Contents lists available at UGC-CARE

International Journal of Pharmaceutical Sciences and Drug Research

[ISSN: 0975-248X; CODEN (USA): IJPSPP]

Available online at www.ijpsdronline.com

Research Article

# Evaluation of Inhibition of Proliferation of SK-Mel-28 Cell Lines by *Acmella ciliata* (Kunth) Cass. Cell Biomass

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ARTICLE INFO

**Article history:**

Received: 29 April, 2021

Revised: 11 February, 2022

Accepted: 19 February, 2022

Published: 30 March, 2022

**Keywords:**

*Acmella ciliata*, Anticancer activity, Cell biomass, Cell suspension culture, HPLC, Spilanthol.

**DOI:**

10.25004/IJPSDR.2022.140204

ABSTRACT

*Acmella ciliata* (Kunth) Cass., a medicinally important plant in the family Asteraceae, contains many phytochemicals like alkaloids, tannins, saponins, flavonoids, including a highly important alkylamide 'Spilanthol' which accounts for most of its pharmacological applications. The plant has rich commercial value because of its traditional phytomedicinal uses. The present study focused on the anticancer potentiality of cell biomass of *A. ciliata* obtained through cell suspension culture. The purplish friable callus raised in MS medium containing 0.5 $mgl^{-1}$ BA and 1.0 $mgl^{-1}$ NAA were cultured in liquid MS medium with same hormone combination. The cells after 28 days of culture were collected and quantification of spilanthol using high performance liquid chromatography (HPLC) revealed that the harvested cells from cell suspension culture of *A. ciliata* recorded 173.702 $\mu gg^{-1}$ spilanthol content in them. The analysis of antiproliferative effect of the methanolic extract of the cell biomass revealed that it is capable of inhibiting or reducing the growth of SK-MEL-28 cell lines and a lower concentration i.e., 24.144 $\mu gmL^{-1}$ of extract was needed to inhibit the growth by 50%. The study suggested the effectiveness of the cell biomass extract for the inhibition of uncontrolled growth of this melanoma cells and thus throw light on the possibility of formulating an anti-cancerous drug from this resource thereby benefiting the pharmaceutical industry.

## Introduction

Cancer, the abnormal growth of cells which tend to proliferate in an uncontrolled way is the second top killer disease after myocardial infarction causing a large number of population worldwide over the past 15 years. The causal factors for the same are environmental causes, mutation, poor eating habits, excess intake of junk foods, lack of exercise, using tobacco and UV-ionising radiations and various other aspects. To some extent it is a preventable disease as the risk can be reduced by the avoidance of causable factors along with habitual consumption of cancer protective foods. There are more than 100 types of cancer that are usually named for the organs or tissues where the cancers form. Cancer of the skin is by far the most common of all cancers, among which melanoma accounts for only about 1% of skin cancers but causes a large majority of skin cancer deaths. Currently, between 2 and 3 million non-melanoma skin cancers and 132,000 melanoma skin cancers occur globally each year. Melanoma is the most serious type of skin cancer, develops in the cells (melanocytes) that produce melanin, the pigment that gives your skin its color. Melanoma can also form in your eyes and, rarely, inside your body, such as in your nose or throat. The treatment for this may depend on the type and stage of cancer and also goes with the overall health of the patient. Several immunotherapy drugs have been approved to fight cancer, and hundred mores are being tested in clinical trials, but like any other treatments, the

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**Relevant conflicts of interest/financial disclosures:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

use of synthetic drugs in chemotherapy for the cure of this disease has got potential risks and side effects. There lies the importance of phytodrug designing which will benefit the patients suffering from this disease with low or no side effects and there are several scientific reports on the pharmacological activity of bioactive components from plants that are cancer suppressants.[1] The use of herbal drugs instead of synthethic formulations are increasing day by day in an alarming rate for the last few decades. It may be due to the disenchantment with modern synthetic drugs which are made by synthetic chemistry, and most of the core structures (scaffolds) for synthetic compounds are based on natural products. Alternative avenues for plant derived compounds for herbal drugs have gained importance in the past decade and the isolation of reserpine from *Rauvofia serpentina* and it pharmacological use as an effective antihypertensive and tranquilizing agent was a major breakthrough in the first half of the 20$^{th}$ century. Later, the isolation of vinblastine and vincristine from *Catharanthus roseus*, an efficient anticancer drug, diosgenin from *Dioscorea deltoidea*, solasodine, from *Solanum aviculare* and *Solanum laciniatum* that are used in the synthesis of steroid hormones indicated the importance of herbal medicine. Currently, there are various plant derived drugs used for cancer treatment which includes vincristine and vinblasine from *Catharanthus*, Paclitaxel from *Taxus brevifolia,* Camptothecin from *Camptotheca acuminata*, Combretastatin from *Combretum caffrum,* Homoharrigtonine from *Cephalotaxus harringtonia,* Roscovitine from *Raphanus sativus* and so on. Most of these are semisynthetic drugs wherein some neoplastic agents were added to the plant derived phytodrug so as to increase the efficacy of the formulation. These anti-cancerous compounds occur at very low levels in the plant tissues and their synthetic production is too expensive because of their complex structure. Consequently, the challenge to produce natural compounds in a sustainable way implies that improved methods of supply of anticancer agents would be desirable and instead of exploring single anticancer molecules, combination of molecules derived from natural extracts seems to be ideal for the development of an anti-cancer experimental approach.

Medicinal plants are the major source of phytochemicals or natural compounds which exhibit therapeutic values and nearly two thirds of the active ingredients in anticancer drugs are of plant origin.[2] Callus cultures and cell suspension cultures are often used successfully to produce secondary metabolites of medicinal significance many of which are used in treating human diseases.[3] Plant cell culture is an effective means for the large scale production of therapeutically relevant bioactive metabolites particularly in circumstances when the natural supply is limited by low yields, slow growth rates or both the cases.[4] Findings regarding the anti-tumor activity of cultured cells of certain plant species revealed growth inhibitory activity against various types of cancers.[5-7] Plant cell suspension culture system is a sustainable way which can be automated to get enhanced production levels of desired compounds more systematically. So here we have explored the potentiality of cells harvested from cells suspension culture system of *Acmella ciliata* (Kunth) Cass. (basionym: *Spilanthes ciliata* Kunth; Asteraceae) against SK-MEL-28 human melanoma cell lines. The plant possesses diverse bioactive properties and immense utilization in medicine, health care, cosmetics and as health supplements.[8] Also, the taxa has got various pharmacological activities like antioxidant, anti-inflammatory, anti-helminthic, antifungal, antimalarial, insecticidal, hepatoprotective, antimutagenic property, immunomodulatory activity and even used as an analgesics all of which are attributed to the presence of an important bioactive compound named 'spilanthol', an alkylamide.[9,10] Alkamides/Spilanthol is a promising secondary metabolite with multipurpose medicinal applications. Several preparations of the spilanthol are available in the market like: Oral gels-Buccaldol® from Alphamega, France and Indolphar® from ID Phar, Belgium,[11] Mouthwash-Dentaforce mouthspray and mouthwash, Antiseptic tincture- Vogel spilanthes tincture containing 65% ethanol from Biohorma, Belgium), Anti- wrinkle cream[12] and Anti-aging products- Gatuline®, SYN®-COLL, ChroNOline™.[13] The intrinsic local pharmacokinetics study of spilanthol on topical application on human skin revealed that it has permeability through skin.[14] Also a transmucosal mode of application has reported rich microcirculation with direct drainage of blood into the internal jugular vein permits systemic effects of permeated alkamide molecules through buccal mucosa.[15,16] Moreover, it has also been reported to have the capacity to cross blood-brain barrier after entering into systemic circulation.[13] One study showed that spilanthol was able to inhibit the tyrosinase enzyme, which have good commercial potential on formulations for topical use (skin and hair), since it may prevent and/or slow skin hyperpigmentation or depigmentation processes.[17] As a study regarding the antiproliferative effect of callus/ cells of this taxa has not yet reported, we focussed on determining the efficacy of cell biomass extract on SK-MEL-28 human melanoma cell lines. So the present study has conducted with a motive in order to check the ability of *A. ciliata* cell biomass to inhibit the proliferation of SK-MEL-28 cell lines.

## Materials and Methods

### Plant Material

*A. ciliata* (Kunth) Cass. (Syn. *Spilanthes ciliata* Kunth) (Herbarium Voucher Nos. TBGT 32710-32711) collected from Aruvikkara, Thiruvananthapuram, Kerala, India, during May 2016 and maintained in the green house of Department of Botany, University College, Thiruvananthapuram, Kerala, India served as the source of explants for establishing the cell suspension culture system.

## Establishment of Callus and Cell Suspension Culture

For callus induction, *in vitro* leaves from established shoot cultures of *A. ciliata* were cut in to appropriate size (1-2 $cm^2$) and inoculated to Murashige and Skoog (MS) medium[18] supplemented with 0.5, 1.0, 2.0 $mgl^{-1}$ NAA/ IAA/IBA/2,4-D either individually or in combination with 0.5 $mgl^{-1}$ BA. Approximately 500 mg callus obtained in MS medium fortified with 0.5 $mgl^{-1}$ BA in combination with 1.0 $mgl^{-1}$ NAA was transferred to 100 ml liquid MS medium supplemented with the same combination and concentration of BA-NAA. The cultures were incubated at 25 ± 2°C with a photoperiod of 8/16 hours light/dark period on a gyratory shaker (Orbitek, Scigenics) at varying rotatory speeds (60, 90, 120 and 150 rpm) in order to standardize the suitable rotation speed for the growth of cells. At regular time interval (4, 7, 10, 13, 16, 19, 22, 25, 28 and 31 days), the cells were collected and the fresh weight and dry weight of the cells were recorded for analyzing the growth kinetics. Finally, growth curve was drawn using the parameters *viz*. culture period (days) in X-axis and dry weight of the suspension cultured cells (g) in Y-axis. After 25 days of inoculation, the cells grown in MS medium containing 0.5 $mgl^{-1}$ BA in combination with 1.0 $mgl^{-1}$ NAA were harvested by vacuum/ suction filtration for further analysis.

## Sample Preparation of Cell Biomass

The cells harvested from cell suspension culture system in MS liquid medium supplemented with 1.0 $mgl^{-1}$ NAA and 0.5 $mgl^{-1}$ BA were collected by vacuum/suction filtration (using 0.45 µm size membrane filters), blot dried and weighed. The weighed samples were then undergone drying at 40°C. The dried samples were ground in to powder using mortar and pestle and again weighed. The cells (0.1 g) were lyophilized and extracted with 1-mL 80% (v/v) methanol for overnight at 150 rpm in a rotating shaker. The resulting homogenate was then centrifuged at 10,000 rpm for 10 minutes and the supernatant was employed for the *in vitro* assays.

## Spilanthol Quantification by HPLC

The amount of spilanthol present in cells harvested from cell suspension culture was determined by HPLC analysis in Varian Prostar HPLC system (Varian, USA) consisting of Ultraviolet (UV) detector, a prostar binary pump and a 20-lL injection loop. Hypersil BDS RP-18 column (Thermo, USA) of dimensions 4.6 9 250 mm was used with acetonitrile:water (93:7) as mobile phase at a flow rate of 0.5 $mlmin^{-1}$. The eluted samples were detected at 237 nm. The peaks obtained in HPLC was identified by comparing with published data. As there was no commercially available standard for spilanthol, it was tentatively quantified on the basis of another compound, dodeca-2(E), 4(E)-dienoic acid isobutylamide (Chromadex, USA), which is structurally similar to spilanthol. Both the compounds contain an isobutylamide group and long carbon chain. Stock solutions (1000 $lgml^{-1}$) of dodeca-2(E),4(E)-dienoic acid isobutylamide, was prepared by dissolving 5 mg of the compound in 5 mL of HPLC-grade methanol. The solution was then stored at -20°C. Quantification was carried out using five levels of external standards obtained by serial dilutions of stock solutions at a concentration range of 250–15 $lgml^{-1}$. Each concentration of standard was filtered through a 0.22 µm nylon membrane filter (Millipore, USA) before HPLC analysis. Method linearity was demonstrated by determining a calibration curve, injecting standard at different concentrations and calculating the regression coefficient ($r^2$). The obtained value through slope equation was used to calculate the amount of the spilanthol in unknown samples. Spilanthol content was reported as $\mu gg^{-1}$ DW of sample.

## Antiproliferative Effect by MTT Method

For *in vitro* antiproliferative effect determination, SK-MEL-28 cell line was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecos modified eagles medium (DMEM) (HiMedia). The cell line was cultured in 25 $cm^2$ tissue culture flask with DMEM supplemented with 10% Foetal bovine serum (FBS), L-glutamine, sodium bicarbonate and antibiotic solution containing: penicillin (100 U/mL),streptomycin (100 $\mu gml^{-1}$) and amphoteracin B (2.5 $\mu gml^{-1}$). Cultured cell lines were kept at 37°C in a humidified 5% $CO_2$ incubator (Galaxy® 170 Eppendorf, Germany). The viability of cells were evaluated by direct observation of cells via Inverted phase contrast microscope and followed by MTT assay method.[19]

## Cells Seeding in 96 Well Plate

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium; 100 µL cell suspension (5×104 cells/well) was seeded in 96 well tissue culture plate and incubated at 37°C in a humidified 5% $CO_2$ incubator.

## Preparation of Plant Extracts and Compound Stock

1-mg of the methanolic cell biomass extract of *A. ciliata* was added to 1 ml of DMEM and dissolved completely by cyclomixer. After that the extract solution was filtered through 0.22 µm Millipore syringe filter inorder to ensure the sterility.

## Antiproliferative Effect Evaluation

After 24 hours, the growth medium was removed, freshly prepared samples in 5% DMEM were 5 times serially diluted by two fold dilution (6.25, 12.5, 25, 50 and 100 µg in 100 µL of 5% DMEM) and each concentration of 100 µL were added in triplicates to the respective wells and incubated at 37°C in a humidified 5% $CO_2$ incubator.

## Antiproliferative Effect by Direct Microscopic Observation

Entire plate was observed at an interval of each 24 hours, up to 72 hours in an inverted phase contrast microscope (Labomed TCM-400 with MICAPSTM HD camera) and

microscopic observation were recorded as images. Any detectable changes especially in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

### Antiproliferative Effect by MTT Method

Fifteen mg of MTT (HiMedia, M-5655) was reconstituted in 3 mL PBS (Phosphate buffer saline) until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30 µL of reconstituted MTT solution was added to all test and control cell wells, the plate was gently shaken well, then incubated at 37°C in a humidified 5% $CO_2$ incubator for 4 hours. After the incubation period, the supernatant was removed and 100 µL of MTT solubilisation solution i.e. dimethylsulfoxide (DMSO) was added and the wells were mixed gently by pipetting up and down in order to solubilise the formazan crystals. The absorbance values were measured by using micro plate reader at a wavelength of 570 nm.

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control group}} \times 100$$

The results of MTT assay were expressed as mean ± SE and were analysed by ANOVA and the means were compared by t test at $p \leq 0.05$. $IC_{50}$ concentration (i.e., the concentration of the sample that causes 50% inhibition) was also calculated using ED 50 PLUS V1.0 Software.

## Results

### Establishment of Cell Suspension Culture System in *A. ciliata*

Callus cultures were established from *in vitro*-derived leaf explants of *A. ciliata* using different concentrations (0.5,

**Table 1:** Effect of plant growth regulators on callus induction in *A. ciliata*

| *Plant growth regulators ($mgl^{-1}$)* | | | | | *% of callus induction* | *Morphology of callus* | *Colour* | *Amount of callus* |
|---|---|---|---|---|---|---|---|---|
| *BA* | *2,4-D* | *NAA* | *IAA* | *IBA* | | | | |
| - | 0.5 | - | - | - | 65.24 ± 0.12$^{h}$ | Friable | Black | ++ |
| - | 1.0 | - | - | - | 72.36 ± 0.58$^{g}$ | Friable | Black | ++ |
| - | 2.0 | - | - | - | 25.41 ± 0.40$^{k}$ | Friable | Black | + |
| 0.5 | 0.5 | - | - | - | 63.28 ± 0.57$^{i}$ | Friable | Black | ++ |
| 0.5 | 1.0 | - | - | - | 58.43 ± 0.64$^{j}$ | Friable | Black | ++ |
| 0.5 | 2.0 | - | - | - | --- | --- | --- | --- |
| - | - | 0.5 | - | - | 91.54 ± 0.45$^{c}$ | Compact, rhizogenic | White | ++ |
| - | - | 1.0 | - | - | 95.24 ± 0.84$^{b}$ | Compact, rhizogenic | White | ++ |
| - | - | 2.0 | - | - | 92.48 ± 0.36$^{c}$ | Compact, rhizogenic | White | + |
| 0.5 | - | 0.5 | - | - | 75.63 ± 0.84$^{f}$ | Compact | Off white | ++ |
| 0.5 | - | 1.0 | - | - | 97.28 ± 0.53$^{a}$ | Friable | White purplish | +++ |
| 0.5 | - | 2.0 | - | - | 95.36 ± 0.64$^{b}$ | Friable | White purplish | +++ |
| - | - | - | 0.5 | - | 89.57 ± 0.72$^{d}$ | Compact more Rhizogenic | White | ++ |
| - | - | - | 1.0 | - | 92.42 ± 0.84$^{c}$ | Compact more Rhizogenic | White | ++ |
| - | - | - | 2.0 | - | 88.63 ± 0.45$^{d}$ | Compact more Rhizogenic | White | ++ |
| 0.5 | - | - | 0.5 | - | 72.27 ± 0.94$^{g}$ | Compact | White | ++ |
| 0.5 | - | - | 1.0 | - | 80.18 ± 0.49$^{e}$ | Compact caulogenic | White, purplish | +++ |
| 0.5 | - | - | 2.0 | - | 82.43 ± 0.63$^{e}$ | Compact | White, purplish | +++ |
| - | - | - | - | 0.5 | 91.68 ± 0.62$^{c}$ | Compact rhizogenic | Cream | ++ |
| - | - | - | - | 1.0 | 94.47 ± 0.35$^{b}$ | Compact rhizogenic | Cream | ++ |
| - | - | - | - | 2.0 | 90.18 ± 0.17$^{c}$ | Compact rhizogenic | Cream | ++ |
| 0.5 | - | - | - | 0.5 | 81.34 ± 0.24$^{e}$ | Compact | Cream | ++ |
| 0.5 | - | - | - | 1.0 | 76.49 ± 0.15$^{f}$ | Compact | Cream | +++ |
| 0.5 | - | - | - | 2.0 | 79.25 ± 0.48$^{e}$ | Compact | Cream | +++ |

+ ~ 0.25 mg callus; ++ ~ 0.5 mg callus; +++ ~ 1.0 mg callus; --- indicates no response

Data represents mean values ± SE of 10 replicates repeated thrice, recorded after 4 weeks of culture. The mean values followed by the same letter in the superscript in a column do not differ significantly based on ANOVA & t-test at $p \leq 0.05$.

1.0 and 2.0 $mgl^{-1}$) of 2,4-D, NAA, BA and Kinetin at either in alone or in combination (Table 1). Relatively better response was noticed in NAA-BA combination. Callus cultures established from MS medium augmented with 0.5 $mgl^{-1}$ BA and 1.0 $mgl^{-1}$ NAA produced purplish friable callus having some embryogenic cells (Fig.1a). So for further suspension culture establishment, this callus served as the stock for the suspension inoculum and approximately 500 mg callus was transferred to fresh liquid medium (100 mL) of the same composition in 250 mL Erlenmeyer flasks and kept in a gyratory shaker at 120 rpm (Fig. 1b).

## Growth Kinetics of Cells During Cell Suspension Culture

Growth analysis was carried out periodically by taking an inoculum of cells after every 3 days interval from $4^{th}$ day onwards. It was observed that from the $5^{th}$ day itself, the cell division started. Maximum cell division and biomass production was noticed after $25^{th}$ days of inoculation and after that stationary phase occurred. The cells grown in MS medium supplemented with 0.5 $mgL^{-1}$ BA and 1.0 $mgL^{-1}$ NAA exhibited better multiplication with 4 fold increase in the cell biomass after 28 days of culture (Fig. 1c). The growth curve exhibited a characteristic S- shaped growth curve (Fig. 2).

## HPLC Analysis for the Quantification of Spilanthol

HPLC revealed that the harvested cells from cell suspension culture of *A. ciliata* recorded 173.702 $\mu gg^{-1}$ spilanthol content in them. Fig. 3 shows the HPLC spectrum of spilanthol in samples obtained from cell suspension cultures.


**Fig.1:** Harvesting of cell biomass from cell suspension culture in *A. ciliata*. a- purplish friable callus MS medium augmented with 0.5 $mgl^{-1}$ BA and 1.0 $mgl^{-1}$ NAA, b- cell suspension culture after 25 days, **c**- Cells harvested from cell suspension culture after 28 days

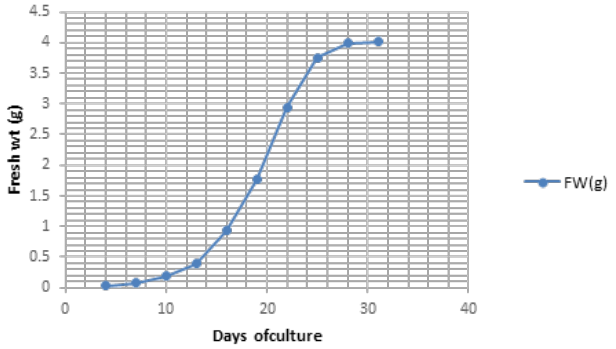


**Fig. 2:** Growth curve of cells during suspension culture in *A. ciliata*

## Antiproliferative Effect of *A. ciliata* Cell Biomass by MTT Assay

*In-vitro* antiproliferative effect of *A. ciliata* cell biomass was determined by MTT assay using SK-MEL-28 cell lines. In this study, when lowest concentration of sample *viz*. 6.25 $\mu gml^{-1}$ methanolic extract of *A. ciliata* cells was added, maximum viable cells were noticed (90.90) (Table 2). As the concentration of extract increased, the cell viability got decreased. Here, the cells got ruptured or dead as the concentration of the sample got increased which shows the efficacy of the plant extract towards the SK-MEL-28 cell lines. As the concentration of the extract got increased the cells lines viability become lower thereby showing the ability of the extract to stop the unlimited proliferation of cells. $IC_{50}$ was noticed in 24.144 $\mu gml^{-1}$ of extract in this experiment.

## Morphological Observation

Light microscopic observation of methanolic cell biomass extract of *A. ciliata* on SK-MEL-28 cell lines showed typical morphological features of apotopsis *viz*. cell shrinkage,

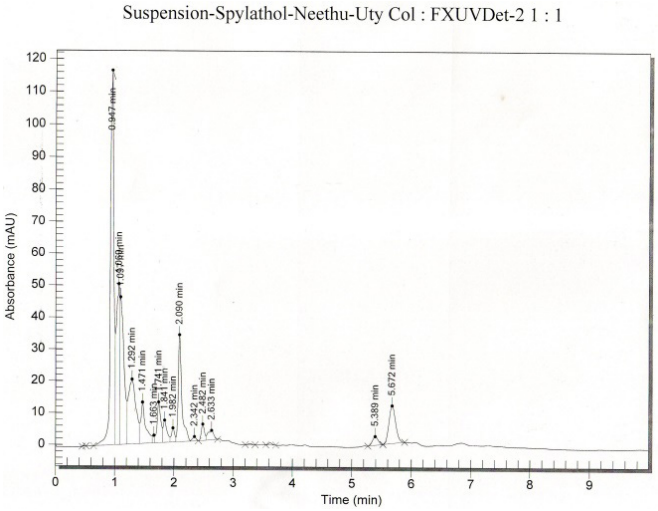


**Fig 3:** HPLC chromatogram of spilanthol in cell biomass from cell suspension cultures of *A. ciliata*

**Table 2:** Anti proliferative effect of *A. ciliata* cell biomass by MTT Assay

| *Concentration ($\mu gml^{-1}$)* | *Percentage viability* |
|---|---|
| Control | $100.00 \pm 0.00^{a}$ |
| 6.25 | $90.90 \pm 0.11^{b}$ |
| 12.5 | $78.78 \pm 0.38^{c}$ |
| 25 | $51.51 \pm 0.82^{d}$ |
| 50 | $36.36 \pm 0.02^{e}$ |
| 100 | $18.18 \pm 0.20^{f}$ |
| $IC_{50}$ value of extract: 24.144 ($\mu gml^{-1}$) | |

Data represents mean ± SE values of triplicate measurements repeated thrice. The mean values followed by the same letter in the superscript in a column do not differ significantly based on t-test at $p \leq 0.05$.

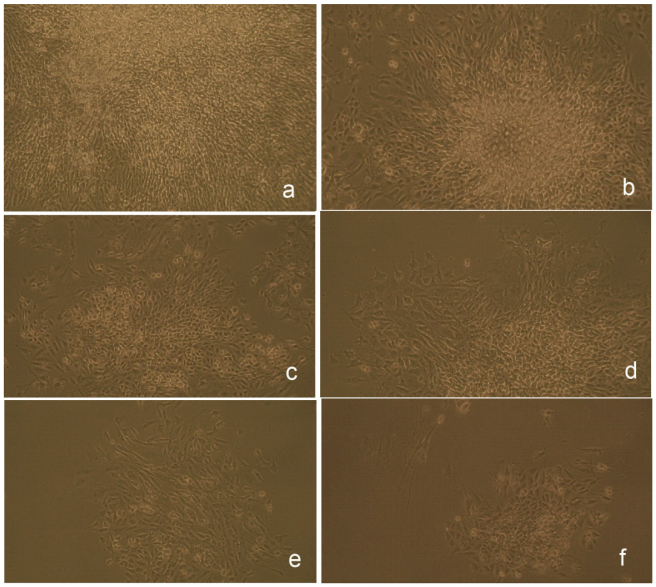


**Fig. 4:** Antiproliferative activity of *A. ciliata* cell biomass extract against SK-MEL-28 cell lines at different concentrations. (a) Control (b) 6.25 µgml$^{-1}$ (c) 12.5 µgml$^{-1}$ (d) 25 µgml$^{-1}$ (e) 50 µgml$^{-1}$ (f) 100 µgml$^{-1}$

reduction in chromatin condensation and cytoplasmic blebs (Fig. 4) thereby describing the potential of *A. ciliata* cell biomass extract as an anticancer drug.

## Discussion

Cell suspension culture technique is more preferable than any other techniques for the large scale production of secondary metabolites naturally due to its rapid growth cycles. They have been widely used for generating large amounts of cells for improving the quantity and production of novel chemicals from the cell cultures within a limited time and area. Nowadays, several cell culture techniques have been developed for the production of valuable secondary metabolites with high medicinal values. In the present study, friable callus procured from MS medium containing 0.5 mgL$^{-1}$ BA and 1.0 mgL$^{-1}$ NAA have succeeded in producing fourfold increase in cell mass after 25 days of culture upon 120 rpm agitation in a gyratory shaker. In *Spilanthes acmella*, maximum viable cultures were obtained at a rotation speed of 120 rpm thereby supporting the present finding.[20] Among various rotation speed tested, cells grown at 120 rpm agitation speed was found to be suitable for better production of withanolides in *Withania somnifera*.[21] In addition to this, for the production of oleanolic acid in *Lantana camara* 120 rpm was most suitable speed of agitation in suspension cultures.[22] For initiating cell suspension cultures in *Scrophularia striata*, 0.5 mgl$^{-1}$ NAA and 2.0 mgL$^{-1}$ BA combination was proved to be better for producing 20 fold increase in the cell mass when compared to initial mass within 15 days of inoculation.[23] In the present experiment, maximum cell mass was received on 25$^{th}$ day of inoculation in the same PGR combination as in *S. striata*. In a previous study conducted in *S. acmella*, 2703 µgg$^{-1}$ spilanthol content was noticed in *in vivo* leaves, 3294.36 µgg$^{-1}$ in *in vitro* leaves, 998.03 µgg$^{-1}$ in callus and 91.4 µgg$^{-1}$ in cell suspension cultures of *S. acmella* upon HPLC analysis.[20] High temperatures and low pressures are essential for obtaining high content of spilanthol in the extracts,[24] whereas the extract taken by supercritical $CO_2$ of *Acmella oleracea* which are lyophilized showed maximum spilanthol content (1.07%) than the dried samples.[8] The present study reported a more effective bioproduction system for the biosynthesis of spilanthol in *A. ciliata via* cell suspension culture and the harvested cells recorded 173.702 µgg$^{-1}$ spilanthol content in them. A highly improved two fold production (239.512 µgg$^{-1}$) of spilanthol in callus compared to the *in vivo* source plant which yielded 92.198 µgg$^{-1}$ spilanthol content was accomplished here which can be further scaled up by appropriate bioreactor technology.

The present study has investigated the cytotoxic property of methanolic extract of *A. ciliata* cell mass on SK-MEL-28 cell lines. Cancer cells treated with any drug shows morphological features of apotopsis that includes, cell shrinkage, membrane blebbing, nuclear condensation and apotoptic body formation. The characteristic widely accepted for an anticancer drug is the capacity of induction of apotopsis rather than necrosis.[25] Light microscopic observation of methanolic cell biomass extract of on *A. ciliata* on SK-MEL-28 cell lines showed typical morphological features of apotopsis thus describing the potential of *A. ciliata* cell biomass extract as an anticancer drug.

Plant extracts of *Spilanthes* spp. has been explored to investigate their anticancer potentiality in different types of cancer cell lines.[26-28] *S. acmella* extract served as a source of bioactive compounds for the treatment of HEP-2 and HT-29 cancer cell lines and may be used as anticancer drug.[29] *S. acmella* plant extract was most potent on the lymphoma (Dalston's lymphoma ascites) cells with an $IC_{50}$ of 147.547 µg/ml, while it has no effect on lung carcinoma (V79).[27] Some other Asteraceae species were reported to exert cytotoxicity on different cell lines. Extracts taken from *Urtica membranacea*, *Artemesia monosperma* and *Origanum dayi* were investigated to test their efficacy on a wide range of cancer cell lines from lung, breast, colon and prostate cancers.[30] The study revealed that the plant extracts with a combination of anticancer compounds were able to have killing activity which was specific to cancer cells and no effect was noticed on normal human lymphocytes and fibroblasts and they suggested these plant extracts were more desirable for using as therapeutic agents than those which are chemically derived which cause toxic complications in cancer treatment. Panc-1 cells treated with 100 µgml$^{-1}$ methanolic extract of *Eclipta prostrate* exhibited cell survival value of 0.50[31] which corroborates the present investigation. Cells treated with 25 µM, 50 µM, 100 µM, 150 µM and 200 µM of caffeic acid

showed a decrease in cell viability, cell death induction by apoptosis, inhibition of colony formation, modulation of cell cycle and alterations in gene expression of caspases and this results suggest an antitumor effect of caffeic acid on SK-Mel-28 cells.[32] In the experiment presented here, different concentrations of the cell biomass extract of *A. ciliata* showed a concentration-dependent cytotoxic effect and the $IC_{50}$ was noticed in 24.144 $\mu gmL^{-1}$ of extract conferring higher cytotoxicity upon SK-MEL-28 cell lines. Similarly potential cytotoxic effects on cancer cell lines were noticed when the cytotoxicity was determined by MTT assay in *A. oleracea* using methanolic plant extract, and higher cytotoxicity was recorded with an $IC_{50}$ of 234 $\mu gmL^{-1}$; while that of spilanthol was determined as 260 $\mu gmL^{-1}$.[33] Spilanthol can exert a variety of biological and pharmacological effects including anticancer effects.[8] Hence the described anti-proliferative effect of *A. ciliata* cell biomass can be attributed the presence of the key component spilanthol. In a very recent study[34] on determination of cytotoxicity of a specific plant extract against human melanoma cells and comparison of the cytotoxicity with some anticancer drugs, the $IC_{50}$ value for the standard anticancer drug cisplatin was found to be 24.84 µg/mL, which is very similar to the IC50 value obtained for *A. ciliata* cell mass extract. The findings shows that the cell mass methanolic extract of *A. ciliata* possess good potential for use as therapeutic agent for treating melanoma and our findings could be recommended for further *in vivo* experiments to confirm the possibility.

## Conclusion

Malignant melanoma accounts for about 75% of the skin cancer-related deaths in the world and there are limited treatment options available for its treatment. A novel bioproduction system for the production of the high value pharmaceutical compound spilanthol from *A. ciliata* was accomplished in this study, which will be a better alternative approach for the conservation and sustainable utilization of this medicinal herb. The antiproliferative analysis by MTT assay reveals that the methanolic extract of A. *ciliata* cell biomass is capable of inhibiting or reducing the growth of SK-MEL-28 cell lines and a lower concentration i.e., 24.144 µg/ml of extract was needed to inhibit the growth by 50%. Mode of administration of any drug/therapeutic plant active compounds may depends upon its pharmacokinetic activity. So further experiments and research on drug formulations are needed for using this cell biomass extract as a novel phytoextract for developing a much improved anticancer drug to augment its effect in phytopharma industry.

## Acknowledgements

We are thankful to 'Athmic Biotech Solutions Pvt Ltd' and Dr. Ajaya Kumar S, Department of Chemistry, Government College for Women, Thiruvananthapuram, Kerala, India for the successful completion of this work.

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HOW TO CITE THIS ARTICLE: Mohan SN, Hemanthakumar AS, Preetha TS. Evaluation of Inhibition of Proliferation of SK-Mel-28 Cell Lines by *Acmella ciliata* (Kunth) Cass. Cell Biomass. Int. J. Pharm. Sci. Drug Res. 2022;14(2):181-188. **DOI:** 10.25004/IJPSDR.2022.140204