RESEARCH COMMUNICATION

Indian Food Ingredients and Cancer Prevention – an Experimental Evaluation of Anticarcinogenic Effects of Garlic in Rat Colon

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Abstract

The major food items of Indian cuisine include rice, wheat, diary products, and abundant fruits and vegetables. Beside these, there are several kinds of herbs and spices as important ingredients, containing many phytochemicals with medicinal properties, adding taste to Indian cuisine. An impressive body of data exists in support of the concept that Indian food ingredients can be used in preventive strategies aimed at reducing the incidence and mortality of different types of cancers because of their antioxidative, antimutagenic and anticarcinogenic properties. Vital ingredients used in Indian cooking include turmeric, cloves, ginger, aniseed, mustard, saffron, cardamom and garlic Garlic is an indispensable ingredient of Indian food and this report concerns the chemopreventive efficacy of garlic in an azoxymethane induced rodent colon carcinogenesis model. The effect of garlic was evaluated in terms of aberrant crypt foci, putative preneoplastic lesions in the colon. In addition, cell proliferation and levels of apoptosis were determined and the expression of cyclooxygenase-2 protein was analyzed. Following treatment, significant inhibition of cell proliferation and induction of apoptosis, as well as suppression of cyclooxygenase-2 activity were observed, associated with significant reduction in the incidence of aberrant crypt foci. The study points to combined protective effects of garlic components on colon carcinogenesis.

Key Words: ACF - Azoxymethane - apoptosis - cell proliferation - cyclooxygenase-2 - Indian food ingredients - Garlic

Asian Pacific J Cancer Prev, **5**, 126-132

Introduction

The increasing magnitude of cancer mortality throughout the world, claiming over six million lives each year, and the failure of conventional chemotherapy of advanced invasive disease to effect major reductions in cancer mortality rates indicate that new approaches are critically needed for control of cancer. In this context, it is essential to adopt a more intensive and imaginative approach towards prevention of this disease. Human epidemiological studies, including both cohort and case-control studies of all cancer sites, supported by experimental observations from animal studies, suggest that cancer risk may be modified by changes in dietary habits or by consumption of naturally occurring antimutagens and anticarcinogens with food, including flavouring agents. The major food items of India include rice and wheat, different diary products like milk, cream and yogurt and plenty of fruits and vegetables. Besides these, there are several kinds of herbs and spices as ingredients of food that add flavouring and taste to the pyramid of Indian food cuisine.

Most of the flavouring ingredients have their use in the Indian system of medicine (Ayurveda) for treatment of various ailments. Asafoetida is a gum obtained from the root of a giant fennel-like plant, and is sold in powder or resin form. It is a rather smelly spice, and usually just a pinch is thrown into hot oil before other ingredients are added. It is considered an aid to digestion and is known for other medicinal properties including nerve disorders. Cinnamon and cardamom, containing many active components like limonene, mannitol, cineole, are among the most aromatic spices that are used in Indian dishes which require a delicate flavour. They help to expel gases from the intestine and can

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relieve diarrhoea.

Cloves, containing eugenol, a salicylic acid, have a strong and distinctive flavour, and are another major ingredient used in Indian cooking. Cloves are also used whole in some curries, pulaus and biryanis. They are widely used in dentistry.

Black cumin seeds are accosinaly used. Black cumin bears no resemblance to white cumin, and the two are not interchangeable. They have certain beneficial activities in digestion.

Garam Masala, an aromatic mixture that incorporates important spices such as black cardamoms, cinnamon, black cumin, and cloves, is considered to be a stimulant.

Garlic is an indispensable ingredient in Indian cookery. It is traditionally used as a spice especially in Asian cuisine and is well known for its medicinal properties with varied pharmacological functions. The major components of garlic include allicin, ajoene, various allyl sulfides, S-allylmercaptocysteine, linoleic acid, selenium and quercetin.

Ginger is also a vital ingredient used in large number of Indian recipes. It contains choline, cineole, sesquiterpene, zingerone, which can act as antioxidants, and is useful in treating indigestion and nausea.

Other spices that are widely used in India include saffron, turmeric, bay leaves, chillies, coriander, curry leaves, fennel seeds, fenugreek seeds, mace, mango powder, mustard seeds, paprika and poppy seeds, all of which contain phytochemicals with known medicinal properties.

Understanding the multidimensional nature of diet and of its relationship with different cancers has led to the identification of various food ingredients having cancer preventive activities. Food ingredients like spices and condiments have chemical constituents which have antioxidant, antimutagenic and anticarcinogenic properties (Krishnaswamy, 1996). Some of them have many other beneficial effects like hypocholesterolaemic, hypoglycaemic, anti-inflammatory and antimicrobial properties. Turmeric, cloves, ginger, thyme, anise, mustard, cinnamon are already reported to have antioxidant and antimutagenic properties. It is therefore apparent that many essential health protective qualities are present in the Indian food and its ingredients.

Extensive researches are now being carried out in several research sectors in India to evaluate the chemopreventive efficacy of various food ingredients in India against several cancer types.

Turmeric (*Curcuma longa*), is used as a spice to add colour, taste and flavour to the food. Curcumin, the active principle in turmeric has strong antioxidant and anti-inflammatory potency. In experimental carcinogenesis turmeric/curcumin has been found to be protective against dimethylbenzanthracene and benzopyrene induced skin and forestomach carcinogenesis respectively. (Krishnaswamy,1998; Annual Report, NIN 1998). Administration of turmeric (1g/day for 9 months) exerted a significant impact on the regression of precancerous lesions of palatal cancers in a specific area of Andhra Pradesh (Krishnaswamy, 1996).

Chemopreventive potential of saffron (*Crocus sativus*), used widely as spice and food colouring agent, has been reported (Fikrat, 2002). The anticarcinogenic effect of saffron is attributed to its inhibitory effect on DNA synthesis and free radical chain reactions (Nair et al., 1992 &1995; Abdullah, 1992).

The antioxidant activities of various other Indian food ingredients, viz., cardamom and cinnamon, have been found to be responsible for their chemopreventive activity in *in vivo* model systems (Khan et al., 1996). Anticarcinogenic effects of bay leaves (*Laurus nobilis)* were observed with human Molt4B and HL60 cells, whereas that of Cumin seeds (*Nigella sativa*), was shown in benzopyrene induced forestomach tumours in animals (Badaray et. al., 1999).

Methanolic extracts of cloves (*Eugenia caryophyllata*), (Moteki et.al., 2002) have been shown to inhibit prostaglandin synthesis, which is a pre-requisite for multistage carcinogenic processes. Fenugreek (*Trigonella foenum gracum*) seeds can decrease lipid peroxidation, a common phenomenon during carcinogenic process, in dimethylhydrazine induced Wister rats (Genet et.al., 2002). Curry leaves (*Murraya koenigii*), can also reduce the malonaldehyde formation and hence can decrease lipid peroxidation (Khan et.al., 1996).

Chemopreventive action of the oriental food-seasoning spices mixture, Garam Masala on dimethylbenzanthracene induced translactational and transplacental carcinogenesis in mice has been established (Rao and Hashim, 1995).

Mustard (*Brassica juncea*) is a spice used for flavouring and as a source of edible oil in India. The leaves of this plant are consumed as a vegetable. Mustard belongs to cruciferous family, other members of which are cabbage and cauliflower. The active principle of mustard is dithiolthione. National Institute of Nutrition studies have shown that mustard has antimutagenic properties. In rats fed 10% mustard powder containing diet, significant reduction in the activity of carcinogen activation enzyme, aryl hydrocarbon hydroxylase and stimulation of the activities of carcinogen detoxification enzymes, namely UDPGT and glutathione-S-transferases, were observed (Polasa et. al., 1994).

Garlic is known to be composed of various anticarcinogenic, antimutagenic compounds and anticarcinogenic properties have been indicated in several studies (Lau, et. al, 1990; Sparnins et.al., 1998; Wargovich, 1987 & 1988). Regular consumption of garlic is reported to be associated with decreased prevalence of adenomatous polyps in the colon and rectum (Witte et. al., 1996). Cancer of the large bowel (colon and rectum) is one of the major cause of cancer mortality in the industrialized world second only to lung (Jass, 2002; Dove-Edwin, 2001). In India also, an increased trend has been noted recently in the incidence of colon cancer. Colon cancer arises from normal cells as a consequence of multistep carcinogenesis and the majority of cases occur sporadically caused by non-inherited factors, such as a combination of diet and other environmental factors (Kinzler, 1996; Davidson, 2000). Diet and nutrition they play

an important role in the etiology and primary prevention of colon cancer.

The present report documents our observations on the effects of garlic on AOM induced colon carcinogenesis in Sprague-Dawley rats, in terms of the incidences of aberrant crypt foci (ACF), preneoplastic lesions in colon formed after carcinogen exposure. Since enhancement of cell proliferation and inhibition of apoptosis are considered to be the prerequisites for multistage carcinogenesis leading to tumour development in the colon (Jenab, 2000), *in situ* cell proliferation and *in situ* cell death (apoptosis) were assessed in the colon tissue.

Cyclooxygenase (COX) is a rate-limiting enzyme in the cellular production of prostaglandins (PGs) from arachidonic acid (Smith, 1996; Ledinghen, 2002). Overexpression of cyclooxygenase-2 (COX-2), an inducible isozyme of COX, causing increased prostaglandin synthesis (particularly, PGE 2), is closely associated with colorectal neoplasia (Pugh, 1996; Crew, 2000; Vogiagis, 2001).

To explore the possible mechanistic pathways of action of a garlic suspension, Western blot analysis for the expression of COX-2 protein in colon tissue was performed.

Materials and Methods

Male Sprague-Dawley rats, 3-4 weeks old (outbred and maintained in our Institution) were housed in plastic cages (~ 4 rats/cage) at an ambient temperature of 22°C - 25°C under a 12 hour light/dark cycle with access to drinking water and pellet diet (Lipton India Ltd.) ad libitum.

AOM (Sigma Chemicals Co. MO, USA) was administered by three weekly subcutaneous injections (15mg /kg.b.w.) between 11am–12 noon.

Garlic (Allium sativum Linn.) was bought from local market (Charnok City, Kolkata, India). The dehusked cloves of garlic were ground in a mortar and pestle and the paste formed was diluted with distilled water as to make a 2.5% aqueous suspension (w/v). The suspensions of garlic used was prepared fresh everyday before oral administration at a dose of 1ml/rat/day, so that each rat in the garlic treated group received 25mg garlic per day, starting from the 1st day of AOM injection. In our previous studies (unpublished), we used three doses, viz., 1.5% (15mg/rat/day), 2.5% (25mg/ rat/day) and 4.5% (45mg/rat/day) (w/v) solutions of garlic to observe their effects on ACF formation during AOM induced colon carcinogenesis. The 2.5% (25mg/rat/day) dose was found to be most effective without manifesting any toxic effects. For this reason, this dose (2.5%, w/v) was considered for the present study.

Experimental Design

The experiment was designed in four sets, each with thirty rats, according to the experimental parameters used. Each set was divided in three groups, viz., Normal, Carcinogen control and Garlic-treated.

The Carcinogen control and the Garlic-treated groups were initiated with AOM (Sigma Chemicals Co., MO, USA) by three weekly subcutaneous injection (15mg/kg. b.w.) administered between 11am – 12 noon. Each rat in the Garlic-treated group received an oral administration of a 2.5% (w/ v) aqueous suspension of garlic at a dose of 1ml/rat/day continuously starting from the 1st day of AOM injection for twelve weeks. The total observation period was 12 weeks. The Normal group received no treatment with AOM or the garlic suspension.

Set I: Assessment of ACF

12 weeks after the 1st AOM injection, the rats in this set were sacrificed to assess the incidences of colonic ACF. The colons of the animals were placed flat between two filter papers and fixed in 10% buffered formalin for about 24 hours. This was followed by staining with 2% methylene blue (Qualigens ltd.) in Ringer's solution for 3-5 min. ACF can easily be identified, after placing the colon on glass slide with mucosal side up under a light microscope at 40X magnification, by their large and elliptical luminal openings (McLellan, 1991). Total number of ACF and the number of ACF with 4 or more aberrant crypts (AC) were counted for each rat.

Set II: In situ Detection of Cell Proliferation

Cell proliferation in colon was measured using 5-bromo-2/-deoxyuridine labelling (Davidson, 2000) with a BrDU Labelling and Detection Kit II, procured from Roche Molecular Biochemicals. For this purpose, rats were sacrificed and the colons were sliced into several parts and placed into BrDU labelling medium when the DNA of proliferated S-phase cells were labelled. The tissue slices were fixed and processed under normal histological procedures and the sections were subsequently incubated with an anti-BrDU Monoclonal antibody (at 37°C for 30 min.). Binding was detected with an alkaline phosphatase-conjugated-antimouse-immunoglobulin antibody (anti-mouse-Ig-AP). The bound anti-mouse-Ig-AP was visualized using nitroblue tetrazolium (NBT), an AP-substrate solution.

The BrDU Labelling Index (BrDU LI) was determined by dividing the number of labelled cells by the total cells counted and multiplying by 100.

Set III: In situ Detection of Cell Death (Apoptosis)

Apoptotic cells in colon were visualized using the terminal deoxynucleotidyl transferase (TdT)- mediated dUTP- biotin nick end labelling (TUNEL) method with the help of *In situ* cell detection kit, AP (Roche Molecular Biochemicals). The deparaffinized tissue sections were permeabilized using Triton-X-100 (Sigma), then incubated with TUNEL reaction mixture containing the TdT and fluorescein-dUTP, at 37°C for 60 min. Slides were rinsed twice in PBS for 10 min. and dried, the AP substrate was added and the sections were covered by a coverslip and incubated at 37°C for 30 min. Slides were again rinsed in PBS for 10 min. and NBT was added and kept for 10-15 min. Finally, the slides were washed and analyzed under a light microscope.

The Apoptotic Index (AI) was determined as the percentage of the labelled nuclei with respect to the total number of nuclei counted (Caderni, 2000).

Set IV: SDS-PAGE Western blotting for COX-2 Expression

Colon tissues were removed and washed in PBS at 0°C. The tissues were cut into pieces and homogenized in ice-cold 5 volume of homogenizing buffer (0.1M NaCl, 0.01M Tris-Cl, 0.001M EDTA) containing 1mM PMSF, 1µg/ml Aprotinin, 0.1 mM Leupeptin at 3000g for one hour at 4°C. The supernatant were estimated for their protein content using Bovine Serum Albumin (BSA) as a standard (Lowry, 1951).

SDS-PAGE and Western blotting were carried out essentially as described by Laemmli (Laemmli, 1970; Singh, 1997). The tissue homogenate equivalent to 200µg protein was mixed with gel loading buffer (100mM Tris-Cl, 200mM ß-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% Glycerol). The samples were then boiled for 5 minutes and resolved in 10% polyacrylamide gel along with Prestained Molecular Weight Markers (Rainbow Markers, Amersham Biochem. Pharmaceuticals).

Electrophoretically resolved proteins were transblotted onto Immobilon-P (PVDF) membranes and subsequently were blocked with 5% TBS blotto A (Santa Cruz Biotech.). The blots were then incubated with anti-COX-2 rabbit polyclonal antibody (Santa Cruz Biotech.) diluted 1:500 in TBS containing 0.5% TBS blotto A (non fat dried milk). After extensive washing, blots were reincubated with HRP-conjugated secondary antibody (Santa Cruz Biotech.). The protein bands were then visualized by using Luminol reagent (Santa Cruz Biotech.) in the dark room and the bands were exposed to X-ray film within an exposure cassette for few minutes and the films were then developed by conventional methods.

Statistical Analysis

The differences in mean values among different groups were tested and the values were expressed as mean ± SD. Data obtained from incidences of ACF were analyzed using Students-t-test and the data obtained from cell proliferation and apoptotic assays were tested by analysis of variance (ANOVA). All the statistical calculations were carried out using Microsoft Excel and a P-value < 0.05 was considered significant.

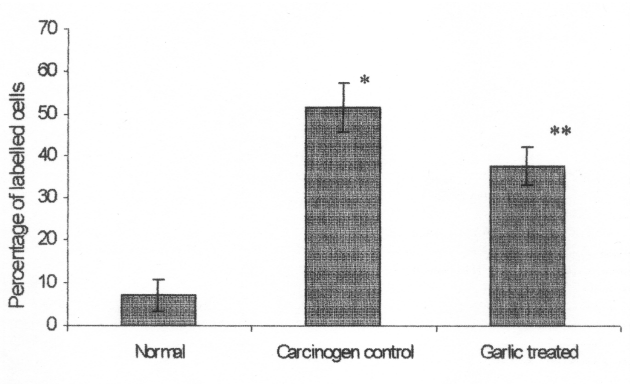

Figure 1. Cell proliferation is expressed as BrDU LI which is calculated as percentages of BrDU labelled cells with respect to the total number of cells counted throughout the distal 5 cm of the colons from each group.

* P= 0.00031 with respect to the Normal group. **P= 0.0003 with respect to Carcinogen Control value.

Results

ACF

At the end of twelve weeks of treatment, after the first AOM injection, the total number of ACF and the number of ACF with four or more aberrant crypts (ACs) were found to be reduced in the Garlic treated group as compared to the corresponding carcinogen control value (Table 1). Total number of ACF was significantly reduced by 42.1% (P= 0.0046) in the Garlic treated group. Number of ACF with four or more ACs was also found to be significantly decreased by 55.5% (P= 0.0054). The colon of rats from Normal group showed no ACF.

Cell Proliferation and Apoptosis

Significant reduction in BrDU LI (Figure 1) and significant increase in the Apoptotic Index (AI) (Figure 2) were observed in garlic treated rat colons. Sections from distal 5 cm of colons were chosen and the cells were counted.

The BrDU LI was found to be increased in the Carcinogen control group (P= 0.00029) from that observed in the Normal group. On the other hand, the Garlic treated group showed significant reduction in the value of Brdu LI (P= 0.00306). AI, indicating the level of programmed cell death, was found to be decreased in the Carcinogen control group (P= 0.0155) with respect to the corresponding value obtained from the

Table 1. Analysis of ACF in Rat Colon after 12 Weeks of First AOM Injection

Groups	Total ACF (mean±SD)	% of inhibition after 12 weeks	ACF (4 or more ACs) (mean±SD)	% of inhibition after 12 weeks
Normal	0	_	0	_
Carcinogen Control	28.5 ± 4.03	_	13.6 ± 2.07	_
Garlic Treated	16.2 ± 2.77*	42.1	7.2 ± 2.88**	55.5

Total number of ACF (mean ± SD) and the number of ACF with four or more Aberrant Crypts (ACs) (mean ± SD); Percentage inhibition was calculated in comparison with the Carcinogen Control value. (*P= 0.0046; **P=0.005).

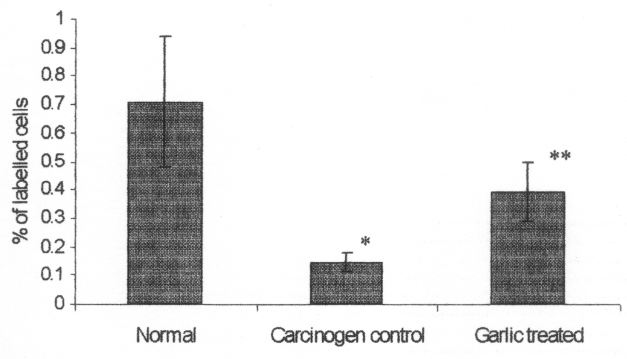

Figure 2. Apoptosis expressed as AI, calculated as the percentage of TdT-labelled cells with respect to the total number of cells counted throughout the distal 5 cm of the colons from each group.

*P= 0.0155 with respect to Normal group. **P= 0.0044 with respect to Carcinogen Control value.

Normal group, whereas, the AI was induced in the Garlic treated group (P= 0.0044) in comparison with that of the Carcinogen control group.

COX-2 Expression

Expression of COX-2 protein in Normal, Carcinogen control and Garlic treated animals has been represented in Figure 3. COX-2 immunoreactivity with polyclonal anti-COX-2 antibody was not detectable in the colonic fraction of Normal rats. In contrast, dark immunoreactive bands of COX-2 at a position of 72KD (Molecular weight of COX-2) were observed in animals treated with AOM only (Carcinogen control). In case of garlic treated animals, though one showed moderate expression of COX-2, the other two showed very little expression of the protein.

Discussion

The present significant reduction in the number of ACF following treatment with aqueous suspension of garlic indicates chemopreventive activity of garlic on chemically induced colon carcinogenesis.

Azoxymethane, a potent colon carcinogen, is metabolized in the liver into methylazoxymethanol (MAM). The reaction is mediated by the action of cytochrome P4502E1(Sohn, 1991). Metabolic activation of MAM to a highly reactive elctrophile (methyl diazonium ion) occurs in liver and colon. This ultimate electrophile can methylate cellular nucleophile, such as, DNA, causing alkylating damage to DNA (Talalay, 1992; Fiala, 1987; Tanaka, 1997)

If colonic epithelial cells no longer respond to DNA damage by undergoing apoptosis, then mutations possibly leading to colon cancer may be acquired and fixed through increased proliferation, which plays a central role during carcinogenesis (Yang, 1996; Jenab, 2000). Since the balance between cell proliferation and cell death is important in the genesis of colon carcinoma (Bedi, 1995), our observation of decreased proliferation, indicated by BrDU LI and

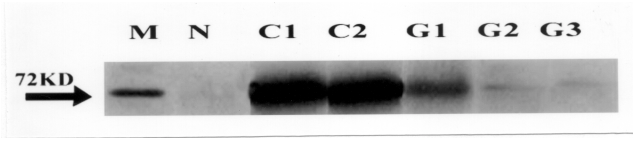

Figure 3. Expression of COX-2 protein after immunoblotting in different groups of animals. Lane M represents the marker giving bands at 250KD, 160 KD, 105 KD, 75 KD, 50 KD, 35 KD and 30 KD. Lane N represents the proteins from Normal group. C 1 and C 2 gives the expression in animals of Carcinogen Control group, whereas, the last three lanes (G 1, G 2 and G3) show COX-2 expression in Garlic treated animals.

increased rate of apoptosis, as reflected by AI, following garlic administration orally assumes significance in this respect.

Earlier studies showed that DAS, a major component of garlic, acts as a potent inhibitor of cytochrome P4502E1 by competing as a substrate for P4502E1 oxidative reactions (Wargovich, 1997; Brady, 1991). Thus it may be suggested that the inhibitory effect of garlic suspension is due to its interference with initial stages of AOM biotransformation. COX-2 has been implicated in the pathogenesis of colorectal cancer (Sharma, 2001). Overexpression of COX-2 results in dedifferentiation, adhesion to extra cellular matrices and inhibition of programmed cell death in untransformed rat intestinal epithelial cells (Tsujii, 1995). COX-2 inhibits apoptosis and increases the invasive potential of malignant cells (Yang, 2000). Inducibility of COX-2 in response to mitogenic stimuli, oncogenes and tumour promoters, link it to cell proliferation (Singh, 1997). Further, nitric oxide (NO), synthesized by nitric oxide synthase (NOS), enhances the activity and expression of COX-2 in various cell types (Rao, 1999).

DADS causes increase in the intracellular calcium which activate Ca2+ dependent endonucleases leading to apoptosis (Sundaram, 1996). Another component of garlic, S-allylcystein (SAC) inhibited NO production through the suppression of inducible nitric oxide synthase (iNOS) mRNA and protein expression (Kim, 2001). Garlic can accumulate selenium in soil and can convert it into monomethylated forms like Se-methylselenocysteine (SAMC) which can trigger apoptosis and inhibit DNA synthesis (Ganther, 1999). Components like SAMC and DATS of garlic cause induction of apoptosis through DNA fragmentation (Sakamoto, 1997; Sigounas, 1997). Moreover, the flavonoidal quercetin of garlic is a potent suppressor of COX-2 transcriptional activity (Mutoh, 2000a). The resorcein moiety within the structure of quercetin seems to be responsible for suppression of COX-2 promoter activities in colon cancer cells (Mutoh, 2000b).

In contrast to the overexpression of COX-2 in the rat colon of the carcinogen control group, the retarded expression of COX-2 protein noted following garlic treatment suggest a combined action of different components

in garlic during inhibition of chemically induced colon carcinogenesis. This effect is reflected at the level of the inhibition of cell proliferation and induction of apoptosis in the garlic treated rats. Further, different active compounds obtained from a single source can have opposing, similar or possibly reinforcing effects on various biological pathways in vivo. The present study therefore supports the contention that it may be more relevant to consider the intake of anticarcinogenic agents present in combination from natural sources, rather than individual chemical components in order to achieve a balanced chemopreventive effects (Manson, 1997).

The growing knowledge from research on cancer control emphasizes the fact that dietary factors are key modulators of carcinogenesis. Much progress in the understanding of functional relationships between nutritional factors has been provided by epidemiological and diet intervention studies, relevant laboratory animal model studies and investigations on mechanisms of tumour induction, progression and inhibition. Chemoprevention has the potential to be a major component of cancer control. Accumulating evidence indicates that various food ingredients may play an essential role in cancer prevention. India is a country well known for its herbal medicinal concept, 'Ayurveda' from ancient times. Traditional use of spices and many other plant parts as food ingredients may confer some protection from cancer reflected in lower incidences of certain forms of cancers in the country in comparison to the Western countries. These food ingredients therefore need to be assessed through cancer chemopreventive studies with special emphasis on the understanding of their mechanism of action at all levels, namely at the molecular, cellular, tissue and organ levels, as well as in the animal as a whole.

Combination chemoprevention, whereby one achieves significant synergism of two or more agents to obtain a desired preventive effect, while minimizing the toxic side effects of the individual components, represents an important challenge in this field. It is likely that the future practical development of chemopreventive regimens will rely on the use of this principle.

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