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Phytol shows anti-angiogenic activity and induces apoptosis in A549 cells by depolarizing the mitochondrial membrane potential

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ABSTRACT

In the present study, the antiproliferative activity of phytol and its mechanism of action against human lung adenocarcinoma cell line A549 were studied in detail. Results showed that phytol exhibited potent antiproliferative activity against A549 cells in a dose and time-dependent manner with an IC_{50} value of 70.81 ± 0.32 μM and 60.7 ± 0.47 μM at 24 and 48 h, respectively. Phytol showed no adverse toxic effect in normal human lung cells (L-132), but mild toxic effect was observed when treated with maximum dose (67 and 84 μM). No membrane-damaging effect was evidenced by PI staining and SEM analysis. The results of mitochondrial membrane potential analysis, cell cycle analysis, FT-IR and Western blotting analysis clearly demonstrated the molecular mechanism of phytol as induction of apoptosis in A549 cells, as evidenced by formation of shrinked cell morphology with membrane blebbing, depolarization of mitochondrial membrane potential, increased cell population in the sub-G0 phase, band variation in the DNA and lipid region, down-regulation of Bcl-2, upregulation of Bax and the activation of caspase-9 and -3. In addition, phytol inhibited the CAM vascular growth as evidenced by CAM assay, which positively suggests that phytol has anti-angiogenic potential. Taken together, these findings clearly demonstrate the mode of action by which phytol induces cell death in A549 lung adenocarcinoma cells.

1. Introduction

Lung cancer is the most frequent and leading cause of cancer death worldwide, accounting for more than 1 million deaths per year. The 5 years survival rate of lung cancer accounts for only 15% even with the therapeutic advances [1]. It has been reported that lung tumor contains approximately 200 nonsynonymous mutations per tumor. Cigarette smoke is identified as the potent mutagen, which plays a vital role in the pathogenesis of lung tumor [2]. Therefore efficient therapeutic strategies are needed to combat this disease. Several studies addressed the specific role of various factors; especially plant-derived bioactive nutrients which play a vital role in cancer prevention. These plant-derived bioactive nutrients, positively affected the cellular proliferation, cell growth, differentiation process and also reverted cancer-related epigenetic dysfunctions, prevented metastasis, reduced tumorigenesis and increased the efficacy of radio and chemotherapy [3]. Vanden Berghe [4] reported that plant-derived polyphenols also have the potential to inhibit tumor progression, prevent metastatic process by reverting the adverse epigenetic mutations in cancer cells. Moreover, it can sensitize the cancer cells to chemo and radiotherapy. In addition, the antioxidant and anti-inflammatory potential of polyphenols has the ability to modulate the transcription factors controlling the expression of genes involved in cell survival and metabolism [3]. Apart from the bioactive compounds, natural food products also have various beneficial effects, for example, Chen and Xu [5] reported that natural food products have the potential to influence three vital epigenetic processes such as DNA methylation, histone methylation and microRNA expression. In that series, essential oils and its constituents have been reported to possess various bioactive potentials in folk remedies [6]. The plant-derived essential oils are complex mixtures of volatile monoterpenes and sesquiterpenes, which protect plants from infection, disease, infestation and predation. Most of the plant terpenoids are cytotoxic to tumor cells, which can be used to develop chemotherapeutic or chemopreventive compounds [7].

Phytol the most abundant acyclic isoprenoid compound (Supplementary Fig. 1) is a constituent of chlorophyll, embedded in the thylakoid membranes of chloroplast [8,9]. It serves as an intermediate in the biosynthesis of chlorophyll, tocopherol and phylloquinone. In human foods, the presence of phytol is mainly restricted to beans, raw vegetables, spinach and asparagus. It is used as a non-mutagenic food additive with satisfactory safety. In addition to the presence in foods, *cis*-phytol was reported to be present in some essential oils of different

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varieties of *Hypericum* sp. and *Tortula muralis* [9,10]. Similarly, phytol was found to be predominant in the flower oils (7.3–15.2%) and leaf oils (36.5–54.5%) of *Onosma echioides* (L.) L. var. *columnae* Lacaita (Boraginaceae family) growing wild in central Italy [11].

It is mainly present as the fatty acid side chain in tocopherols and since phytol is hydrophobic in nature it has the potential to interact with the cell membrane [12]. Regarding toxicity, phytol has not been observed to show any adverse effect; even the phytol-derived compounds have no toxic effect and no cumulative inflammatory effect even in immunocompromised mice [7]. Phytol is reported to have a wide variety of bioactive potentials. Recently Pejin et al. [9] reported that phytol which is freely present in various food products showed enhanced anti-quorum sensing activity against *Pseudomonas aeruginosa* (PAO1). The cytotoxic potential of phytol against certain cancer cell lines from breast, prostate adenocarcinoma, cervical, lung, colorectal and melanoma has been reported recently [13]. Similarly, Song and Cho [14] reported that phytol shows anti-cancer activity in AGS human gastric adenocarcinoma cells. These reports positively suggest that phytol has anticancer potential; however, there are not many reports to substantiate this effect. Hence, the main objectives of this study are to examine the antiproliferative activity and its mechanism of action against human lung adenocarcinoma cell line. Here, we demonstrate that phytol induces apoptosis in A549 cells and show a lesser toxic effect in normal cells, which strongly suggests that phytol might have promising therapeutic effect against lung cancer.

2. Materials and methods

2.1. Chemicals

Phytol and Rhodamine 123 were purchased from Sigma-Aldrich, India. FITC Annexin V/Dead Cell Apoptosis Kit was purchased from Thermo Fisher Scientific. MTT and Acridine Orange were purchased from Himedia, India.

2.2. Cell lines and culture conditions

The lung adenocarcinoma cell line A549 and normal lung cell line L-132 were purchased from National Centre for Cell Science (NCCS), Pune, India. The cells were maintained in Dulbecco's modified Eagle medium (DMEM) at 37 °C supplemented with 10% Fetal Bovine Serum in a humidified incubator supplied with 5% CO_2. The effect of phytol was tested at various concentrations (17, 34, 51, 67 and 84 µM) against A549 and L-132 cells.

2.3. Assessment of antiproliferative activity

The antiproliferative activity of various concentrations of phytol against A549 cell line was tested at 24 and 48 h using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay according to the method of Barbier et al. [15] and Ye et al. [16]. Briefly, after treatment with phytol, the culture medium was removed and the cells were washed with PBS (pH 7.4) and further incubated with MTT solution (1 mg mL^{-1}) for 4 h at 37 °C. After incubation, the MTT solution was removed and the formed formazan salts were dissolved using DMSO. The absorbance was measured at 570 nm using multi-well plate reader (Molecular Device Spectramax M3, equipped with Softmax Pro v5 5.4.1 software). The IC_{50} concentration of phytol against A549 cells was determined using EPA Probit analysis programme v1.5. The percentage of viability was calculated using the following formula.

Cell viability (%) = Absorbance of test / Absorbance of control × 100

2.4. Assessment of cell morphology by phase contrast image analysis

The morphological changes of cells upon phytol treatment were assessed by phase contrast microscope image analysis. After treating the A549 cells with various concentrations of phytol for 24 h, the medium was removed and the cells were washed once with phosphate buffer saline [(PBS) pH-7.4]. The cells were observed and the image was captured using phase contrast inverted light microscope (NIKON ECLIPSE Ti-s).

2.5. Assessment of membrane damage by PI staining

The various concentrations of phytol treated A549 cells were washed twice with PBS (pH 7.4) and stained with Propidium iodide (200 µg mL^{-1}) for 10 min at room temperature. The excess stain was removed by washing the cells with PBS, air dried and observed under fluorescent microscope with excitation and emission wavelength of 536–620 nm.

2.6. Assessment of cytotoxicity in non-cancerous cells

2.6.1. Cytotoxicity testing using L-132 as a model

Cytotoxic effect of phytol was tested using non-cancerous lung cell line L-132 as a model. Cytotoxicity of various concentrations of phytol was tested by MTT assay for 24 and 48 h.

2.6.2. Cytotoxicity testing using PBMC as a model

Peripheral Blood Mononuclear Cells (PBMC) was isolated from healthy human volunteers. Isolated PBMC was incubated with various concentrations (17, 34, 51, 67, 84 and 101 µM) of phytol for 24 h. After incubation, the cells were collected by centrifuging the sample at 2200 rpm for 10 min. Trypan blue exclusion assay was performed to assess the cytotoxicity. The percentage of cell viability was calculated using the following formula.

Viability (%) = Total number of cells − Dead cells / Total number of cells × 100

2.7. Evaluation of mechanism of action of phytol

2.7.1. Assessment of mitochondrial membrane potential

The effect of phytol on mitochondrial membrane potential ($\Delta\psi m$) was measured spectrophotometrically using Rhodamine 123 fluorescent stain. A549 cells treated with various concentrations of phytol was collected and washed with PBS and incubated with Rhodamine 123 (5 µM) for 30 min at 37 °C. After incubation, the cells were washed twice with PBS (pH 7.4) to remove the excess staining solution and the cell pellet was resuspended in PBS. The fluorescence intensity was measured using multi-well plate reader (Molecular Device Spectramax M3, equipped with Softmax Pro V5 5.4.1 software) with the excitation and emission wavelength of 480 and 530 nm respectively; using PBS as a blank. Simultaneously the Rhodamine stained cells were observed under fluorescent microscope (NIKON ECLIPSE Ti-s) and the images were captured.

2.7.2. Scanning electron microscopy (SEM) analysis

The cell surface of control and phytol treated A549 cells were analysed by SEM. The sample preparation was done according to the protocol mentioned in Devi et al. [17]. A549 cells grown on glass slide were treated with phytol for 24 h. After treatment, the cells were washed with PBS (pH 7.4) and fixed overnight at 4 °C in 2.5% glutaraldehyde in PBS. The fixed cells were washed with PBS once and further dehydrated using gradient of alcohol and the dehydrated cells were examined using VEGA3 TESCAN at 10 kV.