Research Article

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Characterization of Lantana Camara Roots (Pentacyclic Triterpenoid) and Mutagenicity Testing of Extracted Oleanolic Acid Using Salmonella Typhimurium

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Abstract

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. 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. 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. 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.

Keywords: Oleanolic Acid, Lantana Camara, Pentacyclic Triterpenoid, HPLC, FTIR, DLC, Ames, Bacterial Reverse Mutagenicity.

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 [1]. It has been stated that its roots contain large quantities of the triterpenoid oleanolic acid [2]. Numerous significant biological actions of Oleanolic acid have been reported, including anti-inflammatory , anti-hyperlipidemic antiulcer, antioxidant activity, and hepatoprotective characteristics [3-11]. Recently, its ability to inhibit the growth of tumors has gained attention [12]. 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 [13,14]. The Structure of Oeanolic acid is as follows (Figure 1):

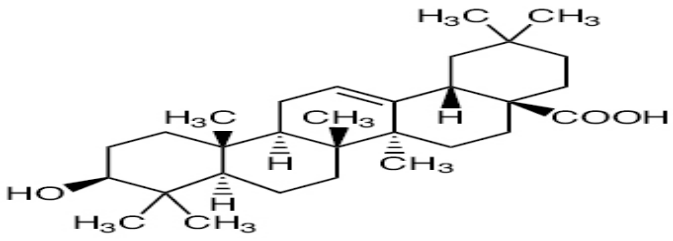

Figure 1: Structure of Oleanolic acid

Material and Methods

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.

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 √10.

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 CHCl3 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.

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

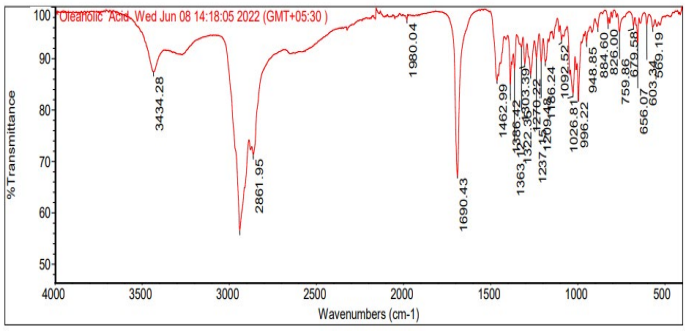

Figure 2: FTIR Chromatography Oleanolic acid

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 (Figure 2).

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 aluminum 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. (Figure 3).

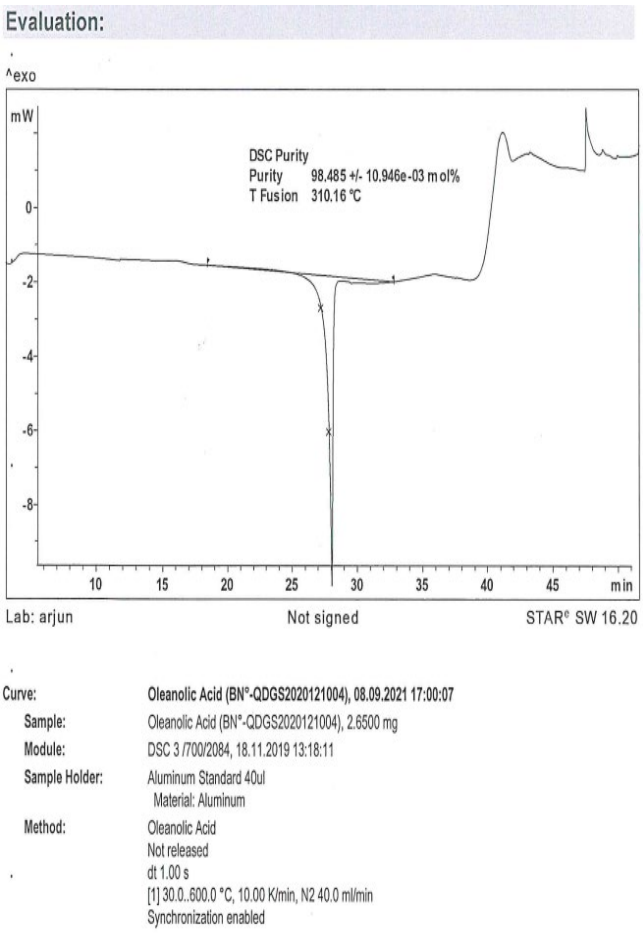

Figure 3: DSC Chromatography Oleanolic acid

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. (Fig.4 & 5.)

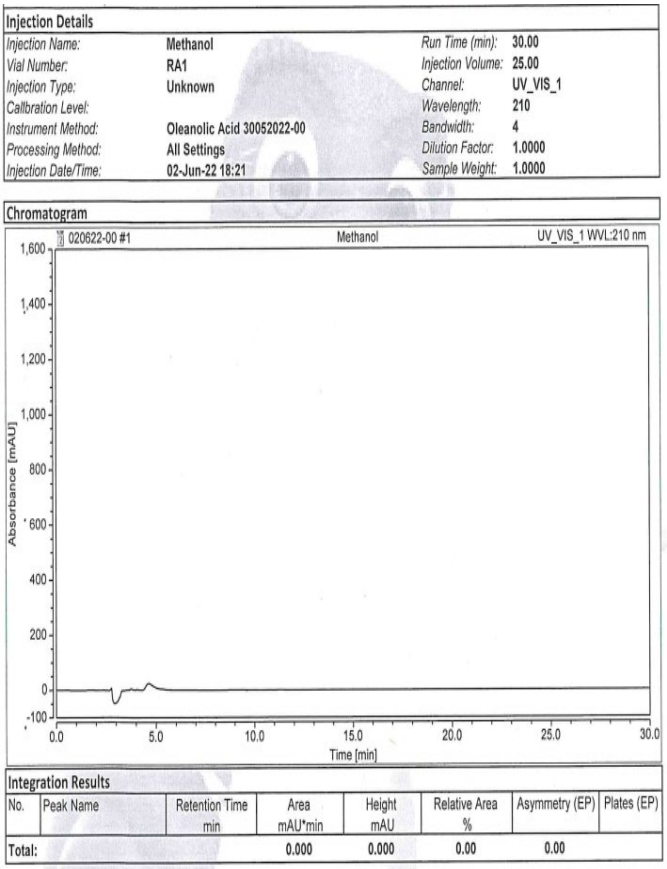

Figure 4: Hplc Chromatography With Methanol

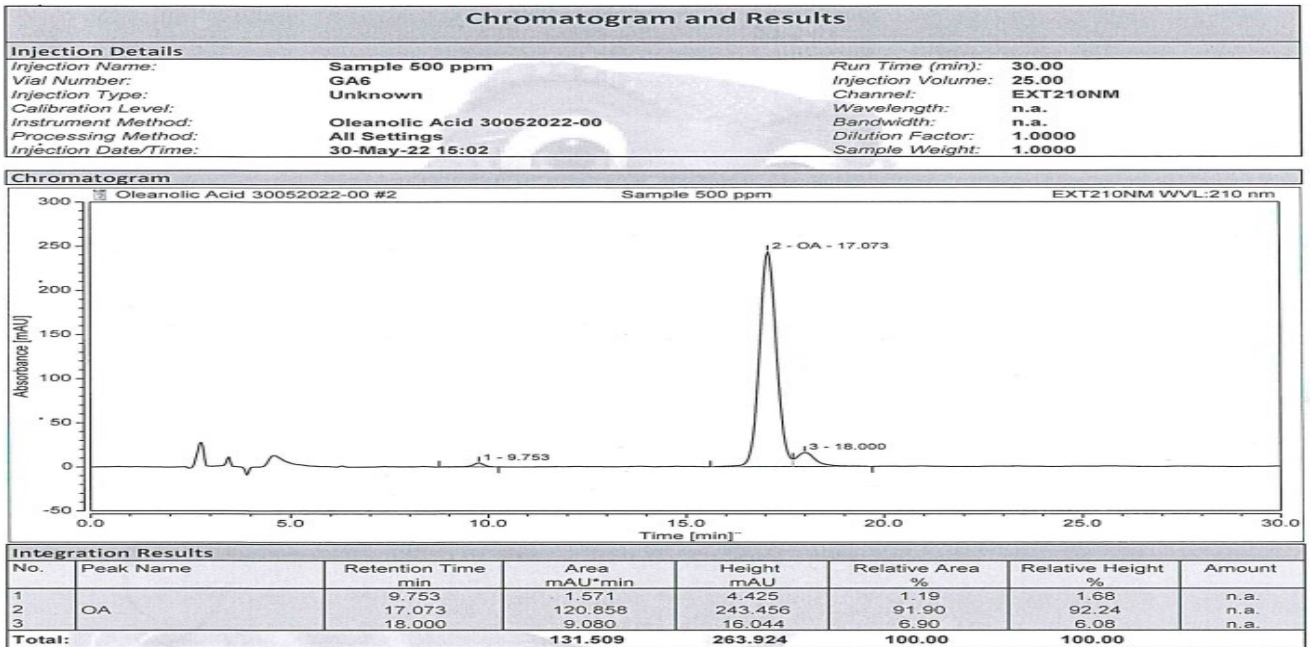

Figure 5: HPLC Chromatography Oleanolic acid (Sample)

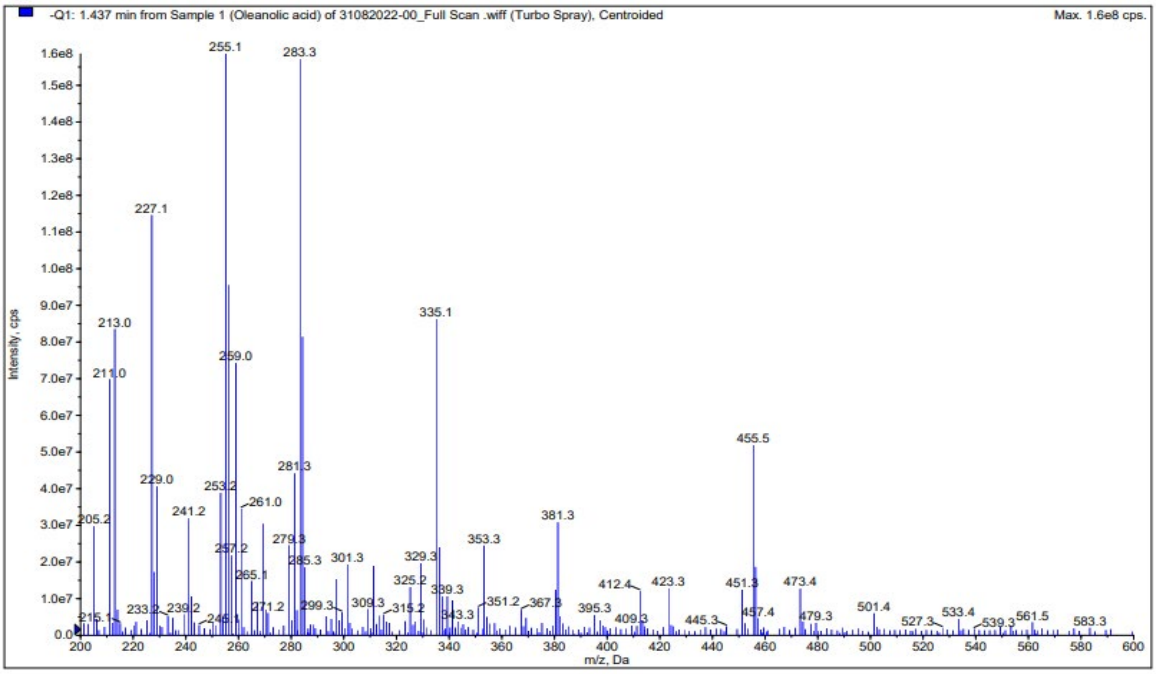

Figure 6: The mass spectrum confirmed the negative ion peak of m/z at 455.5 which is corresponding to molecular weight of oleanolic acid.

Ames Test

Ames test was developed in the 1970s by Dr. Bruce Ames and his associates and was further evaluated by Mortelmans and Zeiger [15-16]. This test is carried out in vitro over a short period of time to assess potential mutagenesis effects of substances.

Metabolic Activation System

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.

S9 Mix

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

Following are the chemicals and S9 homogenate used in the Assay (Table1):

Table1: Chemicals and S9 homogenate Mutagenicity Assay (Plate Incorporation Method):

Chemical		Mutagenicity Study
Cofactor Mix	D-Glucose-6-Phosphate	128.01 mg
	β-NADP	280.02 mg
	Magnesium Chloride	144.01 mg
	Potassium Chloride	216.02 mg
	Sodium Phosphate Buffer	80 mL
Total		80 mL
S9 Mix	Co-factor Mix	54 mL
	S9 Homogenate	6 mL
Total		60 mL
S9 Mix (%)		10

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.

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.

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

Table2: Vehicle Selection Process

S. No.	Vehicle	Quantity of Test Item	Volume of Vehicle Added	Final Concentration	Observation
1.	Milli-Q water	50.0 mg	1 mL	50 mg/mL	Insoluble
2.	DMSO	50.0 mg	1 mL	50 mg/mL	Suspension

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.

Dose Concentration

Mutagenicity Assay was conducted with six groups consisting of Five Oleanolic acid concentrations along with a Vehicle Control in triplicates. (Fig7&8) Concentrations for Mutagenicity Assay (+S9/-S9): 50.02, 158.16, 500.09, 1581.28 and 5000 µg/plate. Concentration Spacing factor: √10.

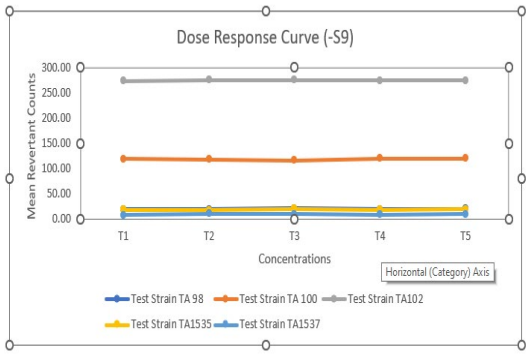

Figure 7: Dose Response Curve Trial-I (-S9)

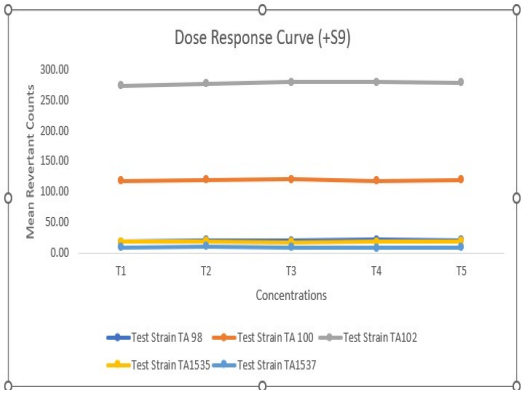

Figure 8: Dose Response Curve Trial-I (+S9)

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.

Preparation of Overnight Cultures

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 ± 1°C and 120 rpm for 16 hours 20 minutes.

Viable Counts

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

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:

Table3: Oleanolic Acid Stock/Dilution

Groups/ Identification	Volume of Stock/ dilution (mL)	Volume of Vehicle (mL)	Final Volume (mL)	mg/mL	µg/plate
T5	Stock	7	10	50	5000
T4	3.16 mL of T5	6.84	10	15.81	1581.28
T3	3.16 mL of T4	6.84	10	5.00	500.09
T2	3.16 mL of T3	6.84	10	1.58	158.16
T1	3.16 mL of T2	6.84	10	0.50	50.02

Concentration Spacing factor: √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).

Plating Procedure

The Test Strain was exposed to the Oleanolic acid via the Plate incorporation method (Table4). The following were the contents of each tube.

Table4: Plate Incorporation

Presence of Metabolic activation (+S9)	Absence of Metabolic activation (-S9)
500 µL S9 mix	500 µL Sodium Phosphate Buffer
100 µL bacterial culture	100 µL bacterial culture
100 µL Test Item solution/vehicle/positive control	100 µL Test Item solution/vehicle/positive control

The tube contents were mixed and poured on to MGA plates. After solidification of top agar, the plates were incubated at 37 ± 1°C for 68 hours. After incubation, MGA plates were taken out from the incubator and were observed for revertant colonies and bacterial background lawn.

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.

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 [14.17,33].

HPLC chromatograms (Fig 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 For instance [18] (Figure 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 an 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 [16,28,30,31,32,35]. Although we have demonstrated the assay method for five strains Table (5,6,7, 8&9) 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 [19,34,36,37,38].

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 [20,21]. oleanolic acid, and their effects on human MCF-7 breast cancer cell line cytotoxicity, cell proliferation, cell cycle, apoptosis, reactive oxygen species (ROS) level, and oxidative DNA damage. According to the findings, oleanolic acid has a strong cytotoxic effect and inhibits proliferation in a dose- and time-dependent way. Cell cycle arrest was caused by oleanolic acid growth suppression at 100 M. Furthermore, while none of the triterpenes tested showed free radical scavenging action in the ABTS and DPPH experiments, they did protect against oxidative DNA damage at a dosage of 10 M. At 10 and 100 M concentrations, oleanolic acid lowered intracellular ROS levels and prevented H2O2-induced oxidative damage. Overall, the findings indicate that the studied triterpenes have the potential to provide strong natural defense against human breast cancer [22]. Oleanolic acid (OA) is an [23] oleanane type pentacyclic triterpene found in almost 200 plant species, including Swertia, Ligustrum lucidum, grape, and elderberry Numerous researches have demonstrated that oleanolic acid has pharmacological actions such as liver protection, antioxidation, hypolipidemia, anticancer, anti-inflammatory, and antiviral properties. Because of its hepatoprotective properties, oleanolic acid is used as an over-the-counter oral medication in China to treat liver problems in humans. Not only does oleanolic acid protect against acute chemical liver injury, but it also protects against liver fibrosis and cirrhosis caused by chronic liver disorders Its mechanism could be linked to alterations in gene expression in the Nrf2-, MT-related genes, and transcription factor farne-soid x receptor (FXR). Oleanolic acid can stimulate Nrf2-dependent gene induction via boosting Nrf2 nuclear accumulation, which protects the liver against acetaminophen-induced hepatotoxicity [25,26,27,29].

Table5: Mutagenicity Assay - Revertant Colony Counts of TA98

Revertant Colony Counts of TA98																		
Groups and Test Concentration (µg/plate)		Absence of Metabolic Activation (-S9)								Groups and Test Concentration (µg/plate)		Presence of Metabolic Activation (+S9)							
		R1	R2	R3	Mean	SD	FI	BBL	ppt			R1	R2	R3	Mean	SD	FI	BBL	ppt
VC	0	19	22	23	21.33	2.08	-	2	Nil	VC	0	22	19	26	22.33	3.51	-	2	Nil
T1	50.02	21	24	18	21.00	3.00	0.98	2	Nil	T1	50.02	20	18	19	19.00	1.00	0.85	2	Nil
T2	158.16	22	19	20	20.33	1.53	0.95	2	Nil	T2	158.16	23	20	21	21.33	1.53	0.96	2	Nil
T3	500.09	23	19	21	21.00	2.00	0.98	2	Nil	T3	500.09	22	21	19	20.67	1.53	0.93	2	Nil
T4	1581.28	22	18	22	20.67	2.31	0.97	2	Nil	T4	1581.28	24	20	23	22.33	2.08	1.00	2	Nil
T5	5000	24	19	20	21.00	2.65	0.98	2	Slight ppt	T5	5000	19	23	22	21.33	2.08	0.96	2	Slight ppt

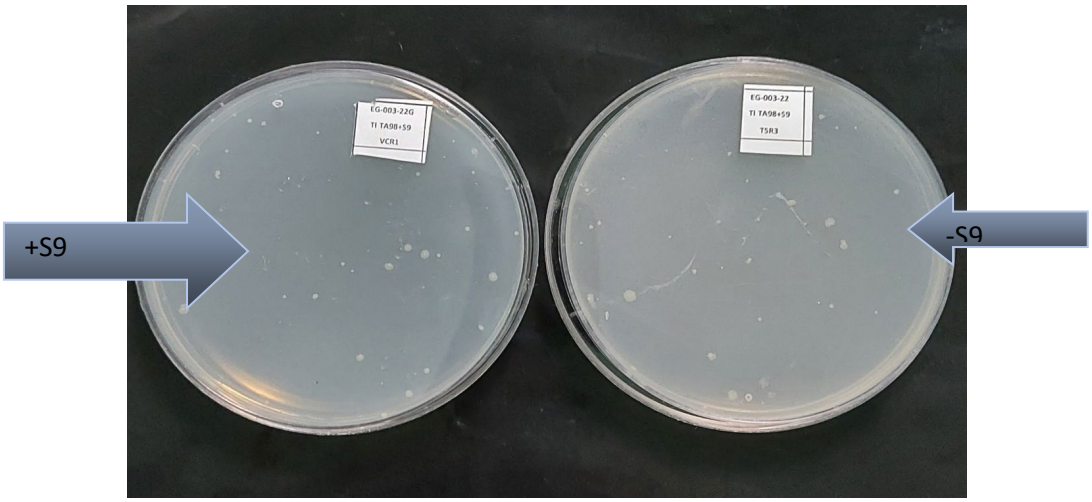

Figure 9: **TA98 Strain**

Table6: Mutagenicity Assay - Revertant Colony Counts of TA100

Revertant Colony Counts of TA100																		
Groups and Test Concentration (µg/plate)		Absence of Metabolic Activation (-S9)								Groups and Test Concentration (µg/plate)		Presence of Metabolic Activation (+S9)							
		R1	R2	R3	Mean	SD	FI	BBL	ppt			R1	R2	R3	Mean	SD	FI	BBL	ppt
VC	0	120	121	115	118.67	3.21	-	2	Nil	VC	0	124	119	120	121.00	2.65	-	2	Nil
T1	50.02	122	119	121	120.67	1.53	1.02	2	Nil	T1	50.02	121	118	122	120.33	2.08	0.99	2	Nil
T2	158.16	116	120	119	118.33	2.08	1.00	2	Nil	T2	158.16	123	120	117	120.00	3.00	0.99	2	Nil
T3	500.09	117	121	122	120.00	2.65	1.01	2	Nil	T3	500.09	121	119	121	120.33	1.15	0.99	2	Nil
T4	1581.28	115	121	123	119.67	4.16	1.01	2	Nil	T4	1581.28	123	116	118	119.00	3.61	0.98	2	Nil
T5	5000	116	122	119	119.00	3.00	1.00	2	Slight ppt	T5	5000	122	120	121	121.00	1.00	1.00	2	Slight ppt

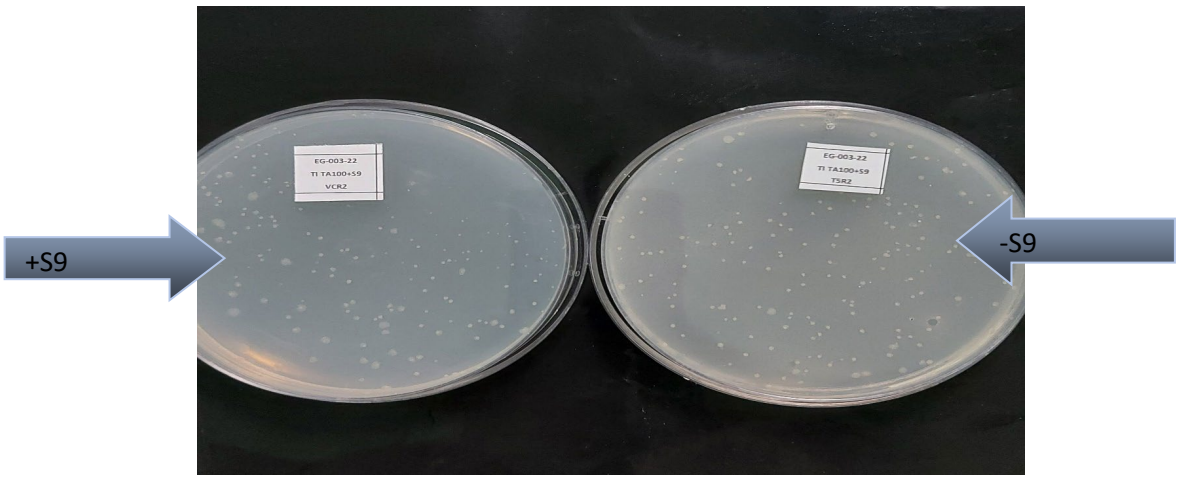

Figure 10: TA100 Strain

Table 7: Mutagenicity Assay - Revertant Colony Counts of TA102

Revertant Colony Counts of TA102																			
Groups and Test Concentration (µg/plate)		Absence of Metabolic Activation (-S9)								Groups and Test Concentration (µg/plate)		Presence of Metabolic Activation (+S9)							
		R1	R2	R3	Mean	SD	FI	BBL	ppt			R1	R2	R3	Mean	SD	FI	BBL	ppt
VC	0	269	272	281	274.00	6.24	-	2	Nil	VC	0	275	279	282	278.67	3.51	-	2	Nil
T1	50.02	273	276	279	276.00	3.00	1.01	2	Nil	T1	50.02	273	274	278	275.00	2.65	0.99	2	Nil
T2	158.16	281	282	270	277.67	6.66	1.01	2	Nil	T2	158.16	270	281	282	277.67	6.66	1.00	2	Nil
T3	500.09	271	272	278	273.67	3.79	1.00	2	Nil	T3	500.09	272	280	274	275.33	4.16	0.99	2	Nil
T4	1581.28	273	280	274	275.67	3.79	1.01	2	Nil	T4	1581.28	281	274	271	275.33	5.13	0.99	2	Nil
T5	5000	270	276	275	273.67	3.21	1.00	2	Slight ppt	T5	5000	276	273	281	276.67	4.04	0.99	2	Slight ppt

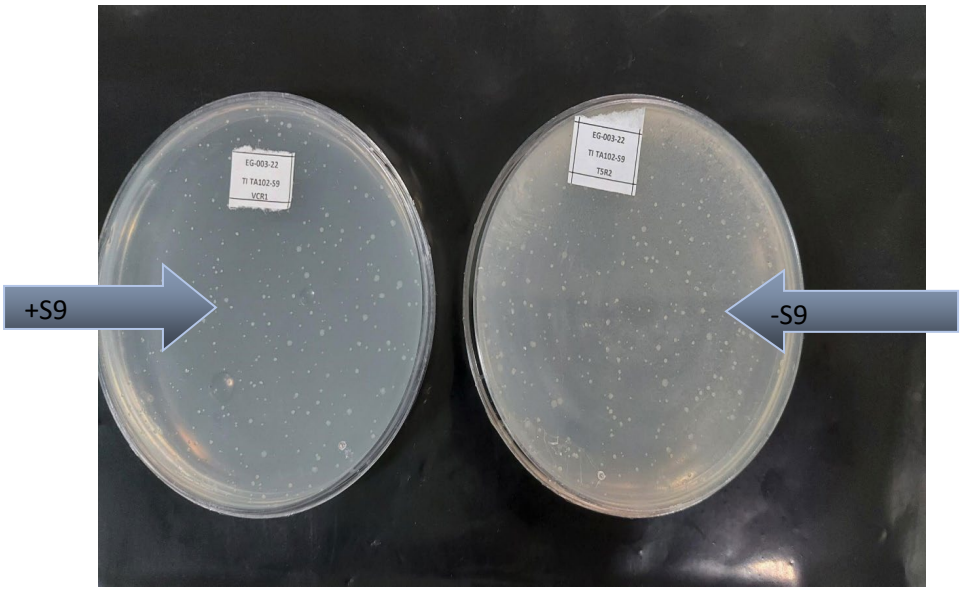

Figure 11: TA102

Table 8: Mutagenicity Assay - Revertant Colony Counts of TA1535

Revertant Colony Counts of TA1535																			
Groups and Test Concentration (µg/plate)		Absence of Metabolic Activation (-S9)								Groups and Test Concentration (µg/plate)		Presence of Metabolic Activation (+S9)							
		R1	R2	R3	Mean	SD	FI	BBL	ppt			R1	R2	R3	Mean	SD	FI	BBL	ppt
VC	0	21	17	19	19.00	2.00	-	2	Nil	VC	0	22	16	20	19.33	3.06	-	2	Nil
T1	50.02	20	16	21	19.00	2.65	1.00	2	Nil	T1	50.02	17	21	19	19.00	2.00	0.98	2	Nil
T2	158.16	15	18	20	17.67	2.52	0.93	2	Nil	T2	158.16	20	21	18	19.67	1.53	1.02	2	Nil
T3	500.09	22	19	20	20.33	1.53	1.07	2	Nil	T3	500.09	16	18	20	18.00	2.00	0.93	2	Nil
T4	1581.28	17	20	18	18.33	1.53	0.96	2	Nil	T4	1581.28	21	19	17	19.00	2.00	0.98	2	Nil
T5	5000	19	17	22	19.33	2.52	1.02	2	Slight ppt	T5	5000	21	17	20	19.33	2.08	1.00	2	Slight ppt

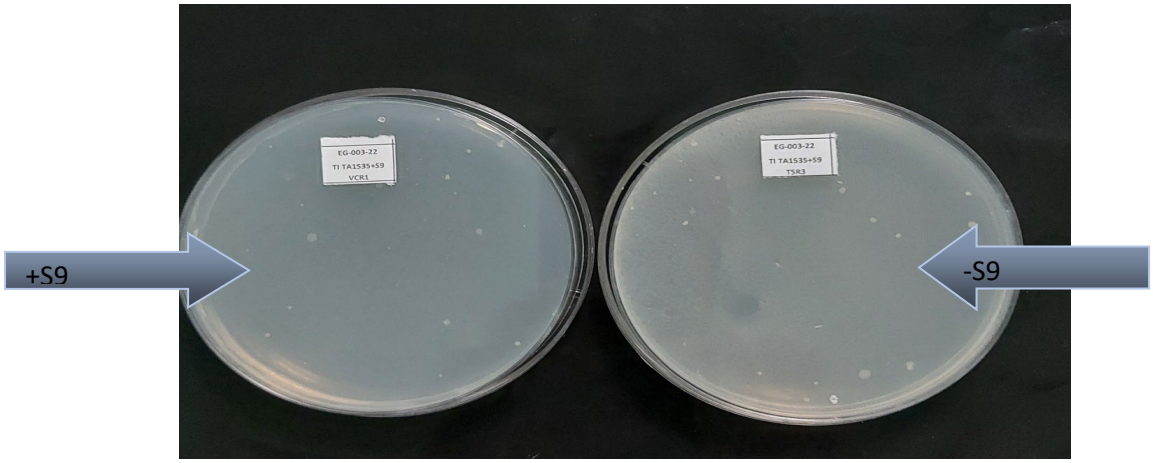

Figure 12 : TA1535 Strain

Table 9: Mutagenicity assay - Revertant Colony Counts of TA1537

Revertant Colony Counts of TA1537																		
Groups and Test Concentration (µg/plate)		Absence of Metabolic Activation (-S9)								Groups and Test Concentration (µg/plate)		Presence of Metabolic Activation (+S9)							
		R1	R2	R3	Mean	SD	FI	BBL	ppt			R1	R2	R3	Mean	SD	FI	BBL	ppt
VC	0	9	11	12	10.67	1.53	-	2	Nil	VC	0	11	8	12	10.33	2.08	-	2	Nil
T1	50.02	10	11	8	9.67	1.53	0.91	2	Nil	T1	50.02	9	10	9	9.33	0.58	0.90	2	Nil
T2	158.16	8	10	9	9.00	1.00	0.84	2	Nil	T2	158.16	11	12	8	10.33	2.08	1.00	2	Nil
T3	500.09	11	9	10	10.00	1.00	0.94	2	Nil	T3	500.09	12	9	8	9.67	2.08	0.94	2	Nil
T4	1581.28	12	8	8	9.33	2.31	0.88	2	Nil	T4	1581.28	11	8	10	9.67	1.53	0.94	2	Nil
T5	5000	9	11	10	10.00	1.00	0.94	2	Slight ppt	T5	5000	7	11	12	10.00	2.65	0.97	2	Slight ppt

Table 10: Appendix – Sterility Check

Constituents checked for Sterility	Microbial Growth Observed Mutagenicity Study
MGA plates	NG
Test Item (Lowest concentration)	NG
Test Item (Highest concentration)	NG
S9 Mix	NG
Solvent / Vehicle	NG
Sodium Phosphate Buffer	NG
Top agar	NG

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.

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Author Contribution

All the authors contributed to the study conception and design. Material preparation, analysis, data collection, research and first draft were drafted by NG, final draft Reviewed by SC, ATS and MJ, all authors read and approved the final manuscript.

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Availability of Data and Materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest

The author has no competing interest to declare that are relevant to the content of this article.

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