• Research Article

Homeopathic mother tincture of *Phytolacca decandra* induces apoptosis in skin melanoma cells by activating caspase mediated signaling via reactive oxygen species) elevation

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OBJECTIVE: Preventive measures against skin melanoma like chemotherapy are useful but suffer from chronic side effects and drug resistance. Ethanolic extract of *Phytolacca decandra* (PD), used in homeopathy for the treatment of various ailments like chronic rheumatism, regular conjunctivitis, psoriasis, and in some skin diseases was tested for its possible anticancer potential not known to have reported earlier.

METHODS: Cytotoxicity of the drug was tested by conducting 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on both normal (peripheral blood mononuclear cells) and A375 cells. Fluorescence microscopic study of 4′,6-diamidino-2-phenylindole dihydrochloride-stained cells was conducted for DNA fragmentation assay and changes in cellular morphology, if any, were also recorded. Lactate dehydrogenase activity assay was done to evaluate the percentages of apoptosis and necrosis. Reactive oxygen species (ROS) accumulation, if any, and expression study of apoptotic genes also were evaluated to pin-point the actual events of apoptosis.

RESULTS: Results showed that PD administration caused a remarkable reduction in proliferation of A375 cells, without showing much cytotoxicity on peripheral blood mononuclear cells. Generation of ROS and DNA damage, which made the cancer cells prone to apoptosis, was found to be enhanced in PD-treated cells. These results were duly supported by the analytical data on expression of different cellular and nuclear proteins, as for example, by down-regulation of Akt and Bcl-2, up-regulation of p53, Bax and caspase 3, and an increase in number of cell deaths by apoptosis in A375 cells.

CONCLUSION: Overall results demonstrate that anticancer potentials of the PD on A375 cells through activation of caspase-mediated signalling and ROS generation.

KEYWORDS: *Phytolacca decandra*; skin neoplasms; reactive oxygen species; apoptosis; gene expression

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1 Introduction

A dynamic increase of skin melanoma mortality rate has been observed in the world in recent years. Though this type of skin cancer comprises just 5 % of all malignant skin cancers, it is responsible for a 75%-80% of deaths caused by these tumors[1]. The high rate of malignancy

of this neoplasm is associated with rapid proliferation, numerous early metastases and high resistance to conventional treatments involving surgery, chemotherapy and radiation. Although preventive measures like chemotherapy are very useful, these often result in manifestation of chronic side effects[2,3]. Therefore, there is a growing demand for the development of alternative anti-melanoma therapies with less side effects. Natural products of various sources, particularly from plants, have been regarded as a precious alternative to modern medicine and investigations on active components with anticancer potential and less side effects have opened up newer avenues[4].

In cancer treatment, one of the approaches to restrain tumor growth is by activating the apoptotic machinery in the tumor cells[5]. Apoptosis, a highly structured and orchestrated process, performs a significant role in regulating cell number for the growth and homeostasis of tissues by eliminating aged, damaged and unwanted cells[6,7].

Phytolacca decandra (PD), commonly known as poke root, is an ornamental plant of the Phytolaccaceae family. Phytochemical characterisation revealed that terpenoids, saponins, alkaloid, resins, phytolacic acid and tannin are the major bioactive ingredients of *Phytolacca* species[8-11]. From a long time *Phytolacca* has been in use to combat different ailments in complementary and alternative medicine (CAM). In homeopathy, mother tincture of *Phytolacca* is used against chronic rheumatism, regular conjunctivitis, psoriasis and in some skin diseases[12]. Although to the best of our knowledge, anticancer potential of the ethanolic crude extract of PD is yet to be systematically studied for scientific validation, stray reports exist in literature enlisting some of the ingredients like saponins and alkaloids extracted and separated from other plant sources to have anticancer potentials[13,14]. Since the crude extract of PD is used in homeopathy, and since no earlier study appears to have been conducted on the anticancer effect on the basis of a reliable investigation, we undertook this research. Therefore, in the present set of experiment our main aim was to explore the anticancer activity, if any, of PD against A375 cells *in vitro* and if positive findings were obtained, to investigate the mode of cell death involved.

2 Materials and methods

2.1 Chemicals and reagents

Plastic wares were obtained from BD Bioscience (USA). Cell culture media Dulbecco's modified Eagle medium (DMEM) and Roswell Park Memorial Institute (RPMI), fetal bovine serum (FBS) and trypsin-ethylene diamine tetraacetic acid (EDTA) were purchased from Invitrogen-Gibco BRL (USA). Primary and secondary antibodies were purchased from Satna Cruz Biotecnology (USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sisco Research Laboratory, India. TRIzol and antibiotics penicillin-streptomycin-neomycin (PSN) were obtained from HiMedia (India). Dichloro-dihydrofluorescein diacetate (H_2DCFDA) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were was purchased from Sigma (USA). RNase and p-nitro phenyl phosphate (PNPP) were purchased from Merck (Germany). All the primers used were obtained from Bioserve (India). Ethanolic extract of PD in the form of homeopathic mother tincture was supplied by Boiron Laboratory, Lyon, France. They prepared the drug adopting the guidelines of *European Pharmacopeaea* and supplied the drug in 65% ethanolic extract. Before the experimental treatment the drug was diluted in the DMEM media.

2.2 Cell culture

A375 and HeLa National Centre for Cell Science (NCCS, India) cells were cultured in DMEM, whereas PC3 (NCCS, India) was cultured in RPMI, supplemented with 10% heat-inactivated FBS and 1% PSN and maintained at 37 ºC with 5% CO_2 in a humidified incubator (Thermo Scientific, USA). Cells were harvested with 1×trypsin-EDTA in phosphate buffered saline (PBS) and plated at required cell numbers and allowed to adhere before treatment.

2.3 Cell viability assay

Cells were dispensed in 96-well plates at a density of 1×10^2 cells per well and treated with various concentrations of PD (0 to 780 μg/mL) for 24 and 48 h respectively and peripheral blood mononuclear cells (PBMCs) were isolated from the human blood sample by conventional ficol gradient method and treated with PD at different concentrations (0 to 780 μg/mL), and incubated for 24 and 48 h. The percentage of cell death was determined by MTT assay[15].

2.4 Selection of drug doses

On the basis of the result of MTT assay, we selected three different drug doses, namely, D1 (100 μg/mL), D2 (150 μg/mL) and D3 (200 μg/mL), for experiments and treated for 48 h on A375 cells. To calculate the concentration of the drug, the dry weight of PD was measured and expressed as drug doses in concentration unit (μg/mL). The positive (vehicle) control set received only ethanol (placebo) and the negative control neither received any drug nor ethanol. As the positive control had no significant change with respect to the negative control, we did not include negative control group in our experiments.

2.5 Morphological changes

Cells were treated with PD at specified concentrations. After 48 h, the cells were observed under an inverted light

microscope (Leica, Germany) equipped with a digital camera and photographs were taken.

2.6 Nucleosomal fragmentation analysis

Treated cells were stained with DAPI (10 μg/mL) and analyzed under a fluorescence microscope (Axiscope plus 2, Zeiss, Germany) and representative photographs were taken.

2.7 Lactate dehydrogenase-based cytotoxicity assay

Lactate dehydrogenase (LDH) activity was assessed using a standardized kinetic determination kit (Enzopak, Recon, India). LDH activity was measured according to the method of Biswas *et al*[16]. LDH activity was measured in both floating dead cells and adherent viable cells for control and drug-treated cells. The floating cells were collected from culture medium by centrifugation (500 × *g*) at 4 ºC for 2 min, and the LDH content from the pellets was used as an index of apoptotic cell death (LDHp) The LDH released in the culture supernatant (designated as extracellular LDH (LDHe)) was used as an index of necrotic death, and the LDH present in the adherent viable cells was designated as intracellular LDH (LDHi). The percentages of apoptotic and necrotic cell deaths were calculated as follows:

Apoptosis percentage = (LDHp × 100%)/(LDHp+LDHi+LDHe)

Necrosis percentage = (LDHe × 100%)/(LDHp+LDHi+LDHe)

2.8 Analysis of intracellular reactive oxygen species generation

Treated fixed cells were incubated with 10 μmol/L H_2DCFDA and photographed under a fluorescence microscope (Leica DMLS, Germany) for qualitative analyses. For the quantitative estimation of reactive oxygen species (ROS) generation, the treated cells were fixed in 70% chilled methanol and incubated with 10 μmol/L H_2DCFDA for 30 min at room temperature in dark. Fluorescence intensity was measured with an excitation wavelength of 480 nm and an emission wavelength of 530 nm by using a FL-1H filter of flow cytometer (BD FACS calibur, USA)[17]. Data were analyzed by using Cyflogic software.

2.9 Assessment of protein activity involved in anticancer signalling pathway

The assay was done according to the manufacturer's protocol (Santa Cruz Biotechnology Inc, USA) using cell lysate. Drug-treated and control cells were lysed in lysis buffer. The lysed material was centrifuged at 13 000 × g for 15 min at 4 °C and the supernatants were collected. Enzyme-linked immunosorbent assay (ELISA) was carried out with the supernatants to study the expressions of p53, Akt/protein kinase B (PKB), BCL-associated X (Bax), B cell lymphoma 2 (Bcl-2) and caspase 3 quantitatively. The assay was standardized by titration of the different concentrations of antigens and antibodies. Wells were coated with 70 μg of proteins in 100 μL of 0.05 mol/L carbonate buffer (pH 9.6) and kept overnight at 4 °C, in a moist chamber. Flicked the plate to remove the unbound antigen solution and wells were blocked with 1% bovine serum albumin (BSA) in 0.1 mol/L PBS (pH 7.2) for 1 h at 4 °C. Flicked and washed wells thrice with 200 μL of PBS containing 0.05% (volume ratio) Tween-20. Wells were then incubated with primary antibody (1 ∶ 500), diluted in PBS (containing 0.05% Tween-20 and 1% BSA) and kept overnight at 4 °C. Plate was again washed and incubated with specific secondary antibodies (1 ∶ 1 000) for 2 h at 37 °C. Wells were washed further three times as described above. PNPP was used as a colour developer and colour intensity was measured at 405 nm with respect to blank. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) served as the housekeeping gene.

2.10 Analysis of mRNA level expressions by semi-quantitative reverse transcriptase-polymerase chain reaction

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions and the gene expressions were analyzed by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)[18] to estimate the expression of p53, Akt, Bcl-2, BAX, and caspase 3, at their mRNA level. G3PDH served as the housekeeping gene. Densitometry was performed using Image J software. Primer sequences of RT-PCR were illustrated in Table 1.

2.11 Blinding

The observers were "blinded" during observations as to whether they were observing the "control" or "drug-treated" materials.

2.12 Statistical Analysis

Statistical analysis was performed by one-way analysis of variance with post-hoc least significant difference tests, using SPSS 14 software to identify if the differences were significant among the mean values of different groups. Results were expressed as mean ± standard error of mean. $P<0.05$ was considered significant.

3 Results

3.1 Effects of PD on the survival of A375 cells

Results of MTT assay revealed that the cell viability gradually decreased from minimal drug concentration to the higher ones in both the cases of 24 and 48 h of incubation after drug treatment. The 50% cell death (LD_{50}) occurred at 348.66 and 203.60 μg/mL of drug employment for the treatment of 24 and 48 h respectively on A375 cells (Figure 1A) and the same for HeLa and PC3 cells were 704.3 μg/mL (24 h) and 653.78 μg/mL (48 h) (Figure 1B), and 595.9 μg/mL (24 h) and 505.44 μg/mL (48 h) (Figure 1C), respectively. The cell cytotoxicity on normal PBMC was also measured in the mentioned dose of PD which showed minimal cytotoxic effects (Figure 1D).

Table 1 The list of the sequences of the primers used

Primer name	Primer sequence
Caspase 3	Forward: 5'-AGGGGTCATTTATGGGACA-3' Reverse: 5'-TACACGGGATCTGTTTCTTTG-3'
AKT	Forward: 5'-CCTGGACTACCTGCACTCTCGGAA-3' Reverse: 5'-TTGCTTTCAGGGCTGCTCAAGAAGG-3'
Bax	Forward: 5'-AGTAACATGGAGCTGCAGAGG-3' Reverse: 5'-ATGGTTCTGATCAGTTCCGG-3'
Bcl-2	Forward: 5'-GTGACTTCCGATCAGGAAGG-3' Reverse: 5'-CTTCCAGACATTCGGAGACC-3'
p53	Forward: 5'-GGAAATTTGTATCCCGAGTATCTG-3' Reverse: 5'-GTCTTCCAGTGTGATGATGGTAA-3'
G3PDH	Forward: 5'-CCCACTAACATCAAATGGGG-3' Reverse: 5'-CCTTCCACAATGCAAAGTT-3'

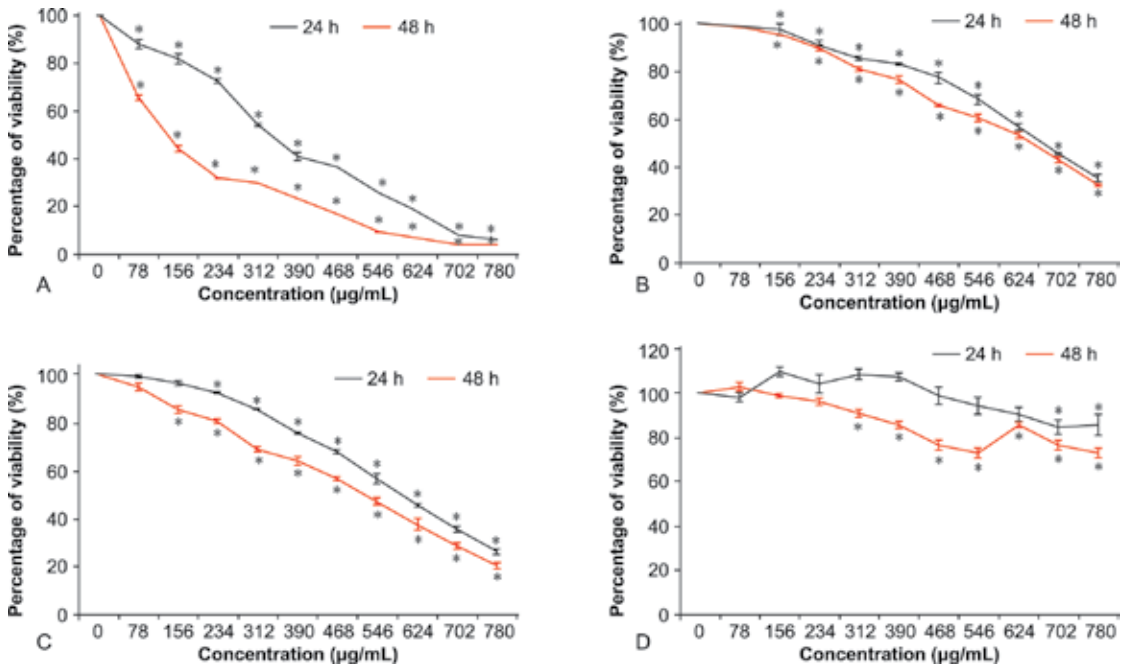

Figure 1 Results of cell viability assay

Different concentrations (0 to 780 μg/mL at the interval of 78 μg/mL) of *Phytolacca decandra* on A375 cells (A), HeLa cells (B), PC3 cells (C) and peripheral blood mononuclear cells (D) incubated for 24 h and 48 h. The cell viability and also the corresponding median lethal dose were then detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at each concentration. The results are expressed as mean ± standard error of mean, n=3; $^{*}P$<0.05, *vs* 0 μg/mL.

3.2 Changes in morphological features

The cellular distortion and the cell blebbing were observed in the drug-treated cells. The cellular distortion was found to be most frequent in cases of higher doses (Figure 2).

3.3 PD-induced nucleosomal fragmentation

Nucleosomal fragmentation was observed in the drug-treated cells but not in the control ones. The DNA breakage mostly occurred in case of the highest drug dose (D3) applied. See Figure 3.

3.4 Determination of apoptosis and necrosis percentages

After drug treatment the apoptotic percentages increased form 10.11% (control) to 54.93%, 65.20% and 66.73% in cases of D1, D2 and D3 doses, respectively. The necrotic percentages were relatively lower in the drug-treated cells and those were 4.76%, 7.08% and 6.80% for D1, D2 and D3, respectively (Figure 4).

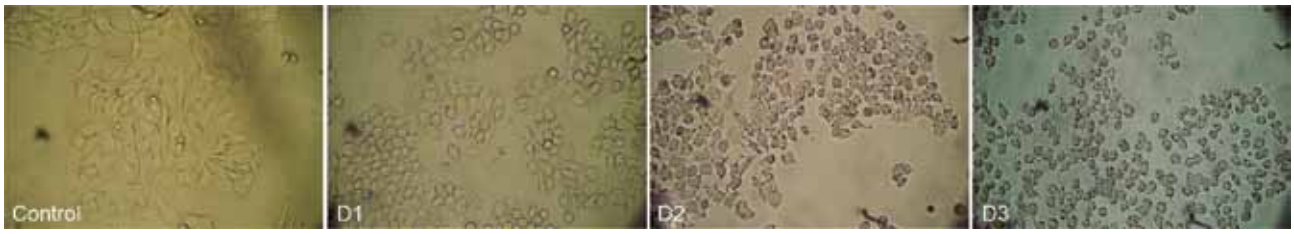

Figure 2 Changes in cellular morphology (Inverted light microscopy, ×20)
D1: 100 μg/mL; D2: 150 μg/mL; D3: 200 μg/mL.

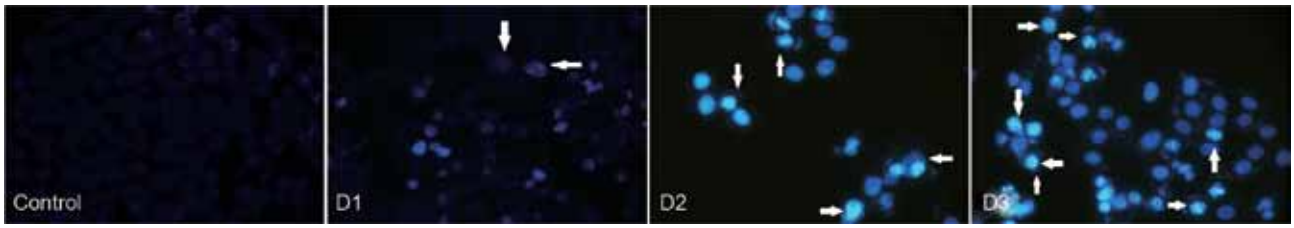

Figure 3 DAPI-stained cells for the detection of DNA damage (Fluorescence microscopy, ×40)
D1: 100 μg/mL; D2: 150 μg/mL; D3: 200 μg/mL; DAPI: 4′,6-diamidino-2-phenylindole dihydrochloride.

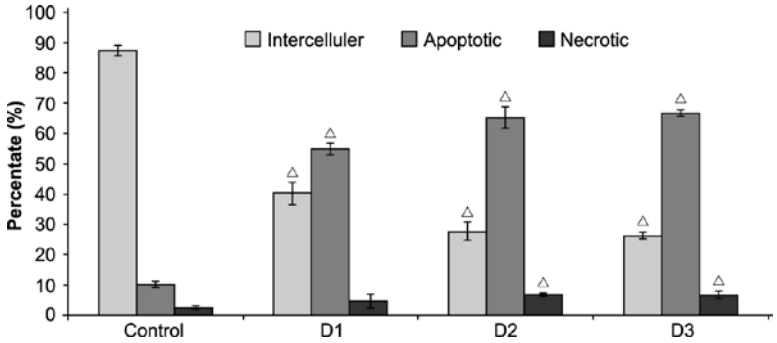

Figure 4 Determination of apoptosis and necrosis percentages by lactate dehydrogenase activity assay of control and drug-treated A375 cells
The drug treatment lasted for 48 h. The results are expressed as mean ± standard error of mean, n=3; $^{\triangle}P$<0.05, *vs* normal control group.
D1: 100 μg/mL; D2: 150 μg/mL; D3: 200 μg/mL.

3.5 Detection of accumulation of intracellular ROS H_2DCFDA staining

The fluorescence intensity gradually increased in the higher drug doses. D3-treated cells had the most ROS accumulation and had the highest amount of florescence (Figure 5A). From the FACS analysis with H_2DCFDA dye, a boost in ROS production was observed (Figures 5B and 5C).

3.6 Changes in expression of pro- and anti-apoptotic genes in both mRNA and protein in A375 cells after *in vitro* treatment with PD

The expressions of p53, Bax and caspase 3 were up-regulated (Figure 6A) and the expressions of Akt and Bcl-2 were down-regulated (Figure 6B) both at mRNA and protein levels upon drug treatment. G3PDH was used as the house keeping gene (Figure 6C). The changes in the Bcl-2/BAX ratio at both mRNA and protein levels are shown in Table 2.

4 Discussion

In the present study, results of cell viability assay with different types of cancer cells, namely, skin melanoma, cervix cancer and prostate cancer for 24 and 48 h, respectively revealed that the treatment with PD reduced the cell viability *in vitro* for all the cancer types; the effect on A375 cells was more remarkable at 48 h of treatment. Hence, we carried out more in-depth studies on A375 cells. Results on cell viability assay showed that PD reduced the viability of the skin melanoma cells significantly but not the normal cells. Incidentally, a potent anticancer drug should have the potential to eradicate the

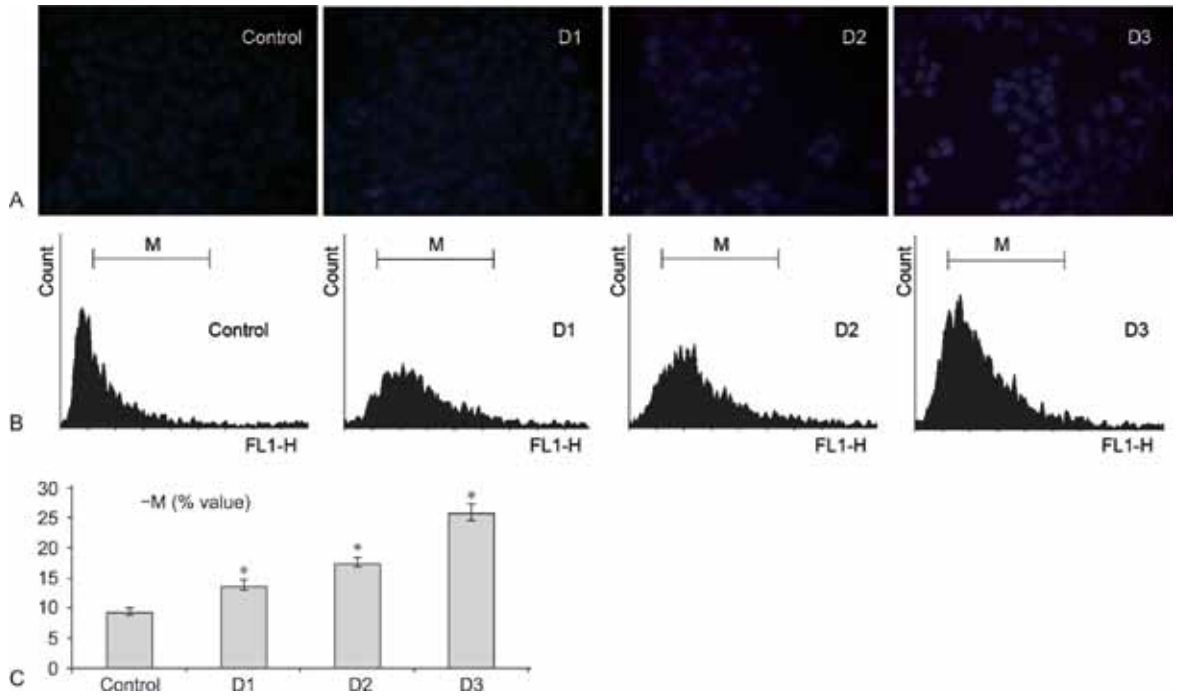

Figure 5 Analysis of accumulation of reactive oxygen species
A: Fluorescence microscopic study (×20); B: FACS analysis; C: H_2DCFDA-stained A375 cells.
In figure B, 'M' represents an arbitrary area. The cell populations (within the area of 'M's) are calculated in percentages considering the cell populations of the total area as 100 % and represented in a graph in figure C.
D1: 100 μg/mL; D2: 150 μg/mL; D3: 200 μg/mL; FACS: fluorescence-activated cell sorting; H_2DCFDA: dichloro-dihydrofluorescein diacetate.

cancer cells without being too cytotoxic to normal cells[19].

Cancer cells are known to have acquired immortality. A noble anticancer drug should normally be expected to have the property of initiating apoptosis (cell death) to the cancer cells[20]. Consistent with this expectation, PD was found to initiate apoptosis in A375 cells as the main cause of cell death, though it also induced necrosis in a few cells. Results of LDH activity assay supported this contention.

As one major way of combating cancer cells is by killing them, one of the approaches to restrain tumor growth is by activating the apoptotic machinery in the tumor cells[21]. The changes in cellular morphology (e.g., membrane distortion, blebbing), condensation and fragmentation of DNA are two significant characteristic features of apoptosis[22]. Nucleosomal fragmentation was evident through microscopic study of cellular morphology and DAPI staining of the cells.

ROS plays a prodigious role in regulating cell proliferation and removal of unwanted cells through apoptosis[23]. The normal human cells possess certain level of ROS activity, but the cancer cells contain a higher level of ROS activity due to its higher metabolic rates. If the ROS level could be elevated to a further extent to the threshold level, the cells push themselves to apoptosis[24]. This could be a way to control the immortality of cancer cells. In this study, the elevated ROS level was found upon deployment of the drug. The accumulated ROS has the capacity to induce DNA damage[25]. The FACS analysis with H_2DCFDA fluorescent dye supported the increased cellular ROS generation upon drug treatment.

ROS regulates cell proliferation by down-regulating Akt gene expression[26,27] which in turn reduces cell proliferation[28]. Expressions both of study mRNA and protein activities (by indirect ELISA) showed down-regulation of Akt. This result was in great support of antiproliferative potential of PD. On the other hand, ROS regulates p53 expression. Elevation in ROS level generally up-regulates the "master gene" p53[29] and this master gene plays a decisive role in whether the damaged DNA will undergo the repair pathway or the fragmentation pathway. When damage is not repairable, generally the cells are led to the apoptotic pathway. Our experimental results, the elevated p53 activity level and DAPI-stained cells, suggest the above event.

Alterations in the levels of Bax and Bcl-2, i.e., the ratio of Bcl-2/Bax, is the decisive factor in determining whether cells will undergo caspase mediated apoptosis[30,31], or be directed towards the survival pathway[32]. In our study, the decrease in Bcl-2 expression and the increase in Bax and

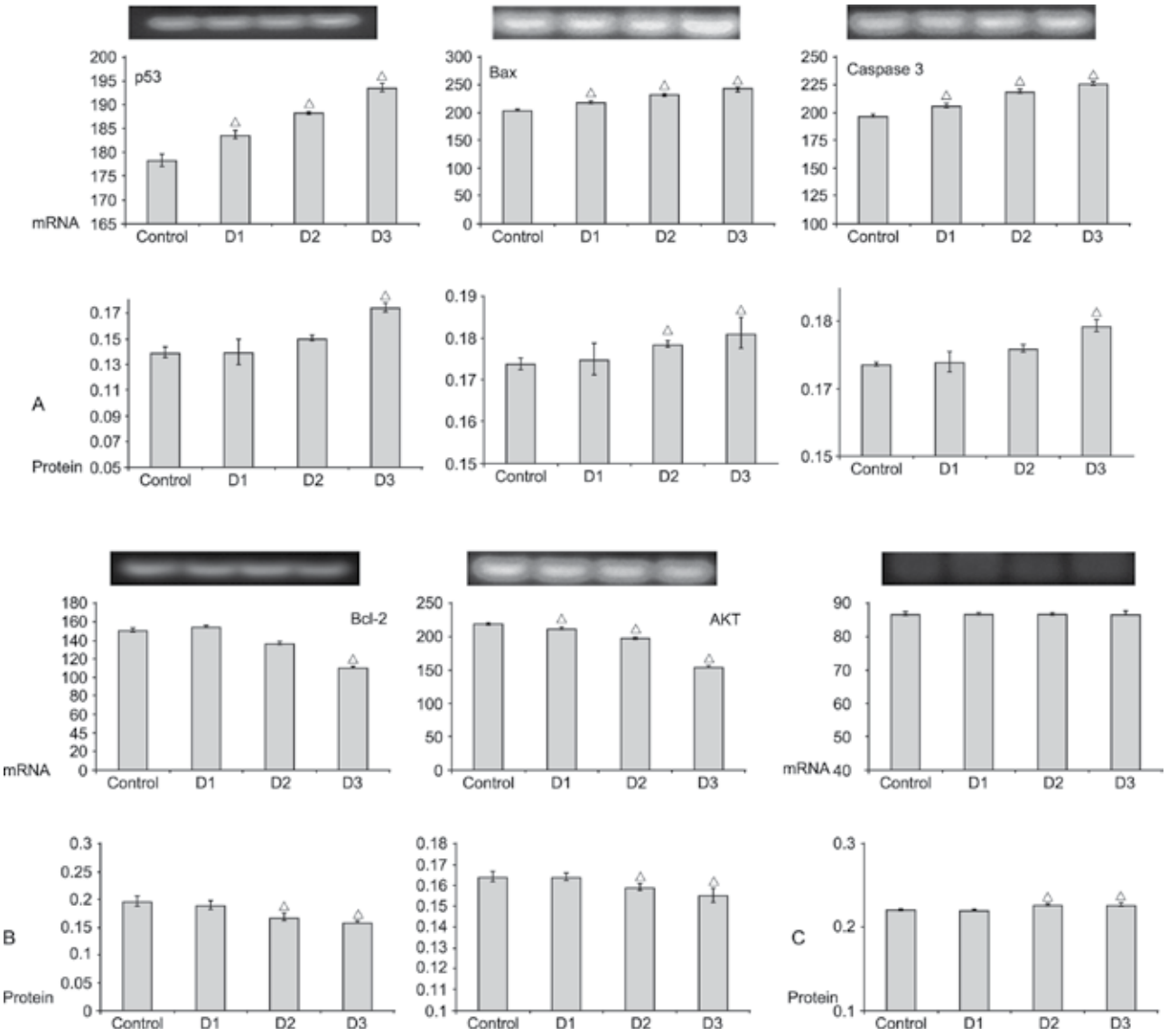

Figure 6 Expression of mRNA and protein

A: Up-regulated gene expression: the bar diagram represents the expression level of mRNA and protein; B: Down-regulated gene expression: the bar diagram represents the expression level of mRNA and protein; C: Expression of G3PHD at mRNA and protein level. G3PDH serves as the housekeeping gene. The results are expressed as mean ± standard error of mean, n=3; $^{\triangle}P$<0.05, *vs* normal control group.

D1: 100 μg/mL; D2: 150 μg/mL; D3: 200 μg/mL; G3PDH: glyceraldehyde 3-phosphate dehydrogenase.

Table 2 The Bcl-2/Bax ratio at the both mRNA and protein levels

Gene	mRNA Expression				Protein Expression			
	Control	D1	D2	D3	Control	D1	D2	D3
Bcl-2	1	0.997 802	0.903 297	0.734 066	1	0.962 838	0.856 419	0.810 811
BAX	1	1.068 293	1.138 211	1.193 496	1	1.005 747	1.026 82	1.042 146
Bcl-2/BAX	1	0.934 016	0.793 611	0.615 055	1	0.957 336	0.834 05	0.778 021

In case of both mRNA and protein activity expressions of Bcl2 and BAX, drug-treated are expressed as fractional values considering the control as the unity.

caspase 3 expression both at mRNA and protein levels (by indirect ELISA) were observed. Hence, the altered Bcl-2/Bax ratio and caspase 3 activation indicate that the cells were directed towards the apoptotic pathway.

Thus, it can be concluded that PD showed profound anticancer effect on skin melanoma by indulging the cells to apoptosis via elevation of ROS. Our study suggested that the ROS might activate p53 and block Akt and induce apoptosis via caspase 3-mediated pathway. Therefore, the results of the present study would validate the efficacy of PD as an anticancer drug and might encourage the CAM practitioners to use *Phytolacca* as a supportive medicine as an anti-melanoma drug.

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6 Competing interests

No authors has any competing interests to declare.

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