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Induction of Apoptosis by a Synergistic Lignan Composition from *Cedrus deodara* in Human Cancer Cells

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AP9-cd, a synergistic lignan mixture from *Cedrus deodara* (Pinaceae) consisting of (–)-wikstromal, (–)-matairesinol and dibenzyl butyrolactol, depicted cytotoxic effects against numerous human cancer cell lines reported previously. The aim of this study was to investigate the mechanism of cell death in human cancer cells. The viability, morphological and ultrastructural changes in Molt-4 cells were investigated. Using the trypan blue exclusion assay, we demonstrated that AP9-cd significantly reduced the viability of Molt-4 cells in a time- and dose-dependent manner. Apoptotic assays using light microscopy revealed that this agent induced Molt-4 cell apoptosis at varied concentrations. The treatment causes a loss in cell viability by activating the apoptotic process as identified by light and electron microscopy. The morphological changes of intracellular organelles in Molt-4 cells treated with 30 μg/ml of AP9-cd revealed the disruption of mitochondrial cristae. Other features included the vacuolization, chromatin condensation and formation of micronuclei. Surface ultrastructural studies of four different tumor cell lines (Molt-4, HL-60, PC-3 and A-549) treated with AP9-cd depicted loss of surface projections, condensation and formation of apoptotic bodies. AP9-cd treatment to transgenic fruit fly, *Drosophila,* carrying human adenomatous polpyposis coli (hAPC) gene enhanced eye phenotypes and therefore may inhibit Wnt/Wg pathway which is important in the aetiology of a number of human cancers.

Keywords: lignan composition; *Cedrus deodara*; *apoptosis*; Molt-4; HL-60; PC-3; A549; SEM; TEM; *Drosophila*.

INTRODUCTION

Although it is now widely accepted that apoptosis induced by many chemotherapeutic drugs is the basis for their antitumor effects (Mesner *et al.*, 1997; Stewart, 1997 and Decaudin *et al.*, 1998), the detailed mechanism of chemotherapy-induced apoptosis remains unknown. DNA damage, p53 activation, altered cell cycle progression or generation of reactive oxygen species (ROS), might be the common trigger by which chemotherapeutic agents induce apoptosis (Mesner *et al.*, 1997). Indeed, there is evidence that many cytotoxic drugs enhance generation of ROS, which in turn mediate the apoptotic process (Decaudin *et al.*, 1998). The mitochondria play an important role in the central control or executioner phase of the cell death cascade (Kroemer *et al.*, 1997; Orrenius *et al.*, 1997 and Reed, 1997). AP9-cd is a novel natural source of lignans derived from waste wood powder of *Cedrus deodara* obtained after organic phase extraction and chromatography on silica gel column. The yield of AP9-cd is about 3–4% on dry weight basis. This agent has been reported to be cytotoxic to numerous human cancer cell lines at concentration between 10 and 30 μg/ml *in vitro* (Rao *et al.*, 2003; Singh *et al.*, 2007), using sulforhodamine B dye for cell-based assay of cytotoxicity (Monks *et al.*, 1991). It is composed of lignans consisting of (–)-wikstromal (75–79%), (–)-matairesinol (9–13%) and dibenzylbutyrolactol (7–11%) (Fig. 1). The individual constituents isolated from chloroform extract showed lesser cytotoxicity than AP9-cd when tested against many cancer cell lines (Rao *et al.*, 2003; Singh *et al.*, 2007). In a call for 'back to the future', the advantage of using 'crude' botanical extracts has been emphasized as opposed to the standard approach which has been to isolate, synthesize and administer the single chemical compound thought to be responsible for the effect of the extract (Vickers, 2002). Based on *in vitro* cytotoxicity assay, AP9-cd inhibited growth of cancer cell lines from breast, cervix, ovary, neuroblastoma, colon, lung and mouth etc. Individual constituents of AP9-cd isolated from different sources have been reported earlier (Rao *et al.*, 2003). Wikstromal is the major ingredient that occurs both in (–) and (+) forms; the (–) form is active against leukemia and HIV while the (+) form is biologically inactive (Khamlach and Brown, 1992). Many lignans and their derivatives have been reported earlier for various biological activities of which methylenedioxyphenyl ring is considered important (Mac Rae and Towers, 1984). In AP9-cd lignan mixture, methylenedioxyphenyl ring is open in all the three isolated constituents and is different from several natural lignans particularly of podophyllotoxin series and its semi-synthetic analogues (Fig. 1). This lignan mixture was also found active *in vivo* using

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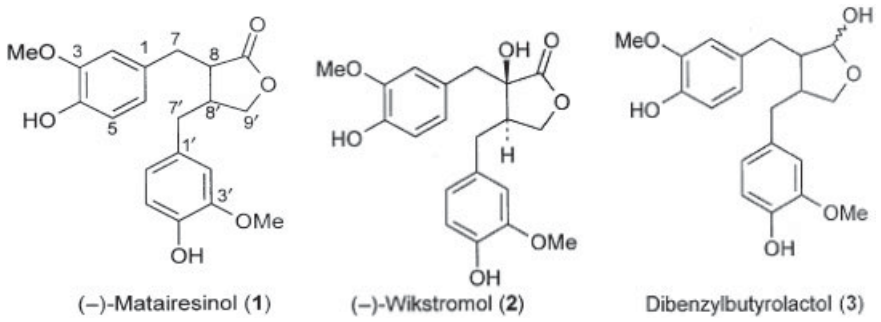

Figure 1. The chemical structures of (–)-Matairesinol (1), (–)-Wikstromol (2) and Dibenzylbutyrolactol (3).

Ehrlich ascites carcinoma and colon carcinoma (CA-51) models in mice (Singh *et al.*, 2007). Since AP9-cd was active both *in vitro* as well as *in vivo,* it was of interest to ascertain its effect on the ultrastructure of cell organelles in tumor cells and also to know the mechanism of cell death in different human cancer cell lines so that the lead might be developed into anti-neoplastic therapeutics. The morphological analysis by light and electron microscopy constitutes a very important and even decisive tool to identify the specific type of cell death unambiguously and thus far, has been the gold standard for the most precise detection of apoptosis based on the original morphological criteria described by Wyllie *et al.* (1980). Recently, the use of a scanning electron microscope (SEM) has been advocated for the precise assessment of mechanism of cell death in human cancer cells (Rello *et al.*, 2005). Indomethacin, an anticolon cancer drug, is earlier known to inhibit Wnt/Wg pathway in the transgenic fruit fly, *Drosophila* thereby specifically enhancing human Adenomatous Polyposis Coli gene (hAPC) induced eye phenotypes (Bhandari and Shashidhara, 2001). The targeted expression of either full-length human hAPC or its ß–catenin binding domain alone negatively regulated the function of the ß–catenin homologue, Armadillo (Arm) and thereby inhibited Wnt/Wg signaling during fly development (Bhandari and Shashidhara, 2001). ß–catenin, a transducer of Wnt/Wg signaling, is a potential oncogene, enhanced activation of which is a cause of different types of human cancers (Bhandari and Shashidhara, 2001; Tickoo and Russell, 2002). First identified in the fruit fly *Drosophila*, Wnt/Wg pathway is important in the aetiology of human cancer. In the present study we show that AP9-cd incubation of four different human cancer cell lines (Molt-4, HL-60, PC-3 and A-549 cells) induced the morphological events characteristic of apoptosis. Trypan blue exclusion assay was used to determine cytotoxicity in Molt-4 cells. Furthermore, using transgenic fruit fly, we document that AP9-cd may inhibit the Wnt/Wg pathway. This study is the first report of AP9-cd on the ultrastucture of tumor cells and on transgenic fruit fly, *Drosophila*.

MATERIALS AND METHODS

Cell culture, growth conditions and treatment. Human T lymphoblast acute lymhoblastic leukemia cell line Molt-4, human promyelocytic leukemia cell line HL-60, prostrate cell line PC-3 and lung cell line A-549 were procured from National Center for Cell Sciences (NCCS), Pune, India. The cells were cultured in RPMI-1640 medium supplemented with 10% FCS glutamine (2 mM), penicillin (50 µg/ml) and streptomycin (50 µg/ml). Standard culture conditions were used. The cell cultures were grown in CO_2 incubator (Heraeus) at 37 °C with 90% humidity and 5% CO_2 gas environment. Both Molt-4 and HL-60 cells were treated during logarithmic growth phase and PC-3 and A-549 were treated during sub-confluent stage, with the test material dissolved in DMSO while the untreated control cultures received only the vehicle (dimethyl sulfoxide <0.5%).

Chemicals and reagents. AP9-cd was provided by Dr Madusudana Rao, Indian Institute of Chemical Technology, Hyderabad, India. The epoxy embedding kit and propylene oxide were procured from Fluka Biochemica (Buchs, Switzerland), Glutaraldehyde and osmium tetroxide were procured from Merck (Darmstadt, Germany). RPMI 1640 was procured from GIBCO-BRL (New York, USA). All these chemicals were stored at 4 °C. Fetal calf serum was obtained from Sera Lab (Crawley Down, GB) and stored at –20 °C. All the other chemicals were of analytical grade.

Cell viability assay. Molt-4 cells were seeded in 6-well tissue culture plates at the density of 2×10^5 cells per ml in complete medium supplemented with 10% FCS in the presence and absence (as controls) of AP9-cd extract at different concentrations as indicated. After 24 h, culture was incubated with different concentrations of AP9-cd (10, 30 and 50 µg/ml) for 6, 12 and 24 h. AP9-cd stock solution was prepared in DMSO and added to the medium to achieve the desired final concentration. Control samples were treated with DMSO vehicle alone. To assess cell viability, after incubation for specified time at 37 °C, aliquots from the cells were mixed with a 0.4% trypan blue solution (1:1) and loaded on to a hemocytometer. The dead cells and the total cells were counted. The percentage of viable cells [(total cells – dead cells) / total cells] × 100 (Jin Wu *et al.*, 2002) was calculated.

Quantification of apoptosis by light microscopy. Molt-4 cells were incubated with 10, 30 and 50 µg/ml AP9-cd for 24 h and apoptosis was assessed using light microscopy. For light microscopy, the cells were spun onto glass slides. The air-dried smears were fixed for 1 min in absolute methanol and stained with modified Giemsa stain (Sigma). The percentage of cells undergoing apoptosis was determined after characterizing 500 cells per slide, using the following morphological criteria: condensation of the nuclear chromatin, vacuolization of the cytoplasm,

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fragmentation of the nucleus and formation of apoptotic bodies (Washo-Stultz *et al.*, 2002) and the percentage of apoptotic cells (number of apoptotic cells/total cell number × 100) was calculated (Salido *et al.*, 2001).

Light and electron microscopy. Molt-4 cells were seeded in complete medium and incubated in 10, 30 and 50 μg/ml AP9-cd for 24 h and studied under the phase contrast microscope. For light (LM) and transmission electron microscopy (TEM), Molt-4 cells were incubated in 30 μg/ml AP9-cd for 24 h and sedimented at 1800 rpm for 10 min. Cell pellets were fixed immediately with 2.5% glutaraldehyde in 0.1M PBS, (pH 7.2) at 4 °C for 1 h, post-fixed with 1% OsO_4 for 1 h in the same buffer, dehydrated with graded ethanol solutions and embedded in Epon 812 (Reno *et al.*, 1999). Semi-thin sections, stained with 1% toluidine aqueous blue at 40 °C, were observed with a Vanox light microscope (Olympus, Tokyo, Japan). Ultrathin sections, obtained with a LKB ultramicrotome (LKB, Bromma, Sweden), collected on copper grids were stained with uranyl acetate and lead citrate and examined with a JEOL 100CXII electron microscope at 60 KV. To evaluate ultrastructural alterations, ~100 cells per sample were examined from two independent experiments. For SEM, HL-60, MOLT-4, PC3 and A549 cells were incubated with 30 μg/ml AP9-cd for 12 and 24 h and processed (Rello *et al.*, 2005). Briefly the cells on a cover slip were fixed in glutaraldehyde, osmicated, dehydrated, dried in a critical point drier using CO_2 (Balzer's Union) and coated with gold using a sputter coater (Polaron). The specimens were examined with a JEOL-100CXII electron microscope with ASID at 40 KV.

The fruit fly, *Drosophila* strains, culture, growth conditions and treatment. The two types of transgenic fly lines *viz*. UAS-hAPC, GAL4/Cy; +/+ and Canton S fly line were provided by Dr L. S. Shashidhara, Centre for Cellular and Molecular Biology, Hyderabad, India. The flies were grown on a standard cornmeal medium having 3% malt extract in a culture room at a temperature of 24 ± 1 °C and 70% R.H. The experiments were, however, conducted at a temperature of 25 ± 0.5 °C and 70% relative humidity in a BOD (Bhandari and Shashidhara, 2001). The dose to be tested on transgenic flies was first standardized on Canton S flies. AP9-cd was mixed at the concentration of 250 μg per ml of *Drosophila* medium at <50 °C and left overnight for drying. The embryos (0 to 2 h) of Canton S flies were collected in embryo collection medium and released 100 per vial containing treated food in triplicates. The controls were run simultaneously. The period of adult emergence and the phenotypes of adults were recorded. The virgin females of UAS-hAPC were collected. The freshly emerged males of ey-GAL4/Cy; +/+ were separately collected. Ten virgin females of UAS-hAPC fly line were released in triplicates and ten freshly emerged males of ey. GAL4/Cy; +/+ were also released in each of the vials having AP9-cd at 250 μg per ml of *Drosophila* medium. Indomethacin at 200 μg per ml of *Drosophila* medium was used as a positive control (Bhandari and Shashidhara, 2001). The untreated controls were run simultaneously. The parental flies were discarded after one week. The progeny of both curly and noncurly flies emerging from the crosses was recorded. The noncurly flies of right type of genotype *i.e.*, ey. GAL4; hAPC/CBD, showing mild as well as enhanced eye phenotypes were categorized in four groups *i.e.*, 1–5, 6–10, 11–15 and 16–20 (Bhandari and Shashidhara, 2001).

Statistical analysis. Statistical evaluation of the results on cytotoxicity and apoptosis was performed by using Student's *t*-test.

RESULTS

AP9-cd induces Molt-4 cell death. Incubation of Molt-4 cells with different concentrations of AP9-cd for 6–24 h resulted in time- and dose-dependent cytotoxicity. After 6, 12 and 24 h incubation, the cell viability at concentrations of 10, 30 and 50 μg/ml was respectively 93.78, 84.36, 76.93; 87.50, 79.86, 68.95 and 73.59, 53.16, 35.93. The decrease in cell viability was significant at all the concentrations from 6–24 h ($p < 0.001$) (Fig. 2).

Quantification of apoptosis. Incubation of Molt-4 cells with different concentrations of AP9-cd for 24 h resulted in dose-dependent increase in apoptosis. The percentage apoptosis at concentrations of 10, 30 and 50 μg/ml was respectively 2.47, 9.92 ($p < 0.01$) and 12.48 ($p < 0.001$) (Fig. 3).

Light microscopic analysis of cell death induced by AP9-cd. Morphological changes of Molt-4 cells in response to AP9-cd were observed. The untreated cells were spherical in shape. The nucleus has nucleolus and is large compared to the cell size, and the cytoplasm is restricted to the periphery of the cells (Figs 4a, e). The incubation for 24 h at 10 μg/ml of AP9-cd revealed condensation both in cytoplasm and nucleus thereby resulting granulation in the majority of cells. The few apoptotic cells were also seen (Fig. 4b). The morphology of cells incubated for 24 h at 30 and 50 μg/ml AP9-cd showed characteristics typical of apoptosis, including cell shrinkage, chromatin condensation and nuclear fragmentation (Figs 4c, d). At 50 μg/ml however a

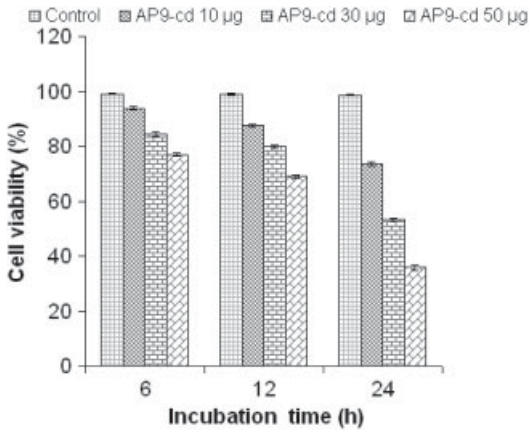

Figure 2. Effect of AP9-cd on Molt-4 cell viability determined by trypan blue staining after 6, 12 and 24 h of incubation. Molt-4 cells were seeded in each well of 6-well plates at a density of 2×10^5 cells/ml. AP9-cd dissolved in DMSO was added to the cells at indicated concentrations while untreated controls cells received the vehicle only. Cells in duplicate wells were used for each treatment. Data are means ± SE ($n = 4$). Experiments were performed in duplicate. Control vs. treatment, $p < 0.001$.

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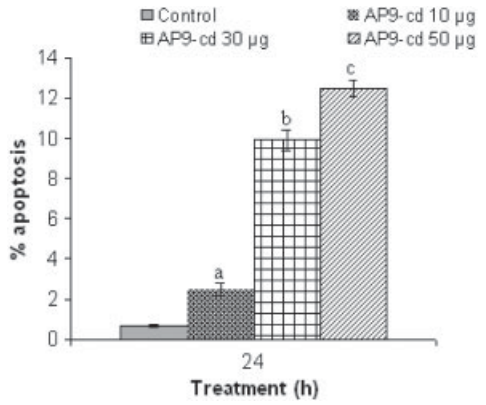

Figure 3. AP9-cd induced apoptosis in Molt-4 cells. Cells were treated with 10, 30 and 50 µg/ml AP9-cd concentrations for 24 h. The percent (%) apoptotic cells were determined using light microscopic criteria. Data are means ± SE ($n = 4$). Experiments were performed in duplicate. Control vs. treatment, [a] not significant, [b] $p < 0.01$ and [c] $p < 0.001$.

few necrotic cells were also seen (Fig. 4d). Semithin sections also provided an overview of the morphology of AP9-cd treated cells. In treated samples after 24 h the apoptotic cells appeared alongside cells with normal morphology. In the apoptotic cells, marginalization of chromatin material, vacuolization in the cytoplasm and formation of micronuclei was clearly visible (Fig. 4b, c, d, f).

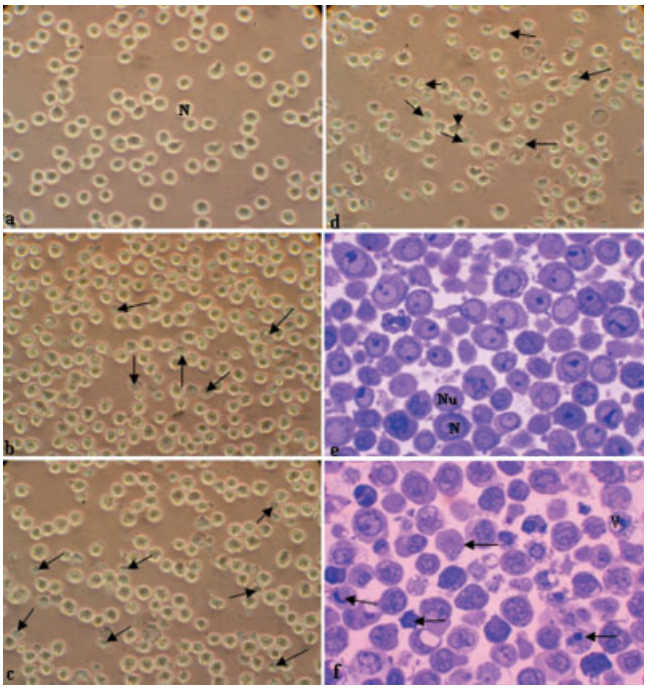

Figure 4. Morphological changes in Molt-4 cells treated with various concentrations of AP9-cd for 24 h: (a-d) cells were observed under phase contrast microscope and (e-f) semithin sections of cells, stained with toluidine blue were observed under light microscope. (a) Untreated control cells showing large sized nuclei (N). (b) Cells treated with 10 µg/ml AP9-cd indicate condensation both in cytoplasm and nuclei and apoptosis (arrow). (c) Cells treated with 30 µg/ml AP9-cd indicate apoptosis (arrow). (d) Cells treated with 50 µg/ml AP9-cd indicate apoptosis (arrow) and necrosis (arrowhead). (e) Untreated control cells showing large sized nuclei (N) with nucleoli (Nu). (f) Cells treated with 30 µg/ml AP9-cd indicate high rate of apoptosis (arrow). Magnification: (a-d) 200X; (e-f) 400X.

Transmission electron microscopic (TEM) analysis of cell death induced by AP9-cd. Ultrastructural investigation revealed the characteristics of both treated and control Molt-4 cells. The cells in the control group had a normal distribution of organelles, with the integrity of organelles well preserved. The mitochondria of different size and shape, lysosomes, rough endoplasmic reticulum (RER) and ribosomes (R) are seen. The nucleus has nucleolus and is surrounded by double membrane envelope. The chromatin material is homogeneously distributed in the nucleus. The nucleocytoplasmic ratio is high (Fig. 5a). The magnified view of mitochondria in control cells shows a double membrane structure with the inner membrane thrown into well developed cristae (Fig. 5e).

The cells incubated with AP9-cd exhibited ultrastructural evidence of apoptosis. These observations include vacuolization, nuclear fragmentation (Fig. 5b), condensation and chromatin margination at the nuclear periphery (Fig. 5c) and formation of micronuclei (Fig. 5d). The nuclear membrane double-layer structure turned into indistinct envelope. The treatment also caused condensation, loss of cristae and vacuolization in mitochondria of apoptotic cells (Fig. 5f).

Scanning electron microscopic (SEM) analysis of cell death induced by AP9-cd. SEM examination revealed that HL-60 cells were spherical in shape with a few surface projections (Fig. 6a). The Molt-4 cells were also spherical in shape and the surface was covered with microvilli (Fig. 6c). The AP9-cd incubation of both the cell lines after 12 h revealed a smoothening of cell surface and a decrease in size in the majority of cells (not shown). After 24 h of incubation, the apoptosis was clearly seen (Fig. 6b, d). Most of A-549 (lung) cells appeared quite homogeneous in morphology, well adherent to the substratum, with short and sparse microvilli projecting from the cell surface (Fig. 6e). The PC-3 (prostate) cells were round with well-preserved cytoplasm and plasma membrane (Fig. 6g). The AP9-cd incubation resulted in a series of morphologically changes in both the cell lines. After 12 h incubation, the cells shrank due to a loss of cytoplasmic volume, became detached from the neighboring cells and from culture substrata and adopted a smooth contour (not shown). After 24 h of incubation, the apoptosis was clearly seen (Fig. 6f, h).

AP9-cd enhanced eye phenotypes in the fruit fly, transgenic *Drosophila*. At 250 µg of AP9-cd per ml of the fly food, the adult emergence was normal in Canton S flies and no morphological phenotypes were induced. This dose was therefore selected for bioassay on transgenic flies expressing human APC gene (hAPC) in developing eyes using eyeless-GAL4. We observed that AP9-cd-enhanced hAPC-induced eye phenotypes *i.e.*, the eye size was further reduced (Fig. 7; Table 1). In untreated controls however, only mild eye phenotypes were observed. The indomethacin used as a positive control, at 200 µg per ml of the fly food also showed enhanced eye phenotypes (Table 1).

DISCUSSION

In recent years, extensive efforts have been made to identify naturally occurring chemo preventive substances

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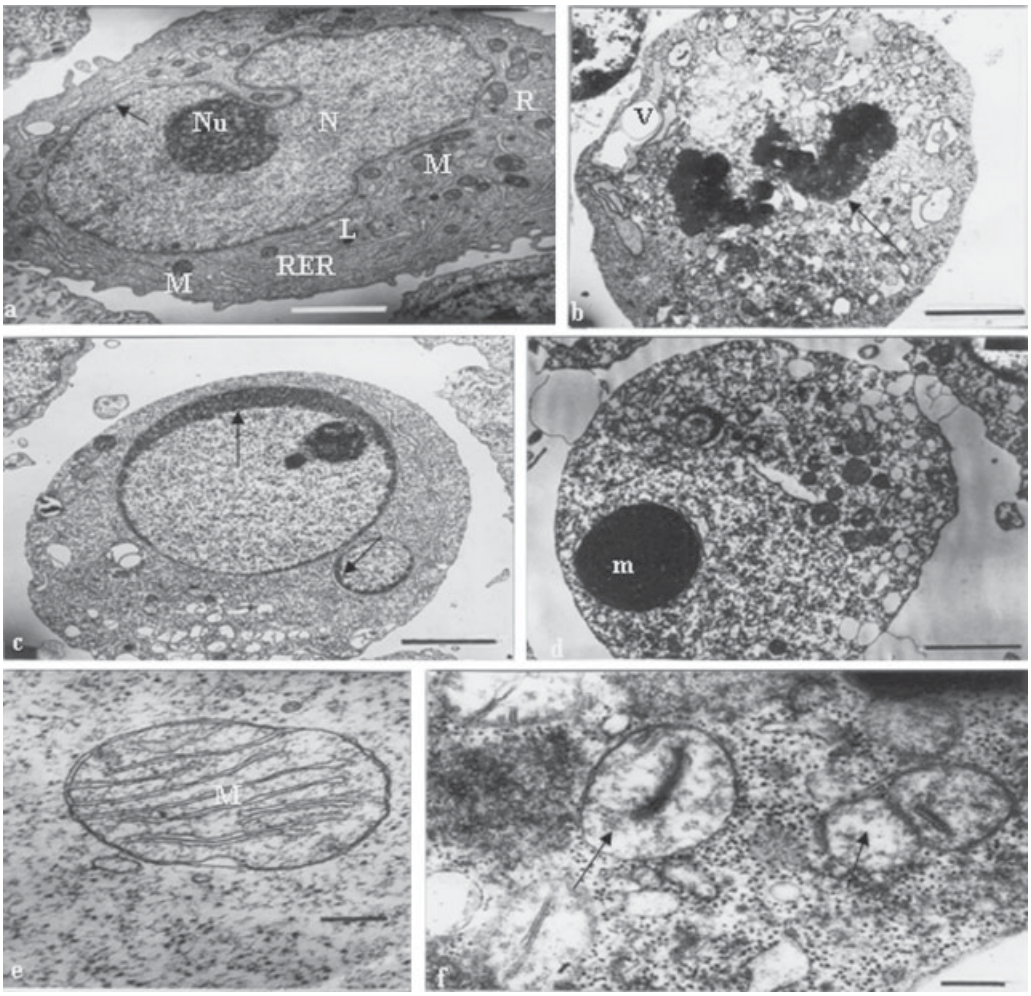

Figure 5. Transmission electron micrographs. Molt-4 cells: control (a, e) and AP9-cd treated (b, c, d, f) 30 μg/ml for 24 h. (a) The nucleus (N) of this control cell contains evenly distributed chromatin and a large nucleolus (Nu). Mitochondria (M) are large and numerous. RER, rough endoplasmic reticulum; L, lysosomes; R, ribosomes; the double membrane nuclear envelope (arrow). (e) Mitochondrion (M) of a control cell shows parallel cristae. (b) The nuclear condensation, fragmentation (arrow) and vacuolization (V) in the treated cell. (c) The chromatin margination (arrow) and vacuolization in the treated cell. (d) The micronuclei (m) in treated cell. (f) Mitochondria of treated cell show vacuolization, condensation and loss of cristae (arrow). The morphological changes in the treated cells (b, c, d, f) are characteristic of apoptosis. Bars: (a-d) 2 μm; (e-f) 0.25 μm.

capable of inhibiting, retarding, or reversing the process of multistage carcinogenesis. In this context, AP9-cd, a lignan mixture, is of special interest since it is cytotoxic to numerous human cancer cell lines *in vitro* and is also active *in vivo* in Ehrlich ascites carcinoma and colon carcinoma (CA-51) models in mice (Rao *et al.*, 2003; Singh *et al.*, 2007). During the present study it was observed that AP9-cd decreased the viability of Molt-4 cells in a time- and dose-dependent manner. The results of the cell count assay revealed that increasing the incubation period to 24 h at 10, 30 and 50 μg/ml, the cell viability was decreased to 73.59, 53.16 and 35.93 respectively ($p < 0.001$) (Fig. 2). Based on changes in cell morphology, a dose-related trend of increased apoptosis was observed in Molt-4 cells. It has been reported that AP9-cd acutely stimulated NO generation in leukemia cells and activated caspase-3 activity as early events leading to the initiation of apoptosis (Bhushan *et al.*, 2006). Caspase-3 is a main executor of apoptosis, playing a central role in its biological processing (Earnshaw *et al.*, 1999). Morphological examination showed that both extra and intra-cellular structures were profoundly affected after treatment with AP9-cd. Mitochondria which supply ATP for the synthesis of proteins and the

Table 1. Distribution of eye phenotypes induced by hAPC/CBD in presence of AP-9cd

	Phenotypic ranks				
Genotypes	1–5	6–10	11–15	16–20	Total
ey-GAL4; hAPC/CBD	11	7	1	0	19(64)*
ey-GAL4; hAPC/CBD in presence of AP-9cd at 250 μg/ml fly food	9	5	6	5	25(60)*
ey-GAL4; hAPC/CBD in presence of Indomethacin at 200 μg/ml fly food	7	7	5	8	27(61)*

* Numbers in parenthesis in the last column indicates total number of flies of right genotype.

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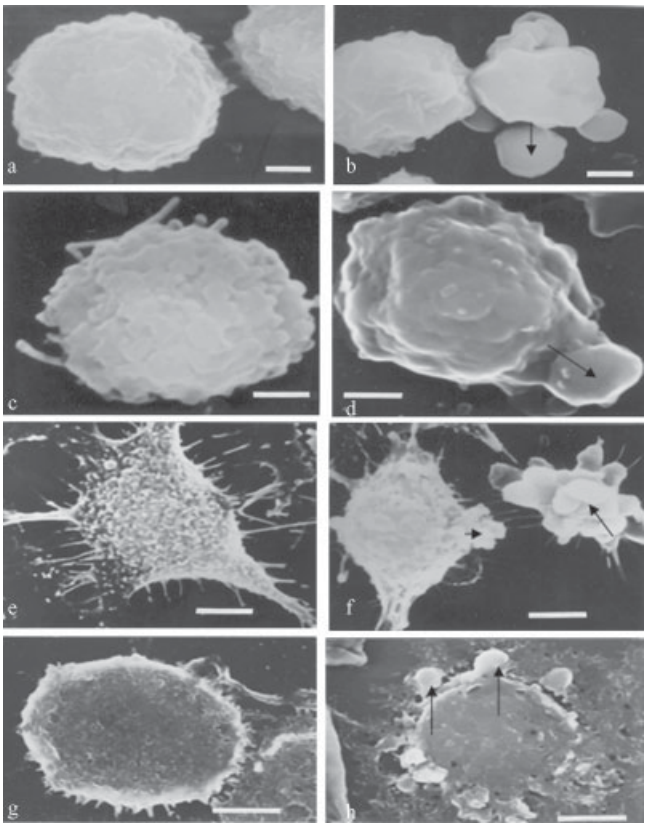

Figure 6. Scanning electron micrographs. Human cancer cells: control (a, c, e, g) and AP9-cd treated (b, d, f, h) 30 μg/ml for 24 h. (a) The control HL-60 cells show rough surface. (b) The treated HL-60 cells reveal reduction in size, smoothening of surface and apoptosis (arrow). (c) The control Molt-4 cells show numerous microvilli and a few projections over the surface. (d) The treated Molt-4 cells reveal shortening of microvilli, projections and apoptosis (arrow). (e) The control A549 cells have polymorphic shape and numerous microvilli over the surface. (f) The treatment in A549 cells causes loss of microvilli, blebbing of the plasma membrane (arrowhead) and apoptosis (arrow). (g) The control PC-3 cells have spherical shape and microvilli over the surface. (h) The treatment in PC-3 cells causes reduction in size, loss of microvilli and apoptosis (arrows). Bars: (a–d) 1 μm; (e–f) 2 μm; (g–h) 5 μm.

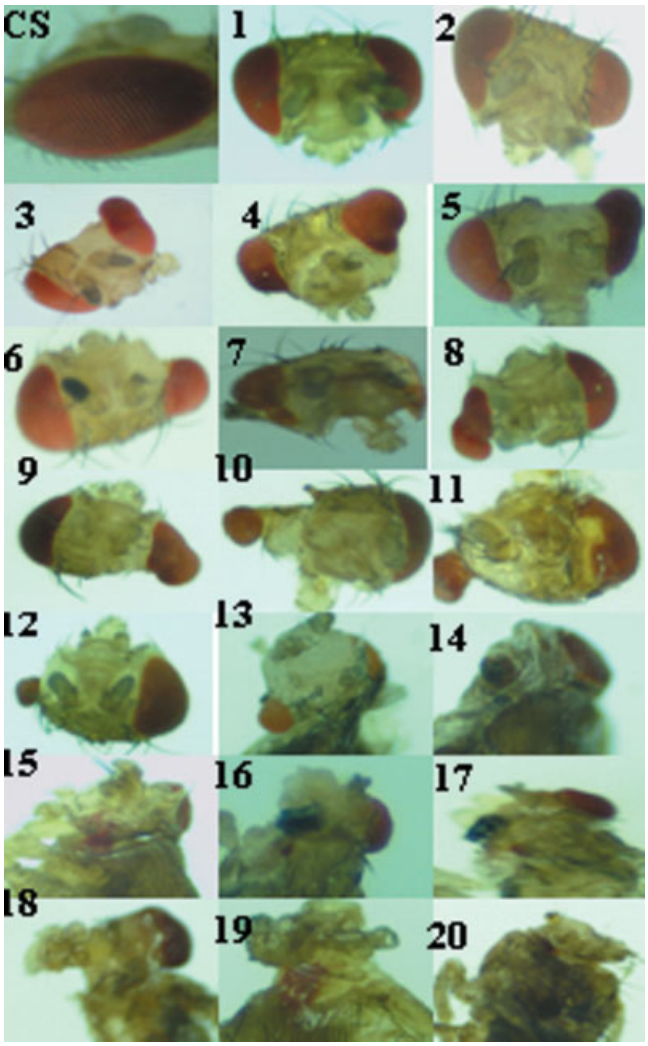

Figure 7. Enhancement of hAPC induced eye phenotypes in transgenic *Drosophila* by treatment of AP9-cd at 250 μg/ml of fly food. Eye phenotypes are classified in the order of severity from 1–20 and thus these images represent phenotypes induced in presence of hAPC/CBD and AP9-cd. Expression of hAPC/CBD in the presence of AP9-cd showed much-enhanced phenotypes (ranging from 1–20) often resulting in eye less flies. CS – Normal eye of Canton-S fly.

duplication of DNA are much more susceptible to a cytotoxic effect. Our study indicated that when Molt-4 cells were incubated with AP9-cd, it could cause mitochondria and other organelle damage morphologically. The nuclei and mitochondria of untreated Molt-4 cells appeared normal. Molt-4 cells incubated with 30 μg AP9-cd for 24 h showed the phenotypic changes associated with apoptosis, including an intact plasma membrane, condensed chromatin and damage of mitochondrial cristae. The untreated Molt-4 cells showed well-preserved mitochondria with well-defined and parallel cristae and a homogeneous matrix (Fig. 5a, e). The AP9-cd incubation of Molt-4 cells caused condensation of mitochondria with vacuolized cristae in a condensed matrix (Fig. 5f). Mitochondrial morphology is important because changes in mitochondrial ultrastructure modulate their function. Thus we may infer that mitochondrial alterations may be crucial for the occurrence of apoptosis in these cells. Mitochondria have been shown to play a major role in programmed cell death. The loss of about 50 and 90% mitochondrial membrane potential in Molt-4 and HL-60 cells respectively after 48 h of 30 μg/ml, AP9-cd treatment has been reported (Bhushan *et al.*, 2006).

SEM analysis of four cancer cell lines revealed that AP9-cd causes the loss of microvilli, shrinkage of cells and apoptosis. The floss microvilli on the surface of the malignant cells contribute to cell movement, such as attachment and invasion (Knyrim and Paveletz, 1977). A reduction in microvilli may potentially inhibit cancer cell invasion. In this sense, SEM analysis justly allows detailed examination of cell surface changes and thus it is a very useful tool to assess the type of induced cell death (Salido *et al.*, 2001; Dini, 2005; Rello *et al.*, 2005). Using Molt-4 cell lines we have found that AP9-cd treatment causes apoptosis in a dose-dependent manner. The morphological criteria allow recognizing the type of death mechanism of tumor cells in a rapid and precise way. We found that AP9-cd had significant effects on inhibiting Molt-4 cell growth and inducing apoptosis. Inhibiting tumor growth has been a continuous effort in cancer treatment. A reduction in cell growth and an induction in cell death are two major means to inhibit tumor growth. In this study, we demonstrated that, at low concentrations, AP9-cd caused significant inhibition of growth in Molt-4 cells. The inhibitory

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effect of AP9-cd on cell growth implies that this compound may have a general function in antitumor cell growth. The cancer cells have developed the capacity of increased proliferation through a variety of growth signal pathways. This includes elevated external growth factors, increased intracellular matrix signal via integrin (Lukashev and Werb, 1998) and Ras protein mutation-derived constitutive mitogenic signals (Medema and Bos, 1993), resulting in growing neoplasm, that causes destruction and atrophy of the surrounding tissue and adjacent organs. In a specific tumor, one pathway may play a more important role than the others. There is strong evidence that tumor growth is a result of uncontrolled proliferation, reduced apoptosis and cancer cells have an acquired capability to evade apoptosis through a variety of ways (Hanahan and Weinberg, 2000; Evan and Vousden, 2001). Inducing tumor cell apoptosis is an ideal way to kill cancer cells. In this study, when four different human tumor cell lines were treated with AP9-cd, they underwent apoptotic death. Apoptosis is modulated by anti-apoptotic and pro-apoptotic effectors, which involve a large number of proteins (Hanahan and Weinberg, 2000; Evan and Vousden, 2001). AP9-cd induced tumor cell apoptosis may involve an array of mechanisms. Our results suggest that the anticancer effect of AP9-cd is a combination of its effects in inhibiting tumor cell growth and inducing tumor cell apoptosis. On the other hand, nuclear modifications induced by AP9-cd were as prominent as cytoplasmic alterations. Nuclear fragmentation and the occurrence of apoptosis in later stages are only observed upon disruption of mitochondrial cristae. Incubation of rat hepatocytes primary culture in AP9-cd did not exert any cytotoxicity. Rather it offered strong protection against carbon tetrachloride (Bhushan *et al.*, 2006) that is known for its hepatotoxicity (Reen *et al.*, 2001). Thus AP9-cd is a promising chemotherapeutic agent and should be investigated further for potential *in vivo* activity against other models. The present study provides the first evidence that AP9-cd induces the apoptosis in four human cancer cell lines. In conclusion, the present data point to the importance of AP9-cd as an emerging potential class of anticancer chemicals, exhibiting an antiproliferative effect on tumor cells and triggering apoptosis. Considering the importance of Wnt/Wg signaling pathway in the contexts of tumor formation, it is important to identify some natural products regulating this pathway. The results of the present study showed that AP9-cd, a natural product may inhibit Wnt/Wg pathway in transgenic fruit fly, *Drosophila*; this is important, as inappropriate switching of this pathway in man is a cause of different types of human cancers. During recent years there have been reports in which fly models have been used to study human genes, diseases and in drug screening (Bhandari and Shashidhara, 2001; Tickoo and Russell, 2002). We observed that AP9-cd enhanced hAPC-induced phenotypes to the extent similar to the enhancement shown by indomethacin (Table 1). The present study thus shows that AP9-cd may inhibit Wnt/Wg pathways in transgenic *Drosophila* but further investigation is necessary to elucidate its mechanism of action in inhibiting this pathway.

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REFERENCES

Bhandari P, Shashidhara LS. 2001. Studies on human colon cancer gene APC by targeted expression in *Drosophila. Oncogene* **20**: 6871–6880.

Bhushan S, Singh J, Rao JM, Saxena AK, Qazi GN. 2006. A novel lignan composition from *Cedrus deodara* induces apoptosis and early nitric oxide generation in human leukemia Molt-4 and HL-60 cells. *Nitric Oxide* **14**: 72–88.

Decaudin D, Marzo I, Brenner C, Kroemer G. 1998. Mitochondria in chemotherapy induced apoptosis: A prospective novel target of cancer therapy. *Int J Oncol* **12**: 141–152.

Dini L 2005. Apoptosis induction in DU-145 human prostate carcinoma cells. *Tissue Cell* **37**: 379–384.

Earnshaw WC, Martins LM, Kaufmann SH. 1999. Mammalian caspases: structure, activation, substrates, and function during apoptosis. *Ann Rev Biochem* **6**: 383–424.

Evan GI, Vousden KH. 2001. Proliferation, cell cycle and apoptosis in cancer. *Nature* **411**: 342–348.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. *Cell* **100**: 57–70.

Jin W, Yaojiong W, Burton BY. 2002. Anticancer activity of *Hemsleya amabilis* extract. *Life Sci* **71**: 2161–2170.

Khamlach MK, Brown R. 1992. D-E. Lignanes. 16. Premieres stynthèses totales du (+)-Wikstromal, de la (–)-Trachelogenine de la (–) nortrachelogenine et des lignoides apparentes. *Tetrahedron* **48**: 10115–10126.

Knyrim K, Paveletz N. 1977. Cell interactions in a 'bilayer' of tumour cells. *Virchows Arch B Cell Pathol* **25**: 309–325.

Kroemer G, Zamzami N, Susin SA. 1997. Mitochondrial control of apoptosis. *Immunol Today* **18**: 44–51.

Lukashev ME, Werb Z. 1998. ECM signaling: orchestrating cell behaviour and misbehaviour. *Tre Cell Bio* **8**: 437–441.

Mac Rae WD, Towers GHN. 1984. Biological Activites of Lignans. *Phytochemistry* **23**: 1207–1220.

Medema RH, Bos JL. 1993. The role of p21ras in receptor tyrosine kinase signaling. *Crit Rev Onc* **6**: 615–61.

Mesner PW, Budihardjo II, Kaufmann SH. 1997. Chemotherapy induced apoptosis. *Adv Pharmacol* **41**: 461–499.

Monks A, Seudiero D, Skeham P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro WA, Gray-Goodrich M, Campbell H, Mayo J, Boyd M. 1991. Feasibility of a high flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Nat Can Inst* **83**: 757–766.

Orrenius S, Burgess DH, Hampton MB, Zhivotovsky B. 1997. Mitochondria as the focus of apoptosis research. *Cell Death Differ* **4**: 427–428.

Rao JM, Srinivas PV, Yadav JS, Raghavan KV, Saxena AK, Shanmugavel M, Kampasi H, Qazi GN. 2003. Herbal chemical composition for the treatment of cancer. *US Patent* **6**: 649–650.

Reed JC. 1997. Double identity for proteins of the Bcl-2 family. *Nature* **387**: 773–776.

Reen RK, Karan R, Singh K, Karan V, Johri RK, Singh J. 2001. Screening of various *Swertia* species extracts in primary monolayer cultures of rat hepatocytes against carbon tetrachloride- and paracetamol-induced toxicity. *J. Ethnopharmacol* **75**: 239–247.

Rello S, Stockert JC, Moreno V, Gámez A, Pacheco M, Juarranz A, Cañete M, Villanueva A. 2005. Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. *Apoptosis* **10**: 201–208.

Renó F, Tontini A, Burattini S, Papa S, Falcieri E, Tarzia G. 1999.

Mimosine induces apoptosis in the HL-60 human tumor cell line. *Apoptosis* **4**: 469–477.
Salido M, Vilches J, Lopez A, Roomans GM. 2001. X-ray mico-analysis of etoposide-induced apoptosis in the PC-3 prostatic cancer cell line. *Cell Biol Int* **25**(6): 499–508.
Singh SK, Shanmugavel M, Kampasi H, Singh R, Mondhe DM, Rao JM, Adwankar MK, Saxena AK, Qazi GN. 2007. Chemically standardized isolates from *Cedrus deodara* stem wood having anticancer activity. *Planta Med* **73**: 519–526.
Stewart BW. 1997. Induced apoptosis as the basis for cancer chemotherapy. *Comments Toxicol* **5**: 541–553.
Tickoo S, Russell S. 2002. *Drosophila melanogaster* as a model system for drug discovery and pathway screening *Curr Opin Pharmacol* **2***:* 555–560.
Vickers A. 2002. Botanical medicines for the treatment of cancer: rationale, overview of current data, and methodological considerations for phase I and II trials. *Cancer Invest* **20**: 1069–1079.
Washo-Stultz D, Crowley-Weber CL, Dvorakova K, Bernstein C, Bernstein H, Kunke K, Waltmire CN, Garewal H, Payne CM. 2002. Role of mitochondrial complexes I and II, reactive oxygen species and arachidonic acid metabolism in deoxycholate-induced apoptosis. *Cancer Lett* **177**: 129–144.
Wyllie AH, Kerr JF, Currie AR. 1980. Cell death: the significance of apoptosis. *Int Rev Cyt* **68**: 251–306.

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