

Research Article

Evaluation of Biological Activities of Exopolysaccharide from *Rhodococcus pyridinivorans* In vitro

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(First received April 12, 2022 and in final form May 23, 2022)

Reference: Ceyhan Güvensen, N., Alper, M., & Taşkaya, A. The Evaluation of Biological Activities of Exopolysaccharide from *Rhodococcus pyridinivorans* In vitro . *The European Journal of Research and Development*, 2(2), 491–504.

Abstract

Microbial exopolysaccharides (EPSs) are biopolymers in the form of carbohydrates produced by many microorganisms and secreted into the external environment. EPS protects the microorganism from drying, phagocytosis, and phage effects, and acts as a barrier in stress environments such as heat, light and sound. EPSs produced for industrial purposes are generally used in areas such as food, cosmetics, petroleum and chemistry. This study was aimed to investigate in addition to basic physical and chemical properties of R. pyridinivorans EPS, in vitro its biological activities such as antioxidant properties and antiproliferative activity. The antioxidant properties of EPS were determined by DPPH and hydroxyl radical elimination. The antiproliferative activity of EPS on HT-29 and MCF-7 cell lines was determined by MTT assay. The results of study indicate that EPS from R. pyridinivorans have important biological activities. Further studies on structural and mechanism elucidation of the bacterial EPSs are still needed being carried out.

Keywords: *Rhodococcus pyridinivorans*, exopolysaccharide (EPS), physical and chemical properties, biological activity, antioxidant activity, antiproliferative activity.

1. Introduction

Recently, there has been increasing interest in the development of sustainable biomaterials and bioplastics from renewable sources.

With their unique structures, physico-chemical properties, chemical stability, excellent selectivity towards aromatic compounds and their ability to form

supramolecular complexes, polysaccharide-based biopolymers have been the focus of attention in many applications such as drug delivery.

It is also used as tissue engineering scaffolds and implants, multifunctional biomaterials, artificial leather, cell carriers and bioactive compounds, nanomedical agents, food additives, bioplastics and films in biopackaging [1]. The widely studied and commercially produced microbial EPSs have film-forming, emulsifying, thickening, structure-modifying, gelling capacities and/or biological activities and are widely used in the food, cosmetic, pharmaceutical and biomedical industries. Examples include xanthan gum, gellan, alginate, pullulan, dextran, glucans, bacterial cellulose, hyaluronan, succinoglycan, and levan [2]. Xing et al., (2018) [3] obtained EPS producer *Leuconostoc mesenteroides* DRP105 strain from sauerkraut. The crude EPS and the purified fraction of the bacterium were found to have unique properties for industrial applications. Interest in the microbial synthesis of polysaccharides with special functional properties has been increasing in recent years. So much so that in another study, *Klebsiella sp.* The physicochemical, rheological, emulsifying and toxicological properties of PHRC1.001 strain and EPS obtained from this bacterium were characterized.

Acute toxicity testing showed that 1.8 g EPS per kg body weight did not cause a toxic effect on mice. In this study, EPS producing *Klebsiella sp.* It has been reported to approach a relatively high EPS yield of 21 g/L. The study showed that EPS could potentially be exploited as a new industrial polysaccharide in food packaging [2]. A few examples of commercial EPSs and their applications are presented in table 1 [4].

Table 1 Commonly used EPSs (homopolysaccharides and heteropolysaccharides): Producer microorganisms and applications.

EPSs	Microorganisms	Applications	References
Homopolysaccharides			

Dextran	<i>Leuconostoc mesenteroides</i> , <i>Streptococcus mutans</i>	Content, drug, chromatography medium in blood plasmas	Mahduri and Prabhakar, 2014; Schmid et al., 2015; Ateş, 2015
Levan	<i>Halomonas smyrnensis</i> AAD6 ^T , <i>Zymomonas mobilis</i> , <i>Bacillus</i> <i>subtilis</i>	Food (prebiotic), feed additives, cosmetics, glue, emulsifier, stabilizer and thickener	Ateş, 2015; Schmid et al., 2015
Pullulan	<i>Aureobasidium pullulans</i>	Thickening, stabilizing, texturing and gelling agents	Ateş, 2015
Curdlan	<i>Agrobacterium radiobacter</i> , <i>A.rhizogenes</i> , <i>Rhizobium trifolii</i>	Food, cosmetics, medicine	Güvensen 2016; Schmid et al., 2015
Heteropolysaccharides			
Xanthan	<i>Xanthomonas compestris</i>	Food additive, animal feed, petroleum industry	Ateş, 2015; Schmid et al., 2015; Mahduri and Prabhakar, 2014
Alginate	<i>Pseudomonas aerugina</i> , <i>Azobacter</i> sp.	Food, feed, pharmaceutical industry	Schmid et al., 2015; Donot et al., 2012.
Emulsan	<i>Pseudomonas fluorescense</i>	Emulsifying agent in various foods	Mahduri and Prabhakar, 2014.
Gellan	<i>Alcaligenes faecalis</i> , <i>Sphingomonas</i> sp.	Food industry, animal feed	Donot et al., 2012; Schmid et al., 2015

The usage areas of commercial EPSs are increasing day by day (Table 1). Especially in the future, their use in medicine is expected to be widely used. It is predicted that it can be used in genetic therapy of cancer, tissue engineering, healing of wounds or diagnosis of diseases [5]. Genetic studies on EPS producing bacteria continue and studies have been carried out to increase EPS production with modified genes and to use them in the food industry [6].

EPSs can be used as an alternative to commercial stabilizers in foods due to their functional properties such as viscosity increaser, structure regulator and water binder. In addition to improving the rheological properties of foods, microorganisms producing EPS can hold onto the host better [7]. In addition, immobilized EPS producing cells are considered as promising therapy for organ transplant patients (eg, diabetes, liver failure). The use of EPSs as a promoter for colonies that stimulate the synthesis of interferon, platelet aggregation and factors, worms' sulphate availability as a promising vaccine adjuvant [8], antitumor, immune response in lactic acid bacteria (LAB) EPSs. stimulant, antibiofilm and antioxidant activity have been reported [4]. The beneficial effects of some microbial EPSs on health are presented in Table 2.

Table 2 Health benefits of EPS producing bacteria (Madhuri and Prabhakar, 2014).

EPS producing bacteria	Health benefits	Reference
<i>Lactobacillus delbrueckii ssp. bulgaricus</i>	Stimulating mouse splenocytes	Kitazawa et al., 1993
<i>Bifidobacterium bifidum</i>	Antiulcer activity	Nagaoka et al., 1994
<i>Lactobacillus rhamnosus</i>	Stimulating lymphocytes in mice	Chabot et al., 2001
<i>B. lactis Bb12</i>	Proliferation of lymphocytes in mice	Amrouche et al., 2006
<i>Bacillus licheniformis</i>	Antiviral and immunostimulatory activity	Arena et al., 2006
<i>L. kefiranofaciens</i>	Increased mucosal immunity	Vinderola et al., 2006
<i>Pantoea agglomerans</i>	Capturing free radicals	Wang et al., 2007b
<i>L. plantarum</i>	Antimutagenic activity	Kodali and Sen, 2008
<i>Bacillus coagulans</i>	Antioxidant and antihyperglycemic activity	Kodali and Sen, 2008

Cancer refers to the rapid replication of abnormal cells with the potential to overgrow, invade nearby tissue, and metastasize to other organs [9]. Cancer is one of the diseases that threaten people and cause death all over the world. Current cancer modalities (surgery, radiation, and chemotherapy) suffer from several limitations in clinical practice, such as multidrug resistance and serious side effects [9,10]. Therefore, the search for new bioactive natural anticancer products has received increasing attention in recent years. Exopolysaccharides (EPSs) have received great attention due to their biotechnological applications [9]. Several previous studies have shown that EPSs have excellent biodegradability and biocompatibility [11], nano drug carriers [12], immunomodulatory and antioxidant properties [13, 14, 15], showed some beneficial effects such as anti-viral effects [16] and anti-tumor activities [17, 18].

2. Materials and methods

2.1. Microorganism, inoculum and culture conditions

ZZ47 *Rhodococcus pyridinovorans* strain, which was isolated from the biofilm causing problems in the wastewater treatment plant and was found to produce EPS, was used. *R. pyridinovorans* strain was activated in TSB medium. It was kept in an incubator at 37 °C for 24 hours. Then it was taken into a +4 cupboard and preserved. For the storage conditions of the studied isolate, a deep mill was placed at -80 °C in 20% glycerol. Bacterial culture was activated in liquid medium in TSB medium predetermined

according to previous studies. 100 ml of liquid TSB and 10 ml of culture were placed in 250 ml flasks and they were kept in a 37 °C incubator for a minimum of 18 hours and a maximum of 24 hours.

2.2. Isolation and purification of EPS

Wang et al. (2015) [19] method was modified and applied for the isolation and purification process of EPS. For purification purposes, EPS producer *R. pyridinovorans* strain was incubated for 24 h at 37 °C. Then, 4% TCA was added to the culture medium and mixed for 30 minutes at room temperature. Centrifugation was performed at 4000 rpm for 33 minutes at 4 °C to precipitate cells and proteins. The supernatants obtained at the end of centrifugation were precipitated with cold propanol. Precipitation was done by mixing the culture medium (100 ml) in 95% isopropanol (300 ml) at the burner flame. The mixture was kept at 4 °C for 24 hours. After 24 hours, EPS was separated from the mixture and lyophilized. The mixing process was done by means of plastic sticks. The resulting EPSs adhered to the plastic rod. Wet EPS separated from the liquid with a plastic stick was placed in petri dishes. Petri dishes, which were covered with parafilm and perforated with a needle, were placed in a lyophilizer at -80 °C. Petri dishes kept in the lyophilizer were removed when the EPS dried. The dried EPSs were pounded in a mortar. The purified EPSs were stored in plastic tubes at -4 °C in the refrigerator.

2.3. pH tolerance

R. pyridinovorans 9 ml TSB medium in glass tubes was incubated at 37 °C for 24 hours. TSB media with pH of 2, 3, 4, 5, 6, 6.8, 7, 7.5, 8, 8.5 and 9 were prepared in glass tubes. Tubes containing 9 ml TSB medium were prepared for each pH, respectively. 1 ml of the activated strain was drawn with a micropipette to transfer it to the first tube. It is poured into the tube that we will dilute first. The dilution process continued in the other tubes. After the process was finished, the tubes were kept in a 37 °C incubator for 2.5 hours. Petri dishes containing TSA medium, which we will use in the last step, were prepared. 100 µl of each tube was inoculated into petri dishes. Spread sowing was done with the help of Drigalski spatula. Petri dishes were placed in a 37 °C incubator. Colony counts were made every 6 hours between 0-30 hours.

2.4. Analysis of the physical and chemical properties of EPS

2.4.1. Appearance and resolution of EPS

The solubility of EPS in solvents such as ethanol, methanol, acetone, propanol and water was investigated. Dry EPS powder (0.02 g) was dissolved in said solvents (5 ml). Magnetic heater (60 °C) was used in the thawing process.

2.4.2. Extraction of EPS

0.05 g of pure EPS was dissolved in 10 ml of water. It was left in an incubator at 35 °C for 24 hours. It was centrifuged at 6000 rpm for 10 min to examine the precipitation of EPS. EPS precipitated was determined and 5 values between 190 nm-550 nm (190 nm, 220 nm, 260 nm, 330 nm, 440 nm, 550 nm) were determined and measurements were made to examine the presence of protein and nucleic acid in the spectrophotometer.

2.4.3. Morphological of EPS

The surface morphology of the polymer samples was investigated by scanning electron microscopy (SEM) and field emission scanning electron microscopy. EPS was washed three times with an aqueous 1% osmium tetroxide solution for 2 hours at 4 °C. The drying process was dehydrated in the ethanol series. Magnetic purification method was used in the coating. 10-15 nm gold plating was applied to the samples. All samples were examined in 5 kV 10 kV and SUBRA 40, Zeiss SMT AG Fesem de Vega TESCAN SEM device. For transmission electron microscopy, samples were fixed for 2 hours at room temperature with 0.15% glutaraldehyde, 2.5% in PBS buffer containing ruthenium red. The fixed material was then washed three times with an aqueous 1% osmium tetroxide for 2 hours at 4 °C and rewashed five times with ethanol dehydration buffer (50, 80, 90 and saturated). Ruthenium red was retained in 0.05% of all solutions up to 80% ethanol. After drying the cast of uranyl acetate stained glass material on a copper grid, it was investigated under a Zeiss TEM at an accelerated voltage of 60 kV.

2.5. Analysis of the biological activities of EPS

2.5.1. Antioxidant activity determination

2.5.1.1. DPPH free radical scavenging activity

The method of Shimada, Fujika, Yahara and Nakamura (1992) [20] was used for DPPH free radical scavenging activity. EPS solution at different concentrations was prepared. For 4 mL reaction mixture, 2 mL DPPH solution and 2 mL EPS solutions at different

concentrations were used. The mixture was kept at 25°C for 15 minutes and the values at 517 nm were measured. The results were calculated according to the formula below [21].

$$(1-A \text{ sample} / A \text{ blank}) \times 100$$

2.5.1.2. Hydroxyl radical scavenging activity

The method of Winterbourn and Sutton (1984) [22] was applied for the Hydroxyl Radical Scavenging Activity. The reaction mixture was adjusted to 4.5 mL in total. To this mixture, 1 mL of PBS, 1 mL of safranin (40 µg/mL), 1 mL of EDTA-Fe(II) (0.945 mM), 1 mL of H₂O₂ (3% (V/V)) and 0.5 mL of EPS were added. It was kept at °C for 30 minutes and measured at 560 nm. The results were calculated according to the equation below [21].

$$(A \text{ blind} - A \text{ sample}) / A \text{ blind} \times 100$$

2.5.2. Cell lines and growth conditions

HT-29 (human colorectal adenocarcinoma) and MCF-7 (human breast adenocarcinoma) cell lines were obtained from the American Type Culture Collection (ATCC). The cell lines were cultured in Roswell Park Memorial Institute-1640 Medium with heat inactivated 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were incubated in a humidified incubator with 95% air and 5% CO₂ at 37°C. When the cells reached 70–80% confluence, they were passaged or used for analysis.

2.5.3. MTT Assay

The antiproliferative activity of *R. pyridinovorans* EPS on HT-29 and MCF-7 cell lines was determined by MTT assay [23]. In this assay, the 200 µl of exponentially growing cells were plated at 2×10⁴ cells/well for HT-29 and 1×10⁴ cells/well for MCF-7 in 96-well microplates as triplicate and incubated for 24 hours in 5% CO₂ incubator at 37 °C. Then, the cells were treated with the EPS at seven different serial concentrations (0.3, 0.6, 0.12, 0.25, 0.5, 1 and 2 mg/mL) and incubated for additional 24 hours. Afterwards, the medium in each well was replaced with 100 µl of fresh growth medium and 10 µl of MTT at 5 mg/ml in phosphate buffer solution was added into each well. After 4 hours incubation at 37°C, the medium with MTT in each well was gently removed and replaced with 100 µl of DMSO to dissolve the formazan crystals. Lastly, the absorbance was measured at 540 nm on a microplate reader. Each independent experiments was repeated at least twice and untreated cells were used as a control. The results evaluated according to % cell viability based on control and treated cells.

2. Results

2.1. SEM images of EPS

The film forming ability of EPS and the control of this film by SEM (Scanning Electron Microscopy) were found to be of good quality. The viscosity was measured at 5.00-500.0 (1/s) shear rates, the average viscosity at 25 °C at 5 g/d L concentration was 0.0057/0.0019 P.a.s. found. It was determined that the viscosity decreased as the shear rate increased, that is, it showed pseudoplastic properties.

Images obtained from scanning electron microscopic examinations for *R. pyridinovorans* Figure 1.1. and presented in Figure 1.2.

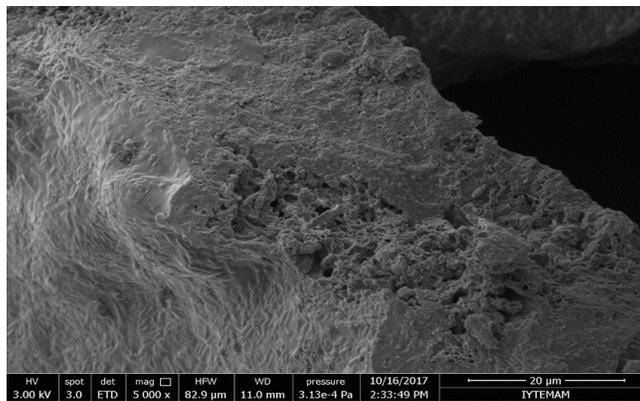


Figure 1.1. SEM photograph of *R. pyridinovorans* EPS (5,000x)

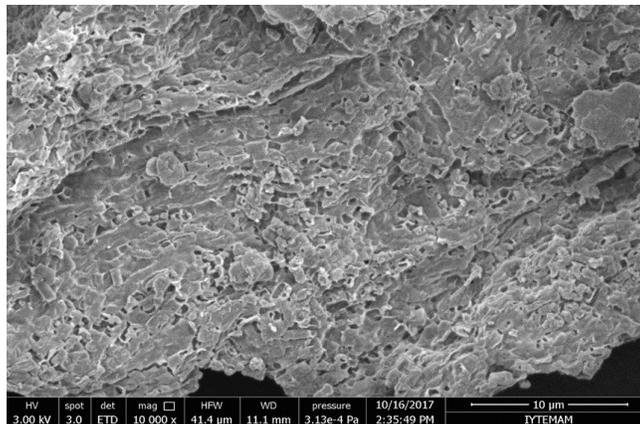


Figure 1.2. SEM photograph of *R. pyridinovorans* EPS (10,000x)

2.2. Antioxidant activity

3.2.1. DPPH free radical scavenging activity

For antioxidant activity, DPPH free radical removal was found to be 54% in 10 different concentrations (0.005-0.25 mg/ml.) of lyophilized EPSs.

In the calculations made according to the DPPH method, free radical removal was found between 64% and 88%. The results obtained are given in Figure 2.1. for the *R. pyridinovorans*.

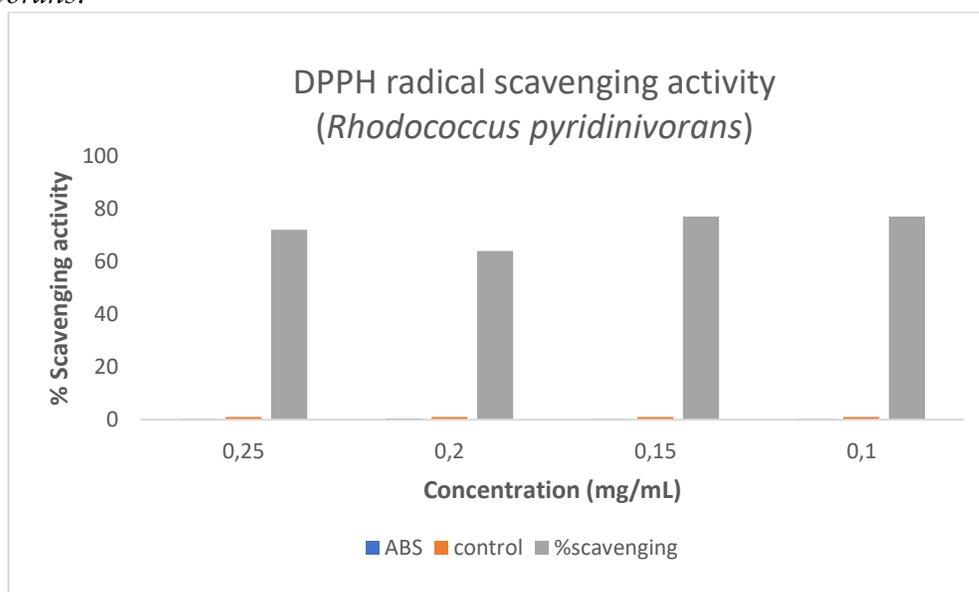


Figure 2.1. Free radical scavenging activity of *R. pyridinovorans* EPS according to DPPH method.

3.2.2. Hydroxyl radical scavenging activity

The radical removal of EPS is higher than the reference biopolymer dextran (51%) at the same concentration. As a result of the experiment, between 22% and 27% hydroxyl radical removal was found in the calculations made between different concentrations. The results obtained are given in Figure 3.1. for the *R. pyridinovorans*.

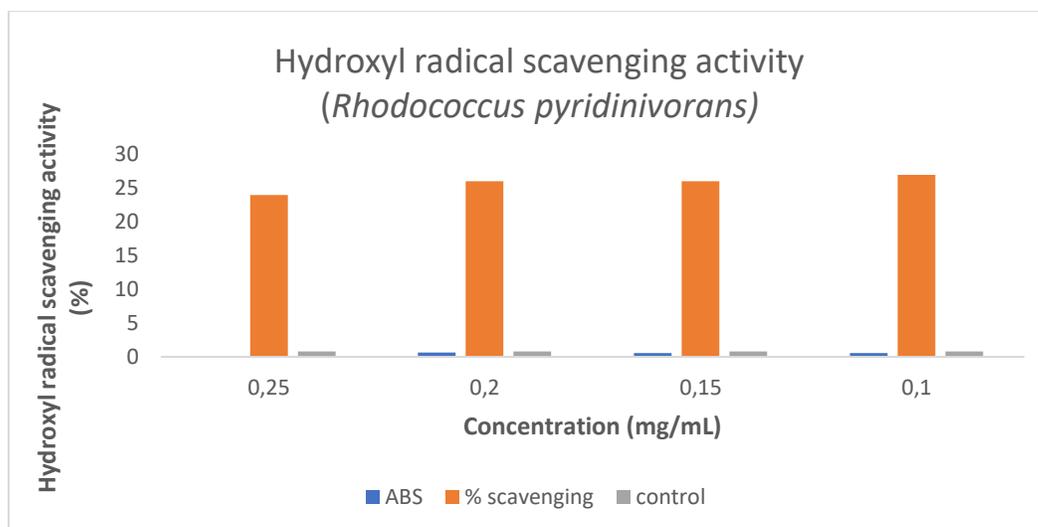


Figure 3.1. Hydroxyl radical scavenging activity of *R. pyridinivorans* EPS.

2.3. The antiproliferative activity of the EPS on HT-29 and MCF-7 cells

Cell viabilities of HT-29 and MCF-7 cells were found to be more than 85% at all tested concentrations of EPS for 24 and 48 hours.

3. Discussion and Conclusion

Many microorganisms can produce EPS. Today, EPS production of many different microbial species such as pathogens and extremophiles still continues. The diversity of EPS producing types and the advantages they provide have become the priorities of industrial production. Today, EPS has been accepted as a new biomaterial. Many superior properties such as gelling, thickening and biofilm formation have caused these biomaterials to have a large market share worldwide [24]. The use of EPS produced from *R. pyridinivorans* ZZ47 strain obtained in isolation from nature in our own laboratory with the research carried out within the scope of Muğla Sıtkı Koçman University Scientific Research Project with numbers 17/121 and 16/066 supports the originality of the study. Important gains with *R. pyridinivorans* ZZ47 EPS are as follows:

- Optimization of the extraction, purification and production conditions of the EPS material with the highest efficiency,
- Physicochemical characterization of EPS,
- Determining that it has antioxidant and cytotoxic activities.

In addition, this bio-product producing bacteria; It is a natural strain isolated from activated sludge, identified as ZZ47 *R. pyridinivorans* by conventional and molecular

techniques, and registered in GenBank with accession number AF173005. Thus, we produce our own bio-material with our own resources and national isolates. Recently, many polysaccharides extracted from natural sources with relatively low toxicity and obvious antitumor activities have been reported, and some of them have shown significant apoptosis-inducing activities [25]. *Bacillus sp.* and *Pseudomonas sp.* have been found to have cytotoxicity for both colon cancer and human breast cancer cell lines, and it has been reported that they may be promising new therapeutic agents because they are active at low concentrations [26]. Di et al., (2018) [27] investigated that EPS obtained from *L. casei* M5, *L. casei* SB27, *L. casei* ×12 and *L. casei* K11, especially acidic EPS produced by *L. casei* SB27, exerted a significant anti-proliferative effect on HT-29 cells through the induction of G0/G1 cell cycle arrest and caspase-3-dependent apoptosis. In a previous study, it was determined that EPS produced by ZZ47 *R. pyridinovorans* caused a 36% cytotoxic effect against MCF-7 cells at a concentration of 2 mg/mL [28]. In the study, both hydroxyl radical scavenging and DPPH free radical scavenging effects of *R. pyridinovorans* EPS were observed. Due to these bioactive properties and wide pH tolerance observed in vitro, EPS material isolated from *R. pyridinovorans* can be used as a natural biological material for health. In future studies, it is aimed to support the findings with in vivo examinations and to present this bioproduct for biotechnological purposes or food supplements use as a natural compound/additive.

4. Acknowledge

The authors gratefully acknowledge Muğla Sıtkı Koçman University Research Support and Funding Office, for their fund support for this research (Research Projects with numbers 17/121 and 16/066).

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