Anticancer Activity of Phenolic Antioxidants against Breast Cancer Cells and a Spontaneous Mammary Tumor

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Phenolics such as ferulic, caffeic, gallic acids and curcumin were tested for their potential anti proliferative and cytotoxic properties in human breast cancer cell line (MCF-7) as well as on a spontaneous mammary adenocarcinoma tumor. As a single agent, caffeic acid showed substantial growth inhibitory activity. In combination with cisplatin it was also found to be effective. For the current study we used a chick embryo model to assess antiangiogenic activity. Curcumin and its beta cyclodextrin complex were observed to interfere with capillary formation. The selected phenolics were structurally related which allowed us to gather additional information regarding the structure – activity relationship underlying the biological activity of these bioactive compounds. It was verified that the hydroxylated acid derivatives yielded better results than the merely hydroxylated ones in these tumor systems.

Globally breast cancer is the most frequent female malignancy. It is the most frequently diagnosed cancer and is the second leading cause of cancer death among women in India. Despite advances in cancer treatment over the past decades, the prognosis of patients with breast cancer has improved only to a small extent. Thus, there is an urgent need to develop new and effective strategies for the prevention and treatment of this form of cancer.

Better description of the molecular abnormalities that occur in breast cancer cells have led to the identification of several new agents with potential therapeutic activity in patients with breast cancer. Although regular cytotoxic agents largely have been successful in inducing disease remission in many patients with breast cancer, the majority eventually develops recurrent disease and new therapeutic strategies are needed[1].

Phytochemicals through intake of vegetables, fruits, herbs and spices in diet can become effective modality. Dietary modification has emerged as a cost-effective approach to control the incidence of cancer. S-allylcysteine (SAC), an organosulfur constituent of garlic, lycopene an antioxidant, tomato carotenoid and several nutrients/non-nutrients are recognized to possess anticancer properties[2].

Experimental studies suggest that consumption of phytoestrogen foods may protect against breast cancer and phytoestrogens have been reported both to inhibit and stimulate the growth of some human breast cancer cells. Extracts of several estrogenic herbs including hops, black cohosh and vitex inhibited growth of T-47 D breast cancer cells. These *in vitro* results suggest that certain herbs and phytoestrogens may have ability in the prevention of breast cancer[3]. Genistein, a bioactive substance (isoflavone) found in soybeans possess strong antiangiogenic activity and exerts multiple suppressive effects on human breast carcinoma cells[4].

The identification of new and effective anticancer drugs has always been a focal point in cancer research. We have undertaken drug development programme to identify antioxidant rich natural resources preparing molecular fingerprints of their chemical composition and study therapeutic properties against multiple forms of cancer. In this context we carried out numerous studies on a bioflavonoid quercetin[5-7]. The present study describes the antiproliferative effects of phenolics on estrogen-positive human breast cancer cells and a spontaneous mammary carcinoma tumor model. Curcumin,

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caffeic acid, gallic acid and ferulic acid were selected as they have attracted widespread interest in biology and also having therapeutic potential. This study may provide new insights in designing the therapeutic tools against breast tumors.

MATERIALS AND METHODS

Ferulic acid (FER) and gallic acid (GAL) were purchased from S. D. Fine Chemicals, Mumbai. Caffeic acid (CAF) and curcumin (CMN) were purchased from Merck, Mumbai. Growth medium, FBS, antibiotics were obtained from Sigma Chemical Co, St. Louis, MO. All other chemicals and reagents were purchased from Gibco Laboratories, Grand Island.

Cell culture:

Human breast cancer cell line (MCF-7) was procured from National Cell Science Centre, Pune. The cells were cultured in IMDM medium containing 10% (v/v) FBS and were maintained at 37° under a humidified atmosphere of 5% CO_2 and 95% air. After appropriate incubation, viability of tumor cells grown in the absence and the presence of phenolics and cisplatin was measured. The concentrations selected for cell culture experiments are based on our ongoing studies. The trypan blue dye exclusion assay was carried out to determine the proportion of living and dead cells[8]. The proportion of control viable cells stands at hundred percent.

Tumor clonogenic assay:

Colony formation assay was carried out by plating 5×10^4 cells/well in 24 – well dishes in culture medium containing fetal calf serum and 0.4% agar on top of a 0.5-0.75 agar cushion[8]. The cells were continuously exposed to test agents during the entire culture time of two weeks. Cells that were killed by these agents or damaged were consequently blocked in the cell cycle and fail to form colonies. The evaluation of colony number in samples treated with agents was compared with untreated controls, which provide a measure of drug activity. For measurements control value was taken as hundred percent.

Preparation of cyclodextrin complex:

The cyclodextrin inclusion complex was prepared in molar ratio of 1:2 by co-evaporation method. Calculated amount of CMN and beta-cyclodextrin (ß-CD) were weighed. CMN (100 mg) was dissolved in methanol (6 ml) and ß-CD (400 mg) in 6 ml of water. This mixture was maintained under stirring for 24 h protected from light. After evaporation of the solvents under reduced pressure, the solid material was dried at room temperature for 2 days in a desiccator and stored until use. The solid material was sieved through 85-mesh sieve and 30-50 mg was used for analysis. The content of CMN was determined by UV spectrophotometer after proper dilution.

Chick choriallantoic membrane assay:

Four-day old chick embryos were purchased from Poultry farm, Aarey Colony, Goregaon, Mumbai and were incubated at 37° with 60% humidity. A standardized procedure[9] developed by us was followed. A small hole was made through the shell at the end of the egg directly over the air sac using a small craft drill. A second hole was drilled on the broader side of the egg directly over embryonic blood vessels, as previously determined by candling. Negative pressure was applied to the original hole, which resulted in the choriallantoic membrane (CAM) pulling away from the shell membrane and creating a false air sac. A 1.0×1.0 cm square window was made that allowed direct assess to the underlying CAM. CMN and its inclusion complex (2.5-10 µg/egg) were applied to the CAMs in a total volume of 100 µl of both solubalized in a suitable vehicle. An equal volume of vehicle was used for the control group. At the end of the 2-day incubation period the vessels were observed using a dissecting microscope (SZ-PT, Olympus) at the magnification of 10. Five eggs were tested for each group and the assay was performed twice to ensure reproducibility.

Evaluation of antitumor activity:

The antitumor activity of CMN and its inclusion complex was determined following the standard protocol[10]. The animal experimentation protocol was approved by the Institutional Animal Ethics Committee. After s.c. transplantation of mammary adenocarcinoma cells into C_3H/J mice, the test materials were administered for days 13-22 orally by gavages. To quantify the solid tumor growth two perpendicular tumor dimensions were measured with a Vernier caliper and the mean tumor surface in mm^2 was calculated for each time period for six mice per group. Tumor weights were estimated from their volume using the formula, Tumor volume (mm^3): [short diameter (mm^2)] × [long diameter (mm)] / 2. The measurements started approximately at day 14 and were performed twice weekly until the first animal dies.

RESULTS AND DISCUSSION

The present study was focused on known specific phenolic antioxidants to investigate their chemotherapeutic

potential. Although tamoxifen showed greater potency in breast cancer patients endometrial cancer emerges after prolonged treatment[1]. Because no predictive biomarker for response is currently available to guide the choice of cytotoxic chemotherapy for breast cancer, the choice of drugs is usually empiric. One important goal of this investigation was to initiate research to determine the cellular target(s) for phenolic antioxidants and to characterize their molecular mechanism of action to rationally design new, more efficient modulators based on their chemical structure. Phytochemicals constitute a wide family of natural components with a considerable range of bioactive properties with potential clinical applications as anticancer drugs.

The four phenolics namely ferulic acid, caffeic acid, gallic acid and curcumin showed selective antiproliferaive effect against estrogen - positive human breast cancer MCF-7 cells (Table 1). The values do show relevant differences between cytotoxic effects of various phenolics among themselves as well as when compared with the chemotherapeutic drug cisplatin. It appeared that breast cancer cells were slightly more sensitive to caffeic acid. Minimal inhibition was observed when MCF-7 cells were exposed to cisplatin. The vehicle dimethylsulphoxide as such had no effect on the proliferation of MCF-7 breast cancer cells.

In a next attempt to enhance the antiproliferative effect of the phenolic compounds, combination studies were carried out. Caffeic acid when co-administered with cisplain was more effective than the remaining combinations (Table 2).

Colony formation assays indicate that caffeic acid is a better *in vitro* growth inhibitor of MCF-7 cells. Interestingly its growth inhibitory effect with cisplatin was greater when a comparison was made with the rest of the combinations with respect to the plating efficiency (fig.1).

There is no doubt that the phenolic acids appear promising while analysing the results obtained in the present investigation and as such they will be more effective in the form of esters as the case with caffeic acid ester[11], nonetheless curcumin follows similar growth inhibitory pattern as a single agent as well as in combination. Besides following observations justifies further studies on this widely consumed dietary supplement.

TABLE 1: EFFECT OF PHENOLIC COMPOUNDS ON BREAST CANCER CELL LINE (MCF-7)

Treatment	Concentration (µg/ml)	Percent viability (Mean±S.E.)
Control	-	100±8.44
Ferulic acid	100	34±2.82*
Caffeic acid	50	18±2.65*
Curcumin	1	61±5.4*
Gallic acid	25	48±3.9*
Cisplatin	1	85±4.21**

(*P < 0.1, **NS)

TABLE 2: COMBINED EFFECT OF PHENOLICS AND CISPLATIN ON BREAST CANCER CELLS (MCF-7)

Treatment	Concentration (µg/ml)	Percent viability (Mean±S.E.)
Control	-	100±5.66
Ferulic acid + cisplatin	100+1	47±2.85*
Caffeic acid + cisplatin	50+1	13±1.91*
Curcumin + cisplatin	1+1	58±1.86*
Gallic acid + cisplatin	25+1	37±1.33*

(*P < 0.1, **NS)

The phytochemical curcumin present in turmeric arrested many cell types in G2/M phase of the cell cycle after treatment. It is one of the potent agents to inhibit nuclear transcription factor NF-kappa B pathway[12]. NF-kB has the ability to suppress apoptosis (programmed cell death) and to induce expression of proto-oncogene such as c-myc and cyclin D1, which directly stimulate cellular proliferation, suggesting that NF-kB may participate in many aspects of oncogenesis[12]. Indeed, constitutive NF-kB activity has been observed in a number of human cancers, including breast cancer and inhibition of NF-kB abrogates tumor cell proliferation. All these augments go well for curcumin suggesting it an ideal candidate in the

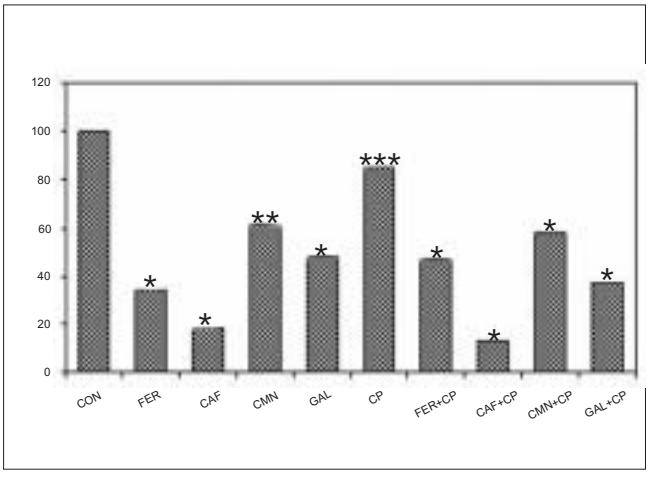

Fig. 1: Effect of Ferulic acid (FER), caffeic acid (CAF), Curcumin (CMN) and gallic acid (GAL) on breast cancer cell line MCF-7 alone and in combination with cisplatin (CP).
Percentage relative plating efficiency plotted as a function of compound on test. Each value represents mean colony number data of 5 plates.

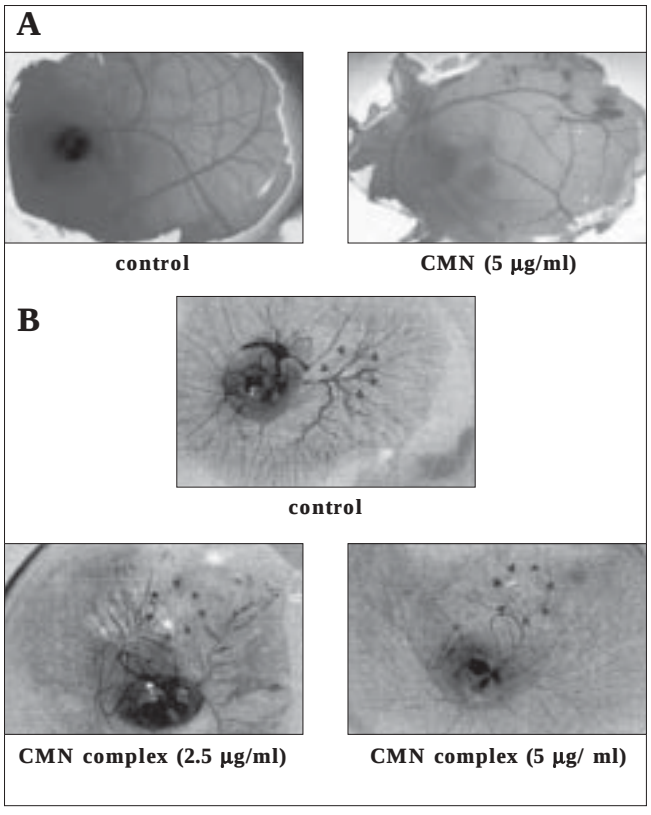

Fig. 2: Antiangogenic activity by CMN and CMN complex in CAM model.
CMN and CMN complex at different concentrations were tested for antiangiogenesis. After 48h exposure to these two test materials, CAMs of five days old embryos were examined under a dissecting microscope and photographed. Arrows show antiangiogenic effect.

treatment of human cancer.

Cyclodextrins are the cyclic oligosaccharides having the ability to form inclusion complex with wide variety of compounds. Cyclodextrins can entrap poorly soluble drug molecules of appropriate size and polarity in their hydrophobic cavity to form reversible non-covalent inclusion complexes. This can improve aqueous solubility, stability and bioavailability of the drug molecule[13].

Angiogenesis, a process by which new blood vessels sprout from existing ones is a prerequisite for out growth and metastasis of tumor. Growth of solid tumors depends on the induction of angiogenesis to provide adequate oxygen and nutrients to proliferating cells. Thus angiogenesis constitutes a very promising therapeutic target[14].

A chick embryo chorioallantoic membrane (CAM) model was used to demonstrate that the administration of physiological relevant doses of curcumin and its beta

TABLE 3: ANTITUMOUR ACTIVITY OF CMN AND ITS CYCLODEXTRIN COMPLEX ON SPONTANEOUS MAMMARY TUMOUR

Treatment	Dose (mg / kg)	T / C% Days after tumor transplantation		
		20	28	40
CMN	100	23	46	51
CMN complex	100	71	39	26

Tumor cells were injected s. c. to C_3H/J mice. Treatment started on day 13 and continued to day 22 orally by gavages. T is the average tumor volume of the treated groups and **C** is the average tumor volume of the control group.

cyclodextrin complex cause significant decrease in induction of angiogenesis (fig. 2). Because angiostatic therapy would require a prolonged maintenance of therapeutic levels *in vivo*, the continuous delivery of CMN would be more effective. In this way its inclusion complex might be a helpful additional therapeutic tool for breast cancer patients.

For tumor growth inhibitory studies curcumin was administered by oral gavages (100 mg/kg) for 10 days for the duration of the experiment. It inhibited the growth of mammary tumor markedly in the beginning of the treatment whereas its cyclodextrin complex showed significantly greater activity within the 5-week period of observations (Table 3). These results provide a basis for the ability of the inclusion complex for in depth studies.

In summary, our results show that caffeic acid and its combination with cisplatin results in greater antiproliferative activity in breast cancer cells in the assays conducted. Curcumin shows similar tendency towards cell growth inhibition. Importantly increased activity of curcumin inclusion complex was observed in spontaneous mammary tumor. Potentially, this effect was due to enhanced oral absorption and oral bioavailability and its antiangiogenic properties.

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