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ORIGINAL PAPER

Dedifferentiation of leaf explants and cytotoxic activity of an aqueous extract of cell cultures of *Lantana camara* L.

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Abstract Several secondary metabolites are present in *Lantana camara* L. as its leaves serve as reservoirs for various bioactive compounds. Callus cultures of *L. camara* were induced from leaf discs incubated on Murashige and Skoog medium supplemented with 5 μM 6-benzyladenine, 1 μM 2,4-dichlorophenoxyacetic acid, and 1 μM α-naphthalene acetic acid (NAA). An aqueous extract (0.23%), obtained from these calli (50 g dry mass), had an apparent cytotoxic effect on HeLa cells with an IC_{50} value of 1,500 μg/ml in 36 h. A dose-time dependent activity of the extract was established wherein higher dosage exhibited increased activity; however, over time cell necrosis was observed.

Keywords *Lantana camara* · Callus · Leaf culture · Aqueous extract · Cytotoxic · HeLa cells

Introduction

Lantana camara L., family Verbenaceae, is an evergreen shrub that contains a wide array of compounds exhibiting diverse range of bioactivity. Leaf extracts are reported to have antimicrobial, fungicidal, insecticidal, and nematicidal activities (Sharma and Sharma 1989; Begum et al. 2000). *L. camara* is used in folklore medicine for treatment of cancer, chicken pox, measles, asthma, ulcers, swelling, eczema, tumors, high blood pressure, bilious fevers, catarrhal infections, tetanus, rheumatism, and malaria (Ghisalberti 2000; Day et al. 2003). Topical applications have been used for treating leprosy and scabies. Leaf exudates of *L. camara* are reported to contain mixtures of flavanoids, lantadene A, and icterogenin (Wollenweber et al. 1997).

Various secondary plant metabolites are involved in plant defense responses and facilitate plant adaptation to their environment by enhancing their general fitness and well-being. These compounds are also sources of pharmaceuticals, pesticides, flavoring agents, fragrances, and food additives. Different plants produce diverse products and their production is often related to a particular developmental stage, and is profoundly affected by seasonal variations. Cell cultures are attractive alternatives to whole plants for production of high value secondary metabolites due to consistency in quality and quantity of the desired product (Rao and Ravishankar 2002).

In the present report, we have established in vitro callus cultures from leaves of *L. camara* and demonstrated anti-cancerous activity of cell derived aqueous extracts on HeLa cells. The low toxicity of this extract on normal BHK-21 cells has also been studied, thereby, verifying the potential of this aqueous extract as an anti-cancer agent.

Materials and methods

Plant material

Healthy leaves of *Lantana* were collected from the campus of Indian Institute of Technology, Guwahati, from the plant bearing pink–yellow colored flowers. Leaves were surface sterilized with 1% (v/v) Tween-20 for 15 min and 0.1% (w/v) mercuric chloride for 10 min, followed by three rinses in the sterile water. Leaf discs explants were prepared by

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punching the sterilized leaves with 5 mm sized cork-borer before being cultured with the adaxial side in contact with the media.

Callus induction and establishment of plant cell cultures

Leaf discs were incubated on MS medium supplemented with different combinations and concentrations of auxins and cytokinins, including 5, 10 μM indole-3-acetic acid (IAA), 5, 10 μM α-naphthalene acetic acid (NAA), 1, 5 μM 6-benzyladenine (BA), and 1, 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The basal medium (BM) used in all experiments consisted of Murashige and Skoog (MS) (1962) macro- and microsalts, MS vitamins, 100 mg/l myoinositol, enriched with 30 g/l sucrose, and solidified with 0.8% (w/v) agar (Hi Media Laboratories, Mumbai, India).

The pH of the medium was adjusted to 5.8 before the agar was added. Media were autoclaved at 121°C at 15 psi for 15 min. Approximately 20 ml of medium was dispensed in each test tube (Borosil, Mumbai, India). Cultures were maintained in 1,000–1,600 lux light intensity at 25°C and were subcultured at every 4-week interval.

Cultured plant cells were harvested, washed in distilled water and filtered under vacuum. Thereafter, cells were dried in an oven at 30°C ± 2°C until a constant weight was achieved. The drying temperature was kept low to prevent thermal decomposition of metabolites.

Preparation of aqueous extract

Dried cell mass (50 g) was soaked in methanol for 48 h, after which cells were sonicated for 40 min at 30% amplitude (pulser 5 s on/off) and then for another 20 min at the same amplitude (pulser 3 s on/off). The mixture was centrifuged in a high speed refrigerated centrifuge (Sigma 4K15C, Osterode Am Harz, Germany) at 10,000 rpm for 10 min. The supernatant was pooled, dried in a rotary evaporator at 40°C, and extract was fractionated into aqueous and organic (ethyl acetate) fractions. The aqueous extract was lyophilized in a freeze dryer and used for further studies. The percentage yield of the aqueous extract was calculated relative to weight of dried cells.

Mammalian cell culture and cell viability assay

The lyophilized aqueous extract was dissolved in a serum-free Dulbecco's modified Eagle medium (DMEM) to prepare a stock solution of 10 mg/ml, and sterilized by filtration through a 0.2 μm filter, prior to use. A curcumin (HiMedia Laboratories, Mumbai, India) sample was used as a positive control.

The human cervical adenocarcinoma cell line HeLa, and the Syrian golden hamster kidney normal fibroblast cell line BHK-21(C-13) were obtained from National Centre for Cell Science (Pune, India). These were grown as monolayer cultures in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% antibiotic antimycotic solution (1000 U/ml penicillin G, 10 mg/ml streptomycin sulfate, 5 mg/ml gentamycin and 25 μg/ml amphotericin-B), and maintained at 37°C in 5% CO_2/95% air atmosphere with 90% relative humidity.

For viability assays, both HeLa and BHK-21 cells were plated at a rate of $\sim 10^4$ cells/well in 96-well culture plates, and incubated for 24 h in a CO_2 incubator to allow confluent growth. Cultures were then exposed to different concentrations of the aqueous extract ranging from 1,000 to 3,000 μg/ml and incubated for 24, 36, 48, 60, or 72 h. The cell viability was estimated using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. At the end of each incubation period, cells were incubated with MTT for 4 h at 37°C for colorimetry based cytotoxicity assay. The yellow colored MTT is reduced to an insoluble purple formazan by mitochondrial reductase that are active only in the mitochondria of living cells. Thus, the conversion can be directly correlated to the number of viable cells. The reaction product, formazan, was dissolved in DMSO, and the absorbance of the purple colored solution was quantitatively measured at 570 nm in a UV visible spectrophotometer (Cary 100, Varian Inc., Middleburg, Netherlands).

Morphological observations

Cells were periodically observed for morphological changes using an inverted light microscope (Nikon, TS 100-F; Tokyo, Japan), then stained with acridine orange/ethidium bromide (AO/EB) and observed under a confocal laser scanning microscope (CLSM) (Carl Zeiss LSM 510Meta, Jena, Germany).

Statistical analysis

All experiments were done in triplicates, along with positive and negative controls. To minimize experimental errors, exponentially growing cells of the same passage were used for each of the experiments. Data on percent cell viability were recorded and subjected to paired *t*-tests. *P*-values less than 0.05 were considered statistically significant.

Results and discussion

Establishment of aseptic leaf cultures and extraction yield

Callus cultures were induced from leaf discs incubated on MS medium supplemented with 5 μM BA, 1 μM 2,4-D and 1 μM NAA. With this combination, explants first turned brown, but after one week, bright green, hard, compact calli began to develop along margins of leaf discs. Although, callus proliferation increased with subsequent subcultures, callus was friable and soft, but remained dark-brown in color throughout seven passages, at 4-week intervals. By 26 weeks, vigorously growing cream and healthy callus was obtained (Fig. 1a–d). Previously, it has been suggested that addition of anti-oxidants such as ascorbic acid, polyvinylpyrrolidone (PVP), polyvinylpolypyrrolidone (PVPP) overcome browning and death of explants (Muhammad and Jaiswal 1987; Naik et al. 1999). Moreover, repeated subculturing at regular intervals was also reported to overcome browning of cultures (Rout et al. 1999). In this study, repeated subculturing reduced phenolic compound accumulation, released from explants.

Recently, cell cultures have been used for the production of various groups of secondary metabolites (Alfermann and Petersen 1995). Competence of undifferentiated callus cultures is critical for production of secondary metabolites (Wewetzer 1998; Kuruvilla et al. 1999; Prakash et al. 2002). As plant cells are biosynthetically totipotent; i.e., cells in culture retain complete genetic information, consequently, they are capable of producing metabolites found in the mother plant (Rao and Ravishankar 2002). Heterogeneity in biochemical activity within a population of cells derived from the same plant species or even various explants from the same plant can be exploited to obtain highly productive cell lines (Evans et al. 1984).

In this study, calli differentiated on leaf explants yielded 0.23% (w/w) of the aqueous extract from 50 g dry weight of cells. The aqueous extract was further utilized for cytotoxic assays on HeLa and BHK-21 cells. Cytostatic effects of whole plant extracts are often superior to particular biologically active compounds on cancer cells (Mishra et al. 2008).

Cell viability assay

Based on MTT assays of treated HeLa and BHK-21 cells, concentrations of aqueous extract below 1,000 μg/ml did not elicit any significant cytotoxic effect (data not shown) and, thus, levels higher than 1,000 μg/ml were

Fig. 1 Establishment of aseptic cell lines of *L. camara*. **a** Dedifferentiating leaf disc explant of *Lantana* after 2 weeks of culture (5×); **b** A 13-week-old culture showing deep brown, soft and friable callus after seven passages (2×); **c** An 18-week-old callus showing a mixture of brown and green cells after ten passages (1.02×); **d** A 26-week-old culture showing healthy, fresh, friable cream callus after 14 passages (2×)

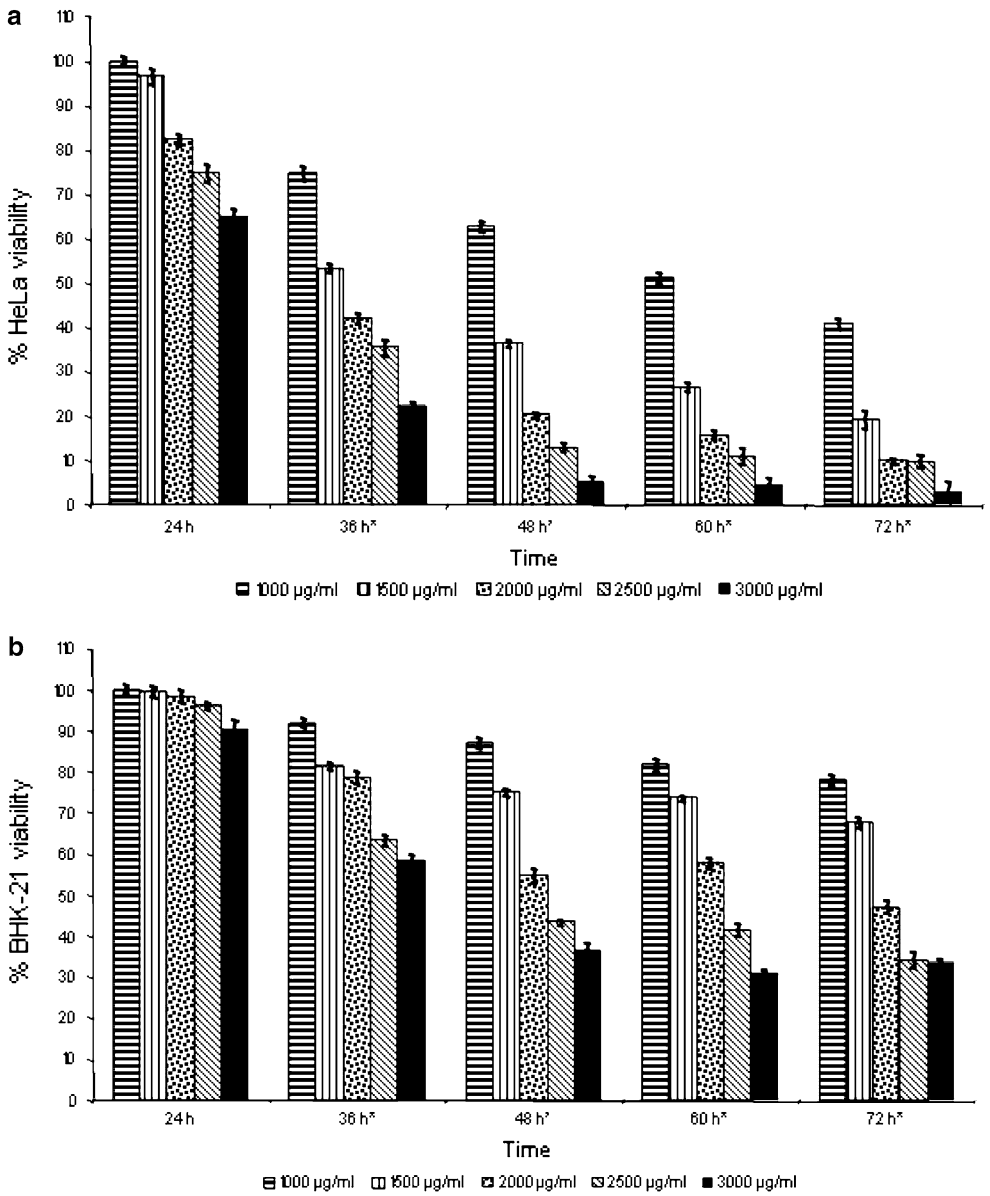

Fig. 2 Frequency of viability of cells treated with aqueous extract of *L. camara* **a** HeLa cells; **b** BHK-21 cells. Means are significantly different at *P*-value < 0.05 (*)

subsequently used. Results indicated that the extract had a dose and time dependent activity (Fig. 2a, b). After 36 h, aqueous extract levels ranging from 1,500 to 3,000 µg/ml showed significant cytotoxicity against HeLa cells. As the duration increased, there was a marked decline in frequency of survival of HeLa cells (Fig. 3); however, necrosis was predominant over apoptosis. At a similar concentration range (1,500–3,000 µg/ml), the effect of the extract on normal BHK-21 cells was negligible ($P < 0.05$ for 36 to 72 h). Curcumin, another plant-derived anticancer agent, was used as a positive control under similar experimental conditions (Fig. 4), for both cell types. IC_{50} values varied from one compound to another and from one cell line to another as there were no set values. Mishra et al. (2008) have reported that although crude extracts have high IC_{50} values, cytostatic effects of whole plant extracts are more efficacious than individual pure compounds due to synergistic effect of various compounds present in the extract.

To our knowledge, this is the first report on exhibition of anticancerous activity from aqueous extract of *Lantana* cell cultures. This provides a controlled system that will support production of uniform quality and quantity of secondary metabolites, all year round. Thus, it may bypass the need to utilize *L. camara* plants growing under variable climatic and geographical conditions.

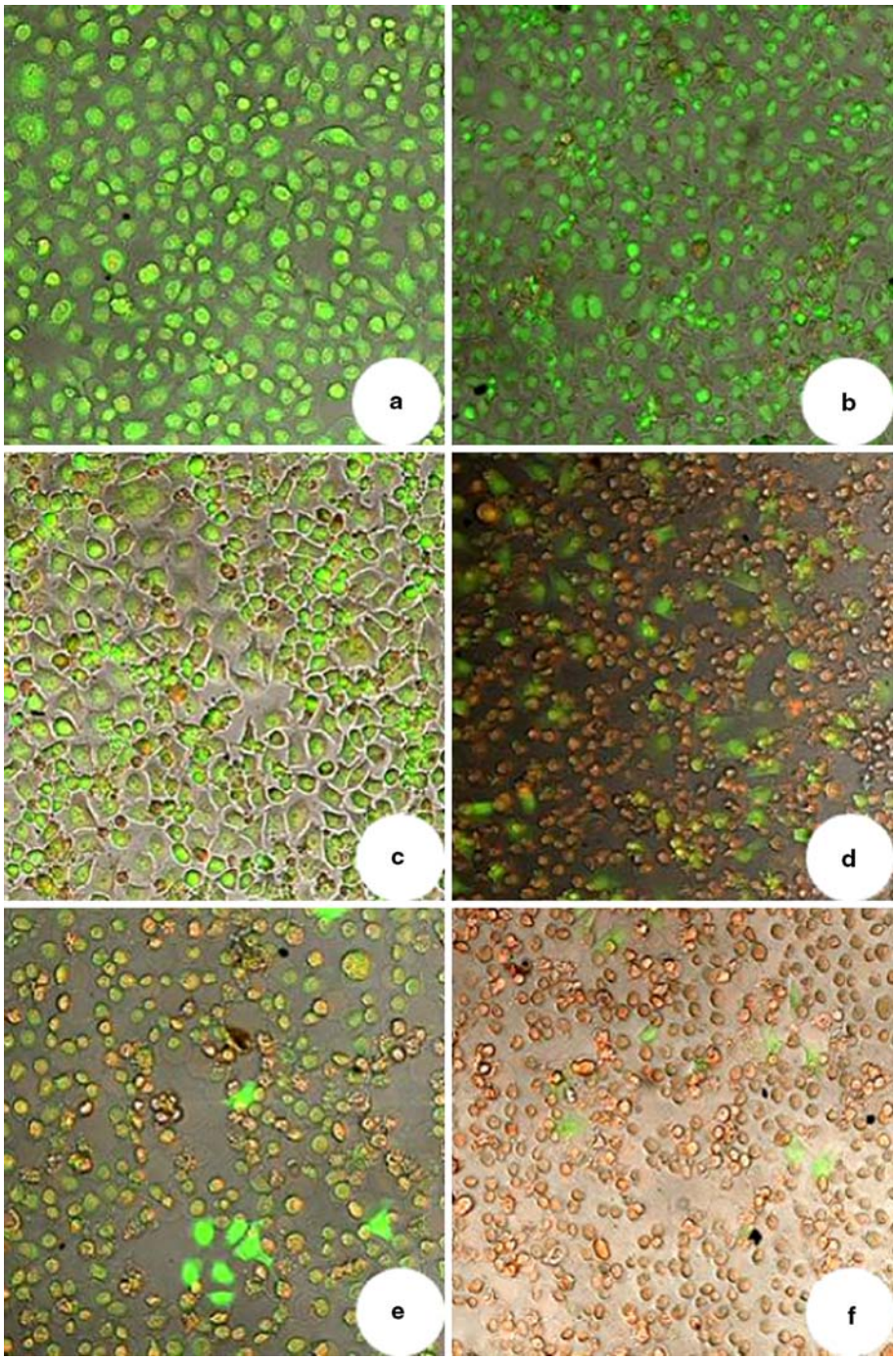

Fig. 3 HeLa cells stained with AO/EB and viewed under confocal laser scanning microscope (50×). **a** Control healthy cells; **b** HeLa cells treated with aqueous extract of *L. camara* for 24 h showing bright green nuclei indicating initiation of chromatin condensation; **c** HeLa cells treated aqueous extract of *L. camara* for 36 h showing zones of cleared monolayer; **d–f** HeLa cells treated with aqueous extract of *L. camara* for 48–72 h showing gradual increase in frequency of dead cells, orange stained

Morphological observations of cell death

Morphological observations of treated HeLa cells showed characteristic features of programmed cell death. These included reduced cell volume, nuclear shrinkage, condensed masses of chromatin, cytoplasmic membrane blebbing, cytoplasmic and nuclear fragmentation resulting in formation of apoptotic bodies. In contrast, a small

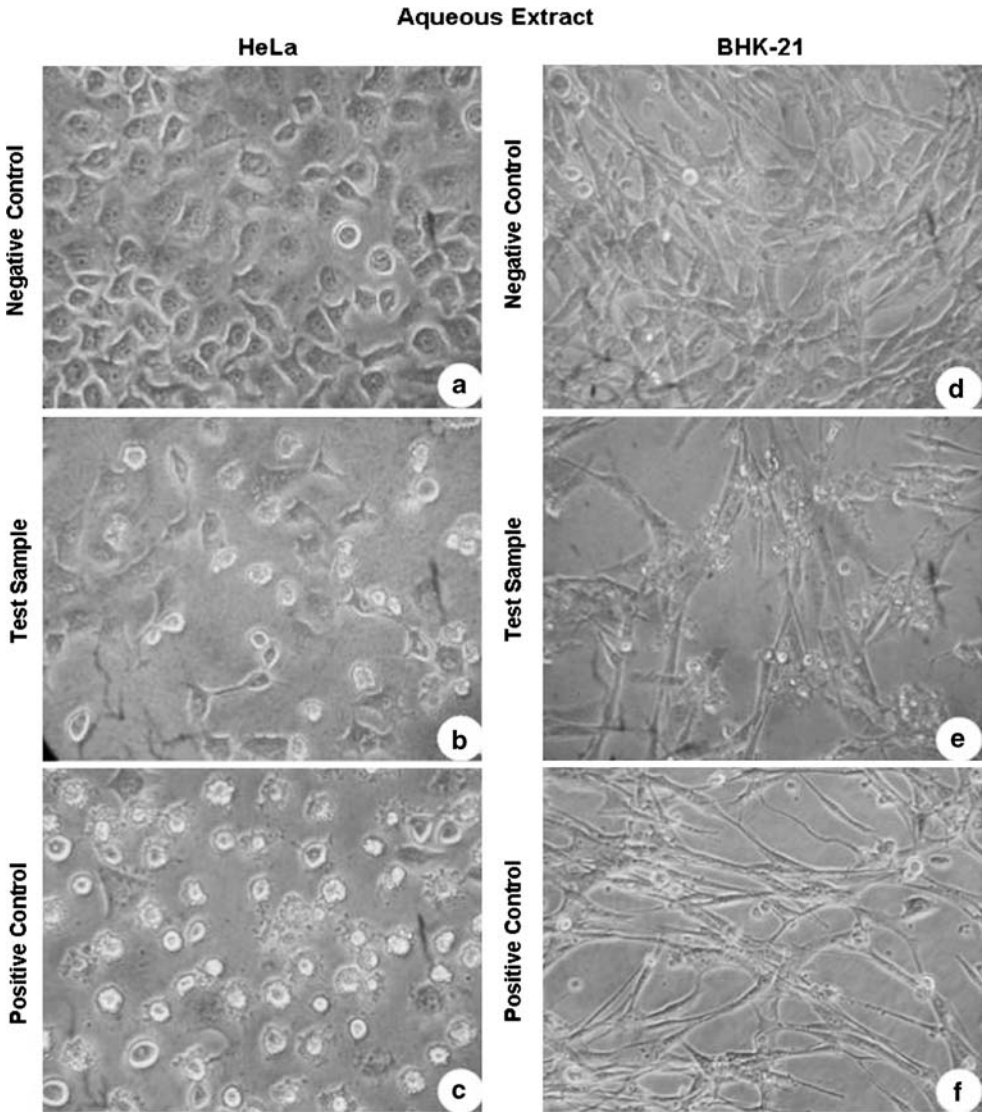

Fig. 4 Morophological observations of HeLa and BHK-21 cells under light microscopy (40×). **a**. Untreated HeLa cells; **b** HeLa cells treated with aqueous extract of *L. camara* showing rounded apoptotic bodies; **c** HeLa cells treated with curcumin showing similar cell death patterns as (**b**); **d** Untreated BHK-21 cells; **e** BHK-21 cells treated with aqueous extract of *L. camara* showing low frequency of apoptotic bodies when compared to HeLa cells; **f** BHK-21 cells treated with curcumin showing similar cell death patterns as in (**e**)

number of cells exhibited characteristic features of necrosis; i.e., increased volume of nucleus and cytoplasm, vacuolization of the cytoplasm, chromatin flocculation, dissolution of nuclear membrane, and dissolution of cytoplasmic membrane leading to cell lysis.

These morphological changes were further confirmed by CLSM wherein increased incubation time resulted in an increase in ratio of dead (stained orange with Ethidium Bromide) to live cells (stained green with Acridine Orange) (Fig. 3). Ability to induce apoptotic mode of death in tumor cells is one of the most desirable properties of any potential anti cancer drug (Reddy et al. 2003). Similar cell death patterns were observed in curcumin treated cells (Fig. 4) used as positive controls.

Conclusion

The aqueous extract isolated from in vitro-derived cell cultures of *Lantana* exhibited promising anti-proliferative activity on HeLa cells. The minimal activity of the extract on normal BHK-21 cells verifies its potential as a feasible

anti-cancer agent and also substantiates the value of callus cultures as source of high value metabolites.

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