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Chemical identification of *Pseudomonas aeruginosa* exopolysaccharide from heavy oil as a solo carbon source

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Abstract: ***P. aeruginosa was*****capable to consum heavy oil as carbon source and produce non insoluble exopolysaccharide. The crude EPS was deprtoenized by tricholoracitic acid naturalized and dialyzed and precipitated by ethanol. One peak was obtained from gel chromatography (G200). By complete acid hydrolysis glucouronic acid, fructose and glucose were detected by HPLC. This polysaccharide show antioxidant activity that possesses DPPH radical-scavenging activity, with an EC50 =0.201**

Keywords: **Exopolysaccharide, *P. aeruginosa*, Heavy Oil, Antioxidant.**

1. INTRODUCTION

The term exopolysaccharide (EPS) was coined by Sutherland (1972) to described high molecular weight carbohydrate polymers produced by many marine bacteria. EPS has also been used to designate more broadly defined compounds called extracellular polymeric substances (Nichols *et al*., 2005). Extracellular polysaccharides produced by microbes are immense and of diverse nature. These are further grouped into four major classes; polysaccharides, slime and microcapsular polysaccharides, polyesters, and polyamides, and have been collectively termed as EPS (Nwodo *et al*., 2012).The utilization of petrochemicals as a carbon source have been reported, about 22 genera of bacteria that can metabolize petroleum hydrocarbons which include- *Pseudomonas, Aeromonas, Bacillus, Flavobacterium, Corynebacterium, Micrococcus* etc. Based on crude oil degradation capacity *Pseudomonas aeruginosa* is the most active hydrocarbon utilizer in crude oil. Previous observations have identified the *Pseudomonas* genus most efficient among hydrocarbon degrading microorganisms [Lal , Khanna , (1996); Banat etal,(2000); Saadoun (2002)]. Based on monomeric composition these EPS are either homopolysaccharides (made up of single monomer linked by glycosidic bond) or heteropolysaccharides (composed of more than two monomeric units joined by glycosidic bond) they also contain a number of different organic moieties like organic acids and amino acids and inorganic constituents like phosphates and sulfates (Nanjani and Soni, 2012). The novel active extracellular polysaccharides from microorganisms hold a great potential application in biology and pharmacology (Du et al. 2009, Kimuran et al. ,2006]. Our work aimed to production exopolysaccharide by *Pseudomonas aeruginosa* using solo hydrocarbon source . The exopolysaccharide was , isolated , purified and the in vitro antioxidant activity was investigated.

2. MATERIALS AND METHODS

Materials

1-Crude heavy oil was obtained from king Abdulaziz University - Faculty of Engineering -Chemical Engineering Department.

2-Other chemicals used will be obtained in analytical grade.

3-Microorganism *Pseudomonas aeruginosa*

Media

Inoculum medium: (g/L)

10g Meat extract, 10 g peptone, 5gNaCl dissolved in 1 liter, at pH 7. The medium was portioned into500ml. Each Erlenmeyer flasks contained100 ml of the medium and sterilized .The flasks were inoculated with a slant of *P. aeruginosa* and incubated at 37°C on a rotary shaker (150 rpm) for 24 h.

Basal medium:

The composition of basal medium (g/L) : (Kita , et al. ,1974)

1 ,KH_2PO_4; 2, K_2HPO_4; 1, $NaNO_3$; 1.5, yeast extract ; 0.21, $MgSO_4$.$7H_2O$.The basal medium will be supplemented with 10 ml heavy crude oil, tween80 3ml and xylene 20 ml. pH of the medium will be set at pH 7.0. The basal medium was portioned into250 ml. conical flasks contained 50 ml of the medium and sterilized. The sterile medium were inoculated at 10% (v/v) level and incubated at 37°C on a rotary shaker (150 rpm) for 48 hour. Samples were removed at intervals for the determination of total sugar and growth of cells.

Chemicals Analyses:

Growth estimation

Growth of cells was estimated by measuring the absorbance of the mixture of 1ml of the basal medium diluted with1ml of distilled water at 600 nm spectrophotometrically. 100 ml of stander culture medium after fermentation were majored at 600nm spectrophotometrically. The cultured medium were centrifuged the precipitated cells were washed several time petroleum ether (40-60 °C) several time to remove the oil residual . The cells were transferred in Petri Dish then it was put oven at 100°C till constant weight. The OD for test medium was compared with OD stander medium; the dry weight of cells can be calculated .The blank was contained of not inoculated fermentation medium (Hoischen and Krämer ,1990).

Analysis of metabolic products by GC-Mass

After 72 h of growth in 100 ml of Basel medium YG at 37 C, cells were removed by centrifugation. The supernatant was then extracted using mixed chloroform–methanol (2:1), after which it was distilled. Next, the final metabolic products were collected bottles and analyzed using a gas chromatograph mass spectrometer (Agilent 7890). The samples were chromatographed by passing them through a GDX103 column (0.25 µm X 0.53 mm X 30 m) using the following gradient temperature program: an initial temperature of 80 C for 2 min, followed by an increase in temperature to 250º C at 10 C/min, where it was held for 10 min. The injection temperature was 280 ºC and the transport line was maintained at 260º C. The helium flow was 0.5 ml/min, and the injection volumes were 2 µL.

Determination of Total Sugars

Total hydrolysable carbohydrates will be determined by phenol sulphuric acid method according to Dubois (1956).

Isolation, purification of exopolysaccharide according to (Feng etal.2012)

A *P. aeruginosa* was be grown aerobically in a production medium at 37°C and 150 rpm on a rotary shaker. The culture broth was centrifuged to separate the cells. Trichloroascetic acid was added to the supernatant to reach a concentration 5 % then the supernatant was neutralized with Na OH. Then the solution was centrifuged the precipitate protein was discarded . The supernatant was dialyzed in dialysis tube against running tap water for 48 hr. then distilled water for 24hr.The dialyzed solution was concentrated reduced pressure at 40 °C. Ethanol was added to reach a concentration of

(Ethanol: Supernatant 5:1 v/v) the mixture was left overnight in the refrigerator. The precipitated polysaccharide was separated by centrifugation and washed with ethanol, acetone and ether then dried under reduced pressure oven at 45°C.

Fractionation of polysaccharide using sephadex column;

The purification was performed by using Econo-column (1.5 cm × 14 cm; BioRad, CA, US) packed with a Sephadex G-200 column (GE Healthcare, Freiburg, Germany). A 0.2 M phosphate buffer solution was used as the mobile phase at a flow rate of 1.0 ml/min the fractions were collected in 5 ml portion. To confirm the EPS in each fraction, the fractionated sample was analyzed by the phenol-sulfuric acid method at 490 nm (Dubois , 1956). After collecting all EPS-containing fractions, dialysis was carried out using a dialysis membrane for 24 h at 4o C against distilled water. An amount of the purified EPS sample was calculated as the dry weight after freeze drying in a lyophilizer . The sample was used for further studies.

Complete hydrolysis of exopolysaccharide :

Hydrolysis of exopolysaccharide was carried out according to the method of Evance and Linker (2000). In this method 50 mg of polysaccharide was hydrolyzed with formic acid 88% in sealed tube at 100 °C overnight. At the end of this period, the hydrolyzed was transferred quantitatively and the solution was evaporated to dryness under vacuum at 40°C. Distilled water was added and the solution was evaporated as described above until the hydrolyzate became free from formic acid. The hydrolyzate was dissolved in ethanol and kept for the analysis.

Detection the monosaccharide by HPLC:

An aliquot of each sample was filtered through 0.22 µm Millipore membranes. For the determining sugars, an Agilent model 1100 Series (Agilent, USA) high performance liquid chromatography equipped with quaternary pump, refractive index detector, and Shim-pack SCR-101N (300 mm X 7.9 mm I.D., 10 µm). The mobile phase was deionized water, degassed under vacuum in an ultrasonic bath. The flow rate was 0.7 ml/min at a temperature of 40ºC.The quantification was achieved by comparison with analytical curves using glucose, fructose and sucrose standards.

Uronic acid content was determined according to m-hydroxydiphenyl method using glucuronic acid as standard (Filisetti-Cozzi , Corpita ,1991)

Total Phenolic Content (TPC) Assay

The total phenolic contents were determined spectrophotometrically according to Folin-Ciocalteu's (FC) procedure used by Kahkonen et al. (1999)

Gallic acid was used as standard unit for total phenolic contentdetermination because it covers a wide spectrum of phenolic compounds. (Fig 1)

Determination of Free Radical-scavenging Activities of the Extracts

The 2) 2 diphenyl-l-picrylhydrazyl (DPPH) test was carried out as described by Burits and Bucar (2000). One milliliter of supernatant extract at different concentration was mixed with 1 mL DPPH reagent (0.002% (w/v) /methanol water solution). After an incubation period (30 min) the absorbance was measured at 517 nm. Ascorbic acid was used as positive control. The results were expressed in percentage of radical scavenging activity, calculated using the following formula:

$$\% \text{ radical scavenging activity} = \frac{(\text{A Blank} - \text{A Sample})}{\text{A Blank}} \times 100\%$$

A Blank refers to the absorbance of the blank control whereas A Sample is theabsorbance of the tested samples.

3. RESULTS AND DISCUSSIONS

Crude heavy oil degradation was first conducted by culturing *Pseudomonas aeruginosa* on basal mineral salt medium supplemented with 3ml tween80 as emulsifier and 5mL/L crude heavy oil. The Basel culture medium was inoculated *P. aeruginosa* and samples were taken intervals (24 hr)for 6 days. Results documented in (Fig.2) very clearly testify that the microbial biomass were approximately increased in the first two days late logarithmic phase and beginning of stationary phase (0.17 g/L) then the bacterial mass stable then bacterial cells decreasing to 0.12 g/L. Further the results presented in

the Fig. (2)also suggest that the optimum exopolysaccharide (total sugars) production output were found after 5 days (100.33mM) then the polysaccharide decreasing to be the 79.5mM after 6 days. Our rustles in accordance with the results of (Hereher, et al 2018) who observed that the exopolysaccharide produced after 4 days .On the other hand , Raza et al (2012) and Conti et al.(1994) who produced exopolysaccharide after 72 h and 50 h respectively .

Biodegradation of heavy oils

Biodegradation often induces changes in the contents of saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes. The effects of induced biodegradation of various heavy oils ,and products of fermentation were shown in Table 1. *P. aeruginosa* was capable of degrading various components of different heavy oils. New compounds (table 1)were formed as result of fermentation .

Isolation, purification and composition of exopolysaccharide

The crude exopolysaccharide produced by *P. aeruginosa* was reached a maximum after 4 days. After centrifugation to separated the cells then 5% Tricholoracetic acid was added to precipitate protein .The supernatant were neutralized by Na OH and dialyzed and precipitated by ethanol. The purified ESP was a brownish powder about 0.5 g , was used for subsequent analysis. It had a negative response to the Bradford test and no absorption at 280 nm or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid.As determined by m-hydroxydiphenyl method, the polysaccharide contains uronic acid.

Purification of the exoplysaccharide by column chromatography:

From the elution profile of Sephadex G-200 column chromatography in (Fig.3), it can be seen that single peak was detected maximum were found in tube number 60 .

Detection of monosugars by HPLC:

The complete acid hydrolysis of the *P. aerougenosa* exoploysaccharide was analyzed by HPLC indicated that EPS was composed of glucouronic acid , fructose and glucose with a molar ratio of 2.2 : 1.3 : 1.0 respectively (Fig .4).

Determination of total phenolic compounds

The contents of total phenols was determined in purified exopoloysaccharide solution produced by *P. aeruginosa* that growing in culture medium contain heavy oil. It was found that the total phenol compounds 0.203 µg exopolysaccharide solution that was measured by Folin-Ciocalteu reagent in terms of Gallic acid equivalent (GAE).

DPPH Radical Scavenging Activity:

DPPH Radical Scavenging Activity

The in vitro antioxidant activity of the isolated EPS was determined by the DPPH free radical scavenging ability. DPPH is one of the compounds that has a proton free radical with a characteristic absorption, which decreases significantly on exposure to proton radical scavengers (Wang, et al. 2011). Furthermore, it is well accepted that the DPPH free radical scavenging by antioxidants is due to their hydrogen donating ability. The present findings showed that EPS isolated from *P. arogenosa* had a noticeable DPPH free radical scavenging activity (Fig.5). It was also observed that the DPPH scavenging activity was increased in a dose dependent (0–135 µg/mL) manner. It was assumed that the isolated EPS somehow donates hydrogen ions to react with the DPPH radical. The greatest scavenging rate of *P. arogenosa* exopolysaccharide was 55%, which was lower than the 72% observed for *Serratia ureilytica* TKU013 (Wang et al. 2010) and 77% for *Paenibacillus* sp. TKU023 (Wang, et al 2011). The half maximal effective concentration *P. arogenosa* exopolysaccharide (IC50 = 0.201) as shown(Fig. 5). TKU032 EPS was a potent and natural antioxidant that could be used as an alternative to synthetic antioxidants.

4. CONCLUSION

The present work dealt with the identification and characterization of exopolysaccharide from *P. aeruginosa*. The results showed that *P. aeruginosa* consumed heavy oil as carbon source for production non insoluble exopolysaccharide. By complete acid hydrolysis glucouronic acid , fructose and glucose were detected by HPLC . This polysaccharide show antioxidant activity that possesses DPPH radical-scavenging activity, with an EC50 =0.201

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APPENDICES - A

List of Table:

Table 1: Biodegradation of heavy oil by *P. aeruginosa*

Heavy oil before fermentation	Heavy oil After fermentation
Trichloroacetic acid, decyl ester	1-sec-butyldiaziridine
2-isopropylpiperazine	2-isopropylpiperazine
1-decanol, 2-ethyl-	Piperazine,2-methyl
Trans-2,3-epoxyoctane	Oxirane,2-methyl-3-propyl, trans-
Carbonic acid, pentadecyl prop-1-en-2-yl ester	Pentane, 2,3-dimethyl
Carbonic acid, dodecyl prop-1-en-2-yl ester	1,2,3-trimethyldiaziridine
Cis-2,3- epoxyoctane	Heptane
Carbonic acid, hexadecyl prop-1-en-2-yl ester	1-(3-Oxobutyl)-3,3-dimethyldiaziridine
Carbonic acid, undecyl vinyl ester	Cyclopentanol,3-methyl-
1,3-propanediol, 2-ethyl-2-(hydroxymethyl)-	1,3-Dimethyldiaziridine
Benzene	9-Octadecenamide,(z)-
Decane	
Tetradecane, 1-chloro-	
Undecane	
Dodecane	
Tridecane	
Tetradecane	
Tetradecane, 2,6,10-trimethyl-	
hexadecane	
Heptadecane	
Heneicosane	
eicosane	
Heptadecane, 9-hexyl-	
Dodecanoic acid, ester	
9-Octadecenamide,(z)-	
Octadecane, 3-ethyl-5-(2-ethylbutyl)-	
Docosanoic acid, 1,2,3-propanetriyl ester	

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List of Figure:

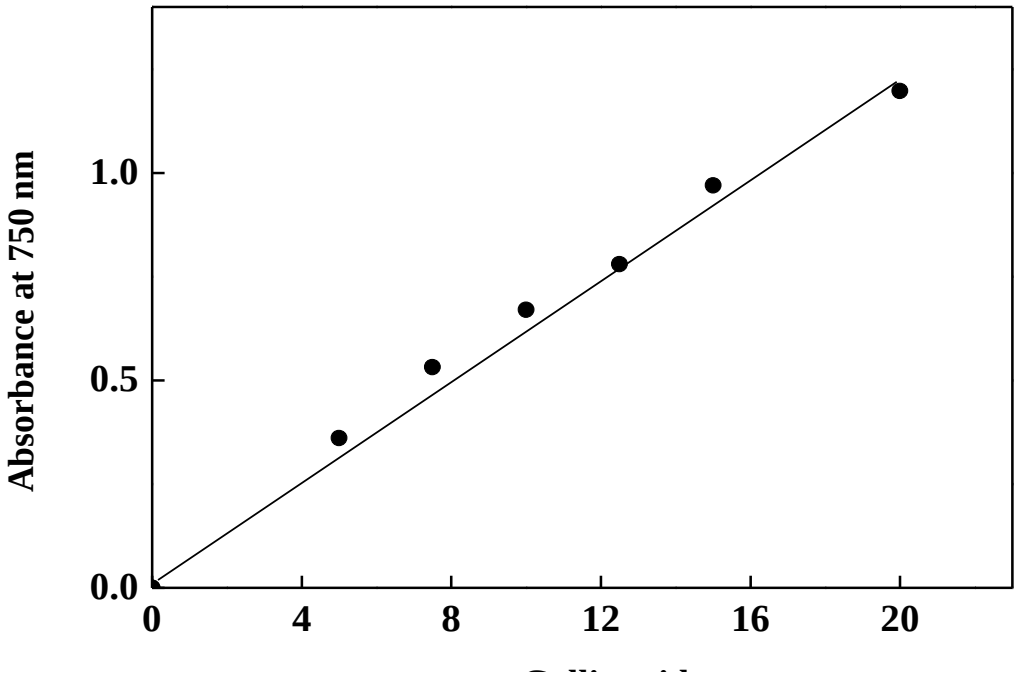

Fig 1: Standard curve of gallic acid

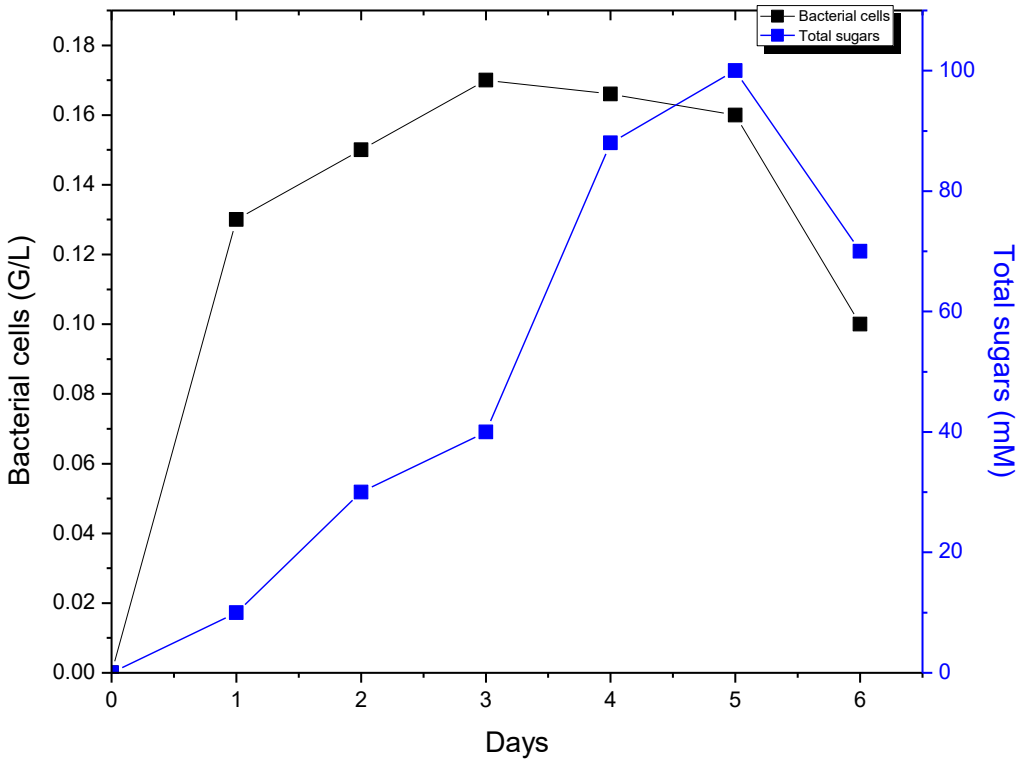

Fig 2: Effect of fermentation periods on the exopolysaccharide production by *P. aeruginosa*

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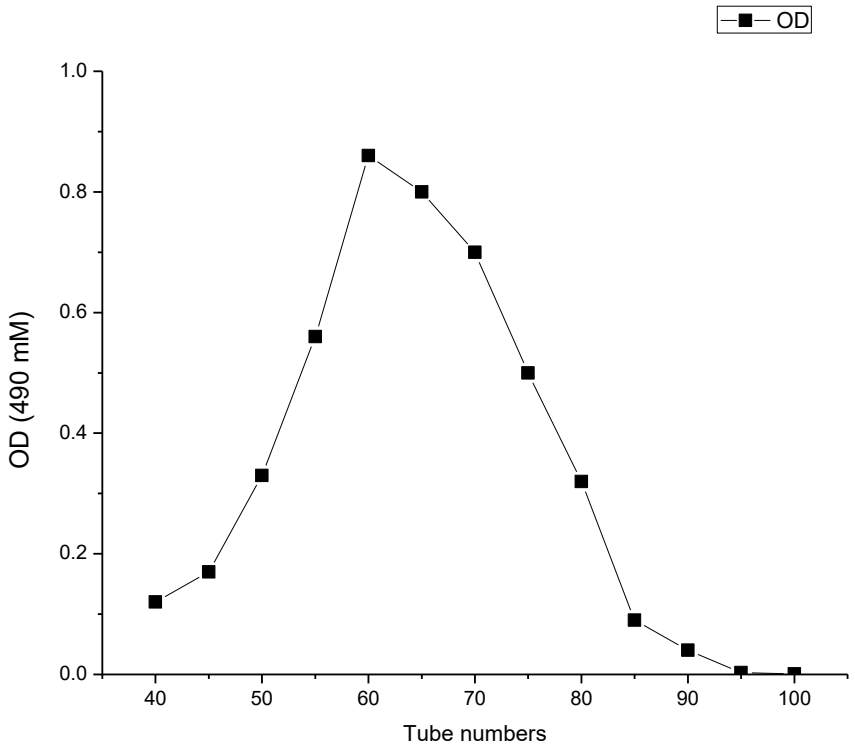

Figure 3: Gel permeation chromatography of *P. aeruginosa* exopolysaccharide on sephdax G200.

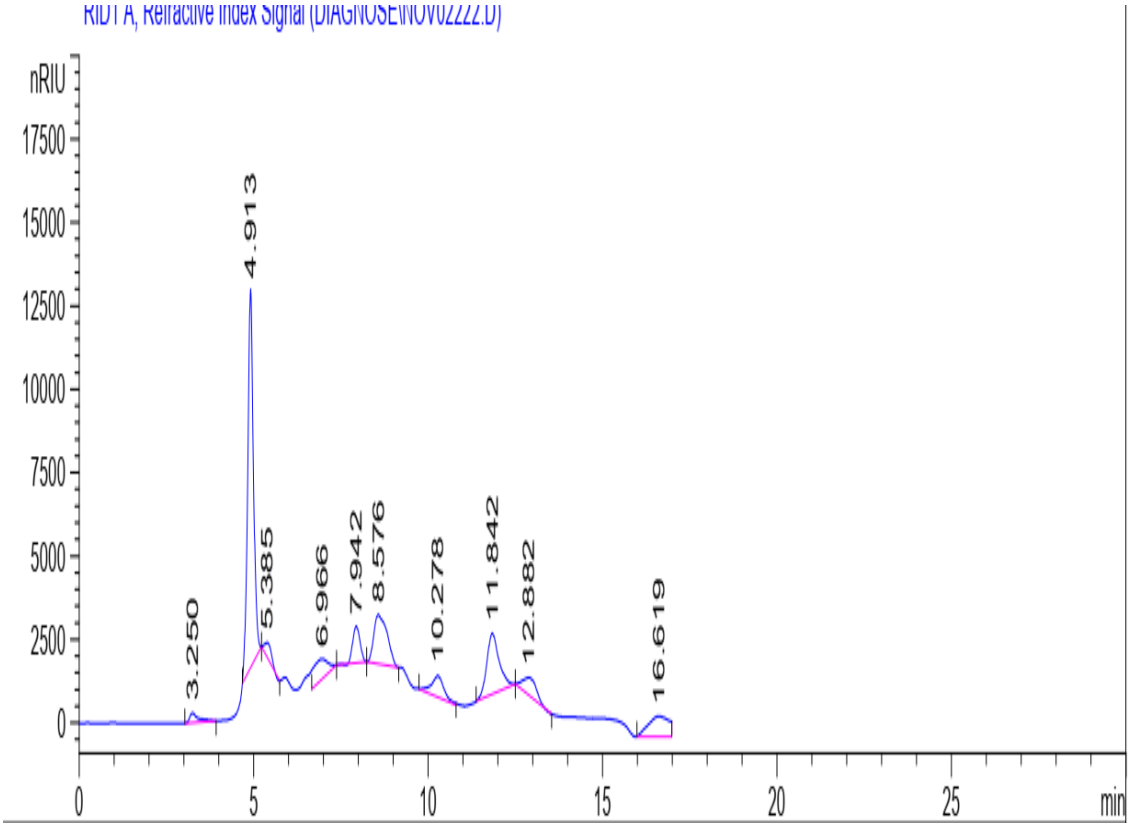

Fig 4: High performance liquid chromatographic (HPLC) separation of monosaccharide

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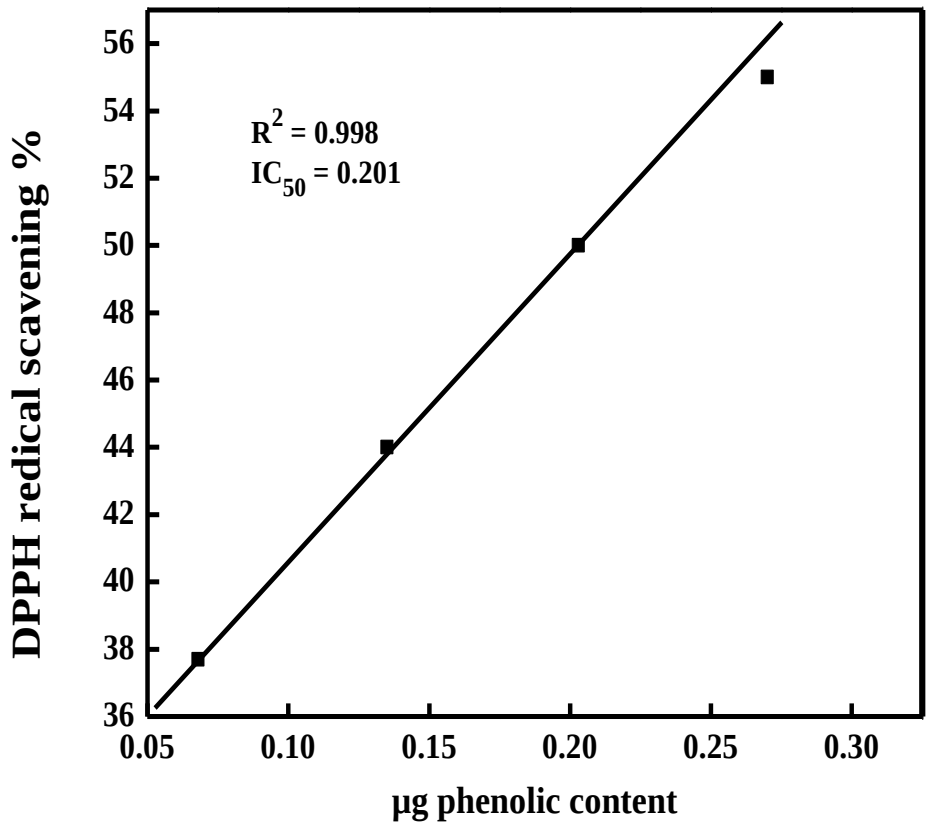

Fig 5: DPPH free radical scavenging ability of *P. aeruginosa* exopolysaccharide produced form heavy hydrocarbon oil