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Study the Chemical Structure characterization, Antibacterial Activity and Anticancer and Cytotoxicity of Exopolysaccharides (EPS) Produced from *Lactobacillus Acidophilus*

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Abstract

Background:Exopolysaccharides (EPSs) from bacteria are being produced in research thanks to the growing trend of utilising natural polymers in numerous fields. Bacterial EPSs are particularly interesting to the domains of science, medicine, and the food business because of their distinctive structural characteristics.

The aim:The current study focused on the Exopolysaccharides from *Lactobacillus acidophilus* and study its chemical structural characterization ,antibacterial activity against isolates as *S.aureus*, *P. aeruginosa*,*E.coli*, *P.mirablis* , *E. feacalis* and its anticancer and the cytotoxicity in cell lines.

The material and methods: EPS extraction from *Lactobacillus acidophilus with* The ethanol precipitation method and determination of carbohydrate content withthe phenol-sulfuric-acid technique, study the chemical structure with FTIR then determination of MIC followed by the antibacterial activity assay by agar diffusion method Finally investigate the anticancer and cytotoxity of EPS by Neutral red uptake assay .

The results: the results of determination carbohydrates rate in purified EPSs were recorded the carbohydrate content was 80% expressed as a percentage of EPS dry weight (w/w%) The antibacterial activity stated there was a favorable effect of EPSs against the isolates employed in the study and demonstrated inhibitory zone and recorded diverse results and significant differences at (p 0.05) between the concentrations withing the isolate as well as with other isolates and the FTIR the chemical structural characterization of EPS by FTIR showed the analysis revealed the presence of hydroxyl, carboxyl, and amide groups.The anticancer activity reoprtedthe concentration causing 50 % proliferation inhibition of MCF7 is (IC50) by EPS from *L. Acidophilus* strain towards MCF**-7** was 97.93 mg/mL^{-1} in same time the cell viability at highst concentration of EPS (200 mg/ml) was 48.03 % while the lowest concentration 6.25 mg/ml showed no effect on the cells while the Cytotoxicitythe results revealed 278.8 mg/ml causing 50 % proliferation inhibition of WRL-68 is

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(IC50) by EPS from *L. Acidophilus* strain towards WRL-68 was 278.8 mg/mL^{-1}.The EPS was an antibacterial that worked against both gram positive and gram negative isolates as antibacterial agent. and the chemical structural revealed that presence of hydroxyl, carboxyl, and amide which similar to other EPSs from lactic bacteria**.**

Conclusion: the findings showed anticancer activity against the breast cancer cell line.

Introduction

Exopolysaccharides (EPSs) from bacteria are being produced in research thanks to the growing trend of utilising natural polymers in numerous fields. Bacterial EPSs are particularly interesting to the domains of science, medicine, and the food business because of their distinctive structural characteristics (2) EPSs are frequently employed in the food sector as viscous, stabilizing, and emulsifying agents to improve the rheological property, texture, and sensibility of bread and fermented milk products including yogurt and cheese [Sing et al., 2017]. (3). EPSs have potential health benefits in addition to their technological capabilities, such as antioxidant, anticancer, anti-inflammatory, antiviral, and cholesterol-lowering actions (5).

Lactic acid bacteria (LAB) have drawn the attention of researchers among EPS-producing bacteria due to their potent ability to produce EPSs. EPSs are typically made by the LAB strains Lactococcus, Pediococcus ,Streptococcus, Leuconostoc, WeissellaleandLactobacillus (6). LAB are capable of creating EPSs with a range of various topologies and are generally regarded as safe microorganisms (GRAS-Generally Recognized as Safe) (7).EPSs participate in the structural elements of extracellular (8) The beneficial properties of EPSs are underlined by their critical role in promoting human health, including their prebiotic, anti-inflammatory, antioxidant, anticoagulant, cholesterol-lowering ,antiviral, and even anticancer action (9).It has been demonstrated that LAB's EPSs have a crucial functional role in preventing blood coagulation. It has been proven that EPSs in sulphate derivatives have potent anticoagulant properties,EPSs give san acidic medium condition to enable Heparin Cofactor II's inhibitory impact on thrombin, which is a powerful inhibitor of thrombin inthe coagulation pathway (10)

Material and method

The microorganism usedin the study

The isolated bacterial as *Lactobacillus acidophilus* , *Escherichia coli,, Staphylococcus aureus* , *Enterococcus feacalis, Proteus mirabilis* ,and *Pseudomonas aeruginosa* and obtained from the

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microbiology laboratory in Babylon university

Extraction and purification

The ethanol precipitation method is employed for EPS extraction (11) *L. Acidophilus* was cultured for 24 hrs in MRS broth then the supernatant was collected and denatured with trichloroacetic acid (final concentration of 14%) after centrifugation at 8,000 g for 20 min at 40C. A two-fold amount of the supernatant added absolute ethanol (cold), which was then maintained at 4C0 for 24 hours before being centrifuged at 8000g for 20 minutes. The precipitated EPS was centrifuged for 15 minutes at 4C0 before the supernatant was collected. The precipitate was then dissolved in deionized water then dialyzed wih a dialysis membrane for 24 to 48 hrs, finally the precipitated EPS dried at 30 C^0 to remove the ethanol before being concentrated and carbohydrate content determined with the phenol-sulfuric-acid technique.

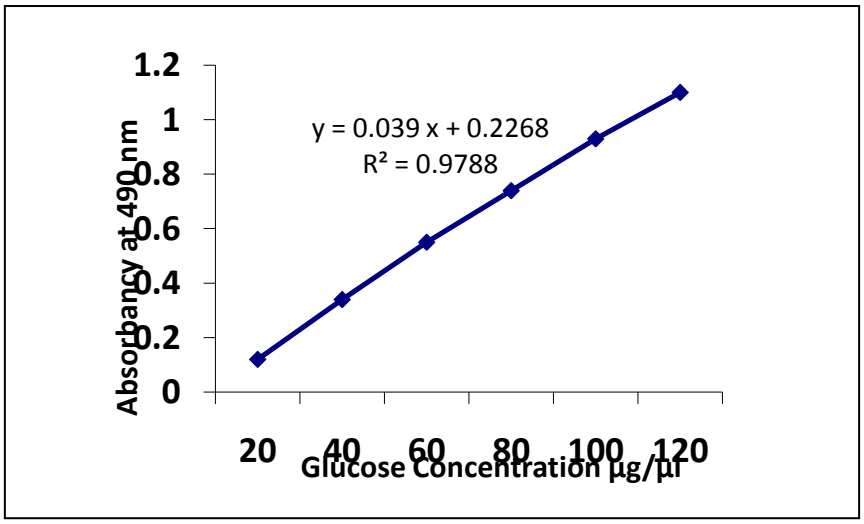

Figure (1): Standard curve of Total carbohydrate using glucose

Structural characterization of the EPSs

Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy, which is often employed as a method for identification of the structural properties of biopolymers, was utilized to undertake the qualitative examination of the sample (12). Thermo Scientific Nicolet iS10 Spectrometer with Smart iTX attachment (Thermo Scientific, Inc., United States) was used to record the samples' FTIR spectra in the 400–4000 cm–1 range with a spectral resolution of 4 cm–1 and 32 scans. The built-in diamond attenuated total reflectance (ATR) sampling method was used to collect FTIR spectra in the reflection mode. For the acquisition, processing, analysis, and management of FTIR data in a graphical environment, the OMNIC

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Software was utilized.

The Antibacterial Activity

Determination MIC of EPSs

Eppendorf tubes were filled with 100 µl of bacterial suspension (adjusted to 0.5 McFarland standard) and 50 µl of the test substance. The tubes were then incubated for 284 hours. The tubes were next checked for turbidity (turbidity implies bacterial growth), which was used to determine the minimal inhibitory concentration (13). The tubes that showed symptoms of turbidity were eliminated.

Determination the Antibiotic activity of EPSs

The isolates were obtained from nutrient broth over the duration of 18 to 24 hours, and they were subsequently calibrated to the 0.5 McFarland standard. roughly $1.5X10^8$ CFU/ml, and subcultured on Mueller-Hinton agar included wells (6 mm in diameter) each well was filled with 0.1 ml of EPS concentrations that have been prepared (13).

The Biological assay

Cytotoxicity and Anticancer assay

Neutral red uptake assay was used to determine cell viability (14). The amount of live cells in a culture can be quantitatively estimated using the neutral red uptake assay. It is predicated on how well-functioning cells can bind and integrate the supravital dye neutral red in lysosomes. At a cell density of 104 cells/well in a 96-well plate, the cells were treated with different concentrations of the test chemicals (125, 250, 500, and 1,000 g ml-1) for 48 hours. The IC50 of the investigated substances was computed using the relationship between the utilized concentrations and neutral red intensity value. Doxorubicin (Mr=579.9), a cytotoxic natural drug utilized as a positive control, produced 100% inhibition. The tested substance was dissolved using dimethyl sulfoxide (DMSO), and its final concentration on the cells was less than 0.2%. The findings of each test and analysis were averaged after being performed in triplicate.

Statically analytics

Using the SPSS software and an ANOVA to account for the least significant difference, the parameter values were shown as means and standard deviations (LSD).

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Results

According to the results of the analysis of the carbohydrates rate in pure EPSs, 12% of the EPS dry weight (w/w%) was made up of other components like hexominase , uronic acids, keto-linked pyruvate groups and acetyl groups, while 80% of the EPS dry weight (w/w%) was made up of carbons,(Hanan*et al*., 2013).The MIC of the EPS showed variations between isolates as in figure 2

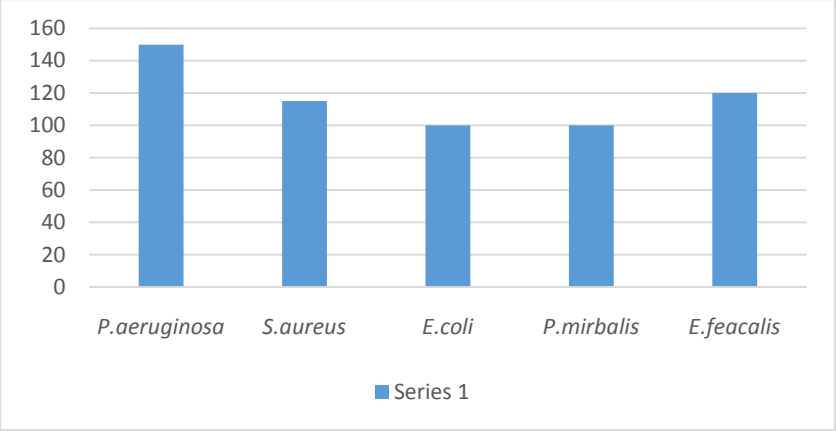

Figure (2): The minimum inhibitory concentration (MIC) of EPSs

According to results of EPSs' antibacterial activity, EPSs had a favorable impact on isolated bacteria with various results and significant differences at ($p \leq 0.05$) as in table (1).

Table(1): Showed the inhibition zone diameter (mm) of Exoploysaccharides against isolates

Conc (µg /ml)	*S.aureus*	*P. aeruginosa*	*E.coli*	*P.mirablis*	*E. feacalis*
200	1.4±0.00 d	1.0±0.00d	1.5 ±0.33d	2.14±1.21d	1.14±1.03d
250	3.18±0.09c	3.26±0.04c	4.05±0.27c	6.06±0.06c	5.19±0.13c
300	7.47±0.27b	5.33±0.07b	6.07±2.03b	7.2±0.11b	6.20±0.16b
350	9.27±0.17a	6.13±0.33a	8.13±0.56a	8.28±0.01a	7.0±0.10a

*The different letters refers to there was a significant differences at($p \leq 0.05$) while the similar letters means there no significant differences

Fourier-transform infrared spectroscopy (FTIR)

By analyzing the functional groups and chemical bonds present in the samples, the FTIR analysis was used to qualitatively identify the purified material as the polysaccharide. Figure(3) presents the sample FTIR spectrum (3).

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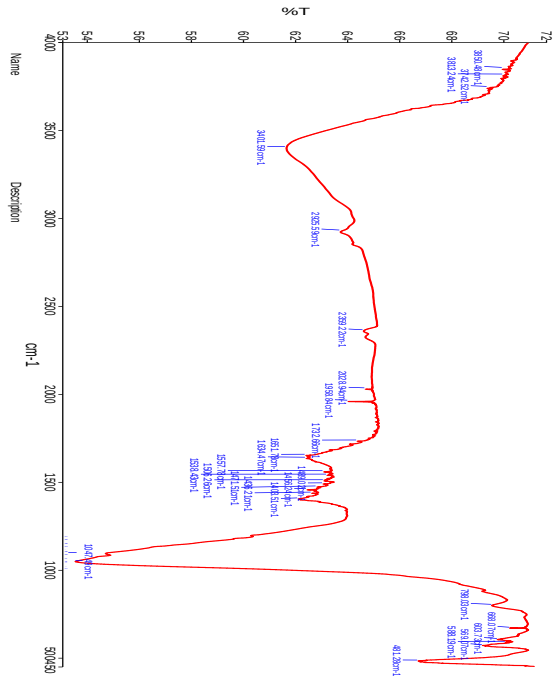

Figure (3): FTIR od EPS extracted from *L. Acidophilus*

Anti-Cancer and Cytotoxicity

The EPS showed anticancer activity against MCF-7cell line (human breast cancer cell line)and Cytotoxicity in WRL-68 cell ine(The human hepatic cell line) depending on IC50 as in figure (4).

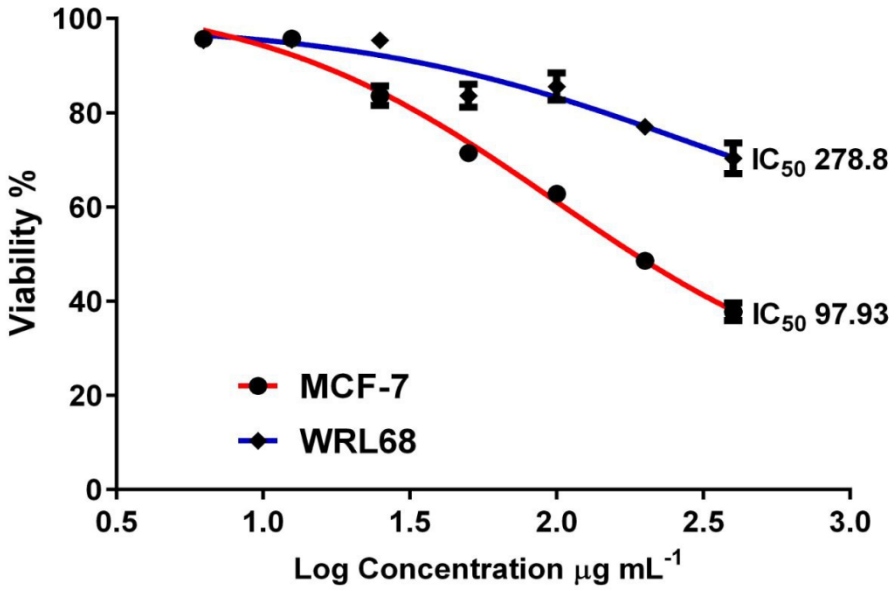

Figure (4): Anticancer activity and Cytotoxicityof EPS against MCF-7 and WRL68 cell line

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The concentration that causing 50 % proliferation inhibition of MCF7 is (IC50) by EPS from *L. Acidophilus* strain towards MCF-7 was 97.93 mg mL^{-1} as in figure (4), in same time the cell cell viability at highst concentration of EPS (200 mg/ml) was 48.03 % while the lowest concentration 6.25 mg/ml showed no effect on the cells as in figure (5).

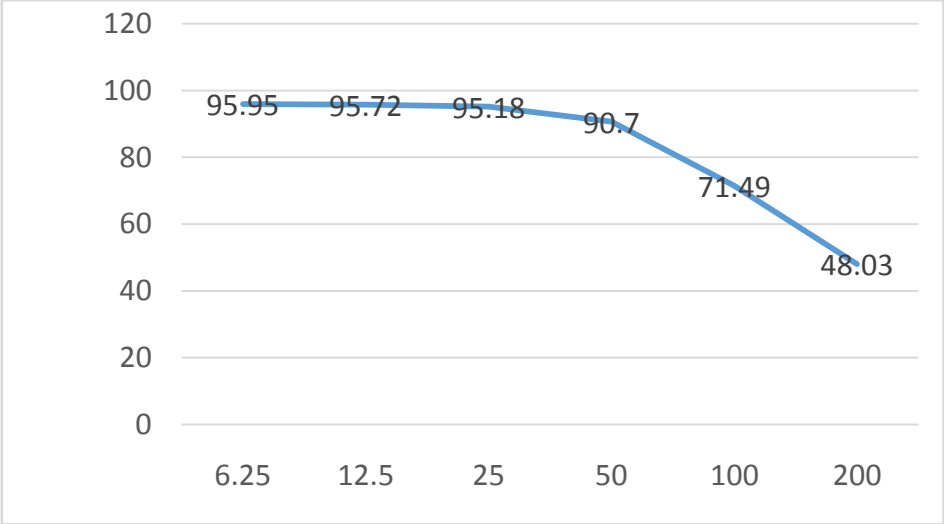

Figure (5): MCF-7 was expressed by the percentage of cell viability after 24h exposure to purified EPS

Anti-Cancer and Cytotoxicity

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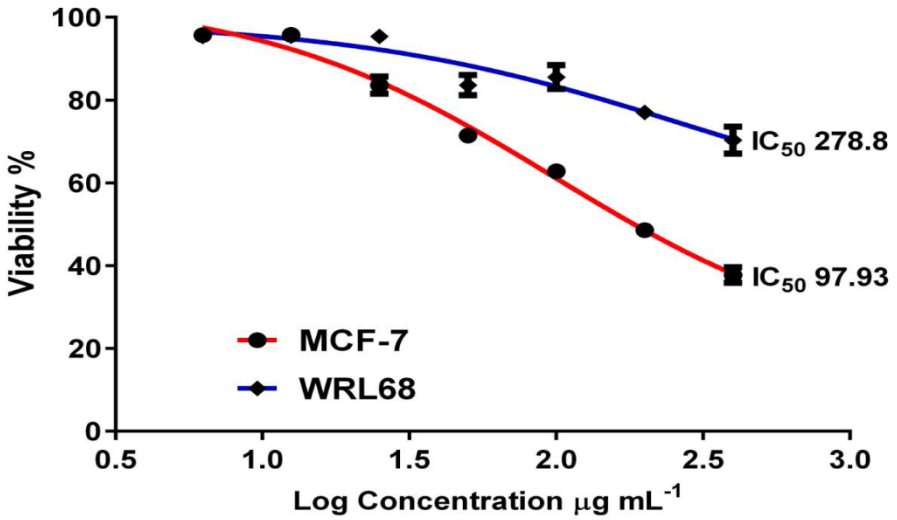

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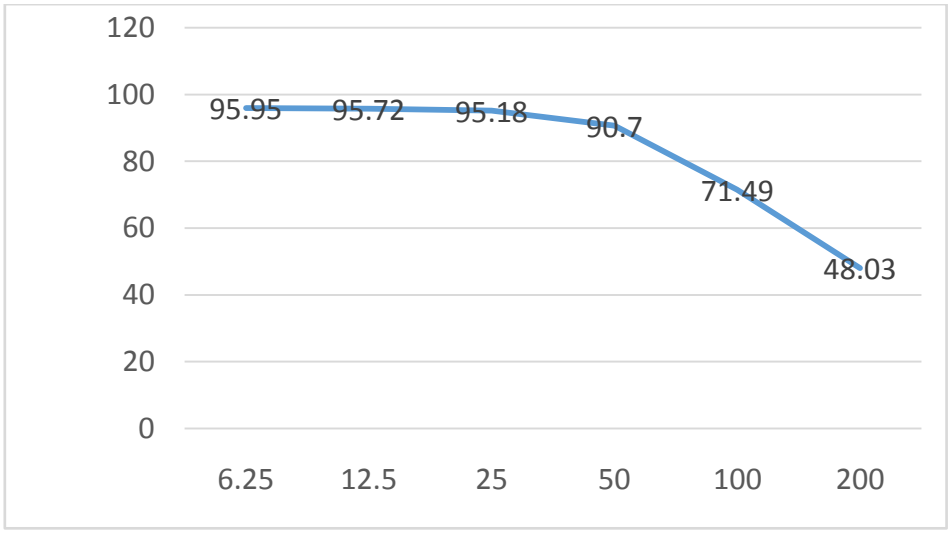

Figure (5): MCF-7 was expressed by the percentage of cell viability after 24h exposure to each purified EPS concentration of L. Acidophilus

Furthermore the results revealed he concentration causing 50 % proliferation inhibition of WRL-68 is (IC50) by EPS from *L. Acidophilus* strain towards WRL-68 was 278.8 mg mL^{-1} as in figure (4), in same time the cell cell viability at highst concentration of EPS (200 mg/ml) was 84.08 % while the lowest concentration 6.25 mg/ml showed no effect on the cells as in figure(6).

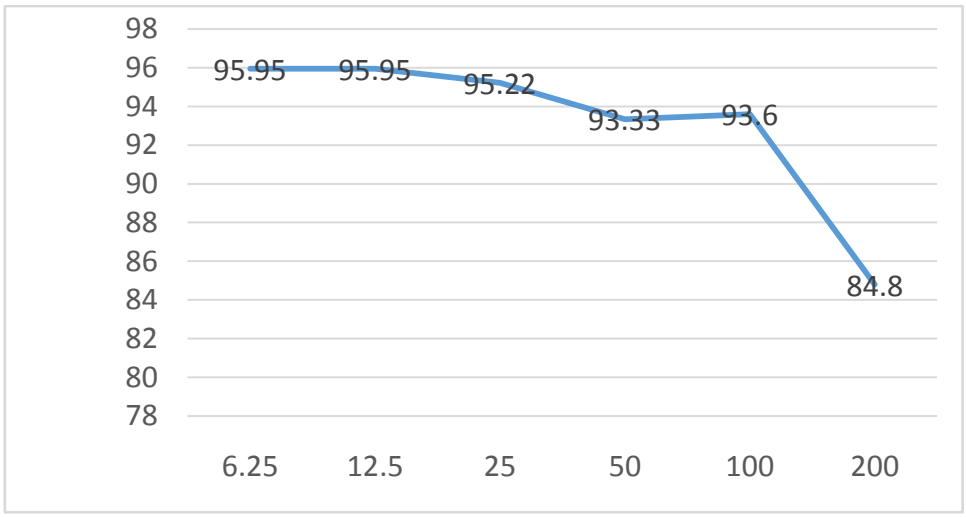

Figure (6): WRL-68 was expressed by the percentage of cell viability after 24h exposure to each purified EPS concentration of *L. Acidophilus*

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Discussion

When EPSs were measured using the phenol-sulfuric acid method, the carbohydrate content was 80% of the dry weight of the EPSs (w/w%), while the remaining 12% was made up of acetyl groups uronic acids, hexominase, ketal-linked pyruvate groups, and (Hanan et al., 2013). However the Figure 2 illustrates how the EPS's MIC varied amongst isolates.

The discovery According to table 2's antibacterial activity results, both gram positive and gram negative bacterial growth were inhibited at various doses. When Khwen and Abed (16) examined the antibacterial activity of EPSs from *L. acidophilus*, they discovered that they had a favorable impact on a variety of isolates, including *S. aureus*, *P. aeruginosa*, and *S. hominis* Also the findings agreed with Rajoka et al.(17)Found the EPSs that *Lactobacillus reuteri* and *Lactobacillus vaginalis* generated after they were isolated from healthy chickens' intestinal cecum samples (hen). In vitro, EPSs showed strong antibacterial activity against *Salmonella typhimurium* and *E. coli*.as well as the results were agreed with Aullybux et al.(18) that found exopolysaccharides from marine bacteria exhibiting antibacterial properties against human bacterial pathogen as *Acinetobacter*, *Bacillus*, *Campylobacter*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Proteus*, *Pseudo monas*, *Salmonella*, *Streptococcus* and *Staphylococcus*.

Majolagbe et al.(19), who reported the of EPS from *Pleurotus tuber-regium* showed activity toward , *E. coli*, *E. feacalis* , *Salmonella*and *S. aureu*s , also t the aqueous EPS extract was active against *S.typhimurium*and *S.epidermis*[20] that revealed in EPS against four isolates against *Staphylococcus* sp, *E. coli* , *Klebsiellaspp*and*S. typhi*.a study reportedEPSs isolated from *B. bifidum*and *L.plantarum*were sjowed antimicrobial activities against *C. sakazakii, E.coli,L.monocytogenes, , S. aureus ,C. albicans, B. cereus. , S. typhimurium, and S* at concentration 300 µg/Ml[21].

Many theories regarding the antibacterial activity of EPS have been attributed to things like inhibiting cell division, cytoplasmic membrane and rupturing the cell wall, and decomposing DNA [22], the second mechanism attributed to presence phenolic compounds and carboxylic that act as in antibacterial (23).

The investigation showed that a typical polysaccharide, as shown in figure, has carboxyl, hydroxyl, and amide groups (3). There is a large absorption band in the spectra at 34013.59 cm-1 that is attributed to -OH or -NH vibrations (24). According to information from the literature, the polysaccharides contain a sizable amount of hydroxyl groups, which show a strong broad stretching

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vibration in that area (25). The distinctive absorption band of the carbohydrate ring, which is what gives polysaccharides their water solubility, is responsible for this. The presence of -CH2 methylene and -CH3 methyl groups, which are frequently found in hexoses like glucose, galactose, and deoxyhexoses like rhamnose, is also indicated by a tiny band at 2925.59 cm-1.In accordance with a normal polysaccharide, as shown in figure, the analysis confirmed the presence of carboxyl, hydroxyl, and amide groups (3). Broad absorption band for -OH or -NH vibrations is present in the spectra at 34013.59 cm-1 (24). The information from the literature showed that the polysaccharides had a substantial amount of hydroxyl groups, which show a strong broad stretching vibration in that area (25). Because of this distinctive absorption band of the carbohydrate ring, polysaccharides are water soluble. A minor band at 2925.59 cm-1 also suggests that the -CH2 methylene and -CH3 methyl groups that are often found in hexoses, such as glucose, galactose, and deoxyhexoses, such as rhamnose, are involved in C-H stretching vibrations.

The anticancer activity results were in agreement with those of Haroun et al. (27), who found that the majority of IC50 values are in the range of 1 mg/mL of EPS has the greatest IC50 for tumor cells is 34.7 mg/mL, the findings were agreed with Di et al. (28) who discovered a LW1high molecular weight fraction of exopolysaccharides isolated from *L. plantarum* NRRL B-4496 acted on Larynx cancer cell lines. For HT29, casei SB27 has the lowest IC50, which ranges from 0.01 to 0.05 mg/mL. Deepak et al.(29) looked into the anticancer efficacy of EPS from *L. acidophilus*The expression of genes involved in tumor angiogenesis and survival may be inhibited the colon cancer cell lines and in EPS, according to research.

Furthermore, the results were consistent with those of Dan et al. (30), who discovered that exopolysaccharide (EPS) isolated from *L. acidophilus* inhibited the growth of CaCo2 colon cancer cell line in a dose-dependent manner and decreased CaCo2 cell line survival in both situations. Peroxisome proliferator activator receptor- (PPAR-) expression was found to be elevated by EPS administration in both normoxia and hypoxia, according to quantitative polymerase chain reaction (qPCR) experiments. Apoptosis induction was one among EPS's cancer-fighting properties. Tumor cells that have received EPS treatment exhibit the typical morphological alterations of apoptosis, such as, cytoplasm condensation ,vacuolation, , chromatin condensation and nuclear disintegration. Additionally, apoptotic bodies around the nucleus and enlarged mitochondria can be seen (29; 28).

Proteases that are specific to cysteine aspartyl are at the heart of the apoptosis mechanism (caspases). Initiator caspases include caspase-2, -8, -9, and -10, while executioner caspases include caspase-3, -6, and -7. The two routes that activate caspases and cause apoptosis are the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. In the extrinsic route, death

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receptors like TNF and FAS receptors (TNFRS/DRS) associated with cell death signals (death ligand) including tumor necrosis factor (TNF), FAS ligand (FASL) and TNF-related apoptosis-inducing ligand (TRAIL). TNF receptor-associated death domain (TRADD) of adaptor proteins and The FAS-associated death domain (FADD) are then recruited. Caspase-8 and Caspase-3 are both activated in this pathway, however the EPS isolated from *L. plantarum*activated the transcription and translation of FAS and its ligand *via* TLR2/MyD88/TRAF6/MKK7/JNK/c-Jun pathway (31).

Furthermore, the results corroborated those of Sharma et al. (32), who discovered that HEp-2C was expressed by the percentage of cell survival following a 24-hour exposure EPS from *L. paraplantarum*the cell viability of the cell lines showed very slight variations between the samples and the negative control (0 g/ ml EPS). But the viability of HEp-2C cells was didn't showed any respond even when exposed to 500 g/ml of EPS, which was significantly tested.

El-Deeb et al(33) who discovered that the IC50 varied between various cell types MDCK, Vero, MRC5, PHK, and PBMC when treated with EPS from *L.acidophilus* , EPS on non-toxic dietary ingredients was discovered in his investigation.. Purified EPS concentration of *L. paraplantarum* was reported to be free of cytotoxic effects and may be significant in the food industry.

Conclusion

The EPS showed strong antibacterial activity the studied bacteria and the chemical structural revealed that presence of hydroxyl, carboxyl and amide groups which similar to other EPSs from lactic bacteria , however the results showed anticancer activity against breast cancer cell line.

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