

***In vitro* chromosomal aberration test of oleanolic acid extracted from the roots of *Lantana camara* in V79 Chinese Hamster lung cell line**

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ABSTRACT

This study was performed in the V79 Chinese hamster lung cell line to check the potential of oleanolic acid for chromosomal aberration effects. Oleanolic acid has medicinal properties, and it is used in the treatment of various diseases. Oleanolic acid was extracted from the roots of *Lantana camara* by the extraction and isolation process using HPLC, DSC, and FTIR techniques. The V79 Chinese hamster cell line is frequently used for *in vitro* chromosomal aberration tests. At the high dose of oleanolic acid, a decrease in the cell viability (cytotoxicity) was observed. At the dose level of 5 mg/mL cytotoxicity (less than 70% cell viability) was observed, whereas more than 90% cell viability observed at maximum dose level of 3.0%, hence non cytotoxic dose 3.0, 1.5, 0.75 and 0.375 mg/mL was selected for the chromosomal aberration test. Short-term and long-term exposure was performed with and without S9 metabolic activation. There were no clastogenic effects observed in the treated V79 Chinese hamster cell line with oleanolic acid at dose levels of 3.0, 1.5, 0.75, and 0.375 mg/mL; hence, it can be concluded that up to the maximum dose level of 3.0 mg/mL of oleanolic acid has no clastogenic effects in the V79 Chinese hamster lung cell line.

Keywords: HPLC, FTIR, S9, cytotoxicity, Oleanolic acid, *Lantana camara*, Chromosomal aberration, V79 cells, Clastogenicity

INTRODUCTION

Lantana camara Linn, derived from the Latin word 'lento' meaning "to bend", is a flowering ornamental plant belonging to family Verbenaceae. They are distributed worldwide which are native to America and found in Mexico, Trinidad, Jamaica, Brazil, Florida, Africa, India, and Nepal (Mane *et al.* 2019), *Lantana camara* Linn commonly called wild or red sage, belongs to the family Verbenaceae. It is an evergreen notorious wild shrub that grows well in tropical and temperate regions (Oladoye *et al.* 2021). It is a woody-smelling plant growing up to 1 – 3 meters; the flowers are often in clusters and diverse colors ranging from red, violet, white, yellow, and pink, often cultivated as an ornament plant but have now become an invasive weed (Ghisalberti, 2000). Folk healers in Asia and South America



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Figure 1: *Lantana camara* Plant

have used *Lantana* species, including *L. camara*, for centuries to treat various human ailments such as dermatological and gastrointestinal diseases, tetanus, malaria and tumors. From ancient times, *L. camara* has been used in the treatment of various ailments such as itches, cuts, ulcers, swellings, bilious fever, catarrh, eczema, tetanus, malaria, tumors, rheumatism, cold, headache, whooping cough, asthma, chicken pox, bronchitis, eye injuries, and arterial hypertension (Pour *et al*, 2011).

Oleanolic acid is a triterpenoid which exists in nature as a free acid or as an aglycone of triterpenoid saponins and it is often ubiquitously found with its isomer, ursolic acid (Janicsák *et al*, 2006)

It has also been served as a framework for additional modifications to achieve semi-synthetic OA derivatives for increased potency, reduced toxicity, increased bioavailability and solubility (Yu *et al*, 2016). Oleanolic acid has been used as a hepatic drug for over 20 years in China because of its hepatoprotective effect. OA is the protection of the liver against toxicity and is currently being used as an over-the-counter hepatic drug in China (Zhao *et al*, 2013). Oleanolic acid widely used on the treatment of hepatotoxicity, diabetes, antimicrobial, cancer, anti-inflammatory, antioxidant and many other treatment, there are very few data available on genotoxic or chromosomal structural effect of oleanolic acid. previous studies on oleanolic acid focused on hepatoprotection, anti-cancer, anti-inflammatory effects, but genotoxicity data are limited. Therefore, the aim of this study was to access in-vitro chromosomal aberration effects oleanolic acid on the V79 Chinese hamster lungs cell line.

MATERIALS AND METHODS

The *Lantana camara* roots were gathered from a hilly region in the Raisen district, which is located in Bhopal, Madhya Pradesh, India, at roughly 23°20' N latitude and 77°48' E longitude.

Prior to accepting the plant for herbarium, plant was verified by department of Botany, Government PG College, Morena. The roots were sliced into little pieces after being gathered in the morning. After being cleaned, the roots were air-dried for several days. After drying roots were crushed and formed in the granular powder for the extraction process. Approx 500 g of crude drug was taken, Petroleum ether was used to extract the powdered form of *L. camara* roots overnight at room temperature. To stop the active ingredients from being thermally degraded, the crude extract was filtered and concentrated under low pressure using a rotary evaporator at temperatures not to exceed 45 °C. After removing the solvent under vacuum, the crude extract was diluted in CHCl₃ and allowed to precipitate overnight. Methanol was used to crystallize the resulting precipitate. Oleanolic acid crystals were produced by repeating the precipitation and crystallization process four times (Srivastava *et al*. 2014; Vyas and Argal, 2014).

FTIR was used to identify the oleanolic acid pattern. Thermo FTIR with ATR was used to record the infrared spectra of isolated oleanolic acid in the 4000–400 cm⁻¹ wavelength range were recorded by using Thermo FTIR with ATR, in infrared zone of wavelength of 4000 to 400 cm⁻¹. The DSC tests were performed using a Mettler Toledo STAR Thermal Analysis System, and the

oleanolic acid pattern and DSC 3 were evaluated for identification. A 40-L aluminium pan with a nitrogen flow rate of 40 ml/min was filled with the sample.

Thermo HPLC system Ultimate 3000 used to analyse the oleanolic acid (OA) that was isolated from *Lantana camara* roots. Chromatographic separation was carried out in isocratic mode on an Intersil ODS column (250 mm x 4.6 mm, 5 µm particle size) using mobile phases A (methanol) and B (0.03 M sodium phosphate in milli-Q water pH at 3) (88:12 v/v).

The mobile phase was supplied to the column at a rate of 1.0 ml/min, and the elution was monitored using a photodiode array detector tuned at 210 nm. 30°C was maintained as the column's temperature. The isolated sample of oleanolic acid from *Lantana camara* roots contains 91.9% purity with very few impurities by HPLC method, moreover when DSC (Differential Scanning Calorimetry) profile was performed the purity check of oleanolic acid was 98.48% remaining 1.52% was unknown impurity.

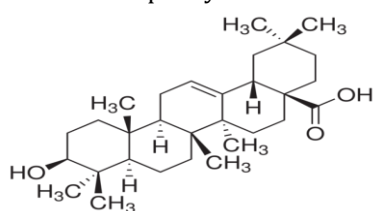


Figure 2: Oleanolic Acid

Test System

Chinese Hamster Lung Fibroblast (V79) cell line purchased from NCCS Pune, India. This cells line was because of their relatively small number of chromosomes (diploid number, $2n = 22$), their high proliferation rate. Morphology of cell line is fibroblast, derived from lungs of Chinese hamster, High proliferation rate (doubling time of about 16 - 18 hours), mycoplasma negative. This strain is also recommended by the OECD 473 guideline for the chromosomal aberration test.

Cell Culture Preparation

For the Chromosomal aberration assay, V79 cells were seeded at approximately 1×10^6 cells/25 cm² flask in 5 mL complete media (DMEM medium supplemented with 10 % FBS and 1 % antibiotic antimycotic) and were incubated at 37°C in a humidified atmosphere of 5 % CO₂ in air for 18-24 hours. The Chinese hamster cell line is widely used for chromosomal aberration testing;

these cells are sensitive to induction of chromosomal aberration by chemical substances.

Dose Preparation:

Test Medium and Solutions

- 1 Dulbecco modified eagle medium (DMEM)
- 2 S9 Mix
- 3 Sodium phosphate buffer; SPB (pH 7.4)
- 4 Giemsa Stain (5 % v/v)
- 5 Fetal bovine serum (FBS)
- 6 Potassium Chloride (0.075 M)
- 7 Cornoy's Fixative (Methanol: Glacial acetic acid; 3:1)
- 8 Colchicine

Preparation of Co-factor component:

D-Glucose-6-phosphate	:0.81 g
β-Nicotinamide adenine dinucleotide Phosphate (β-NADP)	:1.71 g
Magnesium chloride	:0.90 g
Potassium chloride	:1.32 g
Sodium phosphate, dibasic	:6.40 g
Sodium phosphate, monobasic	:1.42 g
Milli Q water	:450 mL

Mitotic Spindle Inhibition

Colchicine solution of 0.2 µg/mL was added to each of the culture tube for short and long term exposure, 2 hours prior to harvesting and incubation at $37 \pm 1^\circ\text{C}$. At the end of incubation, the medium was removed by centrifugation followed by harvesting and processing.

Test Item Preparation: The test item was insoluble in water, so 0.5% DMSO was used as a vehicle. At 5 mg/mL, cytotoxicity was observed; therefore, doses of 3.0, 1.5, 0.75, and 0.375 mg/mL were selected for the chromosomal aberration test.

The stock solution was prepared by mixing 0.5% DMSO and 10% fetal bovine serum in milli Q water to reach the final concentration of 5mg/ml. Formulation was vortex and sonicated to prepared proper solution. As per OECD 473 guidance, different final concentrations of this test were 3.0 mg/mL, 1.5 mg/mL, 0.75 mg/mL and 0.375 mg/mL selected for the study.

Positive control: Cyclophosphamide used as positive control; formulation was prepared with presence of S9 metabolic activation system of concentration 25µg/ml. Mitomycin-C was used as positive control for short and long term exposure with and without S9 metabolic activation at the concentration 0.425µg/ml. Autoclaved

glassware used during the study performance and aseptic conditions were maintained during the handling of cells.

S9 Homogenate

Lyophilized S9 homogenate (liver microsomal enzyme) prepared from male SD rat (Aroclor 1254 induced) was purchased from Krishgen Biosystem, Delhi. Study was carried out in presence and absence of metabolic activation S9 mix (1% v/v). For 1% S9 preparation following composition was used as recommended by OECD 473 guideline (Table 1).

There are two methods for the exposure of cells with test item i.e short term and long-term exposure.

Treatment for Short Duration:

This phase was conducted both in the presence and absence of metabolic activation system. The cells were exposed to the test item with oleanolic for 4 hours at 37°C in a humidified atmosphere of 5 % CO₂. After the exposure period, the treatment medium was removed, the cells washed and replaced with equal volume of fresh complete medium and returned to the incubator for additional 17 hours (Table 2 & 3).

Table 1: Preparation of S9 Homogenate

Volume (mL)				Final Volume (mL)	Final Concentration of S9 (%)
Co-factor mix	S9 fraction	S9 mix	Medium+ Culture		
9.0	1.0	10.0	90.0	100.0	1% (10 mg/ml)

Table 2: Treatment for Short Duration

Positive Control	Concentration	Metabolic Activation	Phase
Treatment Groups	0.375, 0.75, 1.5, 3.0 mg/mL	-	Short term
Positive Control	Concentration	Metabolic Activation	Phase
Mitomycin-C	0.4 µg/mL	Absence of S9 mix	Short term
Cyclophosphamide	25 µg/mL	Presence of S9 mix	Short term

Table 3: Treatment for Long Duration

	Concentration	Metabolic Activation	Phase
Treatment Groups	0.375, 0.75, 1.5, 3.0 mg/mL	-	Long term
Mitomycin-C (Positive Control)	0.4 µg/mL	Absence of S9 mix	Long term

Approx. two hours prior to harvesting of culture colchicine solution of 0.2 µg/mL was added to each of the culture tube in both short term and long-term exposure tubes and incubated at 37 ± 1°C. At the end of incubation, the medium was removed by centrifugation followed by harvesting and processing. After the end of cell culture, the cells were dissociated with 1ml of trypsin solution to make a cell suspension. After cell disaggregation, cells were neutralized with complete DMEM media. All the cells were transferred in marked tubes and centrifuged at 800-1000 RPM. The supernatant was removed after centrifugation and freshly prepared, hypotonic solution (37°C) was added

in cell pellet. The culture tubes were incubated for 30 minutes followed by centrifugation, removal of supernatant and fixation in chilled Carnoy's fixative by washing two times. After fixation the slides were prepared. Some portion of the cell suspension was used for cell counting for the cytotoxicity measurement, and the remaining cell suspension was used for chromosome preparation.

Cytotoxicity was assessed by counting Trypan Blue-stained cells; viable cells exclude the dye, whereas dead cells stain blue due to dye uptake. The relative increase cell count (RICC) was obtained by dividing the

number of viable cells after 48 h (45 hrs exposure to oleanolic acid and three hrs in fresh culture medium) by that of the negative control.

$$RICC (\%) = \frac{\text{Increase in number of cells in treated culture (Final-starting)}}{\text{Increase in number of cells in control culture (Final-starting)}}$$

RICC: Relative increase in cell count

Slide Preparation:

Two replicates of slides were prepared from each tube by dropping 3-4 drops of cell suspension on clean chilled slides and dried. The slides were stained with 5% fresh Giemsa stain solution and then rinsed with tap water followed by mounting with DPX and scoring under microscope. The slides were coded and 300 well spread metaphase cells were counted in different fields of slide per culture. After completion of scoring, the slides were decoded for the analysis of results. A 300 well spread metaphase cells per concentration (150 metaphase in each Replicate) were scored for cytogenetic damage on coded slides. Both Chromatid type and Chromosome type aberrations were scored. Gap were also recorded, but they were not included in the calculation of the aberration frequency. The total number of cells with structural aberrations and the number of aberrant cells were recorded along with vehicle and positive controls.

Study performed as per OECD 473 guideline.

Statistical Analysis:

The data of the oleanolic acid treated groups and negative controls was compared using Fisher's exact

test, and Bonferroni correction was performed for multiple testing of the dose-groups. The P values < 0.05 were considered to be statistically significant for the treatment group as compared with control group.

RESULT

A minimum number of 300 well-spread metaphase cells (two replicated 150 cells per replicate) were counted under a 100x objective using a microscope in different fields of the slide per concentration to determine the different types of chromosomal aberrations. Both chromatid-type and chromosome-type aberrations were scored. Cytotoxicity effect was observed at the dose level of 5 mg/ml, with dose levels of 3.0, 1.5, 0.75, and 0.375 selected for the chromosomal aberration test. No statistically significant differences were observed in the cytotoxicity of all treatment groups as compared to the control group, whereas a statistically significant decrease in the survivability of the cells in the positive control was observed as compared to the control group.

In the short-duration exposure with S9, no statistically significant changes were observed in the structure of the chromatid and chromosome in the treatment group as compared to the control group, whereas statistically significant changes were observed in the structural changes in the chromatid and chromosome of the positive control group as compared to the control group (Table 4).

Table 4: Short Duration Exposure with S9

Concentration mg/ml	Replicates	Chromatid Break	Chromatid Gap	Chromosome Break	Chromosome Gap	Other	Total	Mean
						-	-	-
Control	R1	0	0	1	0	0	1	1
	R2	0	0	1	0	0	1	
Positive Control	R1	2	1	7	0	0	10	10
	R2	3	0	6	1	0	10	
0.375	R1	0	1	0	0	0	1	1
	R2	0	0	0	1	0	1	
0.75	R1	0	0	1	1	0	2	1
	R2	0	0	1	0	0	1	
1.50	R1	0	0	0	1	0	1	1
	R2	0	0	1	0	0	1	
3.0	R1	0	0	1	0	0	1	1
	R2	0	0	0	1	0	1	

Table 5: Short Duration without S9 factor

Concentration mg/ml	Replicates	Chromatid Break	Chromatid Gap	Chromosomal Break	Chromosomal Gap	Other	Total	Mean
Control	R1	0	0	1	0	0	1	1
	R2	0	0	1	0	0	1	
Positive Control	R1	1	0	8	0	0	9	8.5
	R2	1	0	7	0	0	8	
0.375	R1	0	0	1	0	0	1	1
	R2	0	1	0	0	0	1	
0.75	R1	0	0	1	0	0	1	1
	R2	0	0	1	0	0	1	
1.50	R1	0	0	0	1	0	1	1.5
	R2	1	0	1	0	0	2	
3.0	R1	0	0	2	0	0	2	2
	R2	0	1	0	1	0	2	

Table 6: Long term exposure without S9

Concentration mg/ml	Replicates	Chromatid Break	Chromatid Gap	Chromosomal Break	Chromosomal Gap	Other	Total	Mean
Control	R1	0	1	0	0	0	1	1
	R2	0	0	1	0	0	1	
Positive Control	R1	1	0	6	0	0	7	8
	R2	0	1	8	0	0	9	
0.375	R1	0	0	1	0	0	1	1
	R2	0	1	0	0	0	1	
0.75	R1	0	0	1	0	0	1	1
	R2	0	0	0	1	0	1	
1.50	R1	1	0	1	0	0	2	1.5
	R2	0	0	0	1	0	1	
3.0	R1	1	0	1	0	0	2	2.5
	R2	0	1	1	0	1	3	

In the short duration exposure without S9, no statistically significant changes were observed in the structure of chromatid and chromosome in the treatment group as compared to control group, whereas statically significant changes were observed in the structural changes in the chromatid and chromosome of positive control group as compared to control group (Table 5).

In the long-duration exposure without S9, no statistically significant changes were observed in the structure of the chromatid and chromosome in the treatment group as compared to the control group, whereas statistically significant changes were observed in the structural changes in the chromatids

and chromosomes of the positive control group as compared to the control group (Table 6).

DISCUSSION

At the dose level of 5.0 mg/ml cell viability was less than 70% was observed and at the dose level of 3.0 mg/ml cell viability was more than 90% was observed hence 3.0 mg/ml as a maximum dose for the chromosomal aberration test was selected. In this in-vitro chromosomal aberration study, four different concentrations (0.375, 0.75, 1.50 and 3.0 mg/ml) were used along with positive control (cyclophosphamide) and mitomycin group. As result found there was no test item related any abnormality was observed in the

chromatid and chromosomal structure like breakage or gap, however there was an increase in the aberrant effects in chromatid and chromosomal structure observed in the V79 cells treated with Cyclophosphamide in the presence of metabolic activation and Mitomycin-C in absence of S9 metabolic activation on short term exposure and Mitomycin-C in absence of metabolic activation on long term exposure demonstrated the sensitivity of the test system. Cyclophosphamide is well known for the chromosomal aberration. At the different dose level of oleanolic acid has showed minimal or negligible clastogenic effect or alteration in the structure of chromatid or chromosome structure. There were no significant cytotoxic effects observed in the cell viability due to exposure of oleanolic acid in the normal cells at the any dose level, however significant decrease was observed in the tumorigenic cells, Oleanolic Acid did not induce any changes in the MeWo cell viability regardless of the tested concentrations (Barbara Bednarczyk-Cwynar *et al.* 2023). Similar type of results, the chromosomal aberration rates in the mice administered with high, medium and low doses of the ursolic acid extracts were 1.2%, 1.2% and 1.0%, respectively, and these results were not significantly different from those in normal mice in the study of Acute and Genetic Toxicity of Ursolic Acid Extract from *Ledum palustre* L (Jing, 2009; Silva *et al.*, 2019), lack of clastogenicity is consistent with the traditional use of oleanolic acid as a hepatoprotective agent. Oleanolic Acid is effective in protecting against various hepatotoxicants, and one of the protective mechanisms is reprogramming the liver to activate the nuclear factor erythroid 2-related factor 2 (Nrf2) (Liu *et al.*, 2018). A series of new 11-oxoleanolic acid derivatives were synthesized to enhance the anti-inflammatory activity (Jin *et al.* 2021). As the result obtained upto the dose level of 3.0 mg/mL no clastogenic effects was observed, hence it may be correlated with human exposure up to the dose level of 3.0 mg/mL it may not show any clastogenic effect, but more study needs to perform for the confirmation for human exposure.

CONCLUSION

The oleanolic acid extracted from root of lantana camara plant by Differential scanning calorimetry (DSC), High Performance Liquid chromatography (HPLC), and Fourier transform infrared (FTIR) spectrometer. Based on the result obtained in the

study, it is concluded that test item oleanolic acid is 'Non Clastogenic' upto the dose level of 3.0 mg/L. Under the conditions of this study, oleanolic acid did not induce chromosomal aberrations in the V79 chinese hamster cells with or without metabolic activation

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Conflict of interest:

Regarding the publication of this research effort document, the author has no conflicts of interest.

Author Contribution:

Conceptualization, design, planning, test material preparation, data compilation and collection, analysis, and result interpretation were all contributed by the authors. Anu T. Singh, Manu Jaggi and Sumer Singh were guide and reviewing the paper which was performed and written by Ritesh Kumar.

Ethical/Compliance Statement

This study did not involve human or animal subjects; therefore, no ethics approval was required.

AI Usage Statement

No AI tools were used for language editing only or not used at all for any purpose.

Data Availability Statement: Not applicable.

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