Evaluation of the prebiotic activity of ethanolic and polysaccharide extracts of *Spirulina platensis*

Abstract

Prebiotics are indigestible foods that selectively stimulate the growth or activity of one or a limited number of intestine bacteria and affect the host's health. Plant sources and marine Algae can be mentioned as sources of extraction of compounds with prebiotic properties. *Spirulina platensis* is a photosynthetic cyanobacterium that has a very high nutritional and medicinal value. This study aimed to investigate the prebiotic activity of ethanolic and polysaccharide extracts from *spirulina platensis*. The ethanol extract was obtained by the soxhlet method with ethanol solvent and spirulina's polysaccharide after defatting, extracted with hot water, and precipitated with ethanol. Total phenol content was obtained by the folin-ciocalteu method, antioxidant activity of extracts studied by DPPH method, and structural properties and functional groups of extracts were evaluated using Fourier transform infrared spectrometer. The effect of ethanol extracts and polysaccharides was measured at concentrations of 0.5% and 1% compared to the control sample containing inulin 0.5% and 1%, and it was measured on *Lactobacillus plantarum* A7 probiotic bacteria. To determine the antimicrobial properties, we used a modified bilayer culture method on *Escherichia coli* ATCC 25922. The ethanol extract and the polysaccharide extracted from the *Spirulina platensis* have a high phenol content and antioxidant activity based on the results. Also, both concentrations of the extract had comparable prebiotic properties with commercial prebiotic (Inulin). Concentrations of 0.5 and 1 of both extracts in the period of 24 to 72 hr affected the growth and viability of the *Lactobacillus plantarum* A7.

Keywords: Prebiotics, Ethanolic extract, Polysaccharide, Spirulina platensis

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1- Introduction

With the advancement of science and awareness of societies, the role of food in human health and nutrition became important in such a way that most of the importance of food instead of the primary role of food as a source of energy and growth and development was promoted to the biological role of food on human health and the market for food production and consumption was directed more toward pragmatic or functional foods. Functional food is a product that has a health effect on the host except for its specialties as food (good nutritional value, taste, and texture) (1).

Prebiotics alone or with probiotics can have effects such as lowering blood cholesterol levels, affecting glucose metabolism in the body, increasing absorption of minerals, reducing the risk of heart disease and colorectal cancer. One of the main points in defining prebiotics is the selected fermentation by helpful clone bacteria and another problem, either the activity of bacteria in rivers that affect the health of the living organism (2).

Prebiotics are indigestible foods that selectively stimulate the growth or activity of one or a limited number of bacteria in the colon and affect the host's health. Prebiotics selectively stimulate beneficial bacterial groups, including Bifidobacterium and Lactobacillus, residing in the colon (3). Since prebiotics are used as a source of carbon as an energy source for specific bacteria, they can be added to the environment to increase the growth and survival of bacteria. Increasing the viability of probiotics and stimulating their growth and activity are among the benefits of prebiotics (4). Prebiotics are extracted directly from natural sources or produced from polysaccharides during chemical processes or by chemical or enzymatic synthesis processes of disaccharides. Plant polysaccharides or marine Algae are derived from prebiotics (5). *spirulina platensis* is a filamentizing photosynthetic cyanobacterium that grows in warm and semi-hot waters, especially with alkaline pH (6). The Food and Drug Administration of the United States knows *spirulina platensis* and *Chlorella Vulgaris* as GRAS foods (7). Spirulina algae contain nutrients, minerals, pigments, ramenous sugar, trace elements, and absorbable enzymes. Compared to some types of bacteria, the nutritional value of spirulina is due to its low percentage of nucleic acids and, spirulina contains vitamin B12 (8). There is a tendency to extract prebiotic compounds from different sources with functional and bioactive properties among researchers. Veljovic´et al, (2017) studied on Chemical composition, antiproliferative and antioxidant activity of differently processed *Ganoderma lucidum* ethanol extracts (9) and, Salamah et al, (2018) evaluated the total phenolic content and in vitro evaluation of antioxidant activity of ethanol extract of *Ganoderma amboinense*. Also, Rajasekar et al, (2019) worked on Isolation and structural characterization of sulfated polysaccharide from Spirulina platensis and its bioactive potential: In vitro antioxidant, antibacterial activity and Zebrafish growth and reproductive performance (10). This study aimed to investigate the prebiotic activity of ethanolic and polysaccharide extracts from *spirulina platensis*.

2- Materials and Methods

2-1 Materials:

Spirulina platensis was purchased in powder form from Arian Gostar Research Company, and ethanol 96% from Khorasan Distillery Company and antibiotic discs were purchased from a medical antibody company. MRS Agar, MRS Broth, and Dimethyl Sulfoxide (DMSO) were provided by MERK (Germany), MH Broth (Muller-Hinton Broth) by Schalau (Spain), and MH Agar (Muller-Hinton Broth) from conda company (Canada), Inulin from BDH chemicals company in England. Also, the probiotic strain of *Lactobacillus plantarum* PTCC1896 (A7) and *Escherichia coli* ATCC 25922 was provided from Ahwaz medical university.

2-2 Extraction of ethanolic extract of Spirulina platensis:

In order to extract ethanolic extract spirulina, the Soxhlet method was used. 10g spirulina powder was placed on the cover. 250 ml ethanol 70% were poured into the balloons of the device. The extraction was performed at 50 °C for 3-5 hr, then dried at 40 °C (11,12).

2-3 Extraction of a polysaccharide extract of Spirulina platensis:

In order to remove fat, free sugars, and colored compounds, three volumes of alcohol 80% were used at 60 °C for 6 hours. Nylon fabric was used to isolate the dissolved compounds in alcohol and smooth the solution. The residues were dried for 2 hours at 40 °C and were used for water extraction from a water bath at 90 °C with three volumes of hot distilled water for 3 hours. In order to isolate the insoluble particles, the extract was centrifuged for 20 minutes at 3000 rpm. For deposition of polysaccharides, three volumes of alcohol 80% at °C 4 and 48 hours were used. The sediments were separated by centrifugation at 3000 rpm for 10 minutes and washed several times with absolute alcohol to remove impurities. After de-alcoholization, the polysaccharide was dried at 40 °C (13,14). The percentage of efficiency of extracted polysaccharide or ethanolic extract was obtained from dividing the weight of dry polysaccharide or dried ethanol extract into the initial powder weight based on the relationship of 1 that WDP is equivalent to the dry weight of polysaccharide or ethanolic extract, and WDR is equivalent to the dry weight of raw material (14)

Equation

Extraction Efficiency of Polysaccharide or Ethanol Extract=⌊WDP /WDR⌋ × 100

2-4 Determination of polyphenols in ethanol and polysaccharide extracts of *Spirulina platensis*

Determination of total phenolic content (TPC) in the samples of G. lucidum extract was conducted by the Folin-Ciocalteu method. In this method, the extracts' absorption at 765 nm was measured by UV spectrophotometer, and the total amount of phenolic compounds of the extracts was calculated using the standard gallic acid curve. The total phenolic compounds were expressed based on mg equivalent gallic acid in grams of dry samples (9)

2-5 Evaluation of the antioxidant capacity of ethanolic and polysaccharide extracts by DPPH free radical scavenging ability

The basis of this method is the reduction of DPPH free radicals by antioxidants in the absence of other free radicals, the result of which creates a color in the environment that color intensity can be measured by spectroscopy (15). A UV device measured the antioxidant properties of the extracts at a wavelength of 517 nm. Antioxidant activity was calculated according to relationship 2 (16).

Equation 2

$$\text{Scavenging activity}(\%) = \left(\frac{\text{Abs blank} - \text{Abs sample}}{\text{Abs blank}}\right) \times 100$$

Abs blank: Absorption of DPPH methanol solution without ethanol or polysaccharide extract samples

Abs sample: Adsorption of methanolic solution of DPPH with ethanolic or polysaccharide extract samples

2-6 Identification of structural properties of ethanolic and polysaccharide extract of *spirulina platensis* by Fourier transform infrared method (FTIR)

Identification of ethanol and polysaccharide extract structures using Fourier transform infrared spectrometer was carried out in the Wavelength range of 400 to 4000 cm-1 using a spectrophotometer equipped with a total weakened system (16).

2-7 Identification of the effect of ethanolic and polysaccharide extracts on the growth and viability of *Lactobacillus plantarum*A7

For this purpose, each test tube containing the desired percentages of activated bacteria (10^6 CFU/ml) was added. In order to compare the growth of probiotic bacteria, the medium containing 0.5% and 1% inulin was also used. Samples were analyzed at 24, 48, and 72 hours and cultured by pour plate method, and placed in an incubator for 72 hours(37 °C), and then bacteria were counted (17).

2-8 Identification of antimicrobial activity of ethanolic and polysaccharide extracts along with *Lactobacillus plantarum* by a suitable method.

Firstly, culture was prepared for 48 hours, and then it was centrifuged for 10 minutes at 3000 rpm. Sediments were removed under sterile conditions, and soluble was stored in the refrigerator (18).

2-9 diffusion agar method

In this method, the turbidity of half of McFarland was prepared from the microbial suspension of *Escherichia coli* cultured in Mueller-Hinton broth medium. Then, with sterile swap, dense culture was poured on the Müller Hinton Agar medium, then with sterile punch, wells with a diameter of 6 mm were created on the medium at a certain distance. Then, the supernatant was cultured from *Lactobacillus Plantarum*, and the supernatant of extracts and bacteria was poured into wells at a rate of 250 µL. In order to better release the supernatant, the plates were cooled for 30 minutes in the refrigerator at 4 °C. Then, the plates were transferred slowly to the incubator at 37 °C, and after 24 hours, the haze growth of no growth related to each treatment was measured and recorded by a millimeter ruler. In order to compare the antimicrobial effect of supernatant treatments, cotrimoxazole antibiotic discs (tiotropium sulfometoxysol, 10µg), ceftriaxone (30µg), sifexim (5µg), penicillin (10µg) were used (18).

2-10 Data Analysis

The experiments were carried out with three replications and implemented in a completely randomized design. After collecting the data, a one-way analysis of variance was used to determine the difference between treatments. The means were compared with Duncan's test with a confidence level of 95%. All analyses were performed using SPSS25 software, and diagrams were designed by Excel 2019 software .

3- Results and Discussion

3-1 The extraction efficiency of ethanolic and polysaccharide extract of *spirulina platensis*

The extraction efficiency of ethanolic and polysaccharide extract *Spirulina platensis* obtained 3 and 1.2% dry weight, respectively (Table 1). the efficiency could vary according to the extraction method and isolation polysaccharides according to the extraction temperature, solvent, and time variables. Chiklahan et al. (2013) reported the efficiency of spirulina polysaccharide obtained by hot water extraction method equal with 8.3 (19). Chiklahan et al. (2014), in another study, obtained the efficiency of spirulina polysaccharide (by hot water extraction method) equal to 4%, stating that the difference in polysaccharide extraction efficiency depends on different variables such as temperature, time, and solvent to dry matter ratio. In general, the highest extraction efficiency of polysaccharides was related to high temperature (20).

3-2 Total phenol content and antioxidant activity of ethanolic and polysaccharide extract of *spirulina platensis*

The total phenolic amount of ethanol extract of *Spirulina platensis* was 43.78% and for polysaccharide extract was 48.97 based on gallic acid (Table 2). Chiklahan et al. (2013) expressed 45% of phenolic compounds (19). Agustini et al. (2015) expressed the total phenolic extract of dried *spirulina platensis* by 69.2% (11). DPPH was used to evaluate the antioxidant activity of ethanolic and polysaccharide extracts .(11)

Antioxidant activity of ethanolic extract *spirulina platensis* was 76.14% at 0.1% and 67.54% at 0.4% (Table 3) Agustini et al. (2015) obtained 33.07% antioxidant activity of ethanol extract, which is likely to be influenced by phenolic compounds of the extract (8). Antioxidant activity for *spirulina platensis* polysaccharide extract was 70.77 % at 0.1% and 77.4% at 0.4% (Table 3). Kurd and Samavati (2015) obtained the amount of antioxidant activity by the DPPH method at a concentration of 300 µg/ml equal to 78%, which was lower than 96% BHT antioxidant (21). Chiklahan et al. (2013) obtained the antioxidant activity of polysaccharide extract at 90°C equal to 31% (19). Also, Yang et al. (2011) pointed out that the antioxidant activity of polysaccharides was related to their structural properties, including monosaccharides forming glycoside bonds, molecular weight, presence of some groups as carbonyl sulfonyl, amino, and carboxyl (22).

3-3 structural properties of ethanolic and polysaccharide extract of *spirulina platensis* by Fourier transform infrared method (FTIR)

FTIR method was used to evaluate the structural properties of ethanolic and polysaccharide extracts of Spirulina platensis (Fig. 1 and 2). He (2017) considered the vibrations of the approximate wave number for the 632cm-1 band to be related to the C-H bond of alkanes and the 1082 cm-1 band related to the C-O tension band of alcohol and a carboxylic acid, and the 1414 cm-1 band related to the C-O-C bond (23). These bands are consistent with the ethanol extract spectrum (Table 4).
Popovi et al (2013) stated that sulfate groups associated with antioxidant activity are in the range of 1240-1260 cm-band, which is also present in the spectrum of ethanolic extract spirulina (24), He et al. (2017) considered the 1642cm band related to the C-H bond of alkanes, the cm-11045 band related to the C-O-P tensile bond, the 1080 cm-1 to the C-C, and C-O bond, the cm-11172 band. Related to asymmetric tensile bonding CO-O-C, 11464cm11464 band related to CH2 bond, 3022 cm-1 bands related to aromatic C-H stretch bond, and 3458 cm-1 bands related to symmetric NH2 tensile bond reported (23) Li and colleagues (2017) pointed out that polysaccharide index funds are generally seen in the 1200-1200-1 cm-1 zone (25). This spectrum proves that polysaccharides are the dominant compound in the extract extracted from Spirulina platensis. All mentioned bands are compatible with polysaccharide extract absorption bands (Table 5).

3-4 the effect of ethanolic and polysaccharide extracts on the growth and viability of *Lactobacillus plantarum* (A7) and antibacterial activity

In order to evaluate the prebiotic effect of ethanolic and polysaccharide extracts of *Spirulina platensis* on the growth and viability of *Lactobacillus Plantarum* A7 probiotic bacteria during 72 hours of growth, bacterial counting was performed for 72 hours. At 24 hours, both ethanolic and polysaccharide extracts and inulin decreased bacterial population with increasing concentration, but this trend was not repeated for inulin at 48 hours. At 72 hours for both extracts, the highest bacterial population was related to the highest concentration of extracts (1%). Firdaus et al. (2012) stated that in an environment containing prebiotic compounds due to structural complexity, it is possible to produce more enzymes necessary to metabolize food compounds and give the power to microorganisms to maintain stability (14)

However, in the three periods of the test, the bacterial population of samples containing ethanolic and polysaccharide extracts had a significant difference with those containing inulin, the probiotic ability of the extracts was maintained until 72 hours (Fig. 3 and 4). Sheehan et al. (2007) note in their review that the number of live bacteria needed to create probiotic effects was at least 10^{+6} CFU/ml (26). Although there is no information about the prebiotic effect of ethanolic extract and polysaccharide *spirulina platensis*, there have been studies on adding *Spirulina platensis* biomass on the survival and viability of probiotics bacteria in food products. Raposo et al. (2016) stated that some microalgae like *spirulina platensis* could increase the growth of bacteria such as *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Streptococcus thermophilus* (27).

The supernatant obtained from the growth of probiotic bacteria in the presence of 0.5 and 1% concentrations of ethanolic and polysaccharide extracts of spirulina had a suitable antimicrobial effect on the pathogenic strains. The treatments had no significant differences in this method, but there was a significant difference among our extracts and cotrimoxazole and ceftriaxone. Penicillin and cefixime antibiotics did not cause any inhibition zone (Fig. 5). The antimicrobial activity of lactic acid bacteria is due to various factors, including organic acids, hydrogen peroxide, carbon dioxide, and bacteriocins. The presence of lactic acid and acetic acid could decrease the pH of the environment. Furthermore, these compounds deactivate growth conditions for other microorganisms. Also, the non-ionizing form of these organic acids could pass through the cell membranes of pathogenic bacteria and convert to ionized form according to the pH inside the cell. Hydrogen ions can acidify the cytoplasm and disrupt the electrochemical properties of the cell (28).

Conclusion:

This study showed that ethanolic and polysaccharide extracts of Spirulina platensis have total phenol and good antioxidant activity. Also, both concentrations of the studied extract had a prebiotic capability comparable to commercial prebiotics (inulin). Concentrations of 0.5 and 1 of both extracts increased the growth and viability of *Lactobacillus plantarum* A7 between 24 and 72 hours. Due to the increasing resistance of bacteria to chemical antibiotics, supernatant derived from the growth of probiotic bacteria in the presence of 0.5 and 1% concentrations of extracts had a suitable antimicrobial effect on the pathogenic strain.

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Table 1: Percentage efficiency of extracts

The extraction efficiency of ethanol extract(%)	3
The extraction efficiency of polysaccharide (%)	2/1

Table 2: Total phenolic content of ethanol and polysaccharide extracts of spirulina platensis

Extract	Absorption (nm)	Total phenol (mg equivalent percentage of gallic acid per gram of dry sample)
Ethanolic	094/0	78/43
Polysaccharide	099/0	97/45

Table 3: Antioxidant activity of ethanol and polysaccharide extracts of spirulina platensis

Extract	Concentration(%)	Antioxidant activity (%)
Ethanolic	1/0	14/76
	4/0	56/67
Polysaccharide	1/0	77/70
	4/0	40/77

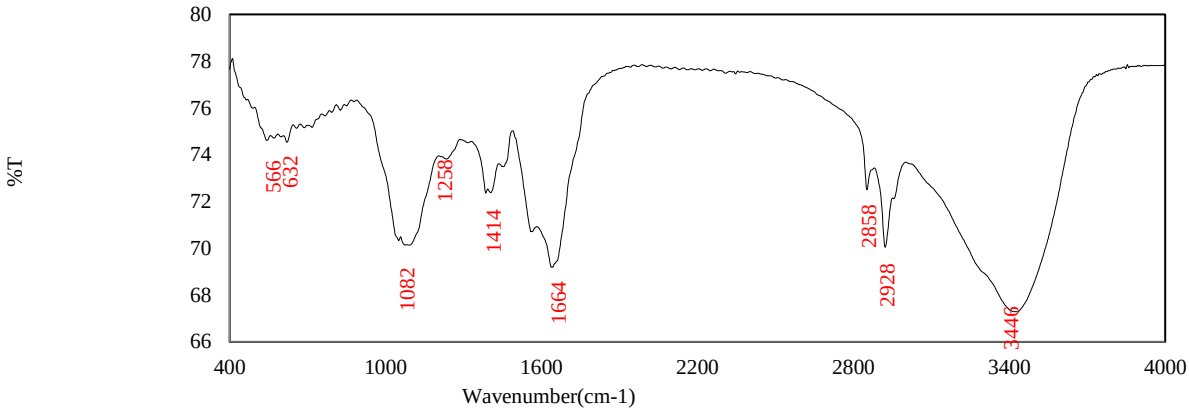

Figure 1: Fourier transform infrared spectrum of Ethanolic Extract of Spirulina Platensis

Table 4: Absorption Bands of Ethanolic Extract of Spirulina Platensis

Absorbed bands of ethanolic extract (cm^{-1})	Index Bands	Type of indicator bands
633	632	C-H
1082	10182	C-O
1414	1414	C-O-C
1258	1260-1240	SO_4^{-2}

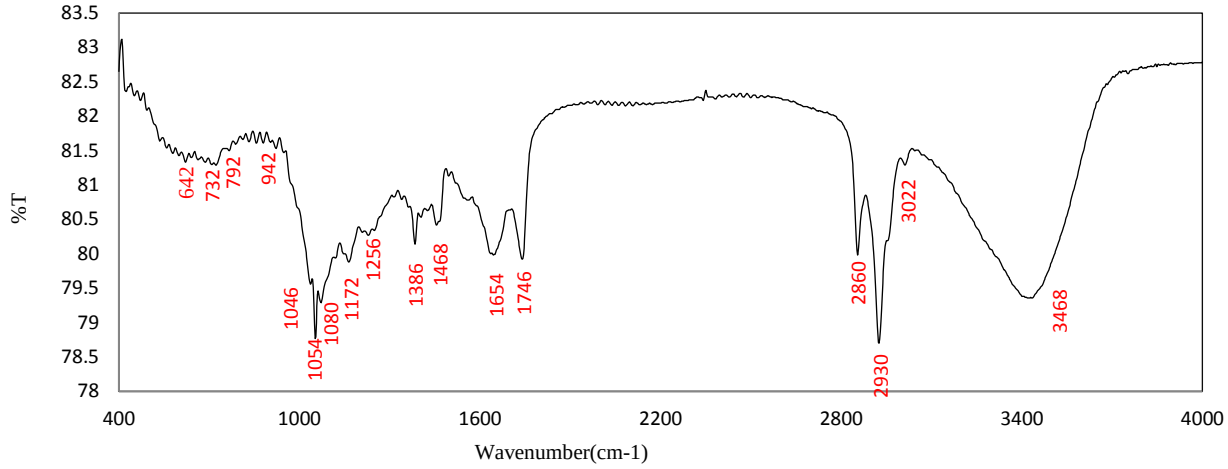

Figure 2: **Fourier transform infrared spectrum of Polysaccharide Extract of Spirulina Platensis**

Table 5: Absorption Bands of Spirulina Platensis Polysaccharide Extract

Absorption bands of ethanol extract$^{(cm-1)}$	Index Bands	Type of indicator bands
642	642	C-H
1045	1045	C-O-P
1080	1080	C-C and C-O
1172	1172	CO-C-C
1464	1464	CH_2
1742	1742	C=O
3022	3022	C-H
3458	3458	NH_2

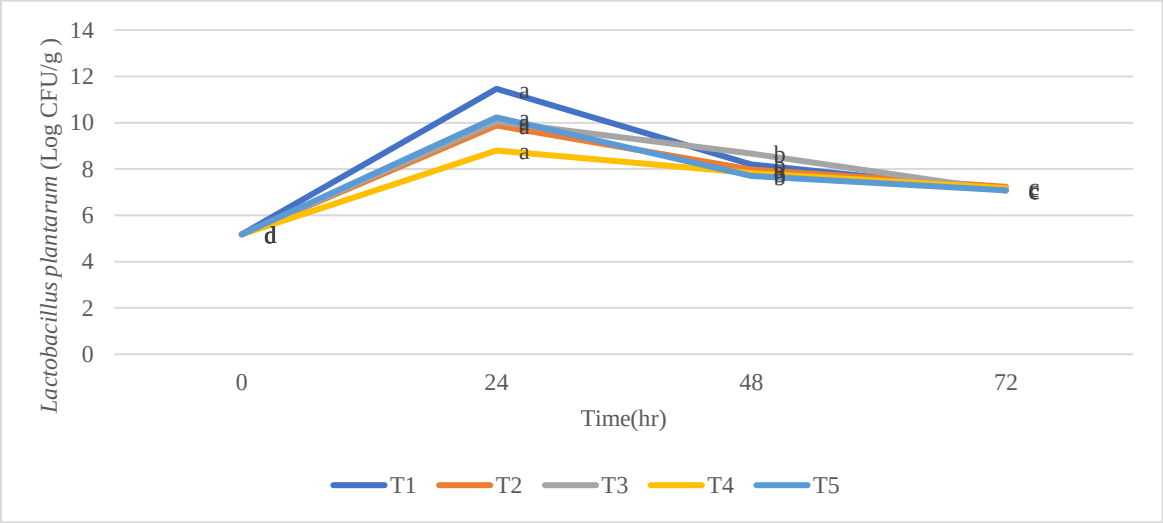

Figure 3. The effect of ethanolic extract of spirulina platensis (0.5 and 1%) in comparison with inulin on the growth of Lactobacillus A7 at 24, 48, and 72 hours

*The samples are:T1(MRS Broth), T2(1% Inolin+1% SP extract), T3(0.5% Inolin+1% SP extract), T4(1% Inolin+1% SP extract), T5(1% Inolin+0.5% SP extract).

*Different Latin letters show a significant difference in the 5% probability level based on Duncan's test

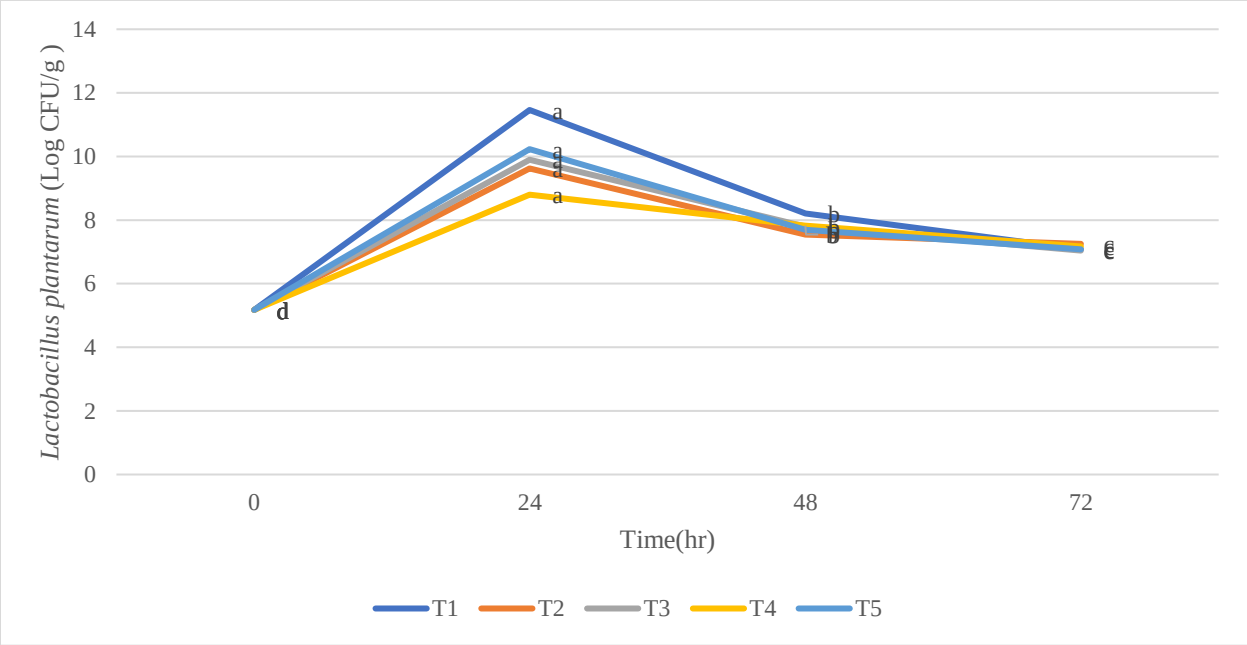

Figure 4. The effect of Polysaccharide extract of spirulina platensis (0.5 and 1%) in comparison with inulin on the growth of Lactobacillus A7 at 24, 48, and 72 hours

*The samples are:T1(MRS Broth), T2(1% Inolin+1% SP extract), T3(0.5% Inolin+1% SP extract), T4(1% Inolin+1% SP extract), T5(1% Inolin+0.5% SP extract).

*Different Latin letters show a significant difference in the 5% probability level based on Duncan's test

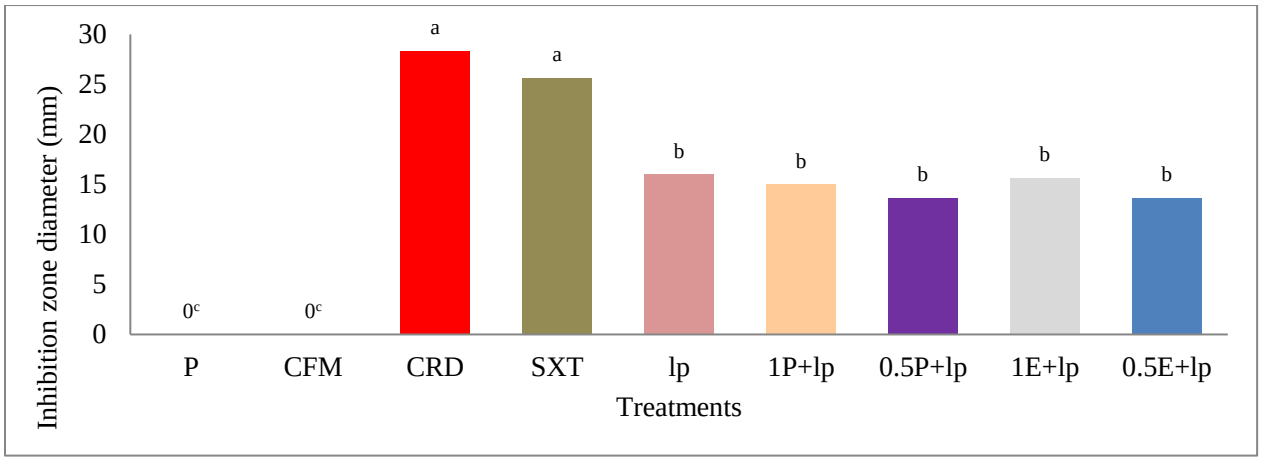

Figure 5: The antibacterial effect of probiotic growth supernatant in the presence of extracts

*In Figure 5, P(penicillin), CFM(ceftriaxone), CRD(cotrimoxazole), SET(sifexim), T1(MRS Broth), T2(1% Inolin+1% SP extract), T3(0.5% Inolin+1% SP extract), T4(1% Inolin+1% SP extract), T5(1% Inolin+0.5% SP extract).

*Different Latin letters show a significant difference in the 5% probability level based on Duncan's test