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A comparative GC-MS analysis of bacterial secondary metabolites of *Pseudomonas* species

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Abstract
The Bacterial metabolites have great importance for humans. The aim of the present study is to determine the potential bioactive components of bacterial metabolites using polar and non-polar solvent by Gas Chromatography Mass Spectrometry analysis. The chemical compositions of ethanol and chloroform extracts of bacterial secondary metabolites were investigated using Perkin-Elmer instrument. The GC-MS analysis provided nine compound with different Retention Time and peak area namely 2,6,10-Trimetyl-12-oxatricyclo[7.3.1.0(1,6)]tridec-2-ene; Nicotinic acid,1,6-dihydro-4-hydroxy-6-oxo-2-propyl-ethyl ester; Benzoic acid,2-amino-6- chloro-methyl ester and Heptadecanoic acid,9-methyl-,methyl ester; 2-(2,6,6-Trimethylcyclohex-1-enyl)cyclopropanecarboxylic acid, methyl ester; 9-Octadecenoic acid,(E)-; Pentadecanoic acid,13methyl-,methyl ester. Out of nine compounds Heptadecanoic acid, 9-methyl-, methyl ester was found as similar in both extracts. Five dissimilar and two similar compounds were obtained from the two solvents.

Keywords: Secondary metabolites, GC-MS, *Pseudomonas*, Chloroform, Ethanol, Heptadecanoic acid, 9-methyl-, methyl ester.

Introduction
Secondary metabolites are molecules required for long term survival of producer organism. Secondary metabolites are synthesized at larger levels during transition between the active and stationary phase [1]. They are produced when growth retards due to depletion of nutrient sources like carbon, nitrogen or phosphate [2]. Producer organism is provided with survival advantages by improving nutrient availability in the form of chelating agents such as siderophores, by protecting against environmental stressors like pigments and osmoprotectants by enhancing competitive interactions with other organisms like antibiotics, but also various signalling molecules or by acting as a metabolic defense mechanism comprising many plant flavonoids and alkaloid toxins. They are extensively used as antibiotics, antitumor agents and antivirals [3]. Pseudomonas have been known for the production of useful secondary metabolites [4, 5, 6, 7, 8].
Organic solvents play an important role in the extraction of secondary metabolites. Solvents like chloroform and ethanol could be used to extract intracellular and extracellular secondary metabolites. The addition of organic solvent to culture medium enhances the activity of secondary metabolites. Dissolving power of solvent denotes the activity of the compound [9].
Gas chromatography (GC) is recognized as the most suitable technique to find out number of components with proportion in a complex mixture of volatile compounds. When it is coupled to mass spectrometry (GC-MS), additional information arises about each separated compound molecular mass, elemental composition when high resolution mass spectrometry is used, functional groups, and in certain cases, molecular geometry and spatial isomerism [10]. Hence the present study is to exploit the usefulness of Bacterial secondary metabolites.

Materials and methods
Isolation of Bacteria
Pseudomonas species were isolated from *Wrightia tinctoria* (Roxb.) R.Br. in nutrient agar medium [11].

Preparation of Bacterial extracts and separation of its compounds
Bacterial isolate was inoculated in an Erlenmeyer flask containing Nutrient broth and incubated for 3 to 4 days. The fermentation flask was incubated at 110 rpm on a rotary shaker

At room temperature for 7 days [12]. After fermentation the culture broth was filtered and the filtrates were added with solvents chloroform and ethanol separately in a ratio of 1:1. Chloroform added culture broth formed two layers; the solvent layer was separated using separating funnel and stored in sterile vials. Ethanol added culture broth was retained as aqueous extract.

GC-MS Analysis of Bacterial Metabolites

Secondary Metabolites were analyzed by Gas chromatography coupled mass spectrometry (GC-MS) to identify the compounds present. GC-MS analysis was performed in a Perkin–Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a Gas Chromatograph interfaced to a Mass Spectrometer (GC-MS) equipped with a Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused capillary column (30 × 0.25 µm ID × 0.25 µm df). For GC-MS detection, an electron ionization system was operated in electron impact mode with ionization energy of 70 eV. Helium gas (99.999%) was used as a carrier gas at a constant flow rate of 1 ml/min, and an injection volume of 2µl was employed (a split ratio of 10:1). The injector temperature was maintained at 250 °C, the ion-source temperature was 200 °C, the oven temperature was programmed from 110 °C (isothermal for 2 min), with an increase of 10 °C/min to 200°C, then 5 °C/min to 280 °C, ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. The solvent delay was 0 to 2 min, and the total GC-MS running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Turbo-Mass Gold-Perkin-Elmer- mass-detector was used, and Turbo-Mass ver-5.2 software was used to handle mass spectra and chromatograms.

Description of components

Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were confirmed.

Results

GC-MS chromatogram of the chloroform extract (Figure 1) showed 4 peaks indicating the presence of four Bacterial constituents. On comparison of the mass spectra of the constituents with the NIST library the four microbial constituents were characterized and identified as 2,6,10-Trimetyl-12-oxatricyclo[7.3.1.0(1,6)]tridec-2-ene; Nicotinic acid,1,6-dihydro-4-hydroxy-6-oxo-2-propyl-ethyl ester; Benzoic acid,2-amino-6- chloro-methyl ester and Heptadecanoic acid,9-methyl-,methyl ester in the following retention time of 11.97, 13.08, 13.32 and 14.17 respectively. The compound Heptadecanoic acid,9-methyl-,methyl ester exhibited highest peak area of 46.80% and 2,6,10-Trimetyl-12-oxatricyclo[7.3.1.0(1,6)]tridec-2-ene showed the lowest peak area of 5.53% (Table 1).

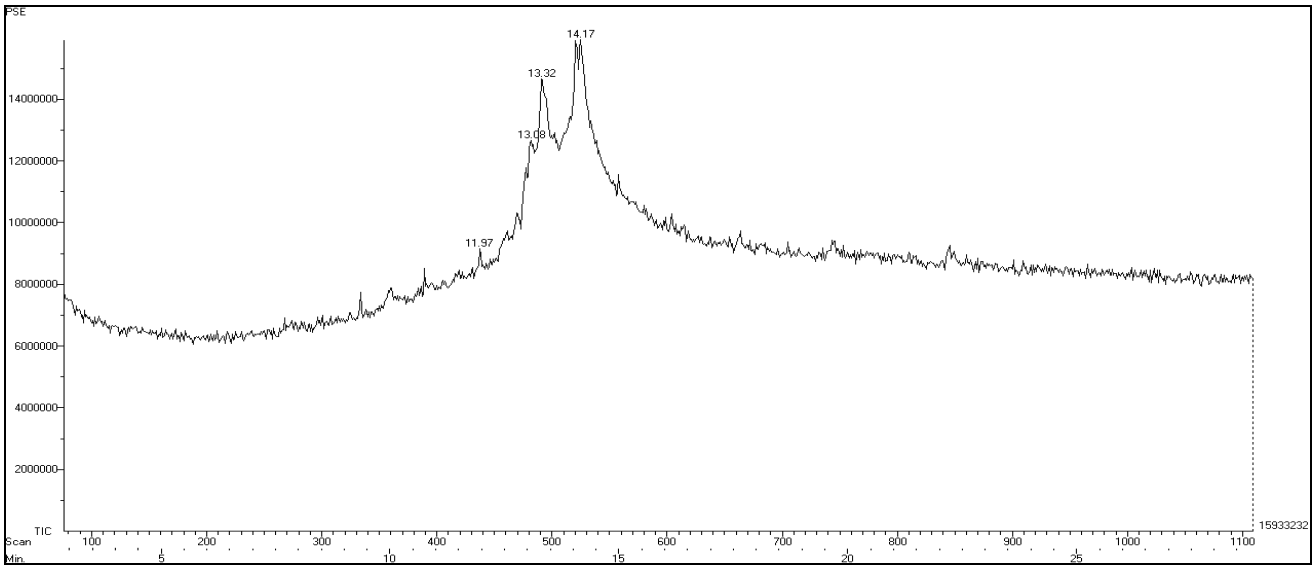

Fig 1: GC-MS chromatogram of *Pseudomonas* (chloroform extract).

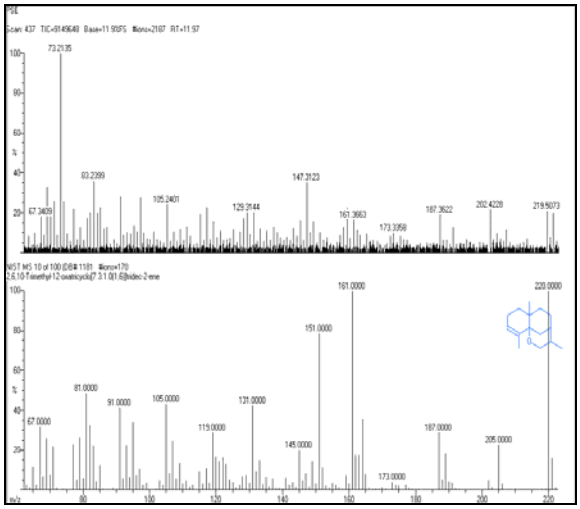

Fig 2A: 2,6,10-Trimetyl-12-oxatricyclo[7.3.1.0(1,6)]tridec-2-ene

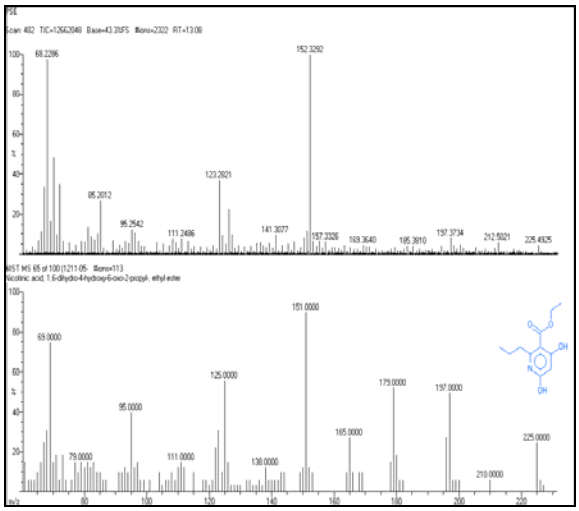

Fig 2B: Nicotinic acid,1,6-dihydro-4-hydroxy-6-oxo-2-propyl-ethyl ester

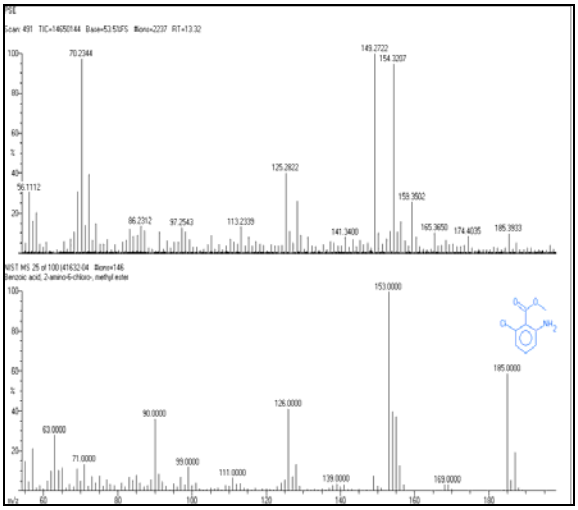

Fig 2 C: Benzoic acid,2-amino-6- chloro-methyl ester

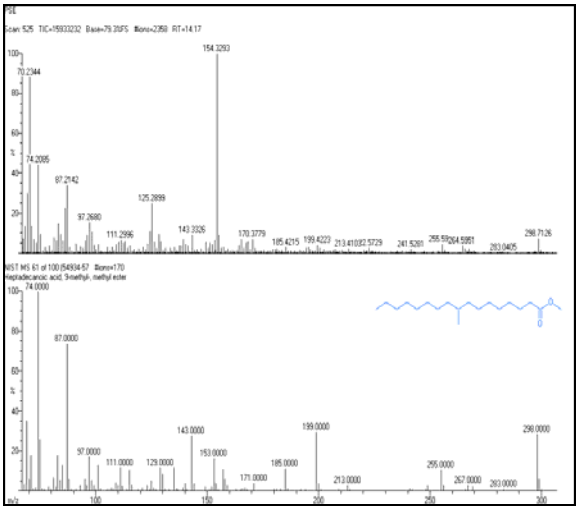

Fig 2 D: Heptadecanoic acid,9-methyl-,methyl ester

Table 1: Bacterial components identified in chloroform extract

S.No.	Retention Time	Compound	Molecular Formula	Molecular Weight	Peak Area %
1.	11.97	2,6,10-Trimetyl-12-oxatricyclo[7.3.1.0(1,6)]tridec-2-ene	$C_{15}H_{24}O$	220.350	5.53
2.	13.08	Nicotinic acid,1,6-dihydro-4-hydroxy-6-oxo-2-propyl-ethyl ester	$C_{11}H_{15}NO_4$	225.241	26.24
3.	13.32	Benzoic acid,2-amino-6- chloro-methyl ester	$C_8H_8ClNO_2$	185.608	21.41
4.	14.17	Heptadecanoic acid,9-methyl-,methyl ester	$C_{19}H_{38}O_2$	298.503	46.80

GC-MS chromatogram of the ethanol extract (Figure 2) showed 5 peaks indicating the presence of five microbial constituents. On comparison of the mass spectra of the constituents with the NIST library the five microbial constituents were characterized and identified as 2-(2,6,6-Trimethylcyclohex-1-enyl)cyclopropanecarboxylic acid, methyl ester; 9-Octadecenoic acid,(E)-; 2,6,10-Trimethyl-12-oxatricyclo[7.3.1.0(1,6)]tridec-2-ene; Pentadecanoic acid,methyl-,methyl ester; Heptadecanoic acid,9-methyl-,methyl ester in the retention time of 9.3, 10.73, 11.95, 13.03 and 14.25 respectively. Heptadecanoic acid,9-methyl-,methyl ester exhibited highest peak area of 44.94% and 2,6,10-Trimetyl-12-oxatricyclo[7.3.1.0(1,6)]tridec-2-ene showed the lowest peak area of 5.56% being the lowest peak area and being the highest peak area (Table 2).

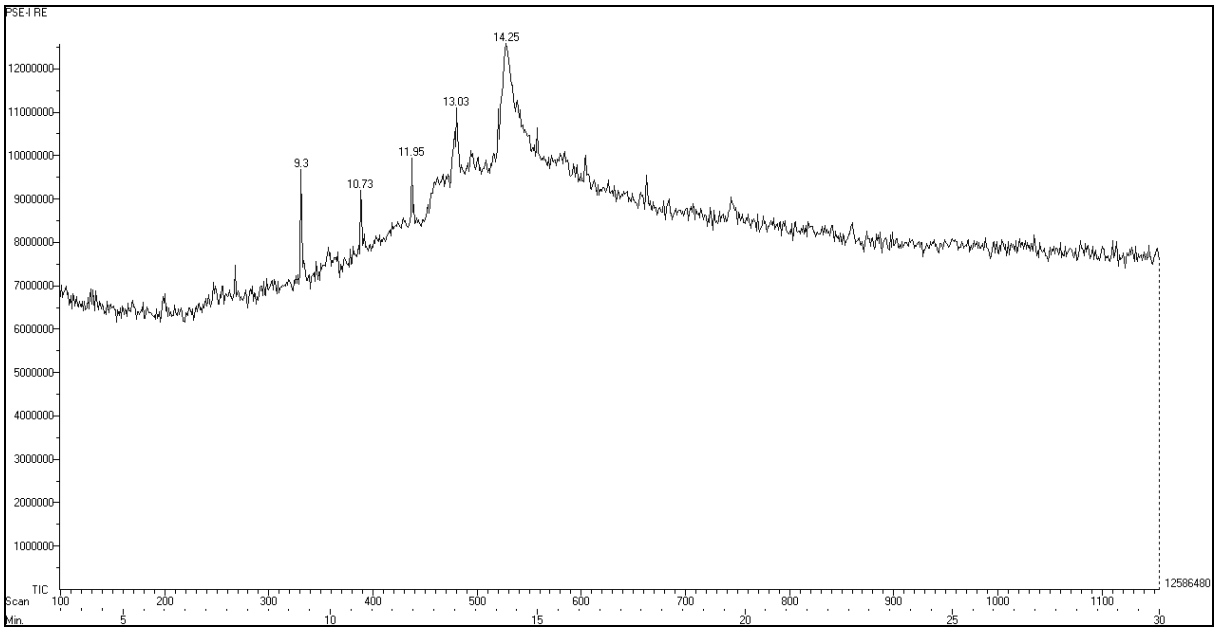

Fig 2: GC-MS chromatogram of pseudomonas (ethanol extract)

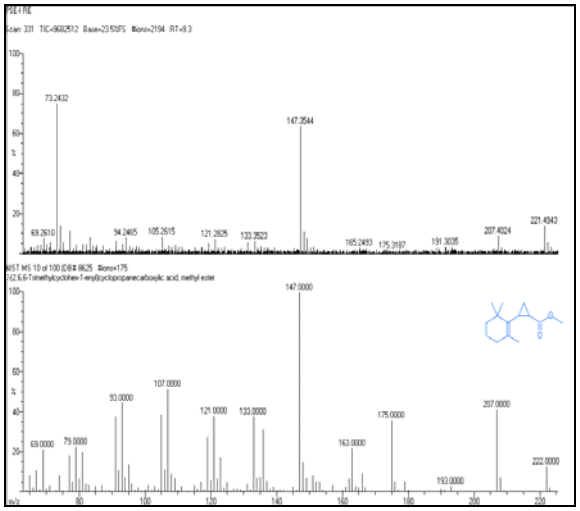

Fig 3 A: 2-(2,6,6-Trimethylcyclohex-1-enyl)

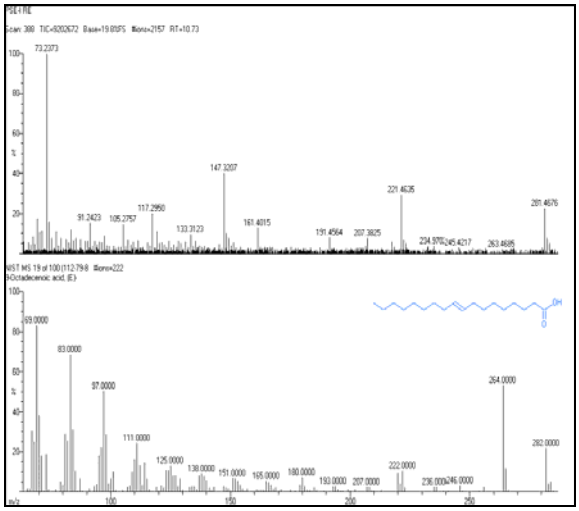

Fig 3 B: 9-Octadecenoic acid,(E)- cyclopropanecarboxylic acid, methyl ester

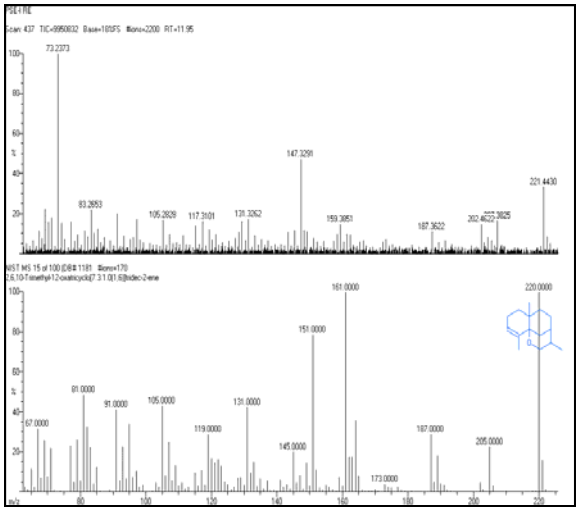

Fig 3 C: 2,6,10-Trimethyl-12-oxatricyclo[7.3.1.0(1,6)

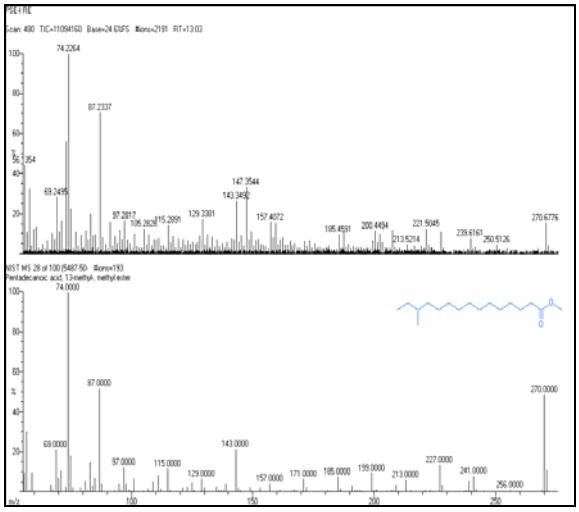

Fig 3 D: Pentadecanoic acid,13methyl-,methyl ester]tridec-2-ene

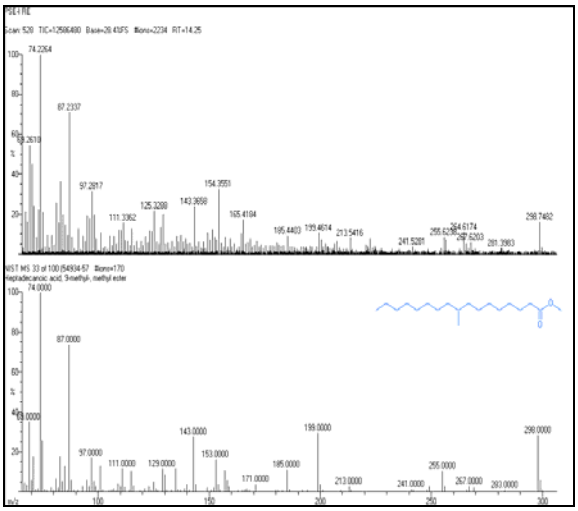

Fig 3 E: Heptadecanoic acid,9-methyl-,methyl ester

Table 2: Bacterial components identified in ethanol extract

S.NO.	Retention Time	Compound	Molecular Formula	Molecular Weight	Peak Area %
1.	9.3	2-(2,6,6-Trimethylcyclohex-1-enyl)cyclopropanecarboxylic acid, methyl ester	$C_{14}H_{22}O_2$	222.323	19.14
2.	10.73	9-Octadecenoic acid,(E)-	$C_{18}H_{34}O_2$	282.461	13.12
3.	11.95	2,6,10-Trimethyl-12-oxatricyclo[7.3.1.0(1,6)]tridec-2-ene	$C_{15}H_{24}O$	220.350	5.56
4.	13.03	Pentadecanoic acid,13methyl-,methyl ester	$C_{17}H_{34}O_2$	270.450	17.20
5.	14.25	Heptadecanoic acid,9-methyl-,methyl ester	$C_{19}H_{38}O_2$	298.503	44.94

Discussion

Solvent extraction has proved to be effective in drawing out the useful compounds from the microbes. Chloroform (Non-polar) and ethanol (Polar protic) has proved to be helpful in extraction of metabolites possessing antimicrobial activity [13, 14, 15, 16]. In this study two solvents had been used to obtain secondary metabolites from *Pseudomonas* species. Solvents were chosen on the basis of their polarity. Chloroform extract resulted in four compounds in comparison to five compounds arise out of ethanol extract. 2,6,10-Trimethyl-12-oxatricyclo[7.3.1.0(1,6)]tridec-2-ene and Heptadecanoic acid,9-methyl-,methyl ester resulted in both solvents. The previous compound resulted in retention time of 11.97 with a peak area of 5.53 in chloroform solvent whereas ethanol solvent showed the same compound at retention time of 11.95 with a peak area of 5.56. The latter compound resulted in retention time of 14.17 with a peak area of 46.80 in chloroform solvent whereas ethanol solvent showed the same compound at retention time of 14.25 with a peak area of 44.94. The compound 9-octadecenoic acid, had been reported in methanolic extract of *Stenotrophomonas maltophilia* at a retention time of 13.9 [17]. The same compound was found in ethanol extract at a retention time of 10.73 in the present study. The impact of oleic acid (cis -9-octadecenoic acid) had been reported on bacterial viability and biofilm production in *Staphylococcus aureus* [18]. According to the literature survey, the following compounds 2,6,10-Trimethyl-12-oxatricyclo[7.3.1.0(1,6)]tridec-2-ene; Nicotinic acid,1,6-dihydro-4-hydroxy-6-oxo-2-propyl-ethyl ester; Benzoic acid,2-amino-6- chloro-methyl ester; Heptadecanoic acid,9-methyl-,methyl ester; 2-(2,6,6-Trimethylcyclohex-1-enyl)cyclopropanecarboxylic acid, methyl ester; Pentadecanoic acid,13methyl-,methyl ester has not been reported previously from *pseudomonas* species.

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