

Characterization of Lantana camara roots (Pentacyclic Triterpenoid) and Mutagenicity Testing of extracted Oleanolic acid Using Salmonella typhimurium.

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Abstract

Objective

The objective of the present study was to isolate oleanolic acid from the roots of *Lantana camara*, and characterize it chemically using DSC, HPLC, and FTIR methods, and additionally carrying out a short term assay for assessment of its mutagenic potential by conducting bacterial reverse mutation test to evaluate the ability of the “Oleanolic acid (Pentacyclic Triterpenoid)” to induce point mutations in tester strains of *Salmonella typhimurium* in both presence and absence of exogenous metabolic activation system (S9) containing microsomal enzymes.

Methods

Differential scanning calorimetry (DSC), High Performance Liquid chromatography (HPLC), and Fourier transform infrared (FTIR) spectrometers were used to chemically analyze the isolated molecule. Oleanolic acid was utilized to carry out Preliminary Cytotoxicity and mutagenicity study.

Results

According to the results of spectrophotometric research, oleanolic acid extracted from *Lantana camara* roots exhibits identical spectrum characteristics to standard oleanolic acid also the mutagenic potential of Oleanolic acid (Pentacyclic Triterpenoid). Oleanolic acid was found to be non-mutagenic in all five test strains of *Salmonella typhimurium*—TA98, TA100, TA102, TA1535, and TA1537 employing plate incorporation assays

Conclusion

It may be determined that oleanolic acid isolated from *Lantana camara* roots gives identical, identifiable signals and absorbance like previously reported reference standard based on the results of DLC, HPLC, and FTIR spectra and their interpretation and was determined that oleanolic acid purified from *Lantana camara* roots is non-mutagenic in *Salmonella typhimurium*.

1) Introduction

An ornamental plant known *Lantana camara* has been historically used to treat a variety of diseases as traditional medicine.. *camara*, is a rich source of numerous bioactive principles, is traditional medicine (Sastri, 1962). It has been stated that its roots contain large quantities of the triterpenoid oleanolic acid (Misra et al., 1997). Numerous significant biological actions of Oleanolic acid have been reported, including anti-inflammatory (Tsuruga et al., 1991), anti-hyperlipidemic (Ma, 1986; Liu, 1995), antiulcer (Gupta et al., 1981), antioxidant activity (Balanehru and Nagarajan, 1991), and hepatoprotective

characteristics (Ma et al., 1982). Recently, its ability to inhibit the growth of tumors has gained attention (Shibata, 2001). As Oleanolic acid, has shown to possess anti-inflammatory, hepatoprotective, anticancer, antioxidant, and anti-hyperlipidemic activity, and is abundantly obtained from the roots of *L. camara*. Both oleanolic acid (3-hydroxy-olea-12-en-28-oic acid) and its isomer, ursolic acid (3-hydroxy-urs-12-en-28-oic acid), are pentacyclic triterpenoids with 30 carbon atoms. They are also synthetically produced by cyclizing squalene, and they are found in a wide variety of plants as free acids or aglycones of triterpenoid sapon (Vyas et al, 2014). The Structure of Oleanolic acid is as follows (Fig. 1):

2) Material And Methods

2.1) Plant Material

Lantana camara Linn. roots were harvested from Bhopal, Madhya Pradesh, India in area of hilly regions. Plant material was harvested early in the morning. The roots were then torn up and left for drying in the air. The end product was a granular powder.

2.2) Plant Identification and Authentication

The Department of Botany, Lucknow verified the plant's authenticity before accepting for the herbarium.

Preliminary Cytotoxicity Assay was performed by using Oleanolic acid and later the Mutagenicity assay was conducted in two phases (Trial-I and Trial-II) with 5 analyzable concentrations of Test Item, concurrent Vehicle and Positive Controls was also be tested in triplicate. The highest concentration tested for Main study from the Preliminary Cytotoxicity results and subsequent lower concentration was selected with spacing factor of 2 or $\sqrt{10}$

2.3) Process of isolation of Oleanolic acid (OA)

The 500g of powdered crude drug was taken, defatted three times over the course of an overnight period with petroleum ether, and then extracted thoroughly four times over the course of an overnight period at room temperature with ethanol. The crude extract was dissolved in CHCl_3 and allowed to stand overnight to precipitate after the solvent was drawn off under vacuum. Methanol was used to crystallize the precipitate that was so produced. Oleanolic acid crystals were produced after four cycles of precipitation and condensation.

2.4) FTIR Chromatography

Pattern of oleanolic acid was run on FTIR for identification. IR spectra of isolated oleanolic acid were recorded by using Thermo FTIR with ATR, in infrared zone of wavelength of 4000 to 400 cm^{-1} . Standard procedure was followed to obtain the IR spectra of the test compound. Results we obtained were illustrated at spectra showed in (Fig. 2).

2.5) DSC Chromatography

A Mettler Toledo STAR Thermal Analysis System was used for the DSC analyses, and the DSC 3 and Pattern of oleanolic acid were assessed for identification. The sample was added to a 40-L aluminium pan with a nitrogen flow rate of 40 ml/min. The sample was run at the rate of 10 k/min from 30 to 600 °C. (Fig. 3).

2.6) HPLC Analysis

We examined the OA (Oleanolic acid) that was extracted from Lantana camara roots using the Thermo HPLC system Ultimate 3000. On an Inertsil ODS column (250 mm x 4.6 mm, 5 m particle size), chromatographic separation was performed in isocratic mode with mobile phase A (Methanol): mobile phase B (0.03 M sodium phosphate in milli-Q water pH at 3) (88:12 v/v). A photodiode array detector set to 210 nm was used to observe the elution after the mobile phase was added to the column at a flow rate of 1.0 ml/min. The column was kept at a temperature of 30°C. (Figs. 4 & 5.)

3) Ames Test

Ames test was developed in the 1970s by Dr. Bruce Ames and his associates (Maron and Ames, 1983) and was further evaluated by Mortelmans and Zeiger (2000). This test is carried out in vitro over a short period of time to assess potential mutagenesis effects of substances (Tagliari et al. 1999).

3.1) Metabolic Activation System

3.1.1) S9 Homogenate

Aroclor 1254-induced Rat Liver S9 homogenate procured from Meshram GenoTox Services, Nagpur (stored in Ultra-freezer below – 60°C) were used for the assay.

3.1.2) S9 Mix

S9 mix was prepared by mixing cofactors solution with S9 homogenate.

Following are the chemicals and S9 homogenate used in the Assay (Table 1):

3.2) Mutagenicity Assay (Plate Incorporation Method):

80 mL of Sodium phosphate buffer was prepared and stored at 2–8°C. On the day of treatment, required amount of cofactor chemicals were weighed and mixed with Sodium phosphate buffer solution and filtered by using 0.22 µm filter. 6 mL of S9 homogenate was mixed with 54 mL (filtered) of Co-factor mix, which resulted in 10% S9 Mix solution. S9 Mix was prepared immediately prior to use in the experiment.

3.3) Test System Selection and Its Justification

The *Salmonella typhimurium* is commonly used test strain for bacterial reverse mutation studies and recommended by the international guidelines (i.e., OECD Test Guideline No. 471). It also meets the regulatory requirement of most of the regulatory agencies.

3.4) Vehicle Selection and Its Justification

Prior to Mutagenicity Assay the solubility test was performed. The following table provides the information for the vehicle selection process at concentration 50 mg/mL of Oleanolic acid slight precipitation was observed which did not interfere in colony count and interpretation of bacterial background lawn Based on the solubility test (Table:2) DMSO was selected as the vehicle for the conduct of the study.

3.5) Dose Concentration:

Mutagenicity Assay was conducted with six groups consisting of Five Oleanolic acid concentrations along with a Vehicle Control in triplicates. (Fig. 7&8) Concentrations for Mutagenicity Assay (+ S9/-S9): 50.02, 158.16, 500.09, 1581.28 and 5000 µg/plate. Concentration Spacing factor: $\sqrt{10}$. Fig. 7: **Dose Response Curve Trial-I (-S9)**

3.6) Mutagenicity Study

The Mutagenicity Assay was conducted with five analyzable concentrations of Test Item. 5000 µg/plate was selected as highest concentration for Oleanolic acid along with vehicle in triplicate.

3.7) Preparation of Overnight cultures

3.7.1) Inoculation

From the thawed ampoules of the strains (stored between -65 to -86°C), appropriate volume of cultures was transferred into flasks containing Oxoid Nutrient Broth No. 2. Cultures were incubated at $37 \pm 1^\circ\text{C}$ and 120 rpm for 16 hours 20 minutes.

3.7.2) Viable counts

The cultures were taken out from the incubator and optical density of the culture was measured at 540 nm.

3.8) Oleanolic acid Formulation:

Stock: 500.0 mg of the Oleanolic acid was procured from TICU, 7 mL of initial volume of vehicle was added, mixed, and transferred into 10 mL volumetric flask. The volume was made upto the mark using vehicle, resulting in 50 mg/mL (stock) was prepared and serially diluted upto 0.50 mg/mL concentration (Table:3). Fresh stocks/ dilutions were prepared on the day of use. Oleanolic acid Stock/ dilution were prepared as follows:

Concentration Spacing factor: $\sqrt{10}$ (3.162). The Research was performed using Test Strains TA98, TA100, TA102, TA1535 and TA1537 in both the presence and absence of metabolic activation (S9).

3.9) Plating procedure

The Test Strain was exposed to the Oleanolic acid via the Plate incorporation method (Table 4). The following were the contents of each tube.

The tube contents were mixed and poured on to MGA plates. After solidification of top agar, the plates were incubated at $37 \pm 1^\circ\text{C}$ for 68 hours. After incubation, MGA plates were taken out from the incubator and were observed for revertant colonies and bacterial background lawn.

3.10) Sterility Check

The sterility check was performed to check the Sterility of MGA plates, Oleanolic acid (Lowest and Highest concentration), S9 Mix, Solvent / Vehicle, Sodium Phosphate Buffer and Top agar.

4) Results And Discussion

As a result of the acquired spectra and their interpretation in this study, it is now appropriate to indicate that oleanolic acid isolated from *Lantana camara* roots displays identical, recognizable signals and an absorbance that is comparable to previously published reference standards. (Elvira et. al., 2009 and Vyas et al. 2014).

HPLC chromatograms (Figs. 4, 5 & 6) of Oleanolic acid showed peak at 17 minutes. From these observations we can attribute the isolated sample of oleanolic acid which showed 91.9% purity by area normalization with some very minor impurities.

The mass spectrum confirmed the negative ion peak of m/z at 455.5 which is corresponding to molecular weight of oleanolic acid.

Thermal analysis has been reported to be a powerful analytical tool for characterization Weijia Huang *et al*/2018 For instance, (Fig. 3) reveals an endothermic peak at (T fusion) 310.16°C in the DSC profile, which denoted the melting point of pure OA. There is sharp peak observed in thermogram denoted that oleanolic acid is 98.485% pure based on %mole basis.

The obtained FTIR spectra showed (Fig. 2) different groups in the following spectral regions 3434 (OH); 2861 (CH₂); 1690 (C = O); 1462 (OH); 1363 (CH₃). At IR spectra of oleanolic acid appears like a adsorption ribbon, which derivates from OH group in the area of 3434 cm^{-1} . A very intensive absorption ribbon in 2861 cm^{-1} derives from symmetric vibrations of CH₂ cm^{-1} group. In the area of 1690 cm^{-1} appears a characteristic ribbon of carbonyl group (C = O). At 1462 cm^{-1} appears absorption ribbon from OH vibrations of planar distortion. In the area of 1361 cm^{-1} appears a characteristic ribbon, which derives from CH₃ group.

Based on the obtained IR spectrums, it is characterized the noticeable functional groups of phenolic which can be confirmed from the structure of oleanolic acid.

Mutagenicity

To mutagenicity, the Ames test is a frequently used bacterial assay (Mortelmans and Zeiger, 2000). Although we have demonstrated the assay method for five strains in, this approach can be utilized to determine the mutagenicity of all substances. While sterile procedures are used in the Ames assay investigations, care must be taken to ensure the sample or plasmid is not contaminated. We can troubleshoot method to analyses the substances tested in clinical trials due to the enhanced methods for detecting the test genotoxicity of various chemical substances (Urvashi et. al., 2018).

In this study we have found Oleanolic acid to be non-mutagenic in Ames test. Certain studies have reported that Oleanolic acid, present in grapes and olives, also has anti-mutagenic properties and rather protects against genotoxicity in human mammary epithelial cells (Cristina Sánchez-Quesada et al. 2015).

5) Conclusion

The present study concluded that the Oleanolic acid was well characterized by Differential scanning calorimetry (DSC), High Performance Liquid chromatography (HPLC), and Fourier transform infrared (FTIR) spectrometer. Oleanolic acid observed to be non-mutagenic up to 5000 µg/plate in both the presence and absence of metabolic activation (S9) under the valid assay conditions. Consequently, it can be stated that Oleanolic acid “(Pentacyclic Triterpenoid)” do not exhibit any mutagenic potential as determined by the Ames test at the concentrations examined.

Declaration

Ethical Approval and Consent to participate:

Ethical approval Not applicable and we have consent letter for all participants.

Human Ethics

Not applicable

Consent for publication

Yes, we have consent for the same from all the Co-authors.

Availability of Supporting data.

Yes, available.

Competing interests

No, there is no competing interest

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Author's contribution

Co-authors have contributed around 20% by first co-author and other 10%(5% and 5%) from 3 and 4th Co-authors.

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Tables

Tables 1 to 10 are available in the Supplementary Files section.

Figures

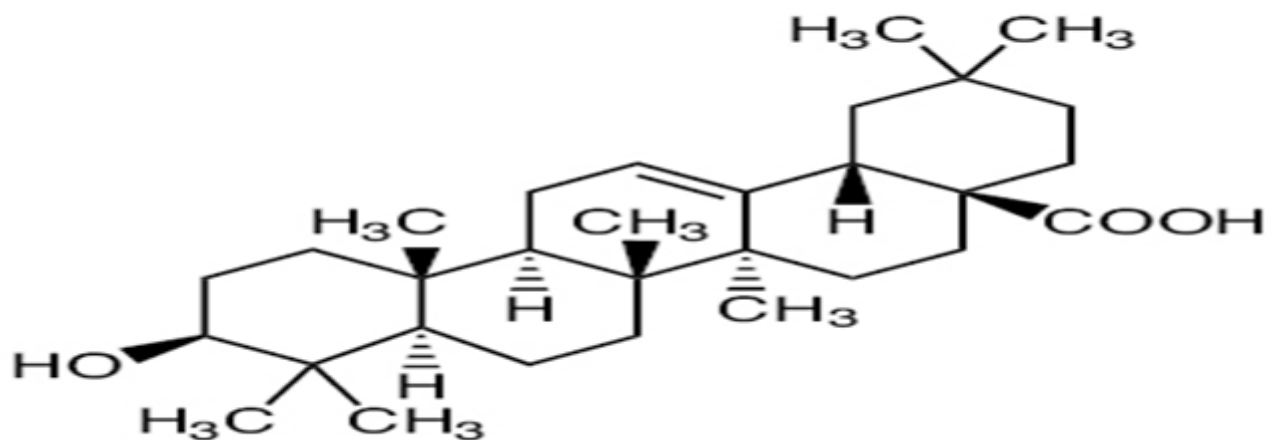


Figure 1

Structure of Oleanolic acid

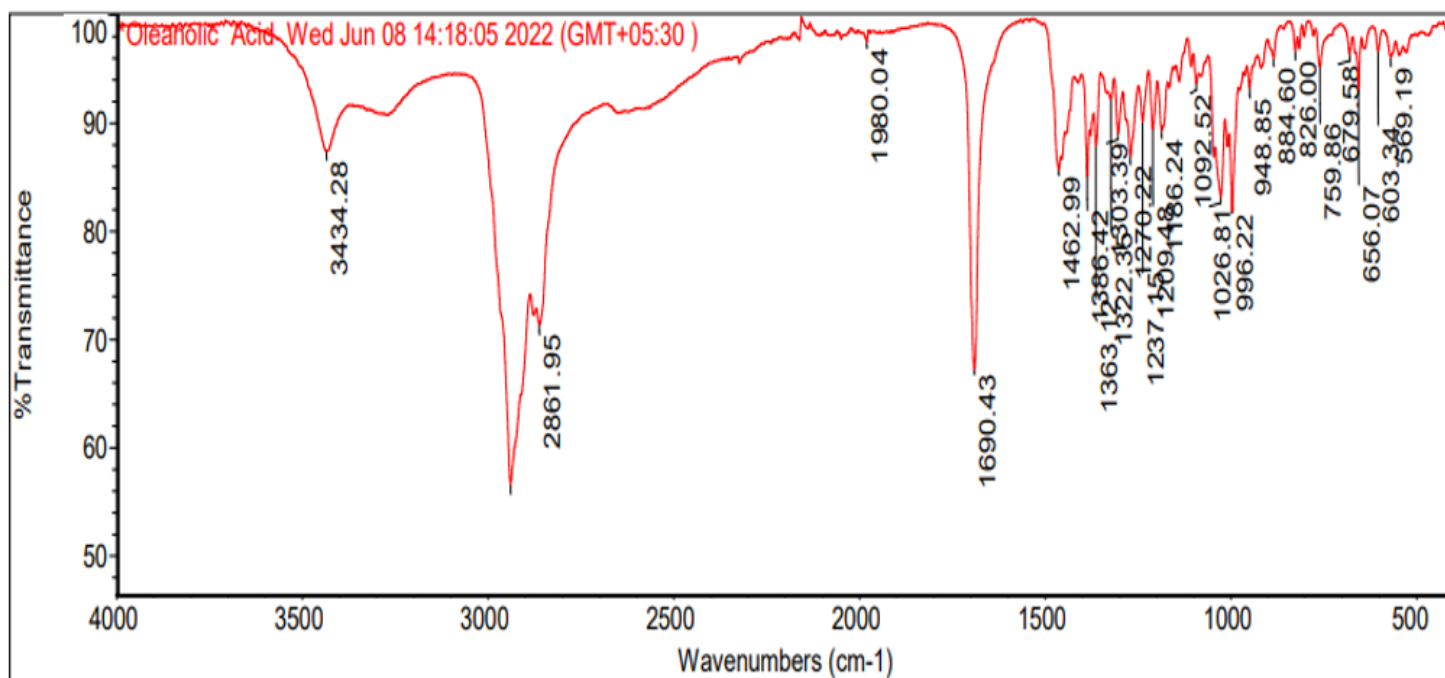
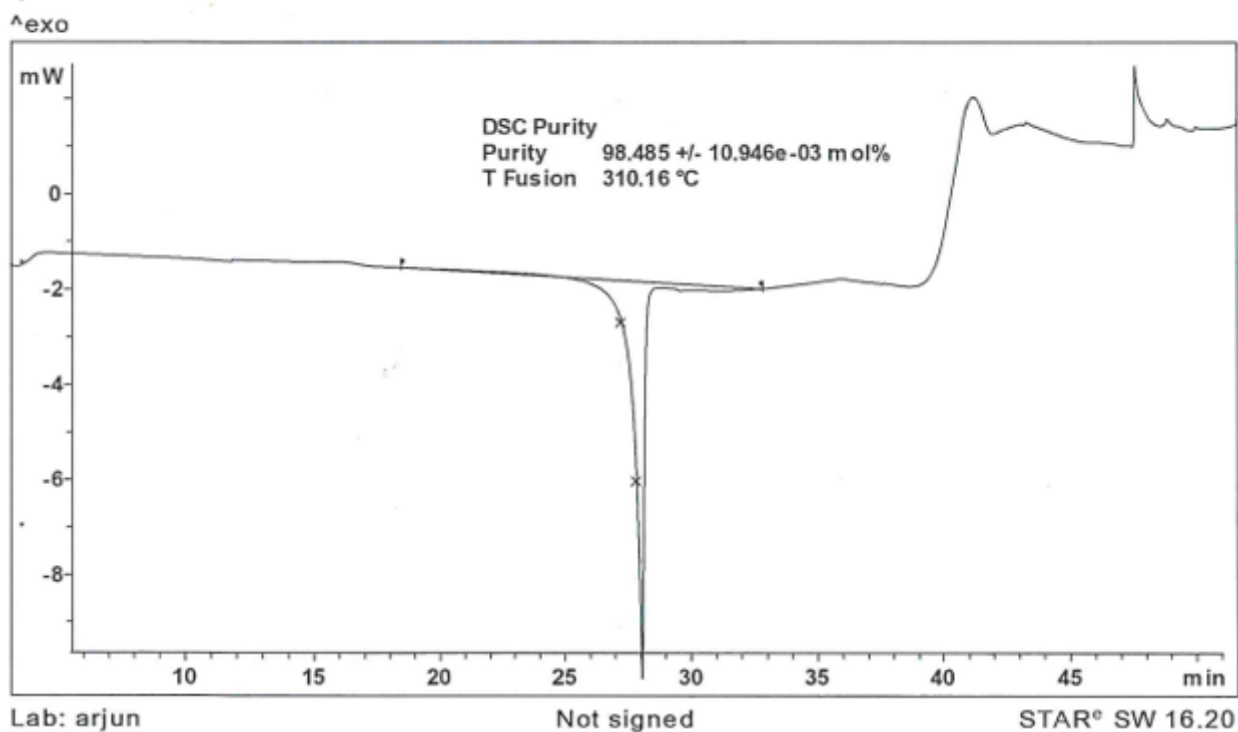


Figure 2

FTIR Chromatography Oleanolic acid

Evaluation:



Curve: Oleanolic Acid (BN®-QDGS2020121004), 08.09.2021 17:00:07
Sample: Oleanolic Acid (BN®-QDGS2020121004), 2.6500 mg
Module: DSC 3 /700/2084, 18.11.2019 13:18:11
Sample Holder: Aluminum Standard 40ul
Material: Aluminum
Method: Oleanolic Acid
Not released
dt 1.00 s
[1] 30.0..600.0 °C, 10.00 K/min, N2 40.0 ml/min
Synchronization enabled

Figure 3

DSC Chromatography Oleanolic acid

Injection Details			
Injection Name:	Methanol	Run Time (min):	30.00
Vial Number:	RA1	Injection Volume:	25.00
Injection Type:	Unknown	Channel:	UV_VIS_1
Calibration Level:		Wavelength:	210
Instrument Method:	Oleanolic Acid 30052022-00	Bandwidth:	4
Processing Method:	All Settings	Dilution Factor:	1.0000
Injection Date/Time:	02-Jun-22 18:21	Sample Weight:	1.0000

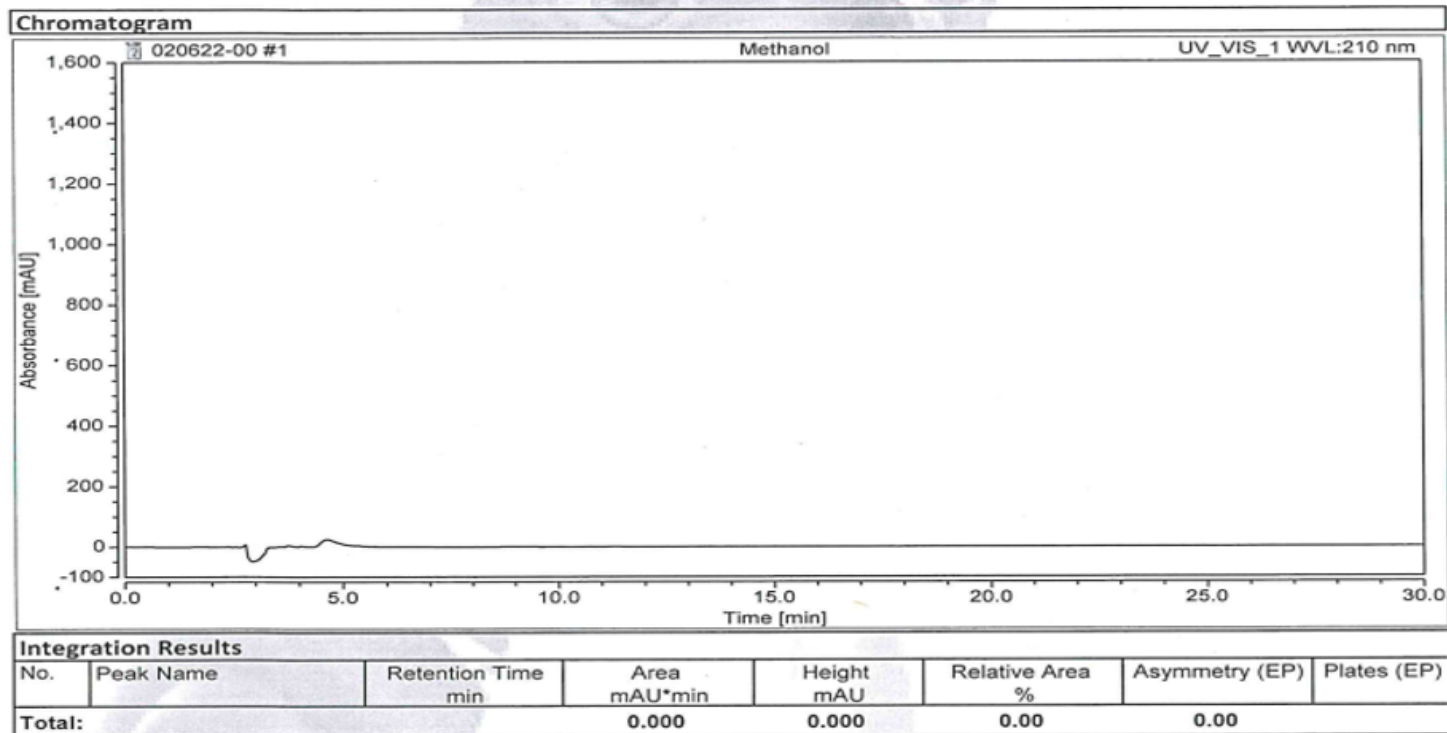


Figure 4

HPLC Chromatography with methanol

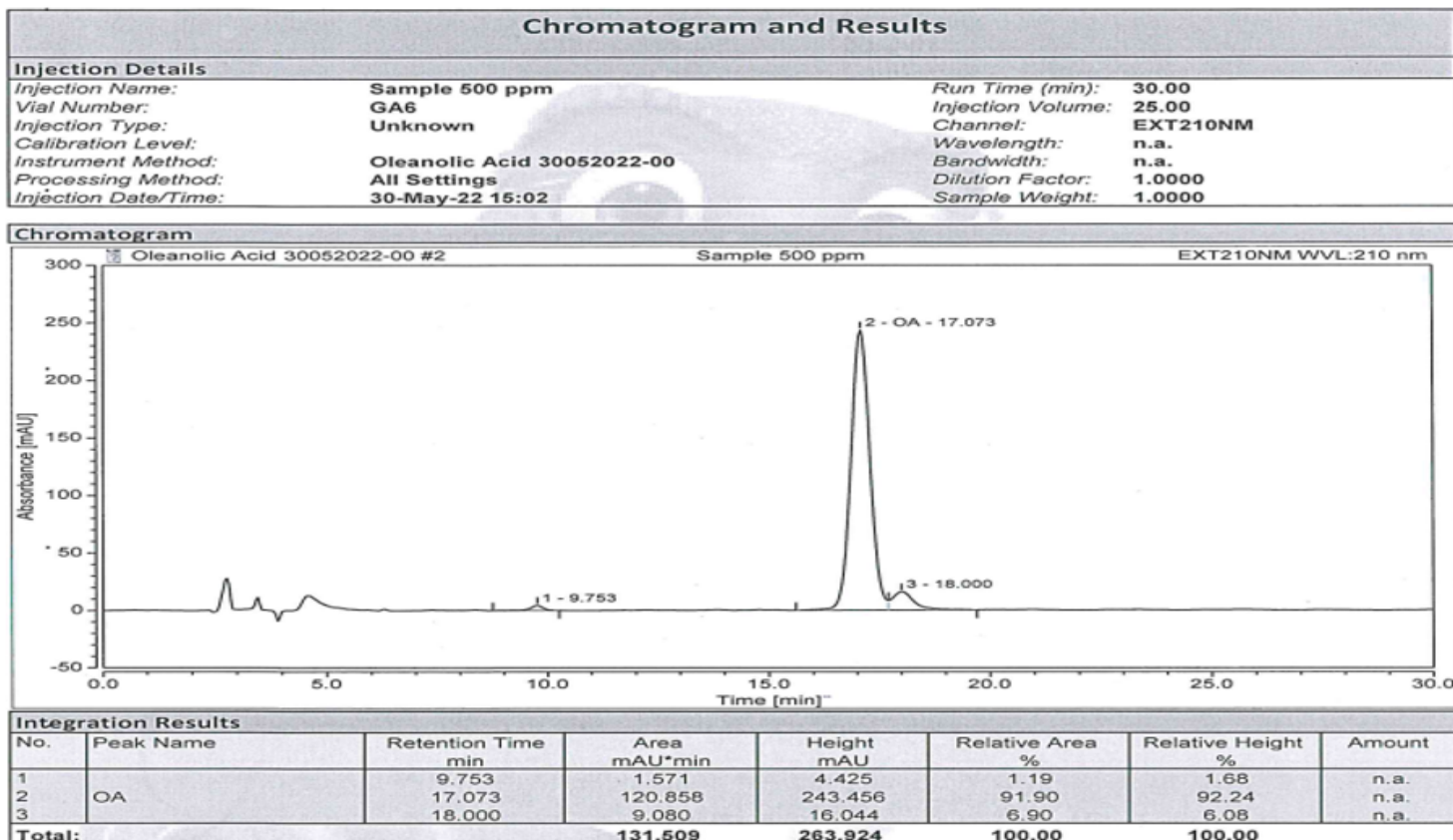


Figure 5

HPLC Chromatography Oleanolic acid (Sample)

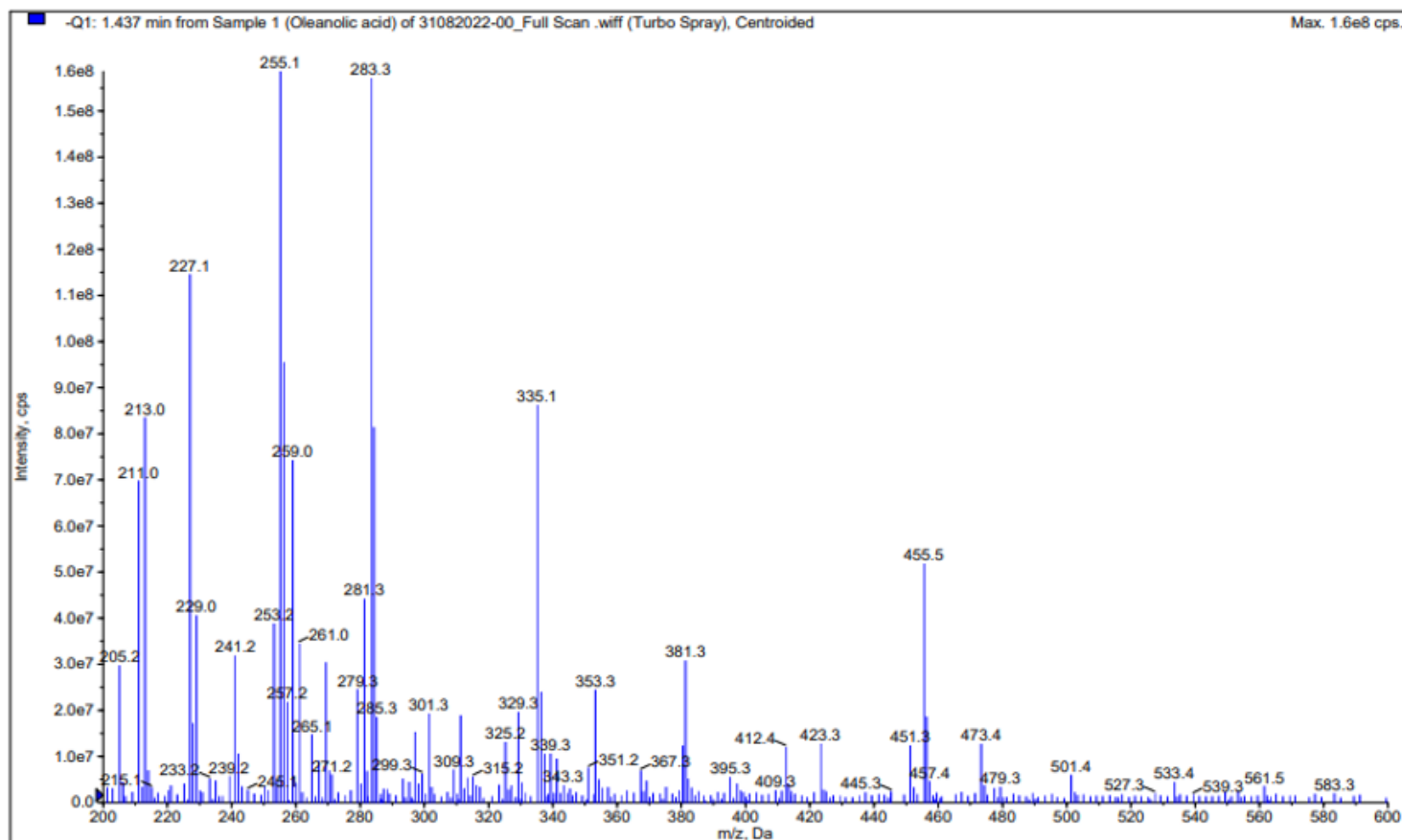


Figure 6

The mass spectrum confirmed the negative ion peak of m/z at 455.5 which is corresponding to molecular weight of oleanolic acid.

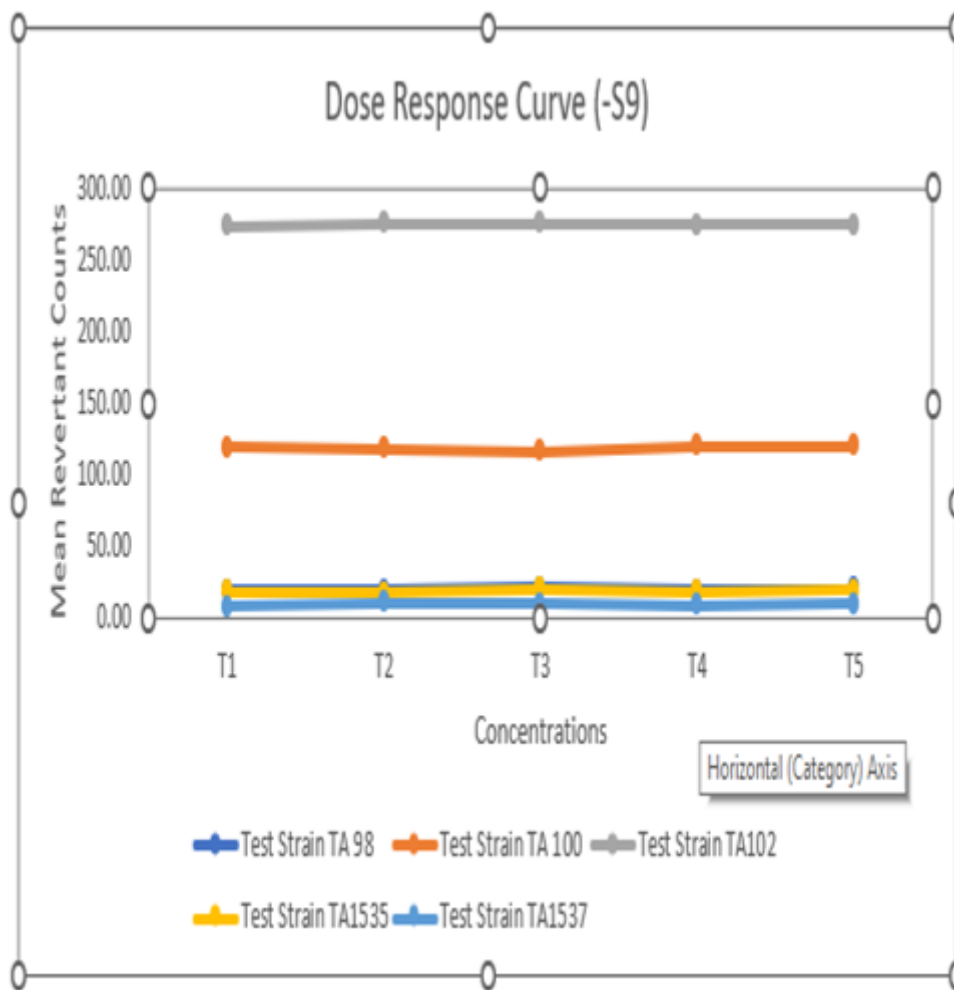


Figure 7

Dose Response Curve Trial-I (-S9)

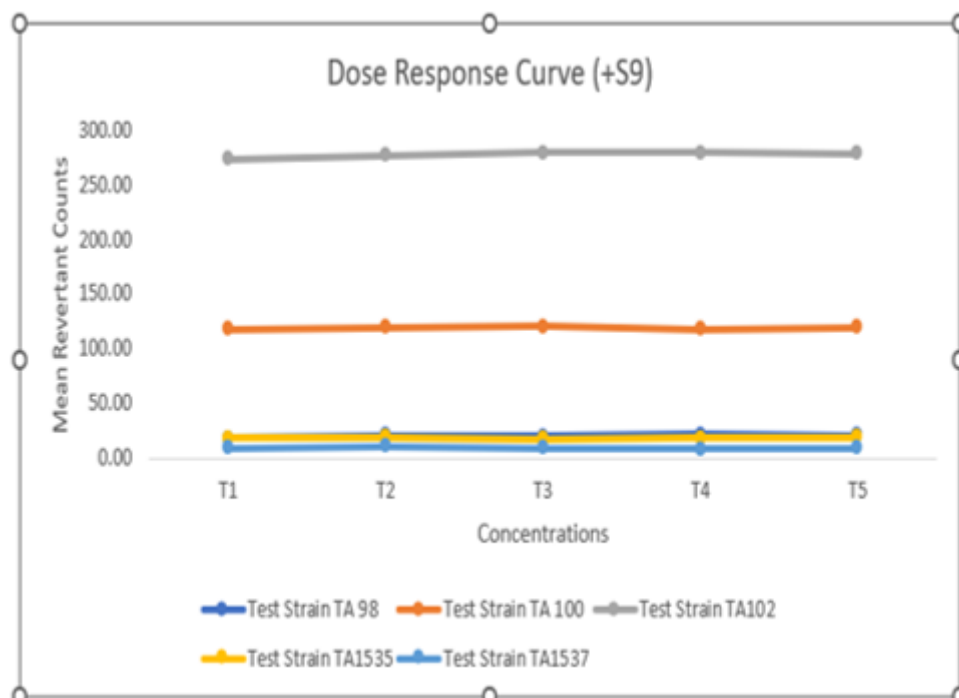


Figure 8

Dose Response Curve Trial-I (+S9)

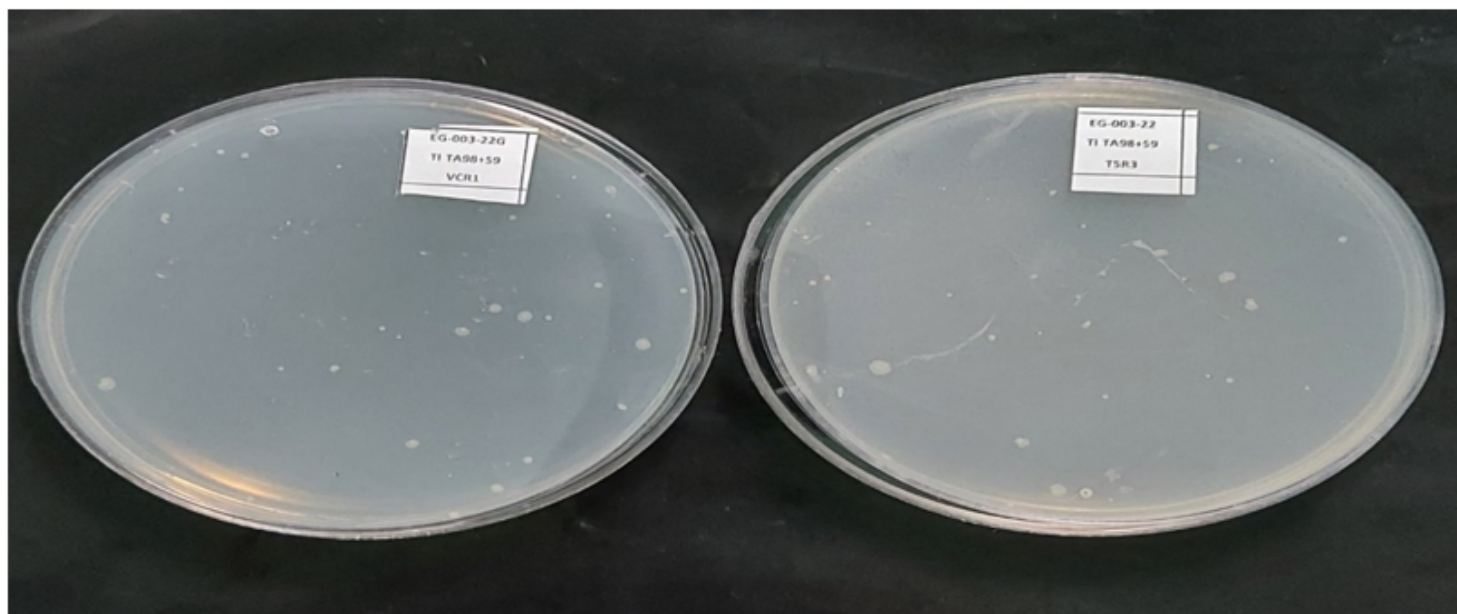


Figure 9

TA98 Strain

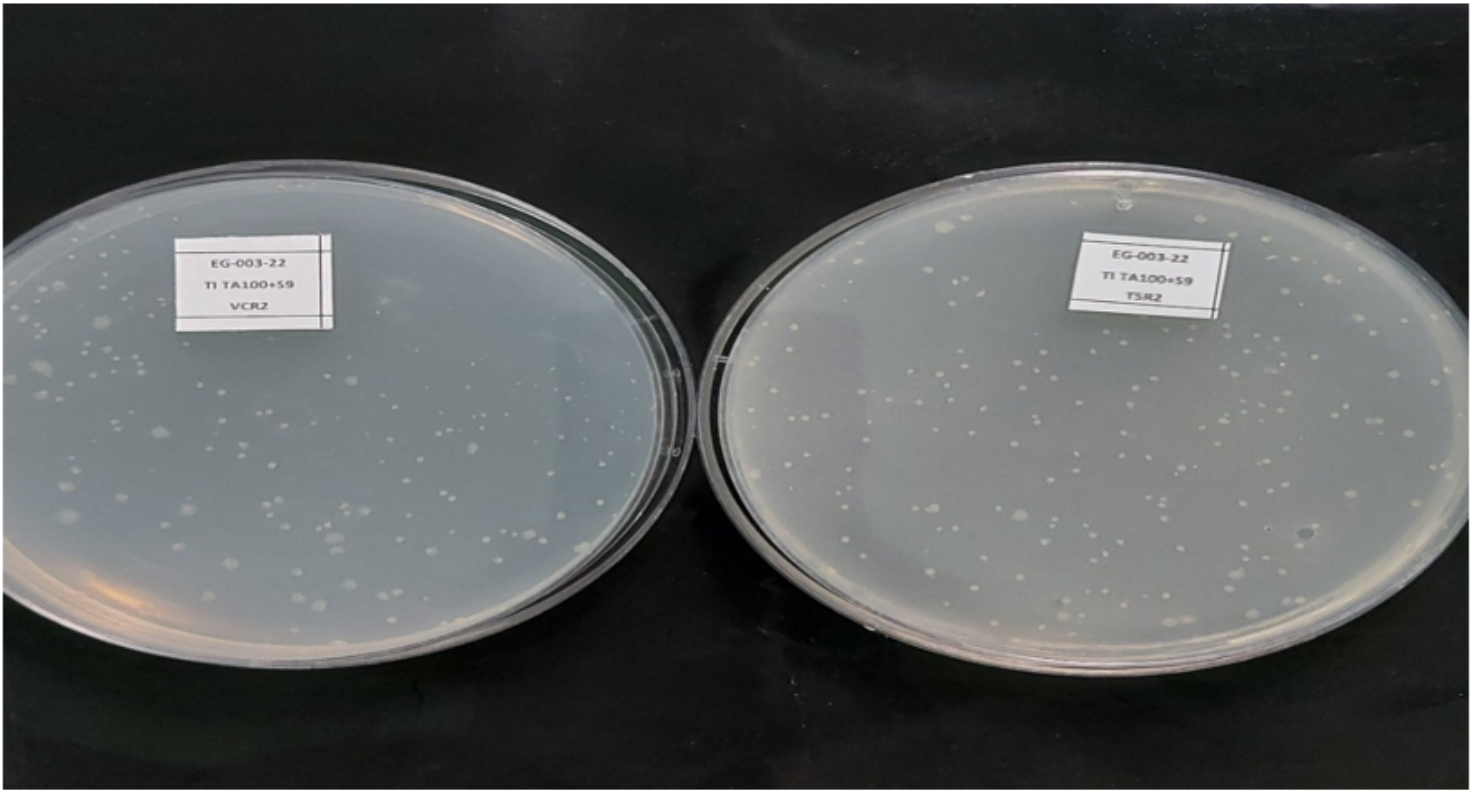


Figure 10

TA100 Strain

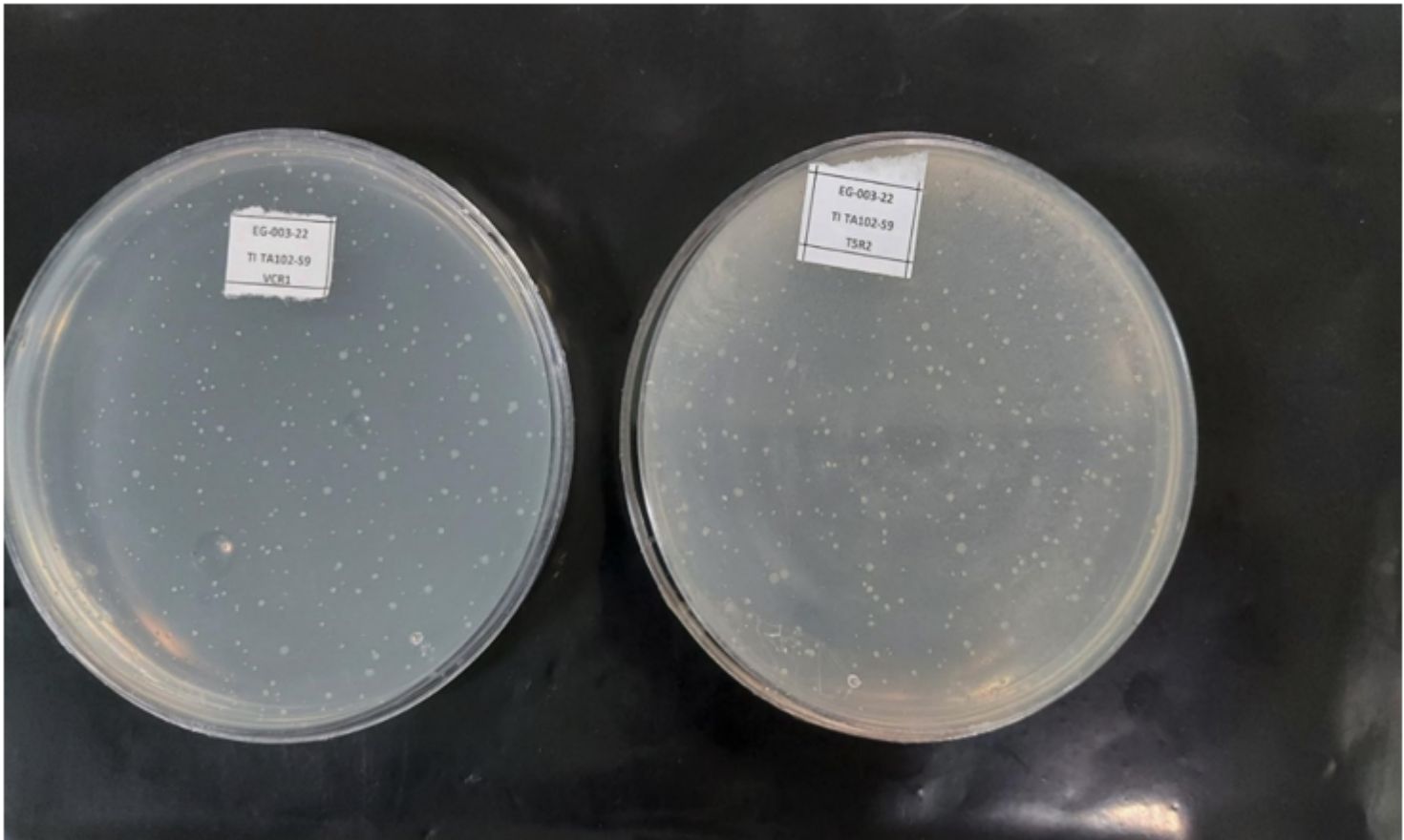


Figure 11

TA102

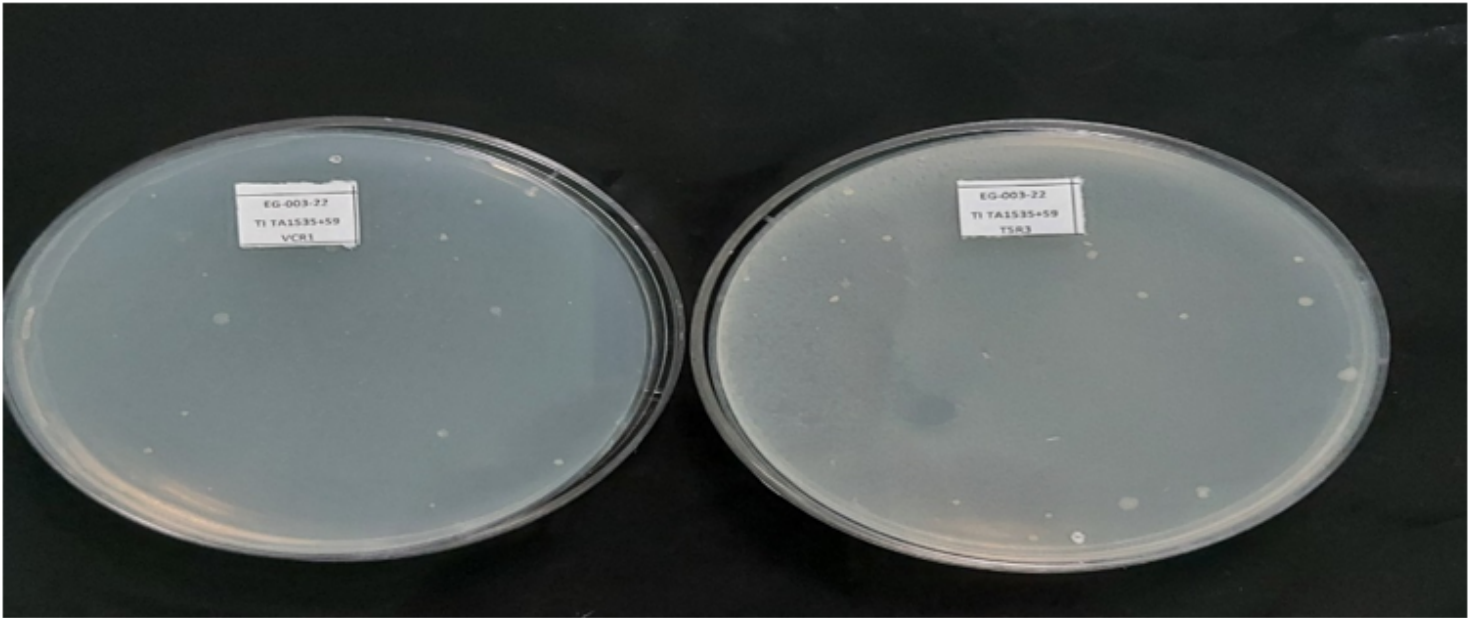


Figure 12

TA1535 Strain

Supplementary Files

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