RESEARCH COMMUNICATION

# Induction of Apoptosis in Tumor Cells by Natural Phenolic Compounds

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## Abstract

**Investigation has been conducted to delineate the action of some phenolic compounds of natural origin in four human tumor cell lines: acute myeloblastic leukemia (HL-60), chronic myelogenic leukemia (K-562), breast adenocarcinoma (MCF-7) and cervical epithelial carcinoma (HeLa). In cells grown in appropriate media the phenolics curcumin, yakuchinone B, resveratrol and capsaicin exhibited growth inhibition as assessed by trypan blue dye exclusion. It was evident from the results of the MTT reduction assay and [$^3$H]thymidine incorporation into nuclear DNA that the phenolics were cytotoxic and inhibited cell proliferation. Dose response studies indicated curcumin to be most cytotoxic towards HL-60, K-562 and MCF-7 but did not show much activity in HeLa cells. On the other hand, yakuchinone B, although less active than curcumin, displayed cytotoxicity towards all four cell lines. Resveratrol was cytotoxic only in leukemic cells, while capsaicin was marginally cytotoxic. All these phenolics did not elicit any cytotoxic activity as judged by the above parameters towards lymphocytes purified from normal human blood. When cells treated with phenolics were stained with propidium iodide and examined under a fluorescent microscope, characteristic apoptotic features such as chromatin condensation and nuclear fragmentation were observed. Scoring of cells with apoptotic and non-apoptotic features showed positive correlation of apoptotic index with dose of phenolic, and fragmented DNA extracted free of genomic DNA displayed on gel electrophoresis a typical ladder pattern. These phenolics which have human exposure are known cancer chemopreventive agents and their action as inducers of apoptosis in tumor cells suggest their potential use in a strategy for cancer control.**

**Key Words :** Phenolics – curcumin - yakuchinone B – resveratrol – apoptosis – tumor cells – DNA fragmentation

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## Introduction

There is considerable evidence that consumption of green and yellow vegetables and fruits reduces the risk of human cancer (Steinmetz and Potter, 1991; Block et al., 1992). A wide variety of naturally occurring substances have been shown to have marked cancer chemopreventive properties (Huang and Ferraro, 1992; Dragsted et al., 1993; Johnson et al., 1994; Surh, 1998). Phenolic compounds have widespread occurrence in nature and are consumed by humans through diet containing fruits, vegetables and beverages. These minor non-nutrient dietary factors elicit considerable chemopreventive activity against experimental carcinogenesis induced by a variety of chemicals (Huang and Ferraro, 1992; Ferguson, 1994; Starvic, 1994; Surh, 1998). These substances also modify critical cellular and molecular events relevant to carcinogenesis. Apoptosis is a programmed cell death and a highly organized physiological phenomenon (Wyllie et al., 1980; Ellis et al., 1991; White, 1996). It plays an essential role as a protective mechanism against carcinogenesis by eliminating genetically damaged cells, initiated cells or cells progressed to malignancy (Barry et al., 1990; Hickman, 1992; Sen and D'Incalci, 1992; Schulte-Herman et al., 2000). Induction of apoptosis thus is a highly desirable mode as a chemotherapeutic as well as a chemopreventive strategy for cancer control. Indeed, many chemopreventive agents of natural origin act through the induction of apoptosis as a mechanism to suppress carcinogenesis (Bursch et al., 1992; Kelloff et al., 1996; Taraphdar et al., 2001). The present study was designed to investigate the role four natural phenolic cancer chemopreventive agents in suppressing tumor cell growth

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by inducing apoptosis.

## Materials and Methods

*Chemicals*

RPMI 1640, MEM, fetal bovine serum, gentamycin, penicillin, streptomycin, trypan blue and agarose were purchased from GIBCO. [methyl-$^3$H]Thymidine (17.2 Ci/mmol) was obtained from BRIT, Mumbai. Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione], resveratrol (3,5,4'-trihydroxy-trans-stilbene), capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide), RNase A, proteinase K, propidium iodide, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide], doxorubicin (adriamycin), NP-40, sodiumdodecylsulfate (SDS) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). Yakuchinone B [1-(4'-hydroxy-3'-methoxyphenyl)-7-phenylhept-1-en-3-one] was a gift from Prof. Young-Joon Surh, Seoul National University, South Korea. Other reagents were of analytical grade and procured locally.

*Cell Cultures*

Four human tumor cell lines routinely maintained in our department were used in these studies. Acute myeloblastic leukemia (HL-60) and chronic myelogenic leukemia (K-562) cells were maintained in RPMI 1640 supplemented with 15% heat inactivated fetal bovine serum and gentamycin (40 µg/ml), penicillin (100 units/ml) and streptomycin (10 µg/ml). Breast adenocarcinoma (MCF-7) and cervical epithelial carcinoma (HeLa) cells were maintained in MEM supplemented with similar concentrations of serum and antibiotics as stated above. Cells were grown at 37ºC in a humidified atmosphere of 5% $CO_2$/95% air.

*Cell Viability and Cytotoxicity*

Viability of cells was determined by trypan blue dye exclusion method and cytotoxicity was assessed by the MTT assay. Exponentially growing cells ($1x10^4$) were plated in 96-well plates and after 48h of growth treated with a series of concentrations of each phenolic compound dissolved in DMSO (final concentration 0.1%). Control cells were treated with DMSO alone and positive controls with various amounts of doxorubicin. Incubation was carried out at 37ºC for 48h. Cells exposed to 0.2% trypan blue were counted in a hemocytometer. MTT solution was added to each well (1.2 mg/ml) and incubated for 4h. The reaction results in the reduction of MTT by the mitochondrial dehydrogenases of viable cells to a purple formazan product. The MTT-formazan product was dissolved in DMSO and the amount was estimated by measuring absorbance at 570 nm in an ELISA plate reader.

*Cell Proliferation*

Exponentially growing cells plated in 35 mm dishes were treated with each phenolic compound at different doses for 48h. Five hour prior to harvest 1 µCi [methyl-$^3$H]thymidine was added to each dish. Harvested cells were washed with phosphate buffered saline (PBS), suspended in PBS and aliquot was spotted on Whatman 3MM filter paper disc. The discs were washed successively in 10% trichloroacetic acid (TCA), 5% TCA, ethanol (2x) and air dried. (Bollum, 1968). Amount of [$^3$H]thymidine incorporation was determined by liquid scintillation counting in an LKB 1217 Rackbeta liquid scintillation counter.

*Microscopic Examination*

Cells treated with each phenolic compound were harvested, washed with PBS and to the cell pellet propidium iodide (50 µg/ml) was added and incubated in the dark at room temperature for 10 min. Cells were spread on a slide and examined under a fluorescent microscope.

*DNA Fragmentation*

The isolation of fragmented DNA was carried out according to the procedure of Hermann et al. (1994). Cells ($2x10^6$) were seeded in 35 mm dishes and treated with each phenolic compound for 24h. After harvesting the cells were washed with PBS and pelleted by centrifugation. The cell pellets were then treated for 10s with 100 µl lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5) and centrifuged for 5 min at 1600xg and the supernatant was collected. The extraction was repeated with the same amount of lysis buffer. To the combined supernatant was added SDS (final concentration 1%) and treated overnight with RNase A (final concentration 5 µg/ml) at 56°C followed by digestion with proteinase K (final concentration 2.5 µg/ml) for 4h at 37°C. After addition of 0.5 vol of 10M ammonium acetate the DNA was precipitated with 2.5 vol of cold ethanol. This method separates only fragmentd DNA and not the genomic DNA. DNA was dissolved in loading buffer (50 mM Tris-HCl, 7.5, 10 mM EDTA containing 0.25% bromophenol blue and 30% glycerol). Aliquot was loaded on 1.5% agarose prepared in 40 mM Tris-HCl, pH 8, 10 mM EDTA containing 3 µg/ml ethidium bromide, and DNA fragments were separated by electrophoresis.

## Results

Treatment of leukemic cell lines HL-60 and K-562 with curcumin, yakuchinone B, resveratrol and capsaicin showed a dose dependent growth inhibition as evident from viable cell counts by trypan blue dye exclusion method (Fig 1). The extent of growth inhibition by these phenolics was somewhat less in MCF-7 and considerably less in HeLa cells. Curcumin and yakuchinone B were most effective in leukemic cell lines and in breast cancer cells (MCF-7). The effect of resveratrol in leukemic cells followed closely and capsaicin showed only marginal growth inhibition in all cell lines. Cytotoxicity data as obtained from MTT reduction assay showed similar trend, curcumin and yakuchinone B being more cytotoxic in HL-60, K-562 and MCF-7 cells. Resveratrol also showed significant cytotoxicity in leukemic cell lines. These results are shown in Fig 2. Based on the

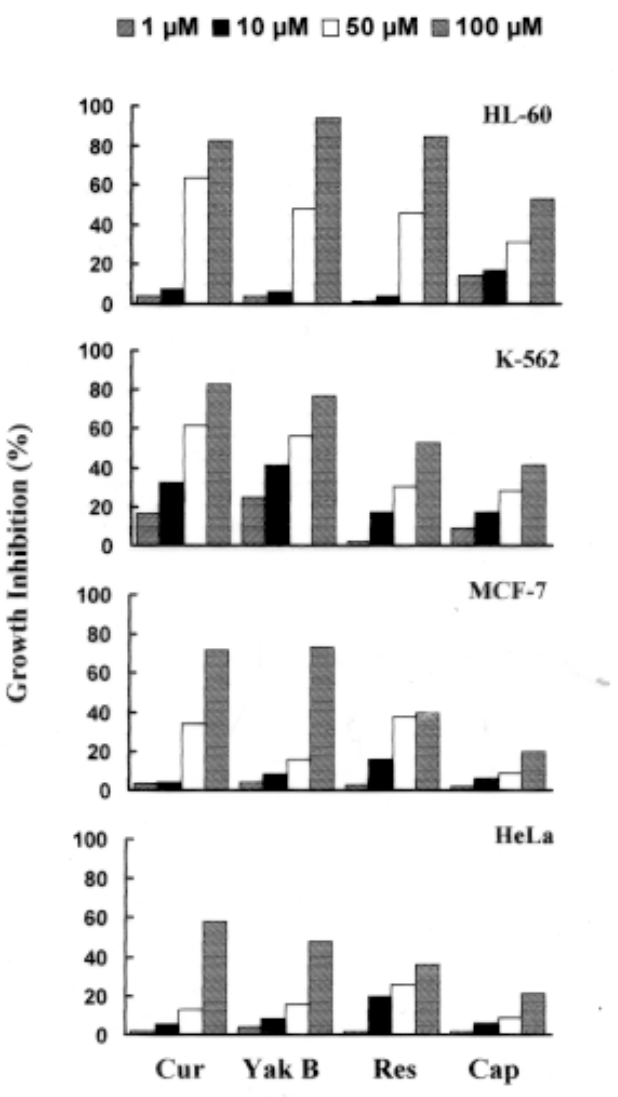


**Figure 1. Dose Dependent Inhibition of Tumor Cell Growth by Different Phenolics.**

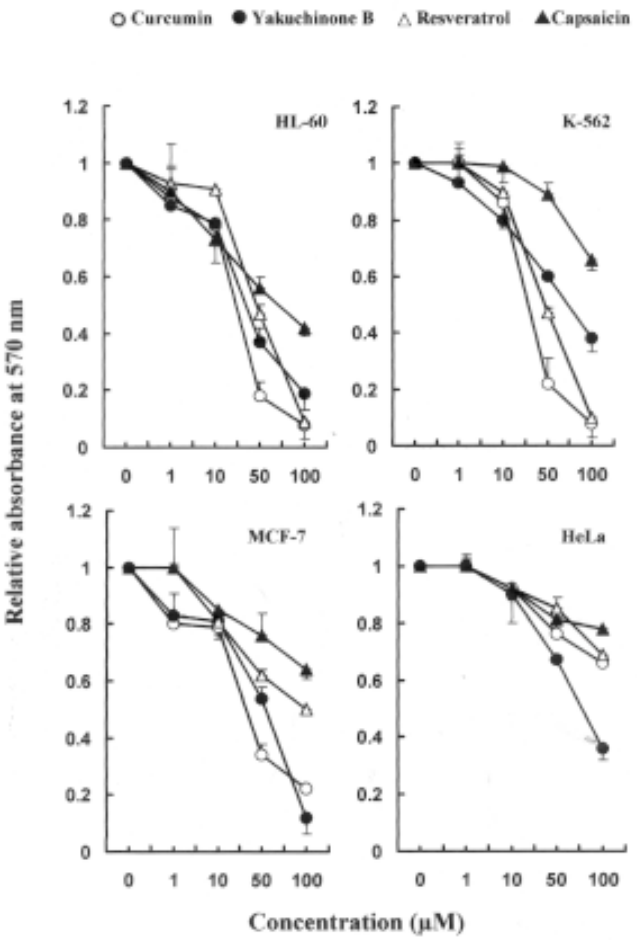


**Figure 2. Cytotoxicity in Tumor Cells as Assessed by MTT Assay by Different Concentrations of Phenolics.**

above criteria $IC_{50}$ values were calculated for all four phenolics and are shown in Table 1. These values indicate relative cytotoxic potency of phenolics. Curcumin was most cytotoxic towards HL-60, K-562 and MCF-7 but did not show much activity towards HeLa. On the other hand, yakuchinone B, although less active than curcumin, displayed cytotoxicity towards all four cell lines. Resveratrol was cytotoxic only in leukemic cells, while capsaicin was marginally cytotoxic. All these phenolics did not elicit any cytotoxic activity as judged by the above parameters towards lymphocytes purified from normal human blood (data not shown).

**Table 1. $IC_{50}$ Values of Different Phenolic Compounds in MTT Cytotoxicity Assay.**

| Phenolics | $IC_{50}$ values (μM) | | | |
|---|---|---|---|---|
| | HL-60 | K-562 | MCF-7 | HeLa |
| Curcumin | 29 | 32 | 35 | >100 |
| Yakuchinone B | 38 | 48 | 52 | 77 |
| Resveratrol | 49 | 68 | 100 | >100 |
| Capsaicin | 75 | >100 | >100 | >100 |

Since capsaicin was marginally cytotoxic it was not included for further studies. In parallel with the growth inhibition and cytotoxicity these phenolics also suppressed cell proliferation as assessed by incorporation of [$^3$H]thymidine in nuclear DNA. In all four cell lines a decrease in the incorporation with increasing amounts of each phenolic was observed. These data are depicted in Fig 3. The extent of inhibition in HeLa cells by the phenolics was, however, much less.

When cells treated with phenolics were stained with propidium iodide and examined under a fluorescent microscope characteristic apoptotic features such as chromatin condensation and nuclear fragmentation were observed. Fig 4 shows fluorescence images of tumor cells in which curcumin, yakuchinone B and resveratrol displayed maximum cytotoxicity. Morphological features of apoptosis such as cell shrinkage and presence of apoptotic bodies were also evident upon examination of cells under a phase contrast

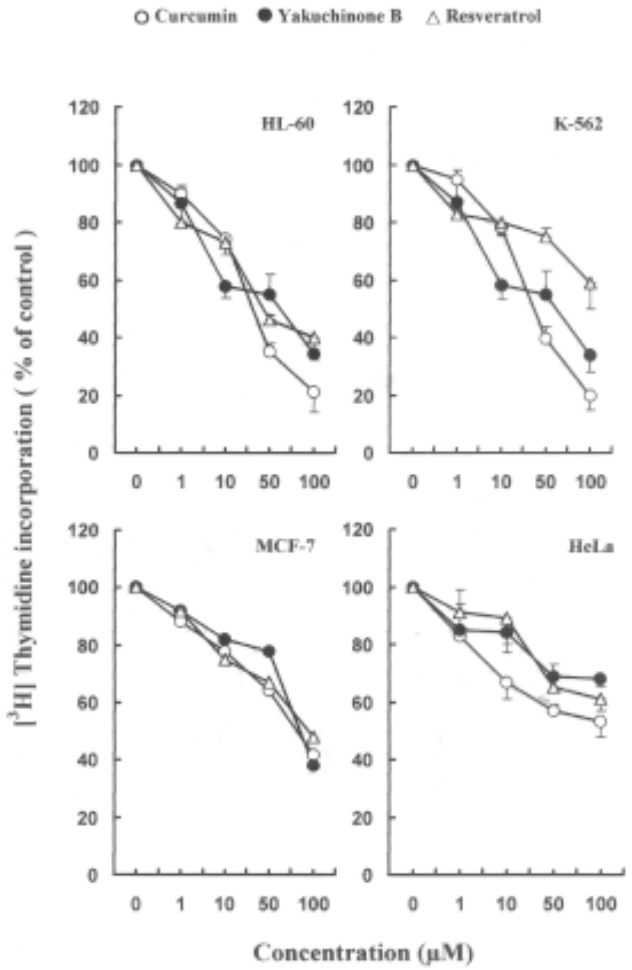


**Figure 3. Effect of Different Phenolics on the Incorporation of [$^3$H]Thymidine in Nuclear DNA of Tumor Cells.**

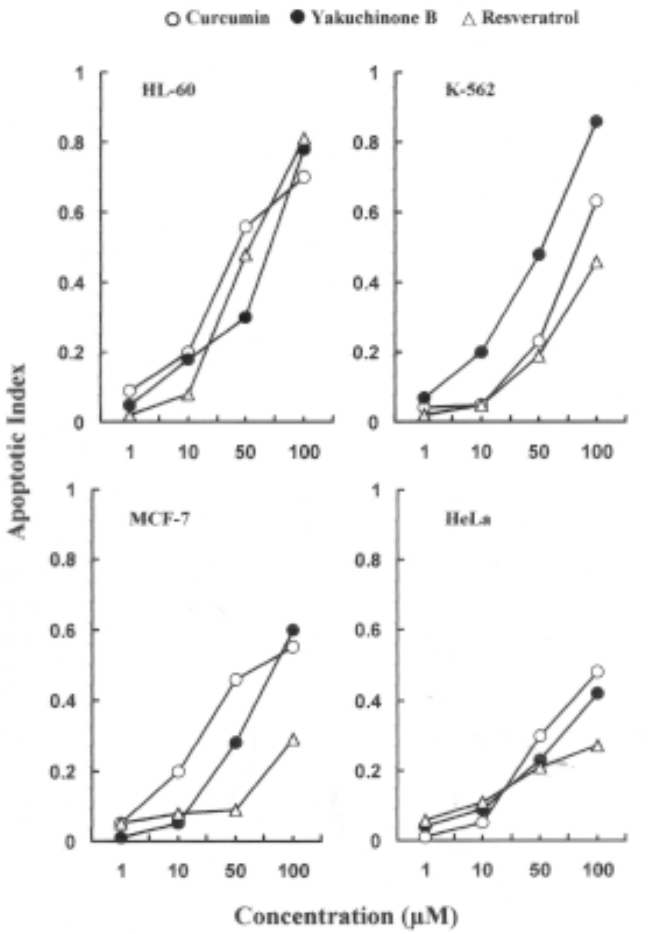


**Figure 5. Apoptotic Indices for Tumor Cells Treated with Phenolics.**

microscope (data not shown). For each treatment 100 cells were scored at random under fluorescent microscope and classified into apoptotic and non-apoptotic cells based on the above criteria. Their ratio designated as apoptotic index was seen to be increased with increasing concentrations of phenolic. The data for all cell lines are shown in Fig 5. Fragmented DNA from treated cells was isolated free of genomic DNA and subjected to agarose gel electrophoresis. Examination revealed a ladder formation characteristic of apoptosis. Representative results are shown in Fig 6.

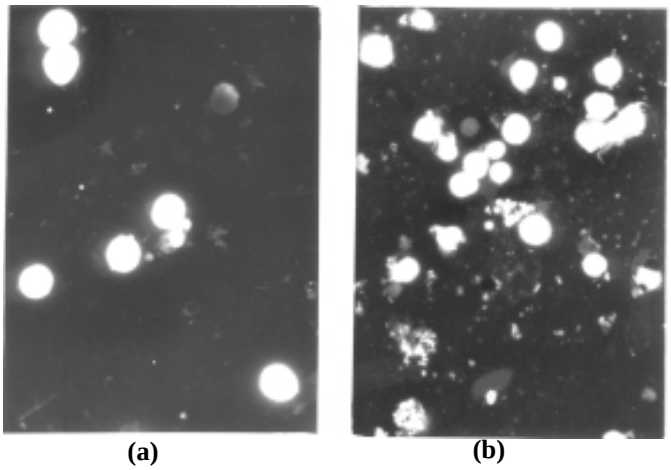


**Figure 4. Fluorescent Microscopic Picture of Tumor Cells Treated with Curcumin in K-562 (a) and MCF-7 (b) Showing Fragmented Nuclei.**

## Discussion

The cytotoxic effects of four natural phenolic compounds were demonstrated in human tumor cell lines HL-60, K-562, MCF-7 and HeLa. These compounds inhibited cell growth and reduced viability as assessed by trypan blue dye exclusion method and MTT assay respectively. Leukemic cells were more susceptible and HeLa cells were relatively resistant. Curcumin was found to be most cytotoxic followed by yakuchinone B, resveratrol and capsaicin. Curcumin and yakuchinone B also reduced the viability of MCF-7 cells significantly. The cytotoxicity was maximum after 48h of treatment of cells grown in the presence of optimum concentration of fetal bovine serum. Cell proliferation as assessed by measuring DNA synthesis showed inhibition by the effective phenolics and this was found to be in parallel

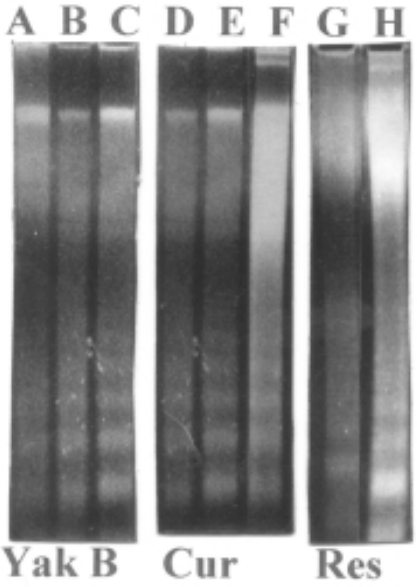


**Figure 6. Electrophoretic Separation of Fragmented DNA of Tumor Cells Treated with Different Phenolics (each 50 μM), Lanes A, D : MCF-7; Lanes B, E & G : K-562; Lanes C, F & H : HL-60.**

with the suppression of cell viability. Morphological characterization of treated cells revealed that the mode of action of cell death induced by the phenolics was mediated through apoptosis. Thus, chromatin condensation and nuclear fragmentation of treated cells were clearly evident and apoptotic index was positively correlated with the treatment dose. Cleavage of DNA at the internucleosomal linker sites yielding DNA fragments in multiples of 180 bp is regarded as a biochemical hallmark of apoptosis (Compton, 1992). Appearance of such fragments resulting in a ladder formation was evident when fragmented DNA free of genomic DNA was isolated from cells treated with three cytotoxic phenolics and subjected to agarose gel electrophoresis.

Curcumin (diferuloyl methane) was previously shown to induce apoptosis in malignant cancer cell lines (Jiang et al., 1996) including leukemic cell lines (Kuo et al., 1996; Kang et al., 1998). Antiproliferative effect of curcumin was also demonstrated against human breast tumor cells (Mehta et al., 1997). Our results on leukemic cells and MCF-7 (breast tumor) cells support these findings. It is interesting to note that curcumin did not exhibit any cytotoxicity towards normal human lymphocytes (present study) and other normal cells in primary cultures (Jiang et al., 1996). Yakuchinone A and yakuchinone B, diarylheptanoid compounds structurally related to curcumin, have been shown to induce apoptotic death in HL-60 cells (Kang et al., 1998). In addition to the apoptotic induction by yakuchinone B in HL-60 cells we have observed similar effects in another leukemic cell line (K-562) as well as in breast tumor cells (MCF-7) and in cervical epithelial cells (HeLa). We found that this was the only compound to which HeLa cells did not elicit any resistance. Resveratrol, a triphenolic stilbene, showed growth inhibitory activity in HL-60 cells through induction of apoptosis (Surh et al., 1999). Apoptosis was also found to be the mode by which resveratrol induced cell death in mouse epidermal cell line (Huang et al., 1999). Our present observation additionally provides evidence for its apoptosis induction potential in various other cell lines derived from human tumors. It has been reported by Morre et al. (1995) that capsaicin, a vanilloid compound, has the capacity to inhibit growth of various transformed cells of human origin including HeLa and HL-60. This inhibition was accompanied by induction of apoptosis. Although we have observed cytotoxic effect of capsaicin in HL-60 cells there was only marginal effect in HeLa cells. The reason for this is not known at present. Capsaicin was also found to induce apoptosis in human and mouse melanoma cell lines (Morre et al., 1996).

Apoptotic pathway is related to up- or down-regulation of distinct genes such as p53 (Oren, 1992) and bcl-2 (Reed, 1994). The induction of apoptosis in HL-60 cells by curcumin (Kuo et al., 1996) and resveratrol (Surh et al., 1999) was found to be associated with down-regulation of bcl-2 gene. Capsaicin-induced apoptosis has also been suggested to be regulated by bcl-2 (Wolvetang et al., 1996). In cells expressing wild type p53 (mouse epidermal cell line) resveratrol induces apoptosis through activation of p53 activity (Huang et al., 1999), and curcumin also has been shown to induce a p53-dependent apoptosis in human basal cell carcinoma cells (Jee et al., 1998). We are presently investigating the expression of bcl-2 and p53 genes in leukemic and breast cancer cell lines treated with curcumin, yakuchinone B and resveratrol. Caspases, the cytoplasmic aspartate-specific cysteine proteases, play an important role in apoptosis (Thornberry, 1997). Activation of caspases appears to be directly responsible for many of the molecular and structural changes in apoptosis including degradation of DNA repair enzyme poly(ADP)ribosepolymerase (PARP) (Lazebnik et al.,1994). Studies are under way to investigate the effects of the above phenolic compounds on the activity of caspase-3 and caspase-8 and on the proteolytic degradation of PARP in tumor cells in culture.

A large body of evidence indicates that many natural phenolic compounds possess considerable cancer chemopreventive property (Huang and Ferraro, 1992; Ferguson, 1994; Starvic, 1994; Surh, 1998). Curcumin is the primary phenolic yellow pigment present in turmeric (*Curcuma longa* Linn) belonging to ginger family. It is commonly used as a coloring and flavoring additive to foods. In various studies it has been shown to exert profound chemopreventive effects on experimental carcinogenesis (Soudamini and Kuttan, 1989; Nagabhusan and Bhide, 1992; Azuine and Bhide, 1992; Huang et al., 1994; Tanaka et al, 1994; Rao et al, 1995; Periera et al, 1996; Conney et al, 1997; Singh et al, 1998; Singletary et al, 1998; Chuang et al, 2000; Ushida et al, 2000). Yakuchinone B, another phenolic , is present in another plant of the ginger family (*Alpinia oxyphylla* Miquel) which has been used in

traditional oriental medicine. This compound as well as other analogues from this plant bear structural similarity with curcumin and because of this reason it is expected that yakuchinones will exhibit cancer chemopreventive activity (Chun et al, 1999). Their anti-tumor promotional properties have been demonstrated (Lee et al., 1998; Chun et al., 1999) and these can contribute to their chemopreventive potential. Resveratrol is a phytoalexin present in grapes and some other fruits. It was shown to have anti-carcinogenic effect in a two-stage mouse skin cancer model (Jang et al., 1997). It also suppressed tumor promoter-induced cell transformation (Huang et al., 1999).

The cancer chemopreventive property of these natural phenolic compounds is attributed to their anti-oxidative, anti-inflammatory and anti-genotoxic activities. Apoptosis results through activation of a pre-programmed pathway of biochemical events that eventually lead to cell death (Ellis et al., 1991; Fisher, 1994; Wright et al., 1994; White, 1996; Vaux and Strasser, 1996). Available evidence suggests that apoptosis may represent a mechanism to counteract neoplastic development, while inhibition of apoptosis may facilitate tumor formation (Barry et al., 1990; Hickman, 1992; Sen and D'Incalci, 1992; Wright et al., 1994; Schulte-Herman et al., 2000). It is evident that a number of chemopreventive agents act through the induction of apoptosis to block neoplastic development (Bursch et al., 1992; Fisher, 1994; Wright et al., 1994; Kelloff et al., 1996; Taraphdar et al., 2001). The results presented here have demonstrated that chemopreventive phenolics curcumin, yakuchinone B and resveratrol are capable of inducing apoptosis in diverse neoplastic cells of human origin, but not in normal cells. These specific effects, together with observations of other investigators, suggest that these three phenolic compounds possess chemopreventive as well as chemotherapeutic properties.

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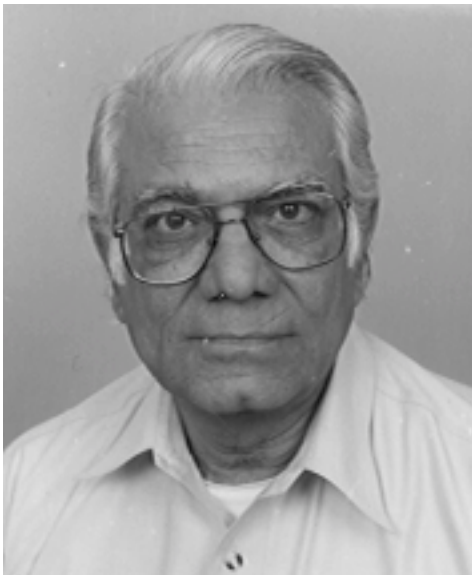

**Personal Profile: Dr RK Bhattacharya**

Dr. R. K. Bhattacharya had his early education in Kolkata. He obtained his B.Sc(Hons) degree from Bombay University (1957), M.Sc. from Nagpur University (1959) and Ph.D. degree in Biochemistry from Calcutta University (1962). He worked as a C.S.I.R. Senior Research Fellow for two years and then proceeded to the University of Minnesota, U.S.A. in early 1964 with the award of an N.I.H. postdoctoral fellowship. After a short tenure he joined the faculty of the Biochemistry department in the St. Paul campus of the same University. Here he pursued research on the molecular mechanism of experimental aplastic anemia. After returning to India Dr. Bhattacharya joined Bhabha Atomic Research Centre in 1975 and developed an independent group on molecular carcinogenesis. His research interests are in molecular aspects of chemical carcinogenesis with special emphasis on oncogene activation and signal transduction, cancer chemoprevention, apoptosis and environmental biochemistry and toxicology. He has published 125 papers in reputed international and national journals and supervised 20 Ph.D. students. He visited Germany, France, Switzerland and Singapore on lecture tours and as visiting scientist. He was honored with AIIMS Silver Jubilee Oration Award in 1994 and Dr. Subodh Mitra Birth Centenary Cancer Research Appreciation Award in 1996. Dr. Bhattacharya retired from Bhabha Atomic Research Centre in 1998 and is presently engaged as an Emeritus Scientist (ICMR) at Chittaranjan National Cancer Institute, Kolkata.