BioScientific Review (BSR)
Volume 6 Issue 4, 2024
ISSN$_{(P)}$: 2663-4198, ISSN$_{(E)}$: 2663-4201
Homepage: https://journals.umt.edu.pk/index.php/bsr

Article QR

Title: **Screening Enterococcus Isolates for Antimicrobial and In-Vitro Antitumor Activity Against Colorectal Carcinoma**

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DOI: https://doi.org/10.32350/bsr.64.09

History: Received: October 26, 2024, Revised: November 22, 2024, Accepted: November 30, 2024, Published: December 20, 2024

Citation: Siddiqa A, Hassan A, Nawaz S, Sajid I. Screening enterococcus isolates for antimicrobial and in-vitro antitumor activity against colorectal carcinoma. *BioSci Rev.* 2024;6(4).134-147. https://doi.org/10.32350/bsr.64.09

Conflict of Interest: Author(s) declared no conflict of interest

A publication of
The Department of Life Sciences, School of Science
University of Management and Technology, Lahore, Pakistan

Screening *Enterococcus* Isolates for Antimicrobial and *In Vitro* Antitumor Activity against Colorectal Carcinoma

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ABSTRACT

Background. *Enterococci* are a part of the natural intestinal flora of humans and animals and play an important role in keeping the microbial balance in the gut. Many species of *Enterococci* are also used as probiotics that produce vitamins, stimulate the immune response, and maintain the integrity of the gut. The use of dietary supplements to reinforce some gut flora components is a current aspect of functional food sciences to treat various diseases.

Methodology. In the current study, 21 *Enterococcus* strains were isolated and identified morphologically, biochemically, and physiologically. The strains were analyzed for their metabolomics potential by using thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) analyses, while the disc diffusion method was employed to assess their antibacterial potential against the known pathogens. The *in vitro* antitumor activity was determined against HCT 116 colorectal carcinoma (CRC) cell lines at different concentrations including 12 mg/ml, 25 mg/ml, 50 mg/ml, and 100 mg/ml.

Results. Out of the 21 strains, 9 showed antimicrobial activity against *Pseudomonas, Enterobacter, Klebsiella*, and *Staphylococcus*. Several strains showed sensitivity against certain antibiotics, such as amoxicillin, norfloxacin, streptomycin, vancomycin, and nalidixic acid. The crude extracts of the isolates also showed high cytotoxicity against *Artemia salina* and significant antitumor activity against HCT 116 colorectal carcinoma CRC cell lines. The crude extracts of these *Enterococcus* strains exhibited the presence of a variety of bioactive metabolites by using TLC and HPLC analysis.

Conclusion. The study revealed that the antimicrobial compounds produced by these bioactive *Enterococcus* strains can be used against *Pseudomonas, Enterobacter, Klebsiella*, and *Staphylococcus*. Moreover, these strains can be investigated as potential probiotic agents to treat colorectal cancer because of their significant *in vitro* antitumor activity against CRC.

Keywords: antimicrobial, antitumor activity, bacterial strains, colorectal carcinoma (CRC), *Enterococcus*, probiotics

Highlights

- The *Enterococcus* strains isolated in this study showed maximum growth inhibition of *Staphylococcus* and *Pseudomonas,* while some strains also showed growth inhibition of *Klebsiella* and *Enterobacter.*

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- The cytotoxicity of the crude extracts of some *Enterococcus* strains against brine shrimps was more than 50%.
- Each *Enterococcus* strain exhibited a unique pattern of secondary metabolites as demonstrated by TLC analysis.

1. INTRODUCTION

Enterococci are commensal bacteria that serve as a vital component of the intestinal flora of humans and animals. They belong to gram-positive, oxidase negative, catalase negative, facultative anaerobic, non-spore forming bacteria that may be single-cellular or create chains. *Enterococci* are lactic acid bacteria (LAB) identified by the presence of low G+C content [1]. The use of *Enterococcus* species as probiotics and in the food industry as starter cultures has increased extensively over the last decade [2].

As a part of the intestinal flora of living organisms including humans and many animals, *Enterococci* play a major role in keeping their gut microbial stability. Many species of *Enterococcus* are also used as probiotics [3–6]. Being a type of LAB, several strains have probiotic properties, such as *Enterococcus durans, Enterococcus mundtii,* and *Streptococcus thermophilus.* These strains are also used to make yogurt in addition to *Lactobacillus delbrueckii* [7–10]. In food industry the production of different compounds, such as organic acids, hydrogen peroxide, and low molecular weight metabolites, including diacetyl and bacteriocins, from these bacteria has a hampering effect on the growth of decaying and pathogenic bacteria; thus, the shelf-life of food products is increased. In addition to the promising effects against diseases caused by the unevenness of the gut microflora, numerous experimental explanations have specified a possible defensive effect of LAB against the progression of colon tumors. Various reports suggest that LAB ingestion prevents the carcinogen-induced pre-neoplastic lesions and tumors. Also, prebiotic intake can decrease the activity of pro-carcinogenic enzymes in humans [11–13].

Supporting evidence has revealed the involvement of colonic microflora in the abnormalities of colonic epithelial cells (CECs). Microorganisms affect the cancer genomic stability, metabolism, and immune receptiveness and could be regarded as groups of gene systems. The properties of altered CECs render them subtle to microbially predisposed carcinogenesis. For drug discovery, microbial extracts represent a major source of many new biologically active molecules. Many new therapeutic targets have now become accessible for recognizing pharmaceutical agents after the development of genomics and high-throughput screening (HTS) technology. In addition to the screening of many microbial fermentation extracts for antibiotic activity, these extracts can now be screened with a variability of novel functional, receptor binding, enzyme inhibition, and protein-protein interaction assays [14–17].

This study aimed at the isolation and screening of *Enterococcus* strains for bioactivity against known bacterial pathogens and antitumor activity against colorectal carcinoma (CRC) due to the potential use of *Enterococcus* as a probiotic and its role in maintaining the microbial balance in human gut. The *Enterococci* strains were isolated from waste water, raw yogurt, and toddler food sample and were characterized morphologically, physiologically, and biochemically. The

isolates were screened for their antimicrobial activity against *Klebsiella, Staphylococcus, Pseudomonas,* and *Enterobacter*. The cytotoxicity of the crude extracts of the selected enterococcal isolates was determined against brine shrimps (*Artemia salina*). While, the evaluation of the anticancer activity of the extracts against CRC was performed using *in vitro* antitumor assay. Chemical screening was performed using TLC and HPLC for the analysis of secondary metabolites.

2. MATERIAL AND METHODS

2.1. Isolation of *Enterococci*

Three different samples of yogurt, waste water, toddler food and containing grinded cereals (wheat, rice, barley) were serially diluted. Then, 50 µl of each sample from dilution 10^{-3} and 10^{-4} was picked and spread on MRS (De Man, Rogosa, and Sharpe) agar separately, followed by incubation under anaerobic conditions in anaerobic jars at 37°C for 36-48 hours. Colonies were then selected and purified by subculturing the isolates on MRS agar.

2.2. Morphological, Microscopic, and Biochemical Characterization

The selected colonies were identified on the basis of their basic morphological, biochemical, and physiological properties. The purified colonies were observed for colony shape, size, margins, elevation, color, texture, form, and pigmentation. For microscopic examination of the selected isolates, Gram staining was performed that divided bacteria into two categories. Biochemical tests including catalase, oxidase, indole, nitrate reduction, voges prausker, hydrogen sulphide production, sugar utilization, tolerance to NaCl (6.5%), and growth at high pH (pH 9) and temperature (10-45°C) were performed for physiological characterization.

2.3. Cultivation of Strains for Preparing Methanolic Crude Extracts

Isolated *Enterococci* were incubated at 37°C for 24-36 hours in MRS broth and growth was achieved in static conditions. After incubation, about 100 ml of culture broth and an equal volume of ethyl acetate was added and sonicated for 15 minutes. Then, the solution was shifted to a separate funnel and shaken vigorously. Two separate layers were formed. The upper layer was removed carefully and evaporated on a rotary evaporator. The dried extract was dissolved in 1 ml methanol.

2.4. Biological Screening

2.4.1. Antimicrobial Activity by Well Diffusion Method. The antimicrobial activity of isolates was determined using the well diffusion method following the previously mentioned protocol [17].

2.4.2. Antimicrobial Activity by Disc Diffusion Method. This method was performed using the discs of filter paper soaked in solvent extracts. About 40 µl of extract with a concentration of 1µg/µl was loaded on each disc [18]. The appearance of zones indicated the growth inhibition of test organisms due to the production of bioactive compounds by bacterial strains.

2.4.3. Antimicrobial Susceptibility Testing by Disk Diffusion Method. The test was performed by inoculating the specific strain onto the surface of MRS agar. Commercially prepared paper discs of antibiotics were positioned on the plate and incubated for 18-24 hours at 37°C. The emergent zones indicating growth inhibition and their diameters were measured using a millimeter scale.

2.4.4. Brine Shrimp Microwell Cytotoxicity Assay. The cytotoxicity of crude extracts was determined against brine shrimps (*Artemia salina*) using microwell cytotoxicity assay. The following formula was used to calculate the mortality rate:

$$\text{Percentage mortality} = \frac{\text{no. of dead larvae}}{\text{No. of dead larvae + no. of live larvae}} \times 100$$

2.4.5. *In vitro* Antitumor Activity. MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was used to determine *in vitro* antitumor activity. This calorimetric method is based on the ability of the mitochondrial dehydrogenase enzyme to reduce a yellow compound MTT to formazan and its spectrophotometric measurement.

$$\text{Percentage mortality} = \frac{\text{O.D (control well)} - \text{O.D (treated well)}}{\text{O.D (control well)}} \times 100$$

2.5. Chemical Screening

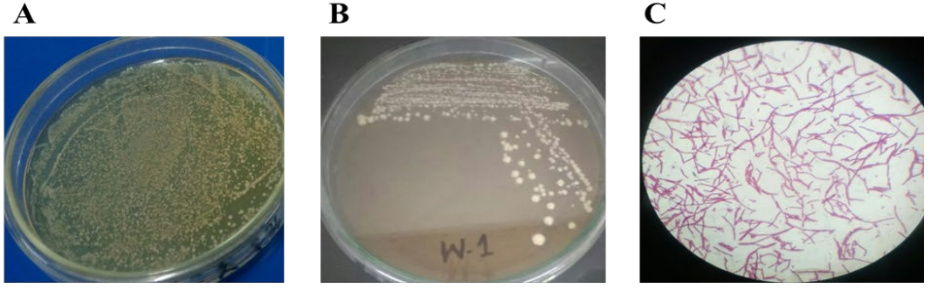
2.5.1. Thin Layer Chromatography (TLC). Silica coated aluminium TLC plates (Merck, Germany) were used. Solvent system comprised 10% methanol in dichloromethane (CH_2Cl_2). The TLC plate was examined under UV light at 366 nm and 254 nm. It was stained with Anisaldehyde/H_2SO_4 and Ehrlich's reagent for the detection of the zones of interest.

2.5.2. High Performance Liquid Chromatography (HPLC). The HPLC system (Sykum HPLC system) and the software Clarity was used. Samples were run for 20 min, while UV absorbance was determined at 254 nm. The comparison of peaks was made at different retention times (RT) with their standard absorbance data of secondary metabolites.

3. RESULTS

3.1. Isolation and Characterization of *Enterococcus* Strains

A total of 21 strains including 11 from toddler food samples, 6 from waste water, and 4 from raw yogurt samples were isolated from the MRS agar plates spread with dilution 10^{-3} and 10^{-4}. The colonies were circular, sticky, pale yellow, and ranging in size from 0.5 to 4 mm. All isolates were observed as gram-positive, Y-shaped rods under the microscope. Most strains exhibited uniform morphological and biochemical characteristics. All the isolates were negative for catalase, oxidase, indole, nitrate reduction, voges prausker, and hydrogen sulfide production tests. They showed growth in high NaCl concentration and at high pH (Fig 1A-F).



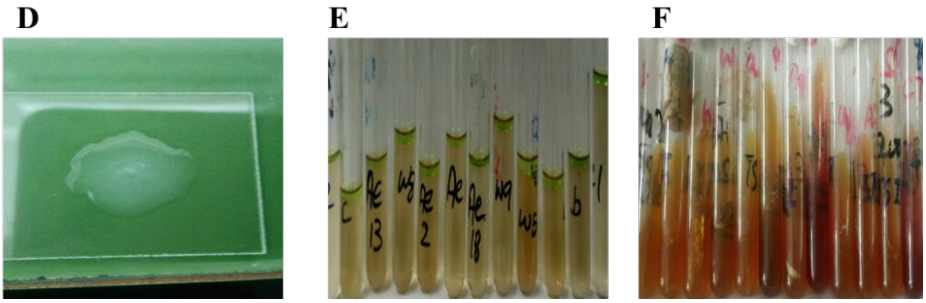


Figure 1: Isolation and Characterization of *Enterococcus* Strains. A. Spread Plate Method for the Selection of *Enterococcus* Strains, B. Streak Plate Method to Obtain Pure Culture of Selected *Enterococcus* strains, C. Gram Staining of Selected Isolates, D. Oxidase test Showing Negative Results, E. Voges-prausker Test Indicating Negative Results, F. H_2S Production Test Showing Negative Results.

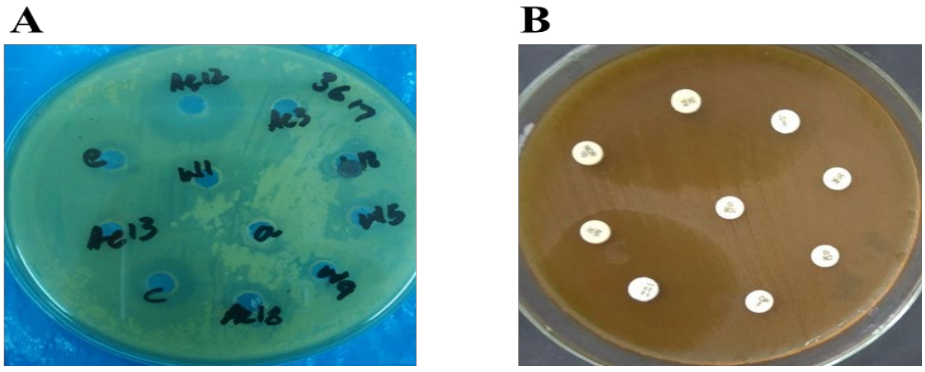


Figure 2: Biological Screening of *Enterococcus* Strains. A. Antimicrobial Activity of *Enterococcus* Isolates against *Pseudomonas, Klebsiella, Staphylococcus*, and *Enterobacter*. B. Antimicrobial Susceptibility Testing of Enterococcus Isolates Against Selected Antibiotics.

3.2. Biological Screening of *Enterococcus* Isolates

The antimicrobial activity of the isolates was evaluated using the well diffusion method. Almost all the strains showed maximum growth inhibition of *Staphylococcus* and *Pseudomonas,* while some also showed the growth inhibition of *Klebsiella* and *Enterobacter*. Antibiotic susceptibility testing of selected strains showed sensitivity towards norfloxacin, streptomycin, vancomycin, nalidixic acid, and amoxicillin (Table 1, Fig 2A, B). Extracts such as AE2, AE12, WT8, and WT9 showed high cytotoxicity towards *Artemia salina* (the percentage was more than 50%). The antitumor activity was checked against HCT 116 carcinoma cell lines at different concentrations, such as 12 mg/ml, 25 mg/ml, 50 mg/ml, and 100 mg/ml. It was found that the percentage cytotoxicity of strains, namely YTC, AE12, YTA, WT1, AE3, YTE, and WT8 was directly related to their concentration (Table 2,3; Fig 3,4).

Table 1. Antimicrobial Susceptibility Testing of *Enterococcus* Isolates by Disc Diffusion Method (CLSI, 2015)

Strain	Susceptibility against antibiotics								
	AX	NOR	S	AK	CN	CIP	VA	NA	TOB
AE12	S	I	I	R	R	R	S	I	R
AE18	S	S	S	R	R	R	S	I	R
WT5	S	S	I	R	R	I	S	S	R
WT8	S	S	I	R	R	R	S	S	R
YTE	S	I	I	R	R	R	S	S	R
YTC	S	I	S	R	R	I	S	I	R

Note. AX= Amoxicillin, NOR= Norfloxacin, S= Streptomycin, AK= Amikacin, CN= Cefalexin, CIP= ciprofloxacin, VA= Vancomycin, NA= Nalidixic Acid, TOB= Tobramycin, I= intermediate, S= sensitive, R= resistant

Table 2. Cytotoxicity of Crude Extracts of Selected *Enterococcus* Stains against Brine Shrimp *Artemia salina*

Strain	Wells	N	A	T	M=[(A-B-N)/G-N]100
AE2	1A	1	32	50	62%
AE12	2A	0	28	50	56%
AE18	3A	0	22	50	44%
WT5	4A	1	15	50	28%
WT8	5A	0	29	50	58%
WT9	6A	1	30	50	58%
YTA	7A	0	41	50	2.8%
YTC	8A	0	10	50	20%
YTE	9A	0	11	50	22%
Blind sample (B)	10A	0	0	50	0

Note. M= percent of the dead larvae after 24 h. A= Number of dead larvae after 24 h. B= Average no. of dead larvae in the blind samples after 24 h.

Table 3. Percentage Cytotoxicity of Methanolic Crude Extracts of the Isolated *Enterococcus* Strains

Extracts	Percentage cytotoxicity (%)			
	12mg/ml	25mg/ml	50mg/ml	100mg/ml
YTC	72.09049	75.18205	75.41986	75.65767
WT5	54.84911	61.03222	68.88002	69.35565
AE13	66.62081	71.37705	72.92283	73.75517
AE12	62.69691	67.57206	72.92283	73.51736
YTA	71.85268	72.80392	73.39845	73.39845
WT1	40.69929	69.59346	73.51736	74.58751
AE3	36.41867	72.80392	76.13329	76.72782
YTE	69.11784	73.99298	76.60892	77.67907
WT8	30.71118	31.0679	51.63864	77.91688

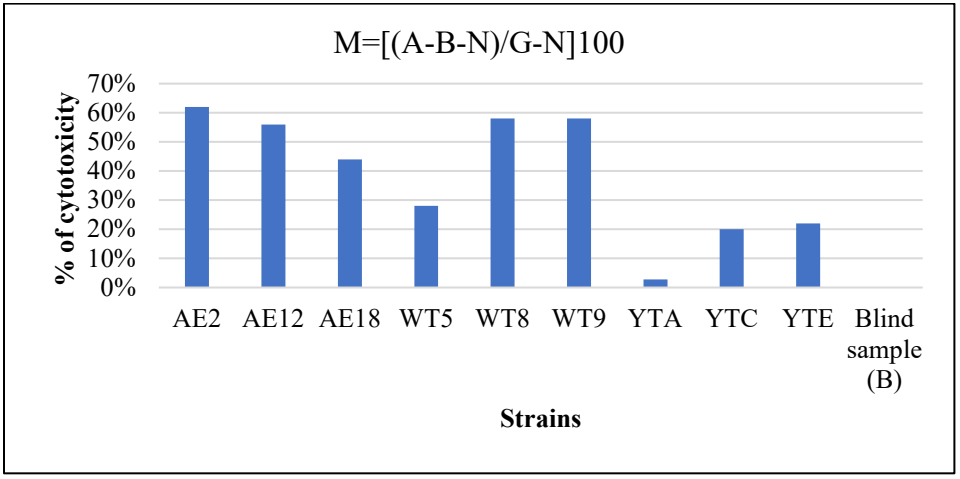


Figure 3. Percentage Cytotoxicity of Crude Extracts against *Artemia salina*

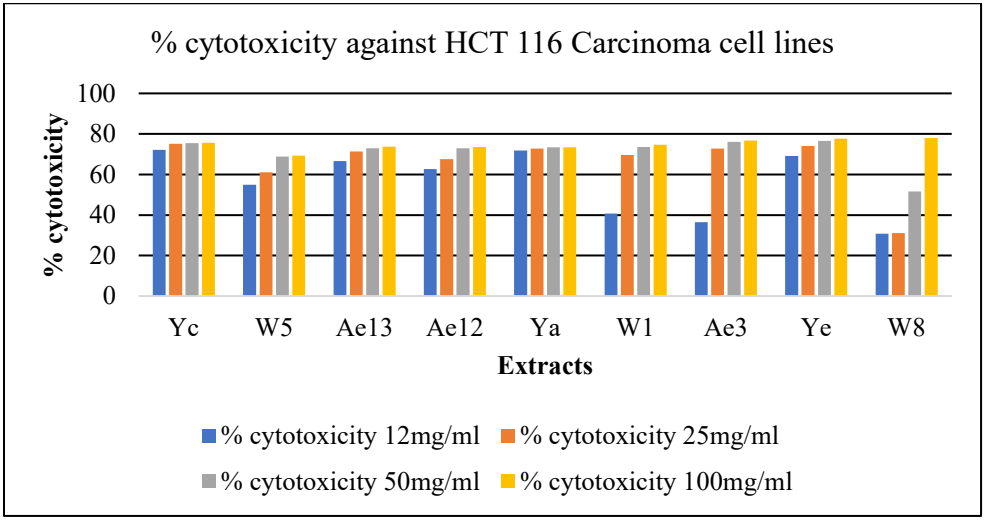


Figure 4: Percentage Cytotoxicity of Crude Extracts against HCT 116 Carcinoma Cell Line

3.3. Chemical Screening of *Enterococcus* Isolates

The TLC of isolates WT1, WT8, YTA, YTC, YTE, AE3, AE12, and AE13 showed a single prominent band along with diffused bands. The HPLC UV chromatogram showed peaks of each sample at different retention times with standard absorption of the data of secondary metabolites. In sample YTE, the major peak occurred at 3.38 min with a peak area of 949.482 [mV.s] (Fig 5A-D).

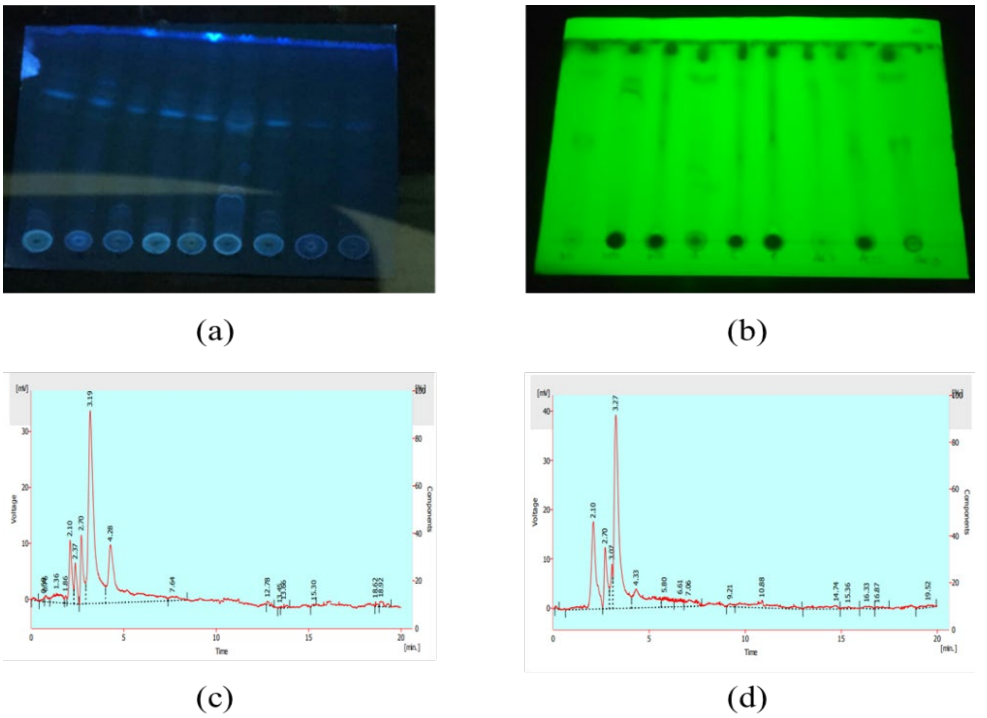


Figure 5. Chemical Screening of Enterococcus Strains. a. TLC Plate under 366nm UV Radiation, b. TLC Plate under 254nm UV Radiation. c. HPLC-UV Chromatogram of Crude Extract of Strain WT5. d. HPLC-UV Chromatogram of Crude Extract of Strain AE12

4. DISCUSSION

Enterococcus is a major member of human gut flora which is a complex community of different types of microorganisms, including a great majority of bacteria, while others comprise yeasts and viruses. The bacteria of the gut flora are beneficial microorganisms. They suppress the growth of the disease-causing bacteria and yeasts and thus have a very important role in human health. However, *Enterococci* can cause disease as well, occasionally. These probiotics are a group of beneficial bacteria that settle in the gut and stop the pathogenic bacterial growth by competition, growth inhibition, and attachment to the gut epithelium. Previous studies showed the beneficial effects of probiotics on the immune system, destruction of pathogenic bacteria, inflammations, gastrointestinal diseases, colorectal carcinomas, anxiety, depression, post antibiotics diarrhea, and some kinds of skin disorders [19–21].

The enterococcal strains isolated in this study were screened for their ability to produce bioactive compounds. The antimicrobial activity of methanolic crude extracts, as well as supernatants, was checked against *Klebsiella, Staphylococcus, Pseudomonas,* and *Enterobacter*. Almost all strains showed maximum growth inhibition of *Staphylococcus* and *Pseudomonas,* while

some strains also showed the growth inhibition of *Klebsiella* and *Enterobacter*. The peptides, that is, enterococcins or enterocins produced by *Enterococcus faecalis* and *Enterococcus faecium* revealed their activity against *Salmonella pullorum* and *Escherichia coli* [22, 23]. Two bacteriocins produced by *Enterococcus mundtii* showed their antimicrobial activity against *Listeria monocytogenes* [24]. In addition to the production of bioactive compounds, the strains were also screened for their resistance against certain antibiotics by antimicrobial susceptibility testing. The majority of strains were found sensitive towards norfloxacin, streptomycin, vancomycin, nalidixic acid, and amoxicillin. Furthermore, the crude extracts of the selected strains of *Enterococcus* showed significant cytotoxicity. Whereas, some strains such as AE2, AE12, WT8, and WT9 showed high cytotoxicity towards *Artemia salina* (the percentage was more than 50%).

The human colon is the most metabolically active organ due to the presence of a diverse microbiota. The main role of these bacteria is to ferment the undigested food materials. The selected isolates were also screened for their antitumor activity against HCT-116 carcinoma cell line. Anti-tumor activity was checked at different concentrations. Then, graphs were plotted where these concentrations occupied the X-axis, while percentage cytotoxicity occupied the Y-axis.

The gut microflora affects the gastrointestinal tract by showing resistance to pathogens, decreases gut tumors, and blood lipids [25]. The use of TLC with certain staining reagents and HPLC-MS/MS for chemical screening is a useful source to visualize an almost complete picture of microbial secondary metabolite pattern. So, new strains could be detected and microbial strain collection may also be modified using these techniques, especially as a fundamental step of efficiently applied biological high through-put assays [26, 27].

The crude extracts of selected *Enterococcus* strains were examined by TLC using Anisaldehyde/HSO and Ehrlisch's reagent as staining reagents. Various bands of fractions were detected under short and long UV (254 nm and 366 nm). The crude extracts constituents displayed UV absorbance. After developing the TLC plate, it was stained with two different staining reagents. The pattern of secondary metabolites, specific for each strain, was observed and recorded. The TLC results demonstrated that each strain exhibited a unique pattern of secondary metabolites. The crude extracts of selected *Enterococcus* strains were analyzed through HPLC-UV. The HPLC chromatogram of the bioactive *Enterococcus* showed major peaks at shorter retention time. Mostly, there were two to three peaks which showed that there is more than one type of secondary metabolites present in the crude extract of the strains. While, the shorter retention time of peaks indicated that metabolites were of low molecular weight and potentially less harmful.

4.1. Conclusion

The study revealed that the *Enterococcus* isolates are a useful source of many bioactive compounds showing antimicrobial activity against many pathogens. Hence, they could inhibit the growth of *Klebsiella, Staphylococcus, Enterobacter,* and *Pseudomonas* strains. The cytotoxicity of the selected strains also indicated that they have high toxicity, more than 50% mortality against brine shrimp

larvae. They could also be used against tumor cells as demonstrated by the *in vitro* antitumor activity against HCT 116 CRC cell lines. So, they can be used in the form of probiotics to treat many diseases, particularly of the gastrointestinal tract. They can also be used to enhance the indigenous gut flora because of their probiotic activity. The TLC and HPLC analyses showed that these isolates produce a variety of secondary metabolites which can be used in combination to inhibit the growth and provide safety against pathogenic bacteria.

CONFLICT OF INTEREST

The authors of the manuscript have no financial or non-financial conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVAILABILITY STATEMENT

The data associated with this study will be provided by the corresponding author upon request.

FUNDING DETAILS

No funding has been received for this research.

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