MECHANISM OF ACTION OF NATURALLY OCCURRING ANTIOXIDANTS

(Studies on oxidative degradation of DNA by green tea)

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

BIOCHEMISTRY

BY

ARSHI MALIK

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF LIFE SCIENCES
ALIGARH MUSLIM UNIVERSITY
ALIGARH (INDIA)

2002

TELEPHONE : 400741
TELEX : 564-230 AMU IN

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF LIFE SCIENCES
ALIGARH MUSLIM UNIVERSITY
ALIGARH-202002 (INDIA)

Ref. No.....

Dated.....31.10.02.....

CERTIFICATE

I certify that the work presented in this thesis has been carried out by **Mr. Arshi Malik** under my supervision. It is original in nature and has not been submitted for any other degree.

Sadhi

(S.M. HADI)
Professor

As a token of love

and

deepest affection

to

my family

Acknowledgements

It is said that a man never gets more than his destiny. But I am indebted to the omnipotent master of the universe – The Almighty – for giving me more than my share. I thank him for giving me parents who sacrificed their own comforts in making me what I am. I thank Him also for giving me teachers who forgave my unpardonable mistakes and guided me out of darkness by their own radiant light.

I owe a deep sense of gratitude to my supervisor, ***Prof. S.M. Hadi*** *for providing excellent research training. It has indeed been an honour and a memorable experience working with the well-learned person that he is.*

Prof. Masood Ahmad*, Chairman and all faculty members of the Department of Biochemistry are acknowledged for their help and cooperation.*

My most sincere thanks to Mr. Suhail Akhtar for all the motivation, encouragement and for the genuine concern and brotherly attitude.

Thanks to my lab colleagues, Dr. Farhan Asad, Dr. Saurabh Singh and Asfar for help at the hour of need. My regards to Dr. Aamir Ahmad for his precious company and valuable suggestions.

Naghma, Ms. Samina Wasi and Dr. Athar H. Siddiqui are sincerely acknowledged for all the support and help.

My sincere thanks to Pawan, Qamar bhai and Kashif for helping me at various stages of the research work.

I gratefully acknowledge Dr. Anees A. Banday, Dr. Shaukat A. Khan, Dr. Sandeep Sinha, Dr. Shakeel A. Khan, Dr. Waseem A. Siddiqui and Dr. Atia Sheereen for their unselfish help and support.

Thanks to Shahid, Amjad, Kaleem, Tariq, Sandeep, Arivarasu, Dr. Moyyad, Dr. Mansour, Dr. Iyad, Sadaf apa, Sabiha apa, Hina apa, Rana, Sonali, Kahkashan, Riffat, Fouzia, Shema, Iram, Irfana and Shamila for their help throughout my stay in the department.

The non-teaching and technical staff of the department as well as the seminar staff shall be remembered for the kind cooperation.

Last but not the least, I would like to acknowledge Abba, Amma, Faizi and Sarah for their prayers and well wishes and it is to them that I dedicate this work.

(Arshi Malik)

CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

Summary

SUMMARY

In recent years a number of reports have documented the chemopreventive effect of green tea consumption on various types of cancers such as those of bladder, prostate, esophagus and stomach. However, such chemopreventive effect is not attributed to the consumption of black tea to the same extent. Gallotannins such as epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG) and epigallocatechin (EGC) are the major polyphenolic constituents of green tea. Of particular interest is the observation that EGCG and its structural constituent gallic acid induce apoptosis in cancer cell lines but not in normal cells. Thus the chemopreventive property of green tea is ascribed to its polyphenol content. Several lines of evidence in the literature strongly suggest that it is the prooxidart action of plant-derived polyphenolics rather than their antioxidant activity that may be an important mechanism for their anticancer and apoptosis inducing properties. Thus a mechanism for the cytotoxic action of polyphenols against cancer cells that involves mobilization of endogenous copper and the consequent prooxidant action has been proposed.

In partial support of this idea, in part I of the thesis I have shown that the water extract of green tea is considerably more efficient than black tea extract in oxidative DNA cleavage in the presence of copper ions. In order to examine, the involvement of Cu(I) in the reaction I have used neocuproine as an agent that sequesters Cu(I) selectively. Green

and black tea extracts reduce Cu(II) to generate Cu(I) as evidenced by the formation of complex with neocuproine absorbing at 450nm. The greater rate of Cu(II) reduction by green tea is possibly accounted by the presence of polyphenols such as epicatechin, epigallocatechin and epigallocatechin-3-gallate. These polyphenols are absent in black tea. Further, the results suggest a possibly redox cycling of copper ions in the reaction. Of the several metal ions tested, only Cu(II) and Fe(II) complemented the tea extracts in the DNA breakage reaction to a significant extent. The higher rate of hydroxyl radical production and superoxide generation by green tea extract correlates with the DNA degradation activity. Inhibition of DNA breakage by tea extracts in the presence of thiourea, catalase, sodium azide and superoxide dismutase established that active oxygen species are involved in the cleavage reaction. Further, I have examined the biological activity of the reaction by examining the viability of bacteriophage T_4. The results indicate that green tea – Cu(II) mediated reaction also causes bacteriophage inactivation through a mechanism similar to DNA breakage. This is particularly suggested by the effect of reactive oxygen scavengers on phage inactivation and the fact that phage inactivation is inhibited by neocuproine which sequesters Cu(I). It was further shown that UV treatment of host bacteria enhances the recovery of the phage, suggesting the involvement of UV inducible pathway in the repair of green tea- Cu(II) mediated damage of phage DNA.

As already mentioned, gallic acid is a structural constituent of tannins and polyphenols of green tea. Similar to green tea it also exhibits antioxidant and prooxidant properties. Thus, with the aim of identifying the structural features of green tea polyphenols, I have compared in part II of the thesis the prooxidant DNA cleavage properties and antioxidant properties of gallic acid and green tea. The rate of oxidative DNA degradation in the presence of Cu(II) by gallic acid is considerably greater than by green tea extract. This correlates with the higher rate of hydroxyl radical and superoxide anion generation by gallic acid. In order to determine the relative antioxidant property of gallic acid and green tea extract, the effect of the two compounds on Fe(II) EDTA (hydroxyl radical generating system) and riboflavin – light (singlet oxygen generating system) mediated DNA degrading system was examined. Green tea was found to be better inhibitor of DNA breakage by both the systems as compared to gallic acid.

As evidenced by its capacity to degrade DNA in presence of Cu(II) and to generate hydroxyl radicals studies in previous section showed that gallic acid possesses potent prooxidant activity. It is a structural constituent of tannic acid present as a digalloyl moiety. Ellagic acid is another plant-derived polyphenol antioxidant which is considered to possess anticarcinogenic properties. Therefore in order to understand the chemical basis of various biological properties of tannic acid, I have studied in Part-III of the thesis, structure activity relationship

between tannic acid, gallic acid and ellagic acid. The techniques used are DNA cleavage assay and Cu(II) reduction. As expected the results indicate that in the presence of Cu(II), these compounds serve as prooxidants. Gallic acid is considerably more efficient as a DNA degrading agent than ellagic acid and tannic acid. The addition of Cu(II) lead to the quenching of the absorption spectrum in case of gallic acid and tannic acid, while an enhancement of the absorption spectrum was observed in ellagic acid, which are indicative of the binding of Cu(II) to all the three compounds. The three polyphenols are also able to reduce Cu(II) to Cu(I) as evidenced by the formation of complex with neocuproine,an agent that sequesters Cu(I) selectively. Such ratios were determined to be 6.0, 6.0 and 4.0 for gallic acid, tannic acid and ellagic acid respectively. Except for tannic acid these values correlate with the relative rates of DNA degradation by gallic acid and ellagic acid. The capacity of gallic acid, ellagic acid and tannic acid to generate hydroxyl radicals in the presence of Cu(II) was also compared. The result shows that ellagic acid is the least efficient in generating hydroxyl radical whereas tannic acid is the most efficient followed by gallic acid. Further I have also compared the relative antioxidant activity of tannic acid, gallic acid and ellagic acid in a hydroxyl radical generating system (Fe(II)- EDTA). The result suggests that tannic acid is a considerable better quencher of hydroxyl radicals than gallic acid which shows least inhibition followed by ellagic acid.

Plant – derived polyphenolic compounds such as flavonoids, tannins, curcumin, stilbene resveratrol, epicatechin-3-gallate, epigallocatechin-3-gallate and epigallocatechin possess a wide range of pharmacological properties, the mechanism of which have been the subject of considerable interest. They are recognized as naturally occurring antioxidants and have been implicated as antitumor compounds. Among the various pharmacological properties of plant – derived polyphenolic antioxidants is their action as preventive agents against cancer. Some evidence suggests that polyphenolic compounds such as tannins and resveratrol are able to traverse cell membranes and may enter the cytoplasmic or nuclear space. Resveratrol is sufficiently hydrophobic and has been shown to be present in such tissues as heart, liver and kidney. A model for the entry and interaction of polyphenols with chromatin-associated copper has been described. Further, the fact that these polyphonelic compounds are ingested by human populations as a part of the normal diet in relatively higher concentrations without adverse reactions points to their great potential as a putative chemopreventive or therapeutic agents.

Introduction

OXYGEN RADICALS AND HUMAN DISEASE

Oxygen is an essential element for aerobes as it is the terminal acceptor of electrons during respiration, which is the main source of energy in these organisms. It has been proposed that known harmful effects of oxygen were due to the formation of free radicals derived from it (Gilbert, 1981). One of the theories of etiology of cancer, which is widely accepted, holds that the major cause is damage to DNA by oxygen radicals and lipid peroxidation (Ames, 1983; Totter, 1980). The presence of free radicals can be advantageous for cells. In fact, they are continuously produced in organisms and many of them are necessary to carry out certain biological reactions. However, when there is a free radical overproduction or antioxidant defence systems are weakened for any reason, cellular damage can appear (Valenzuela and Videla, 1989; Halliwell *et al.*, 1992). Ionizing radiation, tobacco smoke, pesticides, pollutants or some medications are exogenous sources of free radicals. Intracellular systems also produce oxygen free radicals. The autoxidation of small soluble molecules such as catecholamines, flavins, tetrahydropterins, quinones and thiols in the cellular cytoplasm may produce oxygen free radicals by concomitant O_2 reduction (Fridovich, 1983; Proctor and Reynolds, 1984). Reduced flavins and ascorbic acid upon autoxidation produce superoxide anion. This radical further accepts an electron from a reducing agent, such as thiols to yield peroxide (H_2O_2). There is <u>in vitro</u> evidence that H_2O_2

may then react with certain chelates of copper and iron to yield the highly reactive hydroxyl free radical ($OH^{\cdot}$) (Wolff *et al.*, 1986). That the superoxide anion actually appears in metabolism is confirmed by the ubiquitous occurrence of superoxide dismutase. Several cytoplasmic enzymes, for example, xanthine oxidase and aldehyde dehydrogenase generate oxygen free radicals as products of their catalytic cycles. Reactions catalyzed by lipoxygenase and cyclo-oxygenase in the synthesis pathway of leukotrienes, thromboxanes and prostaglandins involve oxygen free radical production. Cyclo-oxygenase is also able to metabolize certain xenobiotics to more toxic species, which may react with oxygen and yield very reactive oxygen species (Yamammoto, 1991; Riendeau *et al.*, 1989). A main source of superoxide anion ($O_2^{\cdot}$) is the respiratory burst of phagocytic cells when they are activated. Compounds that stimulate the biosynthesis of peroxisomes induce overproduction of hydrogen peroxide. Peroxisomes have a great capacity to form hydrogen peroxide because they contain a high concentration of oxidases (Brunk and Cadenas, 1988; Del Rio *et al.*, 1992). When the respiratory chain is highly reduced and its activity is dependent on ADP availability; radicals may be formed at sites different from cytochrome oxidase. Data show that autoxidation of ubiquinone and NADH dehydrogenase produces superoxide radicals (Beyer, 1990; Freeman and Grapo, 1982). Cytochromes P_{450} and b_5 of the microsomic electron transport systems generate superoxide radicals during their catalytic cycle. Cytochrome reductases involved in redox

reactions of cytochromes P_{450} and b_5 can also produce superoxide radicals and hydrogen peroxide when they undergo autoxidation (Sevanian *et al.*, 1990). The high reactivity of free radicals results in their having a short half-life, as well as a short radius for action. These free radicals react in a way that long chains of propagation are established causing biologic effects far from the system, which produced the first radical. All the cellular components, lipids, proteins, nucleic acids and carbohydrates may be damaged by reactions with oxygen free radicals, giving rise to metabolic and cellular disturbances.

Oxygen free radicals have been shown to oxidize lipids. Hydroxyl and hydroperoxyl radicals, but neither superoxide nor hydrogen peroxide, are able to attack unsaturated fatty acids of phospholipids and other membrane lipid compounds initiating, in this way, lipid peroxidation. Lipid peroxidation causes severe damage to the membrane structure and, consequently, alters its fluidity and ability to function correctly (Gutteridge and Halliwell, 1990; Niki *et al.*, 1991; Schaich, 1992). Alcohols, aldehydes, volatile hydrocarbons and hydroperoxides, the final products of peroxidation, inhibit protein synthesis and are also able to alter vascular permeability, inflammatory response and chemotactic activity (Southorn and Powis, 1988; Blake *et al.*, 1987; Del Maestro *et al.*, 1981). In addition, malondialdehyde, a by product resulting from peroxidation of fatty acids with three or more double bonds and a main indicator of lipid peroxidation, has also been found to cause cross-linking and polymerization of membrane

components as well as to react with DNA nitrogenated bases (Nielson, 1981; Valenzuela, 1991).

Oxidized proteins increase their hydrophobicity and sensitivity to proteolysis. Free radicals may react with amino acids containing unsaturated or sulfur groups. These reactions give rise to structural disturbances in proteins as well as cross linking and aggregation phenomena, which are favoured by inter-and intramolecular disulfide bond formation (Gebicki and Gebicki, 1993; Stadtman, 1992). Proteins are fragmented by free radicals involving peptide bond hydrolysis following oxidation of proline residues by hydroxyl radical and superoxide anion (Wolff and Dean, 1986). Oxidative radical damage occurs to a large extent in nucleic acids through alterations in both their bases and their deoxyribose sugars (Davies *et al.*, 1990; Demple, 1990). Carbohydrates are also targets of oxygen free radicals. Consequently, glycosylated proteins are more sensitive to oxidative damage (Freeman and Grapo, 1982; Sies, 1985).

There are many diseases that involve radical reactions in mammalian systems. Free radicals play a major part in inflammation (Randerath *et al.*, 1992; Kunz *et al.*, 1991), the process, which is the response of the host organism to injury. It involves enhanced vascular permeability with edema formation and leukocyte infiltration into the damaged area. Inflammatory response is advantageous for organisms. However, abnormal overactivation of phagocytes with consequent

exacerbation of reactive oxygen metabolite production may damage surrounding tissues and change the viscosity of the extracellular fluid. This is the case in gout and the autoimmune diseases such as myasthenia gravis, systemic lupus erythematosus, dermatomyositis etc. (Southorn and Powis, 1988; Halliwell and Gutteridge, 1989). Patients with rheumatoid arthritis suffer from neutrophil accumulation in their joints (Greenwald and Moy, 1980). These neutrophils overproduce reactive oxygen species responsible for depolymerization of hyaluronic acid, a glycosaminoglycan necessary for maintaining synovial fluid viscosity in joints.

A great number of occular complaints are associated with oxidative damage. Amongst the components of the eye, the retina is the most sensitive to free radical oxidations, due to its high polyunsaturated fatty acid content (Baynes, 1991; Fujiwara *et al.*, 1992). Retrolental fibroplasia (retinopathy of prematurity) is a complication derived from the use of increased oxygen tensions in incubators for premature babies. Hyperoxia inhibits the growth of the retinal blood vessels. The lipid peroxidation and an increased production of thromaboxane A_2 both induced by free radicals may be responsible for this condition.

The lung is an organ greatly affected by free radical production. Long periods of exposure to high oxygen pressures damage the lungs of different animal species, causing many diseases (pulmonary

emphysema, bronchopulmonary displasia, adult respiratory distress syndrome) and even death (Webster and Nunn, 1988; Jackson, 1985). Oxygen free radicals and other toxic products, formed by the lung cells themselves and by activated neutrophils that accumulate in the lungs when pure oxygen is breathed, may possibly contribute to the hyperoxidant damage. Tobacco has been suggested as being a contributory factor in the appearance of lung pathologies. Smoking impairs the ability of antiprotease to protect lung elastine from neutrophil proteases because free radicals contained in cigarette smoke inactivate this protein. The final result is the destruction of lung connective tissue elastine (Pryor and Dooley, 1985). Recent studies suggest that oxygen free radicals might also be involved in the development of atherosclerotic plaques and ischemia-reperfusion injury (Lehr *et al.*, 1992; Gulati *et al.*, 1992).

Numbers of theories have been proposed to explain the nature of aging and one such is the free radical theory (Sohal, 1993; Seppi *et al.*, 1991; Ji *et al.*, 1990). According to the free radical theory of aging, these very reactive species, produced continuously during normal metabolism, eventually accumulate, damaging DNA and other macromolecules. This is due to progressive defects in the defence systems against reactions that generate free radicals. The result is the appearance of degenerative lesions and cellular death. Then the organism ages and finally dies.

It has been suggested that certain promoters of carcinogenesis act by generation of oxygen radicals, this being a common property of these substances. Fat and hydrogen peroxide are among the most potent promoters (Welsch and Aylsworth, 1983). Other well-known cancer promoters are lead, calcium, phorbol esters, asbestos and various quinones. Many carcinogens, which do not require the action of promoters and are by themselves able to induce carcinogenesis (complete carcinogens), also produce oxygen radicals (Demopoulos *et al.*, 1980). These include nitroso compounds, hydrazines, quinones and polycyclic hydrocarbons. Much of the toxic effect of ionizing radiation damage to DNA is also due to the formation of oxygen radicals (Totter, 1980). The mechanism of action of promoters involves the expression of recessive genes and an increase in gene copy number through chromosome breaks and creation of hemizygosity (Kinsella, 1982; Varshavsky, 1981). Promoters also cause modification of prostaglandins, which are intimately involved in cell division, differentiation and tumor growth (Fischer *et al.*, 1982). Most data on radical damage to biological macromolecules concerns with the effects of radiation on nucleic acids because of the possible genetic effects.

MUTAGENS AND CARCINOGENS IN DIETARY PLANT MATERIALS

Plants synthesize a large number of toxic chemicals, presumably as defence against a variety of invasive organism, such as bacteria, fungi and insects (Kapadia, 1982; Clark, 1982). It has been known for many years that plants contain carcinogens, and a number of edible

plants have shown experimental carcinogenic activity for several species and various tissues. Wide use of short term tests for detecting mutagens (Ames, 1979;Stich and San, 1981), and a number of animal cancer tests on plant substances have contributed to the identification of many mutagens and carcinogens in the human diet (Kapadia, 1982). Some examples of most frequently ingested compounds are discussed below.

Pyrrolizidine alkaloids are naturally occurring carcinogens and have been found in some fifty species of the families Compositae, Boraginaceae and Leguminosae (Schoental, 1982), which are used as foods or herbal remedies. Several of these alkaloids are hepatotoxic and certain hepatotoxic pyrrolizindine alkaloids are also carcinogenic (Hirono *et al.*, 1977; Schoental, 1976). However, a number of these alkaloids have been reported to be mutagenic (Clark, 1960) in Drosophila and Aspergillus system (Alderson and Clark, 1966). Mori *et al.*, (1985) have used a hepatocyte primary culture DNA repair test to screen seventeen pyrrolizidine alkaloids for their DNA damaging property. This test is highly responsive to carcinogenic pyrrolizidine alkaloids (Williams *et al.*, 1980). Among the results obtained by these authors is the indication of a species difference in liver bioactivation of these alkaloids. This implies that there may be species difference in the carcinogenic potential of pyrrolizidine alkaloids.

Safrole and estragole are related compounds, which occur in certain species and essential oils and are weak hepatocarcinogens (Fenaroli, 1971; Guenther and Althausen, 1949). Studies have implicated 1'-hydroxysafrole and 1'-hydroxyestragole as proximate carcinogenic metabolites of safrole and estragole respectively (Drinkwater *et al.*, 1976; Borchert *et al.*, 1973). Eugenol and anethole are structurally related to safrole and estragole and are widely used as flavouring agents or as food additives. Black pepper contains small amounts of safrole and large amounts of a closely related compound piperine (Concon *et al.*, 1979). Extracts of black pepper cause tumors in mice at a number of sites at a dose equivalent to 4 mg of dried pepper per day given for 3 months.

Ivie *et al.*, (1981) have reported that linear furocoumarins (psoralens), which are widespread in plants of the Umbelliferae family, are potent light activated carcinogens and mutagens. Three of the most common phototoxic furocoumarins are psoralen, xanthotoxin and bergapten. In addition to Umbelliferae, psoralen also occurs in plants from several other families (Ivie, 1978). Psoralens are potent photosensitizers and highly mutagenic in the presence of activating long wavelength UV light. They readily intercalate into duplex DNA where they form light induced mono- or diadducts with pyrimidine bases. Psoralen in the presence of light is also effective in producing oxygen radicals (Ya *et al.*, 1982).

Edible mushrooms contain various hydrazine derivatives in relatively large amounts. Most hydrazines that have been tested have been found to be carcinogenic and mutagenic. The most common commercial mushroom, Agaricus bisporus contains about 300 mg of agaritine, the glutamyl derivative of the mutagen 4-hydroxymethylphenylhydrazine, per 100 g of mushrooms as well as smaller amount of the closely related carcinogen N-acetyl-4-hydroxymethylphenylhydrazine (Toth *et al.*, 1982). Some agaritine is metabolized by the mushroom to a diazonium derivative, which is a potent carcinogen and is also present in the mushroom in smaller amount. Many hydrazine carcinogens may act by producing oxygen radicals (Hochstein and Jain , 1981).

A number of 1,2-dicarbonyl compounds e.g. maltole, kojic acid, ethylmaltole, diacetyl and glyoxal have been found to be mutagenic in the Salmonella/Microsome assay. Several compounds in this class are of toxicological interest because they occur in various foods. For example, maltole is a product of carbohydrate dehydration and is present in coffee, soyabeans and baked cereals such as bread. Kojic acid is a metabolite of many microorganisms including several fungi used in food production, while diacetyl is an aroma component of butter, beer, coffee, etc. (Fishbein, 1983).

A number of furans, such as 2-methylfuran, 2,5-dimethylfuran, furfural, 5-methylfurfural and 2-furylmethylketone are found in

numerous food products including meat, milk products, various nuts, tea and coffee (Maga, 1979). Stich *et al.*, (1981) have reported that these furans induced relatively high frequencies of chromatid breaks and chromatid exchanges when they were exposed to cultured Chinese hamster ovary (CHO) cells in the absence of a liver microsomal preparation. The clastogenic doses of many of the furans were relatively high (100-3900 ppm), whereas the concentration in food products was relatively low. However, Stich *et al.*, (1981) cautioned that the furans are not the only genotoxic chemicals in the complex mixture of heated, roasted or boiled food products and even if the furans do not pose a serious health hazard by themselves due to their small amounts in most food items, they do contribute significantly to the total genotoxicity of many consumable foods and beverages.

In addition to pyrrolizidine alkaloids, certain glycoalkaloids found in potato, such as solanine and chaconine have been reported to be highly toxic as they are strong inhibitors of cholinesterase (Jadhav *et al.*, 1981). Pyrrolizidine alkaloids and other glycoalkaloids can reach levels, which can be lethal to humans in potatoes that are diseased or exposed to light (Katsui *et al.*, 1982).

Cyclopropenoid fatty acids, present in cottonseed and other oils, have been reported to be carcinogenic and mitogenic having various toxic effect in farm animals. Among these, sterulic acid and malvalic acid are widespread in the human diet. They are also potentiaters of

carcinogenicity of aflatoxins (Hendricks *et al.*, 1980). Human exposure to these fatty acids results from the consumption of products of animals fed on cottonseed. Another major toxin in cotton seed is gossypol, which accounts for about 1% of its dry weight. Gossypol causes male sterility through formation of abnormal sperm and is carcinogenic as well (Xue, 1980). It is a potent initiator and also promoter of carcinogenesis in mouse skin (Haroz and Thomassan, 1980). Gossypol has been tested in China as a possible male contraceptive as it is inexpensive and causes sterility during use. Its mode of action as a spermicide is presumably through the production of oxygen radicals.

A number of quinones and their phenolic precursors are found in the human diet and have been shown to be mutagens (Stich *et al.*, 1981; Brown, 1980; Levin *et al.*, 1982). Quinones are quite toxic as they can act as electrophiles or accept a single electron to yield the semiquinone radicals which can react directly with DNA or generate superoxide radicals (Morimoto *et al.*, 1983; Kappus and Sies, 1981). Many dietary phenols can autoxidize to quinones generating hydrogen peroxide at the same time. The amounts of these phenols in human diet are appreciable, for example, catechol that is mainly derived from metabolism of plant substances and is a potent promoter of carcinogenesis and an inducer of DNA damage (Carmella *et al.*, 1982).

In addition there are many other dietary compounds, which have been shown to be mutagenic and carcinogenic in various test systems. Allylisothiocyanate, a major flavour ingredient of mustard oil, is one of the main toxins of mustard seeds and has been shown to be a carcinogen in rats (Dunnick *et al.*, 1982). Phorbol esters, present in plants of Euphorbiacea family, are potent promoters of carcinogenesis and cause nasopharyngeal and oesophageal cancers (Hecker, 1981). A variety of carcinogens and mutagens are present in mold contaminated food grains, nuts and fruits. Some of these, such as various aflatoxins, are amongst the most potent carcinogens and mutagens known (Hirono, 1981; Tazima, 1982). Nitrosoamines and other nitroso compounds formed from nitrate and nitrites in food have been directly related to the incidence of stomach and oesophageal cancer. Nitrates are present in large amounts in spinach, radish, lettuce and beans (Magee, 1982). Although alcohol is not a constituent of a normal human diet, in view of its widespread use, it would be relevant to mention its toxic role. Alcohol has long been associated with the cancer of mouth, pharynx and liver (Tuyns *et al.*, 1982). Alcohol metabolism generates acetaldehyde, which is a mutagen and possibly a carcinogen (Stich and Rosin, 1983; Campbell and Fantel, 1983). It also generates radicals that produce lipid hydroperoxides and other mutagens and carcinogens (Winston and Cederbaum, 1982; Videla *et al.*, 1982).

ANTICARCINOGENS

The protective defence mechanisms against mutagens and carcinogens include the shedding of surface layer ot the skin, cornea and the alimentary canal. If oxygen radicals play a major role in DNA damage, defence against these agents is obviously of great importance (Totter, 1980). The major sources of endogenous oxygen radicals are hydrogen peroxide and superoxide, which are generated as side products of metabolism (Pryor, 1976-1982). In addition, oxygen radicals also arise from phagocytosis after viral and bacterial infection or an inflammatory reaction (Tauber, 1982). The exogenous oxygen radical load is contributed by a variety of environmental agents (Pryor, 1976-1982). The enzymes that protect cells from oxidative damage are superoxide dismutase, catalase, glutathione peroxidase (Pryor, 1976-1982), D.T. diaphorase (Lind *et al.*, 1982). In addition to these enzymes, some small molecules in the human diet act as antioxidative agent and presumably have an anticarcinogenic effect. Some of these compounds are discussed below:

The polyphenol tannic acid is present in green tea and several plants, including Embellica phyllanthus.The latter has been credited with several therapeutic properties in anemia, jaundice, uremia, cholestreamia, etc. (Tripathi *et al.*, 1988; Thakur, 1985), some of which may be due to antioxidative properties of tannic acid against DNA damage (Devasagayam *et al.*, 1995) and lipid peroxidation

(Ramanathan and Das, 1992). Flavonoids as well as the related polyphenol tannic acid significantly inhibit single strand breaks in plasmid pBR322 DNA induced by singlet molecular oxygen ($^1O_2$). The protective abilities of these compounds were both time and concentration dependent (Devasagayam *et al.*, 1995). Hamamelitannin which contins two galloyl groups and hamamelose has a strong scavenging activity against superoxide anion radicals (Masaki *et al.*, 1993). Studies showed that several flavonoids, tannic acid, gallic acid and fat - soluble vitamins inhibited Hela and Raji lymphoma cell growth and are known to possess anticarcinogenic properties (Mukhtar *et al.*, 1988). All tannic acid extracts tested so far are anti-tumor promoters, but their efficacy may vary considerably depending on their origin and length of their polygalloyl chain (Gali *et al.*, 1993).

Tocopherol (Vitamin E) is an important trap of oxygen radicals in membranes (Pryor, 1976-1982) and has been shown to decrease the carcinogenic effect of quinones, adriamycin and daunomycin which are toxic because of free radical generation (Ames, 1983). Protective effect of tocopherols against radiation induced DNA damage and dimethylhydrazine induced carcinogenesis have also been observed (Beckman *et al.*, 1982). **β-carotene** is a potent antioxidant present in the diet and is important in protecting lipid membranes against oxidation. Singlet oxygen is a highly reactive form of oxygen, which is mutagenic and is mainly generated by pigment mediated transfer of energy of light to oxygen. Carotenoids are free radical traps and are remarkably efficient as quenchers of singlet oxygen (Packer *et al.*,

1981). β-carotene and similar polyprenes are also the main defence in plants against singlet oxygen generated as a byproduct of the interaction of light and chlorophyll (Krinsky and Deneke, 1982). Carotenoids have been shown to be anticarcinogens in rats and mice and may also have a similar effect in humans (Mathews-Roth, 1982; Peto and Schneiderman, 1981). **Glutathione** is present in food and is one of the major antioxidants and is antimutagenic in cells. Glutathione transferases are a major defence against oxidative and alkylating carcinogens (Warholm *et al.*, 1981). Dietary glutathione is an effective anticarcinogen against aflatoxins (Novi, 1981). The cellular concentration of glutathione is influenced by dietary sulphur amino acids (Tateishi *et al.*, 1981). **Selenium**, which is present in the active site of glutathione peroxidase, is another important dietary anticarcinogen. Glutathione peroxidase is essential for destroying lipid hydroperoxides and endogenous hydrogen peroxide and therefore helps to prevent oxygen radical induced lipid peroxidation (Flohe, 1982). Several heavy metal toxins, such as Cd^{2+} (a known carcinogen) and Hg^{2+} decrease glutathione peroxidase activity by interaction with selenium (Flohe, 1982). Other antioxidants include ascorbic acid and uric acid. The former has been shown to be anticarcinogenic in rodents treated with UV light and benzo(α)pyrene (Hartman, 1982). Uric acid is present in high concentration in the blood of humans and is a strong antioxidant (Ames *et al.*, 1981). A low uric acid level has been considered a risk factor in cigarette caused lung cancer; however, too high levels may cause gout.

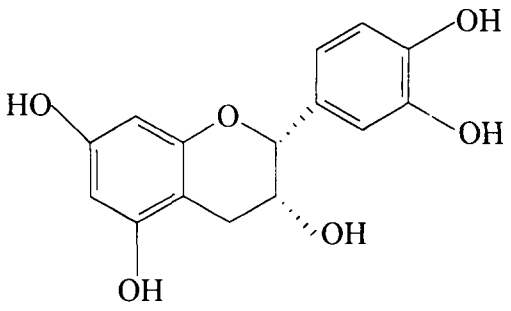
According to Doll and Peto (1981), there are five possible ways whereby diet may affect the incidence of cancer: (i) ingestion of powerful direct acting carcinogens or their precursors, (ii) affecting the formation of carcinogens in the body, (iii) affecting transport, activation or deactivation of carcinogens, (iv) affecting promotion of cells that are already initiated and (v) overnutrition. In summary, most studies involving cancerous tissue or other samples from patients with malignant diseases or diseases associated with an increased risk of cancer show signs of an increased rate of oxidative DNA modification or in some instances deficient repair. This supports the experimentally based notion of oxidative DNA damage as an important mutagenic and apparently carcinogenic factor. However, the proof of a causal relationship in humans is still lacking. In future, the use of biomarkers may provide this evidence and allow further investigation of the qualitative and quantitative importance of oxidative DNA modification and carcinogenesis in humans and also elucidate possible preventive measures (Loft and Poulsen, 1996).

CHEMOPREVENTIVE PROPERTIES OF GREEN TEA AGAINST CANCER

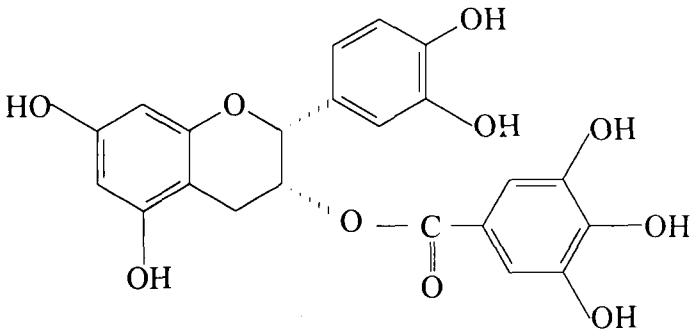
The Chemistry of Tea:

Tea is the second most consumed beverage in the world next to water. Green tea has been used medicinally for centuries in India and

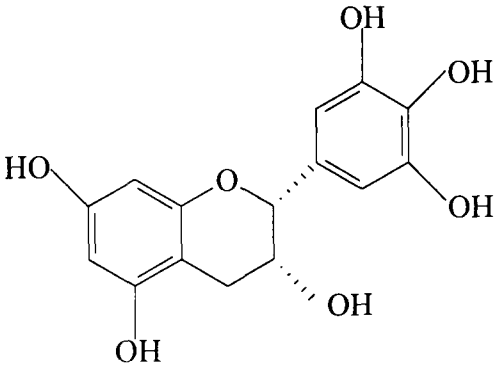
China. A number of beneficial health effects are attributed to regular consumption of green tea and dried/powdered extracts of green tea are available as dietary supplements. Camellia sinensis a member of Theaceae family is an evergreen shrub. Although both green and black teas are derived from Camellia sinensis, it is the production process, which differentiates the two types of teas. Commercial green tea is prepared by picking, lightly steaming and allowing the fresh tea leaves to dry at elevated temperatures. Its chemical composition is similar to that of fresh tea leaves. Green tea contains polyphenols, which include flavanols, flavandiols, flavonoids and phenolic acids. These compounds may account for upto 30% of the dry weight. Most of the green tea polyphenols are flavanols, commonly known as catechins. The primary catechins in green tea are (-) – epicatechin (EC), (-)- epicatechin-3-gallate (ECG), (-)- epigallocatechin (EGC), (-)- epigallocatechin –3-gallate (EGCG), (+)- gallocatechin and (+)- catechin (Yang and Wang, 1993) . The chemical structures of some of these compounds are shown in Figure 1. Other polyphenols include flavanols and their glycosides and depsides, such as cholorogenic acid, coumaroylquinic acid, and one unique to tea, theogallin (3- galloylqunic acid) (Graham, 1992). Caffeine and other methylxanthines such as theobromine and theophylline are the principal alkaloids, which account for about 4% of the dry weight. In addition there are phenolic acids such as gallic acid and characteristic amino acids such as theanine. A cup (200 ml) of green tea contains about 142 mg EGCG, 65 mg EGC, 28 mg ECG, 17



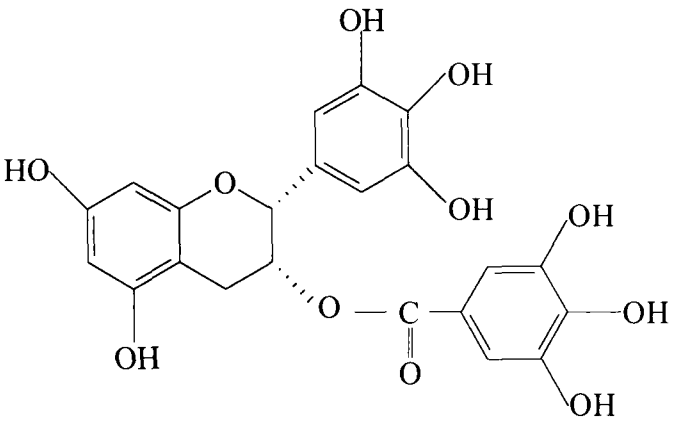
(-) Epicatechin



(-) Epicatechin 3-gallate



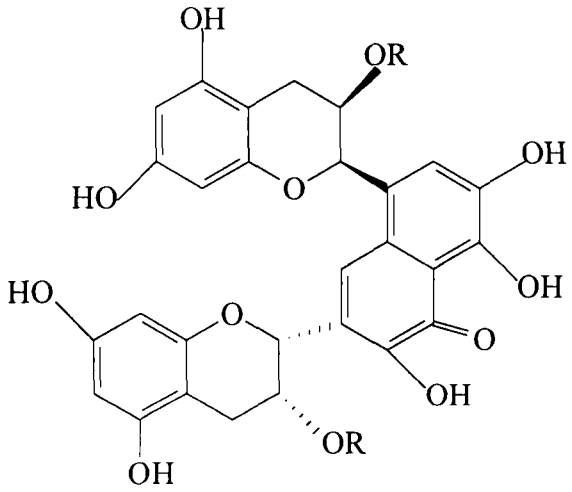
(-) Epigallocatechin



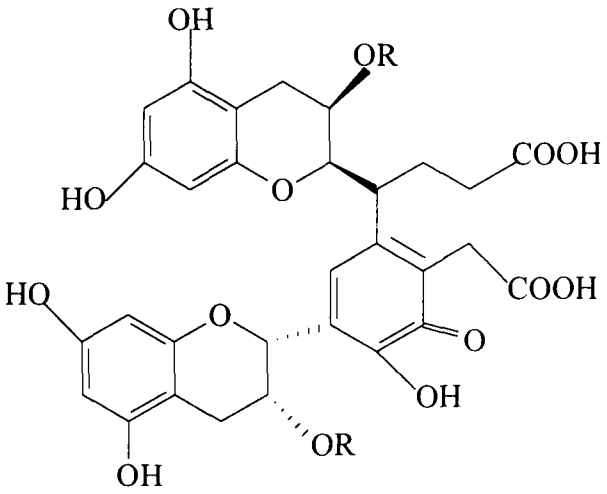
(-) Epigallocatechin-3-gallate

Major Components of Green Tea

Fermentation | **Tea Polyphenol Oxidase**



Theaflavins



Thearubigins
(Possible structure)

R=Galloyl

Major Components of Black Tea

mg EC and 76 mg caffeine (Yang and Wang, 1993). Also present are quinic acids, carotenoids, trigalloylglucose, liginin, chlorophyll and minerals such as aluminum and manganese (Graham, 1992).

During the manufacture of black tea, the monomeric flavan-3-ols undergo polyphenol oxidase – dependent oxidative polymerization leading to the formation of bisflavanols, theaflavins, thearubigins and other oligomers in a process commonly known as 'fermentation'. Theaflavins (about 1% - 2% of the total dry matter of black tea), including theafalvin, theaflavin –3- O – gallate, theaflavin –3'-O-gallate and theaflavin 3,3'-O- digallate, possess benzotropolone rings with dihydroxy or trihydroxy substitution systems, which give the characteristic color and taste of black tea. About 10% - 20% of the dry weight of black tea is due to thearubigins, which are even more extensively oxidized and polymerized, have a wide range of molecular weights, and are less well characterized. The structures of theaflavins and thearubigins are shown in Figure 1.

Green Tea and Cancer Chemoprevention:

The anticarcinogenic and antimutagenic properties of green tea were first elucidated a decade ago (Khan *et al.*, 1988; Wang *et al.*, 1989). Since then, several laboratory and epidemiological studies have been conducted (Ahmad *et al.*, 1998; Katiyar and Mukhtar, 1996; Kohlmeier *et al.*, 1997). It has been demonstrated in mice that oral consumption and topical application of green tea and/or its

polyphenolic constituents affords protection against carcinogenesis induced by chemicals or ultraviolet radiation (Mukhtar *et al.*, 1994). Polyphenols isolated from green tea or water extract of green tea have been shown in other animal models to afford prevention against chemically induced carcinogenesis in forestomach, esophagus, duodenum, pancreas, liver, breast and colon (Ahmad *et al.*, 1998; Katiyar and Mukhtar, 1996; Weisburger *et al.*, 1997). Research also points to other possible roles for green tea extract as a chemopreventive or inhibitory agent in the treatment of skin cancer (Picard, 1996; Record and Dreosti, 1998) bladder tumors (Sato, 1999), ovarian sarcomas (Sugiyama and Sadzuka, 1998) leukemia (Otsuka *et al.*, 1998), liver cancer (Hirose *et al.*, 1993) and oral leukoplakia (Khafif *et al.*, 1998). On the basis of some recent studies, it is now believed that much of the cancer chemopreventive effects of green tea are mediated by EGCG, which is the major polyphenolic constituent of green tea (Ahmad *et al.*, 1998; Katiyar and Mukhtar, 1996). One cup of brewed green tea contains upto 200mg EGCG. A recent bioavailability study (Suganuma *et al.*, 1998) showed that frequent consumption of green tea results in high levels of EGCG in various body organs, indicating that green tea consumption might protect against cancers of multiple body sites.

In addition to imparting preventive and therapeutic effects green tea has also been shown to modulate and increase the efficacy of cancer chemotherapeutic drugs. Sadzuka *et al.* (1998) demonstrated

recently that the oral administration of green tea resulted in enhanced tumor inhibitory effects of doxorubicin on Ehrlich ascites carcinomas implanted in CDF_1 and BDF_1 mice. It is of interest to observe that green tea treatment resulted in an increase in the level of doxorubicin in tumor but not in normal tissue.

Mechanism of Action:

Green tea has been consumed in China to promote health and longevity since 3000 BC. In recent years, much attention has been focused on green tea and more specifically, its polyphenolic content. Despite the growing body of research demonstrating tea polyphenols to be powerful antioxidants with antimutagenic, anticarcinogenic and antiatherogenic properties, understanding of the mechanisms involved in the biological effects of green tea is far from complete. Understanding these mechanism may be helpful in designing better strategies for preventing and treating cancer. The initial mechanistic studies focused on the following areas: (i) antioxidant and free radical scavenging activity which may play a role in lowering LDL-cholesterol, with a consequent decreased risk of cardiovascular disease; (ii) stimulation of detoxification systems, specifically selective induction or modification of phase I and phase II metabolic enzymes which increase the formation and excretion of detoxified metabolites of carcinogens; (iii) Inhibition of biochemical markers of tumor initiation and promotion, including lowering the rate of cell

replication and thus the growth and development of neoplasms; (iv) prevention of mutagenicity and genotoxicity and (v) trapping of activated metabolites of carcinogens. (Ahmad and Mukhtar, 1999).

Yu *et al.*, (1997) suggested that activation of the mitogen-activated protein kinase pathway (MAPK) by green tea polyphenols might be responsible for the regulation of the antioxidant responsive element (ARE). The ARE is believed to mediate the induction of phase II enzymes by many drugs and may be stimulated by green tea polyphenols in the transcription of phase II detoxifying enzymes (Yu *et al.*, 1997; Ahmad and Mukhtar, 1999). Another area, which has been increasingly looked at, is the role green tea catechins play in arresting abnormal cell growth or inducing apoptosis. Apoptosis is a physiologic process involved in maintaining homeostasis in the living system. Also known as programmed cell death, apoptosis is a discrete form of cell death different form necrosis and is regarded as an ideal way of cell elimination. Chemopreventive agents capable of modulating apoptosis and thereby affecting the steady-state cell population may therefore be useful in the management and therapy of cancer. Studies have shown that several cancer chemopreventive agents may induce apoptosis, whereas several tumor – promoting agents inhibit apoptosis (Boolbol *et al.*, 1996; Wright *et al.*, 1994). It is reasonable to assume that chemopreventive agents, which possess proven efficacy in animal tumor bioassay systems and/or human epidemiology and can cause apoptosis of cancer cells, may have a wider use in the management and

control of cancer. Only a limited number of chemopreventive agents are known to cause apoptosis (Jee *et al.*, 1998; Jiang *et al.*, 1996). Ahmad *et al.*, (1997) showed that EGCG induces apoptosis and cell cycle arrest in human epidermoid carcinoma cells A431. Of particular interest was the observation that the apoptotic response of EGCG was specific only to the cancer cells, as the phenomenon of apoptosis was also observed in human carcinoma keratinocytes HaCaT, human prostate carcinoma cells DU145, and mouse Lymphoma cells LY-R, but not in normal human epidermal keratinocytes. The differential effect of EGCG was later verified by another study in which Chen *et al.*, (1998) compared the effect of EGCG on the growth of SV40 virally transformed W138 human fibroblasts (W138 VA) with that of normal W138 cells. Treatment with EGCG inhibited the growth of the transformed W138 VA cells but had little or no inhibitory effect on the growth of normal W138 cells. This study further demonstrated that EGCG induces apoptosis in W138 VA cells but not in W138 cells. In another study Fujiki *et al.*, (1998) demonstrated that EGCG and other tea polyphenols inhibit the growth of human lung cancer cells PC-9 and cause a G2/M phase arrest of the cell cycle. The effect of EGCG on growth factor receptors, specifically epidermal growth factor receptor (EGFR) has also been examined. EGFR tyrosine kinase activation is believed to initiate multiple cellular responses associated with mitogenesis and cell proliferation. The over expression of EGFR might produce a neoplastic phenotype. Liang *et al.*, (1997) found EGCG

inhibited the autophosphorylation of EGFR by its ligand EGF and blocked the binding of EGF to its receptor. EGCG also significantly inhibited DNA synthesis and protein tyrosine kinase activities of EGFR. The results suggest EGCG might inhibit tumor development by blocking growth factor associated signal transduction pathways (Harbowy and Balentine, 1997; Mukhtar *et al.*, 1994).

Previous studies in our laboratory have confirmed that several of the plant derived polyphenolic compounds are themselves capable of prooxidant action particularly in the presence of transition metal ions such as copper. Specifically it has been shown that flavonoids (Rahman *et al.*, 1989), tannic acid and its structural constituent gallic acid (Khan and Hadi, 1998), curcumin (Ahsan and Hadi, 1998) and resveratrol (Ahmad *et al.*, 2000) cause oxidative strand breakage in DNA in the presence of (CuII). In this reaction Cu (II) is reduced to Cu (I) and reactive oxygen species such as hydroxyl radicals are formed, which serve as proximal DNA cleaving agent. Copper is an important metal ion present in chromatin and is closely associated with DNA bases, particularly guanine (Kagawa *et al.*, 1991). In a study involving the DNA degradation by polyphenols Li and Trush (1994) proposed a model that underlines the potential of DNA associated copper in cells to activate phenolic compounds by way of a copper redox reaction producing reactive oxygen and electrophilic phenolic intermediates and ultimately leading to oxidative DNA base modifications, DNA strand breaks and DNA adducts of phenolic intermediates. Further it has been

shown that the flavonoid quercetin (Rahman *et al.*, 1990) and curcumin (Ahsan and Hadi, 1998) are capable of binding to DNA and copper. Evidence in the literature suggests that the antioxidant properties of polyphenols such as gallotannins, curcumin and resveratrol may not fully account for their chemopreventive effects. Therefore, a mechanism has been proposed for the cytotoxic action of these compounds against cancer cells that involves mobilization of endogenous copper and the consequent prooxidant action (Hadi *et al.*, 2000).

Scope of Work

SCOPE OF THE WORK PRESENTED

In recent years a number of reports have documented the chemopreventive effect of green tea consumption on various types of cancers such as those of bladder, prostate, esophagus and stomach (Wei *et al.*, 1999). However, such chemopreventive effect is not attributed to the consumption of black tea to the same extent. Gallotannins such as tannic acid, gallic acid, EGC, ECG and EGCG induce apoptosis in various cancer cell lines (Inoue *et al.*, 1994; Ahmad *et al.*, 1997). Of particular interest is the observation that EGCG (a polyphenol constituent of green tea) and gallic acid induce apoptosis in cancer cell lines but not in normal cells (Inoue *et al.*, 1994; Ahmad *et al.*, 1997). This property is also shared by a number of other plant derived polyphenolic compounds such as curcumin and resveratrol (Clement *et al.*, 1998; Piwocka *et al.*, 1999).

Various pharmacological properties of plants derived polyphenolic compounds are considered to be the effect of their antioxidant action. However, as mentioned above studies in our laboratory have confirmed that these compounds are themselves capable of oxidative DNA damage, particularly in the presence of transition metal ions (Khan and Hadi, 1998). Several lines of evidence in the literature strongly suggest that it is the prooxidant action of plant derived polyphenlolics rather than their antioxidant activity that may be an important mechanism for their anticancer and apoptosis

inducing properties (Piwocka *et al.*, 1999; Mukhtar *et al.*, 1988; Kane *et al.*, 1993). Thus a mechanism for the cytotoxic action of polyphenols against cancer cells that involves mobilization of endogenous copper and the consequent prooxidant action has been proposed (Hadi *et al.*, 2000). In partial support of this idea, in part I of the thesis, I have shown that the water extract of green tea is considerably more efficient than black tea extract in DNA cleavage in the presence of copper ions. Green tea extract also shows a higher rate of Cu (II) reduction and consequent hydroxyl radical production. Further, I have examined the biological activity of the reaction and have shown that green tea–Cu(II) mediated reaction cause inactivation of bacteriophage T_4 through a mechanism similar to DNA breakage.

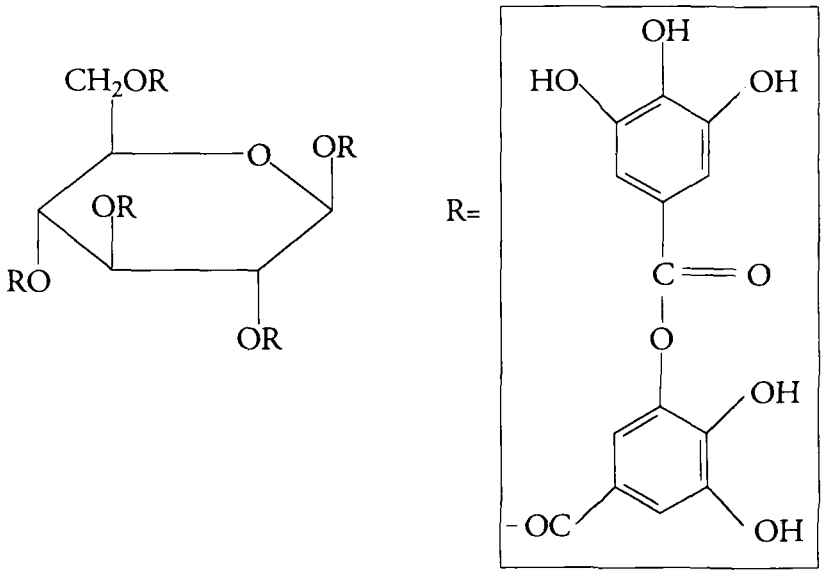
Gallic acid, a naturally occurring plant phenol with antioxidative properties, was found to induce cell death in promyelocytic leukemia HL-60RG cells. Morphological and biochemical studies indicated that the gallic acid induced cell death is apoptosis. Structure activity analysis suggests that gallic acid induced apoptosis in HL-60RG cells depends on its distinctive feature derived from the structure but not on its antioxidative activity. Gallic acid shows selective cytotoxicity to tumor cells compared with normal cells (Inoue *et al.*, 1994). The effect of gallic acid was significantly reduced by blocking the free hydroxyl group with acetyl, methyl, ethyl, n-propyl or isoamyl group. These data suggest the involvement of pro-oxidative action of gallic acid in the induction of apoptosis. Gallic acid is considered to trigger apoptosis by

means of reactive oxygen species such as hydrogen peroxide, superoxide anion in addition to Ca^{+2}-ion and calmodulin dependent enzymes. Gallic acid is a common structural constituent of EGC, EGCG, the most active constituents of green tea therefore in order to identify the structural features of these compounds I have compared in part II, the prooxidant DNA cleavage properties and antioxidant properties of gallic acid and green tea extract.

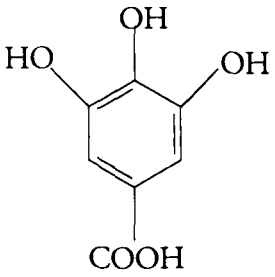
Tannic acid [gallotannic acid, gallotannin or penta-(m-digalloyl-glucose)] is a principal of tannin, derived from Chinese nutgalls (Galle rhois) belonging to Anacardiaceae and constitutes penta to octa-galloyl glucose. The term "tannin" is ordinarily used as a synonym for tannic acid. Tannic acid has numerous industrial, pharmacological and food additive applications. It is used as an additive in medicinal products for humans, including those used for treatment of burns, diarrhea, chemical antidotes in poisoning and as local astringent (Hirono, 1987). It is also used as a flavour enhancer and processing aid in alcoholic beverages (United States Food and Drug Administration, Rocville, 1988). Tannic acid is also used as a clarifying agent in the brewing and wine industries (IARC monograph, 1976) and for colour stabilization of orange fruit juice (Maccarone *et al.*, 1987). When applied topically, injected or added to diet or drinking water, tannic acid has been shown to decrease the risk of tumorigenecity in the skin and other organs (Mukhtar *et al.*, 1988; Athar *et al.*, 1989). Tannic acid and several gallic acid derivatives strongly inhibit the activity of 12-O-

tetradecanoylphorbol-13-acetate (TPA) induced ornithine decarboxylase (ODC) (a biochemical marker of stage 2 promotion) in mouse epidermis in vivo (Gali *et al.*, 1991), suggesting that hydrolyzable tannins inhibit the 2^{nd} stage of tumor promotion phase of skin tumorigenesis. Tannins have high reducing power and form complexes with various metal ions and cofactors, chelate iron and inhibit the iron-catalyzed reactions generating free radicals (Gali *et al.*, 1992). Numerous studies have also demonstrated that tannic acid could induce the DNA excision repair system in bacterial and mammalian cells ((Kuroda, 1988; Shimoi *et al.*, 1985).

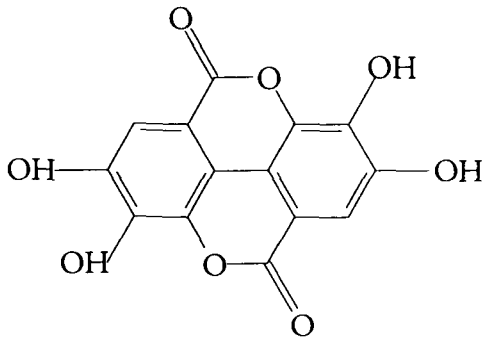
Ellagic acid, another polyphenol has been shown to have an anticarcinogenic effect against an array of nitrosamines, polycyclic aromatic hydrocarbons and fungal toxins, for example skin tumorigenesis induced by 20-methylcholanthracene in mice (Mukhtar *et al.*, 1986), N-nitrosobenzylmethylamine-induced tumors in rat oesophagus (Daniel and Stoner, 1991), N-2-fluorenylacetamide-induced liver carcinogenesis in the rat (Tanaka *et al.*, 1988) and neoplasia caused by benzo(α)pyrene in mice (Lesca 1983). Mutagenicity of aflatoxin B_1 (Mandal *et al.*, 1987) and N-methyl-N-nitrosourea (Dixit and Gold, 1986) was also decreased by ellagic acid. In order to understand the chemical basis of the various biological properties of tannic acid, I have studied in part III the structure – activity relationship between tannic acid, gallic acid and ellagic acid using the DNA cleavage assay and Cu (II) reduction. The structures of these compounds are given in Figure 2.



Tannic Acid



Gallic acid



Ellagic acid

Experimental

MATERIALS

Chemicals used for the present studies were obtained from the following sources:

Chemicals	Sources
Agar powder	Hi - Media, India
Agarose	Koch Light Laboratories, England
Bovine serum albumin	Sigma Chemical Co., USA
Catalase	Sigma Chemical Co., USA
Cupric chloride	S.D. Fine Chem., India
Deoxyribonucleic acid (Calf thymus Type I)	Sigma Chemical Co., USA
Diphenylamine	BDH, India
Ellagic acid	Sigma Chemical Co., USA
Ethidium bromide	Sigma Chemical Co., USA
Ethylene diamine tetra acetic acid	BDH, India
Epicatechin (EC)	Sigma Chemical Co., USA
Epigallocatechin (EGC)	Sigma Chemical Co., USA

Epigallocatechin-3-gallate (EGCG)	Sigma Chemical Co., USA
Gallic acid	Sigma Chemical Co., USA
Mannitol	Qualigens Fine Chemicals, India
Neocuproine hydrochloride	Sigma Chemical Co., USA
Nitroblue tetrazolium	Sisco Research Labs, India
Nutrient broth	Hi-Media, India
Riboflavin	Sigma Chemical Co., USA
S_1 nuclease	Sigma Chemical Co., USA
Salicylate	Qualigens Fine Chemicals, India
Sodium azide	E Merck, Germany
Sodium benzoate	E Merck, Germany
Supercoiled plasmid pBR322 DNA	Isolated and purified in the lab According to the procedure of Maniatis *et al.*, 1982
Superoxide dismutase	Sigma Chemical Co., USA
T_4 DNA	Isolated and purified in the lab According to the procedure of Maniatis *et al.*, 1982

Tannic acid	Aldrich Chemical Co., USA
Thiourea	E Merck, Germany
Tris (hydroxy methyl) aminomethane HCl	Fluka AG, Switzerland
Triton X-100	BDH, India

All other chemicals were commercial products of analytical grade.

MEDIA FOR *E. COLI* B

Nutrient Broth (13.0 g/L) obtained from Hi-Media (India) had the following components

Peptone	:	5.0 g/L
NaCl	:	5.0 g/L
Beef extract	:	1.5 g/L
Yeast extract	:	1.5 g/L
pH (approx.)	:	7.4 ± 0.2

Nutrient Agar (Hard Agar)

Nutrient Broth	:	13.0 g/L
Agar Powder	:	15.0 g/L

Soft Agar/Top Agar

Nutrient Broth	:	13.0g/L
Agar Powder	:	7.0 g/L

METHODS

Preparation of green and black tea extract

Green and black teas (25g) were added to 500ml of boiling water and were steeped for 15 to 20 minutes. Infusion was cooled to room temperature and then filtered. The tea leaves were extracted a second time with 500 ml of boiling water and filtered, and the two filtrates were combined to obtain 2.5% tea water extract (2.5g tea leaves/ 100 ml H_2O). The resulting clear solution is similar to tea brews consumed by humans. The water extracts were dried on a lyophilizer and weighed (Wei *et al.*, 1999).

Thin layer chromatography

Green and black tea extracts were loaded on to silica gel (25 μm layer thickness) for thin layer chromatography (TLC) along with the standard compounds. A mixture of toluene – ethyl acetate (1:8) was taken as the solvent (Wei *et al.*, 1999). Polyphenols were detected by exposure to iodine as well as under UV (254nm).

Reaction of green tea extract, black tea extract, gallic acid, tannic acid and ellagic acid with calf thymus DNA and digestion with S_1 nuclease

Reaction mixtures (0.5ml) contained 10mM Tris-HCl (pH 7.5), 500 μg calf thymus DNA, green tea extract, black tea extract, gallic acid, tannic acid, ellagic acid, cupric chloride and other metal ions. Free

radical scavengers were included in some experiments. All solutions were sterilized before use. After incubation at room temperature for specified time periods, S_1 nuclease digestion was performed. The assay determines the acid soluble nucleotides released from DNA as a result of enzymatic digestion. The reaction mixture in a total volume of 1.0ml contained 0.1 M acetate buffer (pH 4.5), 1mM zinc sulphate, water and enzyme. The mixture was incubated for 2 hours at 42°C. The reaction was stopped by adding 0.2 ml of bovine serum albumin (10 mg/ml) and 1.0 ml of 14% perchloric acid (ice cold). The tubes were immediately transferred to 0°C for at least 1 hour before centrifugation to remove the undigested DNA and precipitated protein. Acid soluble nucleotides were determined in the supernatant using the diphenylamine method of Schneider (1957). To a 1.0ml aliquot, 2.0 ml of diphenyl reagent (freshly prepared by dissolving 1 gm of recrystallized diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of conc. H_2SO_4) was added. The tubes were heated in boiling water bath for 20 minutes. The intensity of blue colour was read at 600 nm.

Treatment of supercoiled plasmid pBR 322 DNA with green tea extract, black tea extract, gallic acid, tannic acid and ellagic acid in the presence of Cu (II)

Reaction mixtures (30 µl) contained 10 mM Tris-HCl (pH 7.5), 0.5 µg of pBR 322 plasmid DNA and other components as indicated in the legends. Incubation at room temperature was performed for specified time periods. After incubation, 10µl of a solution containing 40 mM

EDTA, 0.05% bromophenol blue tracking dye and 5υ% (v/v) glycerol was added and the solution was subjected to electrophoresis in submarine 1% agarose gels. The gels were stained with ethidium bromide (0.5μg/ml), viewed and photographed on a transilluminator.

Stoichiometric titration of Cu (I) production

The concentration of Cu(I) produced in the drug-Cu(II) reaction mixture was determined by titration with neocuproine. Neocuproine complexes with Cu(I) to form Cu (neocuproine)$_2^+$ complex which has an absorption peak at 450 nm (Nebesar, 1961). Green tea extract and black tea extract (6.6 μg/ml); tannic acid, gallic acid and ellagic acid (10μM) in 10 mM Tris-HCl (pH 7.5) were mixed with varying concentrations of Cu (II) ($CuCl_2$) and 0.4 mM neocuproine, in a total volume of 3.0ml. Absorbance was recorded at 450nm after adding Cu (II).

Spectroscopy

The absorption spectra were obtained by using a Beckman DU-40 spectrophotometer fitted with a plotter. The absorption data were obtained in a 1 cm path-length cell. All spectroscopic work was carried out at ambient temperatures.

Detection of superoxide anion ($O_2^{\cdot-}$)

Superoxide anion was detected by the reduction of nitroblue tetrazolium (NBT) essentially as described by Nakayama *et al.* (1983). A typical assay mixture contained 10 mM sodium phosphate buffer pH 8.0,

0.033 mM NBT, 0.1 mM EDTA and 0.06% triton X-100 in a total volume of 3.0 ml. The reaction was started by the addition of the compound. After mixing, absorbance was recorded at 560 nm at different time intervals against a blank, which did not contain the compound.

Determination of hydroxyl radicals

Aromatic hydroxylation of salicylate

This assay is based on the ability of hydroxyl radical to hydroxylate aromatic rings at an almost diffusion controlled rate and the measurement of hydroxylated products by a simple colorimetric method using salicylate (2-hydroxy benzoate) as a detector molecule (Richmond *et al*, 1981). The reaction mixture (2.0 ml) contained the following reagents; 150 mM potassium phosphate buffer (pH 8.0) indicated concentrations of compounds and Cu (II). The mixture was incubated at room temperature for the desired time period. After incubation, the reaction was stopped by adding 80 µl of 11.6 M hydrochloric acid and 0.5 g of sodium chloride followed by 4.0 ml of chilled diethyl ether. The contents were mixed by vortexing for 1 minute. Next, 3.0 ml of the upper ether layer was pipetted out and evaporated to dryness in a boiling tube at 40°C. The tubes were cooled and the residue dissolved in 0.25 ml of cold distilled water to which the following reagents were added in the order stated. (a) .0125 ml of 10% (w/v) TCA dissolved in 0.5M HCl, (b) 0.25 ml of 10% (w/v) sodium tungstate in water and (c) 0.25 ml of 0.5% (w/v) sodium nitrite (freshly prepared). After standing for 5 minutes, 0.5

ml of 0.5 M potassium hydroxide was added and the absorbance at 510 nm was read exactly after 1 minute.

Assay of thiobarbituric acid reactive material

Hydroxyl radical formation was also determined using calf thymus DNA as the substrate. The method of Quinlan and Gutteridge (1987) was followed without modification except that green tea extract, black tea extract and gallic acid replaced rifamycin S.V. The buffer was 10mM Tris-HCl (pH 7.5) and contained increasing concentration of green tea extract, black tea extract and gallic acid. Final concentrations of $CuCl_2$ and calf thymus DNA were 0.1 mM and 400 μg/ml respectively. After incubation for half an hour at 37°C, malondialdehyde formed from deoxyribose was assayed by addition of thiobarbiturate and the resulting adduct was determined colorimetrically at 532 nm.

Antioxidant properties of gallic acid, tannic acid, ellagic acid and green tea extract were assayed by their ability to provide protection to calf thymus DNA breakage by hydroxyl radical generated by Fe (II) – EDTA system (Prigodich and Martin, 1990), containing 0.03% H_2O_2, 1 mM sodium ascorbate and 0.04 mM Fe (II) –0.08 mM EDTA and by singlet oxygen generated by riboflavin – light mediated system containing 20 μM riboflavin with incubation in light for 2 hours.

Maintenance and growth of bacteria

The *E. coli* strain was streaked on a nutrient agar plate. A single colony was picked up from the plate and repurified by streaking. The

culture was tested on the basis of associated genetic markers; raising it from a single colony on a master plate. Having been satisfied with the test clone, the culture was streaked on nutrient agar slants. It was then allowed to grow at 37°C and stored at 4°C. The cultures were transferred onto fresh slants every month.

Preparation of bacteriophageT_4 and treatment with green tea extract and Cu (II)

Bacteriophage stocks were prepared on plates by confluent lysis method (Sambrook *et al,* 1989). Bacteria from the exponential culture were harvested and resuspended in 10 mM $MgSO_4$ solution. 0.3 ml of host cells were infected with phage at a multiplicity of infection (m.o.i) of 5.0. Adsorption was allowed for 20 minutes at 37°C and the suspension plated with 3.0 ml of molten soft agar on hard agar plates. Plates were then incubated at 37°C till confluent lysis was visible to the naked eye. The top agar containing the phage was scraped in a 10 mM $MgSO_4$ solution, 1% chloroform was then added and mixed by gentle vortexing at 37°C. Phage was obtained in the supernatant by centrifugation of the above lysate at 10,000 rpm for 20 minutes at 4°C. The phage stock thus obtained was titered and stored at 4°C over few drops of chloroform.

To assess bacteriophage inactivation, green tea extract and a stock of aqueous $CuCl_2$ previously sterilized by filtration were added to a suspension (0.1 ml) of phage in Tris/Mg^{2+} (0.01 M, (pH 8.0). $CuCl_2$ was added 10 minutes after the addition of green tea and the reaction mixture

was incubated at room temperature for specific time periods during which it was vortexed at 5-minute intervals. In some experiments the quenchers of reactive oxygen species and neocuproine were added before the addition of $CuCl_2$. After incubation, treated phage was diluted with 10mM $MgSO_4$ and 0.1 ml of diluted phage was added to a 0.3 ml suspension of *E. Coli* host strain. T_4 – *E. Coli* complexes were vortexed for 1 minute and then incubated for 20 minutes at 37°C. After incubation, 3.0 ml of soft agar (40°C) was added to the treated phage, vortexed and immediately poured on the nutrient agar plates. Plates were incubated at 37°C for 5-6 hours followed by counting of plaque forming units (PFU).

For experiments in which the indicator bacteria were pre-treated with UV light, cells were harvested, resuspended and washed 3 times by centrifugation in 0.15 M NaCl, 0.015 M potassium phosphate buffer (pH 7.5) and a final suspension (10^8 cells/ml) was poured onto a petridish to a depth of 3 mm. The dish was exposed to an uncalibrated 40 W UV lamp from a distance of 20 cm.

Results & Discussion

Part -I

RESULTS (Part - I)

Constituents of the water extract of green and black teas

The major green tea catechins are (-)-epigallocatechin – 3-gallate (EGCG), (-)- epigallocatechin (EGC), (-)-epicatechin – 3 – gallate (ECG), (-)-epicatechin (EC), (+)- gallocatechin and (+)-catechin (Yang and Wang, 1993). Figure 3 shows thin layer chromatography separation with standard compounds of epicatechin (EC), epigallocatechin (EGC) and epigallocatechin – 3 – gallate (EGCG) in green tea extract prepared as described in 'Methods'. The results indicate that these three polyphenols are present in the green tea extract and are absent from black tea.

Breakage of calf thymus DNA by green and black tea extracts

The reaction was assessed by recording the proportion of double – stranded DNA converted to acid soluble nucleotides by S_1 nuclease. Control experiments (not shown) established that heat denatured DNA underwent 100% hydrolysis following treatment with S_1 nuclease, where as only 8% of native DNA was hydrolyzed. Figure 4 gives the acid soluble material produced from calf thymus DNA with increasing concentrations of green and black tea extracts at a fixed concentration of Cu(II). The rate of DNA hydrolysis and the maximum hydrolysis achieved was greater with green tea extract than with black tea. These results are confirmed by another assay, which uses strand cleavage in

Figure 3: **Thin layer chromatography profile of green and black tea extracts with standard compounds.**

Lane a: green tea extract
Lane b: black tea extract
Lane c: epicatechin
Lane d: epigallocatechin-3-gallate
Lane e: epigallocatechin

a b c d e

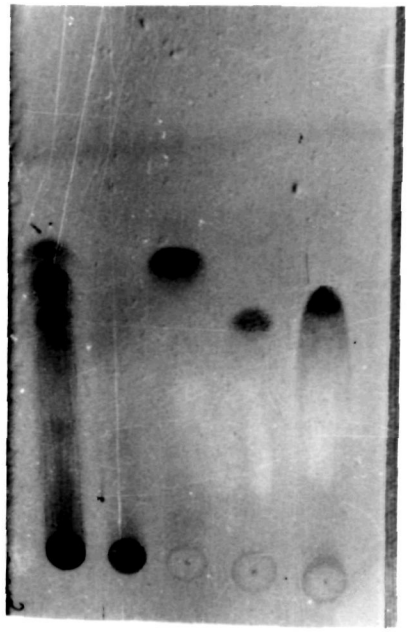
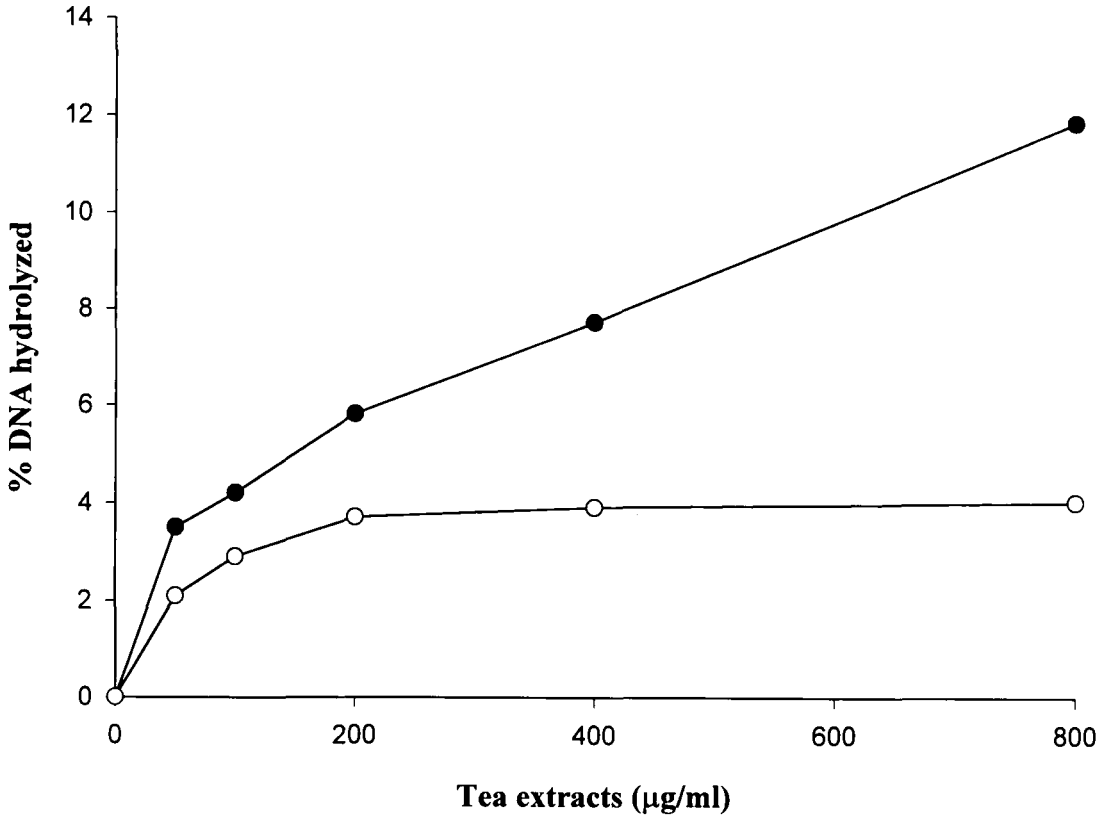


Figure 4: **Degradation of calf thymus DNA as a function of increasing concentrations of green tea extract and black tea extract in the presence of Cu(II) as measured by the degree of S_1 nuclease digestion.**

DNA was incubated with increasing concentrations (50-800μg/ml) of green tea extract (●) and black tea extract (○) in the presence of 0.1mM Cu(II) at 37°C for 1 hour. All points represent triplicate samples and mean values have been plotted.


14
12
10
8
6
4
2
0
% DNA hydrolyzed
0
200
400
600
800
Tea extracts (µg/ml)

supercoiled plasmid DNA as the substrate. This is a sensitive test for DNA breakage as a single nick per molecule of DNA leads to the conversion of supercoiled to the open circular form. Further strand breakage gives rise to the linear form and heterogeneous sized smaller molecules. As seen in Figure 5 the generation of smaller sized molecules is considerably greater in the case of green tea, which is indicated by a trail of smear. Figure 6 gives the kinetics of DNA degradation by the tea extracts and similar relative rates of hydrolysis are seen.

Rate of Cu(II) reduction by tea extracts

In order to examine the involvement of Cu(I) in the reaction I used neocuproine as an agent that sequesters Cu(I) selectively. Green and black tea extracts reduce Cu(II) to generate Cu(I) as evidenced by the formation of a complex absorbing at 450nm (results not shown) (Khan and Hadi, 1998). The reaction possibly leads to the formation of the 'oxidized species' of the components of tea extracts as is the case with other polyphenolic compounds such as flavonoids (Rahman *et al.*, 1989; Utaka and Takeda, 1985). Stoichiometry of the production of Cu(I) by tea extracts was studied (Figure 7) but not clear maximum where absorption plateaued (Khan and Hadi, 1998) was obtained, however the rate of Cu(II) reduction by green tea extract was found to be greater than black tea. The greater rate of Cu(II) reduction by green tea is possibly accounted for by the presence of epicatechin,

Figure 5: **Agarose gel electrophoretic pattern of ethidium bromide stained pBR 322 DNA after treatment with green tea extract and black tea extract in the presence of Cu(II).**

Reaction mixture in a volume of 30 µl containing 0.8 µg plasmid pBR322 DNA, 0.1 mM Cu(II) and 5 µg tea extracts were incubated for 1 hour at 37°C.
Lane a: DNA alone
Lane b: DNA + green tea extract + Cu(II)
Lane c: DNA + black tea extract + Cu(II)

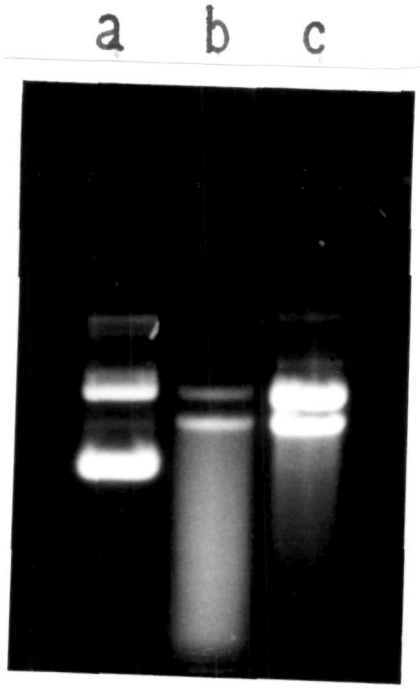

a
b
c

Figure 6: **Effect of time of incubation on calf thymus DNA degradation by the tea extracts.**

DNA was incubated with green tea extract (●) and black tea extract (○) in the presence of Cu(II) for the indicated time periods and then subjected to S_1 nuclease digestion. The concentration of tea extracts and Cu (II) were 800μg/ml and 0.1mM respectively. All points represent triplicate samples and mean values have been plotted.

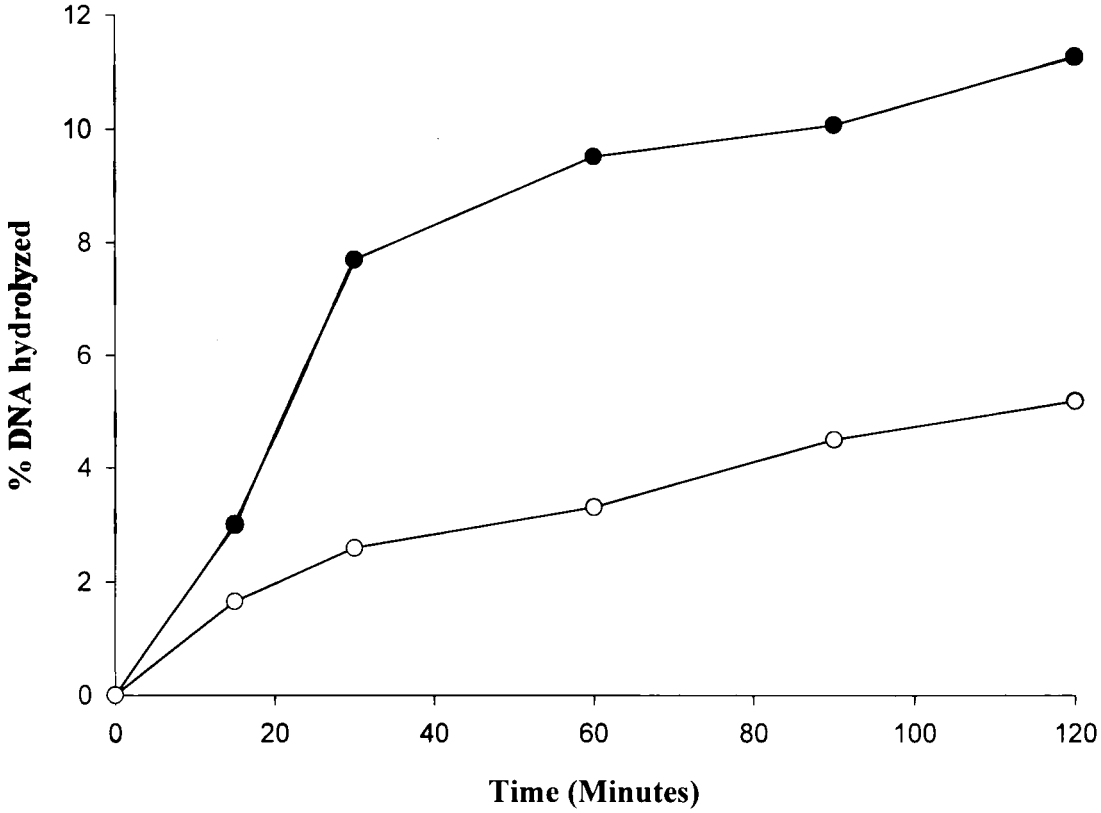

12
10
8
6
4
2
0
% DNA hydrolyzed
0
20
40
60
80
100
120
Time (Minutes)

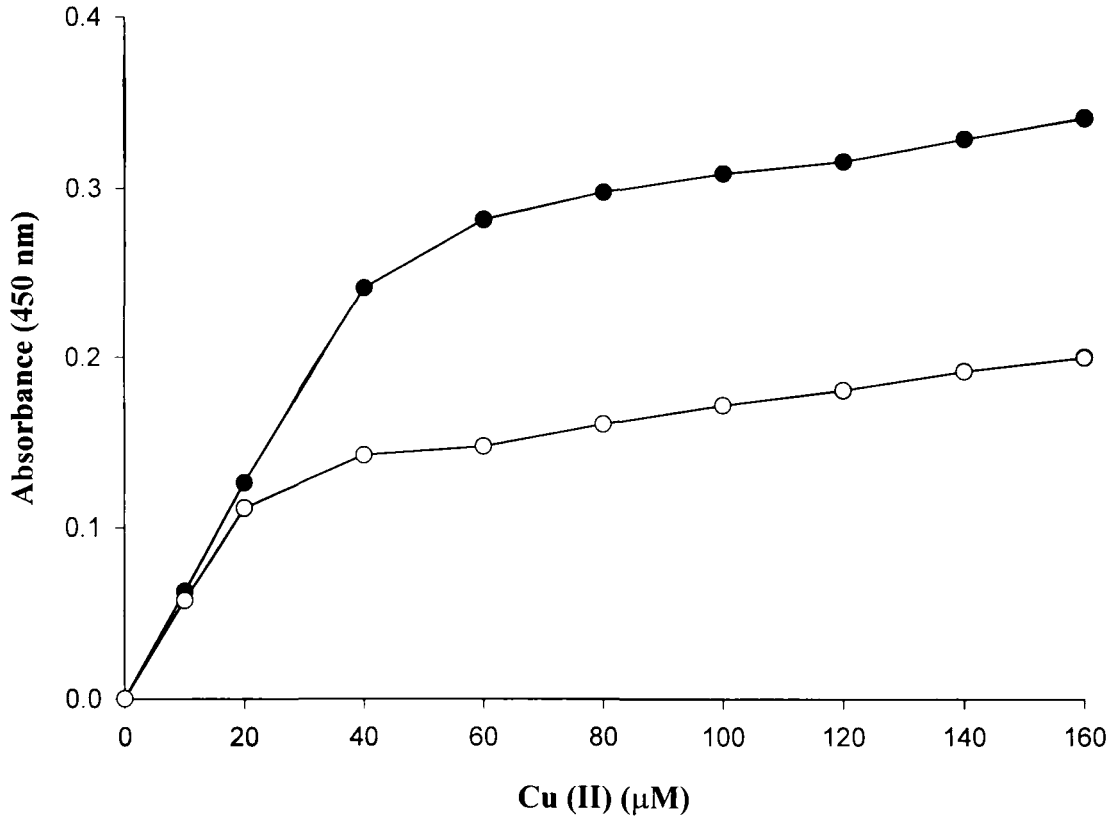
Figure 7: **Rate of reduction of Cu(II) by green tea extract and black tea extract (formation of Cu(I)) using neocuproine.**

The concentrations of neocuproine and tea extracts were 400 μM and 6.6μg/ml respectively. Other details of the procedure are given in 'Methods'.

(●) green tea extract

(○) black tea extract

All points represent triplicate samples and mean values have been plotted.


0.4
0.3
0.2
0.1
0.0
Absorbance (450 nm)
0
20
40
60
80
100
120
140
160
Cu (II) (μM)

epigallocatechin and epigallocatechin-3-gallate. However, it is not excluded that other constituents of tea extracts might act as reductants for Cu(II) as well. Further, the results suggest a possible redox cycling of copper ions in the reaction. It would thus appear that the 'oxidized species' of the components of green and black tea are also able to catalyze the reduction of recycled copper ions [Cu(II)]. In such a case increased copper concentrations at a fixed concentration of tea extract should result in increased rate of DNA hydrolysis. As shown in Figure 8 this is indeed found to be the case. When calf thymus DNA was treated with increasing concentrations of Cu(II) at a fixed concentration of green tea extract concomitant enhancement in the rate of DNA hydrolysis was observed.

Effects of alternative metal ions

Of the several metal ions tested, only Cu(II) and Fe(II) complemented the tea extracts in the DNA breakage reaction to a significant extent (Table I). Although some DNA hydrolysis is also seen with Zn(II), Mg(II), Co(II), Ca(II), Mn(II) and Ni(II). These results are similar to those seen with other polyphenols such as flavonoids (Rahman *et al.*, 1989).

Production of hydroxyl radicals by tea extracts

I demonstrated that tea extracts and Cu(II) generate hydroxyl radicals. The assay involves incorporation of salicylate as a reporter

Figure 8: **Effect of different Cu(II) concentrations at a fixed concentration of green tea extract on calf thymus DNA degradation.**

DNA was treated with (▼) 50 μM, (○) 100 μM, (●) 200 μM of Cu(II) and 400 μg/ml of green tea extract at 37°C for different time intervals and subjected to single strand specific nuclease digestion.
All points represent triplicate samples and mean values have been plotted.

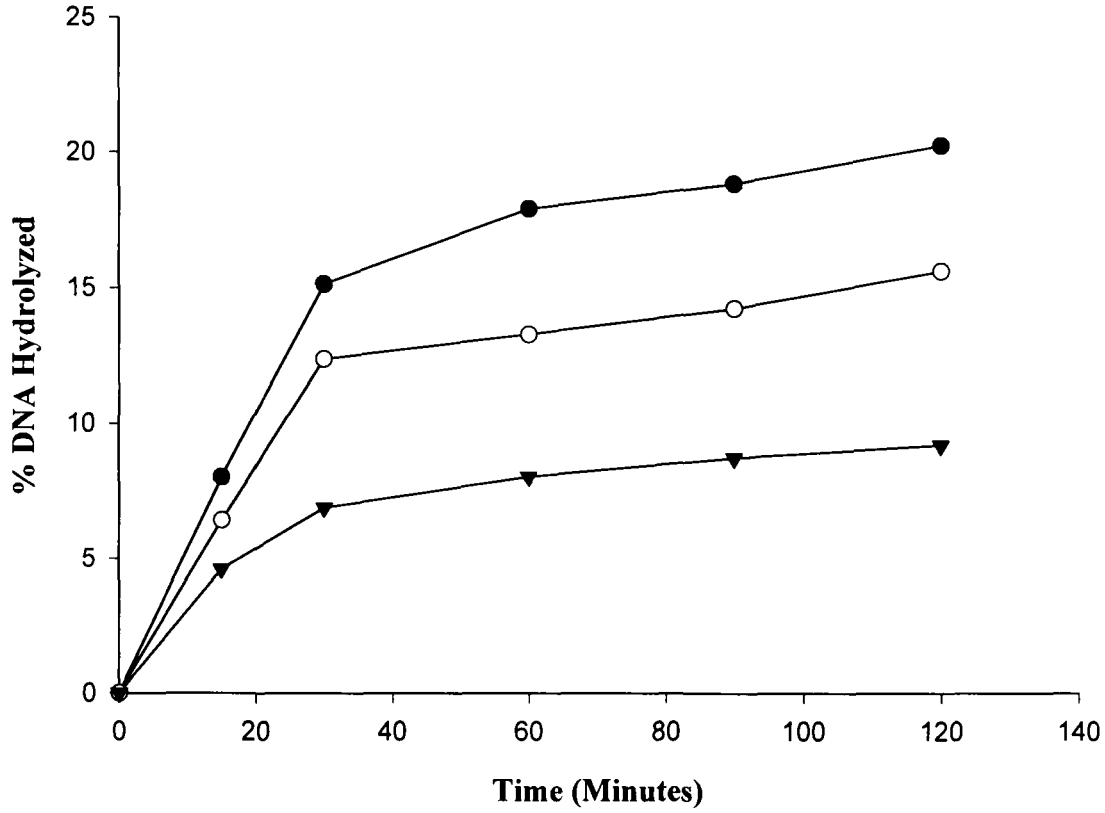

25
20
15
10
5
0
% DNA Hydrolyzed
0
20
40
60
80
100
120
140
Time (Minutes)

Table I: **S_1 nuclease hydrolysis of DNA treated with green and black tea extracts in the presence of different metal ions.**

Metal ion	DNA hydrolyzed (%)	
	Green tea extract	Black tea extract
Cu(II)	22.7 ± 1.38	16.28 ± 1.19
Fe(II)	11.73 ± 1.02	7.65 ± 1.22
Zn(II)	3.82 ± 0.72	3.04 ± 0.64
Mg(II)	3.36 ± 0.44	3.34 ± 0.45
Co(II)	2.63 ± 0.19	2.57 ± 0.19
Ca(II)	2.61 ± 0.23	2.44 ± 0.17
Mn(II)	2.41 ± 0.21	2.40 ± 0.20
Ni(II)	1.29 ± 0.29	1.26 ± 0.23

Treatment of calf thymus DNA (1000μg/ml) in Tris-HCl (10 mM, pH 7.5), with the tea extracts (400μg/ml) was carried out with respective metal ions (0.1 mM) in sterile tubes at room temperature for 1 hour. At the end of the incubation period the reaction mixtures were subjected to S_1 nuclease hydrolysis in the standard assay.

All values are expressed as Mean ± SE for three different experiments.

molecule (Richmond *et al.*, 1981). Table II compares the rate of formation of hydroxyl radicals by green and black tea extracts in the presence of Cu(II). As seen green tea extract is a more efficient generator of the hydroxyl radicals than the black tea. Figure 9 shows that tea extracts – Cu(II) generated hydroxyl radicals that react with calf thymus DNA. The assay is based on the fact that degradation of DNA by hydroxyl radicals results in the release of TBA reactive material, which forms a coloured adduct readable at 532 nm (Quinlan and Gutteridge, 1987). The results confirm the relatively higher rate of formation of hydroxyl radicals by green tea extract and correlate with the rate of DNA degradation by the two extracts.

The most probable mechanism for the formation of hydroxyl radicals would be a Fenton type reaction involving H_2O_2 and Cu(I). However prior presence of H_2O_2 may not be necessary because it is known that generation of superoxide anion may lead to the formation of H_2O_2 (Haliwell and Gutteridge, 1984). The addition of a second electron to the O_2^- anion gives the peroxide ion ($2O_2^-$), which has no unpaired electron and is not a radical. However, at neutral pH the peroxide ion immediately protonates to give hydrogen peroxide (H_2O_2). Alternatively, in aqueous solutions the superoxide anion undergoes dismutation to form H_2O_2 and O_2. I have therefore compared the rate of formation of superoxide anion by green and black tea extracts by the NBT reduction method (see 'Methods'). The results in Figure 10 show that the anion is generated by green tea at a higher rate than black tea.

Table II: **Formation of hydroxyl radical as a function of green and black tea extracts concentrations.**

Green and Black tea extract (μg/ml)	Hydroxylated product formed (n mol)	
	Green tea extract	Black tea extract
5	4.40 ± 0.27	1.89 ± 0.36
25	9.94 ± 1.04	4.33 ± 1.09
50	23.01 ± 3.27	10.16 ± 1.53
100	38.93 ± 2.34	13.61 ± 1.09
200	59.46 ± 0.59	19.42 ± 1.56

Reaction conditions are described in 'Methods' and values of tea extracts are final reaction concentrations.

All values are expressed as Mean ± SE for three different experiments.

Figure 9: **Production of hydroxyl radicals as a function of increasing green tea extract and black tea extract concentrations.**

The reaction mixtures containing increasing concentrations of tea extracts (50-800μg/ml) in the presence of 0.1 mM Cu (II) were incubated for half an hour at 37°C. Reaction conditions are described in 'Methods'.
(●) green tea extract
(○) black tea extract
All points represent triplicate samples and mean values have been plotted.

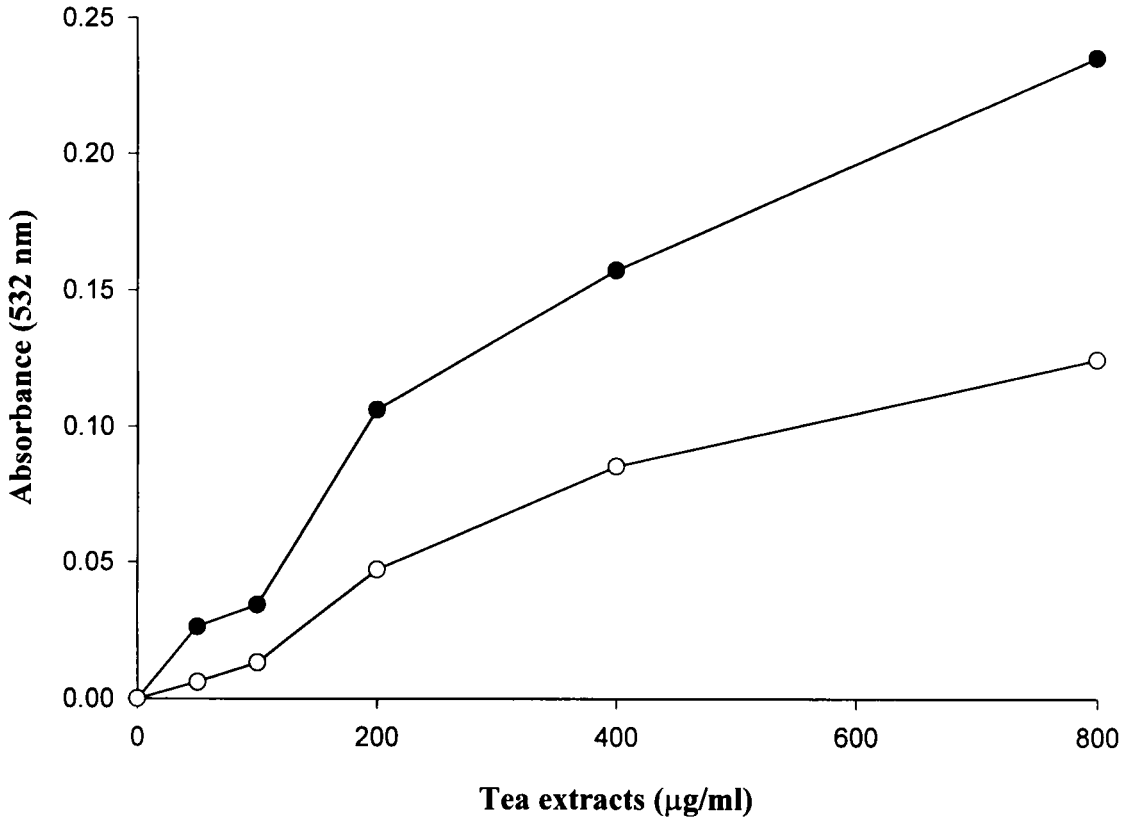
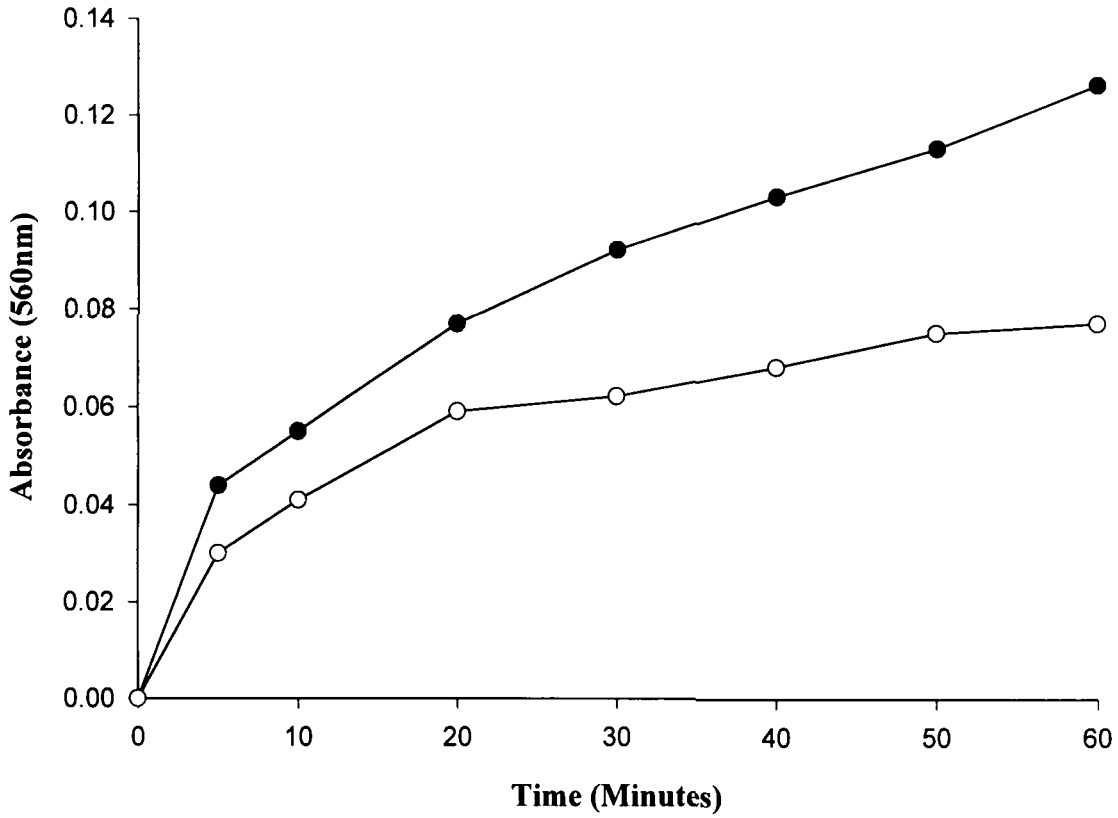

0.25
0.20
0.15
0.10
0.05
0.00
Absorbance (532 nm)
0
200
400
600
800
Tea extracts (μg/ml)

Figure 10: **Photogeneration of superoxide anion by tea extracts as a function of time.**

(●): 66μg/ml green tea extract under fluorescent light
(○): 66μg/ml black tea extract under fluorescent light
The detailed procedure is given in 'Methods'.
All points represent triplicate samples and mean values have been plotted.


0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Absorbance (560nm)
0
10
20
30
40
50
60
Time (Minutes)

This observation is in conformity with the higher rate of hydroxyl radical formation and DNA degradation by green tea extract.

Effect of oxygen radical scavengers on calf thymus DNA breakage by green and black tea extracts

Table III shows the effect of various oxygen radical scavengers on DNA breakage by green and black tea extracts and copper ions. In agreement with the previous results green tea extract was considerably more efficient in DNA degradation in the absence of any scavenger. The scavengers tested showed varying degree of inhibition suggesting the essential role of oxygen radicals. Of the scavengers of the hydroxyl radical, thiourea was the most effective, whereas mannitol showed twice as much inhibition in the case of green tea. Catalase and sodium azide as removers of hydrogen peroxide and singlet oxygen respectively showed significant inhibition. Whereas superoxide dismutase (SOD), which scavenges superoxide anion was more effective in green tea mediated degradation. Neocuproine which sequesters Cu(I) also had a differential effect being considerably less effective in the case of black tea.

Effect of green tea and Cu(II) on the viability of phage T_4

In order to explore the biological consequence of the DNA breakage reaction, inactivation of bacteriophage T_4 by the green tea –

Table III: Percent inhibition of S_1 nuclease hydrolysis of DNA after treatment with 800μg/ml of tea extracts and 100 μM Cu(II) in the presence of scavengers.

Scavenger	Green Tea	Black Tea
	% inhibition	% inhibition
Thiourea (20mM)	85.4 ± 0.71	84.1 ± 0.55
Catalase (0.1mg/ml)	46.1 ± 0.55	42.3 ± 0.76
Sodium Azide (20mM)	45.4 ± 0.41	34.1 ± 0.84
Mannitol (20mM)	41.2 ± 0.69	19.7 ± 0.53
SOD (20 mM)	36.1 ± 0.76	17.3 ± 0.66
Neocuproine (1mM)	29.9 ± 0.56	11.7 ± 0.44
Boiled catalase (0.1mg/ml)	19.95 ± 0.46	3.3 ± 0.24

Concentration of scavengers shown are final reaction concentrations. Details of the reaction mixture are given in 'Methods'.

All values are expressed as Mean ± SE for three different experiments.

Cu(II) system was tested. The inactivating activity was determined by incubating the phage with green tea extract and Cu(II) and then measuring the loss of biological activity. Figure 11 gives the loss of survival of phage as a function of green tea concentration. Increasing concentrations of green tea at a fixed concentration of Cu(II) (100μM) after an hour of incubation at 37°C, resulted in a progressive loss of survival of the phage. The percentage of phage surviving at 200 μg/ml of green tea extract is only about 15%. Increasing concentrations of Cu(II) with a fixed green tea concentration of 200 μg/ml also resulted in a similar loss of survival of the phage (results not shown).

As shown above Cu(I) is an essential intermediate in the DNA breakage reaction. In order to examine its role in phage inactivation I have studied the effect of increasing concentrations of neocuproine on phage survival. The concentration of Cu(II) used was 50 μM. The result given in Figure 12 show that the phage inactivation is inhibited to about 90% indicating the essential involvement of Cu(I) in the reaction. This result indicate that the formation of Cu(I) by green tea is an essential step in the phage inactivation mechanism.

The inactivating activity of green tea – Cu(II) system was substantially but differentially reduced by prior addition of quenchers of oxygen free radicals (Table IV). Maximum inhibition was afforded by sodium benzoate and catalase followed by sodium azide and thiourea indicating the involvement of hydroxyl radical, hydrogen

Figure 11: Kinetics of T_4 phage inactivation by green tea extract -Cu(II).

Effect of increasing concentrations of green tea extract in the presence of 100 μM Cu(II) on the viability of bacteriophage T_4. Phage suspension was treated with indicated concentrations of green tea extract at 37°C for 1 hour in a reaction mixture containing 10mM Tris-HCl/Mg^{2+} (pH 8.0) The detailed procedure is given in 'Methods'.
All points represent triplicate samples and mean values have been plotted.

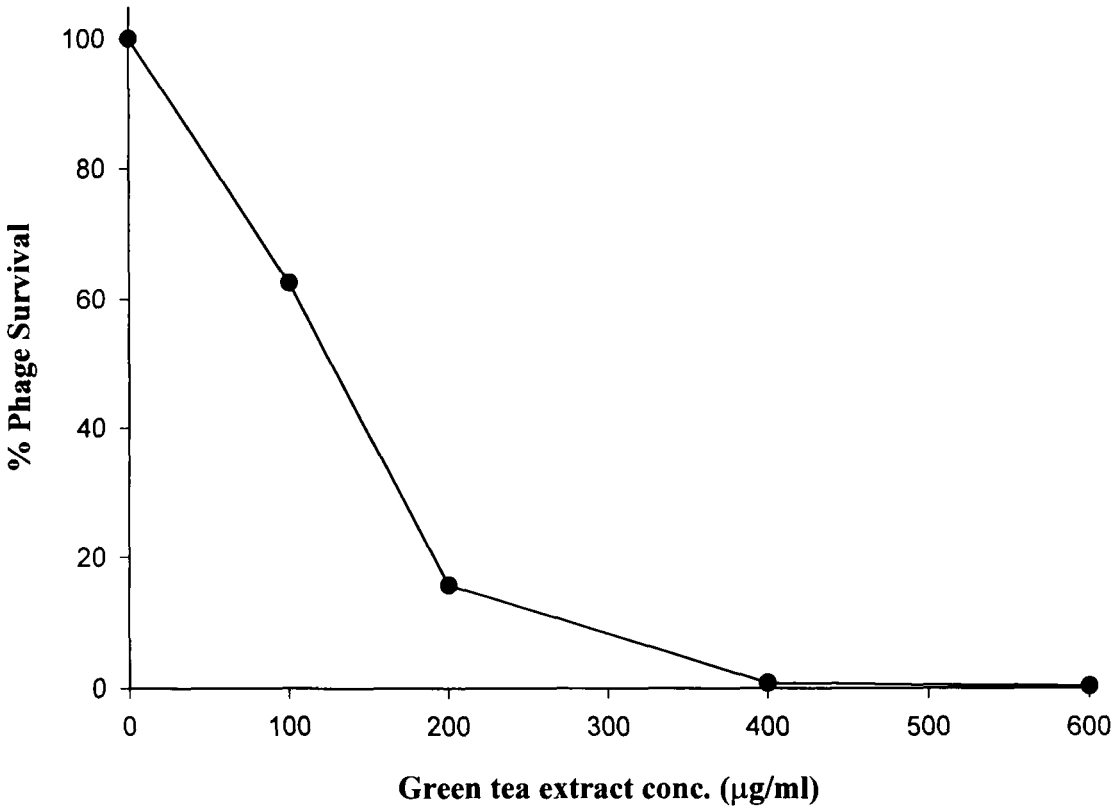

100
80
60
40
20
0
% Phage Survival
0
100
200
300
400
500
600
Green tea extract conc. (µg/ml)

Figure 12: Effect of increasing concentrations of neocuproine on the viability of green tea extract – Cu(II) treated T_4 phage.

The concentrations of green tea extract and Cu(II) were 400μg/ml and 50 μM respectively. Other details of the procedure are given in 'Methods'. All points represent triplicate samples and mean values have been plotted.

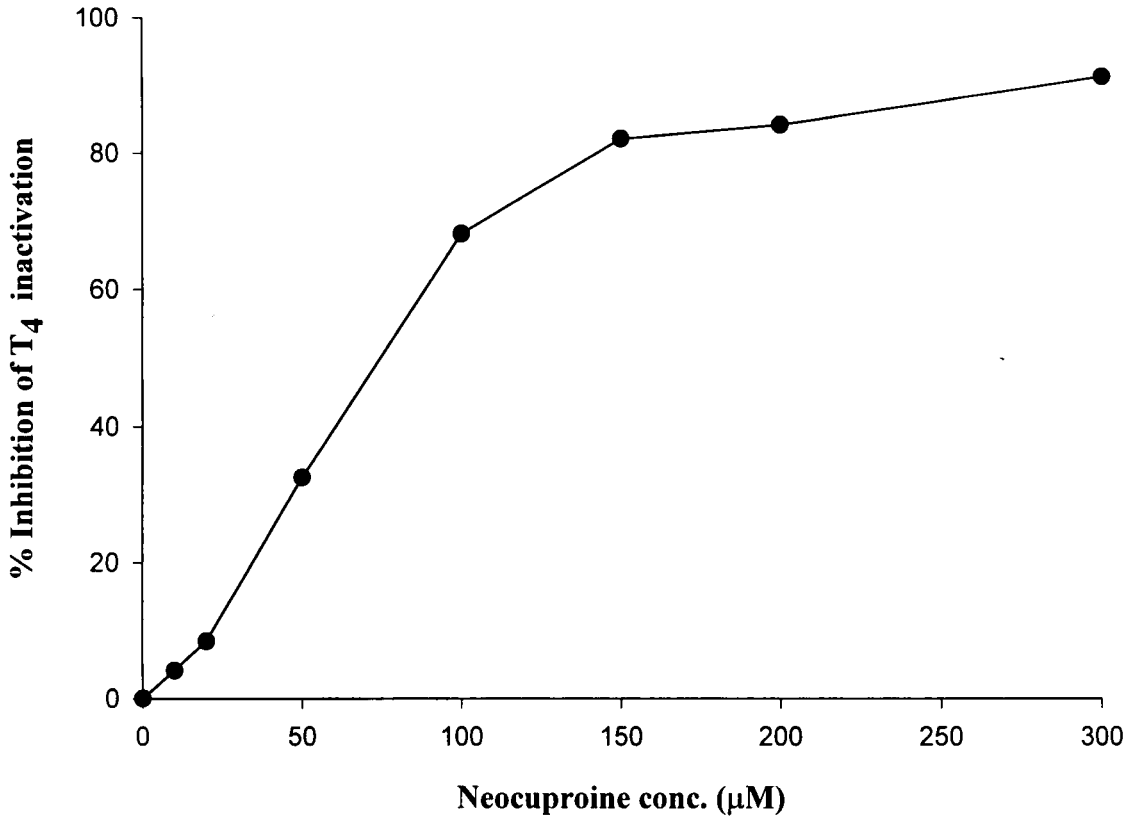

100
80
60
40
20
0
% Inhibition of T_4 inactivation
0
50
100
150
200
250
300
Neocuproine conc. (μM)

Table IV: Percent inhibition of T_4 phage inactivation after treatment with green tea (100μg/ml) and Cu(II) (25μM) in the presence of scavengers of reactive oxygen species.

Conditions	% inhibition of T_4 inactivation
Sodium benzoate (50mM)	56.11 ± 1.51
Catalase (100μg/ml)	49.38 ± 1.83
Boiled catalase (100 μg/ml)	0
Sodium Azide (50 mM)	34.49 ± 1.86
Thiourea (50 mM)	18.2 ± 0.10

All values are expressed as Mean ± SE for three different experiments.

peroxide and singlet oxygen in T_4 phage inactivation reaction by green tea. These results and those of the previous experiments strongly indicate that the DNA degradation and phage inactivation by green tea and copper ions essentially follow a similar mechanism of action.

Table V shows the sensitivity of bacteriophage T_4 to green tea – Cu(II) and the effect of UV irradiation on the host cells. Control incubations of phage with 400 μg/ml green tea alone or 100 μM Cu(II) alone resulted in <5% inactivation. The fractions of surviving phage, after green tea – Cu(II) treatment, were 0.028 and 0.38 in the case of untreated and UV treated host cells, respectively. It may be noted that at 400 μg/ml green tea concentration used in this experiment, the fraction of phage survival (0.028) matches the degree of inactivation in Figure 11 (0.05). This result indicate that UV treatment of the host bacteria enhance the recovery of the phage. One possibility could be that the UV inducible pathway is activated on such treatment leading to enhanced recovery of the phage.

Table V: **Sensitivity of T_4 phage to green tea-Cu(II) and the effect of UV-irradiation on host cells.**

Host Strain	Host pretreatment	Phage pretreatment	PFU/ml	Survival[a]
E.coli BB	None	Control	$(9.51\pm0.049)\times10^9$	1
E.coli BB	None	GTE-Cu(II)[b]	$(2.73\pm0.079)\times10^8$	0.028
E.coli BB	UV	Control	$(7.81\pm0.063)\times10^9$	1
E.coli BB	UV	GTE-Cu(II)[b]	$(3.01\pm0.051)\times10^9$	0.384

Incubation was done for 1 hour at 37°C followed by dilution and plating.

All values are expressed as Mean ± SE for three different experiments.

[a]The value of survival was calculated by dividing values in the presence of green tea-Cu(II) by control values.

[b]Concentrations of green tea and Cu(II) used were 400μg/ml and 100μM respectively.

DISCUSSION (Part I)

The major conclusions of the above experiments may be stated as follows: (i) In agreement with earlier reports (Wei *et al.*, 1999) green tea extract contains catechins which include epicatechin, epigallocatechin, epigallocatechin-3-gallate and these are absent in black tea extract; (ii) the rate of DNA degradation in the presence of Cu(II) by water extract of green tea is considerably greater than by the extract of black tea; (iii) this correlates with the higher rate of Cu(II) reduction and hydroxyl radical generation by green tea extract; (iv) it is further suggested that 'oxidized' polyphenolic constituents of tea extract are also capable of catalyzing the reduction of redoxcycled copper ions; (v) Phage inactivation studies indicate that the DNA breakage reaction is biologically significant.

The structural units of catechins in green tea, shown in Figure 1 are epicatechin and gallic acid. Previously it has been shown that both these polyphenols catalyze strand breakage in DNA in the presence of copper ions (Khan and Hadi, 1998; Ahmad *et al.*, 1992). It was also shown that the rate of DNA degradation correlates with the number of hydroxyl groups present particularly with ortho-dihydroxy configuration. Indeed, when two of the three hydroxyl groups of gallic acid are methylated (syringic acid) the DNA degrading capacity declines sharply (Khan and Hadi, 1998). This result correlates with the

observations (Inoue *et al.*, 1994) which showed that whereas gallic acid exhibits apoptotic activity, syringic acid is inactive. In the manufacture of black tea, the monomeric falvan –3-ol (epicatechin) undergoes polyphenol oxidase dependent oxidative polymerization leading to the formation of bis-flavanols, theaflavins, thearubigins and other oligomers. The formation of these compounds involves the hydroxyl groups, leading to a reduction in the number of copper ion chelating positions relative to the size of the molecule. This possibly accounts for the reduced DNA degradation and copper reducing capability of black tea extract.

The results of bacteriophage T_4 inactivation studies indicate that green tea–Cu(II) mediated action also causes bacteriophage inactivation through a mechanism similar to DNA breakage. This is particularly suggested by (i) the effect of scavengers on phage inactivation and (ii) that the phage inactivation is inhibited by the neocuproine which sequesters Cu(I). These results further indicate that UV treatment of host bacteria enhances the recovery of the phage, indicating the involvement of UV inducible pathway in the repair of green tea – Cu(II) mediated damage of phage DNA.

Most clinically used anticancer drugs can activate late events of apoptosis (DNA degradation and morphological changes) and the essential signaling pathways differ between pharmacological cell death and physiological induction of programmed cell death (Smets, 1994).

Fe^{3+} and Cu^{2+} are the most redox-active of the metal ions in living cells. Although iron is considerably more abundant in cells the major ions in the nucleus are copper and zinc (Bryan, 1979). It has been proposed (Wolfe *et al.*, 1994) that a copper mediated Fenton reaction, generating site-specific hydroxyl radicals, is capably of inducing apoptosis in thymocytes. Further, it has been shown (Burkitt *et al.*, 1996) that the internucleosomal DNA fragmentation might be caused not only by an endonuclease but also by metal chelating agents such as 1,10-phenanthroline (OP), which promotes the redox activity of endogenous copper ions and the resulting production of hydroxyl radicals. Thus, the internucleosomal DNA "laddering" often used as an indicator of apoptosis may also reflect DNA fragmentation by non-enzymatic processes. Several reports indicate that serum (Ebadi and Swanson, 1988) and tissue (Yoshida *et al.*, 1993) concentrations of copper are greatly increased in various malignancies. Indeed, such concentrations have been described as a sensitive index of disease activity of several hematologic and non-hematologic malignancies (Pizzolo *et al.*, 1978). Irrespective of the exact mechanism involved these facts and other observations in literature suggest that the various anticancer effects and apoptotic DNA fragmentation activities of several plant derived polyphenols, including those in green tea, may be explained by their ability to mobilize endogenous or otherwise increased concentrations of copper in cancer cells. However, it is recognized that the results presented above provide only partial support

to our hypothesis (Hadi *et al.*, 2000), which implies a prooxidant effect, involving mobilization of copper ions by green tea polyphenols, on cancer cells. Therefore, further experiments on the effect of green tea extract and the constituent polyphenols, on the induction of oxidative damage to certain critical cellular targets have to be carried out. Further, the fact that these polyphenolic compounds are ingested by human populations as a part of the normal diet in relatively higher concentrations without adverse reactions points to their great potential as putative chemopreventive or therapeutic agents.

Part - II

RESULTS (Part-II)

As already mentioned, gallic acid is structural constituent of tannins and polyphenols of green tea. It shows a number of biological properties including induction of apoptosis. Similar to green tea it also exhibits antioxidant and pro-oxidant properties. Thus, with the aim of identifying the structural features of green tea polyphenols, I have compared the prooxidant DNA cleavage properties and antioxidant properties of gallic acid and green tea.

Breakage of calf thymus DNA by gallic acid and green tea extract

Figure 13 gives the acid soluble material produced from calf thymus DNA with increasing concentrations of gallic acid and green tea extract at a fixed concentration of Cu(II). The rate of DNA hydrolysis and the maximum hydrolysis achieved was greater with gallic acid than with green tea.

Production of hydroxyl radical by gallic acid and green tea extract

Figure 14 shows that gallic acid and green tea – Cu(II) generate hydroxyl radicals that react with calf thymus DNA. The assay is based on the fact that degradation of DNA by hydroxyl radicals results in the release of TBA reactive material, which forms a coloured adduct readable at 532 nm (Quinlan and Gutteridge, 1987). The results confirm the relatively higher rate of formation of hydroxyl radicals by

Figure 13: Degradation of calf thymus DNA as a function of increasing concentrations of gallic acid and green tea extract in the presence of Cu(II) as measured by the degree of S_1 nuclease digestion.

DNA was incubated with increasing concentrations (50-400 μg/ml) of gallic acid (●) and green tea extract (▼) in the presence of 0.1 mM Cu(II) at 37°C for 1 hour.
All points represent triplicate samples and mean values have been plotted.

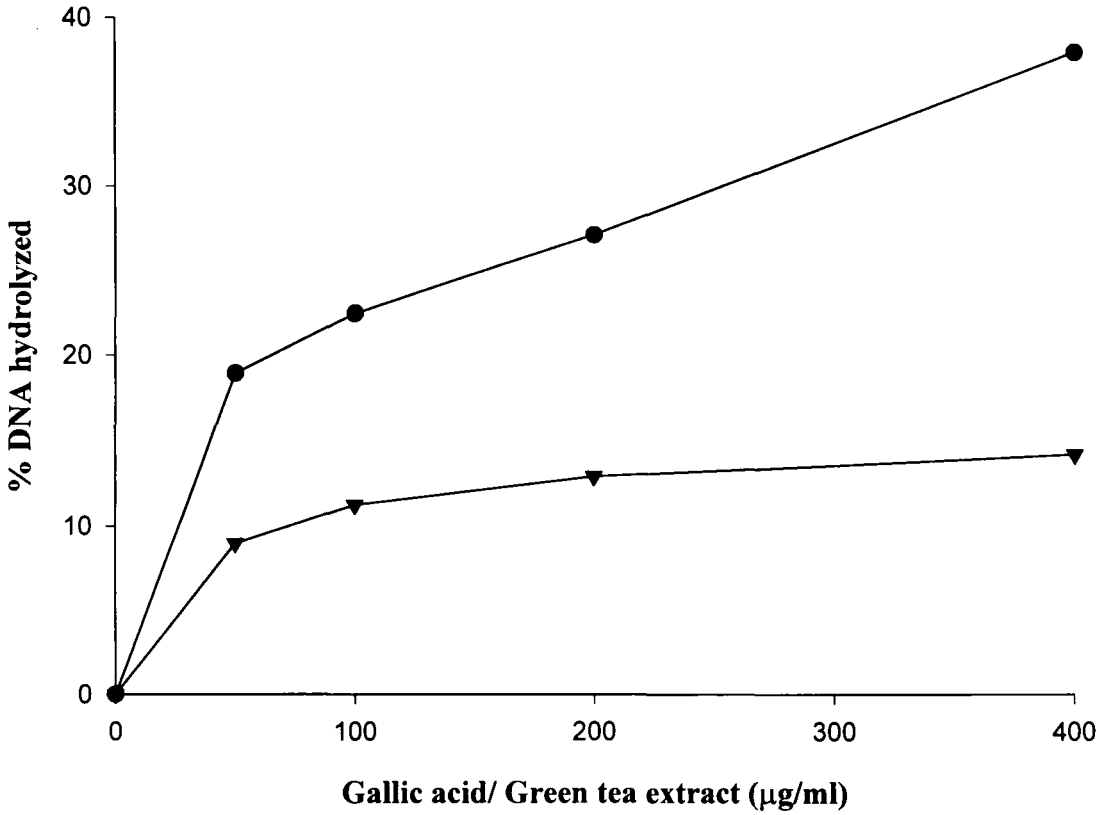

40
30
20
10
0
% DNA hydrolyzed
0
100
200
300
400
Gallic acid/ Green tea extract (μg/ml)

Figure 14: Production of hydroxyl radicals as a function of increasing gallic acid and green tea extract concentrations.

The reaction mixtures containing increasing concentrations of gallic acid and green tea extract (50-400 μg/ml) in the presence of 0.1 mM Cu(II) were incubated for half an hour at 37°C. Reaction conditions are described in 'Methods'.
gallic acid (●)
green tea extract (▼)
All points represent triplicate samples and mean values have been plotted.


0.30
0.25
0.20
0.15
0.10
0.05
0.00
Absorbance (532 nm)
0
100
200
300
400
Gallic acid/ Green tea extract (μg/ml)

gallic acid and correlate with the rate of DNA degradation by the two compounds.

Photogeneration of superoxide anion by gallic acid and green tea extract

I have compared the rate of formation of superoxide anion by gallic acid and green tea extract by NBT reduction method (See Methods). The result in Figure 15 show that the anion is generated by gallic acid at a higher rate than green tea. This observation is in conformity with the higher rate of hydroxyl radical formation and DNA degradation by gallic acid. It may be mentioned that superoxide anion spontaneously leads to the formation of hydrogen peroxide (Halliwell and Gutteridge, 1984), which in the presence of reduced copper (Fenton reaction) can form hydroxyl radicals.

Effect of alternative metal ions

Of the several metal ions tested only Cu(II) and Fe(III) complemented green tea extract and gallic acid in the DNA breakage reaction to any significant extent (Table VI). Although some hydrolysis is also seen with Fe(II), Zn(II), Ca(II), Mg(II), Co(II), Mn(II) and Ni(II). These results are similar to those seen with other polyphenols such as flavonoids (Rahman *et al.*, 1989). However in agreement with the relative DNA hydrolysis by the two systems, gallic acid is considerably more active in the presence of Cu(II) and Fe(III).

Figure 15: Photogeneration of superoxide anion by gallic acid and green tea extract as a function of time.

(●): 66 μg/ml gallic acid under fluorescent light
(▼): 66 μg/ml green tea extract under fluorescent light
The detailed procedure is given in 'Methods'.
All points represent triplicate samples and mean values have been plotted.

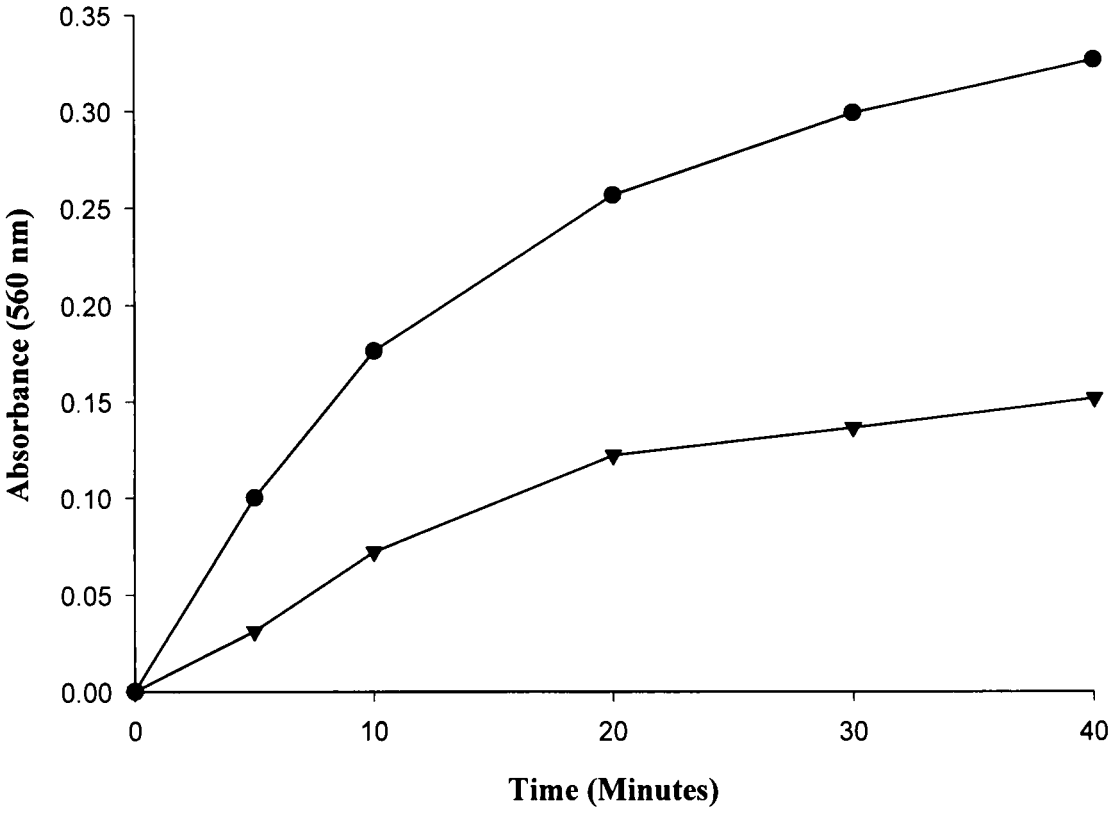

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Absorbance (560 nm)
0
10
20
30
40
Time (Minutes)

Table VI: **S_1 nuclease hydrolysis of DNA treated with gallic acid and green tea extract in the presence of different metal ions.**

Metal ion	DNA hydrolyzed (%)	
	Gallic acid	Green tea extract
Cu(II)	20.23 ± 0.48	14.35 ± 0.41
Fe(III)	11.5 ± 0.71	7.67 ± 0.66
Fe(II)	7.47 ± 0.11	4.64 ± 0.15
Zn(II)	4.38 ± 0.17	3.56 ± 0.13
Ca(II)	3.66 ± 0.14	3.48 ± 0.15
Mg(II)	3.33 ± 0.21	2.86 ± 0.19
Co(II)	2.58 ± 0.13	2.26 ± 0.14
Mn(II)	2.09 ± 0.16	1.64 ± 0.11
Ni(II)	1.26 ± 0.10	0.63 ± 0.04

Treatment of calf thymus DNA (1000μg/ml) in Tris-HCl (10 mM, pH 7.5), with compounds (400μg/ml) was carried out with respective metal ions (0.1 mM) in sterile tubes at room temperature for 1 hour. At the end of the incubation period the reaction mixtures were subjected to S_1 nuclease hydrolysis in the standard assay.

All values are expressed as Mean ± SE for three different experiments.

Effect of gallic acid and green tea extract on Fe(II)-EDTA mediated degradation of calf thymus DNA as measured by the degree of S_1 nuclease digestion

As mentioned above the polyphenolic constituents of green tea and gallic acid are considered to be physiological antioxidants in plants. I have therefore tested these activities on hydroxyl radical mediated degradation of DNA. The generation of hydroxyl radical was carried out by the Fe(II) – EDTA hydroxyl radical generating system (Prigodich and Martin, 1990). Figure 16 shows the effect of increasing concentrations of gallic acid and green tea extract on Fe(II) – EDTA mediated degradation of calf thymus DNA in the presence of H_2O_2 and ascorbate. The result shows that green tea exhibited a greater protective effect than gallic acid.

Formation of hydroxyl radicals by Fe(II) – EDTA and their quenching by gallic acid and green tea extract

Using Fe(II) – EDTA system (Prigodich and Martin, 1990), I have directly tested the effect of increasing concentrations of gallic acid and green tea extract on the generation of hydroxyl radicals. The assay is based on the fact that degradation of DNA by hydroxyl radicals results in the release of TBA reactive material, which forms a coloured adduct with TBA, readable at 532 nm (Quinlan and Gutteridge, 1987). Figure 17 shows that gallic acid has a quenching effect on the production of hydroxyl radicals. Green tea is also shown

Figure 16: **Inhibition of hydroxyl radical [generated by Fe(II)-EDTA-H_2O_2] mediated degradation of calf thymus DNA by increasing concentrations of gallic acid and green tea extract as measured by the degree of S_1 nuclease digestion.**

Calf thymus DNA was treated with 1mM sodium ascorbate (pH 7.0), 40 μM Fe(II), 80 μM EDTA and 0.03% H_2O_2 in the presence of increasing concentrations of gallic acid (●) and green tea extract (▼) (100-600 μg/ml) for 45 minutes at 37°C. At the end of the incubation period the reaction mixture was subjected to S_1 nuclease hydrolysis.
All points represent triplicate samples and mean values have been plotted.

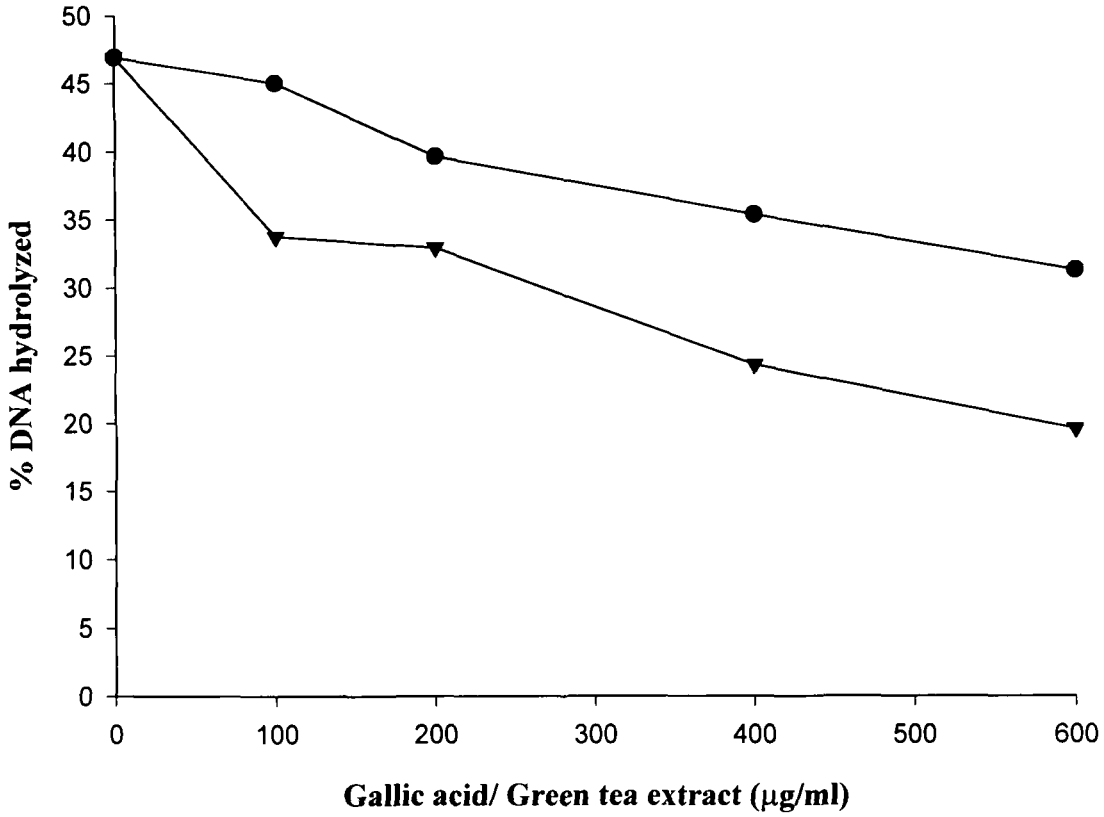
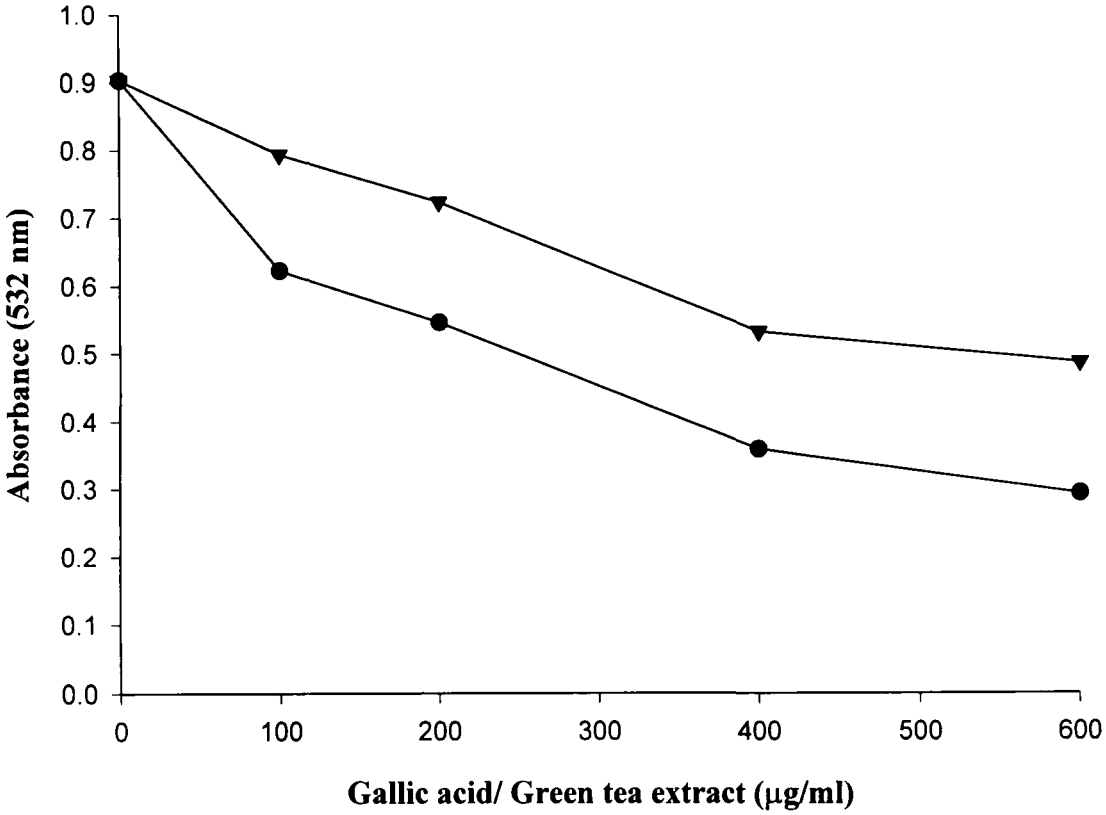

50
45
40
35
30
25
20
15
10
5
0
% DNA hydrolyzed
0
100
200
300
400
500
600
Gallic acid/ Green tea extract (µg/ml)

Figure 17: **Formation of hydroxyl radicals by Fe(II) – EDTA – H_2O_2 system and their quenching by increasing concentrations of gallic acid and green tea extract.**

Calf thymus DNA (400μg/ml) was treated with 0.4mM sodium ascorbate (pH 7.0), 40μM Fe(II), 80μM EDTA and 0.03% H_2O_2 in the presence of increasing concentrations of gallic acid (●) and green tea extract (▼) (100-600 μg/ml) for half an hour at 37°C. Reaction conditions are described in 'Methods'.
All points represent triplicate samples and mean values have been plotted.


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance (532 nm)
0
100
200
300
400
500
600
Gallic acid/ Green tea extract (μg/ml)

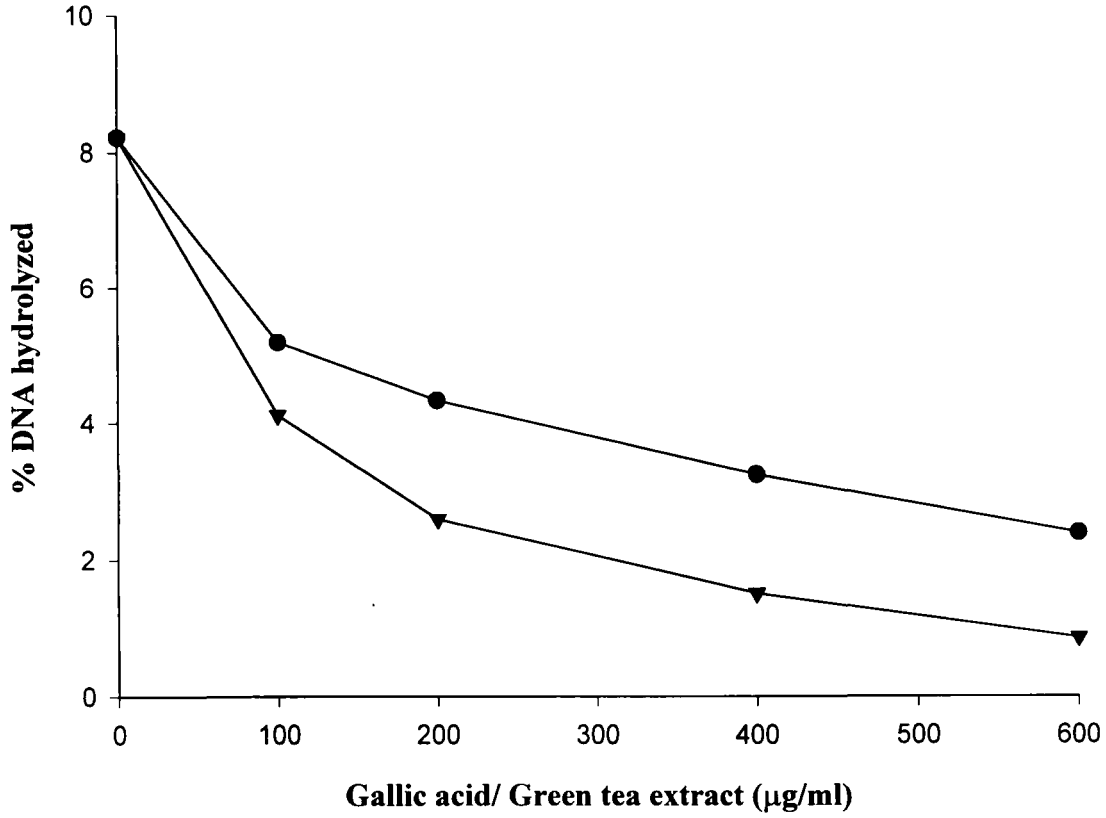
to quench the production of radicals but its effect is relatively less than that of gallic acid.

Effect of gallic acid and green tea extract on riboflavin and light mediated DNA cleavage

Riboflavin being a photo oxidant is known to generate singlet oxygen and cause subsequent DNA degradation in the presence of visible light (Naseem *et al.*, 1993). In order to confirm the relative antioxidant property of gallic acid and green tea extract, the effect of the two compounds on such DNA degrading system was examined. In agreement with the above finding (Figure 16), Figure 18 shows that green tea is a considerably better inhibitor of DNA breakage by riboflavin as compared to gallic acid.

Figure 18: **Inhibition of photosensitized riboflavin mediated degradation of calf thymus DNA by increasing concentrations of gallic acid and green tea extract as measured by the degree of S_1 nuclease digestion.**

Calf thymus DNA in 10mM Tris-HCl (pH 7.5) was incubated with 20 μM riboflavin in the presence of increasing concentrations of gallic acid (●) and green tea extract (▼) (100-600 μg/ml) for 2 hours at room temperature under fluorescent light. At the end of the incubation period the reaction mixture was subjected to S_1 nuclease hydrolysis.
All points represent triplicate samples and mean values have been plotted.


10
8
6
4
2
0
% DNA hydrolyzed
0
100
200
300
400
500
600
Gallic acid/ Green tea extract (μg/ml)

Discussion (Part-II)

The major polyphenolic constituent of green tea, which has shown chemopreventive properties in a number of studies, is epigallocatechin-3-gallate (EGCG). The major structural constituent of EGCG is gallic acid (Figure 1). Similar to EGCG, gallic acid itself is considered to be an antioxidant and induces apoptosis in cancer cells (Inoue *et al.*, 1994). As already mentioned in the 'Introduction' most plant polyphenols also exhibit prooxidant properties particularly in the presence of transition metal ions such as copper, therefore in the above experiments I have compared the prooxidant and antioxidant properties of green tea extract with gallic acid. The major conclusions of these experiments may be stated as follows: (i) the rate of DNA degradation in the presence of Cu(II) by gallic acid is considerably greater than by green tea extract; (ii) this correlates with the higher rate of hydroxyl radical and super oxide anion generation by gallic acid; (iii) gallic acid is also a more efficient quencher of hydroxyl radical generation (Figure 17).

The other polyphenols in green tea that have been implicated in chemoprevention against cancer and cardiovascular disease are epigallocatechin (EGC) and epicatechin (EC). The former also contains gallic acid as a structural component. It is thus possible that gallic acid contributes significantly to the pharmacological action of green tea polyphenols. Structure activity studies carried out in our laboratory

with tannic acid and gallic acid indicate that if two of the three hydroxyl groups of gallic acid are methylated (syringic acid), the DNA degrading capacity decreases sharply (Khan and Hadi, 1998). These results correlate with those of Inoue *et al.* (1994) who showed that modification of phenolic hydroxyl groups, such as that resulting in the formation of syringic acid, abolishes the apoptotic activity of gallic acid. Several lines of evidence in the literature strongly suggest that the antioxidant properties of plant polyphenolics may not entirely account for their chemopreventive effects (Hadi *et al.*, 2000). For example, Burkitt *et al.*, (1996) suggested that the internucleosomal DNA fragmentation might be caused not only by an endonuclease but also by metal chelating agents such as 1, 10-phenanthroline (OP), which promote the redox activity of endogenous copper ions and the resulting production of hydroxyl radicals. In addition, several reports indicate that serum (Ebadi and Swanson, 1988) and tissue (Yoshida *et al.*, 1993) concentrations of copper are greatly increased in various malignancies. Indeed, such concentrations have been described as a sensitive index of disease activity of several hematologic and nonhematologic malignancies (Pizzolo *et al.*, 1978).

Part - III

RESULTS (Part-III)

Breakage of calf thymus DNA and cleavage of plasmid DNA by gallic acid, ellagic acid and tannic acid

Figure 19 gives the rate of S_1nuclease hydrolysis of calf thymus DNA following damage induced by gallic acid, ellagic acid and tannic acid in presence of Cu(II). The reaction was assayed by recording the proportion of double stranded DNA converted to acid soluble nucleotides by S_1 nuclease. Control experiments (data not shown) established that heat denatured DNA underwent 100% hydrolysis following the treatment with S_1 nuclease, whereas only 3% of native DNA was hydrolyzed. As seen in the Figure 19 the efficacy of the compounds tested was as follows: gallic acid > ellagic acid > tannic acid. There is a gradual increase of DNA degradation upon treatment with increasing concentrations of the compounds tested at a fixed concentration of Cu(II). It may be seen that gallic acid causes the largest degree of hydrolysis and lowest degree is achieved by tannic acid.

In order to further substantiate the above results, conversion of supercoiled plasmid DNA to relaxed open circles and linear forms was also examined. This is a sensitive test for just one nick per molecule. Figure 20 shows the ethidium bromide stained banding pattern of pBR 322 DNA with two different concentrations (50 μM

Figure 19: **Degradation of calf thymus DNA as a function of increasing concentration of various compounds in the presence of Cu(II) as measured by the degree of S_1 nuclease digestion.**

DNA was incubated with increasing concentrations (5 μM – 100 μM) of gallic acid (○), ellagic acid (▼) and tannic acid (●) in the presence of 0.1 mM Cu(II) at 37°C for 2 hours.
All points represent triplicate samples and mean values have been plotted.

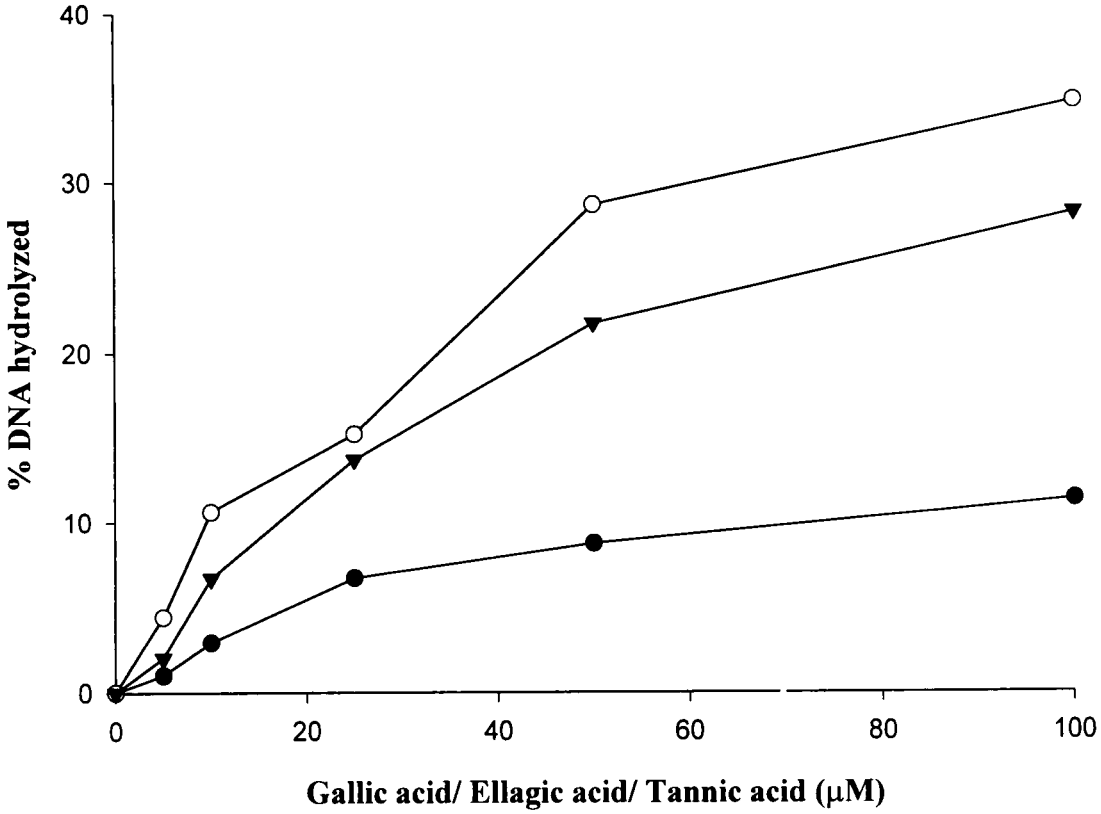

40
30
20
10
0
% DNA hydrolyzed
0
20
40
60
80
100
Gallic acid/ Ellagic acid/ Tannic acid (μM)

Figure 20: **Agarose gel electrophoretic pattern of ethidium bromide stained pBR 322 DNA after treatment with increasing concentration of various compounds in presence of Cu(II).**

Reaction mixtures in a volume of 30 µl containing 0.4 µg plasmid pBR322 DNA, 0.1 mM Cu(II) and increasing concentrations of tannic acid, gallic acid and ellagic acid were incubated for 1 hour at 37°C.
Lane a: DNA alone
Lane b: DNA + Cu(II) + tannic acid (50µM)
Lane c: DNA + Cu(II) + tannic acid (100µM)
Lane d: DNA + Cu(II) + gallic acid (50µM)
Lane e: DNA + Cu(II) + gallic acid (100µM)
Lane f: DNA + Cu(II) + ellagic acid (50µM)
Lane g: DNA + Cu(II) + ellagic acid (100µM)

a b c d e f g

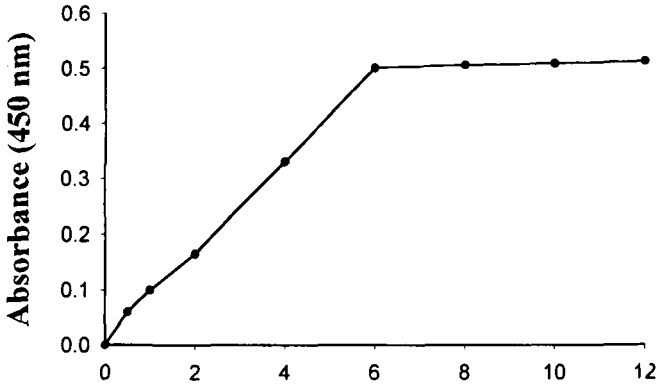
and 100 μM) of gallic acid, ellagic acid and tannic acid. At both the concentrations tannic acid gives partial conversion to relaxed open circle (lanes b and c), gallic acid degrades DNA to small heterogeneous sized molecules giving rise to a smear in lanes d and e and ellagic acid shows conversion to relaxed open circles as well as some linear molecules (lanes f and g). Thus these results are in conformity with the relative DNA degrading capacity of the three compounds seen in Figure 19.

Stoichiometry of Cu(II) reduction by gallic acid, ellagic acid and tannic acid

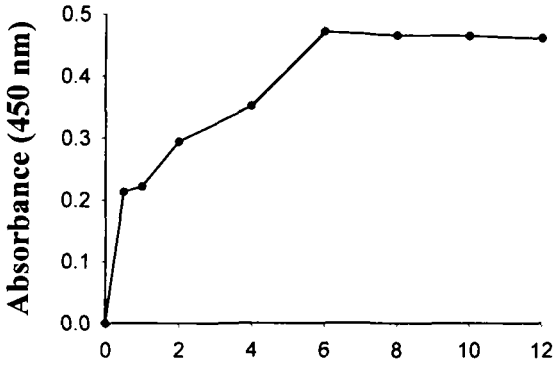
To determine the stoichiometery of Cu(II) reduction by gallic acid, ellagic acid and tannic acid, the Cu(I) sequestering agent namely neocuproine was used. A Job plot (Wong *et al.*, 1984) of absorbance versus [Cu(II)/tannic acid] reveals maximum absorbance at the Cu(II)/tannic acid molar ratio of 6.0 at which maximum conversion of Cu(II) to Cu(I) is achieved (Figure 21) (Bhat and Hadi, 1994). Such ratio was determined to be 6 and 4 for the gallic acid and ellagic acid respectively. The ratio obtained gives the stoichiometry for the reduction of Cu(II) by the free compounds. Except for tannic acid these values correlate with the relative rates of DNA degradation by gallic acid and ellagic acid.

Figure 21: Determination of stoichiometry of Cu(II) reduction by various compounds.

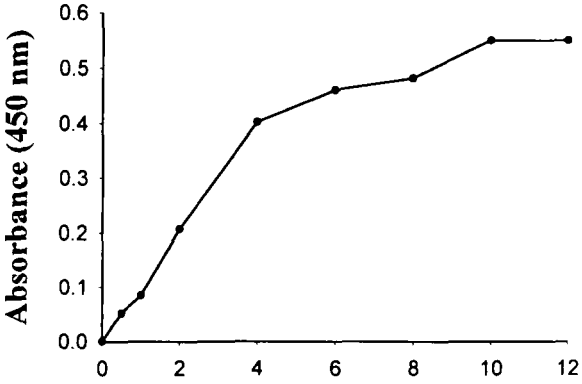
Concentration of compounds used was (●) 0.01 mM in the presence of 0.4 mM neocuproine. The absorbance at 450 nm of samples with added Cu(II) is plotted versus molar equivalents of Cu(II) per molar equivalent of compound. The value of independent variable at the intersection of the two lines is a measure of the moles of Cu(II) converted to Cu(I) per mole of compound. The values obtained for various compounds are:
(a) Tannic acid, 6:1, (b) Gallic acid, 6:1 and (c) Ellagic acid, 4:1.



Eq. [Cu (II)] / [Tannic acid]



Eq. [Cu (II)] / [Gallic acid]



Eq. [Cu (II)] / [Elllagic acid]

Generation of hydroxyl radical ($OH^{\cdot}$) by gallic acid, ellagic acid and tannic acid.

The capacity of gallic acid, ellagic acid and tannic acid to generate hydroxyl radical in the presence of Cu(II) was also compared (Figure 22). This assay uses salicylate as the reporter molecule (Richmond *et al.*, 1981). The result shows that ellagic acid is the least efficient in generating hydroxyl radical whereas tannic acid is the most efficient, followed by gallic acid.

Effect of gallic acid, ellagic acid and tannic acid on Fe(II)- EDTA mediated degradation of calf thymus DNA.

The relative antioxidant effect of gallic acid, ellagic acid and tannic acid was examined by their ability to protect DNA breakage by hydroxyl radicals. The generation of hydroxyl radicals was carried out by the Fe(II)-EDTA hydroxyl radical generating system (Prigodich and Martin, 1990). Figure 23 shows the effect of increasing concentrations of gallic acid, ellagic acid and tannic acid on Fe(II)-EDTA mediated degradation of calf thymus DNA in the presence of H_2O_2 and ascorbate. The result shows that tannic acid is a considerable better inhibitor of such DNA breakage compared to gallic acid, which shows least inhibition followed by ellagic acid.

Figure 22: Production of hydroxyl radicals as a function of increasing gallic acid, ellagic acid and tannic acid concentrations.

The reaction mixtures containing increasing concentrations of compounds (2-25 μM) were incubated for 2 hours at 37°C. Reaction conditions are described in 'Methods'.
(○) gallic acid
(▼) ellagic acid
(●) tannic acid
All points represent triplicate samples and mean values have been plotted.

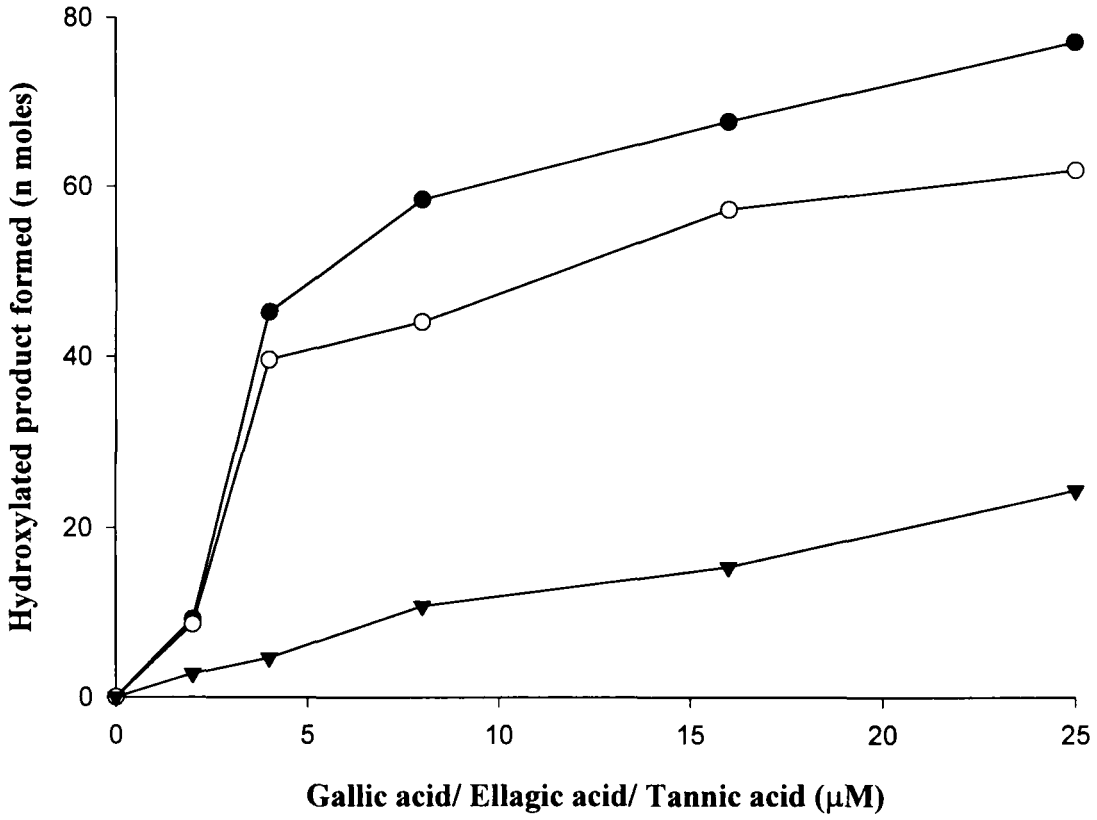
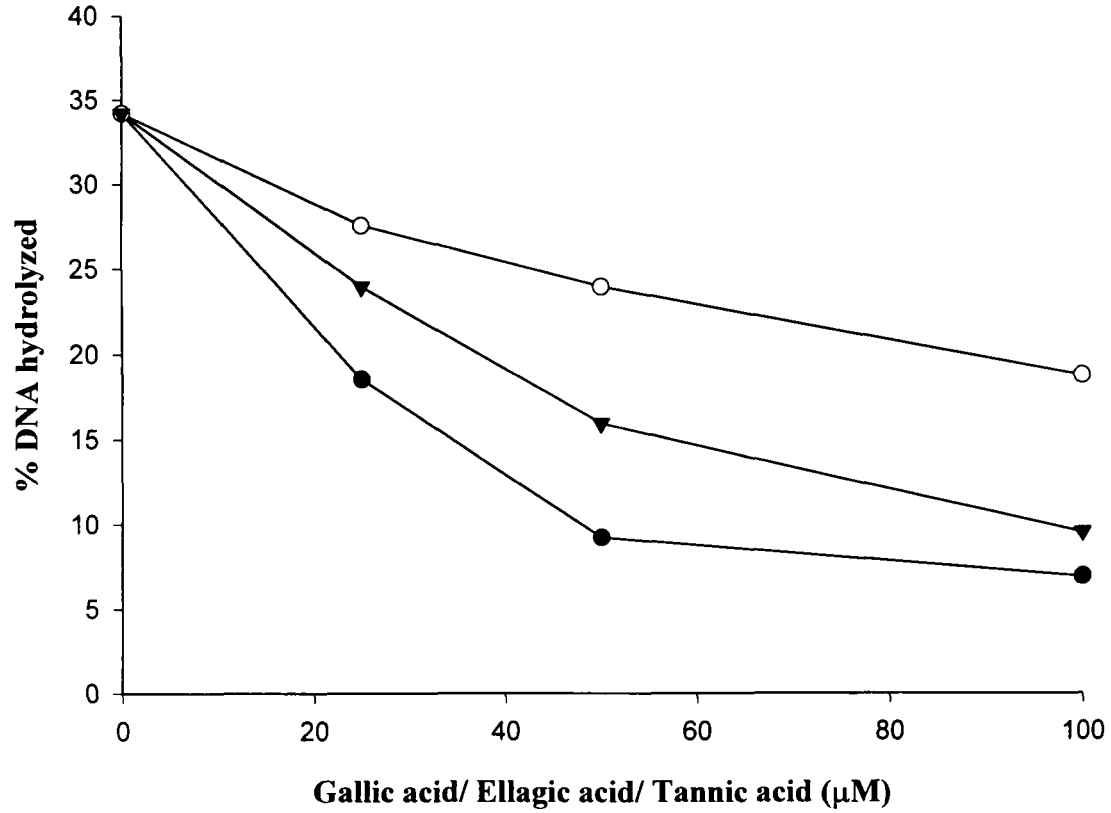

Hydroxylated product formed (n moles)
80
60
40
20
0
0
5
10
15
20
25
Gallic acid/ Ellagic acid/ Tannic acid (μM)

Figure 23: **Inhibition of hydroxyl radical [generated by Fe(II)-EDTA-H_2O_2] mediated degradation of calf thymus DNA by increasing concentration of the compounds as measured by the degree of S_1 nuclease digestion.**

Calf thymus DNA was treated with 1mM sodium ascorbate (pH 7.0), 40 μM Fe(II), 80 μM EDTA and 0.03% H_2O_2 in the presence of increasing concentrations of gallic acid (O), ellagic acid (▼) and tannic acid (●) (25 – 100 μM) for 1 hour 30 minutes at 37°C. At the end of the incubation period the reaction mixture was subjected to S_1 nuclease hydrolysis.
All points represent triplicate samples and mean values have been plotted.


40
35
30
25
20
15
10
5
0
% DNA hydrolyzed
0
20
40
60
80
100
Gallic acid/ Ellagic acid/ Tannic acid (μM)

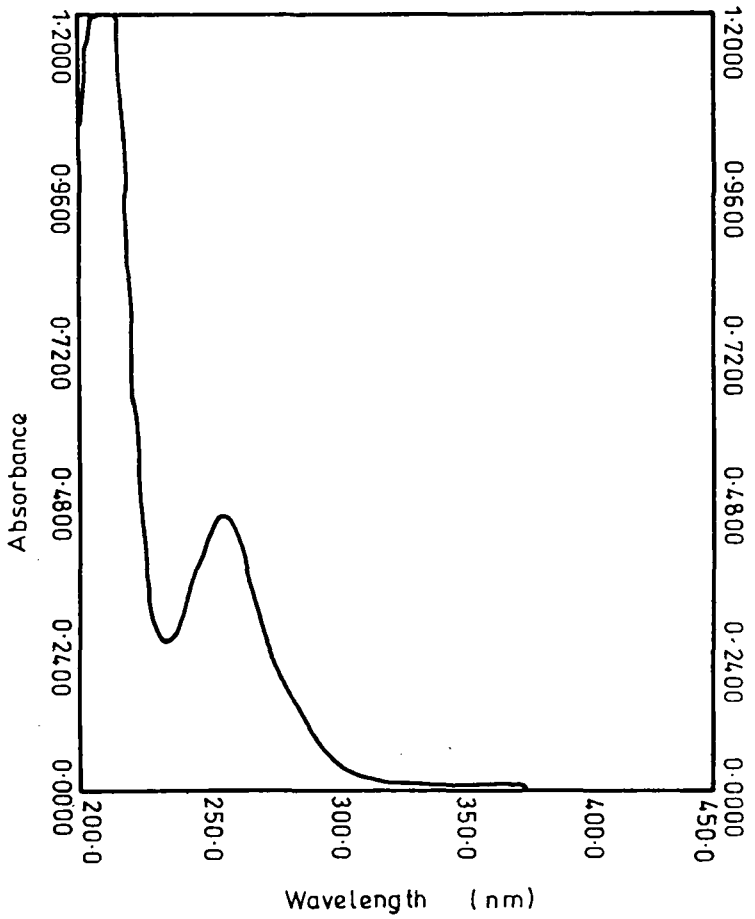
Interaction of gallic acid, ellagic acid and tannic acid with Cu(II)

Absorption spectrum of gallic acid alone gave λmax at 211nm and 260nm (Figure 24(a)). When Cu(II) was added a shift occurred in λmax of gallic acid from 211nm to 226nm and 260nm to 290nm (Figure 24(b)). As with tannic acid when Cu(II) was added there was a decrease in the absorbance at λmax of 215nm and a shift occurred in the λmax of tannic acid from 280 nm to 323nm (Figure 24(c) and 24 (d)). Ellagic acid alone gave peaks at 225nm, 255nm, 275nm and 366 nm (Figure 24(e)) and addition of Cu(II) lead to the formation of a single peak absorbing at 280 nm (Figure 24(f)). These results are indicative of the binding of Cu(II) to gallic acid, tannic acid and ellagic acid.

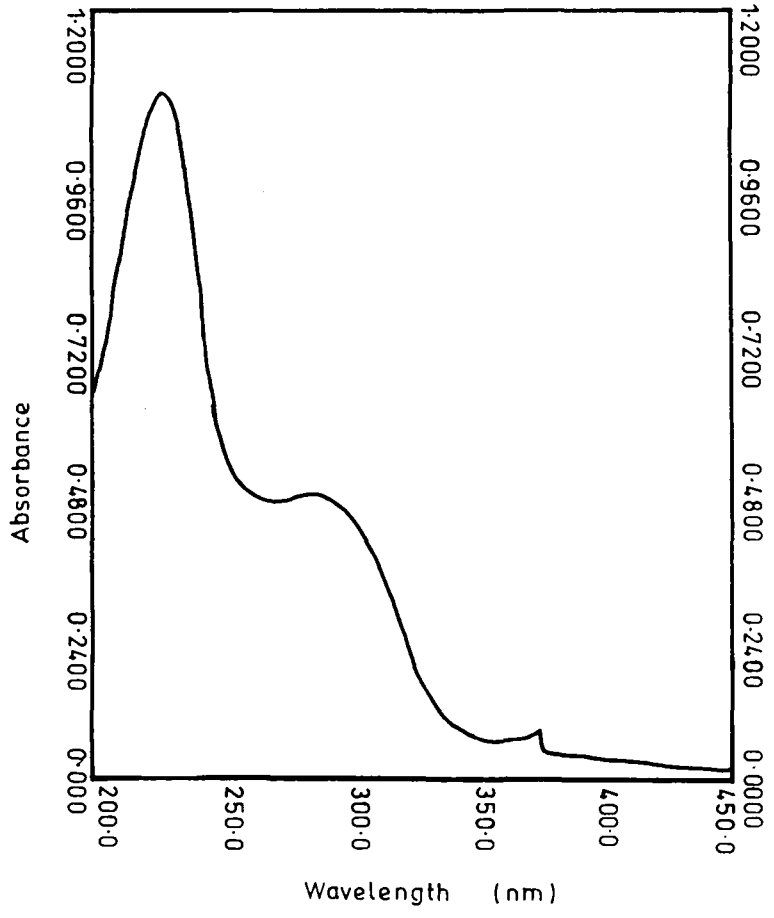
Figure 24: Effect of Cu(II) on the absorption spectrum of gallic acid, tannic acid and ellagic acid.

The absorption solution contained 50μM gallic acid, 10 μM tannic acid, 10 μM ellagic acid and 100 μM Cu(II) in 10 mM Tris-HCl (pH 7.5).

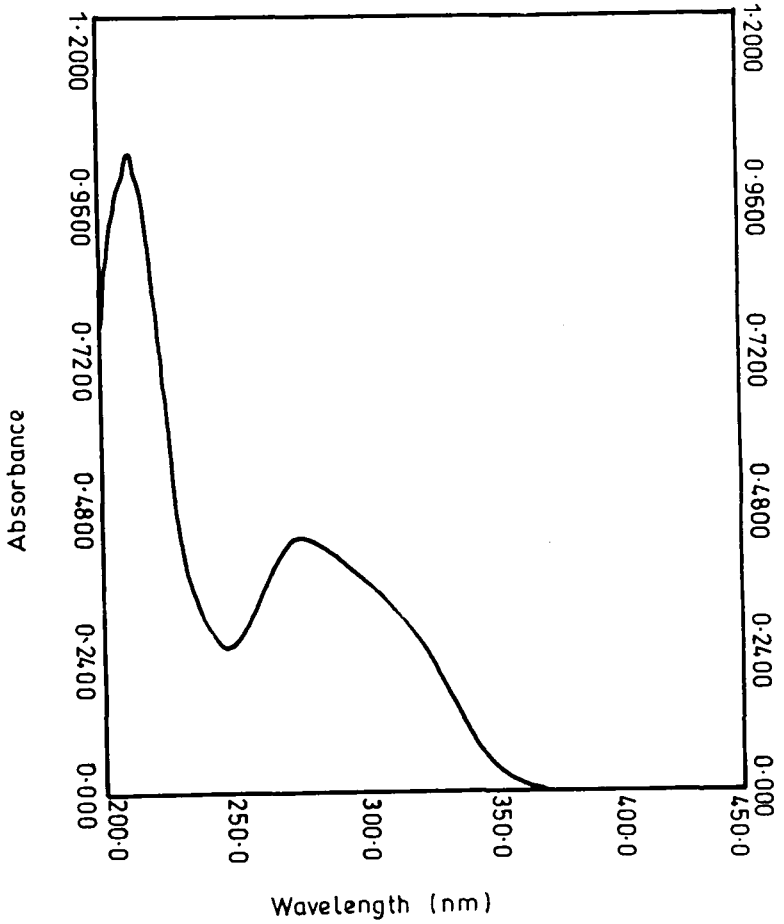
(a) gallic acid alone
(b) gallic acid and Cu(II)
(c) tannic acid alone
(d) tannic acid and Cu(II)
(e) ellagic acid alone
(f) ellagic acid and Cu(II)


Absorbance
1·2000
0·9600
0·7200
0·4800
0·2400
0·0000
200·0
250·0
300·0
350·0
400·0
450·0
Wavelength (nm)

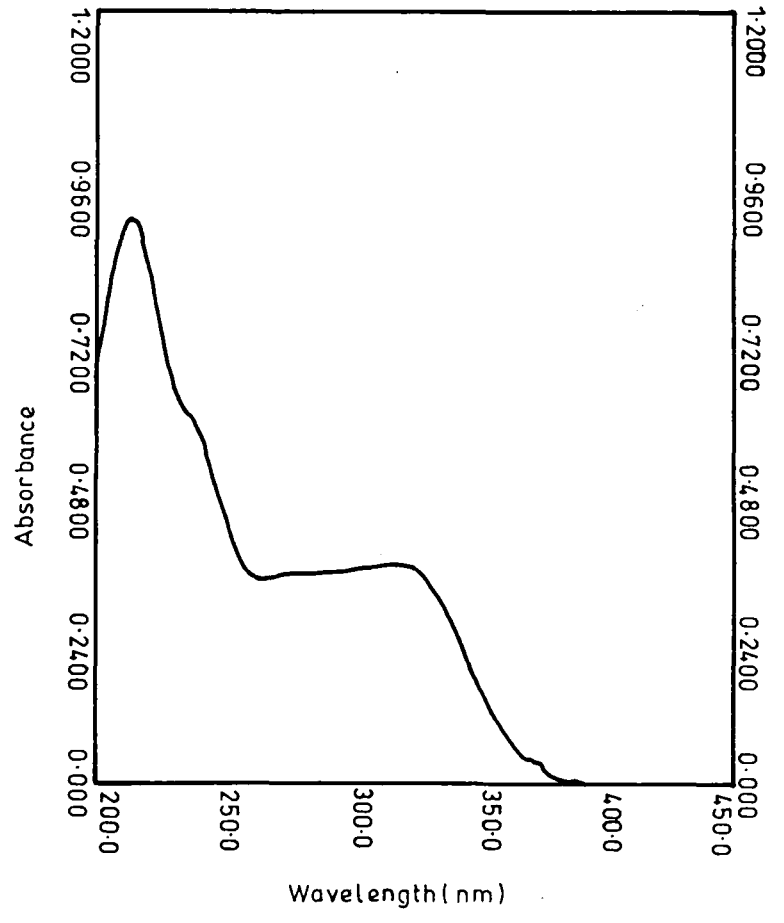
a

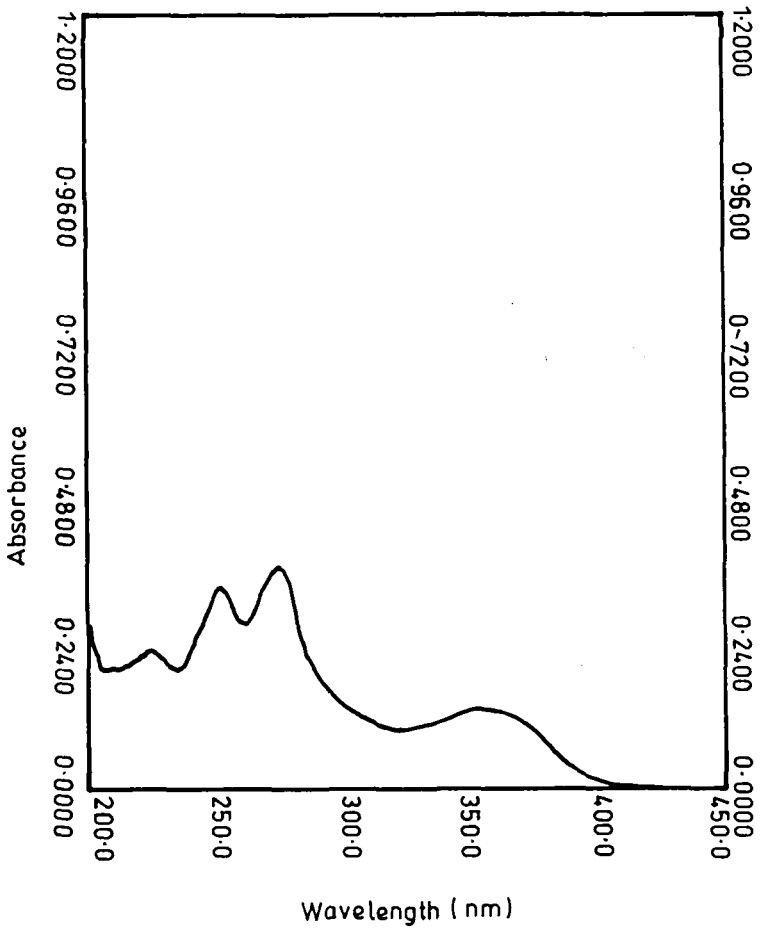

Absorbance
1·2000
0·9600
0·7200
0·4800
0·2400
0·0000
200·0
250·0
300·0
350·0
400·0
450·0
Wavelength (nm)

b

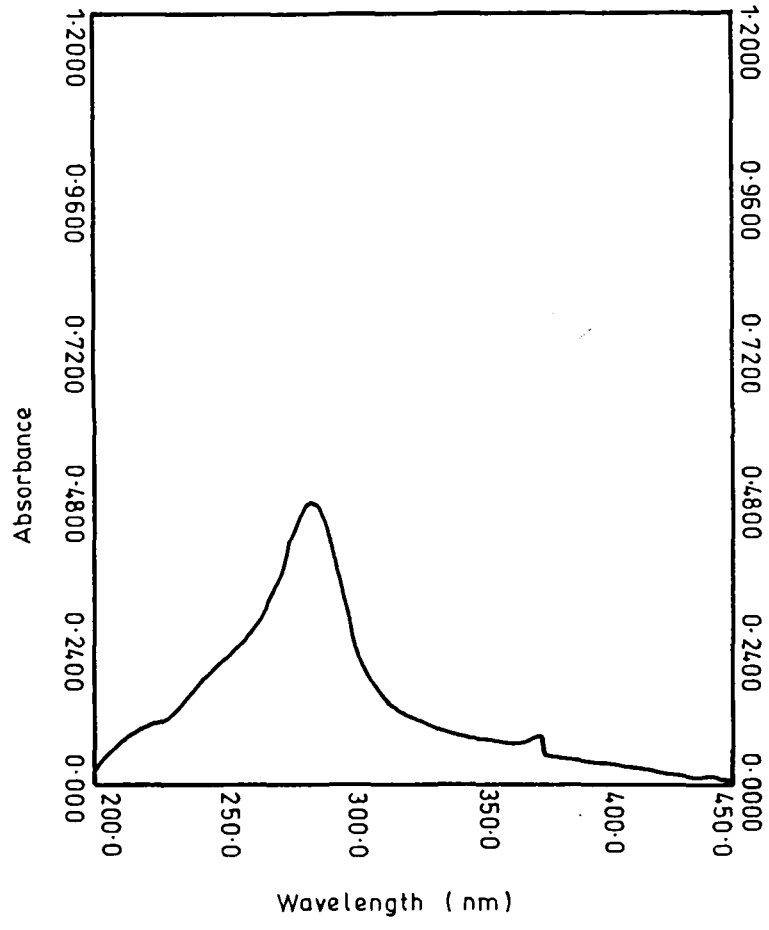

Absorbance
1·2000
0·9600
0·7200
0·4800
0·2400
0·000
200·0
250·0
300·0
350·0
400·0
450·0
Wavelength (nm)

c


Absorbance
1·2000
0·9600
0·7200
0·4800
0·2400
0·000
200·0
250·0
300·0
350·0
400·0
450·0
Wavelength (nm)


Absorbance
1·2000
0·9600
0·7200
0·4800
0·2400
0·0000
200·0
250·0
300·0
350·0
400·0
450·0
Wavelength (nm)

e


Absorbance
1·2000
0·9600
0·7200
0·4800
0·2400
0·000
200·0
250·0
300·0
350·0
400·0
450·0
Wavelength (nm)

f

Discussion (Part-III)

Studies in previous section (Part-II) showed that gallic acid possess a potent prooxidant activity as evidenced by its capacity to degrade DNA in the presence of Cu(II) and to generate hydroxyl radicals. It is a structural constituent of tannic acid present as a digalloyl moiety. Ellagic acid is another plant-derived polyphenol which is considered to possess anticarcinogenic properties (Gali *et al.*, 1992; Khanduja *et al.*, 1999). However, various polyphenols exhibit anticancer and apoptosis inducing properties to variables degrees. Thus it is not clear as to what structural features of polyphenols are important in imparting the various pharmacological properties. For example although ellagic acid is 10 times more potent an antioxidant than is tannic acid, the latter was more effective than the former in inhibiting the promotion of skin tumour by 12-O-tetradecanoyl phorbol-13-acetate (Gali *et al.*, 1992). The authors suggested that the antioxidant effects of those polyphenols may be essential but not sufficient for their activity against tumour promotion. As already mentioned it has been proposed in this laboratory that the cytotoxic action of plant polyphenols against cancer cells possibly involves mobilization of endogenous copper ions and the consequent prooxidant action. I have therefore compared the prooxidant properties of gallic acid (GA), tannic acid (TA) and ellagic acid (EA) with respect to their ability to oxidatively degrade DNA and to reduce copper ions. The relative rates of DNA degradation by GA,

TA and EA was established as GA > EA > TA (Figure 19). Although the stoichiometry of Cu(II) reduction by gallic acid and tannic acid is the same (1:6), gallic acid degrades DNA at a considerable greater rate. On the other hand stoichiometry of Cu(II) reduction by ellagic acid could not be determined as it does not gives a clear maximum of Cu(I) formation where absorption becomes constant. This suggests a possible recycling of copper ions in the reaction. Also the number of free hydroxyl groups in these molecules does not seem to be important as tannic acid would contain a much larger number of such groups compared to both ellagic acid and gallic acid. The rate of hydroxyl radical generation by the three compounds also does not correlate with rate of DNA degradation. The lesser DNA degrading capacity of tannic acid may be possibly explained by the reduced electron density of gallic acid when it is part of the tannic acid molecule. The decreased electron density at phenolic hydroxyls would lead to a reduced Cu(II) chelating efficiency. Steric hindrances in the case of tannic acid may further effect DNA cleavage rate. Similarly a reduced electron density of hydroxyls may explain the decreased DNA degrading capacity of ellagic acid compared to gallic acid.

Bibliography

BIBLIOGRAPHY

Ahmad, A., Asad, S.F., Singh, S. and Hadi, S.M. (2000). Cancer Lett., **154**, 29.

Ahmad, M.S., Fazal, F., Rahman, A., Hadi, S.M. and Parish, J.H. (1992). Carcinogenesis, **13**, 605.

Ahmad, N. and Mukhtar, H. (1999). Nut. Rev., **57**, 78.

Ahmad, N., Feyes, D.K., Nieminen, A.L., Agarwal, R. and Mukhtar, H. (1997). J. Natl. Cancer Inst., **89**, 1881.

Ahmad, N., Katiyar, S.K. and Mukhtar, H. (1998). In: Loannides C, Ed. Nutrition and Chemical Toxicity, West Sussex, England, 301.

Ahsan, H. and Hadi, S.M. (1998). Cancer Lett., **124**, 23.

Alderson, T. and Clark, A.M. (1966). Nature, **210**, 593.

Ames, B.N. (1979). Science, **204**, 587.

Ames, B.N. (1983). Science, **221**, 1256.

Ames, B.N., Cathcart, R., Schwiers, E. and Hochstein, P. (1981). Proc. Natl. Acad. Sci., USA, **78**, 6858.

Athar, M., Khan, W.A. and Mukhtar, H. (1989). Cancer Res., **49**, 5784.

Baynes, J.W. (1991). Diabetes, **40**, 405.

Beckman, C., Roy, R.M. and Sproule, A. (1982). Mutat. Res., **105**, 73.

Beyer, R.E. (1990). Free Rad. Biol. Med., **8**, 545.

Bhat, R. and Hadi, S.M. (1994). Mutat. Res., **313**, 39.

Blake, D.R., Rallen, R.E. and Lunec, J. (1987). Br. Med. Bull., **45**, 371.

Boolbol, S.K., Dannenberg, A.J. and Chadburn, A. (1996). Cancer Res., **56**, 2556.

Borchert, P., Wislocki, P.G., Miller, J.A. and Miller, E.C. (1973). Cancer Res., **33**, 123.

Brown, J.P. (1980). Mutat. Res., **75,** 243.

Brunk, V. and Cadenas, E. (1988). APMIS, **96**, 3.

Bryan, S.E. (1979). Metal Ions in Biological Systems. Marcel Dekker, New York.

Burkitt, M.J., Milne, L., Nicotera, P. and Orrenius, S. (1996). Biochem. J., **313**, 163.

Campbell, M.A. and Fantel, A.G. (1983). Life Sci., **32**, 2641.

Carmella, S.G. Lavoie, E.J. and Hecht, S.S. (1982). Food Chem. Toxicol., **20**, 587.

Chen, Z.P., Schell, J.B. and Ho, C.T. (1998). Cancer Lett., **129**, 173.

Clark, A.M. (1960). A. Vererbungles, **91**, 74.

Clark, A.M. (1982). In: Environmental Mutagenesis, Carcinogenesis and Plant Biology, Klekowsky, E.J. Jr., Ed. (Praeger, New York), **1**, 1977.

Clement, M.V., Hirpara, J.L., Chawdhury, S.H. and Pervaiz, S. (1998). Blood, **92**, 996.

Concon, J.M., Newburg, D.S. and Sweerczek, T.W. (1979). Nutr. Cancer, **1**, 22.

Daniel, E.M. and Stoner, G.D. (1991). Cancer lett., **56**, 117.

Davies, K.J.A., Wiese, A.G., Sevanian, A. and Kim, E.H. (1990). In: Molecular Biology of Aging (Finch C.E., Johnson, T.E. eds.), Wiley-Liss, New York.

Del Maestro, R.P., Bjork, J. and Arfors, K.E. (1981). Microvase Res., **22**, 255.

Del Rio, L.A., Sandalio, L., Palma, J., Bueno, P. and Corpas, P.J. (1992). Free Rad. Biol. Med., **13**, 557.

Demopoulos, H.B., Pietronigro, D.D., Flamm, E.S. and Seligman, M.L. (1980). Environ. Pathol. Toxicol., **3**, 273.

Demple, B. (1990). Mutat. Environ. A., 155.

Devasagayam, T.P.A., Subramanian, M., Singh, B.B., Ramanathan, R. and Das, N.P. (1995). J. Photochem. Photobiol. B. Biology, **30**, 97.

Dixit, R. and Gold, B. (1986). Proc. Natl. Acad. Scien. USA, **83**, 8039.

Doll, R. and Peto, R. (1981). J. Natl. Cancer Inst., **66**, 1192.

Drinkwater, N.R., Miller, E.C. and Miller, J.A. (1976). J. Natl. Cancer Inst., **57**, 1323.

Dunnick, J.K., Prejean, J.D., Haseman, J., Thompson, R.B., Giles, H.D. and McConnel, E.E. (1982). Fundam. Appl. Toxicol., **2**, 114.

Ebadi, M. and Swanson, S. (1988). Prog. Clin. Biol. Res., **259**, 161.

Fenaroli, G. (1971). In: Handbook of Flavor Ingredients, Furia, T.E., Bellanco, N., Eds., Chemical Rubber Co., Cleaveland, Ohio.

Fischer, S.M., Mills, G.D. and Slage, T.J. (1982). Carcinogenesis, **3**, 1243.

Fishbein, L. (1983). Proceedings of the XV International Congress of Genetics, **3**, 3.

Flohe, L. (1982). In: Free Radicals in Biology, Pryor, W.A., Ed. (Academic Press, New York), **5**, 223.

Freeman, B.A. and Grapo, J.D. (1982). Lab. Invest., **47**, 412.

Fridovich, I. (1983). Annu. Rev. Pharmacol. Toxicol., **23**, 239.

Fujiki, H., Suganuma, M. and Okabe, S. (1998). Mutat. Res., **402**, 307.

Fujiwara, H., Takigawa, Y., Suzuki, T. and Nakata, K. (1992). Jpn. J. Opthalmol., **36**, 273.

Gali, H.U., Perchellet, E.M. and Perchaellet, J.P. (1991). Cancer Res., **51**, 2820.

Gali, H.U., Perchellet, E.M., Klish, D.S., Johnson, J.M. and Perchellet, J.P. (1992). Int. J. Cancer, **51**, 425.

Gali, H.U., Perchellet, E.M., Gao, X.M., Bottari, V. and Perchellet, J.P. (1993). Anticancer Res., **13**, 915.

Gebicki, S. and Gebicki, J.M. (1993). Biochem. J., **289**, 743.

Gilbert, D.L. (ED) (1981). Oxygen and Living Processes. An Interdisciplinary Approach. Springer-Verlag, New York.

Graham, H.N. (1992). Prev. Med., **21**, 334.

Greenwald, R.W. and Moy, W.W. (1980). Arthritis Rheum., **23**, 455.

Guenther, E. and Althausen, D. (1949). The essential oils (Van Nostrand, New York), **2**, 499.

Gulati, S., Singh, A.K., Irazu, C., Orak, J., Rajagopalan, P.R., Fitts, C.T. and Singh, I. (1992). Arch. Biochem. Biophys., **295**, 90.

Gutteridge, J.M.C. and Halliwell, B. (1990) Trends Biochem. Sci., **15**, 129.

Hadi, S.M., Asad, S.F., Singh, S. and Ahmad, A. (2000). IUBMB Life, **50**, 167.

Halliwell, B. and Gutteridge, J.M.C. (1984). Biochem. J., **219**, 1.

Halliwell, B. and Gutteridge, J.M.C. (EDS) (1989). Free Radicals in Biology and Medicine, 2nd edn. Oxford University Press, Clarendon, Oxford.

Halliwell, B., Gutteridge, J.M.C. and Cross, C.E. (1992). Clin. Med., **119**, 598.

Harbowy, M.E. and Balentine, D.A. (1997). Plant Sci., **16**, 415.

Haroz, R.K. and Thomassan, J. (1980) Toxicol. Lett. (Suppl.), **6**, 72.

Hartman, P.E. (1982). In: Clinical Mutagen, de Serres, F.Y. and Hollaender Eds., (Planum, New York), **1**, 211.

Hecker, E. (1981). J. Cancer Res. Clin. Oncol., **99**, 103.

Hendricks, J.D. Sinnhuber, R.O., Loveland, P.M., Pawlowski, N.E. and Nixon, J.E. (1980). Science, **208**, 309.

Hirono, I. (1981). CRC Crit. Rev. Toxicol., **8**, 253.

Hirono, I. (1987). In: Naturally occurring carcinogens of plant origin. Elsevier Science Publishers Ltd., Vol. **11**, 161.

Hirono, I., Mori, H., Yamada, K., Haga, M., Tatematsu, H. and Kanie, S. (1977). J. Natl. Cancer Inst., **58**, 1155.

Hirose, M., Hoshiya, T. and Akagi, K. (1993). Carcinogenesis, **14**, 1549.

Hochstein, P. and Jain, S.K. (1981). Fed. Proc. Fed. Am. Soc. Exp. Biol., **40**, 183.

Inoue, M., Suzuki, R., Koide, T., Sakaguchi, H., Ogihara, Y. and Yabu, Y. (1994). Biochem. Biophys. Res. Commun., **204**, 898.

Ivie, G.W. (1978). In: Effects of Poisonous Plants on Live Stock, Keeler, R., Vankampen, K, and James, L., Eds. (Academic Press, New York), 475.

Ivie, G.W., Holt, D.L. and Ivey, M.C. (1981). Science, **213**, 909.

Jackson, R. (1985). Chest., **88**, 900.

Jadhav, S.J., Sharma, R.P. and Sulankher, D.K. (1981). CRC Crit. Rev. Toxicol., **9**, 21.

Jee, S.H., Shen, S.C. and Tseng, C.R. (1998). J. Invest. Dermatol., **111**, 656.

Ji, L.L., Dillon, D. and Wu, E. (1990). Am. J. Physiol., **258**, R918.

Jiang, M.C., Yang-Yen, H.F. and Yen, J.J.Y. (1996). Nutr. Cancer, **26**, 111.

Kagawa, T.F., Geierstanger, B.H., Wang, A.H.J. and Ho, P.S. (1991). J. Biol. Chem., **266**, 20175.

Kane, D.J., Sarafian, T.A., Anton, R., Hahn, H., Butter Gralla, E., Selverstone, V.J., Ord, T. and Bredesen, D.E. (1993). Science, **262**, 1274.

Kapadia, G.J. (1982). In: Oncology overview on naturally occurring dietary carcinogens of plant origin, Ed. (International Cancer Research Data Bank Program, National Cancer Institute, Bethesda, Maryland).

Kappus, H. and Sies, H. (1981). Experientia, **37**, 1233.

Katiyar, S.K. and Mukhtar, H. (1996). Int. J. Oncol., **8**, 221.

Katsui, N., Yagihashi, F., Murai, A. and Masamune, T. (1982). Bull. Chem. Soc. Jpn., **55**, 2424.

Khafif, A., Schantz, S.P. and al-Rawi, M. (1998). Head Neck, **20**, 528.

Khan, N.S. and Hadi, S.M. (1998). Mutagenesis, **13**, 271.

Khan, W.A., Wang, Z.Y. and Athar, M. (1988). Cancer Lett., **42**, 7.

Khanduja, K.L., Gandhi, R.K., Pathanaia, V. and Sayal, N. (1999). Food Chem. Toxicol., **37**, 313.

Kinsella, A.R. (1982). Carcinogenesis, **3**, 499.

Kohlmeier, L., Weterings, K.G.C.and Steck, S. (1997). Nutr. Cancer, **27**, 1.

Krinsky, N.I. and Deneke, S.M. (1982). J. Natl. Cancer Inst., **69**, 205.

Kunz, R., Schoenberg, M.H., Buchler, M., Jost, K. and Beger, H.G. (1991). Klin Wochenschr, **69**, 1095.

Kuroda, Y. (1988). In: Y. Kuroda, D.M. Shankel and M.D. Waters (Eds.), Antimutagenesis and Anticarcinogenesis, Vol. **II**, Plenum, New York, pp. 1-22.

Lehr, H., Becker, M., Marklund, S.L., Hubner, C., Arfors, K.E., Kohlschatier, A. and Messmer, K. (1992). Arteriosclerosis Thrombosis, **12**, 824.

Lesca, P. (1983). Carcinogenesis, **4**, 1651.

Levin, D.E., Hollstein, M., Christaman, M.F., Schwiers, E.A. and Ames, B.N. (1982). Proc. Natl. Acad. Sci., USA, **79**, 7445.

Li, Y. and Trush, M.A. (1994). Cancer Res., **54**, 18955.

Liang, Y.C., Lin-Shiau, S.Y. and Chen, C.P. (1997). J. Cell Biochem., **67**, 55.

Lind, C., Hochstein, P. and Ernster, L. (1982). Arch. Biochem. Biophys., **216**, 178.

Loft, S. and Poulsen, H.E. (1996). J. Mol. Med., **74**, 297.

Maccarone, E., Maccarone, A. and Rapisarda, P. (1987). Int. J. Food Sci. Technol., **22**, 159.

Maga, J.A. (1979). CRC Crit. Rev. Food Sci. Nutr., **11**, 355.

Magee, P.N. (1982). Banbury Report 12, Nitrosoamines and Human Cancer (Cold Spring Harbor laboratory), Cold Spring Harbor, New York.

Mandal, S., Ahuja, A., Shivapurkar, N.M., Cheng, S.J., Groopman, J.D. and Stoner, G.D. (1987). Carcinogenesis, **8**, 1651.

Masaki, H., Atsumi, T. and Sakurai, H. (1993). Free Rad. Res. Commun., **19**, 333.

Mathews-Roth, M.M. (1982). Oncology, **39**, 33.

Mori, H., Sugie, S., Yoshimi, N., Asada, Y., Furuya, T. and Williams, G.M. (1985). Cancer Res., **45**, 3125.

Morimoto, K., Wolff, S. and Koizumi, A. (1983). Mutat. Res. Lett. **119**, 355.

Mukhtar, H., Das, M. and Bickers, D.R. (1986). Cancer Res., **46**, 2262.

Mukhtar, H., Das, M., Khan, W.A. Wang, Z.Y., Bik, D.P. and Bickers, D.R. (1988). Cancer Res., **48**, 2361.

Mukhtar, H., Katiyar, S.K. and Agarwal, R. (1994). J. Invest, Dermatol., **102**, 3.

Nakayama, T., Kimura, T., Kodama, M. and Nagata, C. (1983). Carcinogenesis, **4**, 765.

Naseem, I., Ahmad, M.S., Bhat, R. and Hadi, S.M. (1993). Food Chem. Toxicol., **31**, 589.

Nebesar, B. (1961). Anal.Chem., **36**, 196.

Nielson, H. (1981). Lipids, **16**, 215.

Niki, E., Yamamoto, Y., Komuro, E. and Sato, E. (1991). Am. J. Clin. Nutr., **53**, 2015.

Novi, A.M. (1981). Science, **212**. 541.

Otsuka, T., Ogo, T. and Eto, T. (1998). Life Sci., **63**, 1387.

Packer, J.E., Mahmood, J.S., Mora Arellano, V.O., Slater, T.F., Wilson, R.L. and Wolfenden, B.S. (1981). Biochem. Biophys. Res. Commun., **98**, 901.

Peto, R. and Schneiderman (1981). In: Banbury Report of Quantification of Occupational Cancer, Eds., (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

Picard, D. (1996). Altern. Med. Rev., **1**, 31.

Piwocka, K., Zablocki, K., Wieckowski, M.R., Skierski, J., Feiga, I., Szopa, J., Drela, N., Wojtczak, L. and Sikora, E. (1999). Exp. Cell Res., **249**, 299.

Pizzolo, G., Savarin, T., Molino, A.M., Ambrosette, A., Todeschini, G., and Vettore, L. (1978). Tumori., **64**, 55.

Prigodich, R.V. and Martin, C.T. (1990). Biochem., **29**, 8017.

Proctor, P. H. and Reynolds, E.S. (1984). Physiol. Chem. Phys., **16**, 175.

Pryor, W.A. (1976-82). In: Free Radicals in Biology, Ed. (Academic Press, New York), 1.

Pryor, W.A. and Dooley, M.M. (1985). Am. Rev. Respir. Dis., **131**, 941.

Quinlan, G.J. and Gutteridge, J.M.C. (1987). Biochem. Pharmacol., **36**, 3629.

Rahman, A., Shahabuddin, Hadi, S.M. and Parish, J.H. (1990). Carcinogenesis, **11**, 2001.

Rahman, A., Shahabuddin, Hadi, S.M., Parish, J.H. and Ainley, K. (1989). Carcinogenesis, **10**, 1833.

Ramanthan L., and Das, N.P. (1992). J. Agric. Food Chem., **40**, 17.

Randerath, K., Reddy, R., Danna, T.P., Watson, W.P., Crane, A.E. and Randerath, E. (1992). Mutat. Res., **275**, 355.

Record, I.R. and Dreosti, I.E. (1998). Mutat. Res. **422**, 191.

Richmond, R., Halliwell, B., Chauhan, J. and Darbre, A. (1981). Anal. Biochem., **118**, 328.

Riendeau, D., Denis, D., Chov. L.Y., Nathaniel, D.J. (1989). Biochem. J., **263**, 565.

Sadzuka, Y. Sugiyama, T. and Hirota, S. (1998). Clin. Cancer Res., **4**, 153.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecularcloning: A laboratory manual. New York: Cold Spring Harbor Laboratory Press.

Sato, D. (1999). Int. J. Urol., **6**, 93.

Schaich, Kh. (1992). Lipids, **27**, 209.

Schneider, W.C. (1957). Methods Enzymol., **3**, 680.

Schoental, R. (1976). In: Chemical Carcinogen, Searle, C., Ed. (Am. Chem. Soc., Washington, D.C.), 629.

Schoental, R. (1982). Toxicol. Lett., **10**, 323.

Seppi, C. Castellana, M.A., Minetti, G., Piccinini, G., Balduini, C. and Brouelli, A. (1991). Mec. Ageing Dev., **57**, 247.

Sevanian, A., Nordenbrand, K., Kim, E., Ernester, L.and Hochstein, P. (1990). Free Rad. Biol. Med., **8**, 145.

Shimoi, K., Nakamura, Y., Tomita, I. and Kada, T. (1985). Mutat. Res., 149, 17.

Sies, H. (ed.) (1985). Oxidative Stress, Academic Press, New York.

Smets, L.A. (1994). Anticancer Drugs, **5**, 3.

Sohal, R.S. (1993). Aging Milano, **5**, 3.

Southorn, P.A. and Powis, G. (1988). Mayo. Clin. Proc., **63**, 390.

Stadtman, E.R. (1992). Science, **257**, 1220.

Stich, H.F. and San, R.H.C. (1981). In: Short term test for chemical carcinogens, Eds., (Springer Veralg, New York).

Stich, H.F., Rosin, M.P., Wu, C.H. and Powrie, W.D. (1981). Cancer Lett., **13**, 89.

Stich, H.F. and Rosin, M.P. (1983). Life Sci., **32**, 2641.

Suganuma, M., Okabe, S., and Oniyama, M. (1998). Carcinogenesis, **19**, 1771.

Sugiyama, T. and Sadzuka, Y. (1998). Cancer Lett., **133**, 19.

Tanaka, T., Iwata, H., Niwa, K., Mori, Y. and Mori, H. (1988) Jap. Journ. Cancer Res., **79**, 1297

Tateishi, N., Higashi, T., Naruse, A., Hikita, K. and Sakamoto, Y. (1981). J. Biochem., **90**, 1603.

Tauber, A.I., (1982). Trends Biochem. Sci., **7**, 145.

Tazima, Y. (1982). In : Environmental Mutagenesis, Carcinogenesis and Plant Biology, Klakowsky, E.J. Jr., Ed. (Praeger, New York), **1**, 68.

Thakur, C.P. (1985). Experientia, **41**, 423.

Toth, B., Nogel, D. and Ross, A. (1982). Br. J. Cancer, **46**, 417.

Totter, R.J. (1980). Proc. Natl. Acad. Sci., USA, **77**, 1763.

Tripathi, V.K., Singh, M.B. and Singh, S. (1988). Indian Food Packer, **42**, 60.

Tuyns, A.J., Pequignot, G., Gignoux, M. and Vulla, A. (1982). Int. J. Cancer, **30**, 9.

Utaka, M. and Takeda, A. (1985). Carcinogenesis, **11**, 2001.

Valenzuela, A. and Videla, L.A. (1989). Rev. Med. Chile, **117**, 60.

Valenzuela, A. (1991). Life Sci., **48**, 301.

Varshavsky, A. (1981). Cell, **25**, 561.

Videla, L.A., Fernandez, V., Marinis, A. De. and Valenzuella, A. (1982). Biochem. Biophys. Res. Commun., **104**, 9.

Wang, Z.Y., Khan, W.A. and Bickers, D.R. (1989). Carcinogenesis, **10**, 411.

Warholm, M., Gohenberg, C., Mannervik, B. and Von Bahr, C. (1981). Biochem. Biophys. Res. Commun., **98**, 512.

Webster, N.R. and Nunn, J.P. (1988). Br. J. Anaesth., **60**, 98.

Wei, H., Zhang, X., Zhao, J.F., Wang, Z.Y., Bickers, D. and Lebwhol, M. (1999). Free Radic. Biol. Med., **26**, 1427.

Weisburger, J.H., Rivenson, A. and Garr, K. (1997). Cancer Lett., **114**, 323.

Welsch, C.W., and Aylsworth, C.F. (1983). J. Natl. Cancer Inst., **70**, 215.

Williams, G.M., Mori, H., Hirono, I. and Nagao, M. (1980). Mutat. Res., **79**, 1.

Winston, G.W. and Cederbaum, A.T. (1982). Biochem. Pharmacol., **312**, 2301.

Wolfe, J.T., Ross, D. and Cohen, C.M. (1994). FEBS Lett., **352**, 59.

Wolff, S.P. and Dean, R.T. (1986). Biochem. J., **234**, 399.

Wolff. P.S., Garner, A. and Dean T.R. (1986). Trends Biochem. Sci., **11**, 27.

Wong, A., Huang, C.H. and Crooke, S.T. (1984). Biochemistry, **23**, 2946.

Wright, S.C., Zhong, J.,and Larrick, J.W. (1994). FASEB J, **8**, 654.

Xue, S.P. (1980). Proceedings of the Symposium on Recent Advances in Fertility Regulation (Beijing), 122.

Ya, A., Potapenko, M.V., Mushnin, A.A., Krasnovsky, J.R. and Sukhorunkov, V.L. (1982). Z. Naturforsch, **37**, 70.

Yamammoto, S. (1991). Free Rad. Biol. Med., **10,** 149.

Yang, C.S. and Wang, Z.Y. (1993). Journ. Natl. Cancer Inst., **85**, 1038.

Yoshida, D., Ikada, Y. and Nakayama S. (1993). J. Neurooncol., **16**, 109.

Yu, R., Jiao, J.J. and Duh, J.L. (1997). Carcinogenesis, **19**, 451.

RESEARCH PUBLICATIONS

1. **A. Malik**, S. Azam, N. Hadi and S.M. Hadi, 'DNA degradation by water extract of green tea in the presence of copper ions; implications for anticancer properties'. *Phytotherapy Research* (In press, 2003).

2. N. Hadi, **A. Malik**, S. Azam, N.U. Khan and J. Iqbal, 'Serotonin - Cu (II) mediated DNA cleavage: mechanism of copper binding by serotonin'. *Toxicology In Vitro* (In press, 2002).

3. S. Azam, **A. Malik**, N. Hadi, N. U. Khan and S.M. Hadi, 'Antioxidant and prooxidant properties of caffeine, theobromine and xanthine'. *Food Chemistry* (communicated, 2002).