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RESEARCH PAPER

Accumulation of Betulinic, Oleanolic, and Ursolic acids in *In vitro* Cell Cultures of *Lantana camara* L. and their Significant Cytotoxic Effects on HeLa Cell Lines

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Abstract This study reports the accumulation of three pentacyclic triterpenoids, betulinic acid (BA), oleanolic acid (OA), and ursolic acid (UA), in cell cultures of *Lantana camara* using leaf disc explants. Thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and mass spectroscopy (MS) of organic extract followed by comparison with the standards revealed the presence of the above three triterpenoids. By following the extraction procedure mentioned here the recovery of all these three compounds were > 95%. The bioactivity of the cell-derived extract was demonstrated using cancerous HeLa cell lines. Specifically, the effect of this extract on HeLa cells was noticed from 36 h (at 100 μg/mL) to 72 h (at 25 μg/mL) by employing 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cytotoxicity assay. The results show that an increase in the concentration or duration of the extract treatment was effective in killing cancerous cells. DNA laddering assay confirmed that cell death was due to apoptosis. Further, BA was identified for the first time from an *in vitro* source. Moreover, this was the first report describing this kind of studies conducted using *Lantana* plants.

Keywords: betulinic acid, cell cultures, cytotoxicity, *Lantana camara*, oleanolic acid, ursolic acid

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1. Introduction

Lantana camara L., commonly known as wild or red sage, is an evergreen shrub belonging to the family Verbenaceae. The plant is native to tropical and subtropical America, although it has now become naturalized to many parts of Africa and Asia (India). It is a woody straggling plant characterized by various flower colors, including red, pink, white, yellow, and violet. *Lantana* is regarded as both a notorious weed as well as a popular ornamental garden plant, and it has various uses in folk medicine worldwide [1]. Each and every part of this plant contains a plethora of bioactive compounds. Leaf extract of *Lantana* exhibits antimicrobial, fungicidal, insecticidal, and nematicidal activities [2-5], whereas root extract is used for the treatment of malaria, rheumatism, and skin rashes [6]. Extracts of other plant parts are used for treating fever, common cold, asthma, high blood pressure, rheumatism, ulcers, chicken pox, leprosy, and scabies [1,5,7,8].

Several classes of compounds, including mono- and sesqui-terpenes, triterpenes, iridoid glycosides, flavonoids, furanonaphthoquinones, and phenyl ethanoid glycosides, have been identified in the wild growing plants of the *Lantana* genus [1]. Among these, the pentacyclic triterpenes betulinic acid (BA), oleanolic acid (OA), and ursolic acid (UA) are reported to be of immense pharmacological interest [9-11]. The objective of the present study was, therefore, to screen *in vitro* cultured cell lines of *Lantana* for the presence of bioactive triterpenoids *via* chemical analysis and a bioactivity-based approach.

Our motivation in this study was due to the fact that plant cell cultures offer an attractive alternative to whole plants for the homogeneous, controlled production of high value secondary metabolites, especially when taking com-

mercial demand into account. Plant cell cultures also result in more consistent yields and higher quality products. Additionally, the unavailability of potential biotechnological technique for utilization of large biomasses has limited to explore *Lantana* further. The anti-proliferative potential of organic extract derived from *Lantana* callus against cancerous HeLa and normal BHK-21 cell lines was investigated. This is the first study reporting the simultaneous presence of three acid triterpenoids, BA, OA, and UA, in the *in vitro* cultures of *L. camara* and also their cytotoxic activities against cancerous HeLa cells. Further to this, the presence of BA has not been reported until now from any *in vitro* source.

2. Materials and Methods

2.1. Plant material and initiation of aseptic cultures

Healthy leaves of *L. camara* plants bearing pink-yellow flowers were collected at monthly intervals over three consecutive years from the campus of the Indian Institute of Technology-Guwahati in order to initiate cultures. The leaves were washed with 1% Tween-20 (Merck, India) for 15 min, followed by three rinses in sterile distilled water (SDW). To maintain the sterility, the remaining steps were carried out inside a laminar-air-flow cabinet (Saveer Biotech, India). The leaves were then surface-sterilized with 0.1% mercuric chloride solution ($HgCl_2$) for 10 min, followed by three more rinses with SDW. Leaf-disc explants (5 mm) were prepared by punching the leaves with a cork-borer prior to implantation on Murashige and Skoog (MS) medium [12] containing 3% sucrose that was gelled with 0.8% agar (HiMedia Laboratories, India).

MS medium supplemented with varying combinations of 6-benzylaminopurine (BAP), 6-furfurylaminopurin (Kn), α-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) was used at concentrations ranging from 0.5 to 10 μM for the induction and multiplication of callus of leaf disc explants. After adjusting the pH to 5.8, 20 mL aliquots of the medium were dispensed into individual 150 × 25 mm Borosil rimless glass tubes. The culture tubes were plugged with non-absorbent cotton wrapped in cheesecloth and then autoclaved at 121°C for 15 min. Thermolabile compounds such as IAA were filter-sterilized and added to the autoclaved medium, which was cooled to 50°C. The medium was then dispensed into glass tubes inside a laminar airflow cabinet. All cultures were maintained under diffuse light (1,000 ~ 1,600 lux) conditions with a 16 h photoperiod at 25 ± 2°C and 50 to 60% relative humidity.

At least 24 cultures were raised for each treatment, and each experiment was repeated at least three times. Observations were recorded at weekly intervals. After callus induction, the biomass was subcultured regularly into fresh media at 4-week intervals.

2.2. Determination of dry weight

After 4 weeks, 500 mg calli were inoculated into 150 × 25 mm Borosil glass culture tubes containing 20 mL of fresh medium each. At the end of the passage, 64 g of freshly growing plant cells were harvested from the culture tubes, washed with distilled water, and subjected to vacuum filtration. Thereafter, to determine dry weight, the cells were dried in an oven at 30°C ± 2°C until a constant weight was observed. The drying temperature was kept low to prevent any thermal decomposition of the metabolites.

2.3. Preparation of plant cell extracts

The dried cell mass (50 g) was soaked in methanol (200 mL) for 48 h, after which the cells were sonicated for 40 min at 30% amplitude with pulser 5 sec on/off and was continued for next 20 min at the same amplitude with pulser 3 sec on/off. After filtration, the methanol extract was centrifuged at 10,000 rpm for 10 min, and the resulting supernatant was pooled, filtered, and dried in a rotary evaporator at 40°C. The methanol extract was then further fractionated into organic (ethyl acetate) and aqueous fractions. The ethyl acetate extract was further dried under reduced pressure in a rotary evaporator at 40°C (Buchi Rotavapor R-200, Japan). The % yield of the crude extract was calculated relative to the weight of the dried cells. Recovery experiments were performed by adding known amounts of BA, OA, and UA standards to the cell callus, followed by extraction as described above. The recovery percentage was calculated as:

$$\text{Recovery } (\%) = A - B / C \times 100$$

where A is the quantity of BA, OA, and UA in the spiked powder, B is the quantity of BA, OA, and UA in the powder without the added standards, and C is the quantity of added BA, OA, and UA.

2.4. Thin layer chromatography of organic extract

The dried organic extract was dissolved in chloroform and qualitatively analyzed by thin layer chromatography (TLC) on glass plates coated with silica gel (Merck, India). The extract was then eluted with a chloroform/methanol (24:1) solvent system. Spot visualization was carried out in an iodine chamber, and the R_f values of the spots were calculated.

2.5. High performance liquid chromatography of organic extract

Quantitative estimation of BA, OA, and UA was perform-

ed using a Varian Prostar HPLC system (Varian, USA) equipped with a binary pump and UV detector. A Hypersil BDS RP-C_{18} column (Thermo, USA) with dimensions of 250 × 4.6 mm and a 5 μm particle diameter was used with acetonitrile : water (80:20) as the mobile phase at a flow rate of 1 mL/min. The eluted samples were detected at 209 nm. Identification of the acids was carried out by comparing their retention times with those of authentic standards obtained from Sigma, USA (purity > 90% for BA and UA, > 98% for OA). The crude and standard samples were filtered through a 0.22 mm membrane filter prior to analysis. All solvents used were of HPLC grade.

2.6. Identification of compounds with mass spectrometry

MS detection was carried using a Waters Quadrapole-Tof Premier mass spectrometer equipped with a microchannel plate detector (Waters, USA). MS was operated in negative ion mode with a collision energy of 5 V. The cell entrance and exit voltage were set at 2 and −10 V, respectively. The MS data were obtained in full scan mode (mass range 100 ~ 1,000 amu). Comparison of the mass spectra of the three standard compounds obtained from Sigma with those of the individual fractions eluted by HPLC confirmed the presence of all three triterpenic acids.

2.7. Stock preparation of plant cell extract for cytotoxicity studies

The organic extract was dissolved in dimethyl sulphoxide (DMSO) to a concentration of 10 mg/mL, creating a stock solution from which appropriate dilutions were prepared in culture medium before each experiment. The final concentration of DMSO did not exceed 1% in any experiment. Before use, the stocks were filter-sterilized through a 0.2 μm filter. Curcumin (HiMedia Laboratories, India), a natural polyphenolic compound from *Curcuma longa*, was used as a positive control in the experiments. Stock solution of curcumin was prepared in a manner similar to that of the extract.

2.8. Culture and maintenance of mammalian cell lines

HeLa cells and the Syrian golden hamster kidney normal fibroblast cell line BHK-21(C-13) were obtained from the National Centre for Cell Science, Pune, India. Cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) containing 2 mM L-glutamine, 1.5 g/L of sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate and supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) as well as 1% antibiotic antimycotic solution (1,000 U/mL of penicillin G, 10 mg/mL of streptomycin sulfate, 5 mg/mL of gentamycin, and 25 μg/mL of amphotericin B). The cultures were maintained at 37°C in an atmosphere of 5% CO_2:95% air under 95% relative humidity.

2.9. Cytotoxicity assay

The cytotoxic effects of organic extract and curcumin on cells were measured by dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay [13]. In an independent set of experiments, both HeLa and BHK-21 (C-13) cells grown in T-25 culture flasks were harvested by trypsinization, plated at an approximate density of 1×10^4 cells/well in a 96-well culture plate (Corning®, Sigma), and incubated for 24 h to achieve confluent growth. After 24 h, the medium from each well was removed, and the cells were washed twice with Dulbecco's Phosphate Buffered Saline (PBS) without Ca^{+2} and Mg^{+2}. The cells were then exposed to different concentrations (2.5 μg/well to 200 μg/well) of organic extract as well as curcumin (0.1 μg/well to 10 μg/well). Each well contained 100 μL of serum-free DMEM containing the above-mentioned concentrations of extract. The plates were incubated for different time periods of 24, 36, 48, 60, or 72 h. At the end of each incubation period, the contents were replaced with equal amounts of 0.5 mg/mL of MTT dissolved in serum-free DMEM without phenol red, after which the plates were further incubated for 4 h. The contents were then replaced with equal amounts of DMSO to solubilize the formazan grains formed by viable cells. Finally, the absorbance was read at 570 nm using a Multi-well plate reader.

2.10. Microscopic observations of cell morphology

HeLa and BHK-21 cells were treated with various concentrations of organic extract for different time periods and then examined using an inverted light microscope (Nikon, TS 100-F), confocal laser scanning microscope (CLSM) (Carl Zeiss LSM 510Meta), and scanning electron microscope (SEM) (Leo 1430vp). For CLSM analysis, the cells were stained with acridine orange/ethidium bromide (AO/EB) before observation. For SEM analysis, the cells were initially allowed to grow on a coverslip, followed by extraction treatment. The samples were fixed, dehydrated, dried with a vacuum, and finally sputter-coated with gold prior to examination.

2.11. DNA fragmentation assay

For laddering experiments, the cells were treated with 44.75 μg/mL of extract and incubated for 48 h in a CO_2 incubator. Treated cells were then harvested, washed with ice cold Phosphate Buffered Saline (PBS; pH 7.2), and centrifuged at 3,000 rpm for 6 min at 4°C. The resulting cell pellet was dispersed in 30 μL of lysis buffer (10 mM Tris, pH 7.4; 100 mM sodium chloride; 25 mM ethylene diamine tetra acetic acid; 1% sodium lauryl sulfate) by gentle vortexing. About 4 μL of proteinase K (10 μg/μL)

was then added to the above mixture, followed by incubation at 45°C for about 1 to 2 h. Then, 2 µL of RNase (10 µg/µL) was added to the cell lysates, which were further incubated for 1 h at room temperature. After incubation, cell lysates were mixed with 4 µL of 6X DNA sample dye (50% glycerol; 1 mM EDTA, pH 8.0; 0.25% bromophenol blue), after which the samples were finally subjected to 2% agarose gel electrophoresis at 7 V/cm. The gel was then stained with ethidium bromide (0.5 µg/mL) and visualized under a gel documentation system (BioRad, USA).

2.12. Statistical analysis

All experiments were performed in triplicate along with proper control experiments. To minimize experimental error, exponentially growing cells of the same passage were used. The results are expressed as mean ± standard deviation. Paired *t*-test was performed between the two treatment groups (HeLa and BHK-21). *P* values less than 0.05 and 0.01 were considered statistically significant.

3. Results and Discussion

3.1. Callus induction and multiplication

Leaf disc explants (5 mm) were cultured on a range of media to induce callus formation of *L. camara*. Of all the various growth regulator treatments, MS medium supplemented with 5 µM BAP, 1 µM 2,4-D, and 1 µM NAA induced maximum callus formation. Using this composition, 100% of the explants produced calli, which was profuse after the first subculture. In the responding medium, the leaf disc explants first turned brown. After 1 week, bright-green, hard, compact calli started to develop at the margins of the leaf-disc (Fig. 1A). These compact calli were then dissected and subcultured onto fresh medium of the same growth regulator composition. Although the rate and degree of callus formation increased with subsequent subcultures, the properties of the formed calli did not improve substantially. Calli were friable and soft but remained deep brown in nature (Figs. 1B, 1C, and 1D). In total, it took about 26 weeks of regular subculturing at 4-week intervals to obtain profusely growing, fresh, friable, granulated, and cream-colored calli (Fig. 1E). Thereafter, approximately 500 mg of the leaf disc calli was subcultured every 4 weeks on MS + BAP (5 µM) + 2,4-D (1 µM) + NAA (1 µM) for sustained and massive callus proliferation. Therefore, the calli were allowed to multiply on this medium for more than 20 months.

Browning of the explants and culture media due to phenol exudation has been discussed previously [14,15]. It has been suggested that the addition of antioxidants such as ascorbic acid, polyvinylpyrrolidone (PVP), and polyvinylpolypyrrolidone (PVPP) can be used to overcome explant browning and death. Besides this, repeated subculturing at regular intervals is also an efficient and alternative way of overcoming browning of cultures [16,17]. In the present study, repeated subculturing was adapted as a method of eliminating phenolic exudation from the explant into the medium. This may have helped avoid any changes in the secondary metabolite profile of the cultured cells due to antioxidants.

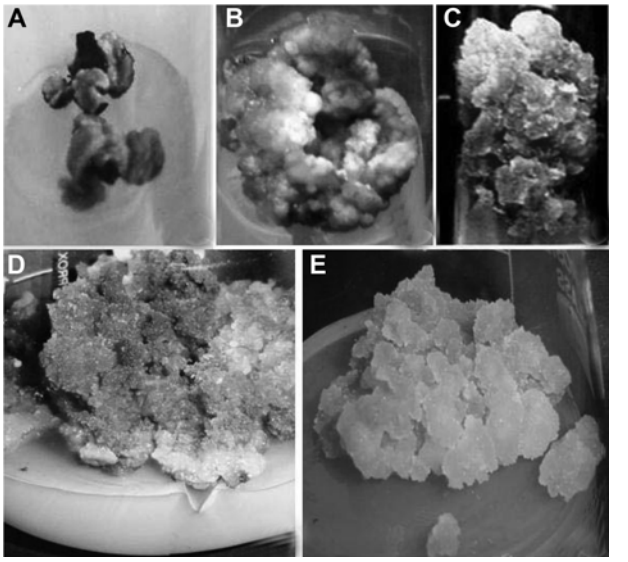

Fig. 1. Establishment of callus cultures of *L. camara*. (A) Leaf disc at culture for 3 weeks, green and brown compact calli seen proliferating from the margins of the leaf disc (2X). (B) Seconed subculture of callus from a; further proliferation of compact and hard callus (2X). (C) Thirteen-week-old culture showing soft and friable calli after 7th subculture, the cells were deep brown in color (1.5X). (D) Eighteen-week-old culture showing improved callus properties after 10th Subculture; light green, fresh cells proliferating among the brown cells (1.2X). (E) Fourteenth subculture showing healthy, fresh, granulated, friable, and cream-colored calli ready for analysis (1.5X).

3.2. Analysis of pentacyclic triterpenoids in plant cell extracts by TLC and HPLC

By following the protocol as described in Materials and methods, the calli (50 g of dry cell mass) proliferated on leaf-disc explants of *L. camara* yielded 0.17% of the organic (ethyl acetate) extract. The ethyl acetate extract was analyzed both qualitatively and quantitatively by TLC and HPLC, respectively, for the presence of three anti-cancerous acid triterpenoids, betulinic acid (BA), oleanolic acid (OA), and ursolic acid (UA). These compounds were identified by comparing their retention factors (R_f value) and retention times with those of authentic standards. TLC revealed that BA, OA, and UA produced spots with R_f values of 0.51, 0.28, and 0.17, respectively. The presence of all three compounds was further confirmed by HPLC, in which BA,

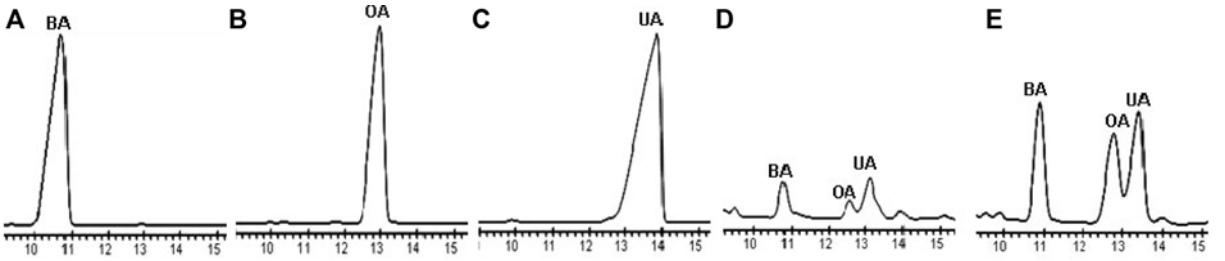

Fig. 2. HPLC of three pentacyclic triterpenoids. (A) Chromatogram of standard Betulinic acid (BA). (B) Chromatogram of standard Oleanolic acid (OA). (C) Chromatogram of standard Ursolic acid (UA). (D) Chromatogram of organic extract. (E) Chromatogram of organic extract spiked with the three standards BA, OA, and UA.

OA, and UA eluted at 10.8, 12.61, and 13.2 min, respectively (Figs. 2A, 2B, 2C, 2D, and 2E). The method also provided excellent yields of 3.1, 1.88, and 4.12% for BA, OA, and UA, respectively, in cell cultures of *L. camara*. When leaves of field-growing plant were extracted and analyzed, they were found to contain OA (ca. 2%) and UA (4.5%) in amounts only marginally higher than those in callus cells, whereas BA was altogether absent (data not shown). The biosynthesis of secondary metabolites in cultured plant cells does not necessarily yield compounds characteristic of the intact plant or in the same amounts as in the whole plant [18-22]. Cells in culture grow under heterotrophic conditions, and their biosynthetic potential depends on the composition of the nutrient medium. Further, since each cell in culture is a separate entity, overall metabolic activity would be faster at a higher level of expression for a shorter duration compared to intact plant or tissues. [21,22]. Using the extraction method reported herein, recovery of all three compounds was above 95% (Table 1).

Apart from its popularity as a garden plant, the large available biomass of *Lantana* has not been explored *via* biotechnological means. In this context, plant cell culture is a promising biotechnological approach that has been adopted for the purpose of producing various categories of secondary plant metabolites [23,24]. *In vitro* culture is a valuable technique because it produces metabolites with high uniformity and stability, and this is independent of plant availability and seasonal variations. Over the past decade, there has been a larger interest in the isolation and purification of BA, OA, and UA from various plants for the treatment of various diseases [11,26-28]. Although there are some reports that screened OA and UA from plant cell cultures, while, until now there are no reports published on BA. To the best of our knowledge, this is the first report on the simultaneous accumulation of BA, OA, and UA from *in vitro* cultures of *L. camara* along with a demonstration of their cytotoxic effects on HeLa cell lines.

3.3. Analysis of compounds by mass spectrometry

Results of mass spectrometry are shown in Table 1. The fragmentation patterns of the fractions eluted by HPLC were similar to those of their respective standards, which further confirm the presence of BA, OA, and UA in the cell lines of *Lantana*.

3.4. Cytotoxicity assay

Results of the MTT assay of HeLa and BHK-21 cells treated with the organic extract are shown in Tables 2 and

Table 1. Results of HPLC and m/z patterns of representative fragments obtained by negative mode electrospray ionization of fractions eluted from HPLC and their respective standard compounds

Compound	Retention time (min)	Content (%)	Recovery (%)	Mass spectra (m/z)
Betulinic acid	10.8	3.1	96.90	*Obtained: m/z* 132, 134, 136, 170, 172, 248 (100), 257, 293, 376, 378, 455 (10), 456, 513 *Standard: m/z* 132, 134, 136, 170, 172, 248 (52), 257, 293, 376, 378, 455 (100), 456, 513
Oleanolic acid	12.64	1.88	95.07	*Obtained: m/z* 132, 134, 248 (7), 255, 299, 310, 343 (100), 455 (2), 456, 523 *Standard: m/z* 132, 134, 248 (10.5), 255, 299, 310, 343 (2), 455 (100), 456, 523
Ursolic acid	13.2	4.12	97.60	*Obtained: m/z* 132, 134, 187, 227, 233, 248, 255 (28), 299 (69), 343 (100), 455 (16), 456, 457, 523, 524, 567 *Standard: m/z* 132, 134, 187, 227, 233, 248, 255 (26), 299 (72), 343 (55), 455 (100), 456, 457, 523, 524, 567

Values in parentheses ( ) indicate percent relative intensities.

Table 2. Effect of organic extract on percentage viability of HeLa cells

Conc. (µg/mL)	Time					
	24 h	36 h	48 h	60 h	72 h	
25	92.76 ± 1.19	85.34 ± 1.03	65.62 ± 1.11	58.48 ± 0.82	50.97 ± 0.85	% Viability**
50	74.43 ± 1.59	62.07 ± 0.63	55.87 ± 1.45	46.28 ± 1.35	38.57 ± 0.98	
100	59.59 ± 1.81	48.80 ± 1.36	41.98 ± 0.95	34.08 ± 1.94	30.49 ± 1.66	
250	48.98 ± 1.36	39.82 ± 1.41	35.67 ± 0.20	29.91 ± 0.95	24.22 ± 0.88	
500	46.08 ± 1.72	35.02 ± 0.53	30.66 ± 1.58	25.74 ± 1.07	20.78 ± 1.84	
1,000	34.02 ± 0.92	27.94 ± 0.93	26.65 ± 1.63	22.02 ± 0.21	19.43 ± 0.90	
1,500	27.14 ± 1.83	22.25 ± 1.44	20.49 ± 1.11	17.86 ± 1.01	15.09 ± 0.53	
2,000	15.44 ± 1.09	13.02 ± 0.72	11.03 ± 1.01	7.59 ± 0.32	5.98 ± 1.59	

**Mean ± SD.

Table 3. Effect of organic extract on percentage viability of BHK-21cells

Conc. (µg/mL)	Time					
	24 h	36 h	48 h	60 h	72 h	
25	100.34 ± 1.16	93.41 ± 1.82	83.06 ± 1.71	75.78 ± 0.28	74.89 ± 1.27	% Viability**
50	83.15 ± 1.05	78.02 ± 1.98	70.39 ± 1.27	60.94 ± 1.24	59.64 ± 0.63	
100	70.02 ± 1.83	66.79 ± 0.69	64.46 ± 1.69	57.42 ± 0.69	53.36 ± 0.63	
250	66.06 ± 1.87	61.78 ± 0.60	55.23 ± 0.19	45.12 ± 0.28	46.19 ± 1.24	
500	52.72 ± 1.16	51.53 ± 0.69	48.89 ± 1.78	46.09 ± 0.28	43.50 ± 0.63	
1,000	48.76 ± 0.88	42.49 ± 1.74	41.46 ± 0.68	38.67 ± 1.38	38.57 ± 1.81	
1,500	36.09 ± 1.52	31.38 ± 1.49	33.20 ± 1.30	26.56 ± 0.70	25.56 ± 0.32	
2,000	27.83 ± 0.96	25.15 ± 0.17	22.59 ± 0.78	17.19 ± 1.52	16.59 ± 1.35	

**Mean ± SD.

Table 4. Results of paired t-tests ($p = 0.05^a$ and 0.01^b) between treatment groups (HeLa and BHK-21) at different time intervals

	24 h	36 h	48 h	60 h	72 h
t	8.3911	8.8888	12.6363	9.0001	9.8795
df	7	7	7	7	7
SED	1.289	1.635	1.299	1.747	1.933
P value	< 0.0001*a,b	< 0.0001*a,b	< 0.0001*a,b	< 0.0001*a,b	< 0.0001*a,b

*a,b Significant at both $p = 0.05$ and $p = 0.01$.

3. The extract had a noticeable effect on the viability of HeLa cells in the concentration range from 25 µg/mL (at 72 h) to 100 µg/mL (at 36 h), in which 50% of the cells were killed. However, at these concentrations and time periods, the effect on BHK-21 cells was not significant ($p < 0.05$, 0.01) (Table 4). We observed a similar pattern in the positive control experiments, in which the effect of extract on BHK-21 cells was negligible at the concentration in which 50% of the HeLa cells were killed. Thus, the organic extract had selective cytotoxicity for the cancer cells. This report is the first to observe that *in vitro* callus cultures of *Lantana* have selective cytotoxic activity due to the accumulation of three powerful bioactive compounds, BA, OA, and UA.

3.5. Microscopic observations of cell morphology

In general, tumor cells undergo cell death by apoptosis and necrosis, which can be detected by morphological and biochemical changes. Of these, morphological study is considered to be the most reliable method of identification [29-32]. The manifestation of molecular events such as nuclear fragmentation is detected only after the appearance of morphological features characteristic of apoptosis [32]. Thus, to assess the pattern of cell death, the morphological features of HeLa and BHK-21 cells were examined by microscopic techniques.

Light microscopy revealed that the organic extract had an adverse effect on cell morphology. A gradual change in the appearance of the treated cells was observed in a dose and time-dependent manner. Most of the dead cells showed characteristic features of apoptosis, including reduced cell volume, cytoplasmic membrane blebbing, and formation of apoptotic bodies (Fig. 3). Further, there were very few populations of cells that showed the characteristic features

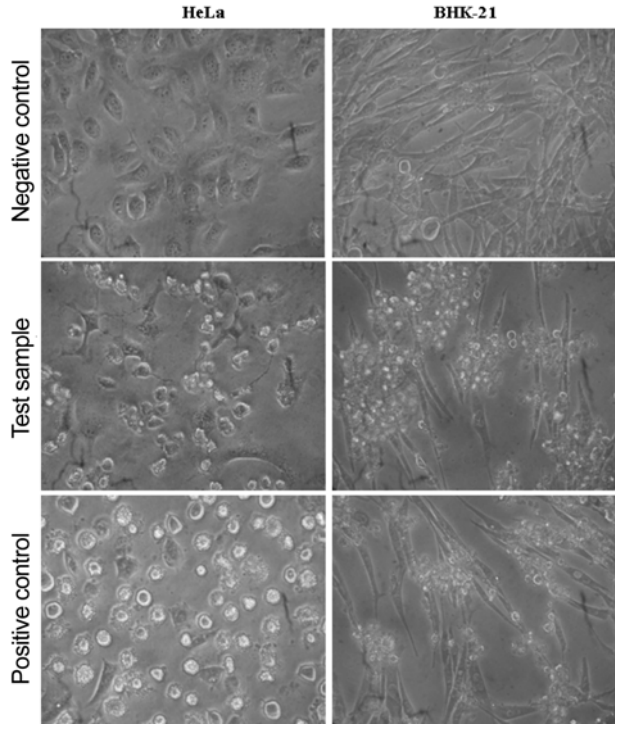

Fig. 3. Images of untreated and treated HeLa and BHK-21 cells as seen under an inverted light microscope. Similar pattern of morphological changes is exhibited by both the test sample and positive control-treated cells (40X).

of necrotic cell death, such as increased cell volume, vacuolization of the cytoplasm, and dissolution of the cytoplasmic membrane leading to the cell lysis. The morphological features of treated and untreated HeLa and BHK-21 cells observed by inverted light microscopy are shown in Fig. 3. It is clear that the cells were prone to apoptosis after treatment with the organic extract. Further, the pattern of cell death was similar to that induced by curcumin, a potent anti-cancer compound of natural origin, which was used as a positive control.

The morphological pattern of cell death was further confirmed by CLSM (Fig. 4) following staining of the cells with AO/EB. AO stains both viable and non-viable cells and emits green fluorescence upon intercalation into double-stranded DNA. It also emits red fluorescence when bound to single-stranded RNA. In contrast, EB stains only non-viable cells upon intercalation and thus emits red fluorescence. The increase in incubation time of the cells treated with the extract from 24 to 72 h resulted in a decreased number of viable cells (Figs. 4A, 4B, 4C, 4D, 4E, and 4F). The viable cells exhibited a bright green nucleus and intact structure, whereas the dead cells showed orange to red fluorescence with characteristic features of apoptosis. As time progressed, the number of viable cells decreased gradually whereas the number of dead cells increased, as

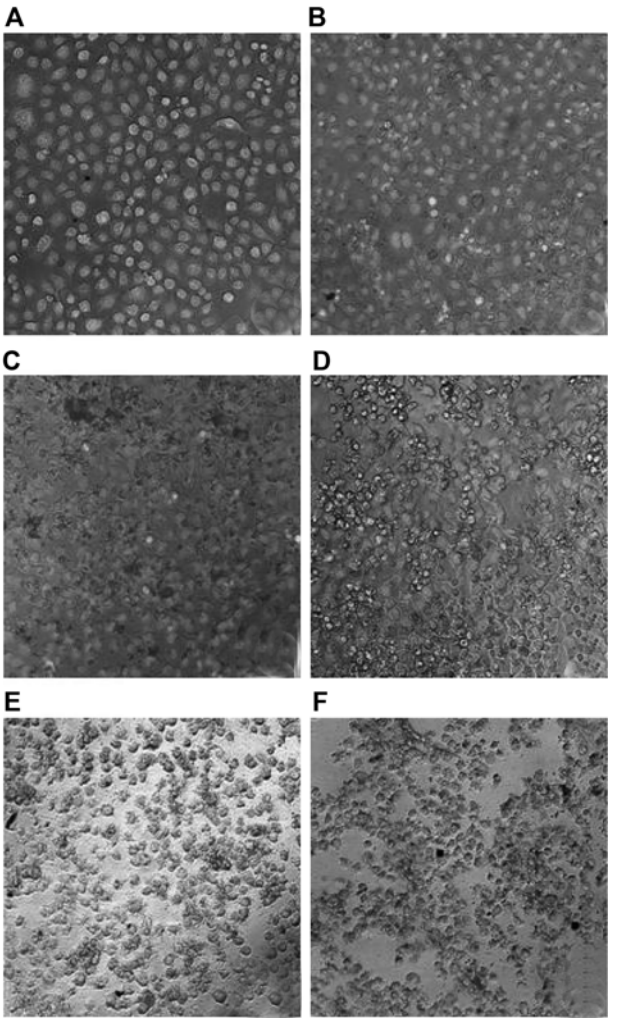

Fig. 4. CLSM images of untreated (A) and treated HeLa cells after 24 h (B), 36 h (C), 48 h (D), 60 h (E), and 72 h (F) (40 X).

evident from the green and red fluorescence.

To further confirm our results, the treated cells were examined under SEM. An overview of treated HeLa cells can be seen in Fig. 5A. Cells in the early stages of the treatment period were found to exhibit normal morphology, including proper size, shape, and cell-cell contacts (Fig. 5B). Subsequent morphological changes included contraction of the extracellular matrix (Fig. 5C), loss of cell-cell contacts (Fig. 5D), detachment of cells from the substratum and membrane blebbing (Fig. 5E), and finally formation of apoptotic bodies (Fig. 5F). Further, a reduction in overall cell size from about 20 to 5 μm was observed by SEM.

Thus, the results of the light microscopy, CLSM, and SEM analyses collectively suggest that the cell morphology of the treated cells underwent significant changes that resemble the features of apoptosis. The ability of a compound to induce apoptosis in tumor cells is considered

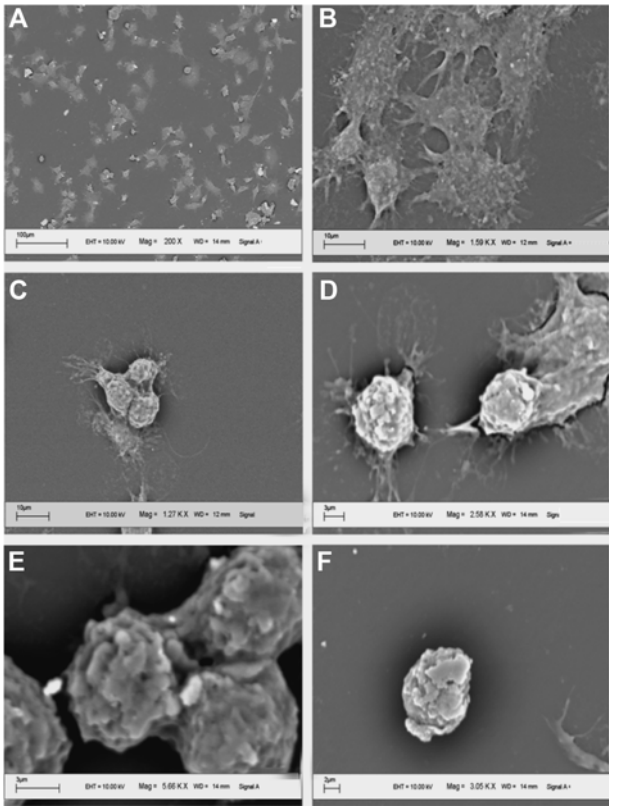

Fig. 5. Images of treated HeLa cells as examined by SEM. (A) Overview of treated cells showing a mixture of live/dead cells; the sequential stage of apoptosis can be seen in Figs. B, C, D, E, and F. (B) Cells in the initial state with intact size, shape, and morphology. (C) Gradual contraction of extracellular matrix. (D) Subsequent loss of cell-cell contacts. (E) Membrane blebbing. (F) Formation of apoptotic bodies.

as an important criterion for its use as an anti-cancer compound.

3.6. DNA fragmentation assay

DNA laddering was performed to confirm that cell death was due to apoptosis. The samples were run on a 2% agarose gel. During apoptosis, endogenous endonucleases cleave nuclear DNA at internucleosomal sites. Subsequently, this results in accumulation of DNA fragments of 180 ~ 200 bp as well as multiples thereof. If this mixture is run at low agarose content (ca. 0.8%), DNA fragments of higher length move comfortably, whereas fragments of shorter length may escape from the gel [33]. Hence, high agarose content (2%), which has a smaller pore size, was used in the current study in order to retain the shorter fragments.

The gel electrophoresis image shown in Fig. 6 clearly shows that the extract resulted in DNA fragmentation and subsequently cell death. Fragmentation of genomic DNA is a late event during apoptosis and is a result of active caspase-3-mediated cleavage of ICAD (Inhibitor of CAD)

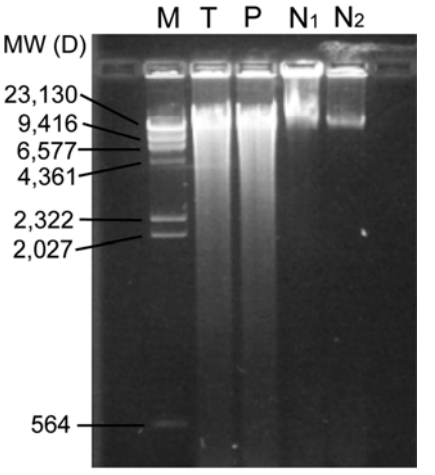

Fig. 6. The gel electrophoresis image obtained after DNA fragmentation assay. The lanes M, T, P, N_1, and N_2, represent marker (lambda DNA Hind III digest), test sample (extract treated), positive control (curcumin treated), negative control – 1 (DMSO treated), and negative control – 2 (without any treatment), respectively. N_1 was performed to verify the effect of DMSO (which was used as a solvent to dissolve the extract) alone on the cells. The molecular weights of the DNA markers ranged from 23,130, to 564 Da.

to CAD (caspase-activated deoxyribonuclease), which is responsible for fragmentation of the DNA [34,35]. The results obtained in the DNA fragmentation analysis conclusively demonstrate the apoptotic effect of the extract.

4. Conclusion

In the present investigation, simultaneous accumulation of three anti-proliferative triterpenoids, BA, OA, and UA, was observed in *in vitro* cultures of *Lantana camara*. Further, the selective cytotoxic effects of the plant cell extract on cancerous HeLa cells demonstrate the potential of using *in vitro* plant cell cultures of *L. camara* to produce bioactive compounds.

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References

1. Ghisalberti, E. L. (2000) *Lantana camara* L. Verbenaceae. *Fitoterapia*. 71: 467-486.
2. Chavan, S. R. and S. T. Nikam (1982) Investigation of *Lantana camara* Linn. (Verbenaceae) leaves for larvicidal activity. *Bull.*

Haff. Inst. 10: 21-22.
3. Sharma, O. P. and P. D. Sharma (1989) Natural products of the *Lantana* plant- the present and prospects. *J. Sci. Indus. Res.* 48: 471-478.
4. Begum, S., A. Wahab, B. S. Siddiqui, and F. Qamar (2000) Nematicidal constituents of the aerial parts of *Lantana camara*. *J. Nat. Prod.* 63: 765-767.
5. Day, M. D., C. J. Wiley, J. Playford, and M. P. Zalucki (2003) *Lantana: Current Management, Status and Future Prospects*. p.102. Australian Centre for International Agricultural Research Monograph, Canberra.
6. Taoubi, K., M. T. Fauvel, J. Gleye, and C. Moulis (1997) Phenylpropanoid glycosides from *Lantana camara* and *Lippia multiflora*. *Planta Med.* 63: 192-193.
7. Anonymous (1962) Lantana Linn. (Verbenaceae). In: B. N. Sastri (ed.). The Wealth of India: A Dictionary of Raw Materials and Industrial products. Council of Scientific and Industrial Research, New Delhi, India, p. 31-34.
8. Kirtikar, K. R. and B. D. Basu (1981) *Indian Medicinal Plants*. p. 984. Panini Office, Bhuwaneswari Asrama, Bahadurganj, Allahabad, India.
9. Hsu, H. Y., J. J. Yang, and C. C Lin. (1997) Effects of Oleanolic acid and Ursolic acid on inhibiting tumor growth and enhancing the recovery of hematopoeietic system postirradiation in mice. *Cancer Lett.* 111: 7-13.
10. Laus, G. (2004) Advances in chemistry and bioactivity of the genus *Uncaria*. *Phytother. Res.* 18: 259-274.
11. Pereira, P. S., F. K. Ticli, S. C. França, B. C. M. de Souza, and M. V. Lourenço (2007) Enhanced triterpene production in *Tabernaemontana catharinensis* cell suspension cultures in response to biotic elicitors. *Quim. Nova.* 30: 1849-1852.
12. Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol. Plant.* 15: 473-497.
13. Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Meth.* 16: 55-63.
14. Amin, M. N. and V. S. Jaiswal (1987) Rapid clonal propagation of guava through *in vitro* shoot proliferation on nodal explants of mature trees. *Plant Cell Tissue Organ Cult.* 9: 235-243.
15. Naik, S. K., S. Pattnaik, and P. K. Chand (1999) *In vitro* propagation of pomegranate (*Punica granatum* L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree. *Scientia Horticult.* 79: 175-183.
16. Mathur, J., P. S. Ahuja, A. K. Mathur, A. K. Kukreja, and N. C. Shah (1988) *In vitro* propagation of *Valeriana wallichii* D C. *Planta Med.* 54: 82-83.
17. Rout, G. R., C. Saxena, S. Samantaray, and P. Das (1999) Rapid clonal propagation of *Plumbago zeylanica* Linn. *Plant Growth Regul.* 28: 1-4.
18. Butenko, R. G. (1975) Eksperimental'nyi morfogenez i differentsiatsiya v kul'ture rastenii, 35-e Timiryazevskie chteniya (Experimental Morphogenesis and Differentiation in Cultures of Plant Cells), Moscow [in Russian].
19. Nosov, A. M. (1994) Functions of plant secondary metabolites *in vitro* and *in vivo*, *Fiziol. Rast.* 41: 873-878.
20. Kol'chugina, I. B. and E. N. Markarova (2009) Photoheterotrophic callus culture *Ficus elastica*. The formation of polyisoprene synthesis. *Moscow University Biological Sciences Bulletin* 64: 28-31.
21. Rao, S. R. and G. A. Ravishankar (2002) Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnol. Adv.* 20: 101-153.
22. Dornenburg, H. and D. Knorr (1995) Strategies secondary for the improvement of metabolite production in plant cell cultures. *Enz. Microbial Technol.* 17: 674-684.
23. Fowler, M. W. and A. H. Scragg (1988) Natural Products from higher plants and plant cell cultures. pp. 166-177. In: M. S. S. Pais, F. Mavituna, and J. M. Novais (eds.). *Plant Cell Biotechnology NATOASI Series*. Verlag, Berlin, Springer.
24. Alfermann, A. W. and M. Petersen (1995) Natural product formation by plant cell biotechnology-Results and perspectives. *Plant Cell Tissue Organ Cult.* 43: 199-205.
25. Ikuta, A., H. Tomiyasu, Y. Morita, and K. Yoshimura (2003) Ursane- and Oleanane- type triterpenes from *Ternstroemia gymnanthera* callus. *J. Nat. Prod.* 66: 1051-1054.
26. Liu, J. (2005) Oleanolic acid and Ursolic acid: Research perspectives. *J. Ethnopharmacol.* 100: 92-94.
27. Pasqua, G., A. Silvestrini, B. Monacelli, N. Mulinacci, P. Menendez, and B. Botta (2006) Triterpenoids and ellagic acid derivatives from *in vitro* cultures of *Camptotheca acuminata* Decaisne. *Plant Physiol. Biochem.* 44: 220-225.
28. Resende, F. A., C. A. M. A. Barcala, M. C. S. Faria, F. H. Kato, W. R. Cunha, and D. C. Tavares (2006) Antimutagenicity of ursolic acid and oleanolic acid against doxorubicin-induced clastogenesis in Balb/c mice. *Life Sci.* 79: 1268-1273.
29. Walker, N. I., B. V. Harmon, G. C. Gobé, and J. F. Kerr (1988) Patterns of cell death. *Methods Achiev. Exp. Pathol.* 13: 18-54.
30. Cotter, T. G., S. V. Lennon, J. G. Glynn, and S. J. Martin (1990) Cell death *via* apoptosis and its relationship to growth, development and differentiation of both tumour and normal cells. *Anticancer Res.* 10: 1153-1159.
31. Renvoize, C., A. Biola, M. Pallardy, and J. Breard (1998) Apoptosis: Identification of dying cells. *Cell Biol. Toxicol.* 14: 111-120.
32. Xiao, J. X., G. Q. Huang, C. P. Zhu, D. D. Ren, and S. H. Zhang (2007) Morphological study on apoptosis HeLa cells induced by soyasaponins. *Toxicol. in Vitro*. 21: 820-826.
33. Zhu, N. and Z. Wang (1997) An assay for DNA Fragmentation in apoptosis without phenol/chloroform extraction and ethanol precipitation. *Anal. Biochem.* 246: 155-158.
34. Eschenfeldt, W. H. and S. L. Berger (1987) Purification of large double-stranded cDNA fragments. *Methods Enzymol.* 152: 335-337.
35. Sakahira, H., M. Enari, and S. Nagata (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 391: 96-99.