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RESEARCH ARTICLE

Exopolysaccharide from *Rhodococcus pyridinivorans* ZZ47 Strain: Evaluation of Biological Activity and Toxicity

Aylin Taşkaya[1] • Nur Ceyhan Güvensen[2]✉ • Cem Güler[3] • Ebru Şancı[4] • Nefise Ülkü Karabay Yavaşoğlu[3,4]

[1]Muğla Sıtkı Koçman University, Faculty of Sciences, Department of Biology, Muğla/Türkiye
[2]Ege University, Tire Kutsan Vocational School, Food Technology Program, İzmir/Türkiye
[3]Ege University, Faculty of Sciences, Department of Biology, İzmir/Türkiye
[4]Ege University, Center for Drug Research and Development and Pharmacokinetic Application, İzmir/Türkiye

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ABSTRACT

Microbial polysaccharides are extracellular polymeric macromolecules excreted in microorganisms. These are widely used in food, cosmetic and pharmaceutical industries. One of them, exopolysaccharides (EPS), plays important role against the factors such as phage attack, antibiotics, toxic compounds or osmotic stress. Recently, this natural polymer has received great attention due to their therapeutic potential. The purpose of the study was to evaluate biological activity and potential toxicity of EPS from *Rhodococcus pyridinivorans* ZZ47 strain isolated from nature. EPS has no genotoxic effect on *Salmonella typhimurium* TA98, TA102, and TA1537 strains by Ames Test. No death occurred with single dose oral toxicity test of EPS and LD_{50} value of it is calculated by >2000 mg/kg in mice. The EPS showed antibiofilm activity on different bacteria. In addition, EPS demonstrated dose-dependent anti-angiogenic properties by HET-CAM test. In conclusion, the isolated EPS has antioxidant activity with no genotoxicity and the biological activities of the polymer indicated that it may be suitable for use in different sectors and industrial applications.

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1. Introduction

The legal discharge of man-made or natural substances threatens public health because of their toxicity (Ahmad et al., 2023). These environmental pollutants, dyes, heavy metals, herbicides and pesticides, cause chronic diseases by transferring to food (Bello et al., 2018). Microbiota existing in nature works to eliminate environmental pollutants. In many studies, it is showed that increased tolerance to toxic pollutants and improved degradation capabilities of bacterial biofilm generally (Chaisuwan et al., 2020 & Ahmad et al., 2023). This biopolymeric matrix obtains stability and refuge to the cells in a biofilm and the main structure of this component is exopolysaccharides (EPS). However, the role of EPS goes

✉ Corresponding author
E-mail address: nur.ceyhan.guvensen@ege.edu.tr

beyond providing protection to microbial cells under stress and appears as a biomaterial in many (Matho et al., 2022). Biofilms can happen on nearly all type surfaces such as food processing surfaces, food and food packaging surfaces, water and food related surfaces. EPS, one of the most commonly used biomaterials, is carbohydrate polymers that can be produced from many plants, fungus, algae and bacteria (Botelho et al., 2014). EPSs have the ability to produce a significant number of microorganisms (bacteria, fungi, yeast) belonging to different taxonomy. The yeast and yeast-like fungi which include the genera of *Candida*, *Cryptococcus*, *Pichia*, *Sporobolmyces*, *Trichosporon*, *Lipomyces* and *Rhodotorula* have been described to produce EPS in the laboratory scale under submerged culture conditions (Ramirez, 2016). While predominantly vegetable polysaccharides were used (Hussain et al., 2017), the industry began using these biopolymers, with the introduction of microbial EPS as well as the benefits to human health (Gürleyendağ, 2006). Microbial EPS are regular, branched or unbranched structures with a high molecular weight of ionic or non-ionic biopolymer capable of dissolving in water and whose repetitive units are merged with glycosidic bonds (Erdoğdu, 2018). Commercially produced microbial EPSs have film-forming properties, emulsifying, thickening, structure-modifying, gelling capacities, and/or biological activities. It has been reported by studies that EPS material is widely used in the food industry, cosmetics, pharmaceutical and biomedical industries. Examples used in all these industries include, alginate, pullulan, glucans, bacterial cellulose, dextran, succinoglycan, xanthan gum and levan (Madhuri & Prabhakar, 2014 & Zhaoa et al., 2017). In the table below, various microorganisms, usage areas and production conditions from which Microbial EPSs are obtained are given (Erdoğdu, 2018).

Table 1. Various microorganisms, usage areas and production conditions from which microbial EPS is obtained

Microbial strains	EPS	Substrates	EPS concentrations (gL^{-1})	Production conditions	Areas of use
Acetobacter xylinum	Cellulose	Fructose Glucose	7-23.6	pH=4-5; 30 °C; 40 h	It is used as a dressing material in the bandaging of wounds, as a binder in high-quality acoustic-diaphragm membranes, ceramic powders and salts, etc.
Pseudomonas aeruginosa	Alginate	Xylose	0.4	30-37 °C; 1 bar 72 h	Food industry, pharmacology etc.
Leuconostoc sp.	Dextran	Sucrose	8.17	pH=5.5; 35 °C 1 bar	Coating of antibiotics, textile industry, blood volume enhancer
Agrobacterium	Curdlan	Glucose/sucrose	5.02-76	pH=7.5; 30 °C	Oligosaccharide, tertiary oil recovery, gelling agent in foods

EPS is used as a biomaterial suitable for medical and biological use due to its chemical structure and physical properties. By looking at the biological activities of EPS produced from different microorganisms in many studies, its applicability as a biomaterial is being investigated with many in vitro and in vivo studies. Basic physical and chemical properties, in vitro antioxidant properties and biological activities such as antiproliferative properties of EPS produced from *R. pyridinivorans* ZZ47 strain isolated from nature. The antioxidant properties of EPS with DPPH and hydroxyl radical elimination have been determined in our previous studies. The proliferation of EPS on HT-29 and MCF-7 cell lines was determined by performing the MTT test. In this study, the genotoxicity, acute toxicity, anti-angiogenesis and anti-biofilm activities were examined in the same EPS material.

2. Materials and Methods

2.1. Bacterial Strain and Culture Media

Rhodococcus pyridinovorans strain ZZ47 has been isolated from a biofilm that is a problem in a wastewater treatment plant. Activation of the *R. pyridinovorans* strain was done in Tryptic Soy Broth medium. The activated strain was optimized by referring to previous studies (Erdoğdu, 2018)

2.2. Growth Curve of Rhodoccocus pyridinivorans ZZ47

Bacterial growth was defined by measuring optical density at 600 nm. *Rhodococcus pyridinivorans* bacteria suspension was diluted in TSB in order to obtain a suitable initial OD for the experiments (0.01 to 0.05 at 600 nm). Triplicates of bacteria were grown in TSB at 37 ˚C in Erlenmeyer flasks shaken at 200 rpm and OD measurements were performed for 24 h with both spectrophotometers (Thermo Multiscan Microplate Spectrophotometer) until stationary phase was reached. The aerobic bacteria, inoculated culture tubes were incubated in duplicate in a water bath at 37 ˚C. The OD was measured using the experimental manifold at 0, 1, 2, 4, 6, 8, 10, 12, 22 and 24 h (Castellane et al., 2017 & Maia et al., 2016).

2.3. Isolation and Purification of EPS

Wang et al. (2015) studies were optimized and EPS isolation was performed. Purification of the *R. pyridinovorans* strain, and precipitation of cells and proteins in the culture

medium were performed according to Güvensen et al., (2022). EPS material, which was left to dry in the lyophilizer, was kept in the lyophilizer until it dried. The dried EPSs were pulverized in sterile air. The purified EPSs were stored in sterile plastic Eppendorf tubes under refrigerator conditions at -4 ˚C.

2.4. Yield Rate Calculation of EPS

The yield rate was calculated by weighing the wet weight after production and the dry weight after the lyophilizer of EPS produced from the harvest, which was made from the EPS producer *R. pyridinovorans*. The % EPS wet and dry yield rate was calculated with the formula below.

(%) Wet EPS yield rate = 100 ml culture x wet EPS weight (g) / culture in total volume (ml)

(%) Dry EPS yield rate = 100 ml culture x dry EPS weight (g) / culture in total volume (ml)

2.5. Biological Activities of EPS

2.5.1. Anti-biofilm activity determination

In order to determine the antibiofilm activity, Venkatesh et al. (2016)'s method has been modified. The reaction mixture was prepared using a 96-well microplate. 180 µl of TSB and 10 µl of biofilm-forming pathogenic bacteria culture were placed in each well, for a total of 200 µl. 10 µl EPS at different concentrations (0.025, 0.050, 0.1 mg/ml) was added to each well. Bacteria used for monitoring biofilm removal; *Salmonella typhimurium* CCM 583, *Aeromonas hydrophila* ATCC 19570, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, *Shigella dysenteriae* ATCC 11835, *Staphylococcus aureus* ATCC 6538/P and *Bacillus subtilis* CCM 99. After the wells were prepared, they were incubated at 37°C for 48 h. At the end of the incubation, the reaction mixture was removed from the wells and washed with 200 µl of PBS. Biofilms were fixed with 50 µl of methanol and incubated for 10 minutes. After removal of methanol, it was washed again with PBS. After staining the biofilms with 0.2% crystal violet, they were washed with deionized water (dH2O). The microplates were left to dry in the crystal violet and 100 µl of acetic acid (0.5 M) was added. The absorbance of the microplates at 570 nm was measured. The % removal was calculated with the formula below.

(%) = Control-Test/Control x 100

2.5.2. Anti-angiogenic activity (HET-CAM test)

In order to determine the anti-angiogenic effects of EPS, a chorioallantoic membrane model was applied on fertilized chicken eggs (Krenn & Paper, 2009). Leghron white chicken eggs used for HET-CAM analysis were purchased from Izmir Veterinary Control Research Institute. Fertilized eggs were incubated in a horizontal position at 37.5±1 °C in 70±2% humidity through 7 days. On the seventh day, CAM was analyzed by cutting a window (2 cm^2) on one side of the egg. Normal developing embryos were included in the assay; malformed or dead embryos were excluded. Eggs were divided into three groups. 0.9% NaCl (300 µl) was used as the negative control, Suramin (50 µg/pellet) was used as the positive control and EPS in different concentrations (2, 1 and 0.5 mg/ml) were used. After the experiment, the eggs were numbered and photographed. The opening in the egg shells was covered with lab film and the eggs were incubated for 24 hours. All compounds were tested in triplicate at different intervals.

After the incubation, eggs were photographed. Angiogenesis scores and anti-angiogenic effects of the compounds on CAM were evaluated according to the scoring system given in Table 2. Finally, the results obtained are calculated by placing them in the formula given below.

Average score = [numbers of egg (score 2) x 2 + number of egg (score 1) x 1] / [total number of eggs (score 0, 1, 2)].

According to this system, a score < 0,5 indicated that there was no anti-angiogenic effect, 0,5-1 indicated a low anti-angiogenic effect, and > 1 indicated a powerful anti-angiogenic effect.

Table 2. Score values used in the evaluation of HET-CAM.

Score	Effect	Impression
< 0.5	No effect	Normal embryo development. There is no change according to the surrounding capillaries. No hemorrhage, vascular lysis or coagulation was detected.
0.5 - 1	Low	The area without the covers is low or the density of the capillary is reduced in a specific area. The effects are not more than 2 times the field of matter
> 1	High	There is space without capillaries. Normal embryo formation is not observed.

2.6. Toxicity of EPS

2.6.1. Genotoxicity assay (AMES test)

Salmonella typhimurium TA98, TA102 and TA1537 strains were used to determine the genotoxic potential of EPS according to Maron and Ames (1983). The design of experiment was performed using Araclor 1254 (CAS 48586) Sigma Aldrich (Germany) induced rat liver S9. Strain-specific positive control chemical Sodium azide (10 µg/ml) was used as strain-specific positive control agent.

For this experiment, the bacteria cultures were incubated at 37 °C shaking incubator (at 100 rpm continuously) for 12-14 hours using growth medium [Xenometrix (PMM-GMOO, LOT: K05672P)]. Subsequently, 100 µl cultures and 100 µl EPS (final concentrations at 2000 µg/plate) were added to 2 ml melted top agar supplemented with 0.5 mM histidine and 0.5 mM biotin. In the presence of S9 activation was added to the suspension of tested strains. The tubes were shaken and then

inoculated on the previously prepared Minimal Glucose Agar (MGA) petri dishes, which were kept at 37 °C for half an hour and heated. The petri dishes were incubated for 48 hours at 37 °C and after incubation, colonies observed in petri dishes were counted and the number of his+ revertants were determined. The experiment was done in triplicate.

2.6.2. Single dose toxicity test

This study was approved by the Ege University, Local Ethical Committee of Animal Experiment (2022-033). To determine potential single dose acute toxicity of EPS, 6 BALB/c female mice (8-10 weeks old, 20-25 g range) were used within the scope of OECD Guideline 423 Acute Oral Toxicity (Acute Toxic Class Method). Dosing was started as 300 mg/kg body weight due to the lack of literature information about the substance. The test substance was administered in such a way that the maximum fluid volume that could be administered at one time did not exceed 1 ml/100 g body weight. Doses were prepared shortly before administration. The test substance was administered orally as a single dose. Before dosing the animals will be fasted overnight, after the fasting period the animals are weighed and the test substance administered. After administration of the test substance, the mice were not given food for 3 hours. The test substance at a dose of 300 mg/kg was administered to 3 female mice. According to the clinical and death findings of the animals, the dose administration order specified in the Guideline was followed by either a 300 mg/kg dose again or a higher dose (2000 mg/kg). 3 female mice were used each time and a minimum of 24 hours was allowed between dosing of each animal. Animals were checked 30 minutes and 24 hours after test substance administration. The animals were followed for 14 days and after this period the animals were euthanized.

2.7. Statistical Analysis

Statistical analysis of the results was applied to SPSS version 25.0 (IBM Corp., Armonk, New York, USA). Values were expressed as mean ± SEM.

3. Results and Discussion

Due to bacteria live in harsh conditions, they have various protective molecules to survive their environment. One of them, EPS, which is high molecular weight carbohydrate polymers secreted by bacteria into extracellular environment, plays crucial defensive role against biotic and abiotic stress factors such as phage attack, antibiotics, toxic compounds, heat or osmotic stress (Limoli et. al., 2015 & Angelin & Kavitha, 2020). The EPS, which has synthesized by bacteria homo- or heteropolysaccharides structure, has biocompatible, biodegradable and non-toxic properties. In addition, it has invaluable biological activities such as anticancer, antioxidant, antiviral, anti-biofilm, and immunomodulatory activities (Angelin & Kavitha, 2020 & Barcelos et al., 2020 & Mohd Nadzir et al., 2021). Thanks to these features, it has great interest food, cosmetic, pharmaceutical and biomedical industries. Among them, in biomedical applications, it is used many aims such as a scaffold, drug carrier, diagnostic agent and surgical sealant (Mohd Nadzir et al., 2021). For these purposes, EPS is extracted from various bacteria. Bacterial EPS production has many advantages such as defined and reproducible rapidly, higher and quality production, and well-known isolation methods. Also, produced EPS amount is depending on several factors; these are bacteria strain, medium composition and culture conditions (Moscovici, 2015 & Barcelos et al., 2020 & Mohd Nadzir et al., 2021). Determination of potential toxic effect of produced EPS is extremely important particularly in terms of usability in food and health areas. With the preliminary studies obtained from *R. pyridinivorans*, high efficiency extraction of EPS material was achieved and purification and production conditions were optimized (Güvensen et al., 2022). The yield rate of our EPS material, whose production conditions were optimized, was calculated. For this purpose, the yield rate was calculated by weighing the wet weight after production and the dry weight after the lyophilizer of EPS from the 6th harvest, in this work. The wet and dry EPS ratios obtained from the culture in total (10 ml x 176 = 1760 ml) are given in the table below (Table 3).

Table 3. The wet and dry EPS ratios.

EPS	EPS amount obtained from 1760 ml culture	100 ml EPS yield rate from culture
Wet EPS	30.13 g	% 1.71
Dry EPS	17.84 g	% 1.01

The growth curve of *R. pyridinivorans*, the wild strain isolated from activated sludge, was formed by OD adjustment considering the study of Castellane et al. (2017) and the experiment was performed in 3 replications and doublets. The OD measurements of this bacterium made every hour are shown in the table. The graph formed according to the results of the OD measurements shows the growth curve of our strain (Fig. 1).

With our preliminary studies obtained from *R. pyridinivorans*, high efficiency extraction of EPS material was achieved and purification and production conditions were optimized. In addition, high film forming, viscosity and gelling capacity were determined and physicochemical characterization of EPS was revealed. Studies have shown that it has high antibiofilm activities (Fig. 2).

Time/OD	OD1	OD2	OD3	Average
0	0,231	0,231	0,231	0,231
1	0,226	0,281	0,202	0,2363333
2	0,301	0,306	0,298	0,3016667
3	0,448	0,446	0,45	0,448
4	0,622	0,611	0,631	0,6213333
5	0,821	0,822	0,825	0,8226667
6	0,916	0,912	0,906	0,9113333
7	0,936	0,941	0,939	0,9386667
8	0,945	0,946	0,952	0,9476667
9	0,945	0,951	0,951	0,949
10	0,951	0,949	0,952	0,9506667
11	0,951	0,953	0,955	0,953
12	0,953	0,957	0,955	0,955
13	0,958	0,965	0,962	0,9616667
14	0,957	0,958	0,961	0,9586667
15	0,956	0,957	0,956	0,9563333
16	0,976	0,981	0,978	0,9783333
17	0,995	0,997	0,991	0,9943333
18	0,999	0,985	0,992	0,992
19	0,987	0,988	0,986	0,987
20	0,992	0,991	0,991	0,9913333
21	0,995	0,997	0,992	0,9946667
22	0,997	0,989	0,991	0,9923333
23	0,986	0,987	0,987	0,9866667
24	0,995	0,994	0,995	0,9946667
	0,995	0,994	0,995	0,9946667

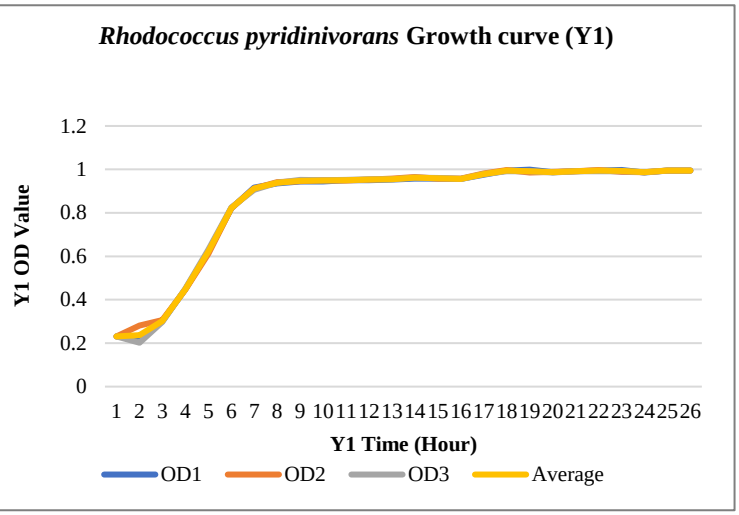

Figure 1. *Rhodococcus pyridinivorans* Growth curve (Y1).

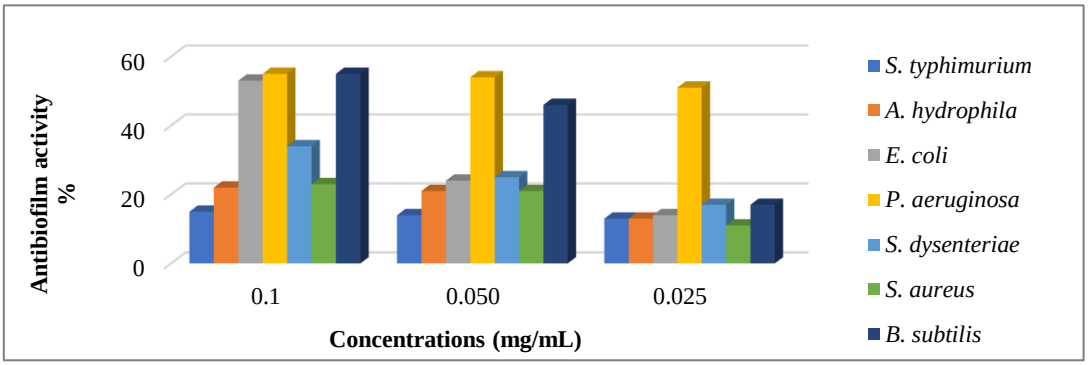

Figure 2. Percent antibiofilm activities.

R. pyridinivorans showed antibiofilm activity between 11-55%. It was observed that biofilm removal was highest in *B. subtilis* and *P. aeruginosa* bacteria at a concentration of 55% at a concentration of 0.1 mg/ml. Again, at the same concentration, it is 53% in *E. coli*. The lowest activity was seen in *S. aureus* at a rate of 11% at 0.025 mg/ml. It was then observed in *S. typhimurium* at a rate of 13% at 0.025 mg/ml. Thanks to the antibiofilm activity of EPS, it will be possible to delay microbial spoilage and extend shelf-life in foods. In addition, our natural strain, R. *pyridinivorans* ZZ47, isolated from activated sludge, was identified by conventional and molecular techniques. Our strain has been registered in GenBank with accession number AF173005. The originality of the study is increasing in terms of toxicity studies of *R. pyridinivorans* ZZ47, which is our national isolate, in order to bring it using in different sectors.

Anti-angiogenic therapy is one kind of popular and common strategy for cancer treatment. Since cancer cells have high energy consume to realize proliferation and metastasis, they need to new blood vessels. Angiogenesis is a complex mechanism the formation of new blood vessels, and several molecules play a role in the phenomenon (Oguntade et al., 2021 & Al-Husein et al., 2012). In our study, the EPS was showed strong and weak anti-angiogenic activity at 2 mg/ml and 1 mg/ml, respectively. On the other hand, no anti-angiogenic effect was seen at 0.5 mg/ml. Hence, we were demonstrated that the EPS has dose-dependent anti-angiogenic properties by HET-CAM test (Table 4, Figure 3). In parallel with our results, an EPS isolated from *Bacillus velezensis* OM03 has inhibited nearly 95% vascularization at 400 µg/ml concentration by chick CAM. In addition to this, this EPS has significant cytotoxic effect on PA-1 (human ovarian teratocarcinoma) and SKOV-3 (human ovarian cancer) cells without damage normal human ovarian surface epithelial cell line T1074, and has triggered apoptosis of PA-1 cells (Chirakkara & Abraham, 2023). In another study, EPS from *Lactobacillus acidophilus* with antioxidant properties has inhibited tumor angiogenesis related genes expressions and upregulated anti-angiogenic genes in colon cancer cell lines (Deepak et al., 2016).

Table 4. Anti-angiogenic effect results of EPS.

Concentration (mg/ml)	Score	Anti-angiogenic effect
2	1.33 ± 0.47	Strong
1	0.66 ± 0.23	Weak
0.5	0.33 ± 0.23	No
Suramin (50 µg/pellet)	1.32 ± 0.05	Strong
NaCl (%0.9)	0.10 ± 0.05	No
Water	0.10 ± 0.05	No

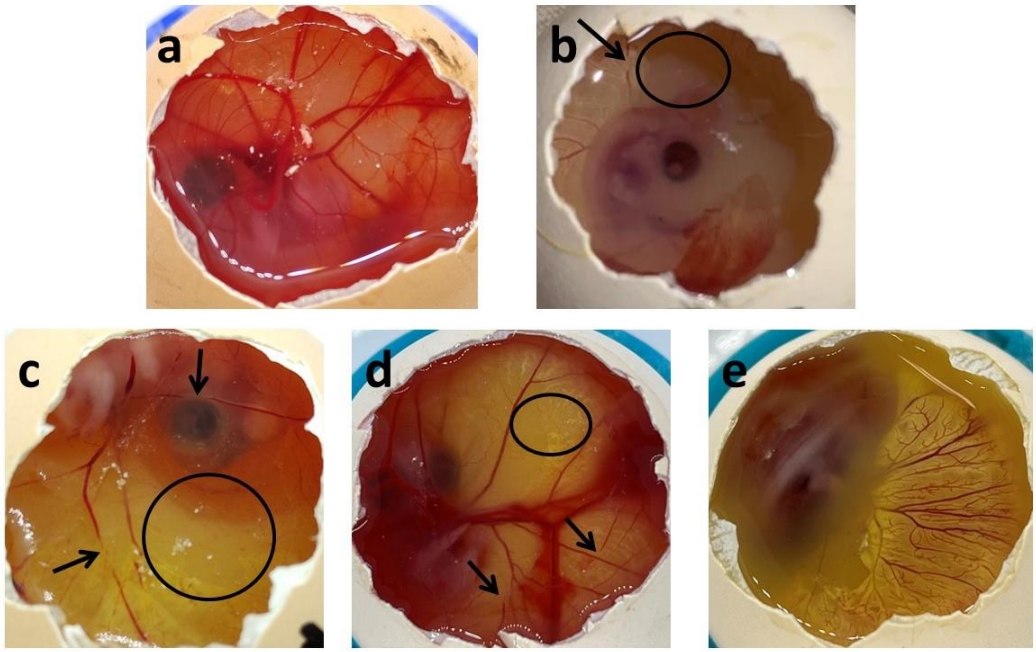

Figure 3. Anti-angiogenic properties results of EPS by HET-CAM test. a. NaCl (negative control); b. Suramin (positive control); c. 2 mg/ml EPS; d. 1 mg/ml EPS; e. 0.5 mg/ml EPS administration. Arrow shows prevented vessel formation. Circle shows area without capillaries.

In recent years, natural products are very attractive research area to find a solution against especially in cancer. Among them, many studies are carried out with bacterial EPS due to it has various biological effect particularly cytotoxic effect. Recently, novel exopolysaccharide EPSR4, isolated from *Bacillus subtilis* strain AG4, has showed remarkable cytotoxic effect on T-24 (bladder carcinoma), A-549 (lung cancer) and HepG-2 (hepatocellular carcinoma) cancer cells and IC_{50} values were 244 µg/ml, 148 µg/ml and 123 µg/ml, respectively. Also, it has strong antioxidant activity cause scavenging DPPH and hydrogen peroxide free radicals (Abdel-Wahab et al., 2022). In a study, EPS, produced from *Rhodococcus erythropolis* HX-2, has demonstrated significant cytotoxic effect on A-549, SMMC-7721 liver cancer cells and HeLa cervical cancer cells without harm to L929 normal cells (Hu et al., 2020). In our previous studies, EPS *R. pyridinivorans* ZZ47 was showed low cytotoxic activities on human colorectal adenocarcinoma (HT-29) and human breast adenocarcinoma (MCF-7) cells (Güvensen et al., 2018 & Güvensen et al., 2022). Although several cytotoxicity tests have performed for different bacterial-source EPS in the literature, there are not genotoxicity test enough. In present study, revertant colonies numbers of EPS treatment were not exceed spontaneous revertant numbers on *S. typhimurium* TA98, TA102 and TA1537 strains (Table 5). Therefore, EPS has not genotoxic effect. Likewise, in a recent study showed that EPS_{KC27L}, isolated from *Lactobacillus salivarius* KC27L, has not genotoxic effect by chromosome aberration, sister chromatid exchange, micronucleus, and comet assays (Yildiz et al., 2023).

Table 5. Revertant colonies numbers by AMES test after EPS administration.

Treatment	Concentration	Revertant colonies numbers (Mean±SD)					
		TA 98		TA 102		TA 1537	
		+ S9	- S9	+ S9	- S9	+ S9	- S9
EPS	2 mg/ml	37±23	17±4	200±5	170±6	33±4	19±3
Distilled water	100 µl	17±4	30±9	130±32	115±20	6±5	6±4
Sodium azide	10 µg	1600±42*	1720±55*	1590±72*	1150±85*	3360±54*	485±35*

*Deviation from the number of spontaneous revertant colonies.

During the single-dose toxicity test, no adverse reactions such as death, general appearance or behavior were observed in the mice. There was no weight loss in the body weight between before and end of the study (22,63±0,17 g. and 25,21±1,56 g, respectively). After a single dose toxicity study, organ (heart, lung, liver and kidney) weights and relative organ weights of the mice are presented in Table 6. Rodents show variation in many clinical chemistry values and therefore reference values cannot be specified for many parameters. However, in the evaluation made by considering the reference values specified for the BALB/cByJ breed (Loeb and Quimby, 1999), according to Total protein (TP) and Albumin (ALB) values, which are indicators of general physical condition, the general health status of the animals was good (Table 7). At the end of the single dose toxicity study, LD_{50} value of the EPS was determined as >2000 mg/kg in BALB/c. In parallel our results, Pinto et al. (2016) showed that bacterial cellulose, which is one kind of cellulosic exopolysaccharide obtained from sugarcane molasses, has not acutely toxic effect on rats with no cytotoxic or genotoxic effect on cells.

Table 6. Acute toxicity test result organ weights and relative organ weights (mean±SD).

	Heart	Lung	Liver	Kidney
Organ weight (g)	0.17±0.01	0.23±0.03	1.48±0.02	0.44±0.03
Relative organ weight	0.006	0.008	0.049	0.015

Table 7. Effect of EPS on biochemical parameters of mice.

Biochemical Parameters	EPS	Ref. range*
Total Protein (g/dL)	4.1	4.4-7.6
ALB (g/dL)	2.2	2.7-4.9
ALP (U/L)	<14	-
GLU (mg/dL)	283	114-279
TBIL (U/L)	1.9	0.5-1.1
TCHO (mg/dL)	67	80-219
ALT (U/L)	203	-
GGT (U/L)	<10	-
Ca (mg/dL)	<4.0	8.5-10.9
IP (mg/dL)	7.2	-
CRE (mg/dL)	0.39	0.2-0.7
BUN (mg/dL)	21.5	-
GLOB (g/dL)	1.9	-

* BALB/cByJ reference range (Loeb & Quimby, 1999).

In conclusion, this study showed that our EPS material, which has antibiofilm, antiangiogenic and antioxidant activities, does not have genotoxicity and acute toxic effects. Due to the biological activities of the polymer it may be suitable for use in different industries such as pharmaceutical, diagnostic, therapeutic and food. Also, thanks to the antibiofilm activity of EPS, it will be possible to delay microbial spoilage and extend shelf-life in foods. It is thought that EPS can activate the defense system more thanks to its antioxidant effect and prevent metastases due to its anti-angiogenic effect. In addition, although promising results have obtained for the EPS, further toxicological studies are required to elucidate the potential of it for use in cancer therapy.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

Abdel-Wahab, B. A., F. Abd El-Kareem, H., Alzamami, A., A. Fahmy, C., H. Elesawy, B., Mostafa Mahmoud, M., & M. Saied, E. (2022). Novel exopolysaccharide from marine bacillus subtilis with broad potential biological activities: Insights into antioxidant, anti-inflammatory, cytotoxicity, and anti-alzheimer activity. *Metabolites*, *12*(8), 715. https://doi.org/10.3390/metabo12080715

Ahmad, S., Tanweer, M. S., Mir, T. A., Alam, M., Ikram, S., & Sheikh, J. N. (2023). Antimicrobial gum based hydrogels as adsorbents for the removal of organic and inorganic pollutants. *Journal of Water Process Engineering*, *51*, 103377. https://doi.org/10.1016/j.jwpe.2022.103377

Al-Husein, B., Abdalla, M., Trepte, M., DeRemer, D. L., & Somanath, P. R., (2012). Antiangiogenic therapy for

cancer: An update. *Pharmacotherapy*, *32*(12), 1095-1111. https://doi.org/10.1002/phar.1147

Angelin, J., & Kavitha, M. (2020). Exopolysaccharides from probiotic bacteria and their health potential. *International Journal of Biological Macromolecules*, *162*, 853-865. https://doi.org/10.1016/j.ijbiomac.2020.06.190

Barcelos, M. C., Vespermann, K. A., Pelissari, F. M., & Molina, G. (2020). Current status of biotechnological production and applications of microbial exopolysaccharides. *Critical Reviews in Food Science and Nutrition*, *60*(9), 1475-1495. https://doi.org/10.1080/10408398.2019.1575791

Bello, K., Sarojini, B. K., Narayana, B., Rao, A., & Byrappa, K. (2018). A study on adsorption behavior of newly synthesized banana pseudo-stem derived superabsorbent hydrogels for cationic and anionic dye removal from effluents. *Carbohydrate Polymers*, *181*, 605–615. https://doi.org/10.1016/j.carbpol.2017.11.106

Botelho, P. S., Maciel, M. I., Bueno, L. A., Maria de Fátima, F. M., Marques, D. N., & Silva, T. M. S. (2014). Characterisation of a new exopolysaccharide obtained from of fermented kefir grains in soymilk. *Carbohydrate Polymers*, *107*, 1-6. https://doi.org/10.1016/j.carbpol.2014.02.036

Castellane, T. C. L., Campanharo, J. C., Colnago, L. A., Coutinho, I. D., Lopes, É. M., Lemos, M. V. F., & de Macedo Lemos, E. G. (2017). Characterization of new exopolysaccharide production by *Rhizobium tropici* during growth on hydrocarbon substrate. *International Journal of Biological Macromolecules*, *96*, 361-369. https://doi.org/10.1016/j.ijbiomac.2016.11.123

Chaisuwan, W., Jantanasakulwong, K., Wangtueai, S., Phimolsiripol, Y., Chaiyaso, T., Techapun, C., Phongthai, S., You, S., Regenstein, J. M., & Seesuriyachan, P. (2020). Microbial exopolysaccharides for immune enhancement: Fermentation, modifications and bioactivities. *Food Bioscience*, *35*, 100564. https://doi.org/10.1016/j.fbio.2020.100564

Chirakkara, S. P., & Abraham, A. (2023). Exopolysaccharide from the mice ovarian bacterium *Bacillus velezensis* OM03 triggers caspase-3-dependent apoptosis in ovarian cancer cells. *Journal of Applied Pharmaceutical Science*, *13*(6), 154-164. https://doi.org/10.7324/JAPS.2023.110355

Deepak, V., Ramachandran, S., Balahmar, R. M., Pandian, S. R. K., Sivasubramaniam, S. D., Nellaiah, H., & Sundar, K. (2016). In vitro evaluation of anticancer properties of exopolysaccharides from Lactobacillus acidophilus in colon cancer cell lines. *In Vitro Cellular & Developmental Biology-Animal*, *52*(2), 163-173. https://doi.org/10.1007/s11626-015-9970-3

Erdoğdu, T. (2018). *Enterobacter sp. KF052587 (ZZ40) ve Rhodococcus pyridinivorans AF173005 (ZZ 47) izolatlarından elde edilen ekzopolisakkaritlerin çeşitli yöntemlerle karakterizasyonu ve biyoteknolojik uygulamalarının değerlendirilmesi* (Master's thesis, Muğla Sıtkı Koçman University). (In Turkish)

Gürleyendağ, B. (2006) *Polisakkarit üreten ekstremofillerin belirlenmesi ve ekzopolisakkarit üretimi* (Master's thesis, Marmara University). (In Turkish)

Güvensen, C. N., Erdoğdu, T., Alper, M., & Güneş, H. (2018). *Evaluation of antibiofilm and cytotoxic potential of exopolysaccharides from ZZ40 Enterobacter sp. and ZZ47 Rhodococcus pyridinovorans strains*. Kastamonu Üniversitesi International Ecology 2018 Symposium. Kastamonu.

Güvensen, N. C., Alper, M., & Taşkaya, A. (2022). The evaluation of biological activities of exopolysaccharide from *Rhodococcus pyridinivorans* in vitro. *The European Journal of Research and Development*, *2*(2), 491-504. https://doi.org/10.56038/ejrnd.v2i2.46

Hu, X., Li, D., Qiao, Y., Wang, X., Zhang, Q., Zhao, W., & Huang, L. (2020). Purification, characterization and anticancer activities of exopolysaccharide produced by *Rhodococcus erythropolis* HX-2. *International Journal of Biological Macromolecules*, *145*, 646-654. https://doi.org/10.1016/j.ijbiomac.2019.12.228

Hussain, A., Zia, K. M., Tabasum, S., Noreen, A., Ali, M., Iqbal, R., & Zuber, M. (2017). Blends and composites of exopolysaccharides; properties and applications: A review. *International Journal of Biological Macromolecules*, *94*, 10-27. https://doi.org/10.1016/j.ijbiomac.2016.09.104

Krenn, L., & Paper, D. H. (2009). Inhibition of angiogenesis and inflammation by an extract of red clover (*Trifolium pratense* L.). *Phytomedicine*, *16*(12), 1083-1088. https://doi.org/10.1016/j.phymed.2009.05.017

Limoli, D. H., Jones, C. J., & Wozniak, D. J. (2015). Bacterial extracellular polysaccharides in biofilm formation and function. *Microbial Biofilms*, 223-247. https://doi.org/10.1128/9781555817466.ch11

Loeb, W. F., & Quimby, F. W. (1999). *The clinical chemistry of laboratory animals, second edition*. CRC Press.

Madhuri, K., & Prabhakar, V. (2014). Microbial exopolysaccharides: Biosynthesis and potential applications. *Oriental Journal of Chemistry*, *30*(3), 1401-1410. http://dx.doi.org/10.13005/ojc/300362

Mahto, K. U., Priyadarshanee, M., Samantaray, D. P., & Das, S. (2022). Bacterial biofilm and extracellular polymeric substances in the treatment of environmental pollutants: Beyond the protective role in survivability. *Journal of Cleaner Production*, *379*, 134759. https://doi.org/10.1016/j.jclepro.2022.134759

Maia, M. R., Marques, S., Cabrita, A. R., Wallace, R. J., Thompson, G., Fonseca, A. J., & Oliveira, H. M. (2016). Simple and versatile turbidimetric monitoring of bacterial growth in liquid cultures using a customized

3D printed culture tube holder and a miniaturized spectrophotometer: application to facultative and strictly anaerobic bacteria. *Frontiers in Microbiology*, *7*, 1381. https://doi.org/10.3389/fmicb.2016.01381

Maron, D. M., & Ames, B. N. (1983). Revised methods for the Salmonella mutagenicity test. *Mutation Research/Environmental Mutagenesis and Related Subjects*, *113*(3-4), 173-215. https://doi.org/10.1016/0165-1161(83)90010-9

Mohd Nadzir, M., Nurhayati, R. W., Idris, F. N., & Nguyen, M. H. (2021). Biomedical applications of bacterial exopolysaccharides: A review. *Polymers*, *13*(4), 530. https://doi.org/10.3390/polym13040530

Moscovici, M. (2015). Present and future medical applications of microbial exopolysaccharides. *Frontiers in Microbiology*, *6*, 1012. https://doi.org/10.3389/fmicb.2015.01012

OECD. (2002). *OECD guideline for the testing of chemicals*. https://www.oecd-ilibrary.org/test-no-423-acute-oral-toxicity-acute-toxic-class-method_5lmqcr2k7mzp.pdf?itemId=%2Fcontent%2Fpublication%2F9789264071001-en&mimeType=pdf

Oguntade, A. S., Al-Amodi, F., Alrumayh, A., Alobaida, M., & Bwalya, M. (2021). Anti-angiogenesis in cancer therapeutics: The magic bullet. *Journal of the Egyptian National Cancer Institute*, *33*(1), 1-11. https://doi.org/10.1186/s43046-021-00072-6

Pinto, F. C. M., De-Oliveira, A. C. A., De-Carvalho, R. R., Gomes-Carneiro, M. R., Coelho, D. R., Lima, S. V. C., & Aguiar, J. L. A. (2016). Acute toxicity, cytotoxicity, genotoxicity and antigenotoxic effects of a cellulosic exopolysaccharide obtained from sugarcane molasses. *Carbohydrate Polymers*, *137*, 556-560. https://doi.org/10.1016/j.carbpol.2015.10.071

Ramirez, M. A. J. R., (2016). Characterization and safety evaluation of exopolysaccharide produced by *Rhodotorula minuta* BIOTECH 2178. *International Journal of Food Engineering*, *2*(1), 31-35. https://doi.org/10.18178/ijfe.2.1.31-35

Venkatesh, K. S., Gopinath, K., Palani, N. S., Arumugam, A., Jose, S. P., Bahadur, S. A., & Ilangovan, R. (2016). Plant pathogenic fungus *F. solani* mediated biosynthesis of nanoceria: Antibacterial and antibiofilm activity. *RSC advances*, (48), 42720-42729. https://doi.org/10.1039/C6RA05003D

Wang, J., Zhao, X., Yang, Y., Zhao, A., & Yang, Z. (2015). Characterization and bioactivities of an exopolysaccharide produced by *Lactobacillus plantarum* YW32. *International Journal of Biological Macromolecules*, *74*, 119-126. https://doi.org/10.1016/j.ijbiomac.2014.12.006

Yildiz, B. M., Yuzbasioglu, D., Yuksekdag, Z., Cetin, D., Unal, F., & Suludere, Z. (2023). In vitro genotoxic and antigenotoxic effects of an exopolysaccharide isolated from *Lactobacillus salivarius* KC27L. *Toxicology in Vitro*, *86*, 105507. https://doi.org/10.1016/j.tiv.2022.105507

Zhao, M., Cui, N., Qu, F., Huang, X., Yang, H., Nie, S., Zha, X., Cui S. W., Nishinari, K., Phillips, G. O., & Fang, Y., (2017). Novel nano-particulated exopolysaccharide produced by *Klebsiella sp.* PHRC1.001. *Carbohydrate Polymers*, *171*(2017), 252-258. https://doi.org/10.1016/j.carbpol.2017.05.015