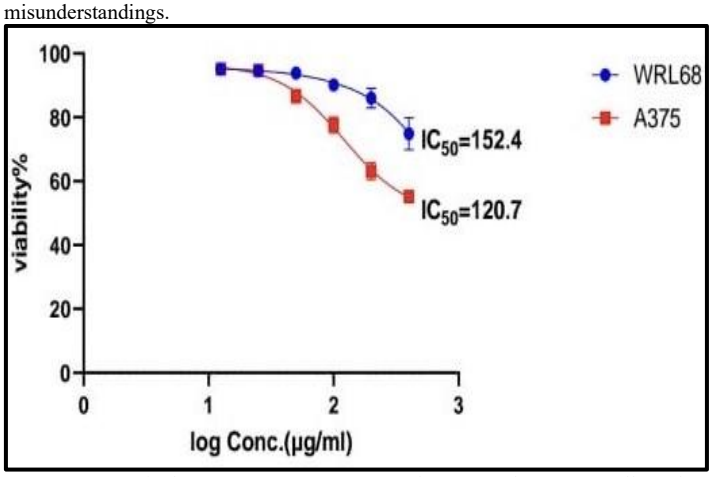
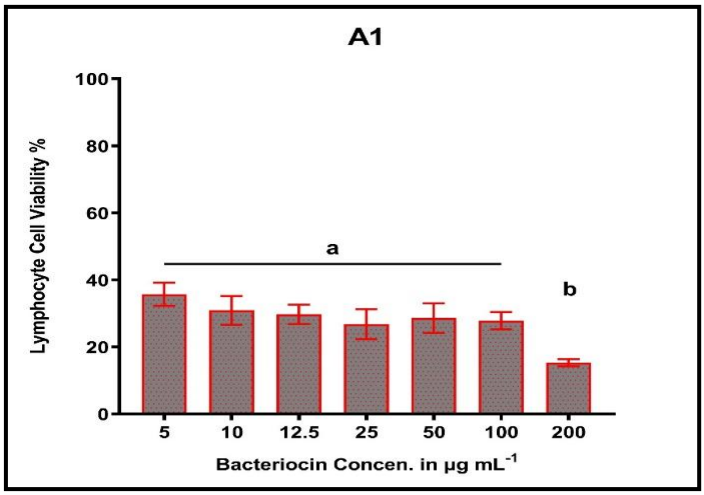


Figure 13 Survival curves (means±SDss%) of A375 and WRL68 cells. After treatment with the synergistic agent using an MTT in vitro assay at 37°C and 5% CO2 for 24 hr. **: $p \leq 0.01$, NS: not significant, SD: standard deviation, n = 3.

Cytotoxicity Test

Finally, the MTT test was used to assess the cytotoxicity of the two drugs on lymphocytes. These findings indicated that these chemicals are safe at active concentrations. The results of the cytotoxicity assays revealed no significant reduction in the viability of human lymphocytes treated with the synergistic agent. The cytotoxic effect on lymphocyte viability was evaluated after the cell cultures were exposed to the components for approximately 24 hours. The results of the MTT assay confirmed the presence of bacteriocin. An almost remarkable reduction in viability was observed in isolation, which exhibited a range of 35% cell viability, as shown in Figure 14. These findings showed that bacteriocin purification could be a good alternative to the current physical and chemical methods associated with environmental toxicity.



**: $p < 0.01$, NS: not significant, SD: standard deviation.

Figure 14 Mean (± SD) viability of human lymphocytes after treatment with the synergistic agent at various concentrations (5, 10, 12.5, 25, 50, 100, and 200 µg mL-1) compared with that of untreated cells (standard deviation, n = 3).

DISCUSSION

In contrast to the usual yellow pinhead colonies of S. aureus, colonies on mannitol salt agar were smaller and had a range of reactions. Bacterial growth occurred on mannitol salt agar media as a selective and differential medium. Some strains displayed yellow fermenter colonies, and other strains showed whitish-pink, non-fermenting colonies for Staphylococcus spp. Isolation from cosmetic tools (**Sara H. Seddiq *et al*., 2023**). These findings generally align with a study conducted by **Faiq & Ahmed (2024)** that showed that 100% and 95% of the isolates were resistant to oxacillin and benzylpenicillin, respectively. In a follow-up investigation, 82.05% and 94.87% of the isolates were resistant to benzylpenicillin and oxacillin, respectively. The present results agree with **Zahraa H *et al.*,2022**, who reported that the well diffusion assay was the best method for identifying the antagonism of microorganisms. Conversely, it has been demonstrated that the bacterium Bacillus, which is derived from Bacillus, exhibits significant efficacy against other types of bacteria. Several recent studies have shown the necessity for novel antimicrobial agents that can effectively combat multidrug-resistant bacteria and are surface-active for use in food technology. P. aeruginosa and MRSA produce antimicrobial proteins known as bacteriocins and crude bacteriocins.

This finding is similar to that of a study performed by **Mais *et al*. (2020).** The composition of growth medium affects the synthesis of bacteriocin, as also noted by (**Ahmed and Kadhim, 2020**), who reported that the primary nutrient sources that significantly impact bacteriocin production are galactose and raffinose. According to response surface methodology, the culture conditions were optimized for a range of carbon and nitrogen sources at different temperatures. The reduction in bacteriocin synthesis at different temperatures is indicative of delayed growth, which in turn causes a delay in bacteriocin production. These results are consistent with previous findings The maximum production of the extracellular bacteriocin niacin by the isolates occurred in a De Man–Rogosa–Sharpe agar (pH 5.0–7.0) at 28°C, and it strongly correlated with bacterial growth. On the other hand, in a study by **Abed *et al*. (2021). Baños *et al*. (2019**) reported that in vitro cytotoxicity assays can be used to determine the range of concentrations at which a drug is safe and effective for causing cell death.

Although the exact mechanism by which lentuscin produces its antibacterial and anticancer properties is still unknown, our results provide some first understanding. Lenuscin has not yet been structurally or mechanistically classified, in contrast to several bacteriocins whose modes of action are well-characterized, such as nisin generating membrane pores or microcin targeting intracellular activities. Our study's selective cytotoxicity against A375 melanoma cells and low toxicity to WRL68 normal liver cells raise the possibility of intracellular targeting specific to tumor cells or selective membrane contact. Additionally, the observed synergy with vancomycin would suggest that lentuscin modifies the permeability or characteristics of the cell surface, which would improve vancomycin binding or uptake. This is especially noteworthy since vancomycin has historically demonstrated little activity against non-bacterial cells, and its observed effectiveness in this case most likely stems from lentuscin-induced modifications to the intracellular environment or tumor cell membrane. Although our viability assays and MIC findings provide indirect support for these concepts, more experimental validation is crucial. Future research using molecular docking, ROS production, apoptosis/necrosis detection, and membrane integrity assays may help clarify whether lentuscin works through a new pathway, causes programmed cell death, or damages membrane structures.

According to previous results (**Kadhim, Zahraa *et al.*, 2023**), however, there are numerous in vivo and in vitro applications of bacteriocin, including food perception, medical field treatment, cytotoxicity, and water contamination treatment. The relationship between the overall cellular uptake of copper and cytotoxicity, which could be directly related to particle stability, is well established; however, it is unknown why intermediate-sized particles, when contrasted with both larger and smaller particles, display these particular characteristics. Finding natural products to help cancer patients overcome their treatment resistance is becoming increasingly popular (**Anwer & Ahmed, 2024**). This investigation investigated the cytotoxic effects of neem plants in three distinct biological models (breast cancer cell lines). However, the in vitro cytotoxic activity of a bactericidal compound against prostate cancer cells has demonstrated the efficacy and perfection of the bacteriocin derived from Staphylococcus hemolysis (**Jasem & Mahmood, 2023**). Targeted mechanistic research is necessary to completely comprehend how lentuscin causes cell death and increases vancomycin action. Future research should specifically look into whether necrosis, apoptosis, or other cell death processes are the cause of the reported cytotoxicity. Annexin V/PI staining if lentuscin causes direct membrane breakdown or programmed cell death. These tests would assist in establishing whether lentuscin, as proposed, alters the composition of membranes or promotes antibiotic penetration. To explain its selectivity and synergistic effects, proteomic or lipidomic profiling may also show whether lentuscin interacts with certain surface receptors or lipid rafts that are specific to cancer cells.

According to MARTIN, **Anandi, *et al*. (2023**), it is well-recognized that Enterococcus faecalis is responsible for several dangerous infections and that it is becoming increasingly resistant to antibiotics, particularly vancomycin. Potential alternative treatments for strains of bacteria resistant to antibiotics include lytic bacteriophages and their endomysia, which can focus on and lyse particular bacterial cells. The results of **Wani *et al*., (2023**). This may result in extremely promising therapeutic effects. A promising bacteriocin candidate was utilized in combination with anti-tuberculosis pharmaceuticals (**Ahmed, M. E *et al*.,2025**). Novelty and relevance: The study addresses the important issue of antibiotic resistance by exploring the potential synergistic effects of bacteriocin and vancomycin on pathogenic bacteria, particularly. This topic is highly relevant in the context of the global challenge of antimicrobial resistance.

The effects of these bacteriocins on the production of biofilms and pre-formed biofilms of both test microorganisms were assessed by evaluating the synergistic activities of a few chosen conventional antibiotics (vancomycin and ciprofloxacin) with these bacteriocins. The findings demonstrated that, in contrast to the strains' capacity to create biofilms, greater concentrations are required to eliminate the metabolic activities of cells within pre-formed biofilms (**Fugaban, *et al*.,2022**). Despite lentuscin's encouraging antibacterial and anticancer properties, the discussion fails to adequately contextualize these results in relation to other bacteriocins that have comparable bioactivities. By triggering apoptosis, compromising membrane integrity, or targeting particular cell surface receptors, several bacteriocins, including nisin, pediocin, and colicin E1, have demonstrated selective cytotoxicity against cancer cells (**Gupta *et al*., 2021**).

On the other hand, For instance, because of its capacity to generate pores, the well-known bacteriocin (VRSAcin) has shown anticancer benefits against head and neck squamous cell carcinoma (**Al-Awadi *et al*., 2025**). Due to a dearth of structural and sequence information, the anticancer mechanism of lentuscin is still primarily conjectural in comparison to these bacteriocins. Additionally, no mechanistic tests were conducted for lentuscin, despite the fact that certain bacteriocins, such as microcin E492, show mitochondrial-dependent apoptosis (**Hetz *et al*., 2002**). Therefore, it is challenging to ascertain if lentuscin belongs to a new class of anticancer bacteriocins or shares conserved characteristics with well-known peptides in the absence of comparable functional evidence. Future research should focus on molecular characterization of lentuscin and a direct comparison of its mechanism and activity spectrum to those of known bacteriocins. To the best of our knowledge, this is the first study to demonstrate a synergistic anticancer effect of lentuscin and vancomycin. Although the antibacterial or cytotoxic qualities of both drugs have been investigated separately, their combined impact on cancer cell lines has not been investigated in any prior studies. This new synergy points to a promising treatment path, especially when it comes to aggressive or drug-resistant tumor forms. Our research expands the possible uses of bacteriocins beyond their traditional antibacterial range and presents a novel combinatorial strategy that may improve efficacy while reducing the dosage of each agent, which would lessen toxicity. This innovative approach opens new possibilities for developing dual-action antimicrobial–anticancer therapies.

CONCLUSION

Matrix screening of antibiotic combinations revealed that vancomycin and lentuscin worked synergistically to prevent bacterial infections. An in vivo cell line tumor and lymphocyte further demonstrated the effectiveness of these two combinations. To better understand medication interactions, studies concentrating on the synergism and mechanism of the combinations should be conducted. Enhanced efficacy, preventing the evolution of resistant strains, enhanced bactericidal activity, fewer side effects, and decreased treatment costs are some of the potential benefits of antimicrobial combination therapy.

Overall Assessment

- Impact: High potential for antimicrobial/anticancer applications but requires deeper mechanistic and in vivo validation.
- Originality: First report on lentiscus-vancomycin synergism against cancer cells.
- Language: Requires moderate editing for clarity and precision

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Code availability: Not applicable.

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Author Contribution: Mais Emad. Ahmed and **Zainab Hussein Mahdi** carried out the experiment and wrote the manuscript with input from another authors. **Lina Abdulameer S. Alsaadi** and **Rana S. Aboud** supervise the project and conceived the original idea designed the model and the computational framework and analysed the data

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