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# In Vitro Cytotoxicity and Anti-tumor Properties of the Total Alkaloid Fraction of Unripe Fruits of *Solanum pseudocapsicum*

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## Abstract

The total alkaloid fraction of the methanol extract of unripe fruits of *Solanum pseudocapsicum* was tested for *in vitro* cytotoxicity on HEp-2, RD and Vero cell lines and anti-tumor activity using DLA and HEp-2 cell lines. Cell viability and morphological changes were assessed. The total alkaloid fraction exhibited strong cytotoxic activity against all the cell lines tested. The $CTC_{50}$ (50% of cytotoxicity inhibition) was found to be 1.65 µg/ml for the RD cell line, 6.32 µg/ml for the HEp-2 cell line and 12.01 µg/ml for the Vero cell line. In the clonogenic assay, no colony formation was observed even up to a concentration of 25 µg/ml. In the short term, anti-tumor, studies using DLA cells, the total alkaloid fraction was associated with 50% viability in the concentration range of 6.25–12.5 µg/ml. In long term, anti-tumor activity using the HEp-2 cell line, no colony formation was observed up to a concentration of 20 µg/ml. Hence, there is a correlation between the results obtained in the cytotoxicity and anti-tumor studies carried out. Morphological observation by phase contrast microscopy revealed intense damage on all the cell lines. The total alkaloid fraction has the potential for further investigation in animal models.

**Keywords:** *Solanum pseudocapsicum*, total alkaloid, cytotoxicity, anti-tumor.

## Introduction

*Solanum pseudocapsicum* Linn. (Solanaceae) is an erect, highly branched non-spiny, bushy shrub and has become naturalized in the gardens of Dun valley and the Nilgiris (Anonymous, 1962). Its antimicrobial (Mitscher et al., 1976), antiviral (Van den Berghe et al., 1978) antispasmodic and antihypertensive (Dhar et al., 1973) properties are known. A 50% ethanolic extract of the entire plant of *Solanum pseudocapsicum* has also been reported to possess potent cytotoxic activity in a preliminary study by Dhar et al. (1973). Several plants and plant constituents belonging to the genus *Solanum* exhibited potent cytotoxic and anti-tumor properties (Cham et al., 1987; Ke et al., 1999; Lin et al., 1990; Mohanan & Devi, 1997; Nakamura et al., 1996). These activities were found to be present in the alkaloidal part of these plants. So far, no cytotoxic and anti-tumor activities have been carried out on the total alkaloid fraction of the plant *Solanum pseudocapsicum.* The present paper describes the *in vitro* cytotoxicity and anti-tumor properties of the total alkaloid isolated from the methanolic extract of the unripe fruits of *Solanum pseudocapsicum*. This was assessed on malignant human rhabdomyosarcoma (RD) cells, caucasian male larynx epithelium carcinoma (HEp-2) cells, Dalton's lymphoma ascites (DLA) cells and normal African green monkey kidney (Vero) cells.

## Materials and methods

### Plant material and isolation of total alkaloids

The unripe fruits of *Solanum pseudocapsicum* were collected in and around Ootacamund, during December 1999. Authentication of the plant material was carried out by the Survey of Medicinal Plants and Collection Unit, Government Arts College, Ootacamund, where voucher specimens are preserved. The fresh unripe fruits were powdered mechanically and extracted (150 g) with methanol (50%) in a Soxhlet extractor for 18 h. The extract was concentrated to dryness under reduced pressure and controlled temperature to yield a light brown residue (16 g). The total alkaloid fraction (yield

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800 mg) was isolated following a conventional procedure (Trease & Evans, 1978). The total alkaloid of *Solanum pseudocapsicum* was dissolved in DMSO and the volume was made up to 10 ml with DMEM/RPMI medium to obtain a stock solution of 1 mg/ml concentration and stored at –20 °C until use.

### Cell lines and culture medium

The HEp-2, RD, Vero and DLA cell cultures used in these experiments were obtained from the National Center for Cell Sciences, Pune and Pasteur Institute of India, Coonoor. Stock cells of HEp-2, RD and Vero cell lines were cultured in RPMI-1640 and DMEM supplemented with 10% inactivated sheep serum, penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% $CO_2$ at 37 °C until confluent. The cells were dissociated with 0.2% trypsin, 0.02% EDTA, in PBS. The stock cultures were grown in 110 ml flat bottles and all experiments were carried out in 96-well microtiter plates, where the cell population was adjusted to 10,000 cells per well. DLA cells used were maintained in Swiss albino mice.

### Cytotoxic assay

The cytotoxicity assays were carried out using 0.1 ml of cell suspension, containing 10,000 cells seeded in each well of a 96-well microtiter plate (Nunc and Tarson). Fresh medium containing different concentrations of the total alkaloid was added after 24 h seeding. Control cells were incubated without the test compound and with DMSO (solvent). The very little percentage of DMSO present in the wells (maximal 0.2%) was proved not to affect the experiment. The microtiter plates were incubated at 37 °C in a humidified incubator with 5% $CO_2$ for a period of 3 days. Twelve wells were used for each concentration of the total alkaloid fraction. Morphological changes were examined using an inverted microscope. The cells were observed at different time intervals after incubation in the presence or absence of the total alkaloid fraction. Cellular viability was determined by using the standard MTT assay (Ke et al., 1999; Francis & Rita, 1986), Trypan blue dye exclusion method (Moldeus et al., 1978) and cell metabolic function by protein estimation (Lowry et al., 1951) from the treated culture of 4 wells of each concentration.

### MTT assay

MTT assay is based on the reduction of the soluble MTT into a blue purple formazan product, mainly by mitochondrial reductase activity inside living cells. The number of viable cells was found to be proportional to the extent of formazan production for the cell lines used in this study. After incubation, the solutions in four wells of each concentration were decanted and 50 µl of a solution of 2 mg/ml of MTT (Sigma, St. Louis, MO, USA) in DMEM (without phenol red) was added and the cultures were incubated for an additional 3 h at 37 °C. The supernatant was removed and the cells were dissolved in propanol (100 µl/well) and kept aside for 10 min at room temperature. The plate was read on a microtiter plate reader (Bio-Rad, Model 550) at a wavelength of 540 nm and the mean absorbance from four wells was recorded. Mean absorbance taken from cells grown in the absence of the total alkaloid fraction was taken as 100% cell survival (control). The percentage inhibition was calculated using the following formula:

$$\text{Growth Inhibition } \% = 100 - \left[\frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100\right]$$

The percentage inhibition was plotted against concentration and the $CTC_{50}$ (concentration required to reduce viability by 50%) value for each cell line was calculated.

### Dye exclusion method

The solutions in the other 8 wells of each concentration were decanted and cells were trypsinized with 100 µl of TPVG for 3 min at 37 °C. To stop the trypsinizing activity, 100 µl of growth medium was added, the cells were flushed and cells from four wells pooled concentration wise into Eppendorf tubes. The pooled cells from four wells of each concentration tested were then subjected for dye exclusion and protein estimation methods.

For testing viability using the dye exclusion method, the pooled cells from four wells of each concentration were mixed with 0.4% Trypan blue in the ratio of 1 : 1 and the number of stained, non-stained and total number of cells were counted using haemocytometer. The percentage inhibition and $CTC_{50}$ were calculated.

### Protein estimation method

The pooled cells from the remaining four wells of each concentration tested were centrifuged at 10,000 rpm in a cooling centrifuge. The cell pellets were then treated with 0.5 ml of 11% trichloroacetic acid and the precipitated proteins were assayed by the Lowry method. The % inhibition and $CTC_{50}$ values were calculated.

### Clonogenic assay

Cytotoxicity was assayed by determining the ability of HEp-2 cells to form colonies following total alkaloid fraction treatment (Christopher et al., 1985). The monolayer cell culture was trypsinized and the cell count was adjusted to 5000 cells/ml. To each well of a 2-well microtiter plate, 1 ml of the diluted cell suspension was added and incubated overnight at 37 °C in an atmosphere of 5% $CO_2$. The supernatant was decanted and the cells were exposed to different concentrations of the total alkaloid fraction for 2 h. Afterwards, the

supernatant was decanted and the cells were washed with fresh medium, followed by addition of 1 ml growth medium. Colonies were allowed to grow for 2 weeks at 37 °C, 5% $CO_2$. The medium was then removed, the colonies were stained with 1% crystal violet (Sigma Chemical Co., St. Louis, MO, USA) in 70% ethanol and then counted manually. The tolerance limit was determined by the ability of a cell to form a colony containing more than 50 cells.

### Short term anti-tumor activity

Short-term anti-tumor activity of the total alkaloid was assayed by determining the percentage viability of DLA cells using Trypan blue dye exclusion technique. DLA cells were cultured in the peritoneal cavity of healthy albino mice weighing between 25 to 30 g by injecting a suspension of DLA cells ($1 \times 10^6$ cells/ml) intraperitoneally. The cells were aspirated aseptically from the peritoneal cavity of the mice on the 15th day. The cells were washed with Hank's balanced salt solution (HBSS) and centrifuged for 10 to 15 min at 1500 rpm in a refrigerated centrifuge. The pellet was re-suspended with HBSS and the process was repeated for three times. Finally, the cells were suspended in known quantity of HBSS and the cell count was adjusted to $2 \times 10^6$ cells/ml. The diluted cell suspension, 0.1 ml was distributed into Eppendorf tubes and exposed to 0.1 ml each of the different concentrations of the total alkaloid fraction and incubated at 37 °C, 5% $CO_2$ for 3 h. After 3 h, the Trypan blue dye exclusion test was performed to determine the percentage viability.

### Long term anti-tumor activity

This assay was carried out using the method of Freshney (1987) by determining the ability of HEp-2 cells to form the colonies after treatment with the total alkaloid fraction. Series of cultures were prepared in 25 $cm^2$ flasks, three for each concentration and three controls by seeding with $2 \times 10^5$ cells in 4 ml of growth medium and incubating at 37 °C for 48 h. Growth medium was decanted and cultures were exposed to different concentrations of the agent and incubated further for 72 h. Morphological changes in the cultures were observed under the microscope. At 72 h post incubation, the medium was decanted and the cultures were trypsinized, cell density was adjusted to 5000 cells/ml required for cell growth. The seeded cultures were incubated for 2 weeks until colonies were formed. The colonies were fixed, stained and counted.

## Results

Dose dependent morphological changes and destruction of monolayer were observed in all cell lines treated with the total alkaloid fraction of the methanol extract of unripe fruits of *Solanum pseudocapsicum* (Table 1). The total alkaloid fraction showed strong cytotoxic activity against all the three cell lines tested. The $CTC_{50}$ of the total alkaloid fraction was found to be 1.65 µg/ml for the RD cell line, 6.32 µg/ml for the HEp-2 cell line and 12.01 µg/ml for the Vero cell line (Table 2). In the clonogenic assay, no colony formation occurred over a concentration of 25 µg/ml. In the short term anti-tumor studies using DLA cells, the treatment with the total alkaloid fraction has resulted in 50% viability in the concentration range of 6.25–12.5 µg/ml. In long-term anti-tumor studies using the HEp-2 cell line, no colony formation was observed above a concentration of 20 µg/ml (Tables 3 and 4).

## Discussion

In the present study, the total alkaloid fraction of a methanol extract of *Solanum pseudocapsicum* was evaluated for *in*

*Table 1.* Morphological changes induced by the total alkaloid fraction of *Solanum pseudocapsicum* on HEp-2, RD and Vero cells.

| | | Morphological Changes* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | HEp-2 Cells | | | RD Cells | | | Vero Cells | | |
| Treatment | Concentration (µg/ml) | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| Total | 25.00 | 0 | ++ | ++++ | 0 | +++ | ++++ | ++ | +++ | ++++ |
| Alkaloid | 12.50 | 0 | + | +++ | 0 | +++ | ++++ | 0 | + | ++ |
| Fraction | 6.25 | 0 | + | ++ | 0 | ++ | +++ | 0 | 0 | + |
| | 3.12 | 0 | 0 | + | 0 | + | ++ | 0 | 0 | 0 |
| | 1.56 | 0 | 0 | 0 | 0 | + | ++ | 0 | 0 | 0 |
| | 0.78 | 0 | 0 | | 0 | 0 | + | 0 | 0 | 0 |
| | 0.39 | 0 | 0 | | 0 | 0 | 0 | | | |
| | 0.19 | 0 | 0 | | 0 | 0 | 0 | | | |
| Control | – | 0 | 0 | 0 | 0 | 0 | 0 | | | |

* Average of three independent experiments; toxic effects were judged to have occurred when there was cell shrinkage, cell wall breakage and/or leakage of cell components, 0 = normal, + = 25% toxic effect, ++ = 50% toxic effect, +++ = 75% toxic effect, ++++ = 100% toxic effect.

*Table 2.* Cytotoxic effect of the total alkaloid fraction of a methanolic extract of unripe fruits of *Solanum pseudocapsicum* on HEp-2, RD and Vero cell lines (No. of independent experiments = 3, 6 replicates, mean ± SEM).

| Cell Line | Total alkaloid fraction Concentration (µg/ml) | Cytotoxicity after 72 hours by MTT Assay % Inhibition | MTT Assay $CTC_{50}$ | Dye Exclusion Method % Inhibition | Dye Exclusion Method $CTC_{50}$ | Protein Synthesis % Inhibition | Protein Synthesis $CTC_{50}$ | Mean $CTC_{50}$ |
|---|---|---|---|---|---|---|---|---|
| HEp-2 | 50.00 | 91.03 ± 4.26 | 6.35 | 100 ± 5.97 | 6.46 | 95.03 ± 4.46 | 6.15 | 6.32 |
| | 25.00 | 86.15 ± 4.12 | | 91.23 ± 5.13 | | 90.15 ± 4.12 | | |
| | 12.50 | 80.24 ± 3.81 | | 85.12 ± 4.85 | | 81.12 ± 3.84 | | |
| | 06.25 | 41.95 ± 2.04 | | 46.05 ± 1.98 | | 58.05 ± 3.62 | | |
| | 03.12 | 28.30 ± 1.20 | | 21.02 ± 1.72 | | 28.45 ± 2.36 | | |
| | Control | 0 ± 0.01 | | 0 ± 0.01 | | 0 ± 0.01 | | |
| RD | 6.25 | 89.29 ± 4.06 | 1.63 | 85.72 ± 5.21 | 1.76 | 73.71 ± 4.81 | 1.55 | 1.65 |
| | 3.12 | 69.39 ± 3.12 | | 66.67 ± 3.97 | | 68.97 ± 4.75 | | |
| | 1.56 | 47.96 ± 2.21 | | 35.72 ± 1.68 | | 50.44 ± 3.69 | | |
| | 0.78 | 38.78 ± 1.55 | | 28.58 ± 1.89 | | 20.25 ± 1.78 | | |
| | 0.39 | 36.74 ± 1.54 | | 22.20 ± 1.56 | | 17.15 ± 1.58 | | |
| | 0.19 | 35.72 ± 1.45 | | 15.19 ± 1.01 | | 12.16 ± 1.42 | | |
| | Control | 0 ± 0.01 | | 0 ± 0.01 | | 0 ± 0.01 | | |
| Vero | 25.00 | 66.95 ± 3.06 | 12.12 | 100 ± 5.98 | 12.16 | 64.75 ± 3.82 | 11.75 | 12.01 |
| | 12.50 | 54.24 ± 2.21 | | 57.15 ± 3.22 | | 57.91 ± 3.44 | | |
| | 06.25 | 09.24 ± 0.41 | | 15 ± 0.65 | | 30.22 ± 2.11 | | |
| | 03.12 | 08.48 ± 0.34 | | 0 ± 0.01 | | 29.50 ± 2.09 | | |
| | 01.56 | 02.55 ± 0.12 | | 0 ± 0.01 | | 17.62 ± 1.19 | | |
| | Control | 0 ± 0.00 | | 0 ± 0.01 | | 0 ± 0.01 | | |

*Table 3.* The anti-tumor effect of the total alkaloid fraction of *Solanum pseudocapsicum* on DLA cells after 3 hours exposure.

| Concentration (µg/ml) | Percentage Viability* |
|---|---|
| 100.00 | 5.32 ± 0.1 |
| 50.00 | 14.73 ± 0.8 |
| 25.00 | 36.14 ± 2.0 |
| 12.50 | 46.38 ± 2.1 |
| 06.25 | 59.78 ± 3.1 |
| 03.12 | 81.81 ± 4.4 |
| Control | 97.82 ± 6.0 |

* Average of three independent determinations.

*Table 4.* The anti-tumor effect of the total alkaloid of *Solanum pseudocapsicum* on HEp-2 cell line by long term test for survival method.

| Concentration (µg/ml) | No. of colonies* |
|---|---|
| 100.0 | 0 ± 0.0 |
| 20.0 | 0 ± 0.0 |
| 04.0 | 50 ± 2.7 |
| 00.8 | 53 ± 2.7 |
| Control | 55 ± 2.9 |

* Average of three independent determinations.

*vitro* cytotoxicity and short term and long-term anti-tumor activity. The total alkaloid exhibited strong cytotoxic activity and dose-dependent structural alterations against all the cell lines tested. Among these, the maximum cytotoxicity was observed against the RD cell line. In the clonogenic assay, no colonies were observed over a concentration of 25 µg/ml. The clonogenic assay confirms the cytotoxic activity of the total alkaloid fraction, which occurs only after a short exposure to the alkaloids. The total alkaloid fraction of *Solanum pseudocapsicum* also strongly inhibited the growth of cancer cell lines in both short term (DLA cells) and long term (Hep-2 cells) anti-tumor studies. The latter demonstrates that there is a strong inhibitory effect on cancer cells at a low concentration.

In an earlier study, the 50% ethanolic extract of the entire plant of *Solanum pseudocapsicum* showed potent cytotoxic activity with an $ED_{50}$ of 20 µg/ml (Dhar et al., 1973). Our studies confirm the cytotoxic and anti-tumor properties of the unripe fruits of this plant and also show that the total alkaloids are the active principles responsible for the activity. Solamargine from *Solanum nigrum* (Ke et al., 1999), glycoalkaloids from *Solanum sodamaeum* (Cham et al., 1987),

incanumine from *Solanum incanum* (Lin et al., 1990) and several other steroidal alkaloidal glycosides (Mohanan & Devi, 1997; Nakamura et al., 1996) have been reported to possess cytotoxic and anti-tumor properties. Phytochemical tests indicated that the total alkaloids of *Solanum pseudocapsicum* are steroidal in nature. Several steroidal alkaloids, including solanocapsine, solacasine, solacapine, episolacapine, isosolacapine and *o*-methylsolanocapsine, have been isolated from the arboreal part of *Solanum pseudocapsicum* (Hohne et al., 1970; Chakravarty et al., 1984; Gan & Lin, 1997). Hence, the observed cytotoxic and anti-tumor properties of the total alkaloid of *Solanum pseudocapsicum* unripe fruits may be due to the presence of steroidal alkaloids. In conclusion, the total alkaloids of *Solanum pseudocapsicum* merit further investigation to identify the active principles, and in animal models the nature of the anti-tumor activity.

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