Inhibitory effect of cinnamoyl compounds against human malignant cell line

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In the present study, anti-proliferative effects of dietary polyphenolic compounds have been observed and demonstrated the strong anticancer efficacy of curcumin (CMN), an active constituent of dietary spice (turmeric) using human leukemia cancer cell line. CMN inhibited the proliferation of K562 leukemic cells by induction of apoptosis. The current study demonstrated synergy with combination of drug therapy, and suggested that combination of ferulic acid and cisplatin synergistically inhibited cellular proliferation. Cytotoxic synergy was observed independent of the sequence of addition of two drugs to cultured cells. The synergized growth inhibitory effect with cisplatin was probably associated with G2-M arrest in cell cycle progression. These findings suggested that among the cinnamoyl compounds, CMN was most potent and FER appeared to be a better modulating agent on human malignant cell line.

Keywords: Antitumor, Caffeic acid, Cinnamoyl compounds, Curcumin, Ferulic acid, Gallic acid, Platinum drugs.

Microconstituents derived from fruits, vegetables and dietary plants have been shown to exert antitumor activity against variety of rodent tumors. The mechanism by which they exert antitumor effects has not been fully elucidated, although a number of potential targets have been implicated. Some interesting biological targets are nuclear factor kappa B (NF-kB), angiogenesis and EGFR which are involved in diverse cellular processes. We have been working on polyphenolic compounds occurring abundantly in food products like grapes, peanuts and various herbs. Polyphenolics are generally believed to be antioxidant in nature. The mechanism by which these antioxidants exert tumor suppressing activity is likely to involve modulation of cell signaling pathways resulting in inhibition of cell proliferation which leads to induction of apoptosis, the biological end point. Cinnamoyl compounds of many plants have been studied — (1) Ferulic acid (FER): It is a major constituent of rice bran or germ. Its anti-proliferative effects on K562 and lymphocytes derived from ALL and CML are reported. It is less studied hydroxycinnamate; (2) Caffeic acid (CAF): It is found to be widely present in many members of plant kingdom. Caffeic acid and its esters inhibited cell growth of colon adenocarcinoma HT-29 and HCT-116 and exhibited differential toxicity to cancer cells; (3) Curcumin (CMN): One of its richest sources is the traditional herbaceous plant Curcuma longa (turmeric) and related Curcuma species of the family Zingiberaceae. The yellow color turmeric is being used as a spice, food preservative, coloring agent and an additive in cosmetic and drug preparations. Curcumin possesses antioxidant, anti-inflammatory and anti-carcinogenic properties. CMN treatment causes induction of apoptosis in cancer cells and reduces the expression of vital factors such as C-Jun, C-myc, NF-kappaB, AP-1 etc; (4) Gallic acid (GAL): It is a trihydroxybenzoic acid, naturally occurring plant phenol, and showed selective cytotoxicity against tumor cells with higher sensitivity than normal cells. Although not a cinnamoyl compound, its hydroxy and carboxic acid groups are important for anticancer activity.

In order to investigate the effects of these compounds and their combined use with chemotherapeutic drugs on cellular functions including cell viability, survival and cell cycle, we used human erythroleukemic cell line K562. In the present report, we present our findings of the studies carried out on some interesting dietary constituents emphasizing more on cinnamates.
Materials and Methods

Chemicals
Ferulic acid and gallic acid were purchased from s.d. Fine Chemicals, Mumbai. Caffeic acid and curcumin were purchased from Merck, Mumbai. Cisplatin (CDDP) and carboplatin (CBDC) were purchased locally. Growth medium, fetal bovine serum (FBS), antibiotics were obtained from Sigma Chemical Co., St. Louis, M. O. All other chemicals and reagents were purchased from Gibco Laboratories, Grand Island (USA).

Cell lines and cell cultures
Human erythroleukemic cell line K562 was procured from National centre for cell sciences, Pune. Cells were maintained at 37°C in IMDM medium (Gibco BRL) containing 10% FBS besides penicillin G (100μg/ml) and streptomycin (100μg/ml) under a humidified atmosphere of 5% CO2 in air.

Assessment of cell viability
Cells (1×10^5cells/ml) were seeded into pertidishes (35mm) and incubated with different concentrations of compounds for 24-72 h. Cell viability was measured by trypan blue assay and also by 3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) colorimetric dye assay.10.

Measurement of GSH and GST activity
Total GSH was measured from cells growing in culture as described previously.10 Total GST activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate. Reactions were performed with cytosolic extracts and the conversion of 1-chloro-2,4-dinitrobenzene by GST was measured with spectrofluorometer. The data are expressed as amount of dinitrophenyl glutathione formed/min/mg of protein at 37°C using extinction coefficient 9.6 mM/cm.

Cell cycle analysis
Analysis of cell cycle effects was performed as follows. Cells were collected in PBS, fixed in 70% ethanol (in PBS) at 4°C and were incubated for 5 min in citrate buffer (750mM, Na2HPO4 750μM, sodium citrate; pH 7.8) to permeabilize the cells. The cells were resuspended in PBS (1ml) containing 20μg/ml of propidium iodide (Sigma) plus 5 units of Rnase A (Sigma) and incubated for 30 min at room temperature. Flow cytometric analyses were carried out in a fluorescence activated sorter with a 360 nm Argon-Ion laser. Analysis of cell cycle and measurement of subdiploid (<2N) DNA content of propidium iodide stained nuclei was performed as published previously.10.

Cyclodextrin complexes
Preparation and characterization of beta cyclodextrin inclusion complexes of the compounds under investigation was carried out as reported earlier.11.

Results and Discussion
The main polyphenolic dietary sources for human are fruits and beverages (fruit juice, tea, coffee, coco, beer and wine) and to a lesser extent vegetables, legumes and cereals. Recently, various purified polyphenolic compounds were shown to display beneficial effects in vitro or in vivo, including antioxidant, chemopreventive and antitumoral properties. However, mechanisms by which these polyphenolics inhibit tumor cell growth and their therapeutic potential are poorly understood. The present study was, therefore, undertaken to define the anti-proliferative doses and the mechanism of action of a few selected polyphenolics namely ferulic acid, caffeic acid, curcumin and gallic acid.

The phenolics were tested for their effect on the growth of human erythroleukemic cell line K562. As shown in Figure 1, FER CAF, CMN, GAL induced a dose dependent decrease of leukemia cell member, compared with cells incubated in medium alone (control group). The data also indicated that leukemia cells differ in their sensitivity to these compounds. Inhibitory doses of FER were relatively higher compared with CAF, CMN and GAL (Fig. 1). It was clear from the data that CMN was the most potent compound. Data clearly showed that phenolics mediated inhibition of leukemia cell proliferation was dose and time dependent (Fig. 2). Besides K562, CMN also inhibited the growth of estrogen responsive MCF7 mammary carcinoma cell line (data not shown). CMN can, therefore, be considered as a potent inhibitory agent for human malignant cell lines taking into consideration the early reported work.

Besides growth inhibition, we checked whether a phenolics mediated decrease of leukemic cell numbers was related to cell cycle arrest. Data showed FER-mediated cell cycle modification in K562 cell line (Table 1). K562 cells showed clear accumulation in G2/M phase, correlations with increasing concentration and consequently decreased numbers of cells in Go/G1 phase were noticeable.
Defects in G2-M arrest checkpoint may allow a damaged cell to enter mitosis and undergo apoptosis and efforts to enhance this effect may increase the cytotoxicity of chemotherapy. Many reported herbal derivatives capable of G2-M arrest may be mediating their effects through microtubules, which induce mitotic arrest beyond the late G2 checkpoint. Discovery of agents with synergy in combination with chemotherapy and associated effects on cdk1 function, suggests that modulation of checkpoint regulators may contribute to cytotoxicity in clinical trials.

Cellular glutathione (GSH) has been shown to be crucial for regulation of cell proliferation, cell cycle progression and apoptosis. We, therefore, analyzed changes of GSH levels of K562 cells by using standard procedure. GSH level was depleted by 72 h exposure of K562 cells to FER (data not shown). Depletion of GSH by FER may explain why exposure to FER leads to cell cycle arrest and cytotoxicity.

Data showed CMN mediated cell cycle modification in K562 cell line (Table 2). CMN induced apoptosis (33%) in K562 cells. Genotoxic damage induces cell cycle arrest and/or apoptosis. Apoptosis or programmed cell death is an active physiological mode of cell death. A number of anticancer drugs are genotoxic and their damaging effects upon cells are mediated by this mechanism. CMN has been reported to

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<th>Group</th>
<th>Concentration (μg/ml)</th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
<th>M1 (%)</th>
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*Significant at P<0.01, FER vs control

Table 2—Cell cycle analysis of K562 cells after exposure to CMN

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<th>Group</th>
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<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
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*Significant at P<0.01, CMN vs control

Fig. 1—Dose dependent effect of phenolic compounds. K562 cells were incubated for 72h with various concentration of compounds and the IC50 of each compound was determined from the dose response curve. Each value represents mean ± SD from 3 independent experiments.
induce apoptosis through mitochondrial pathway involving caspase-8, cytochrome C release and caspase-3 activation in human neoplastic cells.

Increase in systemic toxicity and drug resistance, the major drawbacks of cancer chemotherapeutic agents have led to a new challenge in the field of cancer research. To overcome this problem, extensive research has been directed toward reducing systemic toxicity and increasing drug activity in cancer therapy. In this regard, combination chemotherapy has received more attention for the purpose of finding compounds with a known mechanism of action that could increase the therapeutic index of clinical anticancer drugs. Some dietary supplements possess anticancer activity apart from their other biological efficacy, which is an added advantage in combination chemotherapy.

Combined effect of FER/CDDP and FER/CBDC showed effectiveness on K562 cell line (Figs 3A, B, 4). These results implied that FER could potentiate the activity of platinum complexes. The biological mechanism responsible for increased therapeutic gain might deserve further investigation.

CMN showed significant antitumor activity that was comparable to the activity of CDDP (Figs 1-3). Further study was conducted to observe the enhancement of the antitumor effect of CMN by preparing its beta cyclodextrin inclusion complex. The antiproliferative activity was investigated by treating the cells with various concentrations of CMN alone or in combination with FER or CDDP.

Fig. 3—Effect of polyphenolics and cisplatin alone and in combination on K562 cell growth (A). Also shown are the co-operative efforts of FER with CDDP (B). Cells were cultured and treated with DMSO, cisplatin, polyphenolics alone or their combinations and the % viability was determined at the end of 72h of treatment by trypan blue dye exclusion test. Experiments were conducted in duplicates and repeated thrice. The values represent mean ± SD.
The ferative property of CMN complex was clearly visible on K562 cancer cells (Fig. 5).

In conclusion, the present study showed that curcumin, an anti-inflammatory antioxidant compound consumed widely as a dietary supplement, possessed strong anticancer activity against human cancer cell lines in vitro.

Among the cinnamoyl compounds, FER appeared to be a better modulating agent and CMN was found to be the most potent on human malignant cell lines. For further studies, we propose to include cinnamaldehyde, which may contribute to the development of new directions in anticancer therapies. Depletion of GSH by FER led to an increase in the sensitivity of the K562 cells to platinum complexes. Similarly, G2/M cell cycle arrest of K562 cells was also contributory in enhancing the tumor cell killing affected by cisplatin. It could be concluded that CMN was capable of demonstrating the most pronounced effect on cell viability. Additional work on water soluble derivatives of CMN and its various cyclodextrin complexes must be undertaken to determine in what manner CMN will be more effective in vivo.

References
9. Isuzugawa K, Oghara Y & Inoue M, Different generations of inhibitors against gallic acid induced apoptosis produce different sensitivities to gallic acid, Biol Pharm Bull, 24 (2001) 249.