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Ethanolic extract of *Thuja occidentalis* blocks proliferation of A549 cells and induces apoptosis *in vitro*

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**OBJECTIVE:** To study the possible anticancer and antiproliferative activities of ethanolic leaf extract of *Thuja occidentalis* (TO) or A549 non-small lung carcinoma cells *in vitro*.

**METHODS:** Cell viability was ascertained through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay after treatment of TO in different doses. The half maximal inhibitory concentration (IC50) dose (C98.2 μg/ml) was determined, and two other doses for dose-dependent study, one below the IC50 dose (IC0.1–188 μg/ml) and one above the IC50 dose (IC1, 278 μg/ml) were selected. Bromodeoxyuridine (BrdU) incorporation assay and migration studies were performed to elucidate and proferative activity of the drug, if any. Fluorescence-activated cell sorting analysis after annexin V-fluorescein isothiocyanate and propidium iodide (annexin V-FITC-P) d.i.d staining method was done to ascertain early stage of apoptosis, if any. DNA fragment assay was done through l beschel (32P-32P) and acridine orange-stained bromide staining. DNA damage was confirmed through comet assay. Box-Box regulation and expression studies were performed through indirect cDNA expression molecular assay (ELISA). Caspase-3 activity was measured through reverse transcription-polymerase chain reaction (RT-PCR) analysis. Apoptosis at protein level was assessed through indirect ELISA and Western blot analysis.

**RESULTS:** TO demonstrated a dose-dependent decrease in viability of A549 cells after 24 h of exposure. Cell proliferation was reduced in a time-dependent manner of drug exposure as revealed from BrdU incorporation and migration studies. Annexin V-FITC positivity of cells up to 11.7% as compared to the untreated controls revealed early stage of TO-induced apoptosis. Percentage of comet tail and increased fluorescence of Hoechst after 24 h of drug exposure revealed significant.

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DNA repair activity and chromatin condensation. Bar- up-regulation and Bel-2 down-regulation suitably altered ratio of Bax/Bel-2 in favor of apoptosis. Flow cytometry, indirect LISA and Western blot studies, caspase 3 activity was also found to be significantly increased along with cleaved only ADP-ribosylpolymerase expression.

CONCLUSION: Ex vivo c leaf extract of TO demonstrated apoptotic and antiproliferative potentials against A549 cell line.

KEYWORDS: Thuya; plant extracts; carcinoma, non-small-cell lung; cell proliferation; apoptosis; antineoplastic agents; physiology; in vitro

Lung cancer is one of the major threats among all types of cancer, accounting for almost 1.4 million cases per year.1 Lung cancer is mainly differentiated into two categories, namely, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). Although several chemotherapeutic approaches can be attempted against SCLC, therapeutic scope for NSCLC is limited as it has a very narrow range of chemotherapeutic sensitivity. The drugs which are found to be relatively effective are generally cytotoxic against normal cells, precluding their therapeutic use. So new drugs which are relatively less cytotoxic to normal cells but have anticancer and antiproliferative effects on NSCLC are highly sought after.

*Thuya occidentalis* (TO) is one of the major herbs largely produced in part of Europe for its medicinal value. In homeopathy, medicinal leaf extract of this plant is used as a mother tincture for treating several diseases including cancer. Pharmacological studies revealed that Thuya extract is rich source of terpenoids, flavonoids, and main-like components. Many of these components have been reported to have anticancer property individually. TO has been reported earlier to have anticancer property against skin melanoma cell line. It has also been reported as an antimutagenic agent. But activity of TO against NSCLC had not been reported so far.

Apoptosis is a common phenomenon through which a cell undergoes programmed death of itself. Extrinsic and intrinsic pathways either alone or in combination, regulate this apoptotic signalling pathway.1 However, the basic target of any anticancer drug is to induce these apoptotic pathways in a cancer cell. They serve such a function by targeting apoptotic signalling genes and the respective proteins.

Thus, the hypothesis to be tested in the present study are: if TO has demonstrable antiproliferative and anticancer potentials in vitro against an NSCLC cell line A549; if it had, whether it was cytotoxic to the cancer cells only, but not against normal human embryonic lung cells (L-132 cell line); whether it is possible to ascertain the signalling pathway by which it accomplished its functions. Incidentally, A549 cells, derived from NSCLC, have several mutations in the genes like KRAS, CDKN2A, etc., that make them more resistant to any anticancer chemotherapeutic drugs, thereby denying practically all chemotherapeutic approaches made so far through the orthodox drug regimen.6 This is one of the reasons why we became interested to test the efficacy of TO leaf extract on NSCLC cell line A549.

1 Materials and methods

1.1 Chemicals and reagents

Ethanol extract of TO, which is also available in the market as a homeopathic mother tincture, was procured from Botton Laboratory, France. For culture of A549 and L-132 cells, Dulbecco’s modified Eagle’s medium (DMEM) with fetal bovine serum (FBS), penicillin-streptomycin-neomycin (PSN) antibiotic, trypsin and ethylenediamine tetraacetic acid (EDTA) were purchased from Gibco BRL (Grand Island,


NY, USA). Tissue culture plastic wares were obtained from FASONS. All the antibodies used were obtained from Santa Cruz Biotechnology (USA). All organic solvents used were of high-performance liquid chromatography grade. Hoechst 33258, acridine orange (AO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ethidium bromide (EB) and all other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyvinylidene fluoride (PVDF) membrane for Western blot was procured from Bio-Rad.

1.2 Cell lines Human NSCLC cell line A549 and human normal embryonic cell line L-132 were obtained from National Centre for Cell Science, Pune, India. Cells were cultured in DMEM with 10% heat-inactivated FBS and 1% PSN and maintained at 37 °C with 5% CO_2 in a humified incubator. Cells were harvested with 0.05% trypsin and 0.02 mmol/L EDTA in phosphate buffer saline (PBS), plated at required cell numbers and allowed to adhere for 24 h before treatment.

1.3 Cell viability assay A549 cells were dispensed in 96-well flat bottom microtiter plates at a density of 1 × 10^4 cells per well for 24 h. They were then treated with various concentrations of TO for 24 h to determine the optimum concentration of TO at which the percentage of cell death was nearly 50% (the IC_{50} value of the drug). Based on IC_{50}, two doses, one below and one above the IC_{50} dose, were selected for further study.

The control received no drug. After the incubation 10 μL of MTT solution (1 mg/mL) was added to each well. The intracellular formazan crystals formed were solubilized with acidic isopropanol and the absorbance of the solution was measured at 595 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Multiscan FX, Thermo Electron Corporation, USA). The relative percentage of viability was calculated.

Furthermore, L-132 cells were treated in the same manner and cell viability was analyzed after MTT assay to ascertain whether the drug has any cytotoxicity on the normal cells or not.

1.4 Bromodeoxyuridine incorporation assay A549 cells were dispensed in 96-well flat bottom microtiter plates at a density of 1 × 10^4 cells per well. After 24 h of incubation, they were treated with bromodeoxyuridine (BrdU) solution along with three different drug concentrations for 6, 12 and 24 h of exposure, respectively.

After their respective hour of exposure, cells were washed with PBS and fixed with 1% paraformaldehyde solution for 1 h; after washing it again with PBS it was incubated with primary anti-BrdU antibody (1 : 500 dilution) for 4 h. After that it was incubated with secondary monoclonal antibody (1 : 500 dilution) for 2 to 3 h. Then, para-nitrophenylphosphate (pNPP) was used as a colour developer and colour intensity was measured at 405 nm with respect to blank.

1.5 Wound-healing assay Cell migration was examined by using wound-healing assay. Cells were cultured in a 6-well plate to almost 80% confluency. At the center of the well, a wound was generated by scratching it with a plastic pipette tip. Cell debris was removed by washing it then with PBS. After the drug treatment with less than IC_{50} dose (188 μg/mL) for 6, 12 and 24 h, the plate was placed under a phase-contrast microscope. Reference point was marked on the photograph of the first image and aligned and then the second image was obtained.

1.6 Observation of morphological changes A549 cells (1 × 10^4 per well) were plated in 6-well culture plates in DMEM supplemented with 10% FBS. Cells were treated with or without TO at a specified concentration. After 24 h, the cells were observed under inverted phase-contrast microscope (Axioskop plus 2, Zeiss, Germany) and the photographs were taken.

1.7 Determination of early apoptosis after annexin V-fluorescein isothiocyanate and propidium iodide dual staining fluorescence-activated cell sorting analysis A549 cells (1 × 10^4 per well) were taken into small centrifuge tubes after drug treatment. Cells were spun and pellets were washed with 500 μL binding buffer (10 mmol/L 1,4-dithiothreitol) and binding buffer (100 mmol/L sodium chloride; 5 mmol/L potassium chloride; 1 mmol/L magnesium chloride and 1.8 mmol/L calcium chloride). Then cells were harvested and cell pelter was resuspended in 100 μL binding buffer. Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI)-labelling solutions were added and the cell suspension was incubated for 15 min at room temperature in the dark using Caliber flow cytometer (BD FACS ARIA II) and analyzed by flow cytometric cytof 2.

1.8 Determination of nuclear fragmentation Cells of 1 × 10^4 were treated and then were stained separately with Hoechst 33258 (10 μg/mL) and AO-EB. Then the cells were analyzed under a fluorescence microscope (Axioskop plus 2, Zeiss, Germany) and representative photographs were taken for further qualitative analysis.

1.9 Comet assay After treatment and incubation, the cells were trypsinized and washed in ice-cold PBS. The cell suspension was mixed with an equal amount of 50% low-melting agarose and kept at 37 °C. The suspension (100 μL) was pipetted on to agarose-precipitated microscope slide, then covered with cover slip and placed on a glass tray on ice. Thereafter, the slides were immersed in cold lysis solution (2.3 mol/L sodium chloride; 100 mmol/L EDTA, 10 mmol/L Tris, pH 10, with freshly added 1% Triton X-100, 2% dimethyl sulfoxide (DMSO)) followed by incubation at 4 °C for 1 h. The electrophoresis in weak alkaline (0.93 mmol/L sodium hydroxide, 1 mmol/L EDTA, pH 12) at 1 V/cm and 30 mA for 15 min was preceded by a
25 min immersion of the slides in electrophoresis buffer for chromatin unwinding. After deionisation, the slides were neutralized in 0.1 M Tris buffer. Then it was stained with ethidium bromide (50 μg/mL) for 10 min, washed in distilled water and examined under a fluorescence microscope (Lycra, USA).

1.10 Reverse transcription-polymerase chain reaction
study Reverse transcription-polymerase chain reaction (RT-PCR) method was performed to evaluate mRNA expression levels of caspase 3, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) as a housekeeping gene control. Total RNA was extracted according to manufacturer's instructions using TRIzol reagent. RNA concentration was determined by Oxyplot method. One microgram of total RNA was subjected to a RT reaction. Preparation of cDNA and amplification with primer sequences (caspase 3, forward: 5'-AGCGCTTTGCTTCTGCC-3', reverse: 5'-TACACGATGCTTTTCTG-3'; GAPDH, forward: 5'-CCATGCAGGTGTCTTCTTGAGG-3', reverse: 5'-GACACGTTGCTTCTCAGGACT-3') were done according to the method of Chakraborty et al.14. Fluorescence intensity of band on the agarose gel was measured by using the Image J software.

1.11 Indirect ELISA Cells of 1×10^6 were seeded in 50 mm petridish and allowed to adhere for 21 h. Then T0 was treated for 18 h in case of Bax/Becl protein expression and 24 h in case of caspase-3 activity assessment. The assay was done according to the manufacturer's protocol (Santa Cruz Biotechnology Inc, USA). Protein activity level of Becl-2, Bax, and caspase 3 was measured by using an ELISA reader. GAPDH served as the housekeeping gene. 1nPP was used as a colour developer and colour intensity was measured at 405 nm with respect to blank.

1.12 Western blot analysis Cells of 1×10^6 were seeded and allowed to adhere for 48 h. Then the cells were treated with T0 for 24 h and homogenates were used for Western blot analysis. Equal amounts (70 μg) of protein were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrophoretically transferred to PVDF membrane. Bound antibodies of caspase 3 (1:10,000) and poly ADP-ribose polymerase (PARP) (1:1,000) were developed by 5-bromo-4-chloro-3-indolyl-phosphate-nitro blue tetrazolium and densitometeric quantification of proteins was done by using Image J software. GAPDH (1:1,000) was used as housekeeping gene.

1.13 Statistical analysis The data were presented as mean±standard deviation. Statistical analysis was performed by one-way analysis of variance using SPSS software. P<0.05 was considered significant.

2 Results

2.1 Effects of T0 extract on viability of A549 The effect of T0 on cell viability of A549 cells was measured by MTT assay. The results showed viability reduction in response to increasing drug concentrations: the IC_{50} value was determined to be 282 μg/mL after 24 h of exposure (Figure 1A). Ethanol was used as a drug vehicle control and no significant viability reduction was observed on exposure of it in A549 cells. Meanwhile, exposure of T0 on I-132 cells showed cytotoxicity at a negligible level (Figure 1B). For further study on dose-dependence, 188 μg/mL (IC_{50} designated as D1), 282 μg/mL (IC_{50} designated as D2) and 375 μg/mL (IC_{50} designated as D3) of drug doses were taken.

![Figure 1](https://example.com/figure1.png)

**Figure 1** Effects of T0 extract on viability of A549 cell line (A) and I-132 cell line (B).

Cells were exposed to T0 extract and colour was measured for 24 h at different concentrations and cell viability was determined by MTT assay. Results are expressed as percentage of viability and data are expressed as mean±standard deviation. n=5. **P**<0.05, as compared to control group. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
2.2 Effects of TO on proliferating activity of A549 cells

Proliferation activity was blocked after drug induction as revealed from BrdU incorporation assay in a time-dependent manner, rather than in a dose-dependent manner. While there was a palpable amount of inhibitory effect of TO on proliferating activity of A549 cells at 6, 12 and 24 h of exposure, apparently increasing in magnitude with the time of exposure, there was a concomitant destruction of BrdU activity (Figure 2) at a significant level (P<0.05). From wound-healing assay it was further evident that TO administration blocked migration of A549 cells successfully and in a time-dependent manner (Figure 3), thus proving evidence of anti-proliferative activity of TO extract.

Figure 2  Effects of Thaia arvidiiacei ethanolic extract on cellular proliferation tested by BrdU incorporation assay

Cells were seeded in three aliquots standardized at Thaia arvidiiacei ethanolic extract for 6, 12 and 24 h along with incorporation of BrdU. Incubated BrdU was pulsed with anti-BrdU antibody and then reacted to 1:50 diluted DNP4 as a color developer. Data are expressed in mean ± standard deviation, n=3; P<0.05, vs normal control group. D1: 350 µg/ml; D2: 298 µg/ml; D3: 275 µg/ml; D4: 100 µg/ml; DNP4: horseradish peroxidase; DNP: diaminobenzidine; P: probability.

2.3 Effects of TO on cellular morphology of A549 cells

The morphological changes of A549 cells treated with TO were observed with respect to untreated ones. Cell shrinkage and blebbing, which are the hallmark features of apoptotic cell death, were observed after drug induction (Figure 4).

2.4 TO-induced apoptosis shown by annexin-V positivity

The early state of apoptosis was evaluated by flow cytometry after annexin-V-FITC assay. To confirm the preceding observations, we found an increase of annexin-V-positive cells in TO-treated A549 cells as compared to the untreated control. This increase is dose-dependent in nature as the highest drug dose shows highest level of annexin-V positivity than the others (Figure 5).

Figure 3  Effects of Thaia arvidiiacei ethanolic extract on A549 cell migration (Light microscopy, ×20)

Wound healing assays were performed to assess cell migration. Cells were cultured on treated with Thaia arvidiiacei ethanolic extract (100 µg/ml) for 6, 12, and 24 h. Representative images were taken of treated and untreated cells. Black arrows indicate migratory cells or unstuck ones which found to be seen in parallel to the tested area.

Figure 4  Analysis of cellular morphology by a phase-contrast microscope (Light microscopy, ×20)

Figure 5  FACS analysis after annexin-V-fluorescin isothiocyanate and propidium iodide dual staining assay
2.5 Effects of TO on nick generation and fragmentation of DNA  
Hoechst 33258 staining, which is sensitive to DNA, was used to quantify DNA nick generation, if any, after drug administration. An increase in fluorescence of Hoechst 33258 staining was observed after exposure of A549 cells to different drug doses for 24 h (Figure 6A).

Comet assay revealed an increased length of comet tail (Figure 6B). AO-EB staining produced changes in fluorescence pattern from green (normal cellular DNA) to orange (nicked cellular DNA) along with increased fluorescence intensity of EB (Figure 6C). Collectively, these results indicate that on administration of TO, cellular DNA gets fragmented and nick generation occurs in a dose-dependent manner.

2.6 Effects of TO administration on Bax-Bcl2 protein expression  
ELISA assay showed significant ($P<0.05$) up-regulation of Bax and down-regulation of Bcl2 (Figure 7) after drug induction for 18 h; this kind of change in Bax-Bcl2 ratio leads the cells into apoptosis.

2.7 Effects of TO on expression level of caspase 3 and PARP activity  
Results of RT-PCR analysis revealed that caspase 3 gene activity was up-regulated after drug induction (Figures 8A and 8B). This was further confirmed by results of the ELISA assay (Figure 8C) and Western blot analysis (Figure 9).

Subsequently, increased expression of cleaved PARP was also observed in the drug-treated cells as compared to the untreated ones (Figure 9). The expression of cleaved PARP was generally found to be increased in the drug-treated lots when compared with the untreated ones, but when they were further critically analyzed, the degree of increase was not found to be strictly dose-dependent, as the higher doses did not produce proportionately greater effect when compared with the lower doses. This would possibly imply that the initiation of DNA fragmentation could be triggered at all the concentrations of the drugs, including the one at the lowest dose.

3 Discussion

Complementary and alternative medicines are now considered to play an emerging role as cancer chemopreventive therapy. Since cancer cells are known to attain immortalization, induction of apoptosis in them by a drug is considered to have a great anticancer impact, and a drug showing specific killing effect on the cancer cells, but sparing the normal ones, is the one which is highly solicited in anticancer drug formulation. In the present study, the administration of TO appeared to increase the incidence of apoptosis in A549 cells, but it was not significantly cytotoxic to the normal human embryonic lung cell line L-122. Therefore, we were tempted to study the exact signalling
Figure 7  Effects of TO on Bax/Bcl2 expression at protein levels
Significant Foxp3 up-regulation and Bcl2 down-regulation were observed by immunohistochemical staining after drug treatment for 8 h in A549 cells. Bars are expressed as percentage of control and data are expressed as mean ± standard deviation. n=6; **P<0.01, compared to control group. TO: 128 μg/mL; D1: 288 μg/mL; D2: 736 μg/mL.

Figure 8  Caspase 3 expression analysis after drug treatment at different doses
Caspase 3 gene expression was detected after drug treatment measured from RT-PCR band (A) and their relative intensity (B). Caspase 3 expression at protein level was also increased as revealed from western blot analysis (C). Fold intensity of RT-PCR and protein expression by ELISA assay were expressed as percentage of control and data are expressed as mean ± standard deviation. n=4; **P<0.01, compared to control group. GAPDH was used as a non-changing gene control. D1: 158 μg/mL; D2: 264 μg/mL; D3: 762 μg/mL. RT-PCR: reverse transcription polymerase chain reaction; ELISA: enzyme-linked immunosorbent assay; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Figure 9  Immunohistochemical analysis of caspase 3 and PARP
Expression of caspase 3 and PARP was increased as revealed from Western blot band (A) and their relative intensity (B). Fold intensity of Western blot analysis was expressed as percentage of control and data are expressed as mean ± standard deviation. n=6; **P<0.01, compared to control group. GAPDH was used as a non-changing gene control. D1: 98 μg/mL; D2: 89 μg/mL; D3: 285 μg/mL. PARP: poly ADP-ribose polymerase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.
mechanism by which TO accomplishes this task. TO administration showed cytotoxicity against A549 cells in vitro in a dose-dependent manner. It showed membrane blebbing and distinct morphological changes as observed from cell morphological analysis. Suggesting thereby that the cytotoxicity originated possibly more due to apoptosis than due to necrosis. This contention could be validated by the data of FACS analysis after annexin V-FLIC-PI staining which confirms early stage of apoptosis and furthermore DNA fragmentation, as suggested in earlier studies[12]. Successful DNA nick generation after drug exposure was revealed from increased fluorescence of Hoechst and AO-488 staining against untreated control. Additionally, comet tail formation showed significant amount of DNA fragmentation as well.

Proliferation is the major property of cancer cells that pushes them to migrate and invade other tissue. Therefore, the therapy can be effective if this proliferation of cells can be blocked, and apoptosis can be induced in them as a result of drug treatment[19]. In this investigation, TO was also found to block proliferation significantly in a time-dependent manner, as revealed from BrdU incorporation study. TO appeared to block proliferation at 6, 12 and 21 h time points of drug exposure. This modulation is very much time dependent in section, rather than dose dependent. Migration assay helps us also to conclude that at different time points of fixation, the drug blocks A549 cell proliferation and migration.

Pro- and anti-apoptotic proteins regulate cell survivability by maintaining its proper balance in a regulated manner. Therefore creating alteration in major pro- and anti-apoptotic proteins is the major target area of any anticancer drug[34]. Consistent with this, indirect ELISA assay was revealed that up-regulation of Bax and down-regulation of Bcl-2 occurred after drug exposure in A549 cells.

Caspase activation is also a marked phenomenon of apoptotic cell death[34]. From RT-PCR, indirect ELISA assay and Western blot analysis it was found that expression of caspase 3 gene and its protein product in A549 cells increased significantly after drug treatment. Subsequently cleaved PARP, which is the downstream effectors of caspase 3 was also found to be increased in its expression after drug induction. Similarly, results of comet assay and Hoechst fluorescence study also revealed that DNA fragmentation was possibly enhanced due to caspase 3 and cleaved PARP expression after administration of TO.

In summary, our study shows that TO extract induces apoptotic cell death by altering the balance between Bax/Bcl2 expression and activation of caspase 3. TO also blocks cell proliferation in a time-dependent manner. Through blocking proliferation and activating caspase 3 it induced significant DNA fragmentation that could lead to A549 cell death. Therefore, it could be concluded that TO extract has antiproliferative and anticancer activities against A549 cells having several somatic mutations which raise their immortality and resistance against several drugs. Thus, the results of this study would validate its use as a supportive medicine against NSCLC. However, further in-depth studies are needed to ascertain if any particular isolated compound from this plant extract could have more specific anticancer property or not.

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5 Competing interests

The authors declare that they have no competing interests.

REFERENCES

北美香柏叶的乙醇提取物阻断 A549 细胞增殖

并引起细胞凋亡的体外研究

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目的：研究北美香柏叶的乙醇提取物对小鼠肺癌细胞 A549 细胞的增殖及凋亡影响。方法：通过细胞增殖实验结果对北美香柏叶的乙醇提取物对细胞活性的影响，确定最大抑制浓度为 250 μg/mL。另选择 2 个浓度 188 μg/mL, 573 μg/mL 进行光致分光性能检测，分别进行 DNA 含量、活性细胞和死亡细胞检测，采用流式细胞术检测细胞分裂指数和细胞周期分布，采用分析软件进行检测。结果：A549 的增殖活性在接触着床香柏叶的乙醇提取物 24 h 后明显降低，达到 50% 以上。乙醇提取物对 A549 细胞的增殖活性抑制率为 50% 时，对应的浓度为 250 μg/mL。乙醇提取物对 A549 细胞的细胞周期分布影响显著，对 G1 期细胞的抑制作用最强，对 S 期细胞的抑制作用最弱。乙醇提取物对 A549 细胞的凋亡率也显著增加，对细胞周期的 G1 期、S 期和 G2/M 期均有显著的抑制作用。

关键词：北美香柏叶；乙醇提取物；A549 细胞；细胞增殖；细胞凋亡
1 Introduction

Homeopathy, a popular branch of complementary and alternative therapy, uses ultra-highly diluted natural substances originating from plants, minerals or animals\(^\text{[1]}\). Homeopathic remedies are claimed to cure several diseases around the world. In homeopathy, drugs are used in the form of dynamized preparations obtained from serial dilutions and repeated agitations. Thus, when the drug attains the potency 12C, it becomes diluted to \(10^{-24}\), which is beyond the Avogadro’s limit. Such dilution beyond Avogadro’s limit raises the controversy about the lack of physical existence of any drug molecule, and therefore, its...
efficacy is questioned. However, several studies have tried to define the efficacy of such highly potentized homeopathic remedies in biological systems\[2,3\].

*Thuja occidentalis* is a major plant largely produced in part of Europe. Earlier reports suggested that *Thuja occidentalis* mother tincture and its potentized forms are effective in treating several diseases like lung cancer, breast cancer, etc\[4-7\]. Thuja is reported to be an attractive drug for treating skin diseases including lesion and is also effective against diarrhea\[8,9\]. Earlier, Frenkel et al\[9\] and Banerji et al\[10\] claimed highly potentized form of *Thuja* such as Thuja 30C as an effective remedy against several diseases. In our previous study, we have shown that *Thuja occidentalis* extract, used as a homeopathic mother tincture, was effective against non-small cell lung cancer cell line, A549\[10\]. For further evaluation, we have tested the efficacy of potentized form of the same against lung cancer cell lines, A549 and H460. However, when used directly on the lung cancer cells, Thuja 30C was found to be less effective in killing these cancer cells at lower doses. Such results motivated us to test if the remedy plays any protective role against toxicity induced in normal perfused lung cells exposed to a major carcinogen, benzo(a)pyrene (BaP). BaP is one of the major lung carcinogens and constituents of cigarette, coal tar, etc\[11,12\]. It has previously been reported that it exerts its cytotoxic effect via deregulation of stress balance and thereafter damaging cellular DNA\[13-15\], which in turn enhances cellular mortality.

Therefore, our main objectives of the study were to check whether treatment of highly diluted homeopathic remedy Thuja 30C could be effective in protecting BaP-intoxicated lung cells to give them better survivability, to check if Thuja 30C ameliorates BaP toxicity in lung cells through regulatory control over stress responses, and to know if Thuja 30C has any demonstrable role in combating BaP-induced cellular DNA damage through a retrieval process.

## 2 Materials and methods

### 2.1 Reagents and drugs

Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), and penicillin-streptomycin-neomycin (PSN) antibiotic were purchased from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were obtained from Tarsons (USA). All organic solvents were of high-performance liquid chromatography grade. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI), 2',7'-dichlorofluorescein diacetate (H$_2$DCFDA), calf thymus DNA, BaP, secondary antibodies, etc. were purchased from Sigma Aldrich (USA). Anti-heat shock protein (hsp)-90 monoclonal antibody was purchased from Cell Signaling Technology (USA). Ultra-high dilution of Thuja 30C and potentized alcohol (vehicle media of Thuja 30C) were obtained from Boiron Laboratory, France.

### 2.2 Animals

We housed a large group of healthy inbred strain of Swiss albino mice (6-8 weeks; body weight 20-25 g) for at least 14 d in an environmentally controlled room (temperature (24 ± 20) °C, humidity 55% ± 5%, 12-h light/dark cycle) with access to food and water *ad libitum*. The experiments were conducted under supervision of the Animal Welfare Committee of the Department of Zoology, University of Kalyani, India, and as per the approved ethical committee guidelines of the Institutional Ethical Committee, University of Kalyani, India (vide-892/OC/05/CPCSEA).

### 2.3 Lung cell perfusion

A total of 48 healthy Swiss albino mice were randomized and selected for the experiment. Mice were chloroformed before sacrifice. Mice lungs were dissected out and washed with Hank’s buffered salt solution with 0.1% collagenase. Then the tissues were minced into small pieces within 5% RPMI media and the lung cells were flushed out gently in the media with the aid of hypodermic syringe. Then the cells were passed through a thin nylon mesh (0.22 µm) and homogeneous single lung cell population was obtained. Then the cells were spun down at 1 000 × g for 3-4 min. The blood cells were pelleted out and the supernatant containing lung cells was taken. Viable lung cells were counted in haemocytometer by trypan blue exclusion test and used for further analysis\[16\].

### 2.4 Treatment of BaP and drug dose selection

For MTT assay, BaP at the concentration of 2.2 µmol/L, its half maximal inhibitory concentration (IC$_{50}$), was intoxicated at 37 °C in RPMI supplemented with 5% FBS. As positive control, equal volume of medium was added to the untreated cells\[17\].

For dose-dependent study, BaP-intoxicated cells were simultaneously treated with Thuja 30C for 24 h at the dose of 1-7 µL (for 100 µL culture media). Minimum dose (3 µL in 100 µL solvent media) that started showing an increase in cell viability was selected and used for subsequent experiments. The experiment was performed in triplicate, where each group was six in number.

### 2.5 Cell viability assay

Cell viability was determined using MTT assay. Briefly, perfused lung cells were seeded in 96-well plates at a density of 1×10$^5$ cells per well. They were allowed to settle for 24 h before treatment. Thereafter, the cells were intoxicated with BaP (2.2 µmol/L) with 5% CO$_2$ at 37 °C. Then the cells were simultaneously treated with Thuja 30C in an increasing concentration for 24 h. Cell viability was measured by MTT assay\[18\]. The control values corresponding with the untreated cells were taken as 100% and the viability data were expressed as percentage of control.

To observe whether Thuja 30C has cytotoxic effects on
normal lung cells, we performed MTT assay after treating the cells with only Thuja 30C at the highest concentration.

We also treated the BaP-intoxicated lung cells with the drug vehicle media to evaluate only the drug’s action, if any. The experiment was performed in triplicate, where each group was six in number.

2.6 Intracellular reactive oxygen species accumulation assay

Levels of reactive oxygen species (ROS) generation in cells were assessed fluorometrically using H$_2$DCFDA molecular probe. Cells of $1 \times 10^4$ were intoxicated with BaP with simultaneous treatment of Thuja 30C (3 $\mu$L for 100 $\mu$L dose) for 1 to 6 h or left untreated. After incubation with 10 $\mu$mol/L H$_2$DCFDA for 30 min at 37 $^\circ$C in the dark, fluorescence was measured at excitation and emission wavelengths of the oxidized form at 504 and 529 nm, respectively.[29]. Hydrogen peroxide (H$_2$O$_2$)-treated cells were taken as loading control. The experiment was performed in triplicate, where each group was six in number.

2.7 Protein isolation

Cells ($5 \times 10^4$) were plated in a 90-mm culture dish and were allowed to grow for 24 h. Following BaP intoxication and simultaneous treatment with Thuja 30C, cells were collected and lysed in 20 $\mu$L of ice-cold lysis buffer (10 mmol/L Tris-HCl, pH 7.5, 1 mmol/L MgCl$_2$, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/L phenylmethylsulfonyl fluoride, 5 mmol/L β-mercaptoethanol, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate and 10% glycerol). Cells were incubated for 30 min on ice and centrifuged for 30 min at 5 000 x g at 4 $^\circ$C. After centrifugation the supernatant was collected and stored in −20 $^\circ$C for further use.

2.8 Glutathione estimation

Briefly, drug-treated (3, 6, 9 and 12 h) and untreated cells ($1 \times 10^4$) were taken and the cell extracts were prepared. We undertook spectrophotometric analysis to determine glutathione (GSH) content according to a method of Tietze.[20]. The experiment was performed in triplicate, where each group was six in number.

2.9 Hsp-90 activity assay by indirect enzyme-linked immunosorbent assay

Totally $1 \times 10^4$ cells were seeded in a 90-mm petridish and allowed to adhere for 24 h. Then the cells were intoxicated with BaP and simultaneously treated with Thuja 30C for 3, 6, 9 and 12 h. After respective time interval of drug exposure, protein was isolated. Thereafter, indirect enzyme-linked immunosorbent assay (ELISA) of hsp-90 protein was done according to the manufacturer’s protocol (Santa Cruz Biotechnology, Inc., USA) and quantified using an ELISA reader (Thermo Scientific, USA). We used para-nitrophenyl phosphate (pNPP) as a color-developing agent and measured the color intensity at 405-nm wavelength. The experiment was performed in triplicate, where each group was six in number.

2.10 Circular dichroism spectral analysis

Circular dichroism (CD) analysis was done by taking calf thymus DNA as a standard control. The analysis was done to explain whether Thuja 30C was capable of damaging DNA by interacting with it or not. The calf thymus DNA (ctDNA) concentration in the experiments was 100 $\mu$mol/L. CD spectra were recorded on a JASCO J720 CD spectrometer at 37 $^\circ$C using 1 mm cuvette.[21]. The spectral recording was performed from 200 to 500 nm, repeated thrice and averaged automatically.

Induced CD spectral reading was performed resulting from the interaction of the Thuja 30C or its vehicle media and ctDNA at 37 $^\circ$C, which was obtained by subtracting the CD spectrum of the native DNA and mixture of both ctDNA and Thuja 30C or its vehicle media from the CD spectrum of the buffer and spectra of both buffer and Thuja 30C or its vehicle media solutions, respectively.

2.11 DNA damage assay by DAPI staining

Cells ($2 \times 10^4$) were plated in 6-well culture plates and allowed to grow for 24 h. Then cells were intoxicated with BaP and simultaneously treated with Thuja 30C for 3, 6, 9 and 12 h, respectively. After staining the cells with DAPI (10 $\mu$mol/L) for 15 min, fluorescence of DAPI was observed under a fluorescence microscope (Leica, Germany). The experiment was performed in triplicate, where each group was six in number.

2.12 Blinding

The observers were “blinded” during observations as to whether they were observing the “control” or “treated” materials.

2.13 Statistical analysis

All the experiments were performed in triplicate, where each group was six in number. Results were expressed as mean ± standard deviation, unless otherwise stated. Statistical analysis was made by one-way analysis of variance with LSD post-hoc test using SPSS 16.0 software. $P<0.05$ was considered statistically significant.

3 Results

3.1 Effects of Thuja 30C on cell viability

Normal mouse lung cells incubated with 2.2 $\mu$mol/L of BaP for 24 h showed almost up to 50% of cell mortality as compared to the untreated control. But when these BaP-intoxicated cells were simultaneously treated with Thuja 30C (1-7 $\mu$L for 100 $\mu$L culture media), the cell viability was increased to 99.53% ± 0.59% at the dose of 3 $\mu$L (for 100 $\mu$L culture media) (Figure 1). However, at the dose range of 1-2 $\mu$L (for 100 $\mu$L culture media) the cell viability was 93.31% ± 0.49% and 93.04% ± 0.80%, respectively, but the differences in the percentage of viability as compared to BaP-intoxicated control were still at a statistically significant level.

Cells treated with drugs did not show any cellular cytotoxicity and reduction in viability. This however indicated the
drug’s ability of being effective in adverse cellular condition, not in any normal circumstances. Though after treatment with the vehicle of the drug, viability of BaP-intoxicated cells was increased to 86.49% ± 0.69%, the viability was still much less than the lowest Thuja 30C dose (1 µL for 100 µL culture media)-treated set where the viability was 93.31% ± 0.49%. Therefore, this result demonstrated greater effectiveness of the homeopathic remedy in BaP-intoxicated cells, as compared to only vehicle-treated cells.

3.2 Effects on cellular ROS generation
Fluorimetric results indicated that cellular ROS level got increased after BaP intoxication at an early time (3-12 h). However, cellular ROS level got down-regulated when those cells were simultaneously treated with Thuja 30C (3 µL per 100 µL culture media). At 3-6 h of exposure, Thuja 30C was unable to decrease the ROS level, but later at 9-12 h of exposure, BaP-induced elevated level of ROS was down-regulated (Figure 2), apparently looking like a biphasic effect.

3.3 Effects on GSH level
Enzymatic estimation revealed that intracellular GSH level was down-regulated after 3-12 h of BaP intoxication. However, simultaneous treatment of Thuja 30C (3 µL per 100 µL culture media) for 3-12 h significantly up-regulated cellular GSH level (Figure 3).

3.4 Effects on hsp-90 level
Indirect ELISA assay showed that hsp-90 got up-regulated after BaP intoxication, which presumably helped in controlling the stress level. Simultaneous treatment of Thuja 30C, however, helped in down-regulating hsp-90 level at 3-12 h (up to 62.17% ± 2.06 % after 12 h), suggesting some reduction in stress level (Figure 4).

3.5 Drug-DNA interaction
CD spectral analysis results indicated that Thuja 30C apparently failed to interact with calf thymus DNA, so did the drug vehicle media (Figure 5). The CD spectra were observed to remain unchanged upon either drug or its vehicle media treatment; this would indicate that neither drug nor its vehicle media could trigger any conformational change of the DNA through direct interaction or had any damaging effect on DNA by itself.

3.6 Effects on DNA damage
DAPI staining revealed that BaP-intoxicated cells showed brighter fluorescence which indicated nicked and damaged DNA. It is interesting to note, however, that Thuja 30C-treated cells showed less fluorescence, indicative of the ability of the drug to reduce BaP-induced DNA damage (Figure 6).

Figure 1 Effects of Thuja 30C on the viability of BaP-intoxicated mice lung cells
Cells were either treated with only Thuja 30C (7 µL per 100 µL culture media) or exposed to 2.2 µmol/L BaP. BaP-exposed cells were simultaneously treated with drug vehicle media (7 µL per 100 µL culture media) and Thuja 30C (1 µL per 100 µL to 7 µL per 100 µL culture media). After 24 h of treatment cell viability was determined by MTT assay. Data are expressed as percentage of viability and each point expressed as mean ± standard deviation (n = 6). **P < 0.01, vs untreated group.

BaP: benzo(a)pyrene; MTT: 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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Discussion

Results of MTT assay indicated that cell viability decreased almost up to 50% when the normal lung cells of mice were intoxicated with BaP at a dose of 2.2 µmol/L. However, the cell viability of the intoxicated cells was found to be increased significantly in a dose-dependent manner when they were simultaneously treated with Thuja 30C, particularly after administration of 3 µL (in 100 µL culture media) or a higher dose of Thuja 30C. On the contrary, there was no significant change in the percentage of cell viability when the BaP-intoxicated cells were treated with drug vehicle media. However, the treatment of normal lung cells with Thuja 30C did not show any cytotoxic effect, which
implies the potentiality of Thuja 30C to have a regulatory effect only when the cells were under the carcinogen-induced stress.

An efficient regulatory balance on oxidative stress helps a cell to survive in an adverse condition. Thus the key regulatory act could possibly have been mediated through the regulatory control over the oxidative stress generated by BaP at certain stages to reduce the cytoxicity induced by the carcinogen. This led us to make a more elaborative study on stress generation pathway and its possible modulation by the homeopathic remedy. The data on ROS generation, GSH content and hsp-90 activity also supported this regulatory process. Earlier, Kumar et al. reported that BaP mediates its toxicity by deregulating cellular redox state balance, particularly by generating ROS and down-regulating major antioxidants like GSH. From the results of this study, it seems that BaP at early hours modulated redox potential of normal lung cells by up-regulating the level of ROS and down-regulating the level of antioxidant, GSH. However, treatment of Thuja 30C at a minimal dose (3 µL per 100 µL culture media) was able to decrease ROS level from 9 to 12 h and increase GSH level from 3 to 12 h. At the earlier phases (3 to 6 h) of drug exposure alteration in oxidative stress response was dependent on GSH up-regulation rather than ROS depletion. However, from 9 to 12 h of drug exposure the oxidative stress alteration was carried out both by up-regulation of GSH and depletion of ROS. This would bear testimony to the ability of the potentized homeopathic remedy to normalize the stress condition generated by the BaP. The apparent bi-phasic effect on ROS generation has a similarity with certain effects mentioned as “hormetic effects” by some workers. However, the hypothesis that equated “hormesis” as the cause of homeopathic effect is supported by some, but not by others. In fact, the debate is still on and remains inconclusive, because hormesis is more of a phenomenon of favorable responses elicited by low dose toxin, but it does not really account for the precise molecular mechanism that is involved and it does not also clearly explain the homeopathic doctrines.

Hsp-90 is a part of the molecular chaperones family that is responsible for protein folding and activation. Hsp-90 is also involved in regulating protein degradation. In many a case of chemical toxicity hsp-90 level is up-regulated. This elevation is attributed to an increase in cellular stress. In this study, we also observed an elevated level of hsp-90 after BaP intoxication. This may be the primary reason of cellular stress developed by BaP in turn generated cytotoxicity. However, treatment of Thuja 30C reduced cellular level of hsp-90 in carcinogen-intoxicated cells as indicated by ELISA results. At 3-12 h of Thuja 30C exposure BaP-induced elevated level of hsp-90 got down-regulated. Thus, the other effective way to ameliorate stress could also be its ability to reduce hsp-90 generation by BaP that could render adequate protection to the cells towards their increased survivability.

Increased level of stress is also known to cause nuclear DNA damage. In this study, increased level of DAPI fluorescence was observed after BaP intoxication which indicates cellular DNA damage. However, after simultaneous treatment with Thuja 30C, fluorescence level of DAPI was lowered. This suggests that DNA damage induced by BaP was partially recovered by Thuja 30C treatment. It is reported that some chemicals induce DNA damage itself by interacting with DNA. However, if any agent (drug) bears the capacity to interact with DNA then it might be able to make some conformation changes in the DNA. In our present findings, CD spectral analysis revealed that Thuja 30C was unable to interact with calf thymus DNA. As the drug was not able to interact with calf thymus DNA in a cell-free system, therefore it would not presumably be able to interact with cellular DNA. Therefore, it may be extrapolated that this drug does not bear the self-reactive ability to damage DNA by interaction, rather bear the capability to reduce BaP-induced DNA damage. So the protection was apparently not mediated through direct conformational change in DNA. An overall scheme of important regulatory events that presumably occurred to render protection to intoxicated lung cells has been summarized in Figure 7.
Thus, Thuja 30C, an ultra-highly diluted remedy above Avogadro’s limit, showed quite convincing evidence of anti-carcinogenic effect in lung cells, possibly through its action at the molecular level of gene regulation as proposed by Khuda-Bukhsh\cite{1,2} earlier, presumably because of the reported presence of nanoparticles of the drugs even in their highly diluted forms\cite{30-32}. Further, amelioration of BaP-induced lung cell damage would signify this potentized remedy as a major putative agent in combating lung toxicity, which can be used as a supportive remedy in lung cancer.

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6 Conflict of interests

The authors declare that they have no competing interests.

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Flavonol isolated from ethanolic leaf extract of *Thuja occidentalis* arrests the cell cycle at G2-M and induces ROS-independent apoptosis in A549 cells, targeting nuclear DNA

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**Abstract**

**Objectives**: The K-ras gene mutation commonly found in lung adenocarcinomas contributes to their non-invasive expansion. Our main objective here was to develop a chemopreventive agent against K-ras-mutated lung adenocarcinoma cell line like-A549.

**Materials and methods**: We isolated flavonol from ethanolic leaf extract of *Thuja occidentalis*, and evaluated its apoptotic potentials on A549 cells. They were treated with 1–10 μg/ml of flavonol and viability was tested retaining normal lung cells L-132 as control. We performed assays such as TUNEL, annexin V, cell-cycle and mitochondrial membrane potentials, by FACS analysis. ROS-mediated oxidative stress and drug–DNA interactions were analysed along with gene expression studies for p53, Bax-Bcl2, cytochrome c, the caspase cascade genes and PARP.

**Results**: Flavonol reduced A549 cell viability in a dose- and time-dependent manner (IC$_{50}$ value = 7.6 ± 0.05 μg/ml following 48 h incubation) sparing normal L-132 cells. It effected G2-M phase cell cycle arrest and apoptosis, as indicated by progressive increase in the sub-G1, annexin V and TUNEL-positive cell populations. Apoptotic effects appeared to be mitochondria-dependent, caspase-3-mediated, but ROS-independent. Analysis of circular dichroism data revealed that flavonol intercalated with nuclear DNA. **In vivo** studies on non small cell lung carcinoma (NSCLC)-induced mice confirmed anti-cancer potential of flavonol.

**Conclusion**: Flavonol-induced apoptosis apparently resulted from intercalation of cells’ nuclear DNA. Flavonol inhibited growth of induced lung tumours in the mice, indicating its potential as an effective agent against NSCLC.

**Introduction**

Targeted therapy based on genetic makeup of a tumour plays an emerging role in anti-lung cancer drug design. Development of oncogenic mutation in non-small cell lung carcinoma (NSCLC) cells makes them more adaptive, proliferative and fit to grow in all adverse conditions, defying all attempts towards chemopreventive measures. Among them, K-ras mutations are more frequent in NSCLC (1). With this mutation, tumour cells constitutively activate synthesis of mutated K-ras protein that continuously stimulates related downstream signalling molecules such as EGFR, PI3K, and more. Because of this, K-ras mutated NSCLC patients tend to be resistant to EGFR and PI3K targeted therapeutic agents such as erlotinib and gefitinib (2). These critical regulations of K-ras mutated NSCLC cells give such patients poor prognosis.

At present in anti-cancer research, ability of an anti-cancer drug to interact directly with nuclear DNA is considered to be an added advantage (3). Such a drug would then gain potential to modulate several downstream molecules including pro-apoptotic ones (4,5), avoiding interference with some constitutively activated proteins such as K-ras that might otherwise interfere with the drug’s action. Redox regulation and stress balance have also been shown to be important components for cancer cell survivability (6). Thus, through
pharmacologic intervention, efforts are directed towards producing oxidative stress imbalance, so that drugs endowed with such a capability can elevate cytotoxicity and induce apoptotic cell death.

*Thuja occidentalis*, an ornamental plant that grows in north-eastern areas of the United States and in south eastern parts of Canada is known to contain 1.4–4% essential oils, flavonoids, coumarins, tannic acid and more. (7,8). In an earlier study, we reported that thu-jone, a major essential oil-rich fraction isolated from *Thuja occidentalis* had apoptotic effects on the skin melanoma A375 cell line (9). Ethanolic extract of *Thuja occidentalis* also was shown to exert anti-proliferative and pro-apoptotic activity on the NSCLC A549 cell line (10). Thereafter, we became interested in testing whether active components could be separated and tested for possible preferential anti-cancer potential without significa-

cantly affecting normal cells.

In the study described here, we isolated flavonol from ethanolic leaf extract of *Thuja occidentalis*. Our main objective was to evaluate and elucidate its anti-
cancer potential, if any, against A549 cells that bear sev-
eral target-based therapeutic limitations due to *K-ras*
gene mutation at its 12th codon (11). Furthermore, if the target area of this fraction in DNA intercalation could be highlighted, it would be more meaningful for future drug design. Anti-cancer potential of flavonol was also tested by studying its possible ability to inhibit benz[a]pyrene-induced non-small cell lung tumour growth in a mouse model, so that a more comprehensive assessment could be made to rate its candidature in future drug formulation against NSCLC.

**Materials and methods**

**Reagents**

Dulbecco’s modified Eagle’s medium (DMEM), foetal bovine serum (FBS), penicillin, streptomycin, neomycin (PSN) antibiotic, trypsin and ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were obtained from BD Bioscience (San Jose, CA, USA).

All organic solvents used were of HPLC grade. MTT [3-(4, 5-dimethyl-thiazol-2-yl)-2, S-diphenyl-
tetrazolium bromide], propidium iodide, colchicine, DAPI (4’, 6-diamidino-2-phenylindole), rhodamine 123, MitoRed, 2′,7′ dihydrodichlorofluorescein diacetate (H2-DCFDA), glutathione reductase (GSH), calf thymus DNA, benzo[a]pyrene were purchased from Sigma Aldrich (St. Louis, MO, USA). Caspase-3 inhibitor (Ac-devd-cho), annexin V-FITC, anti-p53, anti-Bax, anti-Bcl2, anti-PARP, and anti-GAPDH monoclonal antibodies were purchased from Santa Cruz Bio-
technology Inc, Dallas, TX, USA. Primary antibodies to caspases -3,-8,-9, cytochrome c, and FITC-conjugated secondary antibody were obtained from BD Bio-
science. Anti-BrdU antibody was procured from Abcam, Cambridge, MA, USA.

**Isolation of flavonol from ethanolic leaf extract of *Thuja occidentalis***

Fresh leaves of *Thuja occidentalis* (1 kg) were col-
lected and allowed to dry under shed. Dried leaves were then powdered and extracted successively with 65% ethanol by soxhlation for 24 h (Boiron Labora-
tory, Lyon, France). The product was then placed under vacuum and dry extract was obtained (yield 17.2% w/w), this was then mixed with petroleum ether (60–80 °C) (50 ml v/v) and miscible component was taken out and dried on a hot plate at 60 °C (yield ~1–2% w/w). After evaporation, the result was a semisol brownish mass (12). Total mixture obtained was then mixed with a minimum quantity of silica gel (60–120 mesh) and loaded on to a silicic acid column (60–120 mesh) using n-hexane and ethyl acetate as solvent system. Flavonol was collected at the polarity 8:2 (v/v) of n-hexane: ethyl acetate (250 ml) mixture. We then purified it chromatographi-
cally using the same solvent system.

Preliminarily, after addition of 10% NaOH to that isolated fraction, a yellow colouration (13) was obtained, confirming it to contain flavonols. Thereafter, by mass spectral analysis, it was confirmed that it was actually a mixture, which primarily contained flavonols and in which 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-
4H-chromen-4-one (molecular weight 302.23) and 3, 5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (molecular weight 286.23) commonly called querce
tin and kaempherol, respectively, were identified as the major active components (Fig. 1a). In this study, the desired amount of isolated fraction was mixed with solvent media and sonicated using a probe sonicator, for use in subse-
quent experiments.

**Cell lines**

Human non-small cell lung carcinoma cell line A549 and human embryonic normal lung cell line L-132, were collected from the National Centre for Cell Science, Pune, India. Cells of both lines were cultured in DMEM with 10% heat-inactivated FBS and 1% antibiotic (PSN), and maintained at 37 °C with 5% CO2 in a humidified incubator. Cells were harvested with 0.025% trypsin and 0.52 mM EDTA in phosphate-buffered
saline, plated at required cell numbers and allowed to adhere for minimum ~24 h before treatment.

**Treatment of isolated flavonol fraction and inhibitors**

For MTT assay, the cells were exposed to flavonol fraction at 1 µg/ml to 10 µg/ml concentration at 37 °C, in DMEM supplemented with serum. As positive control, equal volume of medium was added to untreated cells.

Cisplatin concentrations of 0.1–1.0 µg/ml were used to determine IC₅₀ values. For further cell viability assessment, N-Ac-Asp-Glu-Val-Asp-CHO (Ac-devd-cho) as caspase 3 inhibitor, ascorbic acid as reactive oxygen species scavenger and GSH were used at 50 and 300 µM concentrations, respectively, pre-incubated for 2 h before drug treatment. For dose-dependent study, three drug doses designated as D₁ < IC₅₀, D₂ IC₅₀, D₃ > IC₅₀ values were selected and used for subsequent experiments, unless otherwise specified.

**Cell viability assay**

Cell viability was determined using MTT assay. Briefly, A549 cells were seeded in 96-well plates at 1 × 10⁴ cells per well and were allowed to settle for 24 h before treatment. Cells were then treated with flavonol (1–10 µg/ml) for 24 and 48 h with 5% CO₂ at 37 °C. Cell viability was measured by MTT assay (14). Control values corresponding to untreated cells were taken as 100% and viability data of treated ones were expressed as percentage of controls. IC₅₀ values were determined, as concentration that reduced cell viability by 50%. Drug dose of IC₅₀ value at 48 h exposure was taken for further time-dependent study. For dose-dependent analyses,
three drug doses (IC$_{40}$ as D1, IC$_{50}$ as D2 and IC$_{60}$ as D3) were considered.

Cisplatin of preferred concentration (0.1–1 μg/ml) was used against A549 (NSCLC) and L-132 (normal embryonic lung cell line) cell lines both to compare and to evaluate chemopreventive nature of flavonol vis-à-vis cisplatin as another arm of control.

To observe whether flavonol had cytotoxic effects on normal cells, we performed MTT assay taking cells from normal embryonic lung cell line L-132. Flavonol doses of preferred concentrations were used to assess their effect on L-132 cells.

Furthermore, caspase-3 inhibitor (Ac-devd-cho), a reactive oxygen species (ROS)-scavenger (ascorbic acid) and an antioxidant (GSH) were used to ascertain whether flavonol fraction acted in the pathway mediated by caspase-3 and/or ROS.

Morphological analysis and DAPI staining

Cells (~2 × 10$^4$) were plated in 40 mm culture dishes and allowed to proliferate for 24 h. Morphological analysis of cells was performed after flavonol treatment, by phase-contrast microscopy (Leica, Wetzlar, Germany).

Cells were collected and fixed in 2% paraformaldehyde for 1 h. Thereafter, fixed cells were rinsed twice in PBS and stained with 10 μM DAPI for 30 min to determine by fluorescence microscopy, any chromosomal degradation (Zeiss Axiosvert 40 CFL, Thornwood, NY, USA).

DNA fragmentation assay

1 × 10$^4$ cells were treated with flavonol for 24, 36 and 48 h. DNA lysis buffer was used to lyse the cell pellets. These were then incubated overnight with proteinase K (0.1 mg/ml). DNA extraction was performed by routine phenol-chloroform (1:1) method. Extracted DNA was separated in 2% agarose gel containing ethidium bromide (1 mg/ml) and visualized by UV trans-illuminator.

Additionally, we performed DNA fragmentation assay after flavonol treatment along with caspase-3 inhibitor (Ac-devd-cho, 50 μM), to confirm whether the mode of cell death was mediated by caspase-3.

Analysis of apoptosis by flow cytometry using terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay

DNA strand breaks were analysed using the 5-bromo-2′-deoxyuridine 5′-triphosphate (BrdUTP) labelling method of Darzynkiewicz et al. (15) with slight modification. Briefly, ~2 × 10$^4$ drug-treated cells along with untreated ones, were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. Cells were then washed and labelled with DNA labelling solution containing terminal deoxynucleotidyl transferase (TdT) reaction buffer, TdT enzyme and BrdUTP, for 1 h at 37 °C. At the end of incubation, cells were rinsed and pellets were re-suspended in mouse anti-BrdU antibody solution (1:1000). Cells were then incubated with FITC-conjugated mouse secondary antibody (1:500) for 30 min at 37 °C in the dark. After incubation, fluorescence emissions were analysed by flow cytometry [FACS calibur; BD Bioscience].

Annexin V binding assay

To ascertain and confirm cells undergoing apoptosis, we performed annexin V binding assay through flow cytometry. Briefly, cells were treated with flavonol for 12, 18 and 24 h, respectively. Thereafter, treated and untreated cells were harvested by trypsinization. Harvested cells were then incubated in annexin V-FITC (100 ng/ml) and propidium iodide (50 μg/ml), at room temperature for 15 min in the dark, and analysed using a FACS Calibur flow cytometer (BD Bioscience) taking a minimum 10 000 cells in each sample (16).

Cell cycle analysis

Briefly, ~2 × 10$^4$ cells were seeded and treated with flavonol for different times. Cells were then recovered, washed twice in cold PBS and fixed in 70% chilled ethanol. They were then washed twice in PBS, incubated for 1 h at room temperature with 100 μg/ml RNAse A. Thereafter, 50 μg/ml PI was added, and cells were incubated for 15 min in the dark, and were analysed using a FACS Calibur flow cytometer (BD Bioscience). Colchicine (1 μg/ml) was used as treatment for 24 h, taken as a positive G2-M phase cell cycle blocker. Ten thousand events were analysed for each sample using appropriate gating to select single cell population; the same gate was used for all samples.

Drug–DNA interaction study by circular dichroism (CD) spectral analysis

All CD spectra were recorded on a JASCO J720 CD spectrometer at 37 °C using 1 mm cuvette. Spectral recording was performed from 500 nm to 200 nm, repeated three times and averaged automatically.

Calf thymus DNA (ctDNA) concentration in experiments was 100 μM. Induced CD spectral reading was performed resulting from interaction of flavonol (IC$_{50}$ dose) and ctDNA at 37 °C, obtained by subtracting CD spectrum of native DNA and mixture of both ctDNA...
and flavonol from the CD spectrum of buffer and spectra of both buffer and flavonol solutions, respectively.

For assessment of flavonol–nuclear DNA interaction, 2 × 10⁴ A549 cells were either left untreated or treated with flavonol (IC₅₀ dose) for 2, 4, 6, 12, 18 and 24 h, respectively. After their consecutive time exposures, cells were collected and nuclear DNA was extracted and purified, using GeneiPure Mammalian Genomic DNA Purification Kit, Bangalore, India. Collected DNA of equal concentration was used to analyse CD spectra for determination of extent of bound drug with the nuclear DNA.

**Intracellular ROS accumulation study**

Levels of ROS generation in cells were assessed fluorometrically using 2′,7′-dihydrodichlorofluorescein diacetate (H₂DCFDA, Molecular Probe). ~1 × 10⁵ cells were treated with the isolated fraction (IC₅₀ dose) for 1–6 h, 12, 18, 24, 36 and 48 h or left untreated. Cells were then washed in PBS and further incubated with 10 μM H₂DCFDA for 30 min at 37 °C in the dark. After incubation, cells were washed twice in PBS at room temperature for 5 min each. Fluorescence was measured at excitation and emission wavelengths of the oxidized form at 504 and 529 nm, respectively. Hydrogen peroxide (H₂O₂)-treated cells were taken as loading control.

**Determination of intracellular GSH content**

We undertook spectrophotometric analysis to determine intracellular GSH content (17). Briefly, drug-treated (1–6 h of exposure) and -untreated cells (~1 × 10⁵) were taken and cell extracts were prepared. 100 μl cell extract was added with 400 μl sulphosalicylic acid and after centrifugation, 500 μl supernatant was taken and mixed with 4.5 ml DTNB solution. Immediate measurement was taken against reagent blank (4.5 ml DTNB + 500 μl distilled water).

**Mitochondrial membrane potential (ΔΨm) assay**

Changes in ΔΨm after flavonol treatment were examined after 2, 4, 6, 12, 18 and 24 h time points. Changes in ΔΨm were examined by both fluorescence microscopy (Zeiss Axiovert 40 CFL) and flow cytometry using rhodamine 123 (18). Mean fluorescence intensity was detected by FL1 channel of BD FACS calibur (BD Bioscience).

**mRNA level expressions by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)**

RT-PCR was performed according to the method of Sarkar et al. (19) to evaluate mRNA expression levels of p53, Bax, Bcl2 after 12, 18 and 24 h of flavonol treatment. GAPDH was taken as housekeeping gene control. Fluorescence intensity of agarose gel bands was measured using ‘Image J’ software (National Institute of Health, Bethesda, MD, USA). Oligonucleotide (primer) sequences are provided in Table 1.

**Immunofluorescence studies for detection of cytochrome c and caspase-3 activation**

Both untreated and treated cells were washed in PBS and fixed in 1% paraformaldehyde for 1 h. They were washed again in PBS and incubated in anti-caspase-3 primary antibody (1:500) overnight at 4 °C. Next, samples were incubated in FITC-conjugated secondary antibody (1:1000) for 2 h at 4 °C and visualized using fluorescence microscopy (Zeiss Axiovert 40 CFL).

For cytochrome c translocation assay, cells were incubated in MitoRed (200 nM) solution at 37 °C for 30 min to stain mitochondria. Thereafter, cells were washed in PBS and fixed in 1% paraformaldehyde for 1 h. Samples were then incubated in anti-cytochrome c antibody (1:500) overnight at 4 °C and further incubated with FITC-conjugated secondary antibody (1:1000) for 2 h at 4 °C. Specimens were then visualized using fluorescence microscopy (Zeiss Axio Scope A1).

**Cell extract and protein isolation; preparation of cytosolic and mitochondrial extracts**

Cells (~5 × 10⁵) were plated in 90 mm culture dishes and were allowed to proliferate for 48 h. Following flavonol treatment, cells were collected and washed twice in ice-cold PBS. They were then lysed in 20 μl of ice-cold lysis buffer, made up of 10 mM Tris–HCl, pH 7.5, 1 mM MgCl₂, 1 mM EDTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 0.5% CHAPS, and 10% glycerol. Cells were incubated for 30 min on ice and centrifuged for 30 min at 5000 g at 4 °C. After centrifugation,
supernatant was collected and stored at −20 °C for further use.

To carry out cytochrome c translocation studies, cell subfractionation was performed and mitochondrial and cytosolic proteins were isolated as previously reported by Phaneuf et al. (20) with minor modifications.

**Indirect ELISA for activity measurement of different proteins**

Equal amounts of protein (30 µg) were taken and indirect ELISA in both mitochondrial and cytosolic proteins was performed for cytochrome c translocation. We also assayed caspase-8 and caspase-9 activities by the indirect ELISA method according to the manufacturer’s protocol (Santa Cruz Biotechnology Inc, USA) and quantified them using an ELISA reader (Thermo Scientific, Rockford, IL, USA). We used paranitrophenylphosphate (pNPP) as a colour-developing agent and measured colour intensity in 405 nm wavelength.

**Western blot analysis**

For western blot analyses, equal amounts (70 µg) of protein were loaded and samples were denatured in 12% SDS-PAGE for p53, PARP and 15% SDS-PAGE for Bax, Bcl2, cytochrome c and caspase-3. Separated proteins were transferred individually onto PVDF membranes and were probed with anti-p53 (1:500), anti-Bax (1:500), anti-Bcl2 (1:500), anti-cytochrome c (1:1000), anti-caspase-3 (1:1000) and anti-PARP (1:1000) primary antibodies overnight at 4 °C, followed by 1 h incubation with ALKP-conjugated secondary antibody (1:500); they were developed using BCIP-NBT. GAPDH (1:1000) was used as housekeeping gene control. Quantification of proteins was performed by densitometry using Image J software. Immunoblots of proteins of interest were analysed in three separate sets of experiments.

**Animals**

We housed a large group of healthy inbred Swiss albino mice (*Mus musculus*) (6–8 weeks; body weight – 20–25 g) for at least 14 days, in an environmentally controlled room (temp 24 ± 2 °C, humidity 55 ± 5%, 12 h light/dark cycle) with access to food and water *ad libitum*. Experiments were conducted under supervision of the Animal Welfare Committee of the Department of Zoology and as per approved ethical committee guidelines of the Institutional Ethical Committee, University of Kalyani (vide-892/OC/05/CPCSEA).

**Acute toxicity studies in mice**

For acute toxicity testing, mice (*n = 6*) were fed 25, 50, 100, 150, 200 mg/kg body weight (bw) of flavonol dissolved in its solvent media. We watch for signs of mortality, clinical signs and behavioural changes in mice for 24 h for any sign of acute toxicity.

**In vivo anti-tumour activity**

The Swiss albino mice (*Mus musculus*) were randomized and healthy individuals weighing between 20 and 25 g were selected for the experiment. Benzo[a]pyrene (a carcinogen – dissolved in olive oil – induces non-small cell lung carcinoma) at a dose of 50 mg/kg body weight was fed to the animals for one month, twice a week, to develop lung tumours in 4 months (21). Thereafter, flavonol at a dose of 100 mg/kg body weight was fed daily along with the carcinogen for a further 4 months. Six other mice received the carcinogen only, but no drug. Thus, the following five groups served as materials of the present study:

- **Group 1**: Flavonol only-treated group: animals received only flavonol.
- **Group 2**: Normal control: animals received no treatment and only standard food and water.
- **Group 3**: Olive oil only-fed group: received only olive oil.
- **Group 4**: Benzo[a]pyrene fed group: animals received benzo[a]pyrene dissolved in olive oil at a dose of 50 mg/kg body weight twice a week for one month.
- **Group 5**: Flavonol-treated group (carcinogen + flavonol): animals received drug at a dose of 100 mg/kg body weight. Drug was fed orally for 4 months once daily along with carcinogen for first 1 month.

Flavonol only-fed mice (group 1) were considered for toxicity analysis, if any, and thereafter, this group was excluded from further in-depth studies. Photographs were taken and tumour lengths were calculated and expressed in mm.

**Statistical analysis**

All the data reported were the arithmetic mean of data of independent experiments performed in triplicate where each group was six in number. Results were expressed as mean ± SD (standard deviation), unless otherwise stated. Statistical analysis was made by the one-way analysis of variance (ANOVA) with LSD *post-hoc* test using SPSS 16 software (IBM Corporation, Armonk, NY, USA) and paired Student’s *t*-test using GraphPad QuickCalcs.
software. Statistical significance was considered at *P < 0.05, **P < 0.01, ***P < 0.001.

Results

Flavonol reduces A549 cell viability
Flavonol reduced A549 cell viability in a dose- and time-dependent (Fig. 1b) manner with IC$_{50}$ values of 9.34 ± 0.06 μg/ml and 7.6 ± 0.05 μg/ml at 24 and 48 h exposure, respectively (Table 2). After 48 h flavonol exposure, doses were determined to be 5.8 μg/ml as IC$_{40}$, 7.6 μg/ml as IC$_{50}$ and 9.4 μg/ml IC$_{60}$.

In contrast, flavonol displayed a non-significant cytotoxic effect on normal human lung fibroblast cell line L-132 (Fig. 1c). Moreover, when we exposed both A549 and L-132 cells to cisplatin at 0.1 μg/ml, pronounced cytotoxicity occurred by 24 h to both cell lines, inducing almost 50% cell death at 0.44 ± 0.001 and 0.41 ± 0.006 μg/ml, respectively (Fig. 1d). Thus, the drug had a preferential cytotoxic effect on cancer cells while having negligible cytotoxic effects on normal cells.

Flavonol induced A549 cell and nuclear morphological changes
Detectable changes were found after 48 h in A549 cells treated with different doses of flavonol, including altered cell morphology, cell shrinkage and membrane blebbing, the characteristic features of apoptotic cell death (Fig. 2a). After staining with DAPI, chromatin condensation and DNA nick generation were observed in A549 cells specially after long flavonol exposure, initiated after 24 h (Fig. 2b).

Flavonol induced DNA fragmentation
Significant numbers of TUNEL-positive cells were present after flavonol treatment, in a time-dependent manner (Fig. 2c). DNA fragmentation assay also revealed that flavonol treatment could bring about DNA laddering, indicative of apoptosis (Fig. 2d).

Flavonol initiated apoptosis by phosphatidylserine exposure
Translocation of phosphatidylserine (PS) to external surfaces of plasma membranes is a characteristic feature of early apoptosis, which can be detected by binding to annexin V-FITC. If cell death occurs, fragmented DNA becomes permeable for binding with PI (22). Flow cyto-

| Table 2. IC$_{50}$ values of flavonol and cisplatin for A549 and L-132 cell line |
|-----------------|-----------------|-----------------|
| Compounds       | Cell lines A549 (NSCLC) | L-132 (Normal lung cell line) |
| FRF            | 9.3 ± 0.06 μg/ml (24 h of treatment) | Not determined |
|                | 7.6 ± 0.05 μg/ml (48 h of treatment) |                  |
| Cisplatin      | 0.44 ± 0.001 μg/ml (24 h of treatment) | 0.41 ± 0.006 μg/ml (24 h of treatment) |

Cytotoxic activity of flavonol was determined in A549 and L-132 cell line for 24 and 48 h of exposure. Cisplatin activity against A549 and L-132 cell line was determined after 24 h of exposure. All the data are representative of three similar experiments and values are presented as mean ± SD.

Flavonol blocked proliferation of A549 cells by arresting the cell cycle
Flow cytometric analysis indicated G$_2$-M block in flavonol-treated cells along with significant increase in the sub-diploid cell population (sub-G$_1$), in a time-dependent manner (Fig. 2f). By this, uncontrolled cell proliferation was reduced. Onset of G2-M cell cycle arrest along with increase in the sub-diploid cell population (sub G1) suggests that the isolated fraction was potent enough to induce both G2-M phase cell cycle arrest and apoptosis.

Flavonol intercalated with DNA as revealed from CD spectral analysis
CD spectroscopic results revealed that isolated flavonol intercalated with native B-conformation of both ctDNA and nuclear DNA. The interaction was more pronounced in A549 DNA with higher time intervals of flavonol exposure. Observed CD spectrum of both ctDNA (Fig. 3a) and nuclear DNA (Fig. 3b) consisted of a positive band at around 275 nm (ctDNA-flavonol), 267–275 nm (nuclear DNA-flavonol) and a negative band at around the position of 245 nm (ctDNA-flavonol) and 240–245 nm (nuclear DNA-flavonol). Band intensities were observed to gradually change upon drug treatment, indicating positive drug–DNA intercalation.
and G2-M as P3. Colchicine (1 μg/ml, designated as dose1 (D1)), 7.6 μg/ml (IC50), designated as dose2 (D2)), 9.4 μg/ml (IC50), designated as dose3 (D3) of flavonol and incubated for 48 h. (b) Internucleosomal DNA fragmentations of A549 cells after 7.6 μg/ml of flavonol treatment for 24, 36 and 48 h were assessed after DAPI staining. Nicked DNA with brighter fluorescence was indicated by white arrows. (c) TUNEL assay was performed. 6% (UT), 76.7% (24 h), 80.5% (36 h) and 87.1% (48 h) cell populations were found to be TUNEL-positive. (d) DNA fragmentation assay was performed. In drug-treated lanes (24, 36 and 48 h, respectively), DNA ladder reflected the presence of fragmented DNA, whereas untreated cells showed intact nuclear DNA in the 1st lane. (e) A549 cells were treated with 7.6 μg/ml of flavonol for 12, 18 and 24 h, then stained with Annexin V-FITC and PI and FACS analysis was performed. The number of Annexin V-positive cells was determined and the percentage was represented in lower right quadrant (Annexin V+/PI+) and upper right quadrant (Annexin V+/PI−). (f) Cell cycle analysis was performed after treatment of A549 cells with flavonol for 12, 18, 24, 36 and 48 h. The percentage of cells in sub-G1 (apoptotic) was designated as P5, GO-G1 as P2, and G1-S as P4 and G2-M as P3. Colchicine (1 μg/ml) was treated for 24 h and was taken as a positive G2-M phase blocker.

Figure 2. Flavonol-induced cell cycle arrest and apoptosis in A549 cells. (a) Cell morphology assay was performed after A549 cells were treated with 5.8 μg/ml (IC20), designated as dose1 (D1), 7.6 μg/ml (IC50), designated as dose2 (D2), 9.4 μg/ml (IC50), designated as dose3 (D3) of flavonol and incubated for 48 h. (b) Internucleosomal DNA fragmentation of A549 cells after 7.6 μg/ml of flavonol treatment for 24, 36 and 48 h were assessed after DAPI staining. Nicked DNA with brighter fluorescence was indicated by white arrows. (c) TUNEL assay was performed. 6% (UT), 76.7% (24 h), 80.5% (36 h) and 87.1% (48 h) cell populations were found to be TUNEL-positive. (d) DNA fragmentation assay was performed. In drug-treated lanes (24, 36 and 48 h, respectively), DNA ladder reflected the presence of fragmented DNA, whereas untreated cells showed intact nuclear DNA in the 1st lane. (e) A549 cells were treated with 7.6 μg/ml of flavonol for 12, 18 and 24 h, then stained with Annexin V-FITC and PI and FACS analysis was performed. The number of Annexin V-positive cells was determined and the percentage was represented in lower right quadrant (Annexin V+/PI+) and upper right quadrant (Annexin V+/PI−). (f) Cell cycle analysis was performed after treatment of A549 cells with flavonol for 12, 18, 24, 36 and 48 h. The percentage of cells in sub-G1 (apoptotic) was designated as P5, GO-G1 as P2, and G1-S as P4 and G2-M as P3. Colchicine (1 μg/ml) was treated for 24 h and was taken as a positive G2-M phase blocker.

Flavonol induced apoptosis in an ROS-independent manner

Fluorometric results revealed that flavonol in short exposures (1–6 h) and also in long exposures (12, 18, 24, 36 and 48 h respectively) did not elevate significant intracellular ROS levels (Fig. 4a). Using ROS inhibitor ascorbic acid (300 μM), pre-treated for 2 h, significant changes in viability of flavonol-treated cells were observed (Fig. 4b).

Analysis of enzymatic data reveal that flavonol was not able to deplete intracellular GSH level (Fig. 4c) fully, but could only partially deplete it, specially by 2–5 h exposure. Thereafter, when cells were pre-treated with extraneous GSH (300 μM) along with flavonol treatment, their viability was found to be reduced (Fig. 4d). The redox state therefore was not easily dysregulated by a ROS-mediated pathway needed by cells for apoptosis.

Flavonol altered mitochondrial membrane potential and translocated cytochrome c

From ELISA assays, we found significant time-dependent increase in caspase-9 activity specially at long exposures (after 24 h) to drug induction, whereas caspase-8 was found to have non-significant activation (Fig. 5a). These data indicate that apoptosis triggered by flavonol may be through the mitochondria-mediated intrinsic pathway.

A key step of the intrinsic pathway is alteration in mitochondrial membrane potential and release of cytochrome c into the cytosol, which turns on the caspase cascade (20). As shown in Fig. 5b, increase in mitochondrial permeability transition occurred accompanied by collapse/depolarization of mitochondrial membrane potential, significantly, at around 12–24 h after being hyperpolarized at 2–6 h of drug exposure.

From the immunofluorescence study, upregulation of cytochrome c expression in the cytosol was observed (Fig. 5c). ELISA analysis confirmed increased expression of cytochrome c in the cytosolic fraction, whereas its expression declined in the mitochondrial fraction (Fig. 5d) after drug induction, suggesting cytosolic translocation of cytochrome c. Expression of COX-4, used as loading control for mitochondrial proteins, was the same in mitochondria even after drug treatment, whereas no COX-4 expression was detected in the cytosolic fraction (data not shown). This would indicate that
we had successfully separated mitochondrial and cytosolic fractions of cell lysate. Furthermore, western blot data also revealed significant upregulation of cytochrome c in the cytosolic fraction (Fig. 5e, f) by 12–24 h drug exposure.

Flavonol modulated A549 gene and protein expressions related to cell death

From RT-PCR and western blot analysis, we observed upregulation of Bax and downregulation of Bcl2 along with overexpression of p53, after 12, 18, 24 h flavonol treatment (Fig. 6a–d). Bcl2/Bax imbalance (Fig. 6e) and p53 upregulation were coincident with cytochrome c release. These data are indicative of caspase cascade activation with long time period drug exposure.

Flavonol promoted activation of caspase-3

The immunofluorescence study indicated upregulated expression of caspase-3 by 24–48 h drug exposure (Fig. 6f). From western blot analysis, we also observed upregulated expression of cleaved caspase-3 at 17 kDa and a cleaved PARP fragment at 89 kDa (Fig. 6g, h). To explore whether flavonol-induced apoptosis was specifically associated with caspase-3-mediated pathways or not, we exposed cells to 7.6 μg/ml flavonol along with caspase-3-specific inhibitor, Ac-dvd-cho (50 μM). Intact cell DNA with no internucleosomal fragmentation (Fig. 6i) and greater cell viability (Fig. 6j) were found after drug exposure along with caspase-3 inhibitor. This would tempt one to suggest that apoptosis may be mediated via activation of caspase-3.
Figure 5. Effect of flavonol on mitochondria-mediated intrinsic cell death pathway. (a) A549 cells were treated with flavonol for 24, 36 and 48 h. Indirect ELISA of caspase-8,-9, GAPDH was done. Data are represented as per cent of control and are presented as mean ± SD. **P < 0.01, ***P < 0.001 was considered statistically significant. (b) A549 cells were left untreated or treated with flavonol for 2, 4, 12, 18 and 24 h of exposure and stained with Rhodamine123. Microscopic observation was performed under fluorescence microscope and mean fluorescence intensity of rhodamine 123 was measured by FACS analysis. (c) Effect of flavonol on cytoplasmic release and activation of cytochrome c. A549 cells were treated with flavonol for 12, 18 and 24 h and cytochrome c expression was detected by immunofluorescence assay after dual staining with MitoRed and FITC-tagged secondary antibody specific for cytochrome c. Increased expression was observed with brighter fluorescence of cytochrome c in the cytoplasmic portion. (d) Cytochrome c translocation assay was performed. Data are represented as percentage of control and mean ± SD. **P < 0.01, ***P < 0.001 was considered statistically significant. (e) Western blot analysis of cytochrome c and GAPDH was performed. GAPDH was used for loading correction. (f) Band intensities of cytochrome c were calculated. Data are represented as percentage of control and are presented as mean ± SD. ***P < 0.001 was considered statistically significant.
**Figure 6. Flavonol-induced gene expression analysis.** A549 cells were left untreated or treated with optimum dose of flavonol for 12, 18 and 24 h and (a) RT-PCR of p53, Bax, Bcl2 and GAPDH was performed. GAPDH was used as loading control. (b) Relative band intensities of p53, Bax and Bcl2 were calculated. Values are represented as percentage of control and presented as mean ± SD. *P < 0.05 and ***P < 0.001 were considered statistically significant. (c) Western blot analysis of p53, Bax, Bcl2, GAPDH was performed. GAPDH was used as loading control. (d) Relative band intensities of p53, Bax and Bcl2 were calculated. Values are represented as percentage of control and presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 were considered statistically significant. (e) Bcl2/Bax ratio at both gene and protein levels was calculated. Results are presented as mean ± SD. **P < 0.001 was considered statistically significant. (f) A549 cells were treated with flavonol and caspase-3 activities were detected by immunofluorescence assay. (g) Western blot assay of caspase-3, PARP, GAPDH was performed. GAPDH was used for loading correction. (h) Relative band intensities of caspase-3 and cleaved PARP fragment was measured. Data are represented as per cent of control and presented as mean ± SD. *P < 0.05, **P < 0.001 were considered statistically significant. (i) A549 cells was either kept untreated or treated with 7.6 μg/ml of flavonol after pre-treatment of caspase-3 inhibitor (CI) Ac-devd-cho (50 μM, 2 h) or treated with only flavonol (drug dose 2, i.e. D2 = 7.6 μg/ml). Thereafter, DNA fragmentation assay was performed. Untreated cellular DNA, only caspase-3 inhibitor-treated cellular DNA and flavonol-treated cellular DNA, pre-treated with caspase-3 inhibitor showed no internucleosomal DNA fragmentation, whereas only flavonol-treated ones induced DNA fragmentation. (j) Cell viability assay was analysed. Cells when pre-treated with caspase-3 inhibitor and thereafter exposed to flavonol did not show any reduction in viability, whereas only flavonol-treated cells showed. Data are represented as percentage of control and presented as mean ± SD. ***P < 0.001 was considered statistically significant.

**Flavonol was relatively non-toxic to mice in vivo**

No significant changes in mortality, occurrence of abnormal clinical signs, symptoms, gain or loss of body weight, organ weight or similar signs were observed in respect of acute toxicity analysis of flavonol, in mice (group 1) in vivo. Partial signs of behavioural change were observed, specially by the dose of 200 mg/kg bw of flavonol. Thus, we preferred to use 100 mg/kg body weight as the optimum dose of flavonol for further study.

**Flavonol inhibited benzo[a]pyrene-induced lung tumour growth in mice**

The capability of flavonol to induce apoptosis in A549 NSCLC cell line in vitro suggested that it might have the ability to suppress tumour growth in vivo. To ascertain this, Swiss albino mice were induced to become NSCLC hosts by treatment with benzo[a]pyrene (50 mg/kg bw) followed by feeding with flavonol at optimum dose (100 mg/kg bw). Flavonol inhibited benzo[a]pyrene-induced non-small cell lung tumour growth in a significant manner. Tissue architecture and lung morphology tended to be gradually normalized (Fig. 7a), showing signs of significant regression of tumour numbers and sizes of lesions (Fig. 7b).

**Discussion**

Results show that isolated flavonol fraction was a highly potent agent, which reduced A549 cell viability in a dose- and time-dependent manner. More importantly in our study, L-132, normal human embryonic lung cells exposed to flavonol, showed no significant cell death. However, when L-132 or the A549 cells were separately

![Figures](image-url)

**Figure 7. Effect of flavonol on lung tumour growth reduction, in vivo.** (a) Lung morphology of normal, Benzo[a]pyrene (50 mg/kg body weight)-induced and flavonol (100 mg/kg body weight)-treated mice. (b) Lung tumour length (mm) of Benzo[a]pyrene-induced group and flavonol-treated group was measured. Tumour length differences between Benzo[a]pyrene-induced group and flavonol-treated ones were significant, considering ***P < 0.001, by paired Student’s t test.
exposed to cisplatin (common anti-cancer agent used in the treatment of lung cancer), both types of cell died in significantly high numbers. Flow cytometry studies revealed that this cytotoxic effect of flavonol against A549 cells was by apoptosis, as a good number of the cell population had annexin-V positivity early in treatment (12–24 h), and inter-nucleosomal DNA fragmentation with TUNEL positivity with longer (24–48 h) exposure. Thus, apoptotic induction by flavonol was found to be very much target-specific and chemo-preventive.

To explore underlying mechanisms of flavonol-induced apoptosis, we analysed expression of signal proteins related to apoptosis. The caspase protein cascade, specially caspase-3, plays a central role in apoptosis - which is responsible for DNA fragmentation (23). From western blot data, we showed positive activation of caspase-3 (17 kDa fragment) with long exposure. Furthermore, using caspase-3 inhibitor (Ac-dvd-cho), we did not find any internucleosomal DNA fragmentation in flavonol-exposed cells; therefore, it can be concluded that flavonol-induced apoptosis was mediated by caspase-3. We also obtained high expression of cleaved PARP fragment (89 kDa), which indicates activation of the caspase-3-mediated downstream signalling pathway that leads to DNA fragmentation. Elmore, 2007 (24), in an earlier study, suggested that caspase-3 is generally activated by caspase-8 and caspase-9 mainly through two main pathways of apoptosis, the extrinsic pathway (receptor-mediated) and the intrinsic pathway (mitochondria-mediated). However, from results of the ELISA assay performed in the present study, we observed a significant increase in caspase-9 expression, but there was no increase in caspase-8. This would possibly indicate that flavonol might act possibly through the mitochondrial intrinsic pathway of apoptosis.

In the intrinsic pathway of apoptosis, mitochondria play a central role (25). Disruption of mitochondrial membrane potential (ΔΨm) is known to induce release of cytochrome c into the cytosol. Release of cytochrome c along with Apaf-1 allows formation of the apoptosome complex and activates caspase-9 (26). Activated caspase-9 then cleaves and activates effector caspases such as caspase-3, which executes the ultimate apoptotic process (23,27). Our flow cytometry studies detected that after hyperpolarization during the period from 2 to 6 h, mitochondrial membranes depolarized, mainly by 12–24 h drug exposure. Thereafter, immunofluorescence study, ELISA assay and western blot analysis revealed translocation of cytochrome c from mitochondria to the cytosol and its significant upregulation in the cytosolic fraction at 18–24 h drug exposure. All these data collectively confirm that apoptotic induction by flavonol is possibly mitochondria-mediated.

Release of cytochrome c from mitochondria into the cytosol is known to be regulated by pro- and anti-apoptotic Bcl2 family proteins, which regulate mitochondrial membrane permeability and polarization (28). From RTPCR and western blot studies, we obtained evidence of significant Bax upregulation and Bcl2 downregulation after flavonol exposure, indicating thereby an ultimate imbalance of Bcl2/Bax ratio leading cells to apoptosis, through induction of alteration in mitochondrial membrane potential, which in turn causes cytochrome c release.

Balance between oxidative stress and antioxidative forces helps in maintaining cell homeostasis. Interfering force disrupting this balance when a shift in redox state of a cell occurs may play a mediating role in apoptotic induction (6,29). Proper balance between generation of free radicals such as reactive oxygen species (ROS) and generation of its scavenger antioxidants, helps in maintaining the homeostatic state. From quantitative data, we confirmed that ROS did not increase significantly, but there was depletion of major antioxidant such as GSH early (2–5 h) in drug exposure. On the other hand, ascorbic acid, a potent ROS scavenger (30), and glutathione reductase (GSH) were unable to interfere with flavonol-induced apoptosis; this would indicate that although the drug was able to deplete GSH levels, apoptosis was possibly induced by flavonol by a ROS-independent pathway and not fully dependent on antioxidant depletion. As flavonol bears such ROS inhibitory capacity, it may be effective as an anti-cancer drug against any carcinogen that exerts its effect through ROS generation pathways.

Drugs which intercalate with DNA are known to trigger apoptosis effectively (4). From circular dichroism spectral analysis, we observed that flavonol intercalated with nuclear DNA of A549 cells from early exposure, as well as with calf thymus DNA, which was considered as the experimental control. From RT-PCR and western blot data, we found time-dependent upregulation of p53 after flavonol exposure. This observation would tempt one to suggest that upregulation of p53 was possibly caused by DNA damage through intercalation of flavonol with DNA, in conformity with the proposition of Lakin and Jackson, 1999 (31) for a similar result. Haupt et al. (32) have suggested that upregulation of p53 helps in induction of the apoptotic pathway to a further extent, which might in turn result in the reduction in proliferation of A549 cells by arresting the cell cycle at the specific stages observed in this study.

Cell cycle analysis further showed that cells arrested in G2-M phase of the cell cycle along with an increment in sub-diploid cell population (sub-G1) after flavonol induction, in a time-dependent manner. This result indicates...
that flavonol served dual functions: apoptosis and cell cycle arrest, when A549 cells were exposed to it, thereby reducing uncontrolled cell proliferation. However, this dual response might possibly happen because flavonol–DNA intercalation at the early stages, resulting in a late response in subG1 cell accumulation along with G2-M stage arrest.

Moreover, as flavonol has cell proliferation reduction and apoptotic properties, we conducted a standardized study on its effects by applying it in the murine body system. Benzo[a]pyrene-induced lung tumour-bearing mice, when treated with flavonol at optimum dose of 100 mg/kg bw, had significantly reduced tumour growth, proliferation and improved tissue architecture. Administration of drug alone at that optimum dose neither showed any acute toxic symptoms nor produced any behavioural changes in the animals.

Overall data collectively suggest that flavonol was non-toxic to normal cells, but acted as a chemopreventive agent, which selectively triggered G2-M phase cell cycle arrest and apoptotic cell death in K-ras-mutated NSCLC cell line A549. Apoptosis appeared to be ROS-independent, mitochondria-dependent and caspase 3-mediated. At early stages of exposure, flavonol targeted nuclear DNA rather than relevant signalling molecules, which may render it to be an advantage over other drugs, in producing desired anti-cancer effects. In that case, interference of any relevant signalling molecule, particularly such as K-ras which is mutated in A549 cells, could be strategically avoided. Moreover, as the flavonol-induced apoptotic pathway is ROS-independent, it may be extrapolated that this flavonol may act as a putative agent in combating several carcinogens, as many of them elicit their carcinogenicity by elevating cell ROS level. Beside this, the isolated fraction of flavonol proved to be non-toxic in mice and also capable of showing signs of anti-cancer potential by inhibiting cell proliferation and growth of lung tumours in mice, in vivo. Moreover, flavonol at its specific dose was neither cytotoxic to normal L-132 lung cells, in vitro, nor was able to raise any toxicity in mouse bodies, in vivo. This makes the drug more potent and suitable for therapeutic use against lung cancer.

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Conflict of interest

None declared.

References


[6]-Gingerol isolated from ginger attenuates sodium arsenite induced oxidative stress and plays a corrective role in improving insulin signaling in mice

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ABSTRACT

Arsenic toxicity induces type 2 diabetes via stress mediated pathway. In this study, we attempt to reveal how sodium arsenite (As) could induce stress mediated impaired insulin signaling in mice and if an isolated active fraction of ginger, [6]-gingerol could attenuate the As intoxication hyperglycemic condition of mice and bring about improvement in their impaired insulin signaling. [6]-Gingerol treatment reduced elevated blood glucose level and oxidative stress by enhancing activity of superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and GSH. [6]-Gingerol also helped in increasing plasma insulin level, brought down after As exposure. As treatment to primary cell culture of β-cells and hepatocytes in vitro produced cytodegenerative effect and accumulated reactive oxygen species (ROS) in pancreatic β-cells and hepatocytes of mice. [6]-Gingerol appeared to inhibit/intervene As induced cytodegeneration of pancreatic β-cells and hepatocytes, helped in scavenging the free radicals. The over-expression of TNFα and IL6 in As intoxicated mice was down-regulated by [6]-gingerol treatment. As intoxication reduced expression levels of GLUT 4, IRS-1, IRS-2, PI3K, Akt, PARs signaling molecules; [6]-gingerol mediated its action through enhancing the expressions of these signaling molecules, both at protein and mRNA levels. Thus, our results suggest that [6]-gingerol possesses an anti-hyperglycemic property and can improve impaired insulin signaling in arsenic intoxicated mice.

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1. Introduction

Arsenic is a naturally occurring heavy metal that is present in food, soil and water. It is released in the environment from both natural and man-made sources (Trushenski et al., 1995). Inorganic arsenic and their metabolites (both As(III) and As(V) forms) are known to exert their toxic effects by a variety of mechanisms which may lead to some serious health problems. Epidemiological data have shown that chronic exposure of inorganic arsenic compounds to humans are associated with liver injury, peripheral neuropathy and an increased incidence of cancer of the lung, skin, and liver (Leonard and Lauwerys, 1980). In Asia alone, including Bangladesh, West Bengal, India, Vietnam, Thailand and China, more than 30 million people are chronically exposed to arsenic (Tseng et al., 1968). This arsenic induced toxicity arises and sustains by generating stress response through reactive oxygen species formation and antioxidant depletion (Jomova et al., 2008). According to a recent study, sodium arsenite (As) is found to be associated with increased blood glucose level in experimental rats (Yousef et al., 2008).

Hydroarsenicism is a major public health problem since millions of people worldwide are exposed to arsenic by drinking of contaminated water (Jones, 2007). Studies on mouse bone marrow cells have predicted an increased level of chromosomal abnormality and micronucleus formation after treatment with arsenic (Banerjee et al., 2007) and thereby have confirmed its cytotoxic and cytodegenerative effects. One of the plausible modes of action of arsenic toxicity is by oxidative stress since it can stimulate production of reactive oxygen species (ROS), resulting from an imbalance between antioxidants and oxidants during arsenic metabolism (Goering et al., 1999; Sun et al., 2006).

On the other hand arsenic has been recently proposed as an additional risk factor for diabetes (Silbergeld et al., 2008; Longnecker and Daniels, 2001). According to recent surveys it is found that the occurrence of diabetes is significantly higher in arsenic endemic villages in Taiwan and India than in the general population (Zimmer, 1982; Wang et al., 1997; Belen et al., 2008). The prevalence of diabetes mellitus was 2-fold higher in these areas than in Taipei City and the Taiwan area in general.

From in vitro studies, the impairment of insulin secretion (Diaz-Vilasecor et al., 2006) and the induction of oxidative stress (Izquierdo-Vega et al., 2006) have been postulated for arsenic-induced type 2 diabetes. Induction of stress via generation of free oxygen radicals and antioxidant depletion led to this process and
Ethanolic extract of Condurango (Marsdenia condurango) used in traditional systems of medicine including homeopathy against cancer can induce DNA damage and apoptosis in non small lung cancer cells, A549 and H522, in vitro

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ABSTRACT
In traditional systems of medicine including homeopathy, the Condurango extract (Coe) is often used to cure stomach cancer mainly, without having any scientific validation of its anti-cancer ability. Con has therefore been tested against non-small-cell lung cancer cells (NSCLC) A549 and NCI-H522 (H522) known to contain the KRS4 mutation, making them resistant to most chemotherapeutic agents. As cancer cells generally defy cytotoxicity developed by chemopreventive agents and escape cell death, any drug showing the capability of preferentially killing cancer cells through apoptosis is worth consideration for judicious application. A549 and H522 cells were exposed to 0.35 µg/ml and 0.25 µg/ml of Coe, respectively, for 48 h and analysed based on various protocols associated with apoptosis and DNA damage, such as MTT assay to determine cell viability, LDH assay, DNA fragmentation assay, comet assay, and microscopical examinations of DNA binding fluorescence stains like DAPI, Hoechst 33258 and acridine orange/ethidium bromide to determine the extent of DNA damage made in drug-treated and untreated cells and the results compared. Changes in mitochondrial membrane potential and the generation of reactive oxygen species were also documented through standard techniques. Con killed almost 50% of the cancer cells but spared normal cells significantly. Fluorescence studies revealed increased DNA nick formation and depolarized membrane potentials after drug treatment in both cell types. Caspase-3 expression levels confirmed the apoptosis-inducing potential of Coe in both the NSCLC lines. Thus, overall results suggest considerable antitumor potential of Coe against NSCLC in vitro, validating its use against lung cancer by practitioners of traditional medicine including homeopathy.

Keywords: antitumor potential, Condurango extract, A549 and NCI-H522 non small-cell lung cancer cells, apoptosis, DNA damage, reactive oxygen species, mitochondrial membrane potential

INTRODUCTION
Cancer is one of the dreaded diseases and affects more than ten million people annually [Moss et al., 2010], of which lung cancer is the most commonly diagnosed cancer with 1.4 million new cases reported each year [http://www.who.int/mediacentre/factsheets/fs297/en/index.html]. It is currently the leading cause of cancer-related deaths in the United States [Whitehead et al., 2003]. Different types of carcinogens present in tobacco smoke are the main suspects to cause lung cancer [Kometani et al., 2009; Yang et al., 2000]. Clinically, lung cancer is of two types, namely, small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC). NSCLC accounts for >80% of all lung cancers and is responsible for more deaths from cancer than any other tumor types (Jennal et al., 2005). This type of cancer hardly responds to chemotherapy, primarily because of its KRAS mutation that often makes it resistant to chemotherapy (Guo et al., 2008). Traditionally, surgery, radiation therapy, and chemotherapy are the ultimates to treat patients with NSCLC. But these treatments are usually accompanied by many side-effects. Many cancer patients are now treated with complementary and alternative medicine (CAM) for its less toxic side-effects. Different preparations of herbal extracts including homeopathy are some of the most popular CAM modalities for cancer patients in seven out of 14 European countries today (Rostock et al., 2011). However, many of these herbal extracts used as homeopathic mother mixtures have not been scientifically validated. Therefore, scientific approaches to test the efficacy of such plant extracts for their anti-cancer potentials, in vitro, are of utmost importance for the initial screening. The drugs that show such potentials should further be tested in animal models in vivo, so that the benefits from their therapeutic use can be justified in human cases. Furthermore, many of these natural products are known to have less adverse side-effects, and therefore, a further study of their possible cytotoxicity in normal and cancer cell lines may become more meaningful and significant.

Traditional herbal medicines are rich sources of new drugs. There is increasing interest in investigating different species of plants used as traditional medicine to identify effective ingredients using the most modern chemical and molecular methods (Wang et al., 2004). Literature suggests that the methanolic extract of Marsdenia condurango bark exhibits differentiation-inducing activity in M1 cells (Umehara et al., 1994). Condurango has long been used against a variety of...
Post-cancer Treatment with Condurango 30C Shows Amelioration of Benzo[a]pyrene-induced Lung Cancer in Rats Through the Molecular Pathway of Caspase-3-mediated Apoptosis Induction

Running title: Anti-lung cancer potential of Condurango 30C in rats

Sourav Sikdar, Avinaba Mukherjee, Kausik Bishayee, Avijit Paul, Santu Kumar Saha, Samrat Ghosh, Anisur Rahman Khuda-Bukhsh*

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Key Words
Condurango 30C, benzo[a]pyrene (BaP), lung cancer, apoptosis, caspase-3, homeopathy

Abstract

Objectives: The present investigation aimed at examining if post-cancer treatment with a potentized homeopathic drug, Condurango 30C, which is generally used to treat oesophageal cancer, could also show an ameliorating effect through apoptosis induction on lung cancer induced by benzo[a]pyrene (BaP) in white rats (Rattus norvegicus).

Methods: Lung cancer was induced after four months by chronic feeding of BaP to rats through gavage at a dose of 30 mg/kg body weight for one month. After four months, the lung cancer-bearing rats were treated with Condurango 30C for the next one (3rd), two (5th - 5th) and three (5th - 7th) months, respectively, and were sacrificed at the corresponding time points. The ameliorating effect, if any, after Condurango 30C treatment for the various periods was evaluated by using protocols such as histology, scanning electron microscopy (SEM), annexin-V-FITC/PI assay, flow cytometry of the apoptosis marker DNA fragmentation, reverse transcriptase polymerase chain reaction (RT-PCR), immunohistochemistry, and western blot analyses of lung tissue samples.

Results: Striking recovery of lung tissue to a near normal status was noticed after post-cancerous drug treatment, as evidenced by SEM and histology, especially after one and two months of drug treatment. Data from the annexin-V-FITC/PI and DNA fragmentation assays revealed that Condurango 30C could induce apoptosis in cancer cells after post-cancer treatment. A critical analysis of signalling cascade, evidenced through a RT-PCR study, demonstrated up-regulation and down-regulation of different pro- and anti-apoptotic genes, respectively, related to a caspase-3-mediated apoptotic pathway, which was especially discernible after one-month and two-month drug treatments. Correspondingly, Western blot and immunohistochemistry studies confirmed the ameliorative potential of Condurango 30C by its ability to down-regulate...

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Ethanolic extract of the Goldenseal, Hydrastis canadensis, has demonstrable chemopreventive effects on HeLa cells in vitro: Drug–DNA interaction with calf thymus DNA as target

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ABSTRACT

This study tested chemotherapeutic potential of Hydrastis canadensis (HC) extract in HeLa cells in vitro, with emphasis on its drug–DNA interaction and apoptosis induction ability. Nuclear uptake of HC by DAPI, AO/EB staining and internucleosomal DNA damage by comet assay was studied through fluorescence microscopies. Possible changes in VMP and apoptotic signalling events were critically analyzed. Cell cycle progression studied through FACS and fragmented DNA through "TUNEL" assay were critically analyzed. RT-PCR studies were conducted for analyzing Cyt-C and Bax translocation in mitochondrial and cytosolic extracts, and Caspase 3 in whole cell lysate. Role of p53-mediated regulation of NF-κB and TNF-α were elucidated by Western blot analysis. Data of CD 4 and Tm profile of C5-DNA were analyzed. Overall results indicated anti-cancer potential of HC through its ability to induce apoptosis, interaction with CT-DNA that changed structural conformation of DNA, proving HC to be a promising candidate for chemoprevention.

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Quercetin induces cytochrome-c release and ROS accumulation to promote apoptosis and arrest the cell cycle in G2/M, in cervical carcinoma: signal cascade and drug-DNA interaction

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Abstract

Objectives: Small aromatic compounds like flavonoids can intercalate with DNA molecules bringing about conformational changes leading to reduced replication and transcription. Here, we have examined one dietary flavonoid, quercetin (found in many fruit and vegetables), for possible anti-cancer effects, on HeLa cells originally derived from a case of human cervical cancer.

Material and methods: By circular dichroism spectroscopy we tested whether quercetin effectively interacted with DNA to bring about conformational changes that would strongly inhibit proliferation and migration of the HeLa cells. Cytotoxic effects of quercetin on cancer/normal cells, if any, were determined by MTT assay and such depolarization of mitochondrial membrane potential, as a consequence of quercetin treatment, and accumulation of reactive oxygen species (ROS) also were studied, by FACS analysis and expression profiles of different anti- and pro-apoptotic genes and their products were determined.

Results: Quercetin intercalated with calf thymus cell DNA and HeLa cell DNA and inhibition of anti-apoptotic AKT and Bcl-2 expression were observed. Levels of mitochondrial cytochrome-c were elevated and depolarization of mitochondrial membrane potential occurred with increase of ROS; upregulation of expression of p53 and caspase-3 activity were also noted. These alterations in signalling proteins and externalization of phosphatidyl serine residues were involved with initiation of apoptosis. Reduced AKT expression suggested reduction in cell proliferation and metastasis potential, with arrest of the cell cycle at G2/M.

Conclusion: Quercetin would have potential for use in cervical cancer chemotherapy.

Introduction

Flavonoids compose a large class of low-molecular weight natural products, of plant origin, found ubiquitously in foodstuffs, including tea, capers, lovage, apple, onion, grapes, citrus fruits, tomatoes, and in all green vegetables (1) (Fig. 1a). Quercetin (also known as flavin melanin), present in various food plants, has been known to provide dietary antioxidants which exert significant anti-tumour, anti-allergic and anti-inflammatory effects (2). Of the tumours, quercetin has been reported to have a potent anti-cancer role in cases of colon, breast and lung cancers, and to play an anti-invasive role in prostate cancer (3,4). To our knowledge, no precise study has been undertaken to delineate molecular mechanisms underlying depolarization of mitochondrial membrane potential, with ROS accumulation in mitochondria, in cells of cervical carcinoma. Earlier work carried out has reported: (i) general anti-cancer effects of quercetin (5,6); and (ii) quercetin-induced cell cycle arrest and mitochondria-mediated apoptosis, in HeLa cells (7). Thus, the present investigation was undertaken with a view to understanding molecular mechanisms of quercetin’s action, which have not been studied earlier.

Chemotherapy to treat cancer was introduced more than 50 years ago, and has generally shown considerable efficiency for the treatment of testicular cancer and certain leukemias, but in general its success rate against breast, colon, lung and cervical cancers has been less

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Chelidonine isolated from ethanolic extract of *Chelidonium majus* promotes apoptosis in HeLa cells through p38-p53 and PI3K/AKT signalling pathways

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**OBJECTIVE:** To evaluate the role of chelidonine isolated from ethanolic extract of *Chelidonium majus* in inducing apoptosis in HeLa cells and to assess the main signalling pathways involved.

**METHODS:** Cells were initially treated with different concentrations of chelidonine for 48 h and the median lethal dose (LD₅₀) value was selected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Morphological analysis of nuclear condensation and DNA damage and fragmentation were measured by 4',6-diamidino-2-phenylindole staining and comet assay. Further, reactive oxygen species (ROS) generation, cell cycle arrest and change in mitochondrial membrane potential were also examined and analyzed by flow cytometry. Evaluation of interaction of drug with CT DNA was investigated by circular dichroism (CD) spectral analysis to find any possible drug-DNA interaction. The mRNA and protein expression of major signal proteins like that of p38, p53, protein kinase B (AKT), phosphatidylinositol 3-kinases (PI3K), Janus kinase 3 (JAK3), signal transducer and activator of transcription 3 (STAT3) and E6 and E7 oncoproteins as well as the pro-apoptotic genes and antiproliferative genes were also estimated by reverse transcription-PCR-polymerase chain reaction and Western blot.

**RESULTS:** Based on LD₅₀ value (30 µg/mL) of chelidonine, three doses were selected, namely, 22.5 µg/mL (D1), 30.0 µg/mL (D2) and 37.5 µg/mL (D3). Results showed that chelidonine inhibited proliferation and induced apoptosis in HeLa cells through generation of ROS, cell cycle arrest at sub-G₁ and G₀/G₁ stage, change in mitochondrial membrane potential...
**Helonia dioica extract enhances the anti-diabetic effect of Metformin hydrochloride through increased insulin responsiveness and activation of PI3K/AKT signaling in liver of obese hyperglycemic mice**

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Antihyperglycemic Drug Gymnema sylvestre Also Shows Anticancer Potentials in Human Melanoma A375 Cells via Reactive Oxygen Species Generation and Mitochondria-Dependent Caspase Pathway

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Abstract
Objective: Ethnologic extract of Gymnema sylvestre (GS) leaves is used as a potent anti-diabetic drug in various systems of alternative medicine, including homeopathy. The present study was aimed at examining if GS also had anticancer potentials, and if it had, to elucidate its possible mechanism of action. Methods: We initially tested possible anticancer potential of GS on A375 cells (human skin melanoma) through MTT assay and determined cytotoxicity levels in A375 and normal liver cells. We then thoroughly studied its apoptotic effects on A375 cells through protocols such as Hoechst 33342, H2DCFDA, and rhodamine 123 staining and conducted ELISA for cytochrome c, caspase 3 and PARP activity level. We determined the mRNA level expression of cytochrome c, caspase 3, Bcl2, Bax, PARP ICAD, and EGFR signaling genes through semiquantitative reverse transcription polymerase chain reaction and conducted Western blot analysis of caspase 3 and PARP. We also analyzed cell cycle events, determined reactive oxygen species accumulation, measured annexin V-FITC/PI and rhodamine 123 intensity by flow cytometry. Results: Compared with both normal liver cells and drug-uninjured A375, the mortality of GS-treated A375 cells increased in a dose-dependent manner. Additionally, GS induced nuclear DNA fragmentation and showed an increased level of mRNA expression of apoptotic signal related genes cytochrome c, caspase 3, PARP, Bax, and reduced expression level of ICAD, EGFR, and the anti-apoptotic gene Bcl2. Conclusion: Overall results indicate GS to have significant anticancer effect on A375 cells apart from its reported antidiabetic effect, indicating possibility of its palliative use in patients with symptoms of both the diseases.

Keywords
Gymnema sylvestre, A375 melanoma cells, reactive oxygen species, DNA damage, apoptosis, anticancer potential

Introduction
The burden of cancer is increasing worldwide despite advances in diagnosis and treatment with the orthodox system of medicine. Skin cancer is the third most common among the human malignancies arising from the pigmented cells of the skin called melanocytes. Epidemiological studies have shown that many types of cancers may be avoidable,\(^1\) and in many, chemotherapy and radiation are highly ineffective necessitating other options for the cure of this disease.\(^2\) Evidence-based studies reported that melanoma is highly resistant to conventional treatments such as radiation and chemotherapy.\(^3\) Complementary and alternative medicine is increasingly becoming popular particularly in oncology, which often applies various complementary and alternative medicines to give patients a better quality of living by alleviating their sufferings.\(^4\)

In traditional systems, medicinal plants and plant-derived drugs are extensively used, but many of them need scientific validation to give confidence to the users, particularly when treating chronic life-threatening conditions such as cancer.\(^5\) Gymnema sylvestre is a woody climber tree, commonly known as Gurmee in India, belonging to the order Gentianales, and family Asclepiadaceae. The plant grows in the western and central parts of India, in tropical Africa, and in Australia. Its ethnobiological extract is considered a potent anti-diabetic drug used in folk medicine, Ayurveda, and homeopathic systems of medicine,\(^6\) but whether it has anticancer property has never been tested earlier. Hence, as a part of our routine program of testing extracts of some unconventional and unexplored plants for their

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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.

METHODS: Cytotoxicity of the drug was tested by conducting 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyterrazolium 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, were 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 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 75%—80% of deaths caused by these tumors11. The high rate of malignancy...
Condurango-glycoside-A fraction of *Gonolobus condurango* induces DNA damage associated senescence and apoptosis via ROS-dependent p53 signalling pathway in HeLa cells

Kausik Bisshayee · Avijit Paul · Samrat Ghosh · Sourov Sikdar · Avinaba Mukherjee · Rakthim Biswas · N. Boujedaini · Anisur Rahman Khuda-Bakhsh

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**Abstract** *Gonolobus condurango* plant extract is used as an anticancer drug in some traditional systems of medicine, including homeopathy, but it apparently lacks any scientific validation. Further, no detailed study is available to suggest whether condurango-glycoside-A (CGA), a major ingredient of condurango, serves as a potent anticancer compound. Therefore, we investigated apoptosis-inducing ability of CGA against cervix carcinoma cells (HeLa). β-galactosidase-activity and DNA damage were critically studied at different time points; while induced DNA-damage was observed at 9–12th hours, senescence of cells appeared at a later stage (18th hour after CGA treatment), implicating thereby a possible role of DNA damage in inducing premature cell senescence. Concurrently, the number of cells undergoing apoptosis increased along with increase in reactive oxygen species (ROS) generation. Expression of p53 was also up-regulated, indicating that apoptosis could have been mediated through p53 pathway. DCHIUDA (4',6-Diamidino-2-phenylindole dihydrochloride) assay, acridine orange/ethidium bromide staining and annexin V/PI assay results collectively confirmed that apoptosis was induced by increased ROS generation. Reduction in proliferation of cells was further evidenced by the cell cycle arrest at G0/G1 stage. Expression profiles of certain relevant genes and proteins like p53, Aki, Bcl-2, Bax, cytochrome c and caspase 3 also provided evidence of ROS mediated p53 up-regulation and further boost in Bax expression followed by cytochrome c release and activation of caspase 3. Overall results suggest that CGA initiates ROS generation, promoting up-regulation of p53 expression, thus resulting in apoptosis and premature senescence associated with DNA damage.

**Keywords** Condurango-glycoside-A · DNA damage · Senescence · Apoptosis · HeLa cells

**Introduction**

Condurango or condurango (*Gonolobus condurango*) is native of South America and belongs to the milkweed family (Apocynaceae). It was first introduced into the United States in 1871 to treat stomach cancer and syphilis [1, 2]. While it was never really proven effective for cancer during those early years through any scientific study, its use continued since then in various traditional and complementary medicines as a remedy for many types of stomach and digestive problems [3]. It is also used as a remedy to calm nervous and upset stomach, relieve stomach pain, nausea and indigestion gas [3]. Besides, condurango has also been documented for its anti-inflammatory and anti-oxidant actions in animal studies [4]. The extract contains a group of novel glycosides and steroids; along with tannin, small quantities of a tryptamine-like alkaloid, earucouche, condurangia, condurant, essential oil, phytosterol, resin, and stigmasterol [5, 6]. The anti-tumour activity of some of these novel compounds was first reported in the 1980s [7]. However, the mechanism behind this activity still remains unknown, as its efficacy has not been investigated systematically so far.