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Protocatechualdehyde Induces Apoptosis in Human Non-Small-Cell Lung Cancer Cells by up Regulation of Growth Arrest and DNA Damage-Inducible (GADD) Genes

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Abstract

Growth arrest and DNA damage-inducible (GADD) 45 and GADD153 proteins have been implicated in DNA repair, cell cycle regulation and growth arrest along with numerous other cellular mechanisms. In recent years, evidence has emerged that proteins encoded by these genes play pivotal roles in tumor suppression and apoptotic cell death. Thus, compounds altering the expression of these genes are likely to be of interest in cancer prevention and/or treatment. Protocatechualdehyde is isolated from *Phellinus gilvus* and has been investigated as a promising cancer preventive agent because of its medicinal properties. This mushroom belongs to *Hymenochaetaceae Basidiomycetes,* and has advantages over many *Phellinus* species due to short growth period (3 months), making production cost-effective. The exact molecular mechanisms of protocatechualdehyde are not clearly understood. Based on studies of pro-apoptotic activity of protocatechualdehyde in T-cells and colorectal cancer cells, we examined the relationship between the expression of *GADD45* and *GADD153* and apoptosis induction in human lung cancer cell line PC-9. We report a p^{53}-independent increase in *GADD45* and *GADD153* expression by protocatechualdehyde. Likewise, the proliferation of PC-9 cells is inhibited via a G1/S arrest of the cell-cycle stimulating apoptosis. Further, induction of apoptosis was inhibited in PC-9 cells knocked down for GADD45 and GADD153. Protocatechualdehyde treatment also induced the expression of cell cycle inhibitors p^{21} and p^{27}, while inhibiting Bcl-2, cyclin D1, CDK2, CDK4 and CDK6 genes. These findings suggest that upregulation of GADD45 and GADD153 proteins are the mechanism for protocatechualdehyde's anti-tumor activities.

Keywords: Protocatechualdehyde; Apoptosis; Cell-cycle; Lung cancer; Cancer prevention

Introduction

Growth arrest and DNA damage-inducible (GADD) 45 and GADD153 proteins have been implicated in DNA repair, cell cycle regulation and growth arrest along with numerous other cellular mechanisms. In recent years, evidence has emerged that proteins encoded by these genes play pivotal roles in tumor suppression and apoptotic cell death. The GADD45 expression is rapidly regulated at both transcriptional and post-transcriptional levels in response to genotoxic stress via p^{53} tumor suppressor pathway or by mRNA stability as well as other growth-arrest signals [1]. Several studies have correlated GADD153 expression with cell death by treating the cells to different stress conditions especially with DNA damaging agents. Because of the involvement of GADD45 protein with the cell cycle, apoptosis, DNA repair and stability makes it an obvious target for cancer therapy [2]. Previous studies show that GADD45 and GADD153 proteins play key roles in the induction of programmed cell death and thus we sought to investigate their role in human lung cancer. In this study two GADD genes (*GADD45* and *GADD153*) were studied representing a p^{53}-dependent and a p^{53}-independent pathway respectively.

Lung cancers are carcinomas that derive from epithelial cells with small-cell lung carcinoma (SCLC), and non-small-cell lung carcinoma (NSCLC) being the main types. The estimated deaths from lung cancer is 159,480 (87,260 in men and 72,220 among women), accounting for about 27% of all cancer deaths [3]. Worldwide, there are 2.28 million new cases of lung cancer annually, with 1.59 million deaths, making lung cancer a leading cause of cancer-related mortality and account for an estimate of about 14% of all new cancers [4]. Tobacco use, particularly of smoking, is the main contributor to lung cancer; however, a significant number of non-smoking individuals are reported with lung cancer [5]. Males show higher mortality rate because of increased risk of lung cancer associated with cigarette smoking [6]. approximately reporting 63,000 new lung cancer cases each year in India [7]. Impediment in early detection and diagnosis of advanced stage is the main reason for high mortality [8]. The best strategy is to minimize the development of lung cancer using cancer preventive agents. There is a strong interest in cancer prevention with widely investigated natural products, such as capsaicin, green tea, turmeric, cardamom and resveratrol.

Protocatechualdehyde which is isolated from *Phellinus gilvus*, has been investigated as a promising cancer preventive agent because of its medicinal properties. This mushroom belongs to *Hymenochaetaceae Basidiomycetes,* and has advantages over many *Phellinus* species due to short growth period (3months), making production cost-effective [9-11]. Previous studies have established various biological activities of protocatechualdehyde**.** Among the reported benefits of protocatechualdehyde are inhibition of pulmonary inflammation, prevention of intraperitoneal adhesion under infectious circumstances, promotion of dermal wound healing, anti-platelet aggregation and antitumor activities [9,10,12,13]. Protocatechualdehyde has been

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a subject of continuous interest because of its effective scavenging property for oxygen free radicals [14,15]. DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging and xanthine-oxidase (XO)-inhibitory activities, antioxidant/anti-inflammatory activities of protocatechualdehyde have been evaluated. Apoptotic cell death induced by protocatechualdehyde is demonstrated in different cells. The exact molecular mechanisms of protocatechualdehyde are not clearly understood [16-19]. Since protocatechualdehyde is well studied to have therapeutic effects on various types of diseases, the cancer preventive activity of protocatechualdehyde is of very much interest, and experiments with protocatechualdehyde in animal models indicate the protective nature against several diseases. Specifically, protocatechualdehyde inhibited cell growth of various cancer cell lines, and induced apoptosis of cancer cells [16,20-22]. Moreover, protocatechualdehyde was shown effective on cell-cycle regulation of the mammalian cell line [17]. Based on these findings, we investigated the molecular mechanisms of cancer preventive and anti-cancer activities of protocatechualdehyde for lung cancer prevention in humans, and this study provides the first report on role of protocatechualdehyde in the prevention of lung cancer in human.

Numerous reports have focused on evidence that the cancer preventive activity is stimulated by altering the phases of cell cycle, associated with the modulation of their regulatory components [23,24]. This suggests that these genes are induced by stimulation of GADD45 and GADD153 in response to DNA damage and growth arrest, which is shown in many cell types. In this study we report the anti-proliferative effects of protocatechualdehyde on human lung cancer cell lines PC-9 and A549 by inducing GADD45 and GADD153 by p^{53} independent manner. These results add to our knowledge on importance of protocatechualdehyde on lung cancer prevention.

Materials and Methods

Chemicals and reagents

Protocatechualdehyde, 4', 6-diamidino-2-phenylindole (DAPI), and propidium iodide (PI) were purchased from Sigma-Aldrich Pvt. Ltd. (India). Antibodies specific for GADD45, GADD153 and GAPDH were obtained (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell culture and transfection

The human non-small cell lung cancer cell lines A549 and PC-9 were cultured in DMEM-F12 supplemented with 10% HyClone fetal bovine serum (FBS) (ThermoFisher Scientific, Fremont, CA, USA) in an atmosphere of 5% CO2 at 37°C. Cells were grown in 75 cm^2 culture flasks and harvested in a solution of trypsin-EDTA at the logarithmic growth phase.

PC-9 cells were transfected with human *GADD45* and *GADD153* specific siRNAs prepared by Invitrogen. Stealth RNAi negative control duplex (Invitrogen) was used as a control. The siRNA targeting *GADD45* corresponds to the sequence GAGCAGAAGACCGAAAGGAUGGAUA, and the siRNA targeting *GADD153* corresponds to the sequence GAGAAUGAACGGCUCAAGCAGGAAA. PC-9 cells were transfected using Lipofectamine RNAi max (Invitrogen), according to manufacturer's instruction. In brief, 1.4×10^6 PC-9 cells were transfected with final concentration of 50 nM siRNA in six-well culture plate in triplicates. Cells were treated with protocatechualdehyde or vehicle 24 h after transfection. DAPI staining was performed for apoptosis assay. Cells were lysed in lysis buffer containing 0.2% NP-40. Total RNA was extracted from the cells lysate using TRIzol reagent following the manufacturer's instructions (Invitrogen).

Western blotting

Cells lysate was estimated for total protein content by Bradford assay. Total 10 µg of protein sample was run on 10% polyacrylamide gel. Subsequently transferred to a polyvinylidene difluoride (PVDF) (Millipore) membrane by semi-dry electroblotting. Blocked in 5% nonfat dry milk in TBS with Tween 20 (0.05%). Blots were incubated at RT for 1h in primary antibody at 1:1250 dilutions in blocking solution. Following washing with TBST, membranes were incubated respective horseradish peroxidase (HRP)-conjugated secondary antibody, and visualized with ECL plus western blot detection kit (GE Healthcare).

Cell viability and cell growth assay

Trypan blue dye exclusion method: PC-9 cells (1×10^4/ml) were cultured in 6-well plates for 24 h followed by treatment with different concentrations of protocatechualdehyde or vehicle as control supplemented with 10% FBS for 24-72 h with changing culture medium every 24 h. Protocatechualdehyde was dissolved in DMSO as stock solution and stored at -20°C. Trypan blue dye exclusion test [25] was used to determine the inhibition of cell growth. The result was expressed as a percentage, relative to treatment with vehicle control, and the IC_{50} values were calculated using non-linear regression analysis (percent survival versus concentration). Results shown are from at least three independent experiments performed in replicates.

MTT assay: Cell viability was evaluated by measuring the amount of insoluble formazan formed in live cells based on the reduction of MTT salt. Briefly, 100 µl PC-9 cell suspensions at 2×10^4 cell/ml were seeded in 96 well micro titer plate (BD, USA). Protocatechualdehyde in concentration range of 0-100 µg/ml were added. MTT reagent was added after 72 h exposure followed by dissolution of formed formazan crystal using DMSO (Sigma, USA). Optical density was read with ELISA reader (LX-800) at 550 nm. The dose-response curve is plotted and concentration that exhibited 50% inhibition of cell growth (IC_{50}) is calculated. Concentration that inhibits 50% of cell viability was used as a parameter for cytotoxicity.

Cell-cycle analysis

To compare the effects of different concentration of protocatechualdehyde on the cell cycle, the Cycle TEST™ PLUS DNA Reagent Kit (Becton Dickinson, USA) was used. The cells (1×10^6 cells/ml) were seeded in a 6-well plate and treated with protocatechualdehyde for 24h. After harvesting, made to suspension, permeabilized with trypsin buffer. Incubated with propidium iodide (1mg/ml) for 10 min in dark. Cells were analyzed by flow cytometry (BD FACS Calibur, USA) using BD Cell Quest acquisition and analysis software. Cell cycle phase distribution of nuclear DNA was determined by analyzing at least 10,000 cells per sample and the percentage of cells in G1, S and G2 phases.

Apoptosis assay

Identification and quantitation of apoptotic cells were done by DAPI staining. PC-9 cells were cultured overnight at the density of 5×10^5 cells/ml in a 6-well culture plate and then treated with different concentrations (0-100 µg/ml) of protocatechualdehyde for 24 h. The cells were then fixed with 500 µL fixing solution (acetic acid: methanol; 1:3) for 5 min, dried, and stained with the DNA-specific fluorochrome DAPI (2 µg/ml). Following 10 min of incubation, the cells were washed with phosphate buffered saline (PBS), air-dried, mounted with 90% (v/v) glycerol. The percentage of apoptotic cells was calculated using fluorescence microscopy (Olympus, Japan) with at least 200 randomly selected cells. Results are obtained from at least three independent experiments.

Quantitation of cell cycle and apoptosis related gene expression by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cultured PC-9 cells treated with various concentrations of protocatechualdehyde for 24 h using the TRIzol reagent and then one µg of RNA subjected to reverse-transcription using SuperScript® III First-Strand Synthesis System (Life Technologies, Inc.). To allow a quantitative determination of relative gene expression levels for the apoptosis related genes [26]. The cDNA content of the samples was normalized, and the linear range of amplification was determined using SYBR Green reagent for each primer set PCR on 7300 Real Time PCR System (Applied Biosystems). GAPDH expression was used as normalization control. At least two independent experiments were performed to confirm the results. The sequences of the primers used are provided in Table 1.

Statistical analysis

Statistical analyses were performed with Student's *t*-test. The results were considered to be significant when *p* value was less than 0.05, $^{*}p<0.05$, $^{**}p<0.01$.

Results

Effects of protocatechualdehyde exposure on cell viability and cell growth

Protocatechualdehyde inhibited cell growth in PC-9 cells, when exposed with various concentrations and the inhibition was dose-dependent manner shown in Figure 1. After 24 h treatment with 100 µM protocatechualdehyde, the percentage of viable cells was 47.3% of the vehicle control and after 48h treatment with 75 and 100 µM protocatechualdehyde viable cells reduced to 48.6 and 31.0% of control. The lowest and highest dose with significant changes were observed at 50 µM and 100 µM respectively, above which no considerable changes were observed and therefore these two doses were used in the study for analysis of role of protocatechualdehyde on expression of gene of interest.

Protocatechualdehyde induce apoptosis of PC-9 Cells

We determined the mechanisms of growth inhibition activity of PC-9 cell lines and further investigated apoptosis of PC-9 cells by morphological changes with DAPI staining. Cells were treated with various concentrations of protocatechualdehyde and percentage of apoptotic cells increased dose-dependent manner and cells with condensed and fragmented nuclei were observed. After 48 h treatment of cells with protocatechualdehyde at 25, 50, 75 and 100 µM concentrations, apoptosis was induced as 11.4, 32.5, 41.5 and 62.3% of cells respectively (Figure 2). From the above results it was confirmed that protocatechualdehyde treated cells shows alterations in the expression of GADD genes, growth inhibition, and stimulation of cell cycle arrest and apoptosis.

Effect of protocatechualdehyde on cell cycle regulation in PC-9 Cells

Regulatory effects of protocatechualdehyde on the cell cycle were evaluated in PC-9 cells. Protocatechualdehyde at a concentration of 100 µM in PC-9 cells significantly increased the cells of G1 phase from 46.6 to 63.2% (Figure 3, Table 2). PC-9 cells when treated with protocatechualdehyde at concentrations of 25 and 50 µM for 24 h, an accumulation of S phase respectively was observed (Figure 3, Table 2).The growth inhibition was linked to protocatechualdehyde induced G1 and S phase arrests in the cell cycle in PC-9 cells.

Protocatechualdehyde induced expression of genes associated with cell cycle and apoptosis

The eukaryotic cell cycle is regulated by the periodic synthesis and destruction of cyclins and their cyclin-dependent kinases and

Gene	Sequence Forward (5'–3')	Sequence Reverse (5'–3')
GAPDH	CCAGCAAGAGCACAAGAGGA	TACATGACAAGGTGCGGCTCCC
Bcl-2	CTGCACCTGACGCCCTTCACC	CACATGACCCCACCGAACTCAAAGA
Bax	TCAGGATGCGTCCACCAAGAAG	TGTGTCCACGGCGGCAATCATC
Cyclin D1	*AGCTCCTGTGCTGCGAAGTGGAAAC*	*AGTGTTCAATGAAATCGTGCGGGGT*
CDK2	ATGGATGCCTCTGCTCTCACTG	CCCGATGAGAATGGCAGAAAGC
CDK4	CCATCAGCACAGTTCGTGAGGT	TCAGTTCGGGATGTGGCACAGA
CDK6	ACCTCTGGAGTGTCGGTTGCAT	TTCCTCTCCTGGGAGTCCAATG
GADD45	GCCTGTGAGTGAGTGCAGAA	CCCCACCTTATCCATCCTTT
GADD153	AGAACCAGGAAACGGAAACAGA	TCTCCTTCATGCGCTGCTTT
p21	GGCAGACCAGCATGACAGATT	GCGGATTAGGGCTTCCTCT
p27	AGGAGAGCCAGGATGTCAGC	CAGAGTTTGCCTGAGACCCAA

Table 1: Primer sequences Used in Quantitative Real-Time PCR.

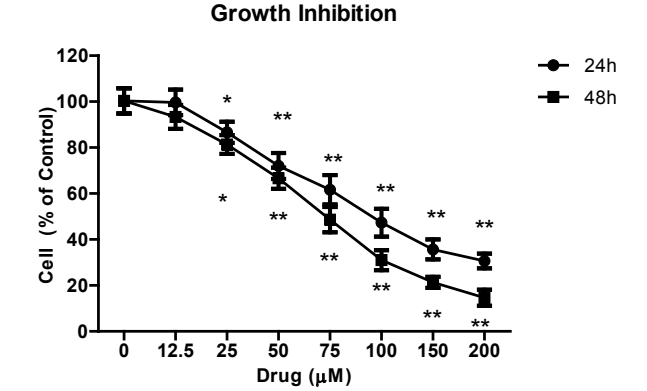

Figure 1: Effects of protocatechualdehyde exposure on cell viability and cell growth: Effects of protocatechualdehyde on cell viability in PC-9 cell lines was tested by treating with increasing concentrations of protocatechualdehyde for 24 and 48 hours. The viable cell numbers were counted using a hemocytometer via trypan blue dye exclusion. Values are expressed as means ± SD of three separate experiments performed in triplicate. *p<0.05. ** p<0.01.a.

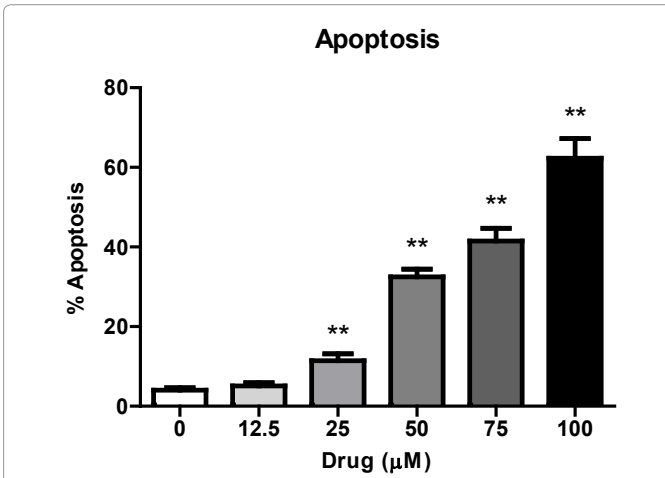

Figure 2: Protocatechualdehyde induce apoptosis of PC-9 Cells: Apoptotic cells were counted in PC-9 cells randomly after DAPI staining and morphological changes showing apoptosis features were measured by fluorescence microscopy. Results of three independent experiments (± SD) shown as apoptotic index. **p<0.01.

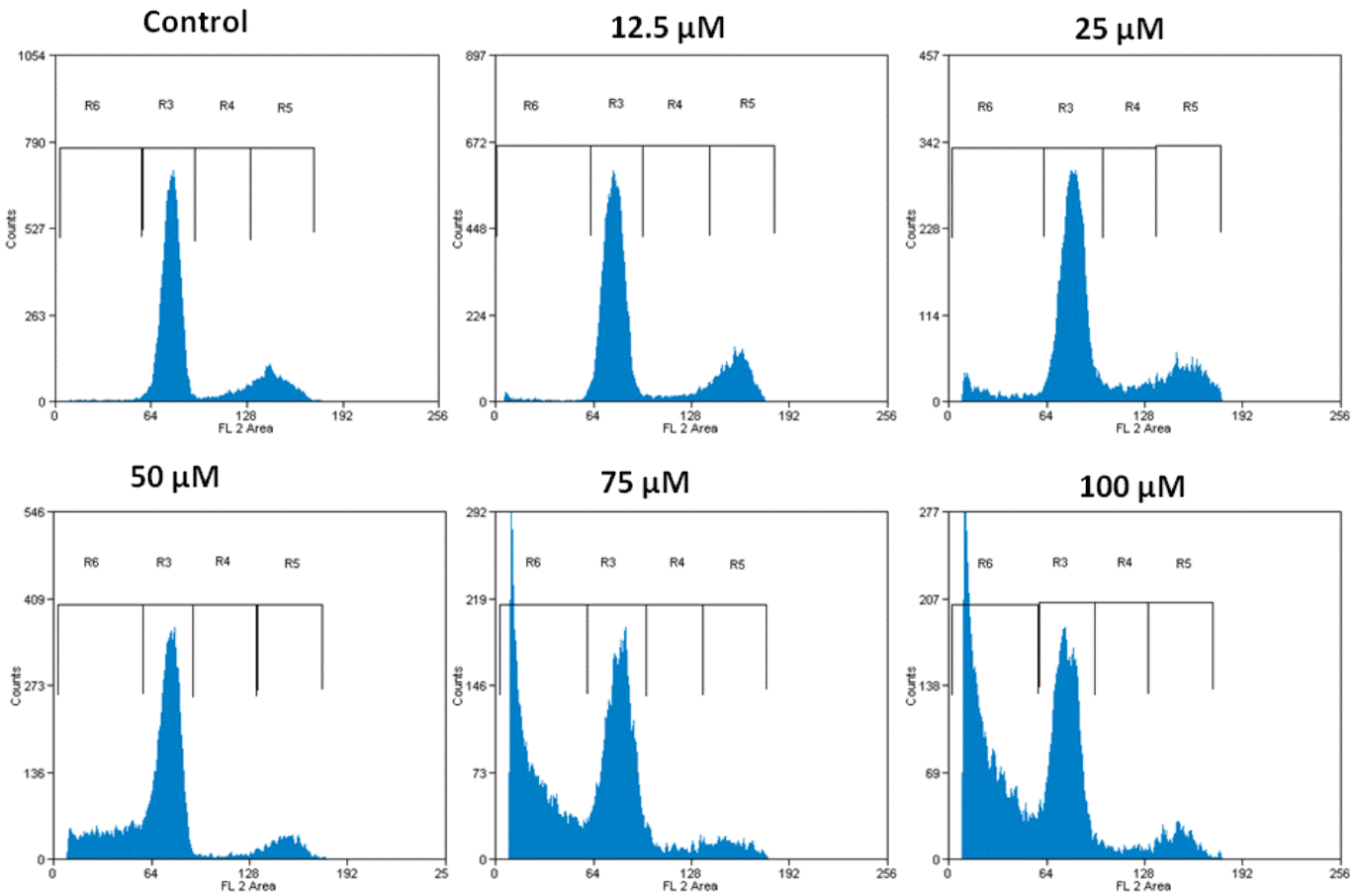

Figure 3: Effect of protocatechualdehyde on cell cycle regulation in PC-9 Cells: PC-9 cells ($1x10^6$) were incubated with protocatechualdehyde for 24 h. Following propidium iodide staining, the DNA contents of about 10,000 cells in each group were measured with BD FACSCalibur flow cytometer and analyzed using BD CellQuest software. Similar results were obtained in two independent experiments.

Protocatechualdehyde (µM)	Cell Cycle Phase			
	G_0/G_1	S	G_2/M	Sub G_0
0	74.2 ± 2.6	6.4 ± 0.9	18.6 ± 1.4	1.2 ± 0.2
12.5	71.8 ± 2.4	5.6 ± 0.6	21.1 ± 2.1	2.5 ± 0.6
25	67.5 ± 2.7	8.2 ± 1.1	17.8 ± 1.9	7.2 ± 1.0
50	65.9 ± 3.6	2.5 ± 0.4	9.6 ± 0.8	22.5 ± 4.1
75	46.5 ± 2.3	4.8 ± 0.9	5.8 ± 0.6	43.6 ± 3.8
100	41.6 ± 3.2	3.5 ± 0.2	6.5 ± 1.3	48.4 ± 1.9

Table 2: Induction of G1/S Cell-Cycle Arrest by Protocatechualdehyde.

negatively controlled by cyclin-dependent kinases inhibitors (called CDIs) [27]. Expressions of these cell-cycle regulatory genes like *Cyclin D1*, *CDK2*, *CDK4* and *CDK6* were determined in protocatechualdehyde treated PC-9 cells for understanding the mechanisms of apoptosis. Expression of genes and fold changes were evaluated by quantitative real-time PCR after 24 h of treatment with protocatechualdehyde in PC-9 cells. Transcript levels of *cyclin D1*, *CDK2*, *CDK4* and *CDK6* genes found to be decreased, as shown in Figure 4. In particular, after 24 h protocatechualdehyde at 100 µM concentration, the mRNA levels of *cyclin D1*, *CDK2*, *CDK4* and *CDK6* were down-regulated 0.28, 0.31, 0.42 and 0.61 fold, compared with that of vehicle control. However, after 24 h treatment of protocatechualdehyde at a concentration of 100 µM significantly increased the mRNA levels of the CDK inhibitors p^{21} and p^{27} to 17.46 and 5.96 fold (Figure 4). Protocatechualdehyde in PC-9 cells decreased the expression of *Bcl-2* (Figure 4) and there was no change in expression of *Bax* gene.

Protocatechualdehyde induces *GADD45* and *GADD153* gene expression

GADD45 and *GADD153* genes were up regulated with protocatechualdehyde treatment in PC-9 cell line. Though the increased expression was time and dose dependent still 100 µM protocatechualdehyde dosages increased 29.2 and 21.6 fold expression after 24 h incubation (Figure 5). Protocatechualdehyde treated human lung cancer cells A549 cells with wild type p^{53} also showed similar increased expression of *GADD45* and *GADD153*. These results suggest p^{53}-independent induction of GADD45 and GADD153 by protocatechualdehyde. Above observation was confirmed at protein

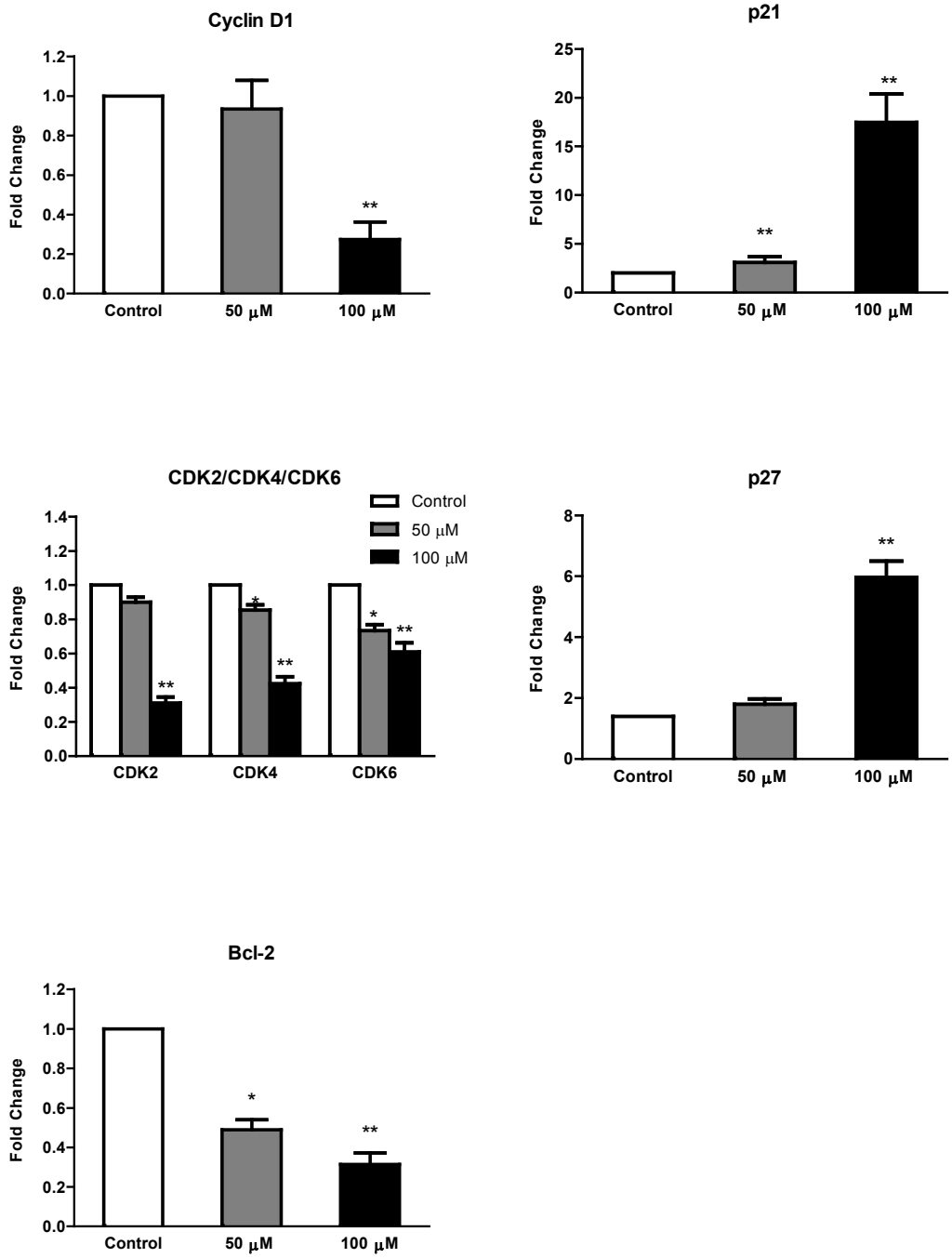

Figure 4: Protocatechualdehyde induced expression of genes associated with cell cycle and apoptosis: Expression levels of genes associated with cell cycle and apoptosis were compared among protocatechualdehyde treated PC-9 cells, by using qRT-PCR. The data are fold difference of the gene expression of two representative experiments performed in triplicate. *p<0.05, **p<0.01. Gene expression levels are normalized to *GAPDH* of control cells, and taken as 1.

level by western blotting for GADD45 and GADD153 proteins and shown increase in protein level as shown in Figure 5.

Protocatechualdehyde on GADD45 and GADD153 knockdown:

From the above observations it was clear that protocatechualdehyde induces the *GADD45* and *GADD153* gene expression with consequential in apoptotic induction. Functions and involvement of *GADD45* and *GADD153* genes were explored to study the mechanisms of apoptotic induction using siRNA-mediated knockdown of *GADD45* and *GADD153*. PC-9 cells were treated with GADD45 and GADD153 siRNAs for 24 h, and expressions of the *GADD45* and *GADD153* genes that were induced by 100 μM protocatechualdehyde was inhibited (Figure 6A). Furthermore, protein production of GADD45 and GADD153 in PC-9 cells induced by protocatechualdehyde (was also inhibited by the siRNAs (Figure 6B). It was apparent to study the relationship between the reduction of GADD45 and GADD153 proteins and its effect on the apoptotic induction by protocatechualdehyde in PC-9 cells.

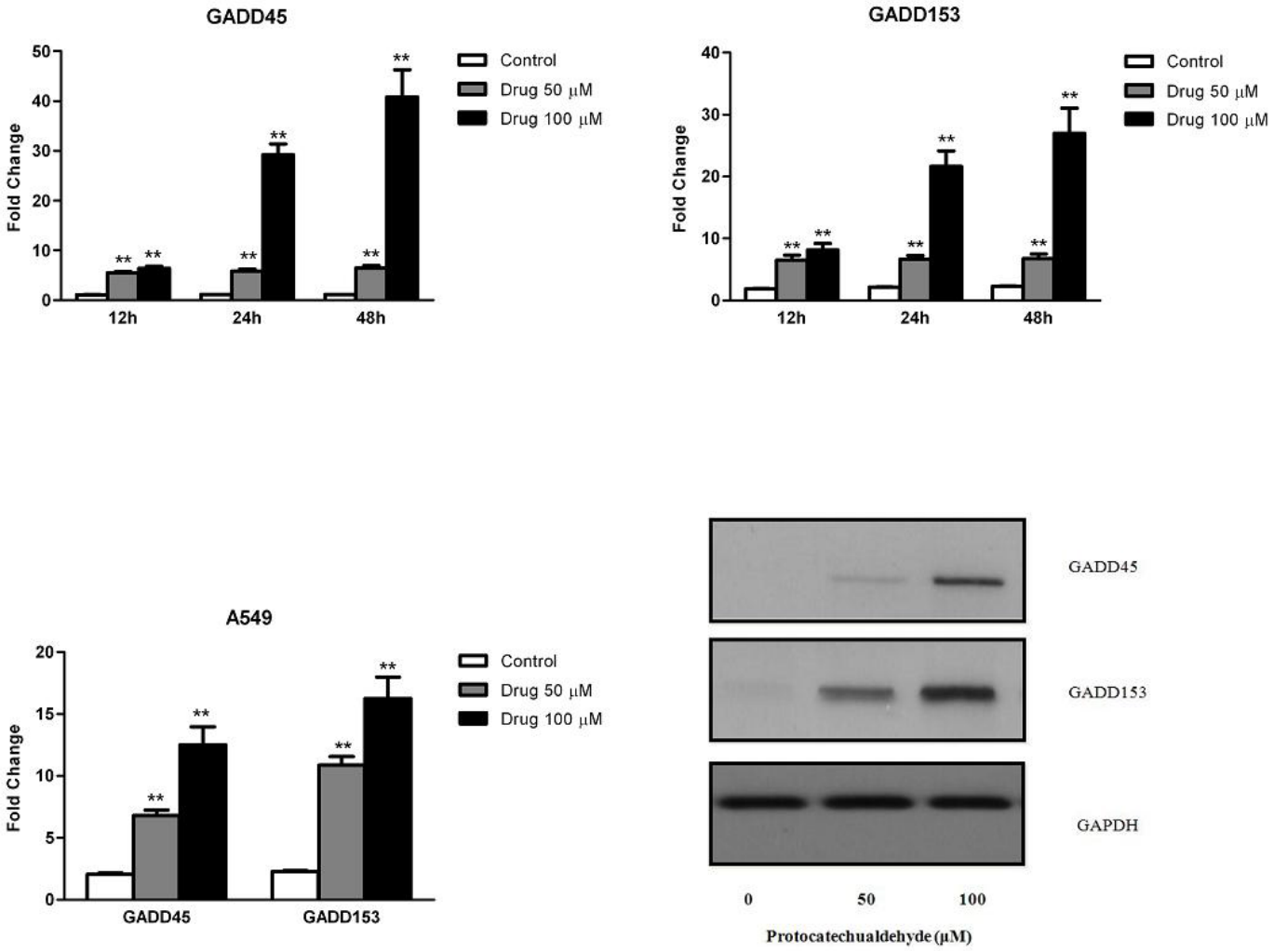

Figure 5: Protocatechualdehyde induces *GADD45* and *153* gene expression: PC-9 cells were induced by treating with protocatechualdehyde (50 or 100 μM) or the same volume of vehicle and then total RNA was harvested and used for gene expression by RT-qPCR performed. The results were analyzed by the delta-Ct method and expression of target genes was normalized to GAPDH expression. Data is presented as fold change to vehicle treated control cells. (**) indicates significant increase, $p < 0.05$, in comparison to control. Bars represent mean ± S.D. of two experiments performed in triplicate. Western blot showing increase in GADD45 and GADD153 protein expression upon treatment, GAPDH served as the loading control.

In comparison with the control experiments where the percentage of apoptotic cells were 20.0 and 33.0%, siRNA induced apoptosis in 24.0 and 35.6% of PC-9 cells treated with 50 and 100μM protocatechualdehyde, respectively, at 24 h (Figure 6C). However, PC-9 cells treated with GADD45 and GADD153 siRNA, showed significantly lower sensitivity towards the apoptotic induction by protocatechualdehyde: 11.0 and 11.8% at 50 μM, and 15.6 and 16.2% at 100 μM, respectively (Figure 6C). From the above observations it was evident that, both the *GADD45* and *GADD153* genes play significant role in protocatechualdehyde-induced apoptosis and shall be targeted for controlling the apoptosis process in the PC-9 cells.

Discussion

In the present study, we determined the growth inhibitory activities and IC_{50} values of protocatechualdehyde and induction of G1/S phase cell cycle arrest and apoptosis in colon cancer PC-9 cells. Earlier studies revealed that protocatechualdehyde was able to exert consistent anti-proliferative activity indicating arrest from G1 to S in various cancer cells, such as human colorectal cancer cells and smooth muscle cells [16,28]. The G1/S checkpoint is primarily controlled by collective kinase activities of the complex of cyclin D with CDK4, CDK6 and cyclin E and these G1 kinases can in turn be regulated by cell cycle inhibitors, which may cause the cells to arrest at the G1 phase [29]. To evaluate the cell cycle inhibitor effect of protocatechualdehyde, we investigated changes in regulation of putative G1 cyclin such as *cyclin D1* and the cyclin dependent kinase *CDK2*, *CDK4, CDK6* and observed that cell-cycle arrest is associated with downregulation of these cell cycle regulators [30]. Nuclear protein Cyclin D1, an essential regulator of G1 to S phase progression, is tightly associated, and aberrantly expressed in numerous human cancers [31] including human lung tumors, and several other types of human cancer [32]. Cyclin D1 is frequently overexpressed in various forms of cancer including non-small cell lung carcinoma (NSCLC) [33] and decrease in the level of expression of Cyclin D1 inhibits growth and reverses the transformed phenotype of human esophageal cancer cells [34]. Phosphorylation of the retinoblastoma (Rb) protein during the G1/S transition of the cell cycle appears to be initiated by Cyclin D/CDK4 and from this study we believe that protocatechualdehyde exerts its effect by cell cycle

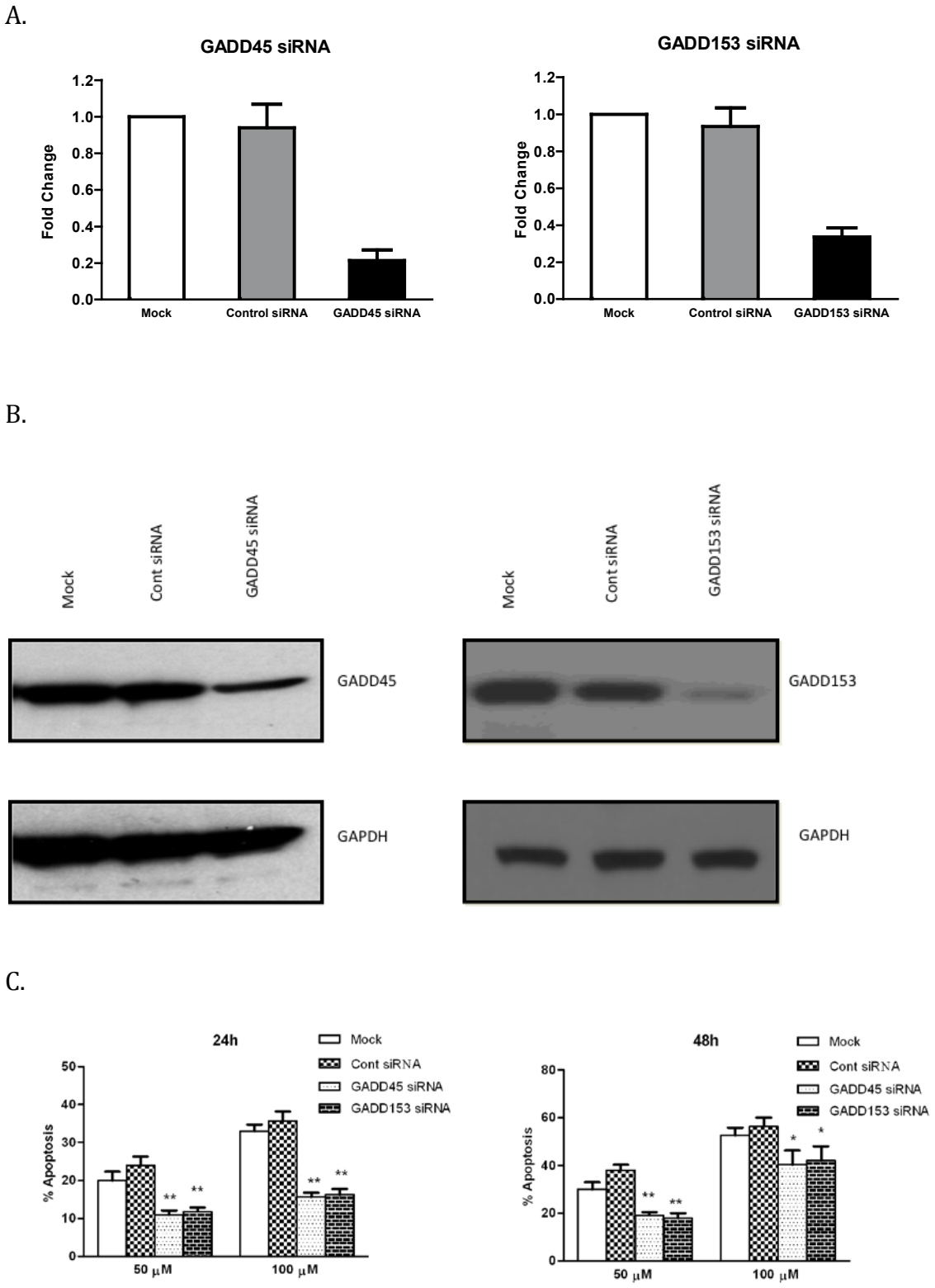

Figure 6: Protocatechualdehyde on GADD45 and 153 knockdown: PC-9 cells were transfected with Lipofectamine with *GADD45*, *GADD153* siRNAs, control siRNAs or no siRNA. Controls for viability included cells mock-transfected with no siRNA or cells transfected with control siRNA. Twenty-four hours after transfection, cells were treated with vehicle or 50 or 100 μM protocatechualdehyde for 24 h. (A) The amounts of GADD45 and GADD153 mRNA with the mean of three separate experiments done in triplicate are expressed as mean ± S.D. Expression level of genes normalized to *GAPDH* of mock was considered as 1 (B) followed by Western blotting with GAPDH as loading control. Three independent experiments produced similar results. (C) In a separate experiment, 24 hours after transfection, cells treated with vehicle, 50 or 100 μM protocatechualdehyde were evaluated for extent of apoptosis, measured by DAPI staining at 24 and 48 h. Bars represent mean ± S.D. of two independent experiments performed in triplicate. *p<0.05, **p<0.01.

arrest in G1 phase in PC-9 cells by reducing the expression of Cyclin D1 and *CDK4/6* genes. Also the expression of *CDK2* is decreased, demonstrating the importance of *CDK2* in the regulation of the cell cycle G1 checkpoint [35]. CDK inhibitor such as p^{21} is found associated with multiple Cyclin-CDK complexes, phosphorylated p^{21} binds simultaneously to both cyclin and CDKs and cyclin/CDK complexes are regulated by p^{21} [23]. Indeed, p^{21} is a potent CDK inhibitor and displays selectivity for G1, and over-expression of p^{21} arrest cells in the G1 phase of the cell cycle [36,37]. Protocatechualdehyde reportedly affects cell proliferation and apoptosis in human colorectal cancer cells and define potential mechanisms by which protocatechualdehyde mediate growth arrest and apoptosis of cancer cells. Protocatechualdehyde decreases Cyclin D1 expression in protein and mRNA level and suppress luciferase activity of Cyclin D1 promoter, indicating transcriptional downregulation of cyclin D1 gene in human colorectal cancer cells. It was recently reported that suppressed cell proliferation by G1 arrest and induced apoptosis in human colorectal cancer cells [16] and the effect may mediated by apoptosis through upregulation of p^{21}, in view of the fact that upregulation p^{21} may promote apoptosis. We hypothesized that protocatechualdehyde inhibited the growth of PC-9 cells and induced G1/S arrest of the cell cycle by upregulation of p^{21} expression. Another member of the CDK inhibitor family p^{27}/Kip1 binding to Cyclin E-CDK2 complexes and the binding dependent phosphorylation of p^{27} is involved in the transition of G1 to S phase resulting in tumor suppression [38,39]. Earlier studies present strong evidence that the lack of functional p^{27} has a profound effect on tumorigenesis and is a major factor in the development of human tumors [40-43]. Up-regulated p^{27} protein activity in PC-9 cells treated with protocatechualdehyde signifies that the p^{27} gene upregulation is associated with cancer preventive activity [23].

To explore the possible association between *GADD45* and *GADD153* genes with apoptosis in PC-9 cells treated with protocatechualdehyde, we examined the knockdown of *GADD45* and *GADD153* gene expressions by siRNA in PC-9 cells. However, no significant change in the expression of cell-cycle regulating gene including p^{21}, p^{27}, *cyclin D1*, *CDK2*, *CDK4*, and *CDK6* were observed. From our result, the observed G1/S cell-cycle arrest by protocatechualdehyde in PC-9 cells may be associated with the variation of cell-cycle regulators by protocatechualdehyde. A significant observation in this study is that, expression of *GADD45* and *GADD153* genes in both p^{53} mutated PC-9 cells and p^{53} wild type A549 cells was up-regulated by treatment with protocatechualdehyde, indicating that p^{53} protein does not have direct stimulatory effect on expression of *GADD45* and *GADD153*. The present study was designed to determine whether protocatechualdehyde increased expression of the gene *GADD 45* and *GADD153*, which are induced by genotoxic stress through p^{53}-dependent and -independent pathways. *GADD45* and *GADD153* gene expressions are induced by deoxycholate in both HCT-116 colonocytes with wild type p^{53} and p^{53} mutant HCT-15 colonocytes [44]. GADD proteins have been shown to inhibit proliferation and stimulate DNA repair and/or apoptosis [45]. The induced effect of protocatechualdehyde on these gene expressions in PC-9 and A549 cells would be independent of p^{53} as it was reported with other genotoxic agents. One observation reported by several researchers involves correlation between upregulation of *GADD45* and *GADD153* and apoptotic induction in actively dividing cells [46-48]. Earlier study indicates that the induction of apoptosis in mice exposed to hyperoxia was associated with the upregulation of *GADD45* and *GADD153* gene expressions [45]. Natural products like curcumin is reported to induce DNA damage, apoptosis by *GADD153* gene in human colon cancer cell lines [48].

In this study, we show that treatment with siRNAs inhibits the expression of *GADD45* and *GADD153* in PC-9 cells and induce apoptosis, suggesting significant role of GADD gene family in the mechanisms of cancer prevention. Protocatechualdehyde is also one of the active components of cardamom, popular dietary spice [49]. Data from earlier studies indicate that regular consumption of medicinal herbs like cardamom enhances activities like fibrinolysis, antioxidant [50] and Natural killer (NK) cell [51] in humans. Therefore, the daily consumption of cardamom will contribute to protocatechualdehyde and protect against the risk of cancer. *GADD45* and GADD153 are new targets for protocatechualdehyde in lung cancer, and may be used in lung cancer prevention and treatment in humans.

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