

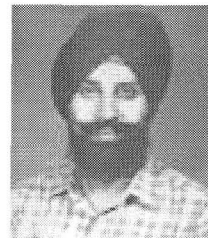
**Technical session 9
Tea and Health
Chairman B.N. Dhawan**

Chapter 40

**PHARMACOTHERAPEUTICS OF TEA :
PROPOSED STRATEGIES AND PHARMACOLOGICAL STUDIES**

Jaswant Singh*

Prof. B. N. Dhawan, ex-director of Central Drug Research Institute, Lucknow and a pioneer pharmacologist of the country, introduced the speaker to the audience. Besides a note of appreciation about the speaker, his ability and commitments to pharmacological studies of herbal products, he apprised the audience of excellent advanced facilities his department at RRL Jammu currently offers and of which advantage can be taken to develop tea products into therapeutics which is the main theme of his talk in this technical session. Tea industry is perhaps the oldest industry and tea is a traditional beverage used in this country for centuries. When I browsed into the literature on tea constituents and their pharmacological activities, I realized that tea has many ingredients that can be developed into therapeutics and value addition products. My present lecture is, therefore, to propose studies as how to develop some of these products as therapeutics against certain diseases. Most of the studies till now have been centered around beneficial health effects of catechin and epigallocatechin, while many other constituents relatively have remained uninvestigated.



ISOLATION OF CHEMICAL CONSTITUENTS

Tea samples (black, green or other tea) should be subjected to extraction and fractionation, and then fractions tested for in vitro biological activity. Based on bioactivity-guided fractions, pure molecules are to be isolated and their biological activity validated in vitro to get the discovery lead molecules. The molecules and their structures are characterized using modern chemical instrumentation. SEPbox is an interesting machine that allows the fractionation of plant extract / fraction into roughly 300 fractions of which many may comprise pure molecules. The amount of individual fraction / isolate is sometimes so prohibitive that it would require in vitro test models employing mammalian cell cultures, in particular, to validate their pharmacological activity. In this way the chances of escaping the pharmacological validation for each constituent appear quite minimal.

BIOLOGICAL SCREENING

Before, a test fraction / compound is tested for pharmacological activity; it should be evaluated in vitro per se for its cytotoxicity and genotoxicity using currently available protocols and various mammalian cell cultures. After this the fractions should be tested against various disease models, using both in vitro and in vivo models. Some of the important areas where pharmacological studies of tea extracts / fractions / isolates proposed are: anticancer, bioavailability enhancers, hepatoprotective, immunomodulation and anti-inflammatory.

ANTI-CANCER SCREENING

In vitro cytotoxicity against human cancer cell lines is evaluated using NIH guidelines. Cells in culture are treated with different concentrations of test material for 24 hr. Cell viability is assessed using sulforhodamine dye. Cells are washed using liquid robotic system for washing and bound dye dissolved is read on ELISA reader. Test compound

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killing more than 50% cells at $<10 \times 10^{-6} \mu\text{M}$ or 10 $\mu\text{g/ml}$ is considered a promising molecule. This discovery lead is further optimized in vivo as anti-cancer activity in nude mice against xenograft of solid human tumor or in transgenic mice expressing human cancer. The test compounds are then subjected to study the induction of apoptosis, inhibition of angiogenesis, or over expression of tumor suppressor genes as influences on mission critical events in cancer cells for understanding the mechanism of action.

BIOAVAILABILITY ENHANCER

Tea is a common beverage and its constituents may affect the disposition of other drugs and their efflux during intestinal first-pass or systemic circulation. An important criterion determining the effect in human is the bioavailability of tea constituents in systemic circulation and influence on drug transporter proteins. Some of the constituents may possess capability of maximizing drug efficacy at reduced doses. This concept is important to provide a cost effective formulation where dose vs toxicity of drug is reduced with enhanced efficacy. This is important against serious disease like tuberculosis that requires prolong treatment and where organisms develop resistance to drugs. Here tea major constituents may play important role and should be investigated. Inhibition potential of cytochrome P3A4 in particular should be studied using human liver microsomes or mammalian cells expressing CYP3A4. Besides one should also look into the efflux component of cells particularly P-glycoprotein inhibition. For medium and high through put screening human colon cancer cell line Caco-2 offer a system of choice to study the inhibition of both CYP3A4 and P-glycoprotein. Once promising leads are obtained, they can be taken further in diseased states and in vivo pharmacokinetics of desired drugs so as to develop potent bioavailability enhancer. Such studies are also important to understand the influence of tea on drugs disposition and drug-drug action.

HEPATOPROTECTIVE ACTIVITY

Culminant hepatitis and chronic liver injury are posing a serious health problem world over. Several chemicals, drugs, virus infections, autoimmune diseases, sustained alcohol abuse, drug –drug interaction, etc. can cause liver damage. Liver is a major organ involved in biotransformation of drugs and chemicals (xenobiotics) mediated by cytochrome P450 (CYP) -dependant oxidases and conjugases. Hepatocytes or parenchymal cells constitute the major mass of liver and isolated hepatocytes as such in primary culture are found to express differentiated functions of liver in terms of metabolic competence. The cells have found ample application in screening anti-hepatotoxicants against chemicals induced toxicity in culture. Other important cells are resident macrophages in the liver (kupffer cells) and quiescent hepatic stellate cells, which are star players in the development of hepatic injury and fibrosis under oxidative stress or in response to certain antigens. Aetiology of hepatitis appears to share some important components of immune system. TNF- α is synthesized and released by macrophages including.

KUPFFER CELLS OF LIVER

LPS induces secretion of several cytokines and plays important role in liver injury when liver cells are sensitized with D-glucoamine. TNF- α and platelets derived growth factor (PDGF) are released in large amounts as early events in cell injury and onset of fibrosis. Thus liver cells are sensitized after D-galactosamine injection following TNF- α or LPS administration. The damage is evident after 24 hr. Prophylactic or curative effect of lead fractions or isolates can be evaluated from liver functions or decrease in the plasma level of TNF- α . Because of free radical scavenging activity, some of the tea polyphenols may be taken up for the development of hepatoprotectives. We have more than 40 herbal formulations used in traditional system as hepatotonic, but there is not a single molecule which

can safely be used as hepatoprotective. Development of such molecule may also be seen as a value addition in drugs that cause liver damage, cardiotoxicity or renal toxicity.

ANTI-INFLAMMATORY ACTIVITY

Anti-oxidant and anti-inflammatory activities are mutually related. TNF- α plays a pivotal role in inflammation. Because of anti-oxidant properties reported with poly phenols, several tea constituents can be examined for their free radical scavenging activities and many of them may prove beneficial in ageing related and degenerative diseases. Besides, the molecules can be tested for their inhibitory potentials of molecular targets in chronic / acute inflammation such as COX-I and COX-II inhibition. Or these constituents may be important value additions to existing non-steroidal anti-inflammatory drugs. Rheumatoid arthritis is an immunological disorder of cytokines deregulation, the etiology of which is not known. Release of certain pro-inflammatory molecules like TNF- α , IL-1 and IL-6 are liable to initiate a plethora of reactions culminating in release of inflammatory molecules such as free radicals, NF- γ , IL-8, PGE2, LTB4, etc. This may result in cellular damage and related clinical problems. Expression of these inflammatory molecules can be investigated in collagen-induced arthritis or LPS-induced inflammatory conditions in mice and in macrophage cell lines RAW 264.7 or JJ74A. Inhibitions of TNF- α and down regulation of NF- γ expression are related to the molecules that have anti-inflammatory conditions. *In silico* docking of pure tea molecules to molecular targets may give early leads that may be improvised in wet lab experiments using both *in vitro* and *in vivo* models.

IMMUNO-MODULATORY ACTIVITY

Tea polyphenols are perhaps the most abundant and efficient anti-oxidants and are the star players in immune system by regulating a delicate balance between the immune cell functions by modulating their secretion of specific cytokines. Tea

polyphenols play interesting roles in the development and activation of immune cells thereby modulating their Th1/Th2 balance. Ethylamine, a degradation product of L-theanine, has shown remarkable effects in priming human V δ 2V γ 2 T cells and further enhancing their memory to abrogate microbial infections. These and other aspects of tea constituents are opening up newer frontiers in their development as therapeutics and nutraceuticals without having any adverse health effects.

Most of the studies so far have been conducted with selected constituents. It is necessary now to isolate each constituent and determine its pharmacological activities, including immunomodulatory functions *in vitro* and in mice. FACScan analysis of various immune cells, using cell flow cytometry, is an important advancement to quantitate various immune cells, their *in situ* cytokines levels and their immuno-phenotyping pattern. Stimulation and activation of immune cells are studied using cell flow cytometry by tagging surface markers with fluorochrome labeled antibodies in whole blood PBMC or mice spleenocytes. NIH protocols can be used for initial *in vitro* evaluation of cellular functions such as lymphocytes proliferation, macrophage and NK cells functions. Various established *in vitro* and *in vivo* models are used to determine the immunomodulatory potentials of the test compound, viz. immunostimulant, immunosuppressive and immunoadjuvant activities. Other related area worth exploring is to examine the influence on diabetes type-II. It is a very complicated disease and there is hardly any model that mimics the *in vivo* situation. One may use a battery of *in vitro* and *in vivo* models for evaluation of anti-diabetic activities.

Since tea does not appear to have any adverse effects, use of reverse pharmacology might be very useful in the development of potent therapeutics for various diseases.

Remarks of the Chairman

Dr. Jaswant Singh raised following important issues.

Good manufacturing practice (GMP) norms must be established and followed. Most CSIR labs are GMP compliant and something like that must be done with tea industry.

Dr. Singh laid down a road map to develop therapeutics (drugs) from tea. He mentioned many areas of which two are very important. (a) Alcohol induced liver diseases are very common and countries like Germany are looking for such a product to try on its population. It is possible that tea drinking can protect humans from alcohol related diseases. (b) The use of bio-availability enhancer can sensitize bacteria and overcome resistance in the anti-bacterial infections. Such product can also be used with anti-cancer drugs. Tea products should be explored for these studies as a priority.

The RRL Jammu has unique facilities, perhaps not found in other parts of country. Tea industry should collaborate with CSIR laboratories that have modern tools and infrastructure. CSIR has a system where if the industry tries to work with CSIR, a part of the cost is born by CSIR while industry has to share IPR and patents arising out of such collaborative studies. With competent scientists like Dr Jaswant Singh and powerful tools available with RRL Jammu and other CSIR labs, industry can collaborate to improve the optimal use of tea and its products for the health benefit of the masses.



Chapter 41**(Manuscript submitted but not presented)****CHEMOPREVENTIVE EFFECTS OF BLACK TEA EXTRACT AGAINST CHEMICALLY INDUCED CANCER****Yogeshwer Shukla, Neetu Kalra, Annu Arora and Y. K. Gupta**

Dr. Yogeshwer Shukla, is presently working as Senior Assistant Director, at Industrial Toxicology Research Centre Lucknow. Born on December 01, 1963, Dr. Shukla did his Masters from Lucknow University and he was awarded Ph.D. from Meerut University in 1983. His field of interest is cancer chemoprevention by natural products. He has more than 75 publications in journals of international repute. Dr. Shukla has also several review articles, book chapters to his credit. Dr. Shukla is in the editorial boards of several international journals. Dr. Shukla also supervised the work of about 40 Ph.D.; M.D. MDS and Masters students. He also served at various important positions in a number of academic bodies.

INTRODUCTION

It is becoming evident that dietary practice and other aspects of lifestyle play an important role in determining the risk for the development of a number of major human cancers. The mechanisms of carcinogenesis involve multiple stages of biochemical and molecular alterations in target cells. Cancer chemoprevention can be defined as the prevention, inhibition or reversal of carcinogenesis by administration of one or more chemical entities, either as individual drugs or as naturally occurring constituents of the diet. Chemoprevention by naturally occurring agents is gaining attention as a newer dimension in the management of neoplastic changes. Flavonoids are naturally occurring low molecular weight polyphenolic compounds widely distributed in fruits, vegetables and beverages including tea. Numerous experimental studies have examined the role of specific flavonoids in disease prevention. For example, increased flavonoids intake was associated with decreased risk of cardiovascular disease and carcinogenesis.

Considerable evidences are now available to establish the inhibitory effects of tea infusions or feeding of tea components in chemically induced

cancers in animals. Anticarcinogenic activities of tea are mostly attributed to its polyphenolic flavonoid components characterized by hydroxylated aromatic rings including epicatechin, epigallocatechin, epicatechingallate, epigallocatechingallate and theaflavin (Graham, 1992). Besides their well-known antioxidant properties, the tea polyphenols show profound biochemical and pharmacological functions including modulation of carcinogen metabolism, inhibition of cell proliferation, induction of cell apoptosis and cell cycle arrest. They intervene in the biochemical and molecular process of multistep carcinogenesis, comprising tumour initiation, promotion and progression. Further recent studies show that both green and black teas contain other flavonoids, such as quercetin, kaempferol and myricetin (Lin et al, 1999). Moreover, theaflavin and thearubigins, major flavonoid component of black tea, also possess antioxidant properties (Challa et al, 1997). As most of the evidence for protective effects of tea is based on studies using green tea, work needs to be undertaken on black tea as well since a majority of world population drinks black tea during their everyday life. Understanding the mechanism of action of tea at different stages of carcinogenesis is thus essential. Furthermore, as theaflavin and thearubigins are the antioxidant flavanols found only in black tea, it would be of

special interest to evaluate the chemopreventive efficacy of black tea.

The relationship between the tea consumption and carcinogenesis is an important health concern and yet it is a complex topic. Epidemiological studies have so far yielded no clear-cut conclusions concerning the effect of tea consumption on human cancer risk. After reviewing the available information in 1989, the International Agency for research on Cancer Working Group concluded that there is inadequate evidence for the carcinogenicity in humans and experimental animals of tea drinking. On the other hand, results from more and more laboratory studies have demonstrated the inhibitory effects of tea against tumor-genesis.

Studies on the anti-carcinogenic role of black tea and some chemical compounds have already been initiated in the Department of Cancer Chemoprevention. The following review offers a critical examination of this topic, covering basic chemistry and epidemiological and laboratory studies, as well as possible directions for future research.

LABORATORY STUDIES ON BLACK TEA AND CANCER

Antigenotoxic Effects

Cytogenetic tests are well identified for evaluation of mutagenic damage caused by environmental toxicants to the chromosomes (Preston et al, 1997). The applicability of *in vivo* cytogenetic assays in determination of antigenotoxic potential of dietary compounds is well documented (Ferguson, 1994; Bronzetti, 1997). In our lab the antigenotoxic potential of black tea and their polyphenols were evaluated using Swiss albino mouse bone marrow for micronuclei induction, sister chromatids exchange and chromosomal aberrations induced by cyclophosphamide. BTE was given as sole source of drinking solution for two weeks prior CP treatment. The percent incidence of aberrant cells

including breaks, fragments, exchanges and multiple chromosomal damages were found to be reduced by black tea extract in a dose dependent manner. Furthermore, CP induced cytotoxicity was also found to be protected by different doses of BTE. Hence the study reveals that administration of black tea has potential in suppressing chromosomal aberrations and cytotoxicity produced by mutagens *in vivo*.

Suppression of Growth of Implanted Tumor Cells

We reported the preventive efficacy of 2% BTE of different cultivars of black tea; orthodox, CTC and dust teas were tested in Ehrlich Ascites (EA) tumor bearing mice. The preventive effects of BTE were observed in terms of increased life span. All the cultivars of the tea showed more than 25% increase in life span of the animals. Cytotoxic effects of various doses of all the cultivars of black tea were also observed *in vitro* on EA cells (Javed & Shukla 2000). The increase in life span and cytotoxicity in EA tumor model are well-established parameters to study the antitumor activity of test compounds (Mitsui et al, 1995). One of the possible explanations for the arrest of EA tumor growth may be due to immunopotentiating property of tea, which was earlier reported by Ruch et al (1989). *In vitro* efflux from EA cells was significantly inhibited by exposure to BTE. At 50 mg dose level 42%-49% cytotoxicity was observed (Javed & Shukla, 2000).

Effect on Regression and Growth of Experimental Skin Tumors

The study was being done in our lab to evaluate the anti-tumorogenic potential of aqueous black tea extract in Swiss albino mice against skin tumors in *in vivo* animal bioassay, using 7,12 dimethyl benz(a)anthracene (DMBA) as carcinogen. In the experimental group, 2% BTE was given orally as sole source of drinking water, while the control was allowed to drink normal water. DMBA was given

topically along with BTE. The results revealed that drinking of 2% BTE could effectively inhibit the onset of tumorigenesis, cumulative numbers of tumors and average number of tumor per mouse. In BTE drinking group, in comparison to the group in which only DMBA was given, 44% animals remained tumor free till the termination of experiment, i.e. 26 weeks (Javed & Shukla, 2000).

Possible Mechanisms for Inhibition of Tumorigenesis by Black Tea

The appropriate use of a chemopreventive agent ultimately depends on the understanding of its mechanism of action at all levels namely at the molecular, cellular, tissue and organ levels. Therefore, we investigated the induction of apoptosis as the possible mechanism of its antiproliferative effect. It is now well recognized that apoptosis is a form of cell death characterized by active suicide of cells. The apoptosis inducing potential of BTE was investigated using multifacet approaches, i.e. flow cytometry, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) assay and formation of formation of DNA ladder on agarose gels, a hallmark of apoptosis in DMBA induced skin tumors. The DNA fragmentation analysis of mouse skin/tumors on agarose gels revealed a higher amount of apoptotic DNA in BTE supplemented groups. Characteristic sub G1 peak appeared in those samples of skin tumors, which were supplemented with BTE. Similarly TUNEL assay of skin tumors of BTE supplemented animals showed a significant increase in the number of apoptotic cells in comparison to the animals exposed to DMBA alone. Suppression of apoptosis is involved in tumor promotion by chemical agents (Taraphdar et al, 2001). Induction of apoptosis is, therefore, being appreciated as an ideal way for the elimination of cancer cells and apoptosis inducing agents are viewed as potential agents for the chemoprevention and for treatment of cancer (Taraphdar et al, 2001).

CONCLUSIONS AND FUTURE DIRECTIONS FOR RESEARCH

The available epidemiologic information does not indicate that tea consumption has a statistically significant causative effect on human cancers. However, possible harmful effects of the consumption of excessive amounts of tea, tea at very high temperature, or salted tea cannot be discounted. On the basis of many epidemiologic observations and numerous laboratory studies, we believe that tea consumption is likely to have beneficial effects in reducing cancer risk in certain populations. Depending on the etiology of the disease, the protective effect may be observed in selected cancers in certain populations but not in other situations.

The present set of investigation revealed the chemopreventive and chemotherapeutic efficacies of black tea. Additional studies on the chemical properties and biologic activities of tea consumption and tea components are needed to provide a sound background for examining the effects of tea on health. Black tea is the major form of tea consumed in western countries. The chemistry and biologic activities of black tea components, however, are poorly understood; research in this area is definitely needed.

Although a great deal of data has been accumulated on the effects of tea on cancer, a clear understanding of the mechanisms by which tea components may effect the genesis, growth and progression of specific cancers is essential. Two important factors are the selection of relevant experimental models and the ability to correlate results obtained in vitro to situation in whole animals and in humans. The dose-response effect is a key issue in such studies. However, the bioavailabilities of tea components are not known. Therefore, studies on the absorption, distribution and metabolism of key components from green and black teas in animals and humans are of great importance.

Definit answers on the deleterious or beneficial effects of tea consumption have to come from studies with human populations. Because the causative factors are different for different cancers, or for the same cancer in different populations, tea consumption may effect carcinogenesis only in selected situations rather than have a general effect on all cancers. Therefore, in future epidemiology studies, it is important to consider the etiologic factors of the specific cancers and to collect more specific information on the qualitative and quantitative aspects of tea consumption.

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Chapter 42

PHARMACOTHERAPEUTICS OF TEA : PROPOSED STRATEGIES AND PHARMACOLOGICAL STUDIES

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Dr. Tanya Das of the Bose Institute, Kolkata. Dr. Das received her Master's degree in Biochemistry and Ph.D. in Protein Chemistry from Calcutta University, followed by Post-doctoral research in Signal Transduction in the Cleveland Clinic Foundation, Ohio, U.S.A. After a short stint as pool officer at IICB, she joined Bose Institute, where she is currently a senior Lecturer in the Animal Physiology Section. Dr. Das is a Visiting Scientist at Cleveland Clinic Foundation where she collaborates with Dr. J. Fink, who is a pioneer in renal cancer and immunity.



Dr. Das' group is exploring the possibility of cancer chemoprevention by dietary phytochemicals and has shown that plant products like black tea can selectively kill cancer cells without harming the immune, hepatic and detoxification systems of the host. Dr. Das is also investigating the molecular mechanisms underlying such novel action of this widely accepted beverage.

ABSTRACT

During cancer, due to the disease itself or due to continuous exposure to various toxic drugs, the intrinsic defense systems, e.g. immune and detoxification systems, of the host become jeopardized thereby making cancer therapy unsuccessful. Thus, identification of any anti-cancer agent having no toxic effect on the host's defense machineries is of immense importance. Next to water, tea is the most ancient and widely consumed beverage in the world. Epidemiological studies have suggested its cancer preventive effect, but the results obtained so far are not conclusive. In the current study, mechanisms of black tea-induced cancer killing were delineated. Our results signified that black tea-induced apoptogenic signals, involving p53 and Bax, overrode the growth-arresting message of p21 in Ehrlich's Ascites Carcinoma (EAC) cells thereby leading to EAC apoptosis. Importantly, this popular beverage was not toxic by itself; additionally it regressed tumor-induced toxicity and improved suppressed immune system in host. Further studies revealed that EAC burden increased the expression of pro-apoptotic proteins, p53 and Bax, in splenic lymphocytes while decreasing significantly the level of pro-proliferative protein Bcl-2. Interestingly, black tea could down-regulate p53 and Bax, while augmenting Bcl-2 in these cells. As a result, Bcl-2/Bax ratio was increased and immunocytes were protected from tumor-induced apoptosis. Moreover, activities of hepatotoxic marker enzymes, e.g., SGOT, SGPT and ALP, that were elevated due to EAC-induced toxic stress, brought back almost to normal values by black tea. Thus black tea was successful in inducing tumor cell apoptosis and reducing immuno-suppression and general toxicity of tumor bearer.

Keywords: Stress; black tea; tumor; toxicity; immunity; apoptosis

The abbreviations used are: Alkaline phosphatase, ALP; Ehrlich's ascites carcinoma, EAC; fluorescein isothiocyanate, FITC; fluorescent-activated cell sorter, FACS; glutathione-S-transferases, GST; propidium iodide; PI; serum glutamate oxalo-acetate transaminase, SGOT; serum glutamate pyruvate transaminase, SGPT; Superoxide dismutase, SOD.

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INTRODUCTION

Cancer is one of the leading causes of death in industrialized nations. The mechanisms of carcinogenesis involve multiple stages of biochemical and molecular alterations in target cells (1). Cancers are caused by the progressive growth of the progeny of a single transformed cell. Therefore, curing cancer requires that all the malignant cells be removed or destroyed without killing the normal cells of the patient.

Cancer is known to cause massive immunosuppression and toxicity (2). Moreover, anti-tumor treatments can also have a detrimental impact on host immunity and detoxification systems (3). The tumor microenvironment also influences the functional potential of immune cells. Certain cancer cells may secrete immunosuppressive factors to modify the host immune responses (4-8), e.g. increased production of immunosuppressive interleukin-10 with simultaneous suppression of IL-2. The impaired anti-tumor immunity can also be related to defective immune regulation (8). Immune-effector cells obtained from the tumor-bearer have also been reported to have a variety of functional abnormalities which may vary in magnitude and may be related to extent of disease (9-11). Thus, it appears that immuno-inhibitory influence of the tumor might become a systemic effect in cancer-bearing host. The immune cells may be actively dying in host with cancer and the rapid turnover of these carcinoma cells occurs as a consequence of the process. Thus, the ability to assess the level of this turnover might be viewed as an important component of or indicator for therapeutic intervention aimed at the protection of immune cells from apoptosis. A number of studies have raised the possibility that tumors of both mouse and human origin can evade immune surveillance activity by delivering apoptotic death signals to lymphocyte (12).

On the other hand, hepatotoxic marker enzymes like serum glutamate oxalo-acetate transaminase (SGOT), serum glutamate pyruvate transaminase

(SGPT) and serum alkaline phosphatase (ALP) activities are often elevated (13). Status of antioxidant enzymes, i.e. superoxide dismutase (SOD), catalase, glutathione-S-transferases (GST), peroxidases etc., which protect cells against oxidative damage, are almost invariably altered during carcinogenesis as well as during drug therapy thereby ultimately leading to physiological catastrophe. Successful cancer therapy has, therefore, still remained an unfulfilled dream to scientists worldwide (14). In this regard, dietary phytochemicals, which are non-toxic to the normal cells of the tumor bearer, have gained immense importance.

Cancer chemoprevention can be defined as the prevention, inhibition or reversal of carcinogenesis by administration of one or more chemical entities, either as individual drugs or as naturally occurring constituents of the diet. Next to water, tea is the most ancient and widely consumed beverage in the world. Epidemiologically green tea has been shown to possess cancer chemo-preventive effects in a wide range of target tumors in rodents (15). Black tea has also been shown to inhibit tumorigenesis in animal model systems, including lung (16), colon (17) and skin (18). However, besides some scattered reports and supporting epidemiological evidences on the protective role of black tea, the detailed molecular mechanisms underlying the anticancer effect are still unclear and inconclusive although the worldwide production and consumption of black tea far exceeds that of green tea (19). Here we report some aspects of black tea in amelioration of perturbations found in tumor bearing host along with its tumoricidal mechanism.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, fetal bovine serum, streptomycin and penicillin were purchased from Gibco BRL (Gaithersburg, MD). RNase A, 4',6'-diamidino-2-phenylindole (DAPI), Ficol-hypaque from Pharmacia Fine Chemicals, Sweden and

general reagents were purchased from Sigma (St. Louis, MO). Black tea leaves were obtained from Lipton Tea Co., India. Cycle TEST PLUS DNA reagent kit and Apo-Direct kit were procured from Becton Dickinson Immunocytometry system, San Jose, CA. Polyclonal anti-Bax, anti-Bcl-2, anti-p21, anti-p53, anti-CD16 antibodies were obtained from Pharmingen, San Jose, CA. The remaining chemicals were purchased from local firms (India) and were of highest purity grade.

Black Tea Preparation

Black tea leaves were brewed in hot water (w/v) to obtain black tea extract. Animals were given either drinking water or black tea extract as sole source of drinking fluid at doses of 0.625%, 1.25%, 2.5%, 3.75%, 5% (w/v) one week before tumor inoculation till sacrifice.

Mice And Tumor Models

All animal experiments were performed following "Principles of laboratory animal care" (NIH publication No. 85-23, revised in 1985) as well as specific Indian laws on "Protection of Animals" under the provision of authorized investigators. Swiss albino mice (~20 g each; ten mice in each group) were randomly divided into different groups including (i) normal set (non-tumor-bearing), (ii) tumor-bearing set (which were intra-peritoneally injected with 1×10^5 exponentially grown EAC and (iii) black tea-treated tumor-bearing set. Animals were given either drinking water or black tea extract as sole source of drinking fluid at different concentrations. Different doses of black tea have been started 7 days prior to tumor inoculation and continued till sacrifice (21st day). Black tea leaves were brewed in 100 ml of hot water (w/v) to obtain black tea extract.

Isolation of EAC and Splenic Lymphocytes

Lymphocytes from spleen and, EAC cells from peri-toneal cavity were removed aseptically and single cell suspension was made in RPMI 1640 medium, freed from adherent cells and viable cells were counted by trypan blue exclusion test. The

non-adherent splenic cell populations were subjected to Ficol-hypaque density gradient separation to obtain mononuclear cell-rich population (20). Spleens were removed aseptically and single cell suspension was made in RPMI 1640 medium. Splenic lymphocytes were purified as described earlier (21). Viable splenic lymphocytes and thymocytes were counted in hemocytometer by trypan blue exclusion test and used for further analysis.

Detection of Cell Cycle by Flowcytometry

For the determination of cell cycle phase distribution, EAC cells or splenocytes as harvested from tumor-bearing mice were fixed with *p*-formaldehyde, permeabilized with TritonX 100, and nuclear DNA was labeled with propidium iodide (PI) using Cycle TEST PLUS DNA reagent kit. Cell cycle phase distribution of nuclear DNA was determined on FACS (fluorescence-activated cell sorter), fluorescence detector equipped with 488 nm Argon laser light source and 623 nm band pass filter (linear scale) using CellQuest software (Becton Dickinson). Total 10,000 events were acquired and analysis of flowcytometric data was performed using Mod Fit software. Histogram display of DNA content (x-axis, PI-fluorescence) versus counts (y-axis) has been displayed.

Annexin v-FITC Assay

To distinguish between apoptosis and necrosis, in a double labeling system, EAC cells (1×10^6 cells in each case) from untreated- or black tea-treated tumor-bearing mice were harvested and PI and Annexin V Fluos were added directly to the medium. The mixture was incubated for 15 min at 37 °C. Excess PI and Annexin V Fluos were then washed off, cells were fixed and then analyzed on flowcytometer (equipped with 488 nm Argon laser light source; 515 nm band pass filter for FITC-fluorescence and 623 nm band pass filter for PI-fluorescence) using CellQuest software. Electronic compensation of the instrument was done to exclude overlapping of the emission spectra. Total 10,000 events were acquired and the cells were properly gated for analysis.

Western Blotting

For Western blot analysis of p53, p21, Bax and Bcl-2, EAC or splenocyte lysate was loaded onto a 10% SDS-poly-acrylamide gel. After electrophoresis the gel was transferred to nitrocellulose membrane and blocked with nonfat milk in PBS containing Tween-20 prior to antibody treatments. The protein of interest was visualized with chemiluminescence. The blot was then stripped and after extensive washing, the blot was used again for probing the next molecule. Equal loading of protein in each lane was confirmed by probing with actin antibody.

Assay of Alkaline Phosphatase

During the treatment, on day 21 blood samples were drawn from the supraorbital sinuses from the mice and serum was separated for the estimation of alkaline phosphatase (ALP) according to standard protocols (22).

Assays of SGOT and SGPT

The marker serum-enzymes of liver toxicity, e.g., serum glutamate oxalo-acetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) were assayed in our tumor-bearing mice model with or without black tea extract treatment following the method protocol used by Talib *et al.* (22).

RESULTS

Effect of Black Tea on Tumor Cell Number

We evaluated the effect of black tea on the EAC cell number in black tea-treated or untreated tumor-bearing mice. Mice who have been given black tea as the sole source of drinking fluid showed a decrease in the number of tumor cells over their control counterparts. It was observed that black tea lessened the tumor burden considerably in a dose-dependent manner showing significant effect at 2.5% (w/v). At day 21 a total of 460×10^6 EAC cells were measured in the peritoneal fluid of untreated mice, whereas in 2.5% dose black tea-treated group only 80×10^6 EAC cells were found (Table I).

Table I. Effect of black tea on Ehrlich's ascites carcinoma in tumor-bearing mice

Doses of Black tea (w/v)	EAC Number ($\times 10^6$)		
	1 st Week	2 nd Week	3 rd Week
0.00%	110.02 \pm 7.51	312.00 \pm 15.34	460.00 \pm 38.51
0.625%	90.06 \pm 7.32	188.04 \pm 8.94	230.00 \pm 12.10
1.25%	77.03 \pm 5.96	164.60 \pm 12.38	146.00 \pm 4.40
2.50%	52.60 \pm 4.88	94.06 \pm 4.58	80.00 \pm 3.04
3.75%	44.06 \pm 8.63	82.04 \pm 11.26	57.00 \pm 10.12
5.00%	31.08 \pm 9.46	60.28 \pm 7.34	46.00 \pm 2.10

Swiss albino mice were intra-peritoneally injected with 1×10^6 EAC. After one week, various doses of black tea were administered orally. Control mice received drinking water as carrier vehicle. At the end of each week viable EAC cells were counted. Each group contained ten mice and the values are mean \pm S.E.M.

Effect of Black Tea on Splenic Lymphocyte Count

We evaluated the effect of black tea on the numbers of splenic lymphocytes in black tea-treated or untreated normal as well as tumor-bearing mice. Normal mice that have been given black tea as the sole source of drinking fluid showed no significant change in the number of splenic lymphocytes and thymocytes over their control counterparts (Table II) indicating that black tea is not immunotoxic itself at the dose used for these experiments. In EAC bearing mice, significant reduction was observed in numbers of splenic lymphocytes in comparison to normal mice (Table II). Such depression in immunocyte numbers in the tumor bearing mice was ameliorated by black tea at its anticancer dose, i.e., 2.5%. Interestingly, at higher doses, again a decrease in the numbers of splenic lymphocytes was observed (Table-II). Therefore, further studies were performed using 2.5% black tea extract.

Table-2. Effect of black tea on splenic lymphocyte numbers in tumor-bearing mice

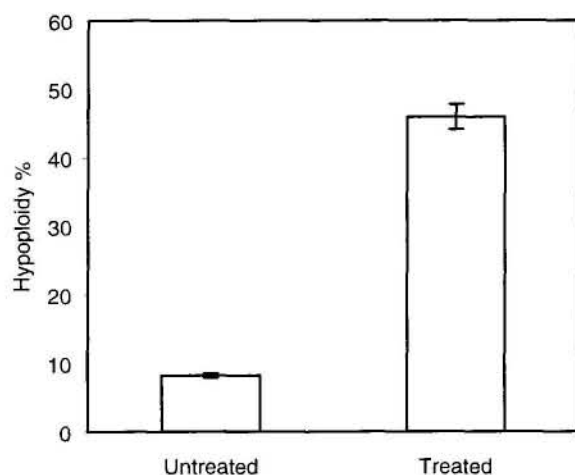
Treatment	Splenic lymphocyte Number ($\times 10^6$)
Normal	164 \pm 10.2
Normal +Tea (2.5%)	158 \pm 5.6
EAC	81.4 \pm 4.4
EAC +Tea (2.5%)	144.6 \pm 8.4

Swiss albino mice were intra-peritoneally injected with 1×10^6 EAC. One week before inoculation, black tea feeding was started and continued up to sacrifice. Normal mice received drinking water instead of black tea. At the end of each week,

mice were sacrificed and viable splenic lymphocytes were counted. Each group contained ten mice and the values are mean \pm S.E.M.

Flowcytometric Analysis of EAC Cell Cycle Phase Distribution

Fig. 1. Flowcytometric analysis of EAC hypoploidy percentages. (EAC cells from tumor-bearing mice orally fed with drinking water as carrier vehicle or black tea were fixed and nuclear DNA was labeled with PI. Cell cycle phase distribution of EAC nuclear DNA was determined by single label flow-cytometry.)



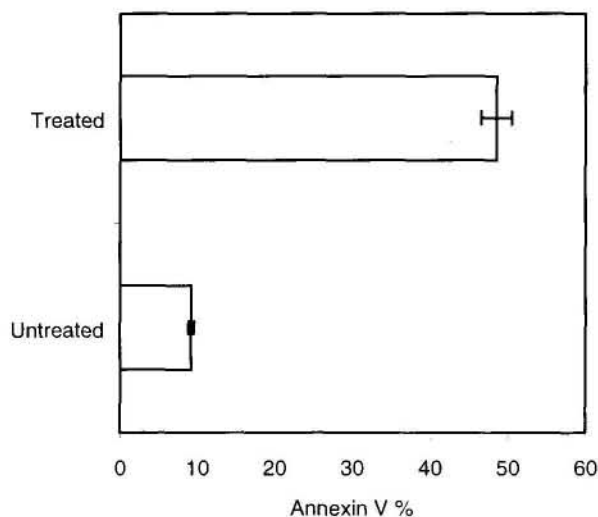
To find out the mechanism of tumor cell killing by black tea extract we exploited fluorescence activated cell sorter and analyzed tumor cell cycle phase distribution. The FACS data described the effect of black tea extract on cell cycle phase distribution of EAC DNA. On 21th day after EAC inoculation, at 2.5% dose, content of hypoploid DNA (8.1%) before treatment was increased (45.8%) (Fig.1). These results suggested the break-down of EAC DNA. The obvious ramification was EAC apoptosis.

Black Tea Induces EAC Apoptosis, Not Necrosis

To further confirm the nature of cell death, we utilized double labeling techniques using Annexin V Fluos/PI to distinguish between apoptotic and

necrotic cells. In early stages of apoptosis, while the cell membrane is still intact and impermeable to PI, phosphatidylserine to which Annexin V binds specifically is translocated to the extracellular side of the membrane. In contrast, during necrosis because the cell membrane is ruptured, these cells take up both the stains. Our flowcytometric data revealed that, in comparison to control untreated EAC cells (Fig. 2), black tea treated unfixed EAC cells showed Annexin V-FITC binding but no PI staining (data not shown) indicating that the mode of cell death is apoptosis but not necrosis. These results indicated that black tea could increase apoptosis in EAC cells. It also suggested that necrosis was not the cause of cellular killing in EAC.

Fig. 2. Distinction between apoptosis and necrosis. (In a double label system, unfixed EACs from control or black tea treated tumor-bearing mice were labeled with PI and Annexin V Fluos and then fixed and analyzed on a flowcytometer. Histogram statistics of percentage Annexin V positive cells have been represented.)

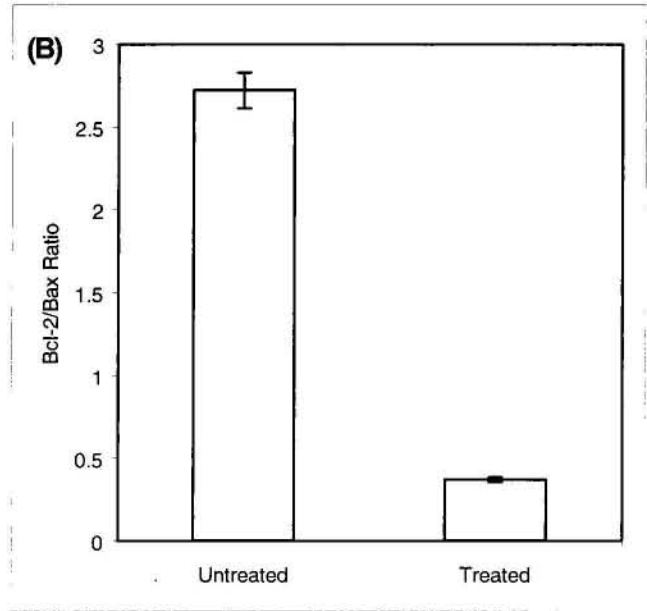
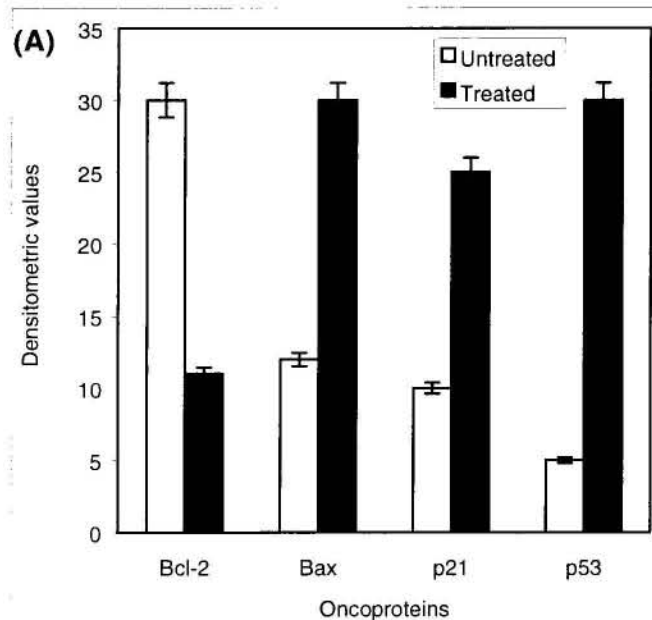


Effect of Black Tea on the Expression of Pro- and Anti-Apoptotic Proteins

After confirming that black tea induces EAC apoptosis, we next attempted to unveil the

mechanism of EAC killing. Since it is well recognized that various pro- and anti-apoptotic proteins play crucial role in programmed cell death, we examined whether or not black tea has any effect on the expression of growth arresting and pro-apoptotic proteins, p53, p21 and Bax as well as anti-apoptotic protein, Bcl-2, in our mice model. Using Western blot analysis, we observed that in EAC, the levels of p53 and Bax expression increased significantly (Fig. 3A) and level of p21 increased moderately (Fig. 3A) after black tea treatment. Interestingly, the level of Bcl-2 decreased upon black tea treatment (Fig. 3A) thereby resulting in a decrease in Bcl-2/Bax ratio (Fig. 3B) that further confirmed our flowcytometric data and supported the notion that as a result of black tea treatment the balance between positive and negative regulators of apoptosis was shifted towards cell death.

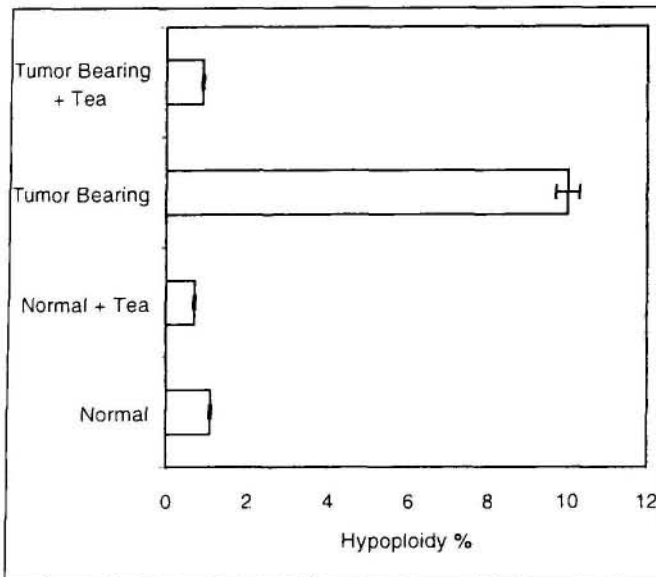
Fig. 3. Effect of black tea on the expression of Bcl-2, Bax, p53 and p21 in EAC by Western blot analysis. (A) Densitometric values are represented as histogram statistics of the different oncoproteins in untreated and treated EAC lysates. (B) Bcl-2/Bax ratio represented in histogram statistics in untreated and treated EAC cells.



Flowcytometric Analysis of Splenic Lymphocyte Cell Cycle Phase Distribution

To find out the mechanism of immuno-restoration by black tea extract we analyzed cell cycle phase distribution of DNA in splenic lymphocytes of normal and tumor-bearing mice by flowcytometry. On 21st day after EAC inoculation, in comparison to the normal one (Fig. 4), content of hypoploid DNA (<2n DNA) was highly increased (Fig. 4). On the other hand black tea, at 2.5% dose, could decrease the percent of sub-G0/G1 population of splenic lymphocytes, and increased the same in G0/G1 phases as well as in S and G2/M (Fig. 4) phases in EAC-bearing mice indicating the amelioration of tumor-induced immunosuppression by black tea at its anti-tumor dose. The obvious ramifications were the survival of splenic lymphocytes. Importantly, black tea itself showed no immunotoxic effect as revealed by flowcytometric data on splenic lymphocytes of black tea-treated normal mice (Fig. 4).

Fig. 4. Flowcytometric analysis of splenocyte hyploidy percentages represented as histogram statistics. (Splenocytes from normal, untreated tumor bearing and tumor-bearing mice treated with black tea were fixed and nuclear DNA was labeled with PI. Cell cycle phase distribution of splenocytes nuclear DNA was determined by single label flowcytometry)

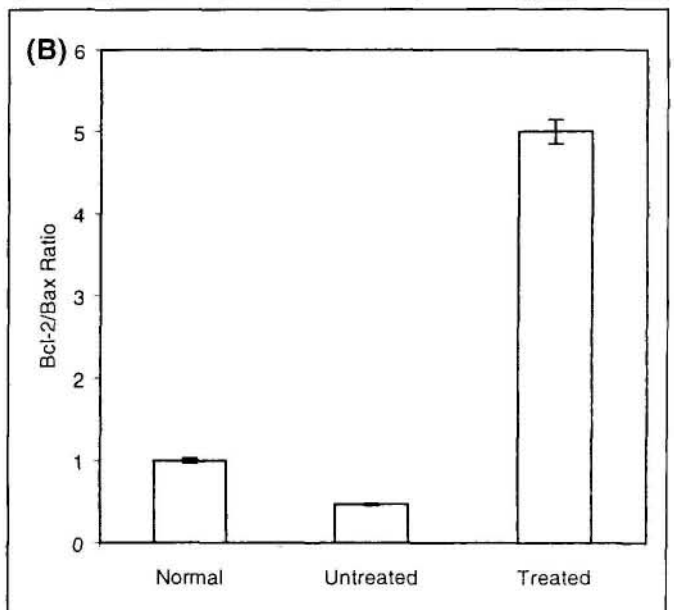
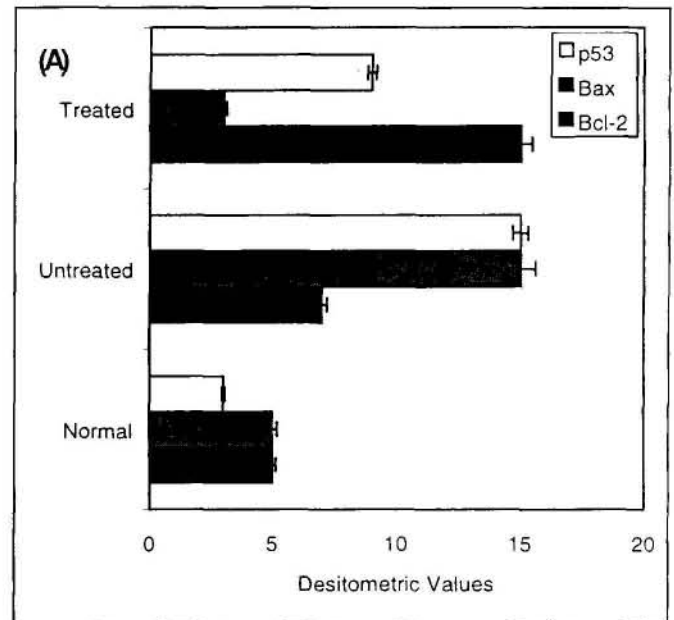


Effect of Black Tea on Expression of Pro- and Anti-Apoptotic Proteins in Splenic Lymphocytes

After confirming that black tea prevents splenic lymphocytes from undergoing EAC-induced apoptosis, we next attempted to unveil the underlying mechanisms. Since it is well recognized that various pro- and anti-apoptotic proteins play crucial role in cell survival as well as in programmed cell death, we examined whether or not black tea has any effect on the expressions of growth-arresting and pro-apoptotic proteins, p53 and Bax as well as anti-apoptotic protein, Bcl-2, in our mice model. Using western blot analysis, we found that in splenic lymphocytes, the levels of p53 and Bax protein expressions increased significantly (Fig. 5A) in tumor-bearing mice in comparison to the splenic lymphocyte of the normal mice. However, black tea treatment (at 2.5% dose) could decrease the levels almost to the normal values. Interestingly, the level of Bcl-

2, which remained same in splenic lymphocytes of normal as well as tumor-bearing mice, increased upon black tea treatment thereby resulting in an increase in Bcl-2/Bax ratio (Fig. 5B).

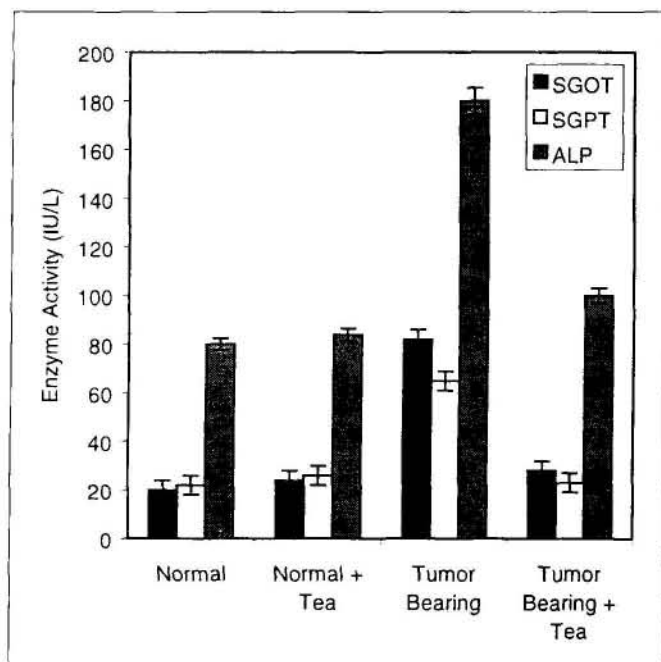
Fig. 5. Effect of black tea on the expression of Bcl-2, Bax, p53 and p21 in splenocytes from normal, untreated tumor bearing and tumor-bearing mice treated with black tea. (A) Densitometric values are represented as histogram statistics of the different oncoproteins. (B) Bcl-2/Bax ratio represented in histogram statistics.



Effect of Black Tea on Tumor-Induced Hepatic Toxicity

To evaluate the effect of anti-tumor dose of black tea on tumor-induced liver toxicity, we measured the activities of different marker enzymes of liver toxicity. As shown in Figure 6, activities of SGOT, SGPT and ALP were significantly increased in EAC-bearing mice serum in comparison to normal mice. This indicates that tumor itself renders toxic insult to these mice. Treatment with the anti-tumor dose, i.e., 2.5%, could reduce the toxicity levels almost to the normal ones. Black tea itself had no effect on the host system when treated to normal mice unlike other traditional drugs (Fig. 6).

Figure 6. Effect of black tea on serum toxicity marker enzymes in tumor-bearing mice. (Serum glutamate-oxaloacetate (SGOT), glutamate-pyruvate transaminase (GPT) and alkaline phosphatase (ALP) activities were biochemically assessed as described in Methods from serum of normal, normal treated with black tea, untreated tumor bearing and black tea treated tumor bearing mice. The data represented are mean \pm SEM of three independent experiments.)



DISCUSSION

Epidemiological surveys and experimental studies have provided evidence that environmental factors, including dietary substances, play a major role in the regression of cancer (23). Various findings suggest that tea and its polyphenolic components possess anti-cancer effect (24). Green tea and its polyphenolic components exhibit growth inhibitory effect in Ehrlich ascites tumor cells (25). Aqueous extract of black tea has been shown to inhibit Ehrlich Ascites tumor growth and increase survival in mice (26). Some reports, although not conclusive, have suggested the protective role of black tea against carcinogenesis. Results of this present study suggest that black tea treatment has apoptogenic effects on EAC cells grown in the peritoneal cavity of Swiss albino mice.

Our studies have shown that black tea was effective in imparting growth inhibition, cell cycle deregulation and apoptosis in EAC cells. It is now well recognized that whether a cell becomes committed to apoptosis partly depends upon the balance between proteins that mediate growth arrest and cell death, e.g., p53, p21, Bax, and proteins that promote cell viability, e.g., Bcl-2 (27, 28). Among these proteins, p53 has been found to facilitate apoptosis in various cell types. In our study black tea increased the p53 expression. It is known that p53 may induce two sets of genes in response to stress signals thereby contributing to the decision making between growth arrest and apoptosis. This tumor suppressor protein may up-regulate the pro-apoptotic protein Bax in one hand, and/or mediate growth arrest involving p21 as a major effector on the other (29). In fact, p53-dependent induction of p21 prevents entry of the cells into S phase (30, 31). Our results indicated that p21 level increases after black tea treatment. The other set of gene products downstream to p53, e.g. Bax, induces apoptosis (32). In fact, overexpression of Bax, an effect that is associated with the formation of Bax/Bax homodimers, has been shown to accelerate the cell death of murine FL5.12 cells after interleukin 3-withdrawal (33). In our system, black tea

treatment decreased the expression level of Bcl-2 and increased Bax concentration thereby decreasing the Bcl-2/Bax ratio in these cells. All these observations establish the relationship between p53 status, p21 induction, Bcl-2/Bax ratio, cell cycle deregulation and apoptosis in black tea-treated tumor cells. To conclude, these results imply an apoptosis enhancing capability of black tea in EAC that was affected by modulating the tumor cell cycle as well as the balance between pro- and anti-apoptotic factors.

Tumor-induced massive depletion in immune cell numbers has already been reported by many scientists (34). In our EAC-bearing mice model, marked reduction in splenic lymphocytes were observed reconfirming that the development of cancer impaired normal lymphocyte development. We have observed that at the doses beyond 2.5%, there was marked reduction in splenic cell counts indicating immunotoxicity. Our report already showed that at doses beyond 2.5%, activities of serum glutamate oxaloacetate transaminase and serum glutamate pyruvate transaminase, the marker enzymes of liver toxicity, increased significantly in mice serum (35). This report is indicative of toxicity in the animals at higher doses of black tea, which is also reflected in our observation here regarding immunotoxicity.

A search for the underlying mechanism revealed apoptosis as the cause of EAC-induced immune cell death in spleen. These results were supported by the report of Nagarkatti *et al.* (36) who also observed apoptosis of immune cells in LSA tumor-bearing mice. Interestingly, 2.5% black tea down-regulated the apoptotic signal and promoted the survival of splenic lymphocytes. Shifting of balance between various signals, rather cross talk between pro and anti-apoptotic signals perturbed the apoptotic environment and helped to enjoy the full gamut of trophic support that may ensure immunocyte survival in cancer bearing host (37). In this regard, it is well accepted that tumor suppressor p53 plays key role in deciding a cell's

fate. In fact, increased apoptosis in spleen and thymus was found to be associated with enhanced expression of p53 (38). Atrophic changes along with apoptosis was observed in spleen in p53(+/+) mice whereas no vigorous cell death was induced in p53(-/-) mice (39). Our study indicated that in splenic lymphocytes of untreated tumor-bearing mice that were underwent apoptotic death, p53 expression was high. Interestingly, 2.5% black tea decreased p53 level in spleen significantly. It is now also well recognized that the fate of any cell also depends, at least partly, upon the balance between the pro- and anti-apoptotic proteins of Bcl-2 family, e.g., Bax and Bcl-2. Bcl-2 deficient mice has been found to undergo severe lymphoid apoptosis in spleen (40, 41). On the other hand, increase in Bax expression has been reported to promote apoptosis in spleen (42). In our tumor model, Bax level was highly increased in splenic lymphocyte as compared to normal mice. Interestingly, black tea treatment again decreased the Bax level almost to the normal in both cell types thereby again increasing Bcl-2/Bax ratio. Considering these observations as well as the previous results on p53 status, it can be concluded that black tea could ultimately change the balance between pro-and anti-apoptotic oncoproteins in favor of cell survival as a result of which immune cells were protected the from tumor-induced apoptosis.

In case of cancer chemotherapy, various popular and effective drugs exert concurrent toxic manifestations, including oxidative stress and liver damage in the tumor bearer. As a result SGOT, SGPT and ALP are elevated in serum (43). Ultimately, the increase in SGOT and SGPT activities of the host gets manifested (44). We have found that in untreated EAC bearing mice, levels of serum SGOT, SGPT, ALP as well as bilirubin were high and were brought down to the normal levels by the continued treatment of black tea. Reversal of tumor-induced hepatotoxicity by black tea indicates its role as a scavenger that provides the hepatoprotective effect to the host to fight the toxicity generated by the developing tumor. Such

protective effect of black tea may be due to the healing of liver parenchyma and regeneration of hepatic cells. Our results directly showing the detoxifying effect of black tea will, therefore, be of immense help in enriching the knowledge so far as the designing of a potential anti-toxic anti-cancer drug is concerned.

Overall, our findings support that black tea not only induces apoptosis in tumor cells, but also ameliorates tumor-induced immunosuppression and decreases general toxicity of the tumor bearer. Reversal of tumor-induced hepatotoxicity by black tea indicates its role as a scavenger that provides the hepatoprotective effect to the host to fight the toxicity generated by the developing tumor and served as a 'protector' at the level of immune cell apoptosis in cancer-bearing host. The knowledge gathered from these studies might lead to recognize this beverage as a successful anti-cancer agent in future.

ACKNOWLEDGEMENTS

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Concluding Remarks by Session Chairman Prof. B.N. Dhawan

Thank you Dr. Das, I could not think of a better finale to these sessions on tea and health. We have learnt so much in the last technical session. All of you would agree that we had so much of new, exciting data. There were a few points that came out in sharp focus. Dr. Tanya Das's presentation confirmed what was earlier said by Dr. Juneja, that the effect of tea is very selective on the test organism. It affects the cancer cells but the affect is much less or different on the normal cells. Secondly, her presentation very clearly brought out the array of powerful tools that we have available now to analyze and to dissect biological activity. In a way this marriage between ancient wisdom and the modern tools is going to be very useful in understanding the intricacies of the mechanisms involved. Finally her presentation showed that nothing is absolutely safe. If you take a very large amount of tea or give a very large concentration of catechins, you do get toxic effects: so there is safety limit, which has to be determined for tea intake.

Dr. Jaswant Singh talked about the importance of GMP norms. Good manufacturing norms should be established, there must be SOP'S and these must be strictly followed. It is for tea industry to see as to how this is to be done because there are some other areas where similar standards have been adopted. For example, most of labs are now GLP (good laboratory practices) compliant. They have their SOP's and they are certified labs. Something similar has to be done with those industries, which are not adopting this extremely important practice. He also gave you a general road map of developing drugs from natural products. However, he did not give you the time frame. It takes several years and the cost is very high. Dr Jaswant Singh was talking of Hepto protective activity. The major problem in the western world is alcohol related liver damage, the incidence of which is increasing. There are good animal models to show that tea drinking prevents alcohol induced liver damage. If data can be generated for a country like Germany, who are looking for agents of this type, it can be tried in their population. This issue should be actively promoted. He also talked about bio-availability enhancer, for which there is data available. With anti-cancer drugs and antibiotics, the compounds from tea prevent the development of resistance to them and increase sensitivity to these drugs. So in the presence of compounds of tea, at least some of the anti-biotics and anti- cancer drugs become more effective. This is the situation where all that one could do was not give tea as a drug. But just tell them that instead of taking their medicine with water take it with a cup of tea, and the efficacy may go up. That's something that needs to be studied. Yet again both of them talked about black tea, which was good from Indian point of view.

A final point, forgotten by Dr. Jaswant Singh, is that CSIR has a system where, if the industry works on developing a product with a CSIR Lab, they cover the cost element of CSIR Lab. The industry meets its own cost. Industry is a partner and CSIR shares the cost of expenditure, which would have been normally borne by the industry. You have seen the very powerful tools that are available at both the labs — Jammu and Cancer Research Center of Bose Institute. Despite the reduced cost, the industry will still share the IPR. So the industry shares the patents and the benefits thereof while a part of cost is met by CSIR. This should be focused again in the valedictory session where Dr. Jain should initiate it. I feel that the industry should seriously assess how they can collaborate with not only competent people like Dr. Jaswant Singh and Dr. Tanya Das but also access some very powerful tools available, without bearing the entire cost burden.

Leaving aside commercial aspects, let us develop the theme of how we can improve the optimal utilization of tea and how tea can play a positive role in providing better health and health care to the people. That is the message I would like to go before concluding this session. I would like to thank the speakers for the very exciting findings. I also thank the organizers who gave me this extra time and the opportunity to say things that I could not mention earlier. Thank you very much indeed. I announce that the session is over.