PUTATIVE ANTICANCER MECHANISMS OF PLANT DERIVED POLYPHENOLIC COMPOUNDS

(A Putative Mechanism of Apigenin, Luteolin, Chrysin, Epigallocatechin-3-gallate, Resveratrol and Genistein)

THESIS

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By

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Certificate

This is to certify that the work presented in this thesis has been carried out by **Mr. Husain Yar Khan** under my supervision. It is original in nature and has not been submitted for any other degree.

S. M. Hadi
(Professor)

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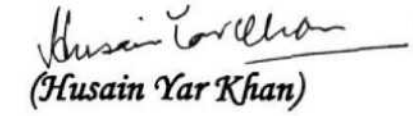

(Husain Yar Khan)

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SUMMARY

During the past few decades our understanding of dietary agents as potential regulators of carcinogenic events has grown, providing opportunities for identifying new targets for therapeutic development. The momentum was based on the findings that as many as 35% of all cancers can be prevented by lifestyle changes including dietary modifications. Consistent with this observation are the epidemiological findings associating high consumption of fruits, vegetables and beverages such as red wine and green tea with lower incidence of cancer. Polyphenolic compounds are considered important bioactive components of these plant-derived human foods and attributed at least in part to the protective effect provided. In fact, numerous reports have documented that plant polyphenols are capable of inducing apoptosis and tumor regression in cancer cell lines and animal models, respectively. Of particular interest is the observation that a number of these polyphenols including EGCG, resveratrol and genistein induce apoptotic cell death in various cancer cell lines but not in normal cells. Moreover, it has also been realized that unlike conventional clinically used anticancer drugs, plant-derived polyphenolics have an extended margin of safety as they exhibit negligible toxicity even at relatively high concentrations. Therefore, various polyphenolic compounds such as gallocatechins, isoflavones, stilbenes, tannins and curcuminoids have been implicated as chemopreventive agents. However, the mechanism by which these compounds inhibit proliferation and induce apoptosis in cancer cells has been the subject of considerable interest. Thus studies related to identification of molecular targets and mechanism of action of plant polyphenols that are relevant to tumor microenvironment are critical steps in cancer chemoprevention.

Most of the pharmacological properties of plant polyphenols are considered to reflect their ability to scavenge endogenously generated oxygen radicals or

those free radicals formed by xenobiotics, radiations, etc. However, some data in literature suggests that antioxidant properties of the polyphenolic compounds may not fully account for their chemopreventive effects. The conventional wisdom holds that these dietary polyphenols act as antioxidants and this action is primarily involved in their role as cancer preventive agents. However, polyphenols have also shown cancer regression and such a therapeutic outcome remains unexplained by an antioxidant mechanism. Most of the plant polyphenols possess both antioxidant as well as prooxidant properties and it has been proposed that the endogenous copper dependent prooxidant cytotoxic action of polyphenols rather than antioxidant effect may be an important mechanism of their anticancer and apoptosis inducing properties. Previous work in our laboratory has provided evidence that these antioxidants may also behave as prooxidants to initiate reactive oxygen species (ROS) mediated cell death. Such a prooxidant mechanism is a result of redox-active microenvironment in cancer cells due to elevated levels of copper. Copper is an important redox active metal ion present in chromatin, closely associated with DNA bases and can be mobilized by metal chelating agents. Several reports in literature have established that both serum and tumor copper levels in cancer patients are significantly elevated. Therefore, cancer cells may be more subject to electron transfer between copper ions and polyphenols to generate ROS responsible for DNA cleavage and cell death.

In order to further explore the chemical basis of the chemopreventive activity of plant polyphenols, in this thesis I have attempted to elucidate one of the important putative anticancer mechanisms of action of dietary polyphenolic compounds that involves endogenous copper mobilization. In chapter I, using fluorescence and absorption studies, it has been shown that the flavones apigenin, luteolin and chrysin are able to bind both DNA as well as copper and are capable of reducing Cu(II) to Cu(I). These polyphenolic compounds are also capable of degrading supercoiled plasmid pBR322, calf thymus and cellular

DNA in the presence of copper ions. Further, using a system of permeabilized cells, it is demonstrated that nuclear copper is mobilized in such a cellular DNA cleavage reaction. The efficiency of DNA breakage induced by the flavones is found to be in the order - luteolin> apigenin> chrysin, which correlates with their relative copper reducing abilities. These results suggest that flavone-Cu(II) system for DNA breakage is physiologically feasible and could be of biological significance.

In chapter II, studies are done on rats by orally administering them with copper. Copper levels are found to be elevated in plasma and lymphocytes of such rats and upon treatment with different polyphenols, an increased degradation of cellular DNA is observed in such copper overloaded lymphocytes as compared to that in lymphocytes from control animals. Further, such DNA breakage is effectively inhibited by scavengers of ROS as well as copper chelator, but not by iron and zinc chelators, suggesting that the polyphenol-induced DNA breakage occurs through an oxidative process involving ROS generation and copper mobilization.

In chapter III, polyphenols have been shown to inhibit cell proliferation and induce apoptosis in different cancer cell lines and that such cell death is prevented to a significant extent by copper chelator neocuproine and various scavengers of ROS such as SOD, catalase and thiourea. Further, normal breast epithelial cells, cultured in a medium supplemented with copper, become sensitized to polyphenol-induced growth inhibition and are found to have an increased expression of copper transporter Ctr1 (as shown by Western blot analysis). Also, formation of ROS in cancer cells treated with polyphenol is detected by confocal microscopy. Copper chelator quenches such ROS production, confirming the conclusion that mobilization of intracellular copper by polyphenols causes ROS generation leading to prooxidant cell death.

Previous studies on anticancer mechanisms of polyphenols have mainly implicated antioxidant action or modulation of signal transduction pathways such as regulation of cellular proliferation, apoptosis, angiogenesis or metastasis. Based on the work presented in this thesis, I would like to conclude that mobilization of nuclear copper by plant polyphenols and the consequent prooxidant action could be one of the important mechanisms for their anticancer and chemopreventive properties. Indeed such a common mechanism better explains the anticancer effects of polyphenols with diverse chemical structures as also the preferential cytotoxicity towards cancer cells.

Introduction

INTRODUCTION

Cancer prevention: Role of dietary constituents

With more than 12 million new cases each year, cancer is, at present, one of the most devastating diseases worldwide. Despite the development of various therapies, cancer remains the second leading cause of death, accounting for nearly one in every four deaths in the United States of America and in many other nations of the world. The American Cancer Society in its recent annual report, "Cancer Statistics, 2012", estimates approximately 1.64 million new cancer cases to be diagnosed and nearly 577,000 mortalities from cancer projected to occur in USA alone in the year 2012 (Siegel et al., 2012). Despite being one of the major causes of death across the world, cancer has been shown to be a largely preventable disease, highly susceptible to modulation by dietary factors. Dietary patterns, foods, nutrients and other dietary constituents are closely associated with the risk for several types of cancer, and in this regard, it has been estimated that 35% of all cancers can be prevented through appropriate dietary modifications (Doll and Peto, 1981; Manson, 2003).

Carcinogenesis in humans is a multistage process involving a series of events and generally occurs over an extended period. During this process, accumulation of genetic and epigenetic alterations leads to the progressive transformation of a normal cell into a malignant cell. Cancer cells acquire several abilities that most healthy cells do not possess: they become resistant to growth inhibition, proliferate without dependence on growth factors, replicate without limit, evade apoptosis, and invade, metastasize, and support angiogenesis (Hanahan and Weinberg, 2000). It is currently accepted that diet can affect the overall process of carcinogenesis in different ways. Its constituents may contain cancer causing substances but can also contain

several cancer preventive agents. These dietary agents can retard or prevent the process of carcinogenesis by multiple mechanisms, namely i) enhanced detoxification of the carcinogenic intermediates through induction of Phase II drug metabolizing enzymes ii) reduced carcinogen activation due to suppression of cytochrome P450- dependent monooxidases, iii) perturbations in cell cycle progression, iv) selective promotion of apoptosis in cancerous or precancerous cells and v) inhibition of angiogenesis and metastasis formation (Stan et al., 2008). Since apoptosis provides a physiologic mechanism for eliminating abnormal cells, dietary factors affecting apoptosis can lead to important effects on carcinogenesis. Conceivably, activation of apoptosis in pre-cancerous cells offers a prevention mechanism of cancer by dietary factors.

Epidemiological studies have consistently shown that diet plays a crucial role in the protection against several chronic diseases (Willett, 1994; Temple, 2000). Consumption of fruits and vegetables as well as grains, has been strongly associated with reduced risk of cancer, cardiovascular diseases, diabetes, Alzheimer's disease, cataract and age related functional decline (Willett, 1994; Willett, 1995; Temple, 2000). The biologically active chemicals present in fruits, vegetables and grains are termed as phytochemicals, many of which provide desirable health benefits beyond nutrition to reduce the risk of a number of chronic diseases (Liu, 2003). It is believed that phytochemicals have the ability to modify the disease process thus relating the food stuffs, beyond their basic nutritional benefits, to disease prevention (Roger et al., 1993; Thomasset et al., 2007). Such foods have also been termed as 'functional foods'. Thus convincing evidence suggests that a change in dietary behaviour such as increasing the consumption of fruits and vegetables is a practical strategy for significantly reducing the incidence of chronic diseases.

Of particular relevance is the consistent cancer protective effect reported for individuals consuming high quantities of fruits and vegetables compared to those with low intakes (Block et al., 1992; Pavia et al., 2006). In fact, multiple epidemiological and animal studies have shown that consumption of foods rich in fruits and vegetables decreased the incidence of cancers, suggesting that certain dietary constituents may thus be effective in preventing the disease (Steinmetz et al., 1996; Reddy et al., 2003; Benetou et al., 2008; Freedman et al., 2008). Similarly, a wealth of information is available, implicating dietary agents in cancers of the skin (Khan et al., 2008), prostate (Syed et al., 2008; Haseen et al., 2009), breast (Bougnoux et al., 2010) and lung (Cranganu and Camporeale, 2009; Goralczyk, 2009).

There are two major diet related prevention strategies that have been involved in combating cancer, i.e. cancer chemoprevention and dietary prevention, with an appreciable overlap existing between them. Generally, cancer chemoprevention is recognized as the pharmacological intervention with synthetic or naturally occurring non-toxic chemicals to prevent, inhibit or reverse carcinogenesis or prevent development of invasive cancer (Boone et al., 1997; Mayne and Lippman, 1997). On the other hand dietary prevention is recognized as the changes in food consumption pattern necessary to reduce the risk of cancer development (Goodman, 1997).

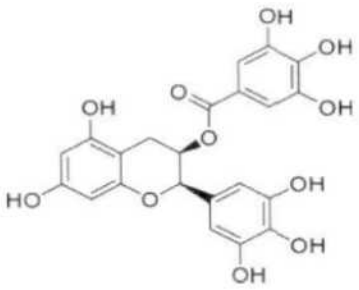
Cancer chemoprevention and dietary polyphenols

Fruits and vegetables contain a wide variety of phytochemicals that are regarded as effective protective agents. One such prominent class of phytochemicals, plant derived foods and beverages are rich in, are dietary polyphenols that have received much attention over the last two decades for their health benefits, including cancer chemopreventive effects. Polyphenols are plant secondary metabolites that serve as a component of plant defense

mechanisms against predation by microorganisms, insects and herbivores. They are widely distributed plant derived dietary constituents and have been implicated as the active components in a number of herbal and traditional medicines (Wollenweber, 1988). Polyphenols are known to possess a wide range of pharmacological properties including cardioprotective, neuroprotective, anti-inflammatory and anticancer properties (Szewczuk et al., 2004; Dai et al., 2006; Thomasset et al., 2007; Ullah and Khan, 2008).

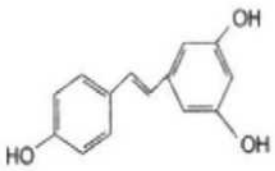
Plant derived polyphenolic compounds are important constituents of human diet which include resveratrol from red grapes and red wines, epigallocatechin-3-gallate from green tea, curcumin from spice turmeric, apigenin from parseley, quercetin from onion and isoflavone genistein from soybean. Figure 1 illustrates some of the popular dietary polyphenols that have been known to possess chemopreventive potential and their dietary sources.

Many studies in different cell lines, animal models and human epidemiological trials suggest a protective role of dietary polyphenols against different types of cancers (Watson, et al., 2000; Wenzel et al., 2000; Yang et al., 2001). Clinical trials have correlated polyphenolic intake with prevention of particular cancers, showing a decreased risk for different types of cancers (Knekt et al., 1997; Key et al., 1999; Arts et al., 2002; Su and Arab, 2002) or a diminished recurrence of cancer (Nakachi et al., 1998; Le Marchand et al., 2000) after the consumption of polyphenols or certain foods or drinks, such as tea and red wine, rich in these phenolic compounds. The most direct evidence of beneficial effects by a particular food rich in polyphenols, or individual compounds, have come from animal models and *in vitro* experiments. In fact, cell culture studies constitute a valuable tool for identifying the molecular targets modulated by dietary polyphenolic compounds in cancer cells.



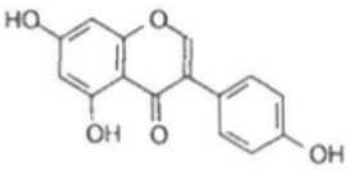
EGCG

Tea



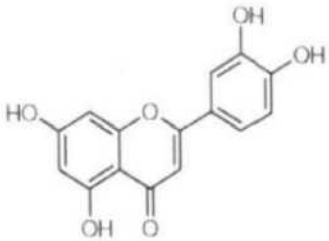
Resveratrol

Grapes



Genistein

Soy



Luteolin

Parseley

Figure 1: Some chemopreventive polyphenolic compounds and their sources

The wide array of chemopreventive phytochemicals present in fruits and vegetables can be of two types, namely cancer blocking and cancer suppressing agents. The former prevent carcinogens from initiation of carcinogenesis by several mechanisms such as (a) enhancing carcinogen detoxification, (b) modifying carcinogen uptake and metabolism, (c) scavenging free radicals and (d) enhancing DNA repair. Cancer suppressing agents, on the other hand, inhibit cancer promotion and progression after the formation of preneoplastic cells by interfering with (a) cell cycle regulation, (b) signal transduction, (c) transcriptional regulation and (d) apoptosis (Greenwald, 2002; Surh, 2003). Many potential chemopreventive polyphenols may interrupt or reverse the carcinogenesis process by acting on intracellular signalling network molecules involved in the initiation and/or promotion, but also these compounds may arrest or reverse the progression stage of cancer (Manson, 2003; Surh, 2003). The anticarcinogenic activity of these plant derived dietary polyphenolic compounds may be attributed to a combination of their cytoprotective effect on normal cells (cancer blocking effect) and their cytotoxic effect on preneoplastic and/or neoplastic cells (cancer suppressing action).

An ideal chemopreventive agent should be selective for damaged or transformed cells, display a significant bioavailability in the target region and have more than one mechanism of action. Moreover, it should be highly effective, easy to administer, and inexpensive. Dietary polyphenols, in this regard, are particularly attractive because of human long standing exposure to them and their relative lack of toxicity. In fact, numerous polyphenolic compounds have been shown to display antiproliferative and cytotoxic effects towards several tumor cells, showing cancer cell specific toxicity in comparison to normal cells (Lepley et al., 1996; Agullo et al., 1997; Skaper et al., 1997; Sergediene et al., 1999). Dietary polyphenols are mainly consumed through fruits, vegetables and beverages such as juice, wine, tea and coffee,

apart from cereals and olive derivatives (Hollman & Katan, 1999; Visioli & Galli, 2002). Their average daily intake has been reported to be around 1g (Scalbert and Williamson, 2000; Scalbert et al., 2005), which is much higher than intake of all other classes of dietary antioxidants. For instance, it is approximately 10 times higher than vitamin C intake and 100 times the intakes of vitamin E and carotenoids (Scalbert and Williamson, 2000). However, an important drawback with polyphenols being effective chemopreventive agents is their low bioavailability after ingestion (Manach et al., 2005; Scalbert et al., 2005). Nonetheless, polyphenols as chemopreventive agents found in human diet are considered a very promising group of compounds, on account of their safety, low-toxicity, and general acceptance.

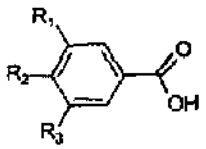
Chemical structure and basic classification of polyphenolic compounds

Polyphenols are one of the most numerous and widely distributed groups of compounds in the plant kingdom. More than 8000 compounds having a polyphenol structure, i.e. several hydroxyl groups on aromatic rings, have been identified in higher plants, of which several hundred are found in edible plants. Polyphenolic compounds are frequently found attached to sugars (glycosides), thus tending to be water-soluble. Occasionally, polyphenols also occur in plants as aglycones.

Most of these heterogeneous compounds may be broadly classified into four different general classes based on the number of phenol rings that they contain and of the structural elements that bind these rings to one another. Distinctions are, therefore, made between the phenolic acids, flavonoids, stilbenes, and lignans (Figure 2). Two subclasses of phenolic acids can be distinguished: derivatives of benzoic acid and derivatives of cinnamic acid (Figure 2). Hydroxybenzoic acids are, furthermore, components of complex

Phenolic Acids

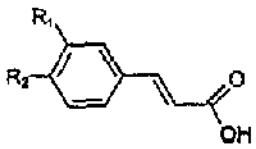
Hydroxybenzoic acids



R_1=R_2=OH, R_3= H: Protocatechuic acid

R_1=R_2= R_3= OH: Gallic acid

Hydroxycinnamic acids

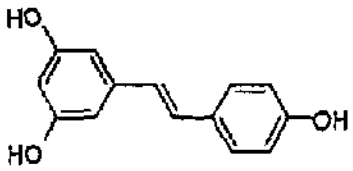


R_1=OH: Coumaric acid

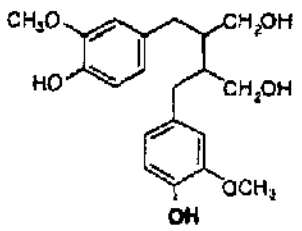
R_1=R_2=OH: Caffeic acid

R_1=OCH_3,R_2=OH: Ferulic acid

Stilbenes



Lignans



Flavonoids

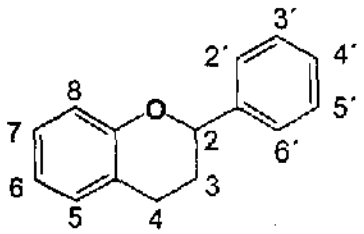


Figure 2: General chemical structures of major classes of polyphenols

structures such as hydrolyzable tannins (gallotannins in mangoes and ellagitannins in red fruit such as strawberries, raspberries, and blackberries) (Clifford & Scalbert, 2000). Because these hydroxybenzoic acids are found in only a few plants eaten by humans, they have not been extensively studied and are not considered to be of great nutritional interest. The hydroxycinnamic acids are more common than the hydroxybenzoic acids and consist chiefly of p-coumaric, caffeic, ferulic, and sinapic acids.

Flavonoids, phenolic acids, stilbenes and lignans are the most abundantly occurring polyphenols in plants, of which flavonoids and phenolic acids account for 60 and 30%, respectively, of dietary polyphenols. More than 5000 different flavonoids have been identified and classified according to their

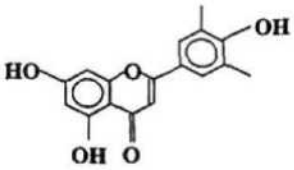
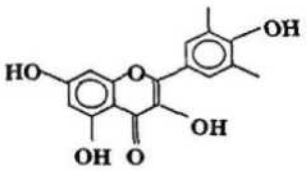
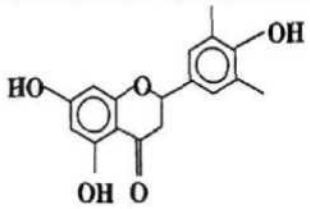
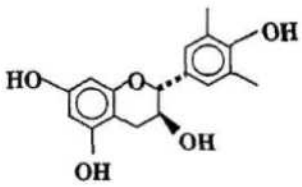
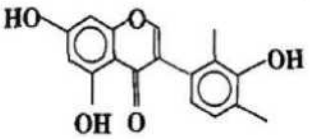
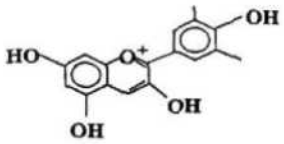
molecular structure (Ross and Kasum, 2002). Flavonoids, therefore, are the major dietary polyphenols which are derived from a wide variety of plant sources. They share a common basic structure, consisting of a heterocyclic skeleton of flavan (2- phenylbenzopyrane). The structure is represented by 2 aromatic rings (A and B) that are joined together by a linear three-carbon chain that forms an oxygenated heterocycle (ring C). The constituent polyphenolic units are derived from the secondary plant metabolism of the shikimate pathway (Dewick, 1995). Flavonoids may themselves be divided into 6 subclasses as a function of the type of heterocycle involved. The six major subclasses of flavonoids include the flavones (e.g. apigenin, luteolin), flavonols (e.g. quercetin, myricetin), flavanones (e.g. naringenin, hesperidin), catechins or flavanols (e.g. epicatechin, epigallocatechin-3-gallate), anthocyanidins (e.g. delphinidin, malvidin), and isoflavones (e.g. genistein, daidzein). Table I gives a classification of flavonoid subclasses along with their important members that are known to possess pharmacological properties.

Flavonoids are often hydroxylated at positions 3, 5, 7, 2', 3', 4', 5'. In addition to this diversity, polyphenols may be associated with various carbohydrates and organic acids and with one another. Usually in the plant system, these flavonoids exist in conjugated forms, the most common being the glycosides. When glycosides are formed, the glycosidic linkage is normally located at position 3 or 7 and the carbohydrate moiety can be L-rhamnose, D-glucose, gluco-rhamnose, galactose or arabinose (Middleson, 1984).

Biosynthesis of plant polyphenols

Plant polyphenols execute a vast array of important functions in plants (Croteu et al., 2000). For example, stilbenes and coumarins serve to defend pathogen attacks, flavonoids act as UV irradiation protectants while isoflavone

Table I: General chemical structure of different subclasses of flavonoids with their important constituent members

Subclass	General Chemical structure	Representative compounds
Flavones		Apigenin, Luteolin, Chrysin
Flavonols		Quercetin, Kaempferol,
Flavanones		Hesperidin, Naringenin
Flavanols		Catechins, Epicatechins, Epigallocatechin-3-gallate (EGCG)
Isoflavones		Genistein, Daidzein
Anthocyanidins		Delphinidin, Malvidin

and anthocyanins serve as flower pigments. The majority of polyphenolic compounds produced by plants are synthesized by a highly branched phenylpropanoid pathway (Figure 3). The initial compound is cinnamic acid, which arises from phenylalanine by the action of PAL (Phenyl-ammonia lyase). Several simple polyphenols with the basic C6-C3 skeleton of phenylalanine are produced from cinnamate via a series of hydroxylation, methylation and dehydration reactions; these include p-coumaric acid, caffeic acid, ferulic acid, siapic acids and other simple coumarins (Dixon and Paiva, 1995). In addition, compounds such as styrenes, benzoic acid and derivatives, acetophenones and gingerols arise from hydroxycinnamic acid by chain shortening and lengthening without ring formation.

The precursor molecules 4-coumaroyl-CoA and malonyl CoA give rise to tetraketide. Tetrakedite may get converted into resveratrol by the enzyme Stilbene synthase (STS). Chalcone synthase (CHS) is the first step in the branch of the pathway that produces the flavonoids. CHS acts on tetrakedite to form tetrahydroxychalcone. Tetrahydroxychalcone provides the basic structural skeleton for biosynthesis of all classes of flavonoids, including flavonones, isoflavones, flavones, flavonols, anthocyanins and flavanols. Chalcone Isomerase (CHI) acts upon tetrahydroxychalcone and converts it into the flavanone, naringenin. Naringenin by different reactions gives rise to isoflavone genistein, flavones apigenin and luteolin, and flavonols such as kaempferol, quercetin etc. Also, naringenin in a series of reactions leads to the formation of leucoanthocyanidins. Anthocyanidin synthase converts leucoanthocyanidins to anthocyanidins which finally lead to the formation of epigallocatechins by anthocyanidin reductase.

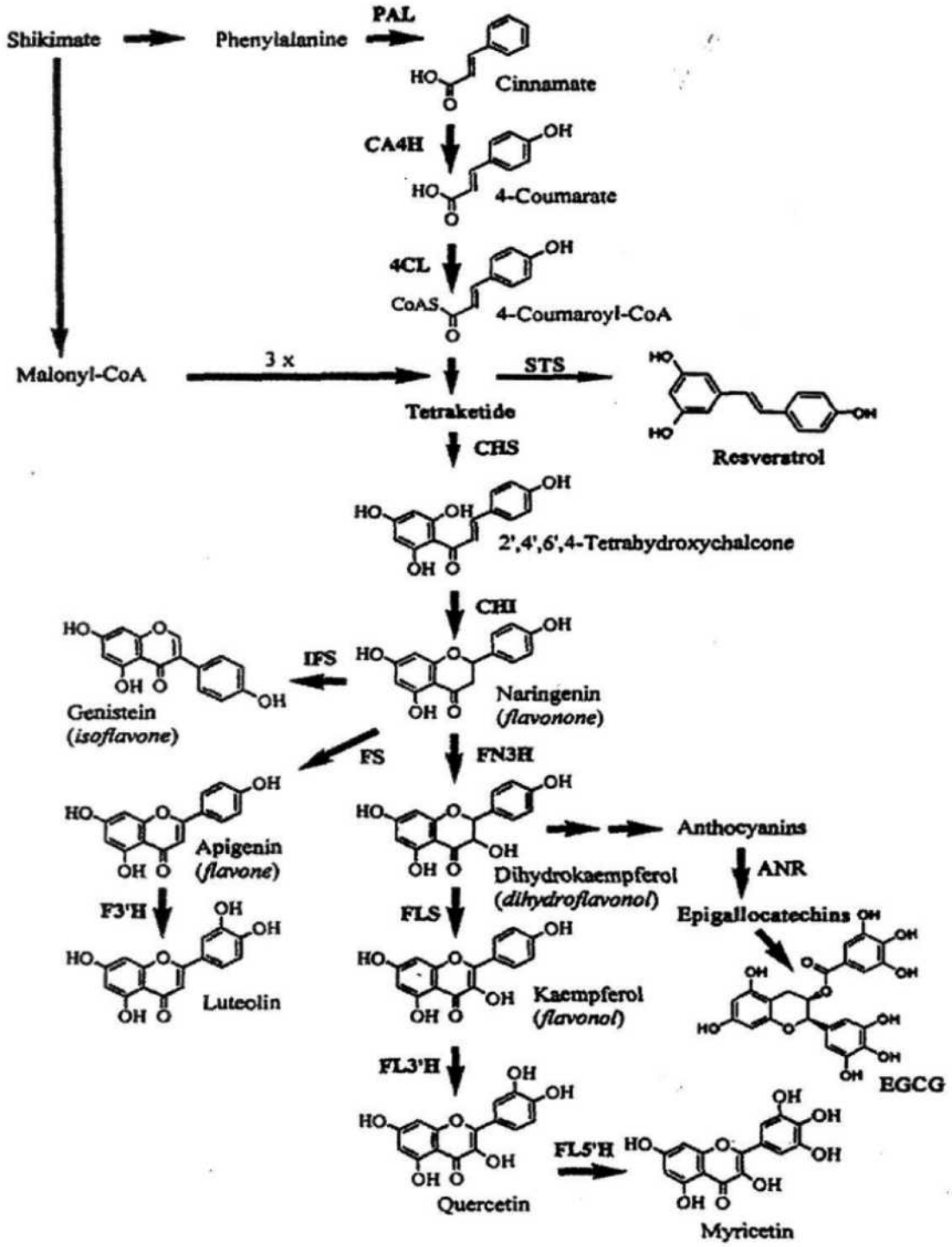


Figure 3: The phenylpropanoid pathway by which plants synthesize a wide range of polyphenols

PAL, phenylalanine amminia lyase; CA4H, cinnamate 4-hydroxylase; 4CL, 4 coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; IFS, isoflavone synthase; FN3H, flavanone 3-hydroxylase; FS, flavone synthase; F3'H, flavone 3'-hydroxylase; FLS, flavonol synthase; FL3'H, flavonol 3'-hydroxylase; FL'CH, flavonol 5'-hydroxylase; STS, stilbene synthase; ANR, anthocyanidin reductase.

Sources of plant derived dietary polyphenolic compounds

A large body of literature exists regarding the polyphenolic content of various plant-derived human foods. Common polyphenols belonging to different subgroups and the various food sources from which they are usually obtained are enlisted in Table II. Flavonols are the most ubiquitous flavonoids in foods, and the main representatives are quercetin and kaempferol. They are generally present at relatively low concentrations of15-30 mg/kg fresh wt. The richest sources are onions (up to 1.2 g/kg fresh wt), curly kale, leeks, broccoli, and blueberries. Red wine and tea also contain up to 45 mg flavonols/L. These compounds are present in glycosylated forms. The associated sugar moiety is very often glucose or rhamnose, but other sugars may also be involved (eg, galactose, arabinose, xylose, glucuronic acid). Fruit often contains between 5 and 10 different flavonol glycosides (Macheix et al., 1990). Flavones are much less common than flavonols in fruit and vegetables. Flavones consist chiefly of glycosides of luteolin and apigenin. The important edible sources of flavones are parsley and celery. Cereals such as millet and wheat contain glycosides of flavones (Sartelet et al., 1996).

In human foods, flavanones are found in tomatoes and certain aromatic plants such as mint, but they are present in high concentrations only in citrus fruit. The main aglycones are naringenin in grapefruit, hesperetin in oranges, and eriodictyol in lemons. Flavanones are generally glycosylated by a disaccharide at position 7. Isoflavones are found almost exclusively in leguminous plants. Soya and its processed products are the main source of isoflavones in the human diet. They contain 3 main molecules: genistein, daidzein,and glycitein, generally in a concentration ratio of 1:1:0.2. These isoflavones are found in four forms: aglycone, 7-O-glucoside, 6-O-acetyl- 7-O-glucoside, and 6-O-malonyl-7-O-glucoside (Coward et al., 1998). The isoflavone content of soya and its manufactured products varies greatly as a function of geographic zone,

Table II: Main groups, representative compounds and dietary sources of common polyphenols

Polyphenol subgroup	Representative polyphenols in diet	Common food sources
Flavonols	Quercetin, Kaempherol, Myricetin, Rutin	Onions, apples, broccoli, tea, red wine, berries, tomato, leek
Flavones	Apigenin, Luteolin, Chrysin	Parseley, celery, thyme, oregano, capsicum peppers
Isoflavones	Genistein, Daidzein, Biochanin A	Soybeans, soy foods, legumes
Flavanols	*Monomers* Catechin, Epicatechin, EGCG *Polymers* Proanthocyanidins	Tea (particularly green tea), Apples, pears, raspberries, chocolate, beans, apricot
Flavanones	Naringenin, Hesperitin, Eriodictyol	Orange, grapefruit, lemon
Anthocyanidins	Cyanidin, Malvidin, Delphinidin	Cherry, Strawberry, red wine, blue berry, black currant, rhubarb
Stilbenes	Resveratrol	Grapes, red wine, peanuts, berries

growing conditions, and processing. Soybeans contain between 580 and 3800 mg isoflavones/kg fresh wt. and soymilk contains between 30 and 175 mg/L (Reinli and Block, 1996; Cassidy et al., 2000).

Flavanols exist in both the monomer form (catechins) and the polymer form (proanthocyanidins). Catechins are found in many types of fruit (apricots, which contain 250 mg/kg fresh wt, are the richest source). They are also present in red wine (up to 300 mg/L), but green tea and chocolate are by far the richest sources. An infusion of green tea contains up to 200 mg catechins (Lakenbrink et al., 2000). Catechin and epicatechin are the main flavanols in fruit, whereas gallocatechin, epigallocatechin, and epigallocatechin gallate are found in certain seeds of leguminous plants, in grapes, and more importantly in tea (Arts et al., 2000a & b). In contrast to other classes of flavonoids, flavanols are not glycosylated in foods. Anthocyanidins are found in red wine, certain varieties of cereals, and certain leafy and root vegetables (aubergines, cabbage, beans, onions, radishes), but they are most abundant in fruit. Cyanidin is the most common anthocyanidin in foods. Food contents are generally proportional to colour intensity and reach values up to 2-4 g/kg fresh wt. in blackcurrants or blackberries. These values increase as the fruit ripens. Anthocyanidins are found mainly in the skin, except for certain types of red fruit, in which they also occur in the flesh (cherries and strawberries).

Stilbenes are found in only low quantities in the human diet. One of these, resveratrol, which been extensively studied, is found in several edible natural products such as grapes, peanuts and berries (blue berries, cranberries, lingo berries etc) and wine. Fresh grape skins contain 50-100 mg resveratrol/g. Resveratrol is much more concentrated in red than in white wine. The average concentration of resveratrol in common varieties of red wine ranges from 2 to 40 μM (Gusman et al., 2001). Free resveratrol, however, occurs in low

quantities in wine (0.3-7 mg aglycones/L Vs 15 mg glycosides/L in red wine) (Bhat and Pezzuto, 2002; Vitrac et al., 2002).

Bioavailability of dietary polyphenols

It is important to realize that the polyphenols that are the most common in the human diet are not necessarily the most active within the body, either because they have a lower intrinsic activity or because they are poorly absorbed from the intestine, highly metabolized, or rapidly eliminated. In addition, the metabolites that are found in blood and target organs and that result from digestive or hepatic activity may differ from the native substances in terms of biological activity. Extensive knowledge of the bioavailability of polyphenols is thus essential if their health effects are to be completely understood.

Metabolism of polyphenols occurs via a common pathway (Scalbert and Williamson, 2000). The aglycones can be absorbed from the small intestine. However, most polyphenols are present in food in the form of esters, glycosides, or polymers that cannot be absorbed in their native form. These substances must be hydrolyzed by intestinal enzymes or by the colonic microflora before they can be absorbed. During the course of absorption, polyphenols are conjugated in the small intestine and later in the liver. This process mainly includes methylation, sulfation, and glucuronidation. This is a metabolic detoxication process common to many xenobiotics that restricts their potential toxic effects and facilitates their biliary and urinary elimination by increasing their hydrophilicity. The conjugation mechanisms are highly efficient, and aglycones are generally either absent in blood or present in low concentrations after consumption of nutritional doses. Polyphenols are able to penetrate tissues, particularly those in which they are metabolized. Polyphenols and their derivatives are eliminated mainly in urine and bile.

For any chemical moiety to exert a biological effect, it should be bioavailable i.e. it must be readily absorbed into the bloodstream and reach concentrations that have the potential to exert effects *in vivo*. Bioavailability is a key factor in the description of polyphenols and is an essential aspect for understanding the role they might play in addressing human disease. Most of the polyphenols are known to be readily absorbed (Scalbert and Williamson, 2000; Rowland et al., 2003) but are prone to be modified into other forms inside the body, one such common chemical modification being conjugation (Lambert et al., 2005). Table III summarises the major bioavailable forms of common dietary polyphenols.

Table III: Major dietary polyphenols and their bioavailable forms in plasma

Polyphenols	Bioavailable forms in plasma
Flavonols	Methyl, sulphate or glucuronic acid conjugates
Isoflavones	Sulphates or glucuronides conjugates; Also occur as glycosides and aglycones
Flavanols	Methyl, sulphate or glucuronic acid conjugates; EGCG occurs in the unconjugated form; Dimers
Anthocyanidins	Glucosides
Stilbenes	Glucuronides, Sulphate conjugates; Unconjugates are also present as product of fermentation

Curcumin undergoes metabolic O-conjugation to curcumin glucuronide and curcumin sulfate and bioreduction to tetrahydrocurcumin, hexahydrocurcumin, and hexahydrocurcuminol in rats and mice *in vivo* and in suspensions of human and rat hepatocytes (Ireson et al., 2001). Certain curcumin metabolites, such as tetrahydrocurcumin, possess anti-inflammatory (Mukhopadhyay et al., 1982) and antioxidant activities (Sugiyama et al, 1996)

similar to those of their metabolic progenitor. Dietary resveratrol is rapidly absorbed and predominantly present in plasma as glucoronide and sulphate conjugates. When administered in food, such as wine or grape juice, resveratrol metabolism is significantly inhibited by other polyphenols due to competitive reactions with metabolizing phase II enzymes resulting in an increased concentration of the free form (Wenzel and Somoza, 2005). Isoflavones such as genistein are also known to undergo conjugation with glycosides and is metabolized in human intestine to dihydrogenistein and 6'-hydroxy-O-desmethylangolensin. Concentration of genistein has been shown to be higher in individuals consuming soy rich diet (Adlercreutz et al., 1993) and consequently genistein and its metabolites have been detected in plasma, breast aspirate and prostatic fluid (Mills et al., 1989). Similarly, other polyphenols are also known to be absorbed and metabolized into various end products which may or may not possess the biological effects of the parent compound.

Therapeutic potentials of dietary polyphenols

A longstanding tenet of nutrition holds that people with diets rich in fruits and vegetables enjoy better health than those eating few such foods. Much of current research shows that free radicals are linked to various chronic diseases. As a result dietary antioxidants such as plant derived polyphenols hold promise in at least delaying the onset/progression of these diseases. An insight into the investigations, both *in vitro* and *in vivo*, reveals the properties of plant derived dietary polyphenols that can form the basis of their use in the prevention and cure of certain human disorders. A large number of pharmacological properties, including cardioprotective, neuroprotective and anticancer effects, are thought to be associated with their beneficial effects. For instance, resveratrol, a polyphenol found in grapes and red wine, is widely

recognized as a bioactive agent with potential benefits for human health (Figure 4).

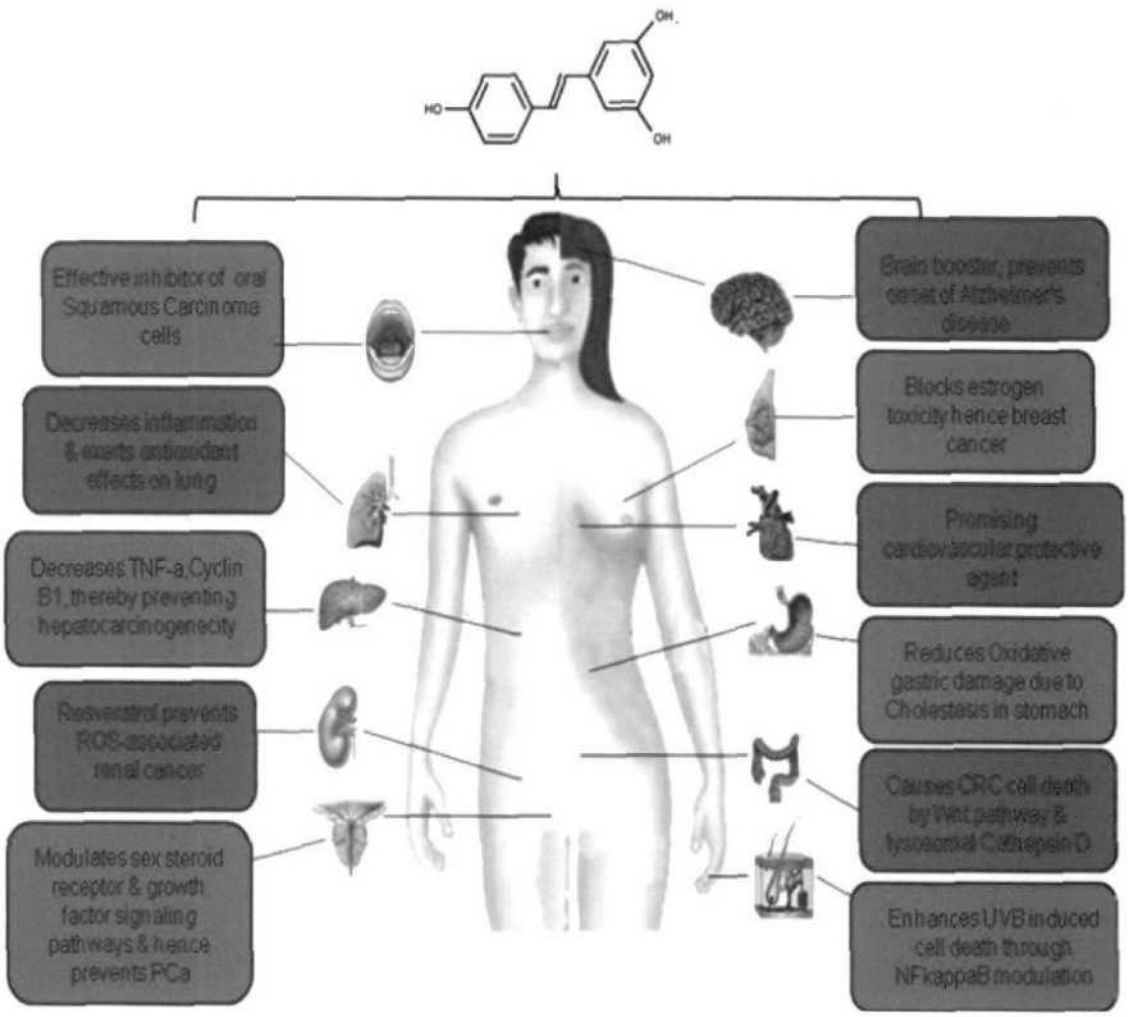


Figure 4: Proposed targets for beneficial effects of dietary polyphenol resveratrol on human health (from Shukla Y and Singh R. *Ann N Y Acad Sci* 2011; 1215: 1-8).

Some of the important therapeutic properties of plant derived polyphenols with some strong evidence from the existing literature have been discussed below.

Cardioprotective properties

The "French Paradox" – the observation that mortality from coronary heart disease is relatively low in France despite relatively high levels of dietary

saturated fat, led to the idea that regular consumption of red wine (rich source of polyphenols) might provide additional protection from cardiovascular diseases (Criqui and Ringel, 1994). Regular, moderate consumption of red wine is linked to a reduced risk of coronary heart disease. Resveratrol, a red wine polyphenol has been linked to a number of potentially cardioprotective effects (Szewczuk et al., 2004). Anthocyanidins have also been found to have antioxidant potential (Falchi et al., 2006). Similarly, green tea consumption has also been associated with a lower risk of coronary artery disease in Japanese populations (Sano et al., 2004). Studies suggest that EGCG (a major polyphenol in green tea) can suppress active oxygen species and thereby prevent the development of cardiac hypertrophy (Li et al., 2006).

Endothelial dysfunction is involved in the initiation and progression of arteriosclerosis. Some polyphenols have been shown to relax endothelium-denuded arteries. There have been reports that extracts from grape and wine induce endothelium-dependent relaxation via enhanced and/ or increased biological activity of nitric oxide (NO) which leads to the elevation of cGMP levels (Andriambeloson, 1997). Resveratrol has been found to promote vasodilation by enhancing the production of NO (Wallerath et al., 2002).

Recently, Lowenstein and co-workers have shown that EGCG has the potential to reduce aggregation of the inflammatory leukocytes that directly contribute to atherosclerosis (Yamakuchi et al., 2008]. Genistein, one of the major isoflavones in soy protein, binds to estrogen receptor β with much higher affinity than to ERα (Kuiper et al., 1998) and can elicit endothelium dependant vasorelaxation *in vitro* (Figtree et al., 2000) and *in vivo* (Walker et al., 2001). Other isoflavones such as dihydrodaidzeins have also been reported to enhance endothelial function (Shen et al., 2006). Flavonoids have also been found to be good hypochlorite scavenger *in vitro* and could have favourable effects in diseases such as atherosclerosis, in which hypochlorite is known to

play a significant role (Firuzi et al., 2004). Increase in LDL is taken as a parameter for the occurrence and susceptibility to cardiovascular diseases. Polyphenols such as dicvertin have been reported to produce a 12% decrease in LDL along with a 14% increase in HDL in coronary heart disease patients (Belaia et al, 2006). Lipid-lowering activity has also been reported in tea flavonoids (Li et al, 2006).

Chronic low levels of ROS are known to promote cardiovascular diseases (Barchowsky et al., 1996). Therefore, in the prevention of cardiovascular diseases, many of the observed effects of polyphenols, can be attributed to their recognized antioxidant and radical scavenging properties, which may delay the onset of atherogenesis by reducing chemically and enzymatically mediated peroxidative reaction (German and Walzem, 2000).

Neuroprotective properties

Neurodegenerative disorders are a heterogeneous group of diseases of the nervous system, including the brain, spinal cord and peripheral nerves, which have different etiologies. The multifactorial etiology of these diseases suggests that interventions such as polyphenols having multiple targets could have therapeutic potential for them. Moreover, epidemiological studies indicate that dietary habits and antioxidants from diet can influence the incidence of neurodegenerative disorders such as Alzheimer and Parkinson's diseases (Morris et al., 2002). The nervous system is rich in fatty acids and iron. High levels of iron can lead to oxidative stress via the iron-catalyzed formation of ROS (Bauer and Bauer, 1999). In addition brain regions that are rich in catecholamines are vulnerable to free radical generation. One such region of the brain is the substantia nigra, where a connection between antioxidant depletion and tissue degeneration has been established (Perry et al., 2002).

There is substantial evidence that oxidative stress is a causative or at least an ancillary factor in the pathogenesis of many neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS) (Ghadge et al., 1997), Huntington's disease (HD) and Schizophrenia (Philips et al., 1993). Neuroprotective capacity is believed to be a characteristic property of plant derived polyphenolic compounds (Yazawa et al., 2006; Ritz et al., 2008). Polyphenols exhibit biological effects such as anti-inflammatory, antioxidant and metal chelating properties, which augment their role in neuroprotection. Reports also suggest that red wine that contains high levels of antioxidant polyphenols reduces the incidence of AD (Wang et al., 2006). Polyphenols such as EGCG, curcumin, extracts of blue berries and *Scutellaria* are also known to help in AD (Dai et al., 2006). *In vitro* studies show that green tea extract rich in catechins could protect neurons from the amyloid beta-induced damages in AD (Bastianetto et al., 2006). EGCG is also found to be of use in ALS (Xu et al., 2006) and PD (Ramassamy, 2006).Extract of *Scutellaria* stem and polyphenols such as curcumin and naringenin also exhibit neuroprotection in PD (Shang et al., 2006). Alzheimer's disease is characterized by chronic inflammation and oxidative damages in the brain. Curcumin possess antioxidative and anti-inflammatory properties and has thus been shown to exert a protective effect against oxidative damages initiated by divalent metals or suppress inflammatory damage by preventing metal induction of NF-kB and also inhibits amyloid beta fibril formation (Kim et al., 2005). Dietary polyphenols have potential as protective agents against neuronal apoptosis, through selective actions within stress activated cellular responses including protein kinase signalling cascade (Schroeter et al., 2006). Several dietary supplements with blueberries extracts have been reported to reduce some neurological deficits in aged animal models (Joseph et al., 1999; Joseph et al., 2003).

Anticancer properties

The role of polyphenols as anticancer agents is complex and extensive research has indicated that their anticancer effects are exerted at multiple levels including inhibition of gene expression (Liu et al., 2008), inhibition of angiogenesis (Shankar et al., 2008), inhibition of metastasis (Shankar et al., 2008; Kushima et al., 2009) and suppression of cell proliferation (Gu et al., 2009). The anticancer properties of dietary polyphenolic compounds, including their subcategory flavonoids, have been widely documented by several studies (Bosetti et al., 2006; Cardenas et al., 2006; Walle et al., 2007). Epidemiologic studies have consistently shown an inverse relation between flavonoid consumption and risks for certain types of cancer (Mukhtar and Ahmad, 2000; Russo, 2007). Several studies have demonstrated that some naturally occurring flavonoids such as genistein (Jeune et al., 2005), apigenin (Lim et al., 2007) and luteolin (Way et al., 2005) possess significant suppressive effects on human cancers.

Furthermore, numerous studies have reported antiproliferative effects mediated by polyphenolic compounds against both human and rodent ovarian, leukemic, intestinal, lung, breast, bladder and prostate cancer cells. For example, it has been shown that genistein could induce apoptosis in MDA-MB-231, MDA-MB-435, and MCF-7 breast cancer cells; PC3 and LNCaP prostate cancer cells; H460 and H322 non-small cell lung cancer cells; HN4 head and neck squamous carcinoma cells, and pancreatic cancer cells (Davis et al., 1998; Lian et al., 1998; Li et al., 1999; Alhasan et al., 2000; Banerjee et al., 2005, 2007). Moreover, Genistein is also reported to be inhibitory at concentrations similar to conventional anticancer drugs such as methotrexate and doxorubicin (Hirano et al., 1994). Moiseeva et al. (2007) reported that physiological concentrations of a dietary phytochemical such as genistein results in reduced growth and induction of apoptosis in cancer cells. Quercetin

and luteolin are shown to induce apoptosis in a wide range of tumor cells such as A431, MiaPaCa-2, Hep G2 and MCF 7 (Huang et al., 1999; Lee et al., 2002) Similarly, Shukla and Gupta (2004) have demonstrated apigenin-mediated growth inhibition and induction of apoptosis in DU145 prostate cancer cells. Genistein and quercetin, in addition, to their antiproliferative action, appear to alter the metastatic potential of rat breast adenocarcinoma cells, measured as a reduced ability to migrate within collagen matrix (Lu et al., 1996).

The citrus flavonoid tangeretin suppresses HL60 proliferation quite strongly, with an IC_{50} of 0.17 μM (Hirano et al., 1995). Also it has been shown that gallocatechins, found in green tea and which include tannic acid, gallic acid, epigallocatechin, epicatechin- 3-gallate and epigallocatechin-3-gallate (EGCG), can potentially induce apoptosis in various cancer cell lines (Ahmad et al., 1997). Curcumin, a natural phenolic compound found in spice turmeric, has been shown to have antiproliferative action against colon cancer, breast cancer and myeloid leukemia (Tsvetkov et al., 2005; Maheshwari et al., 2006). Resveratrol, the polyphenol found in berries and grapes, has been reported to possess anticancer properties (Aggarwal et al., 2004) and is able to inhibit the growth of prostate tumors by acting on the regulatory genes such as p53 (Narayanan, 2006). Androgen independent DU145 human prostate cancer cells manifest resistance to radiation-induced apoptotic death (Yacoub et al., 2001). Scarlatti et al (2007) have reported that pre-treatment with resveratrol significantly enhances radiation induced cell death in DU145 cells. Similarly, isoflavones have also been shown to sensitize cancer cells to radiotherapy (Hillman and Singh-Gupta, 2011).

Further, the capacity of certain dietary polyphenols to protect against both chemically induced or spontaneous formation of tumors in animals is well established. For instance, quercetin administered to rats in combination with dimethyl-benz-(a)-anthracene (DMBA) or N-nitrosomethylurea (NMU)

reduced the incidence and multiplicity of carcinogen induced mammary tumor by 30% and 50% respectively (Verma et al., 1988). Quercetin and luteolin (10 g/Kg diet) decreased fibrosarcoma incidence (52% and 60% respectively) and tumor size in male Swiss albino mice following treatment with the model chemical carcinogen 20-methylcholanthrene (Elangovan et al., 1994). Quercetin (20 g/Kg b.w) also increases the survival and reduces the tumor burden of mice transplanted intrasplenically with ML-3 hepatoma cells (Chi et al., 1997). The citrus flavonoid naringenin inhibits the *in vivo* development of DMBA induced mammary tumors in Sprague-Dawley rats (So et al., 1996). Jang et al. (1997) first reported that topical resveratrol applications prevented skin cancer development in mice treated with a carcinogen In further studies on mice and rats, resveratrol was found to be able to inhibit the carcinogenic activity of DMBA and Neuro-2a cells subcutaneously injected to induce breast cancer (Whitsett et al., 2006) and neuroblastoma (Chen et al., 2004a), respectively.

Several studies have described a protective effect of tea polyphenols against carcinogenesis. Rats fed on a diet containing 10 g green tea catechins/kg b.w have a considerably reduced mortality from mammary tumors following DMBA treatment compared with rats given carcinogen alone (Hirose et al., 1994). In a comprehensive study, Yang et al (1998) described the ability of both green and black tea infusions to inhibit N-nitrosodiethyl-amine-induced lung carcinogenesis in mice model. EGCG, the main tea polyphenolic, in a model of transgenic mice for skin cancer, has exhibited a preventive effect (Meeran et al., 2006). EGCG has also shown beneficial effects in lung cancer by decreasing the growth of the primary tumors and metastasis when mice were intraperitoneally injected with B16-F3m cells (Liu et al., 2001)

In addition to their potential as anticancer agents, an important role of plant polyphenols as natural modulators of cancer multidrug resistance (MDR) has

been documented (Ullah, 2008). Resistance of recurrent disease to cytotoxic drugs is the principal factor limiting long-term treatment success against cancer. Flavonoids have been found to inhibit breast cancer resistance protein (BCRP), an ABC transporter, which plays an important role in drug disposition leading to chemoresistance in breast cancer (Shuzhong et al., 2005). Isoflavones such as biochanin A, daidzein (Chung et al., 2005) and green tea polyphenol EGCG (Feng et al, 2005) have also been shown to exhibit anti MDR activities in various drug resistant cancer cell lines, such as doxorubicin resistant KB-A1 cells through the inhibition of P-glycoprotein transporters. Curcumin has been reported to induce apoptosis in chemoresistant ovarian cancer cell lines SKOV3 and ES-2 (Wahl et al., 2007).

Putative anticancer mechanisms of plant derived dietary polyphenols

While the anticancer properties of several common dietary polyphenols like resveratrol, EGCG, genistein and quercetin have been well established, the underlying molecular mechanisms of their antiproliferative effects are not completely understood and clearly defined. Dietary polyphenols can affect the overall process of carcinogenesis by several mechanisms and their effects may depend on tissue or cell type and may differ at high and low doses. Polyphenols may interfere in several of the steps that lead to the development of malignant tumors, including protecting DNA from oxidative damage, inhibiting carcinogen activation, activating carcinogen detoxifying systems and modulating multiple key proteins involved in diverse signal transduction pathways such as regulation of cellular proliferation, differentiation, apoptosis, angiogenesis or metastasis (Galati et al., 2000; Ren et al., 2003; Surh, 2003; Ramos, 2007).

Several suggested mechanisms by which polyphenols exert anticancer effects are briefly discussed below.

Antioxidant action:

In addition to the natural defence mechanisms of the cell, dietary polyphenols can also act as antioxidants, preventing injury caused by free radicals and blocking the initiation step of cancer (Middleton et al., 2000; Watson et al., 2000; Alia et al., 2006). Polyphenolic compounds can prevent the DNA damage caused by free radicals or carcinogenic agents through different mechanisms, such as (i) direct radical scavenging (Sonee et al., 2004), (ii) chelating divalent cations involved in Fenton reaction (Nakagawa et al., 2004) and (iii) modulation of enzymes related to oxidative stress such as glutathione peroxidase (GPx), glutathione reductase (GR) and superoxide dismutase (SOD) (Alia et al., 2006). It is considered that many of the antiproliferative effects of polyphenols are attributable to their antioxidant properties (Mukhtar and Ahmad, 1999). Polyphenols like EGCG and genistein have been shown to act as powerful radical scavengers, protecting cells from oxidative stress induced toxicity (Murakami et al., 2002; Sonee et al., 2004).

Modulation of enzymes associated with carcinogen activation and detoxification:

One important mechanism by which flavonoids may exert their *in vivo* chemopreventive effects is through their inhibition of phase-I drug-metabolizing enzymes, such as cytochrome P450 (CYP), which metabolically activates procarcinogens to reactive intermediates that trigger carcinogenesis (Ren et al., 2003; Schwarz and Roots, 2003).

Another mechanism claimed to be responsible for the chemopreventive activity of polyphenolic compounds is the induction of phase-II metabolizing enzymes such as glutathione Stransferase (GST), NAD(P)H:quinone oxidoreductase (NQO), and UDP-glucuronyltransferase (UGT) (Galati et al.,

2000; Maliakal and Wanwimolruk, 2001), by which carcinogens are detoxified and therefore more readily eliminated from the body.

Cell cycle arrest:

In cancer, normal cell growth and behaviour is lost and alterations in the regulation of cell cycle have been described (Chen et al., 2004b; Thangapazham et al., 2007). Thus, any perturbation of cell cycle specific proteins by dietary polyphenols can potentially affect and block the continuous proliferation of tumorigenic cells. Studies have shown that polyphenols can inhibit different cells at different cell phases such as G1, S, S/G2, and G2 (Gusman et al., 2001). For instance, EGCG or green tea polyphenol treatments induced G1 phase cell cycle arrest through down-regulation of cyclin D, cyclin E, cyclins-dependent kinase (CDK)1, CDK2, CDK4 and proliferating cell nuclear antigen (PCNA) over time in breast and cervical cancer cells (Thangapazham et al., 2007). Similarly, resveratrol was also shown to induce apoptosis, preferentially in cells arrested in the G0/G1 phase (Surh et al., 1999). Therefore, cell cycle arrest can represent a chemopreventive mechanism by subsequent induction of apoptosis.

Induction of apoptosis:

Programmed cell death or apoptosis is a systems approach to get itself rid of defective cells in order to prevent the defect from invading the normal cells. However cancer cells have evolved mechanisms to evade inherent apoptotic signals of the individuals own immune system. Therefore, natural compounds capable of inducing apoptosis in cancer cells are considered potential chemopreventive agents. In this respect, many dietary chemopreventive polyphenols, including quercetin, EGCG, resveratrol, genistein, apigenin, chrysin, curcumin and ellagic acid, evoke their inhibitory effect on carcinogenesis through the induction of apoptosis (Manson et al., 2003; Surh,

2003). Moreover, tumor cells are found to be more sensitive to the apoptotic action of dietary polyphenols than normal cells (Chen et al., 1998; Ahmad et al., 2000b; Park et al., 2005; Feng et al., 2007). For example, quercetin exerted the apoptotic effect in a selective manner by significantly inhibiting the growth of highly aggressive PC-3 and moderately aggressive DU-145 prostate cancer cell lines, while not affecting the poorly aggressive LNCaP prostate cancer cells and normal fibroblasts (Nair et al., 2004). Similarly, green tea polyphenols have been shown to induce a dose-dependent inhibition of cell growth, G0–G1-phase arrest of the cell cycle and induction of apoptosis in human osteosarcoma cells (MG-63 and Saos-2), but not in normal rat osteoblasts (Park et al., 2005).

The Bcl-2 family is the best characterized component of apoptotic signalling pathways and involves two functional groups including antiapoptotic proteins (Bcl-2 and Bcl- XL) and proapoptotic proteins (Bax, Bak and Bad). It is the fine balance of these two functional protein groups that provide the signal for cell survival or cell death which is transmitted to the downstream effector molecules such as intracellular caspases. Sarkar and co-workers have suggested that a downregulation of antiapoptotic Bcl-2 and up-regulation of proapoptotic Bax may be one of the molecular mechanisms by which polyphenol genistein induces apoptosis (Lian et al., 1998). They showed that the ratio of Bax to Bcl-2 was significantly increased after 24 h of treatment, corresponding with a significant increase in apoptotic cells after 48 h of genistein treatment.

In some studies, inhibition of the proteasome chymotrypsin-like activity has been reported to be associated with induction of apoptosis in tumor cells (Lopes et al., 1997). In one such study, apigenin was shown to inhibit proteasome activity and induce apoptosis in human breast cancer MDA-MB-231 cells (Chen et al., 2007). Moreover, in certain circumstances, polyphenol

induced apoptotic death has been shown to occur as a consequence of an inhibited telomerase activity (Yokoyama et al., 2004) and/or with the activation of both apoptotic pathways (extrinsic and intrinsic routes) (Hayakawa et al., 2001; Kawai et al., 2005). Polyphenols have also been suggested to induce apoptosis by inhibiting DNA topoisomerase-II, thereby preventing ligation of DNA double strand breaks (Lynch et al., 2003).

Modulation of cellular signalling pathways:

The cellular signalling pathways that regulate proliferation, survival and transformation of cells are indirectly involved in the regulation of apoptotic cell death. Components of these pathways include several kinases such as mitogen-activated protein kinases (MAPK) and protein kinase C (PKC) which contribute to the maintenance of cell homeostasis. Abnormal activation or silencing of these kinases or their downstream transcription factors can result in uncontrolled cell growth, leading to malignant transformation. Many dietary polyphenolic compounds can effectively suppress tumorigenic signalling *in vitro*. Some of the possible mechanisms by which polyphenols influence these signal transduction pathways are mentioned below.

(a) Suppression of NF-kB transcription factor activation and its nuclear translocation. (Ahmad et al. 1999; Davis et al., 1999; Alhasan et al., 2000; Manna et al., 2000).

(b) Suppression of AP-1 transcription factor activation (Yoshioka et al., 1995; Dong et al., 1997; Yu et al. 2001)

(c) Suppression of Protein Kinases, such as PKC (Protein kinase C), Akt/PKB (Protein Kinase B) and PTK (tyrosine kinases) (Markovits et al., 1989; Liu et al., 1993; Lin et al., 1997; Atten et al., 2001; Banerjee et al., 2007)

(d) Suppression of Mitogen Activated Protein Kinases (MAPK) (Kuo and Yang, 1995; Siddiqui et al., 2004)

(e) Suppression of Growth-Factor Receptor (GFR)-mediated pathways (Masuda et al., 2001; Masuda et al., 2002; Kaneuchi et al., 2003)

Anti-inflammatory action:

Epidemiological and clinical studies have reported an association between inflammation and cancer (Thun et al., 2004) and key signalling molecules like NF-kB and cyclooxygenase-2 (COX-2) are thought to be involved in this process (Lu et al., 2006). Inhibition of COX-2 and blocking the prostaglandin (PG) cascade may have an impact on neoplastic growth and its development by inhibiting proliferation, angiogenesis and metastasis. Flavone was shown to induce apoptosis in human colon carcinoma cells through changes in mRNA levels of COX-2 and NF-kB (Wenzel et al., 2000).

Anti-angiogenic action:

Angiogenesis, the process during which new blood vessels are formed from preexisting ones, can be classified as either physiological or pathological. Physiological angiogenesis provides a driving force for organ development and wound healing, while it is the pathological angiogenesis which occurs during tumor growth at primary and metastatic sites (Folkman, 2007). The signalling pathway governing tumor angiogenesis is exceedingly complex, involving various angiogenic mediators. The major signalling mediators include VEGF, platelet derived growth factor (PDGF), fibroblast growth factors (FGFs), epidermal growth factor, angiopoietins, endothelins, integrins, cadherins, and notch (Gordon et al., 2010). Many plant polyphenols have shown angiogenesis-modulating properties by targeting one or more of these mediators in the signalling pathway (Brakenhielm et al., 2001; Bagli et al., 2004; Fan et al., 2006)

Anti-metastatic action:

Tumor cell invasion and metastasis are interrelated processes involving cell growth, cell adhesion, cell migration, and proteolytic degradation of tissue barriers such as the extracellular matrix and basement membrane. Many proteolytic enzymes, including MMPs (chiefly MMP-2 and MMP-9) (Jiang et al., 2001; Sternlicht and Werb, 2001) and intercellular adhesion molecule (ICAM), participate in the degradation of these barriers (Kleiner and Stetler-Stevenson, 1993; Aimes and Quigley, 1995). Several polyphenolic compounds have been shown to inhibit tumor cell invasion and metastasis by targeting one or more molecules such as the MMPs and the CAMs (Hung et al., 2005; Yang et al., 2005; Piantelli et al., 2006; Zhen et al., 2006)

Prooxidant action:

It is generally understood that antioxidants counteract ROS production and inhibit the oxidative DNA damage and therefore reduce the risk of cancer. Notwithstanding these observations, growing experimental evidence suggests that polyphenolic antioxidants themselves mediate production of ROS (prooxidant action) which may be responsible for their ability to induce apoptosis of cancer cells (Hadi et al., 2007; Qian et al., 2009). Dietary polyphenols have been shown to exhibit both prooxidant and antioxidant activities. They act as prooxidants in systems containing redox-active metals, such as copper (Cu) and iron (Fe) that catalyze their redox cycling, leading to the formation of ROS and phenoxyl radicals that can damage DNA, lipids, and other biological molecules (Ahmad et al., 1992; Li and Trush, 1994; Decker, 1997).

The anticancer properties of polyphenols are generally believed to reflect their ability to scavenge endogenous ROS. However, the prooxidant action of plant-derived polyphenolic compounds rather than their antioxidant action

may be an important mechanism for their anticancer and apoptosis-inducing properties (Michels et al. 2005), as ROS can mediate apoptotic DNA fragmentation (Hadi et al., 2000; Shen et al. 2004; Noda et al. 2007). The antioxidant properties of dietary polyphenolics may only partly explain their antitumor promotion effects, as ellagic acid is 10 times more potent an antioxidant than tannic acid. However, tannic acid was more effective than ellagic acid in inhibiting the promotion of skin tumor by 12-O-tetradecanoyl phorbol-13-acetate (Gali et al., 1992). It was suggested that the antioxidant effects of these polyphenols might be essential but not sufficient for their antitumor promotion. In any case ROS scavenging properties of plant polyphenols may account for their chemopreventive effects but not for any therapeutic action against cancer cells (Radin, 2003).

Further, it may be mentioned that the apoptotic DNA fragmentation properties of several anticancer drugs are considered to be mediated by ROS (Kaufmann, 1989; Turella *et al.* 2005; Kim *et al.* 2006). Certain properties of dietary polyphenolic compounds, such as binding and cleavage of DNA and the generation of ROS in the presence of transition metal ions (Rahman et al., 1990), are similar to those of known anticancer drugs. A putative mechanism for anticancer and apoptosis-inducing properties of plant derived dietary polyphenolic compounds, which involves mobilization of intracellular copper and consequent prooxidant action, has been proposed (Hadi et al., 2000). Compared with normal cells, preneoplastic cells and neoplastic cells have been shown to contain elevated levels of copper (Gupte and Mumper, 2008) and may be more sensitive to electron transfer with polyphenols to generate ROS. Therefore DNA damage induced by polyphenols in the presence of Cu(II) may be an important pathway through which preneoplatic cells and neoplastic cells can be killed while normal cells may survive (Figure 5).

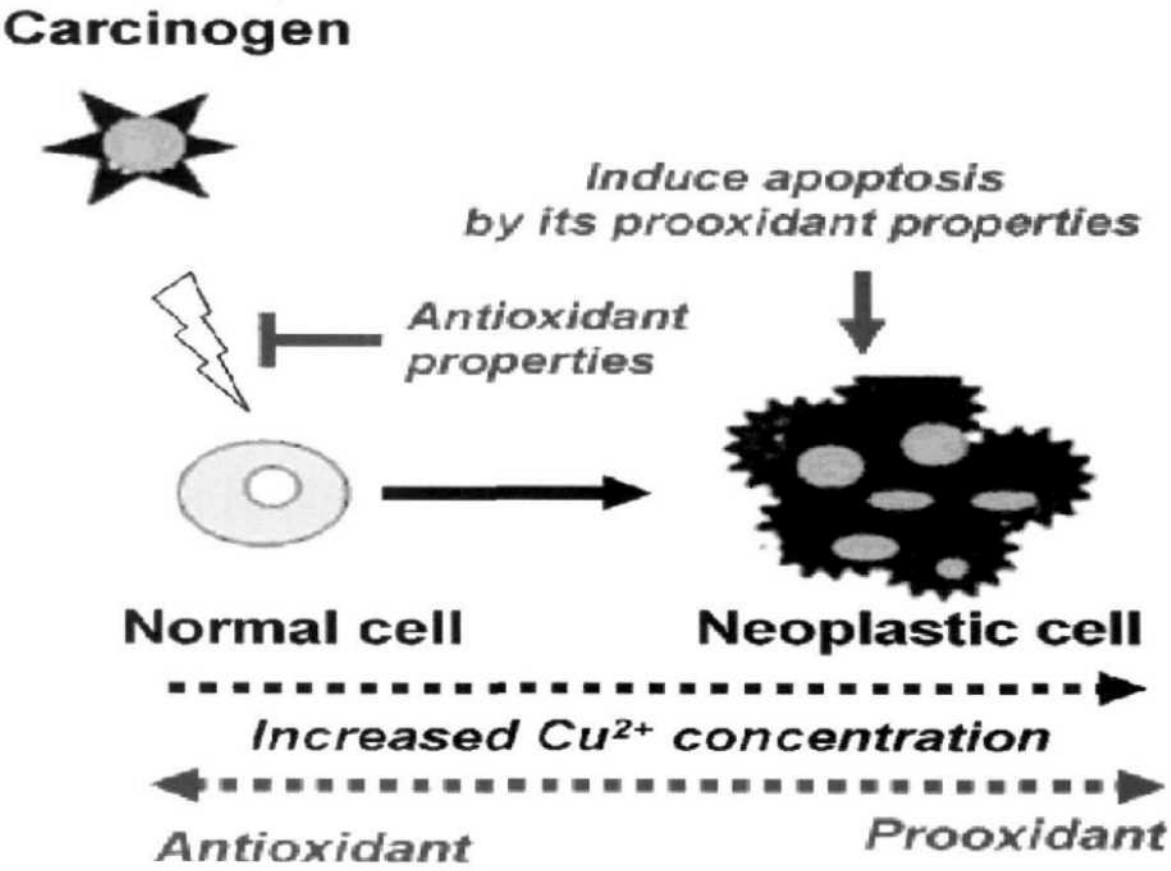


Figure 5: Possible mechanism for anticancer effects of polyphenolic compounds (from Qian et al., *J Med Chem* 2009; 52(7): 1963-1974).

Scope of the work presented

SCOPE OF THE WORK PRESENTED

As mentioned in the previous section, there is evidence in literature suggesting that antioxidant activity of such plant derived polyphenols may not fully account for their chemopreventive effects. Therefore, it is likely that other mechanisms may be responsible for the varied pharmacological properties. Most antioxidants of plant origin are redox (reduction-oxidation) agents, protecting against ROS in some cases and promoting radical generation in others (Herbert, 1996). Studies in this laboratory have shown that plant polyphenols behave as prooxidants in the presence of copper ions catalyzing DNA breakage through the generation of ROS (Ahmad et al., 1992; Bhat & Hadi, 1994; Ahsan & Hadi, 1998; Ahmad et al., 2000a; Azam et al., 2004; Ahmad et al., 2005). Copper is a major metal ion present in the nucleus and is also implicated in tumorigenesis and angiogenesis (Chevion, 1988). Oxidative DNA breakage by these compounds correlates with their apoptosis inducing capacity. Further, properties of polyphenols, such as binding and cleavage of DNA and the generation of ROS in the presence of transition metal ions are similar to those of some known anticancer drugs (Ehrenfeld et al., 1987). It is also known that serum, tissue and cellular copper levels are significantly elevated in a number of malignancies (Linder, 1991; Gupte and Mumper, 2008). Induction of apoptosis by several polyphenols has been shown in various human cancer cell lines and animal models (Lee et al., 2002; Sarkar and Li, 2003; Whitsett et al., 2006; Moiseeva et al., 2007; Noda et al., 2007;Scarlatti et al., 2007). Interestingly, some studies have indicated that apoptosis induction by polyphenols and other anticancer agents is independent of caspases and mitochondria (Piwocka et al., 1999; Leist and Jaattela, 2001) and is accompanied by an increase in the intracellular levels of ROS (Yoshino et al., 2004; Heiss et al., 2007; Noda et al., 2007).

Thus, there is significant data in literature that points to the prooxidant rather than the antioxidant property of polyphenols as the mechanism of their anticancer properties. Taking into consideration our own observations and those of others this laboratory has proposed a mechanism according to which plant polyphenols mobilize endogenous copper in cancer cells leading to cytotoxic action through the generation of ROS (Hadi et al., 2000; Hadi et al., 2007). In confirmation of this hypothesis, the following milestones have been achieved, (i) an *in vitro* reaction between polyphenols, Cu(II) and DNA leading to DNA cleavage has been characterised (Ahmad et al., 2000a; Azam et al., 2004); (ii) as a further step it has been shown that polyphenol-Cu(II) system is indeed capable of causing DNA degradation in a cellular system (Azmi et al., 2005); (iii) it has also been shown that polyphenols are capable of mobilizing endogenous copper ions from cells leading to cellular DNA breakage (Azmi et al. 2006); (iv) further, in the above oxidative cellular DNA breakage nuclear copper has been demonstrated to be mobilized (Shamim et al. 2008); (v) recently, isoflavone genistein induced growth inhibition in breast cancer cell lines has been shown to be inhibited by copper chelator to a significant extent whereas iron and zinc chelators proved to be relatively ineffective (Ullah et al. 2011).

Based on the above hypothesis, in the work presented here, I have attempted to further elucidate and confirm the mechanism of action of plant derived polyphenolic compounds which may be responsible for their anticancer effects. The studies have been carried out using several different types of polyphenols such as the flavones apigenin, luteolin and chrysin; the catechin EGCG; the isoflavone genistein; and the stilbene resveratrol. The work has been divided into three chapters. In the first chapter, absorption and fluorescence studies have been employed to explore the DNA binding and copper reducing abilities of the flavones- apigenin, luteolin and chrysin. Further, using single cell gel electrophoresis (comet assay), the ability of these

flavones to induce DNA breakage in intact as well as permeabilized cells has also been tested. The second chapter deals with studies done on rats by orally administering them with copper and measuring the copper levels in the isolated lymphocytes of such rats. Subsequently, isolated rat lymphocytes have been subjected to treatment with polyphenols *in vitro* and cellular DNA breakage has been analyzed (Khan et al., 2011). Finally, in the third chapter, the effect of different metal specific sequestering agents and the scavengers of ROS such as SOD, catalase and thiourea on the polyphenol-induced antiproliferation and apoptotic cell death in breast, prostate and pancreatic cancer cell lines has been studied.

Materials & Methods

MATERIALS

Chemicals	Source
Agarose	Sigma Chemical Co., USA
Apigenin	Sigma Chemical Co., USA
Bathocuproine disulphonic acid	Sigma Chemical Co., USA
Bovine Serum Albumin	Sigma Chemical Co., USA
Catalase	Sigma Chemical Co., USA
Chrysin	Sigma Chemical Co., USA
Deoxyribonucleic acid (Calf Thymus Type I)	Sigma Chemical Co., USA
Desferrioxamine mesylate	Sigma Chemical Co., USA
Diphenylamine	BDH, India
Dimethyl sulphoxide (DMSO)	Qualigens, India
Epigallocatechin-3-gallate (EGCG)	Sigma Chemical Co., USA
Ethidium Bromide (EtBr)	Sigma Chemical Co., USA
Ethylenediaminetetraacetic acid (EDTA)	Qualigens, India
Genistein	Sigma Chemical Co., USA
Histidine	Sisco Research Lab, India
Histopaque 1077	Sigma Chemical Co., USA
Low melting point agarose	Sigma Chemical Co., USA
Luteolin	Sigma Chemical Co., USA
Neocuproine	Sigma Chemical Co., USA
Nitroblue Tetrazolium (NBT)	Sisco Research Lab, India
Phosphate Buffered Saline (Ca^{2+}&Mg^{2+}free)	Sigma Chemical Co., USA
RPMI 1640 media	Sigma Chemical Co., USA

Resveratrol	Sigma Chemical Co., USA
Single strand specific nuclease	Sigma Chemical Co., USA
Sodium chloride	Qualigens, India
Supercoiled plasmid pBR322 DNA	Genei, India
Superoxide dismutase (SOD)	Sigma Chemical Co., USA
Thiourea	E. Merck, Germany
Tris(hydroxy methyl)aminomethane	Qualigens, India
Triton X – 100	Qualigens, India

All other chemicals were commercial products of analytical grade.

METHODS

Preparation of stock solutions of various polyphenols:

For apigenin, luteolin and chrysin, stock solutions of 2.0 mM were prepared in dimethyl sulphoxide (DMSO). EGCG and resveratrol were dissolved in 3.0 mM cold NaOH before use as fresh stocks of 1.0 mM. Fresh solution of genistein was prepared as a stock of 1.0 mM in absolute methanol. For studies with cancer cell lines DMSO was used as solvent. The solvent controls containing the highest percentage of the solvent used in the studies were tested in each experiment. No difference was observed with or without solvent, indicating that the solvents at the tested concentrations did not influence the results. Upon addition to reaction mixtures, in the presence of buffers mentioned and at concentrations used, all the polyphenols used remained in solution. The volumes of stock solution added did not lead to any appreciable change in the pH of reaction mixtures.

Absorption studies:

The absorption spectra were obtained by using Beckman DU-40 spectrophotometer (USA) equipped with a plotter.

Spectrophotometric study for flavone-Cu(II) interaction:

The effect of increasing concentrations of Cu(II) on absorption spectra of luteolin, apigenin and chrysin was observed. The reaction mixture (3.0 ml) contained 10 mM Tris-HCl (pH 7.5), 50 µM isoflavone and increasing concentrations of Cu(II). The spectra were recorded immediately after addition of all components.

Spectrophotometric detection of Cu(II) reduction by luteolin, apigenin and chrysin:

The selective sequestering agent bathocuproine was employed to detect reduction of Cu(II) to Cu(I) by recording the formation of bathocuproine-Cu(I) complex which absorbs maximally at 480 nm. The reaction mixture (3.0 ml) contained 3.0 mM Tris–HCl (pH 7.5), fixed concentrations (100 µM) of Cu(II) and Cu(I) (for positive control), bathocuproine (300 µM) and of flavones (apigenin, luteolin or chrysin) (50 µM). The reaction was started by adding Cu(II) and the spectra were recorded immediately afterwards.

Detection of superoxide anion generation by flavones:

Superoxide ($O_2^{\cdot-}$) was detected by the reduction of nitroblue tetrazolium (NBT) essentially as described by Nakayama et al. (1983). A typical assay mixture contained 50 mM sodium phosphate buffer (pH 7.5), 33 µM NBT, 100 µM EDTA and 0.06 % triton X-100 in a total volume of 3.0 ml. The reaction was started by the addition of flavones (apigenin/chrysin/luteolin). After mixing, absorbance was recorded at 560 nm at different time intervals, against a blank, which did not contain the compound.

Detection of hydroxyl radical generation by flavones:

In order to compare the hydroxyl radical production by increasing concentrations of luteolin, chrysin and apigenin in the presence of 50 µM Cu(II), the method of Quinlan and Gutteridge (1987) was followed. Calf thymus DNA (100 µg) was used as a substrate and the malondialdehyde generated from deoxyribose radicals was assayed by recording the absorbance at 532 nm.

Flourescence studies:

The fluorescence studies were performed on a Shimadzu spectrofluorophotometer RF-5000 (Japan) equipped with a plotter and a calculator. Luteolin, apigenin and chrysin were excited at their absorption maxima (λ_{max}) of 270 nm, 272 nm and 273 nm, respectively. Emission spectra were recorded in the wavelength range shown in figures.

Treatment of pBR322 DNA with flavones in the presence of Cu(II):

Reaction mixture (30µl) contained 10mM Tris-HCl (pH 7.5), 0.5µg of plasmid DNA and other components as indicated in legends. Incubation was performed at 37°C for 2 hours. After incubation, 10 µl of solution containing 40 mM EDTA, 0.05 % bromophenol blue (tracking dye) and 50 % (v/v) glycerol was added and the solution was subjected to electrophoresis in submarine 1 % agarose gel. The gel was stained with ethidium bromide (0.5 µg/ml), viewed and photographed on a UV-transilluminator.

Reaction of flavones with calf thymus DNA and digestion with single strand specific nuclease:

Single strand specific digestion was performed as described by Wani and Hadi (1979). Reaction mixtures (0.5 ml) contained 10 mM Tris-HCl (pH 7.5), 500 µg of calf thymus DNA and varying amounts of apigenin/luteolin/chrysin and cupric chloride (50µM). All solutions were sterilized before use. Incubation was performed at 37°C for one hour. The assay determines the acid soluble nucleotides released from DNA as a result of enzyme digestion. Reaction mixture in a total volume of 1.0 ml contained 40 mM Tris-HCl (pH 7.5), 1 mM Magnesium Chloride, water and enzyme. The reaction mixture was incubated at 37°C for 2 hours. The reaction was stopped by adding 0.2 ml bovine serum albumin (10 mg/ml) and 1.0 ml of 14% perchloric acid (chilled). The tubes

were immediately transferred to 0°C for 45 min before centrifugation at 2500 rpm for 10 min at room temperature to remove undigested DNA and precipitated protein. Acid soluble deoxyribonucleotides were determined in the supernatant, colorimetrically, using the diphenylamine method (Schneider, 1957). To a 1.0 ml aliquot, 2.0 ml diphenyl reagent (freshly prepared by dissolving 1 gram of recrystallized diphenylamine in 100 ml glacial acetic acid and 2.75 ml of concentrated H_2SO_4) was added. The tubes were heated in a boiling water bath for 30 min. The intensity of blue colour was read at 600 nm.

Evaluation of polyphenol-induced DNA breakage in human peripheral lymphocytes by alkaline single cell gel electrophoresis (Comet Assay):

Isolation of lymphocytes

Heparinized blood samples (2 ml) from a single, healthy, non-smoking donor was obtained by venepuncture and diluted suitably in Ca^{++} and Mg^{++} free PBS. Lymphocytes were isolated from blood using Histopaque 1077 (Sigma Diagnostics, St Louis, USA), and the cells were finally suspended in RPMI 1640.

Viability assessment of lymphocytes

The lymphocytes were checked for their viability before the start and after the end of the reaction using Trypan Blue Exclusion Test (Pool-Zobel et al., 1993). The viability of the cells was found to be greater than 93 %.

Comet Assay

Comet assay was performed under alkaline conditions essentially according to the procedure of Singh et al. (1988) with slight modifications. Fully frosted microscopic slides precoated with 1.0% normal melting agarose at about 50ºC (dissolved in Ca^{++} and Mg^{++} free PBS) were used. Around 10 000 cells were

mixed with 75 µl of 2.0% LMPA to form a cell suspension and pipetted over the first layer and covered immediately by a coverslip. The agarose layer was allowed to solidify by placing the slides on a flat tray and keeping it on ice for 10 min. The coverslips were removed and a third layer of 0.5% LMPA (75 µl) was pipetted and coverslips placed over it and kept on ice for 5 min for proper solidification of layer. The coverslips were removed and the slides were immersed in cold lysing solution containing 2.5M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, and 1% Triton X-100 added just prior to use for a minimum of 1 h at 4ºC. After lysis DNA was allowed to unwind for 30 min in alkaline electrophoretic solution consisting of 300 mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was performed at 4ºC in a field strength of 0.7 V/cm and 300 mA current. The slides were then neutralized with cold 0.4M Tris, pH 7.5, stained with 75 µl Ethidium Bromide (20 µg/ml) and covered with a coverslip. The slides were placed in a humidified chamber to prevent drying of the gel and analyzed the same day. Slides were scored using an image analysis system (Komet 5.5, Kinetic Imaging, Liverpool, UK) attached to a Olympus (CX41) fluorescent microscope and a COHU 4910 (equipped with a 510–560 nm excitation and 590 nm barrier filters) integrated CC camera. Comets were scored at 100X magnification. Images from 50 cells (25 from each replicate slide) were analyzed. The parameter taken to assess lymphocytes DNA damage was tail length (migration of DNA from the nucleus, µm) and was automatically generated by Komet 5.5 image analysis system.

Treatment of intact lymphocytes with flavones and the subsequent Comet assay was performed essentially as described earlier (Azmi et al., 2005). However, since the DNA breakage in intact cells had to be compared with that in permeabilized cells, the treatment of cells was done on slides rather than in eppendorf tubes. Therefore, the lysis of cells was carried out after the flavone treatment. The other conditions remained the same as described above.

Treatment of lymphocytes with flavones was also carried out using permeabilized cells (Czene et al., 1997). For this purpose lymphocytes prior to treatment with flavones, were exposed to the permeabilization solution (0.5% Triton X-100 in 0.004 M Tris-HCl, pH 7.4) for 10 min on ice. For treatment each slide was then transferred to a rectangular dish (8cm × 3cm× 5mm) which contained a reaction mixture of luteolin/apigenin/chrysin and other additions as mentioned in the legends to figures. The slides with the reaction mixture were incubated at 37^{0}C for desired time periods (1 hour for intact cells / 30 minutes for permeabilized cells) and were then washed twice by placing in 0.4 M phosphate buffer (pH 7.5) for 5 min at room temperature before being processed further for Comet assay.

Statistical Analysis

The statistical analysis was performed as described by Tice et al (2000)and is expressed as ±S.E.M. of three independent experiments. A student's t-test was used to examine statistically significant differences. Analysis of variance was performed using ANOVA. P values <0.05 were considered statistically significant.

Studies on animals:

Adult male Wistar rats weighing between 190 and 220 g were used in the study. The rats were housed in isopropelyene cages and acclimatized for a period of one week to laboratory conditions (23 ± 2 °C and 60% humidity). They received a commercial standard diet (Ashirwad Industries, Chandigarh, India) and water ad libitum. After acclimatization, the rats were randomly divided into two equal groups and henceforth, identified as control and test groups. All the animal studies were carried out in compliance with the international practices for animal use according to the guidelines of

Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

Cupric chloride was administered orally to rats from the test group in order to raise their intracellular status of copper. Rats from the control group were gavaged with only drinking water. Twelve hours post-gavaging, blood was drawn from control as well as copper-administered rats by cardiac puncture and kept in heparinised tubes.

Isolation of rat lymphocytes

Heparinized blood samples (2.0 ml) from both untreated rats and their copper-overloaded counterparts were diluted suitably in Ca^{++} and Mg^{++} free PBS. Lymphocytes were then isolated from the blood using Histopaque 1077 (Sigma) and the isolated cells (2×10^6) were subsequently suspended in RPMI 1640.

Viability assessment of rat lymphocytes

The isolated rat lymphocytes were checked for their viability before the start and after the end of the reaction using Trypan Blue Exclusion test (Pool-Zobel et al., 1993). The viability of the cells was found to be almost 94%.

Measurement of copper in plasma and lymphocytes

3.9 ml of nitric acid (2.5%) was added to aliquots of 100 µl plasma/lymphocytes and vortexed. The solutions were then kept at 37°C for 5 hours with regular shaking. The mixture was centrifuged at 3000 rpm for 5 min. Copper was measured in the clear supernatant by means of flame atomic absorption spectrophotometry (FAAS) (Varian Spectra 200 FS, Varian Inc, California, USA) (hollow cathode lamp, Flame type: Air acetylene; replicate 3; wavelength 324.8 nm) as described (US EPA, 1994). Plasma and cellular

copper levels of each animal were measured and a mean value was determined for the whole group.

Treatment of rat lymphocytes with polyphenols

Isolated lymphocytes were exposed to specified concentrations of polyphenols EGCG, genistein and resveratrol in a total reaction volume of 1.0 ml. Incubation was performed at 37°C for 1 hour. In some experiments, lymphocytes were pre-incubated with various concentrations of different metal chelators prior to being treated with polyphenols. In another set of experiments, scavengers of ROS were added to the reaction mixture containing polyphenol at the final concentrations indicated. After incubation, the reaction mixture was centrifuged at 4000 rpm, the supernatant was discarded and pelleted lymphocytes were resuspended in 100 µl of PBS and processed further for Comet assay (as discussed above).

Cell lines and Reagents:

Immortalized non-transformed breast cell line MCF-10A and cancer lines, MDA-MB-231, MDA-MB-468, BxPC-3, PC3 and C42B were obtained from ATCC (Manassas, VA). MDA-MB-231 and BxPC-3 cell lines were maintained in DMEM (Invitrogen,Carlsbad, CA) while MDA-MB-468, PC3 and C42B cells were maintained in RPMI (Invitrogen,Carlsbad, CA). Both of these media were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 µg/mlstreptomycin. All cells were cultured in a 5%CO_2 - humidified atmosphere at 37°C. Stock solution of apigenin, luteolin, EGCG and resveratrol (50mM) were made in DMSO and small aliquots were stored at -20°C. The stock solutions of various chelators of metal ions - Neocuproine/ Desferoxamine mesylate/ Histidine were made in PBS at a final concentration of 50 mM and were always made fresh just prior to experiments.

Normal breast epithelial cell line, MCF-10A, was propagated in DMEM/F12 (Invitrogen) supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 0.1 µg/ml cholera toxin, 10 µg/ml insulin, 100 units/ml penicillin, and 100 µg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. MCF-10A+Cu cells are MCF-10A cells that were cultured in their normal culture media (above) with additional supplementation of 25µM $CuCl_2$ for at least 4 weeks.

Cell Growth Inhibition Studies by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay:

Cells were seeded at a density of 2 x 10^3 cellsper well in 96-well microtiter culture plates. After overnightincubation, normal growth medium was removed and replaced with a freshmedium containing DMSO (vehicle control) or different concentrations of respective polyphenolic compound diluted from a 50 mM stock. Various chelators were added in individual assays as mentioned in respective experiments.After 3 days of incubation, 25 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5mg/ml in PBS) was added to each welland incubated further for 2 h at 37°C. Upon termination, the supernatantwas aspirated and the MTT formazan, formed by metabolically viablecells, was dissolved in isopropanol (100µl) by mixing for 30 min on a gyratory shaker. The absorbancewas measured at 595 nm on Ultra Multifunctional Microplate Reader (TECAN, Durham, NC). Each treatment had eight replicate wells and the amount of DMSO in reaction mixture never exceeded 0.1%. Moreover, each experiment was repeated at least three times.

Histone/DNA ELISA for Detection of Apoptosis:

The CellDeath Detection ELISA Kit (Roche, Palo Alto, CA) was used todetect apoptosis in cancer cells treated with different polyphenols, according to the

manufacturer's protocol. Briefly, cells were treated with polyphenolic compounds or DMSO control for a time period of 72 hours. After treatment, the cytoplasmic histone/DNA fragments from cellswere extracted and incubated in the microtiter plate modulescoated with anti-histone antibody. Subsequently, peroxidase-conjugatedanti-DNA antibody was used for the detection of immobilizedhistone/DNA fragments followed by color development with ABTSsubstrate for peroxidase. The spectrophotometric absorbanceof the samples was determined by using Ultra MultifunctionalMicroplate Reader (TECAN) at 405 nm.

Soft Agar Colonization Assay:

Cancer cells (3×10^4) were plated in 0.5 ml of culture medium containing 0.3% (w/v) top agar layered over a basal layer of 0.7% (w/v) agar (with culture medium and the supplements) in 24-well plates. At the time of seeding, the culture was supplemented withdifferent polyphenols or DMSO, with or without copper chelator. After appropriate culture time (22 days),colonies (>50 cells) were counted. Experiments were carried out in quadruplicate and mean values are reported.

Western Blotting:

For Western blot analysis, cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 8.0, 137mM NaCl, 1% NP-40, 2mM EDTA, 0.5% sodium deoxycholate and 0.1% SDS), containing complete mini EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktails 1 and 2 (Sigma-Aldrich). Protein concentration was measured by BCA Protein Assay (Pierce, Rockford, IL). After the resolution of proteins on 12% polyacrylamide gels under denaturing conditions and transfer to nitrocellulose membranes, appropriate primary antibodies (anti-Ctr1) were added. This was followed by incubation with horseradish peroxidase-conjugated secondary antibody. Proteins were

visualized using the chemiluminescence detection system (Pierce). For re-probing, membranes were incubated for 30 min at 50°C in buffer containing 2% SDS, 62.5 mM Tris (pH 6.7), and 100 mM β-mercaptoethanol, washed and incubated with desired primary followed by secondary antibodies and the signals were detected as described above.

Detection of ROS in cancer cells treated with polyphenol:

The Image-iT LIVE Green ROS Detection Kit (Molecular Probes, Life Technologies, USA) was used to detect the generation of ROS in MDA-MB-231 breast cancer cells treated with polyphenol using the manufacturer's protocol. Briefly, cells were first seeded on slides using Lab-Tek II Chamber Slide System (Nalge Nunc International, Naperville, IL, USA). 75 µM compound was layered on to cells for one hour. Cells were washed twice in PBS and layered with 25 µM carboxy-H_2DCFDA for 30 min in dark. Cells were counter stained Hoechst 33342 at a final concentration of 1 µM at the last five minutes of the 30 min incubation with carboxy-H_2DCFDA. The cells were washed again with PBS and mounted in warm buffer before visualization using a Leica TCS SP5 confocal microscope.

Statistical analysis:

Results are expressed as mean ±S.E. of at least three independent observations. Student's t-test was used to examine statistically significant differences. Analysis of variance was performed using ANOVA. p-values < 0.05 were considered statistically significant.

Chapter - I

DNA binding and its degradation by flavones (luteolin, apigenin & chrysin) in the presence of copper ions

RESULTS-I

Formation of polyphenol-Cu(II) complex:

The possibility for the formation of polyphenol- Cu(II) complex was examined. This was carried out by recording the absorption spectra of luteolin, apigenin and chrysin with increasing concentrations of Cu(II). The results given in figures 6, 7 and 8 show that the addition of Cu(II) to luteolin, apigenin and chrysin results in an enhancement in the absorption peak appearing at their respective λ_{max}. The absorption spectra of these polyphenols in the presence of copper suggests a simple mode of interaction between them and Cu(II).

Formation of complexes involving calf thymus DNA and polyphenolic compounds:

Figures 9, 10 and 11 show the effect of addition of increasing molar base pair ratios of calf thymus DNA on the fluorescence emission spectra of luteolin, apigenin and chrysin at 560 nm, 545 nm and 540 nm, respectively. Such an addition resulted in a progressive enhancement of the fluorescence. There was however, no significant shift in the λ_{max}-emission suggesting a simple mode of binding between DNA and these flavones. When DNA alone was excited at the respective excitation wavelengths (270 nm for luteolin, 272 nm for apigenin and 273 nm for chrysin), no interference with the fluorescence emission spectra of flavone alone/flavone + DNA was found, thus confirming the binding results.

Binding of copper ions to polyphenols:

Binding of copper ions to luteolin, apigenin and chrysin was studied by the effect of increasing Cu(II) molar ratios on the fluorescence emission spectra of these flavones. The results shown in figures 12, 13 and 14 clearly indicate the binding as addition of Cu(II) causes progressive quenching of flavone

fluorescence. These results support the results of absorption studies shown in figures 6, 7 and 8, where formation of polyphenol-copper complex was demonstrated.

Detection of polyphenol induced Cu(I) production by bathocuproine:

The production of Cu(I), formed as a result of reduction of Cu(II) by luteolin/apigenin/chrysin, was analyzed using bathocuproine which is a selective Cu(I) sequestering agent that binds specifically to the reduced form of copper, i.e. Cu(I), but not to the oxidized form (Simpson et al., 1992). The Cu(I)-chelates exhibit an absorption maximum at 480 nm. As shown in figure 15, Cu(II) does not interfere with the maxima, whereas luteolin + Cu(II), apigenin + Cu(II) and chrysin + Cu(II) react to generate Cu(I) which complexes with bathocuproine to give a peak appearing at 480 nm. The results show that these flavones are able to reduce Cu(II) to Cu(I) and contribute to the redox cycling of the metal.

Generation of oxygen radicals by polyphenols:

Superoxide production: The production of superoxide anion was determined by the method of Nakayama et al. (1983), which involves reduction of NBT by luteolin/apigenin/chrysin to a formazan. The time dependent generation of superoxide anion by these flavones, as evidenced by the increase in absorbance at 560 nm, is shown in figure 16. The fact that NBT was genuinely assaying superoxide was confirmed by SOD (100 µg/ml) inhibiting the reaction (results not shown). It is known that superoxide undergoes automatic dismutation at neutral pH to form H_2O_2 which in the presence of transition metals such as copper favours Fenton type reaction to generate hydroxyl radicals which could act as a proximal DNA cleaving agent leading to oxidative DNA breakage.

Hydroxyl radical generation: It has been previously shown that during the reduction of Cu(II) to Cu(I), reactive oxygen species such as hydroxyl radicals are formed which serve as the proximal DNA cleaving agent (Rahman et al., 1989). Therefore, the capacity of luteolin, apigenin and chrysin to generate hydroxyl radicals in the presence of Cu(II) was examined. The assay is based on the fact that degradation of DNA by hydroxyl radicals results in the release of TBA (2-thiobarbituric acid) reactive material, which forms a colored adduct with TBA whose absorbance is read at 532 nm (Quinlan and Gutteridge, 1987). The results given in figure 17 clearly show that increasing concentrations of the flavones lead to a progressive increase in the formation of hydroxyl radicals.

Cleavage of plasmid pBR322 DNA by luteolin/apigenin/chrysin:

In order to examine the efficacy of flavone-Cu(II) system in DNA cleavage, as shown in figure 18, I have tested the ability of luteolin, apigenin and chrysin to cause cleavage of supercoiled plasmid pBR322 DNA in the presence of copper ions. As can be seen from the ethidium bromide stained agarose gel pattern, apigenin/luteolin/chrysin alone show generation of only some open circular form of plasmid DNA. However, addition of copper to these flavones resulted in the generation of greater open circular as well as some linearized (in case of luteolin) forms of plasmid DNA, demonstrating that flavones are capable of plasmid DNA cleavage in the presence of copper ions.

Breakage of calf thymus DNA by polyphenols in the presence of Cu(II) :

Apigenin, luteolin and chrysin in the presence of Cu(II) were found to generate single strand specific nuclease sensitive sites in calf thymus DNA. The reaction was assessed by recording the proportion of DNA converted to acid soluble-nucleotides by the nuclease. Table IV gives the dose response data of such a reaction. However, apigenin, luteolin and chrysin in the absence of Cu(II) did

not show appreciable degradation of calf thymus DNA. Control experiments (data not shown) established that heat denatured DNA underwent 100 % hydrolysis following the treatment with nuclease. In the presence of Cu(II) (50 μM), increasing concentrations of apigenin, luteolin and chrysin resulted in an increase in nuclease sensitive sites in DNA leading to increased DNA hydrolysis.

DNA breakage by polyphenol-Cu(II) system in lymphocytes as measured by Comet Assay:

Increasing concentrations of luteolin, apigenin and chrysin [0-50 μM] either alone or in the presence of 50 μM $CuCl_2$ were tested for DNA breakage in isolated human peripheral lymphocytes using the Comet Assay (Figure 19). The corresponding tail length is plotted as a function of polyphenol concentration. It can be seen in figure 19 that whereas all the three flavones alone were able to cause some breakage of cellular DNA, the degree of such breakage was significantly enhanced in the presence of Cu(II). Cu(II) [50 μM) controls were similar to untreated lymphocyte without any significant DNA breakage. The results clearly establish that flavone-Cu(II) system is capable of DNA breakage in a biological system such as isolated lymphocytes.

A comparison of DNA breakage induced by polyphenols in intact lymphocytes and permeabilized lymphocytes as measured by Comet Assay:

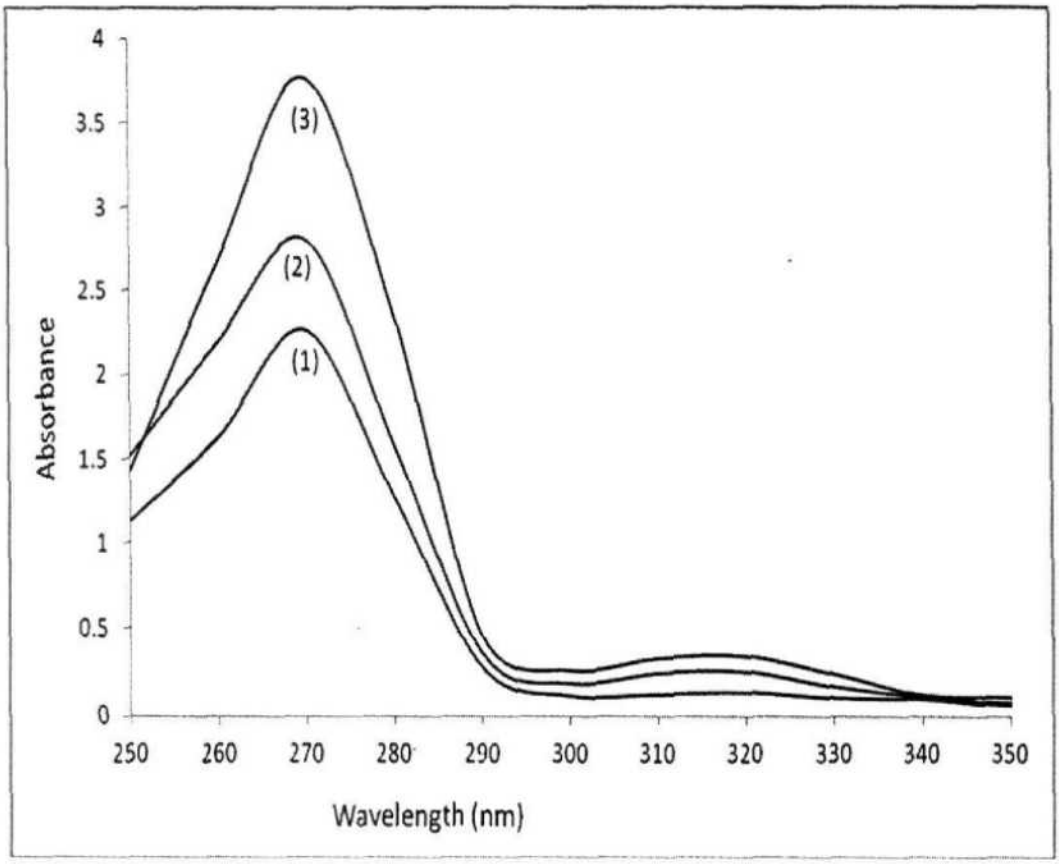
As shown in the previous experiment (Figure 19) flavones luteolin, apigenin and chrysin are able to cause single strand breaks in a cellular system and that the degree of such DNA breakage is greater in the presence of copper. In the present experiment (Figure 20), increasing concentrations [0-50 μM] of luteolin, apigenin and chrysin are tested for DNA breakage in permeabilized lymphocytes and are compared with that observed in intact lymphocytes. Use of permeabilized lymphocytes allows the direct interaction of flavones with

cell nuclei at physiological pH. It also eliminates the need to first lyse the cells at alkaline pH and then resuspend them at neutral pH. Thus, considerably greater DNA breakage should be observed in permeabilized lymphocytes as compared to the intact cells. Figure 20 shows that the rate of comet tail formation induced by all the three polyphenols is indeed greater in the case of permeabilized lymphocytes suggesting that these compounds are able to interact with the cell nuclei when a permeabilized system is used. However, in both intact and permeabilized cells, the rate of DNA breakage is greater in the case of luteolin, followed by apigenin and chrysin.

Effect of neocuproine and bathocuproine on polyphenol induced DNA breakage in intact lymphocytes and permeabilized lymphocytes:

In the experiment shown in figure 21, the DNA breakage induced by luteolin in intact lymphocytes and permeabilized lymphocytes has been assessed in the presence of Cu(I) specific chelators neocuproine and bathocuproine. Incubation of lymphocytes with neocuproine (a cell membrane permeable copper chelator) inhibited luteolin induced DNA degradation in intact lymphocytes. Bathocuproine disulphonate (the water soluble membrane impermeable analog of neocuproine) which is unable to permeate through the cell membrane did not cause such inhibition (Figure 21 A). This study has further shown (Figure 21 B) that luteolin is able to degrade DNA in permeabilized cells and that such DNA degradation is inhibited by neocuproine as well as bathocuproine disulphonate (both of which are able to permeate the nuclear pore complex), suggesting that nuclear copper is mobilized in this reaction. These results indicate that oxidative DNA breakage by flavones involve mobilization of chromatin bound copper and that Cu(I) is an intermediate in the pathway that leads to DNA breakage.

Figure 6: Absorption spectra of luteolin in the presence of Cu (II)



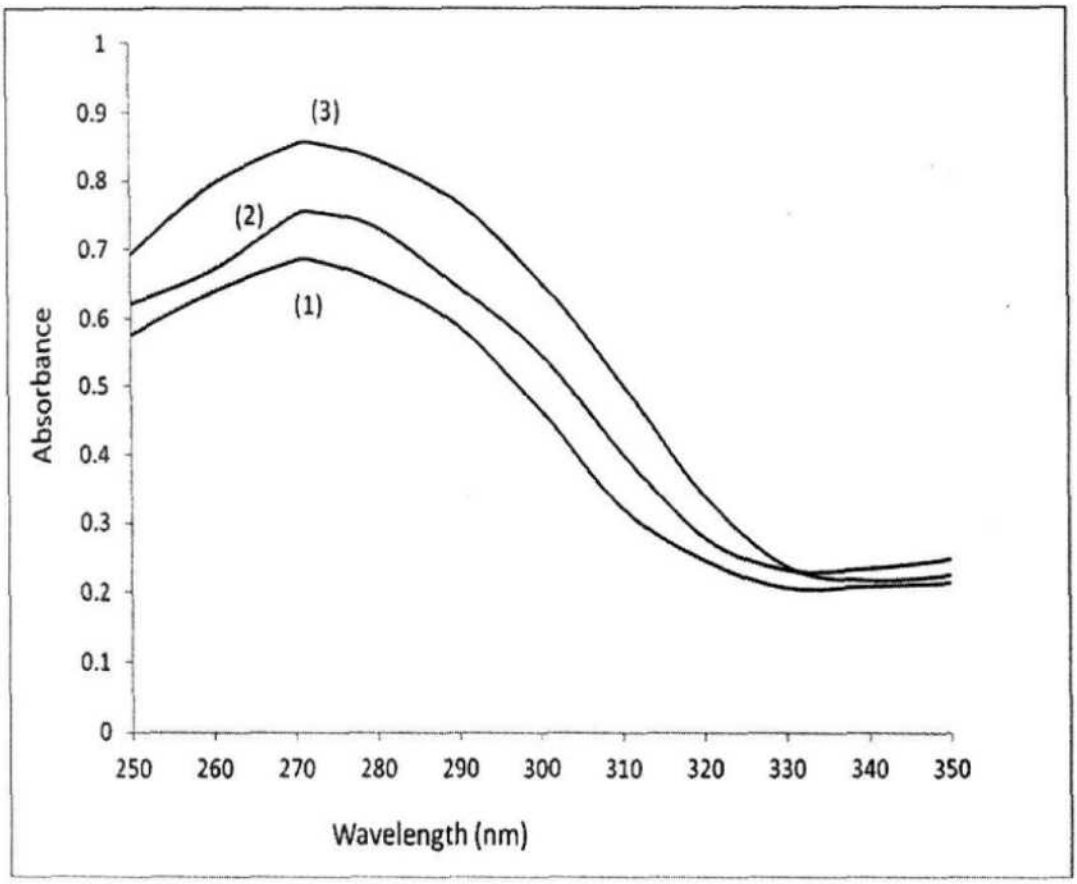
The 3.0 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 µM luteolin and increasing concentrations of Cu(II). The spectra were recorded after addition of components indicated.

(1) Luteolin alone

(2) Luteolin + 50 µM Cu(II)

(3) Luteolin + 100 µM Cu(II)

Figure 7: Absorption spectra of apigenin in the presence of Cu (II)



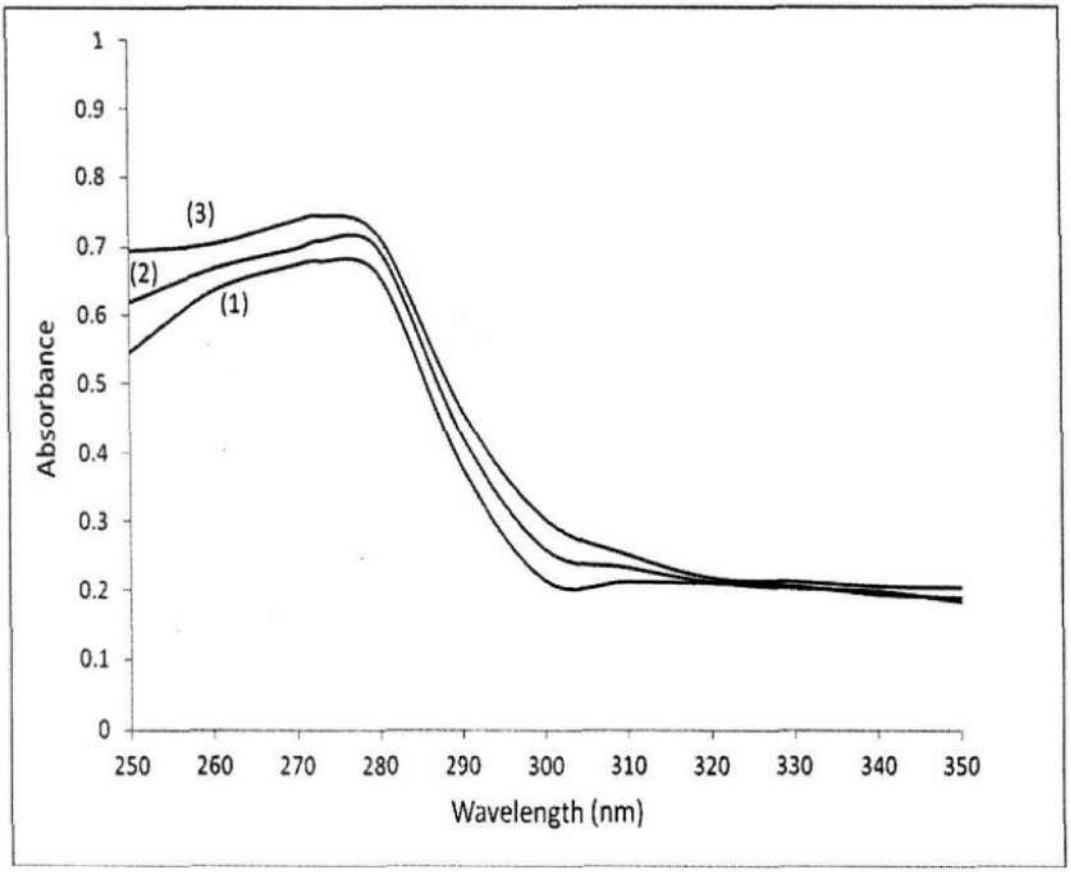
The 3.0 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 µM apigenin and increasing concentrations of Cu(II). The spectra were recorded after addition of components indicated.

(1) Apigenin alone

(2) Apigenin + 50 µM Cu(II)

(3) Apigenin + 100 µM Cu(II)

Figure 8: Absorption spectra of chrysin in the presence of Cu (II)



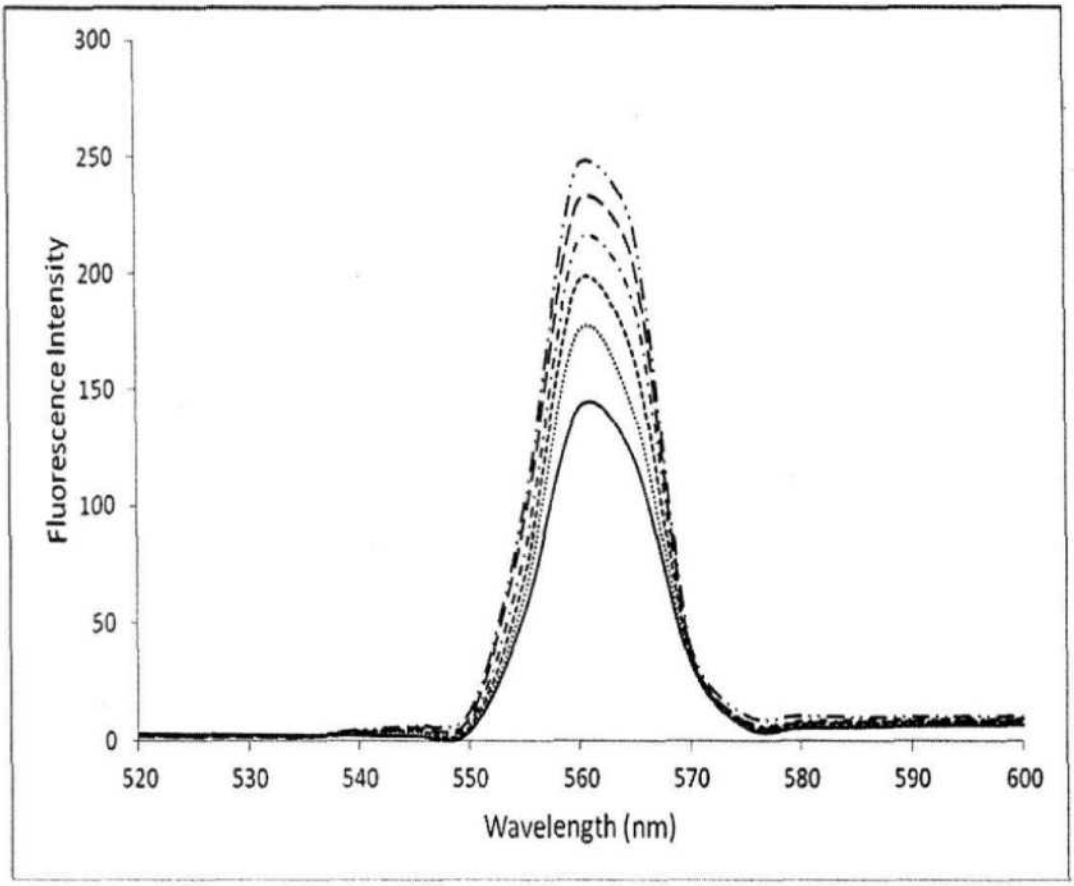
The 3.0 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 μM chrysin and increasing concentrations of Cu(II). The spectra were recorded after addition of components indicated.

(1) Chrysin alone

(2) Chrysin + 50 μM Cu(II)

(3) Chrysin + 100 μM Cu(II)

Figure 9: Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of luteolin



Luteolin (in 10 mM Tris-HCl, pH 7.5) was excited at its λ_{max} (absorption) of 270 nm and the emission spectra were recorded between 520-600 nm.

[__] Luteolin alone (30 μM)

[.....] Luteolin: DNA base pair molar ratio (1:1)

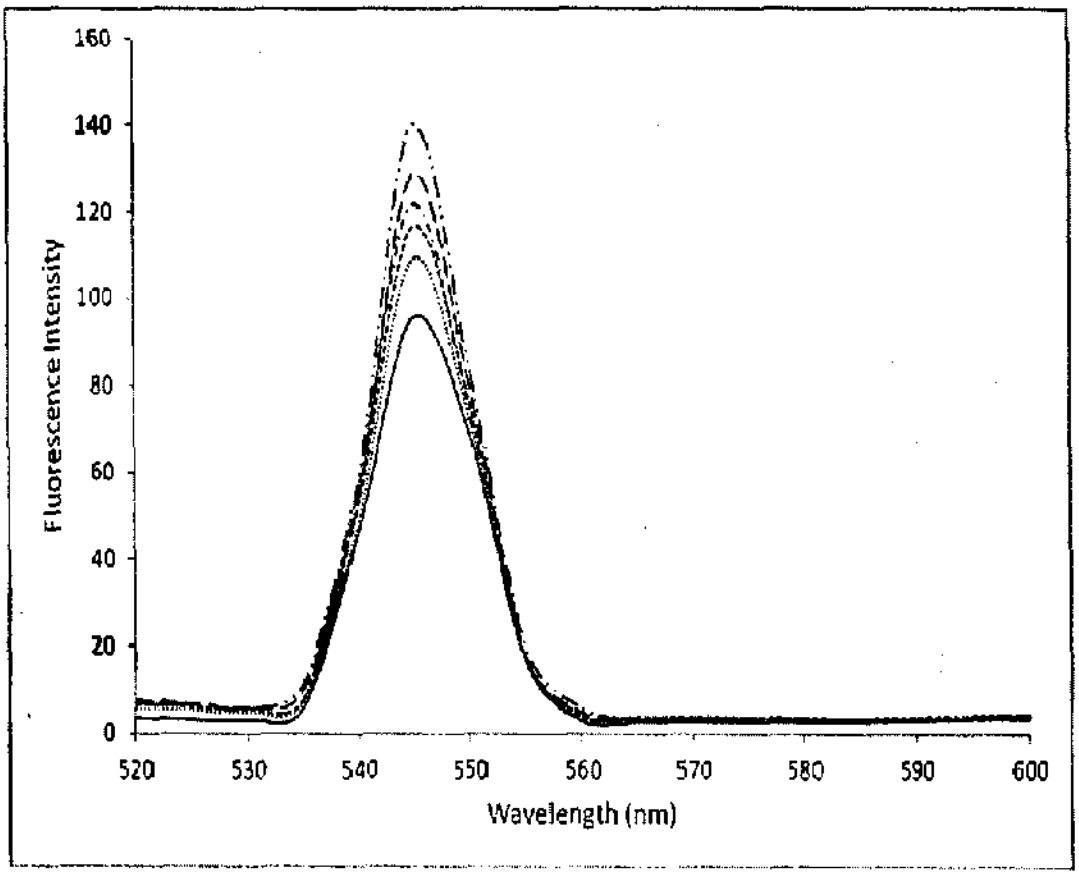
[_ _ _] Luteolin: DNA base pair molar ratio (1:2)

[— · —] Luteolin: DNA base pair molar ratio (1:4)

[__ __] Luteolin: DNA base pair molar ratio (1:6)

[_ .. _] Luteolin: DNA base pair molar ratio (1:8)

Figure 10: Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of apigenin



Apigenin (in 10 mM Tris-HCl, pH 7.5) was excited at its λ_{max} (absorption) of 272 nm and the emission spectra were recorded between 520-600 nm.

[__] Apigenin alone (30 μM)

[.....] Apigenin: DNA base pair molar ratio (1:1)

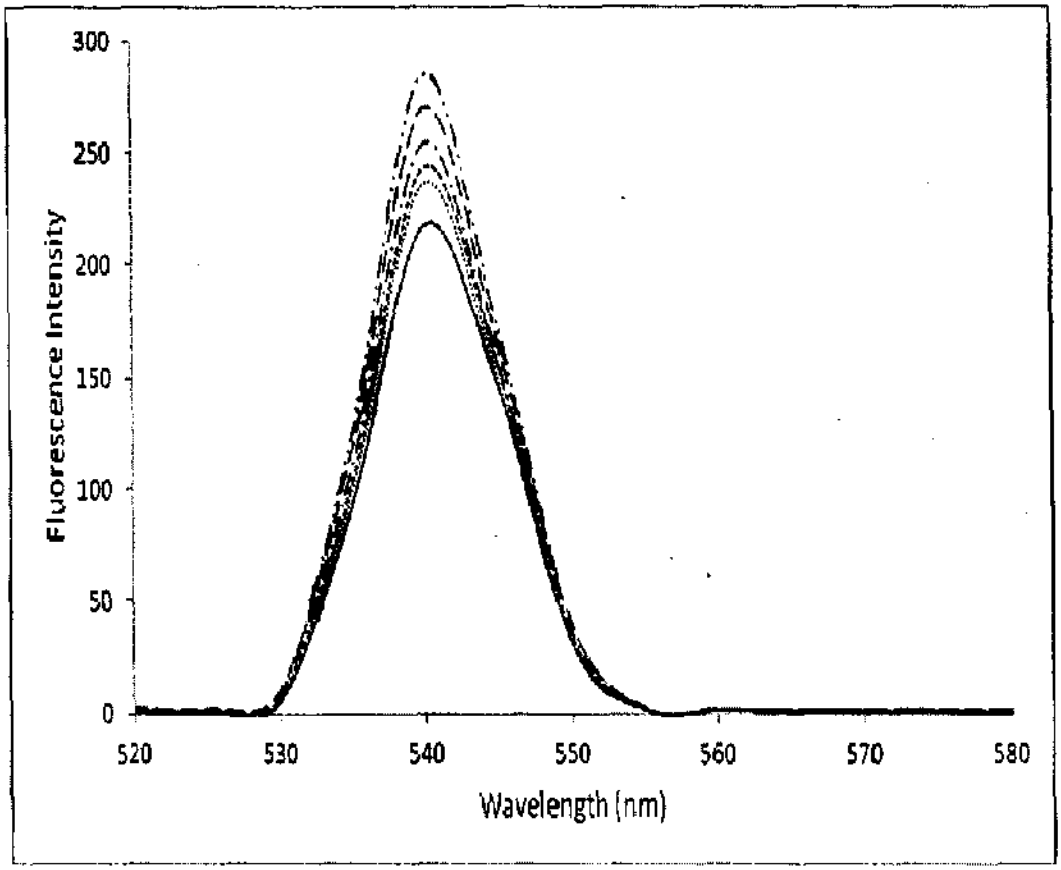
[_ _ _] Apigenin: DNA base pair molar ratio (1:2)

[— · —] Apigenin: DNA base pair molar ratio (1:4)

[__ __] Apigenin: DNA base pair molar ratio (1:6)

[_ .. _] Apigenin: DNA base pair molar ratio (1:8)

Figure 11: Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of chrysin



Chrysin (in 10 mM Tris-HCl, pH 7.5) was excited at its λ_{max} (absorption) of 273 nm and the emission spectra were recorded between 520-580 nm.

[__] Chrysin alone (30 μM)

[.....] Chrysin: DNA base pair molar ratio (1:1)

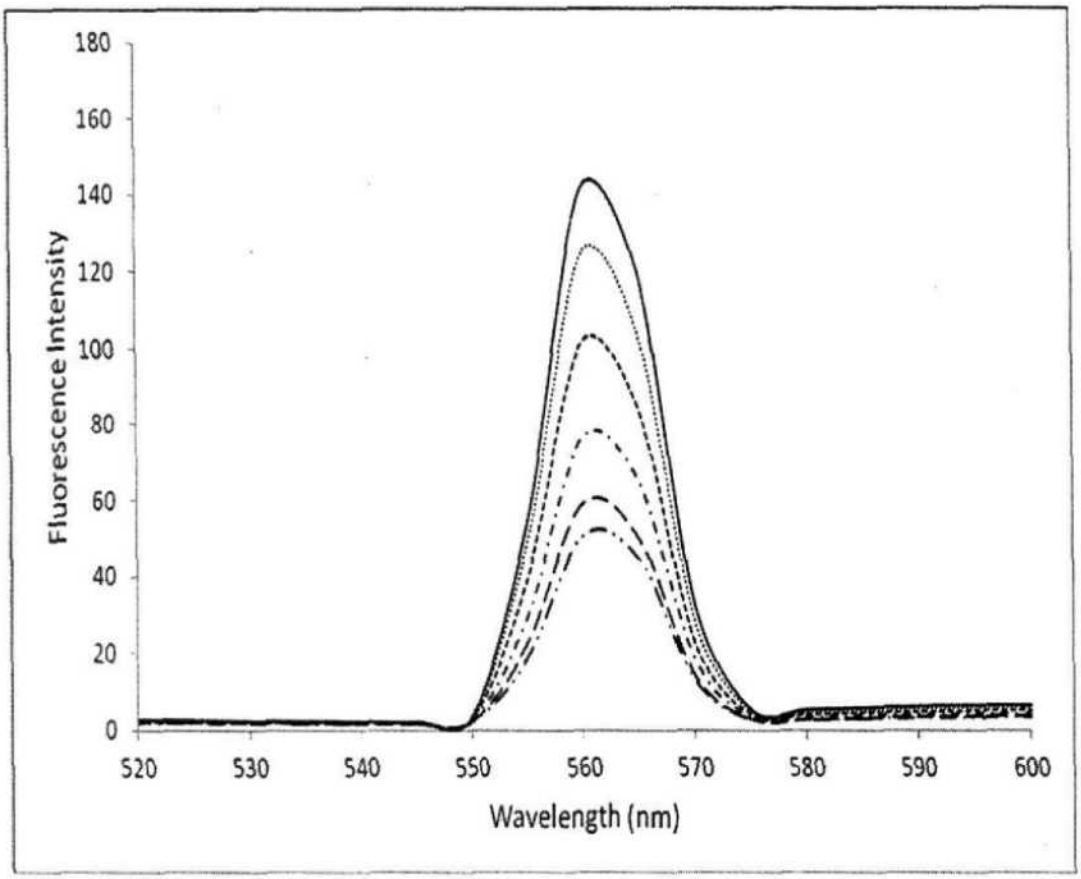
[_ _ _] Chrysin: DNA base pair molar ratio (1:2)

[— · —] Chrysin: DNA base pair molar ratio (1:4)

[__ __] Chrysin: DNA base pair molar ratio (1:6)

[_ .. _] Chrysin: DNA base pair molar ratio (1:8)

Figure 12: Effect of increasing concentration of Cu(II) on the fluorescence emission spectra of luteolin



Luteolin (in 10 mM Tris-HCl, pH 7.5) was excited at its λ_{max} (absorption) of 270 nm and the emission spectra were recorded between 520-600 nm.

[__] Luteolin alone (30 μM)

[.....] Luteolin: Cu(II) molar ratio (1:1)

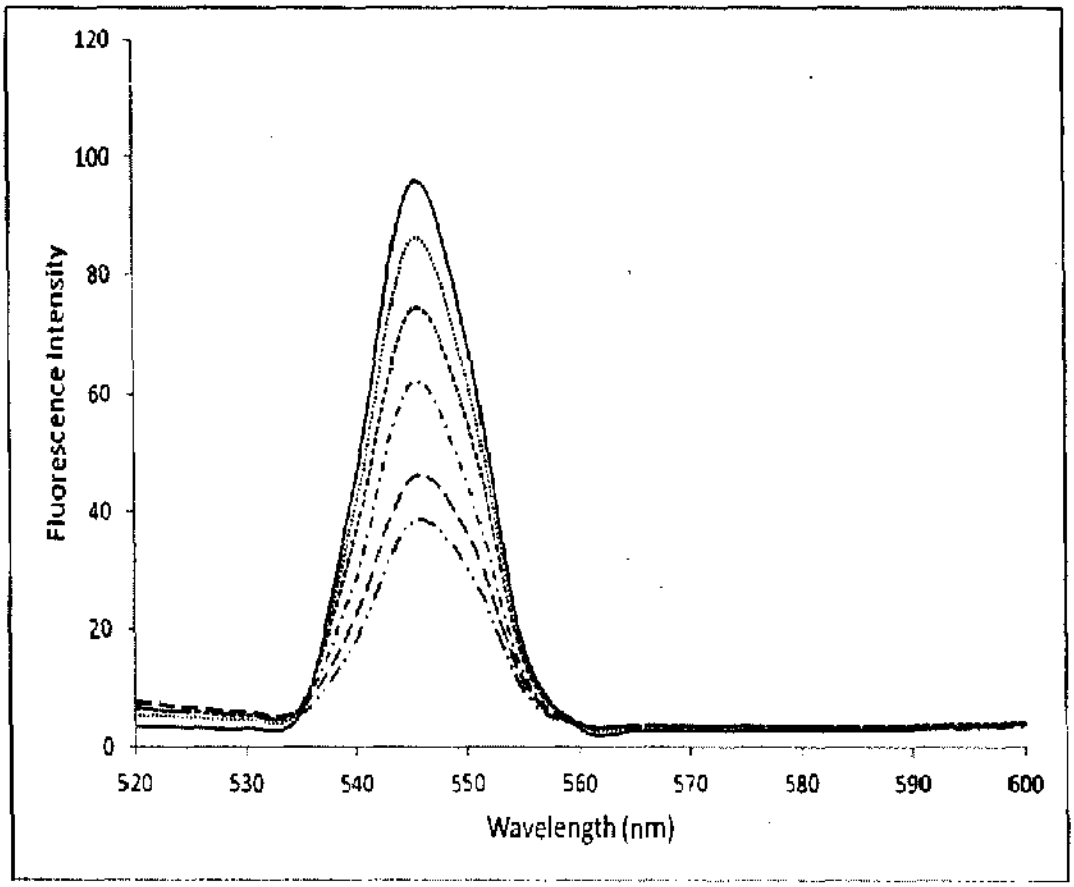
[_ _ _] Luteolin: Cu(II) molar ratio (1:2)

[— · —] Luteolin: Cu(II) molar ratio (1:4)

[__ __] Luteolin: Cu(II) molar ratio (1:6)

[_ .. _] Luteolin: Cu(II) molar ratio (1:8)

Figure 13: Effect of increasing concentration of Cu(II) on the fluorescence emission spectra of apigenin



Apigenin (in 10 mM Tris-HCl, pH 7.5) was excited at its λ_{max} (absorption) of 272 nm and the emission spectra were recorded between 520-600 nm.

[__] Apigenin alone (30 μM)

[.....] Apigenin: Cu(II) molar ratio (1:1)

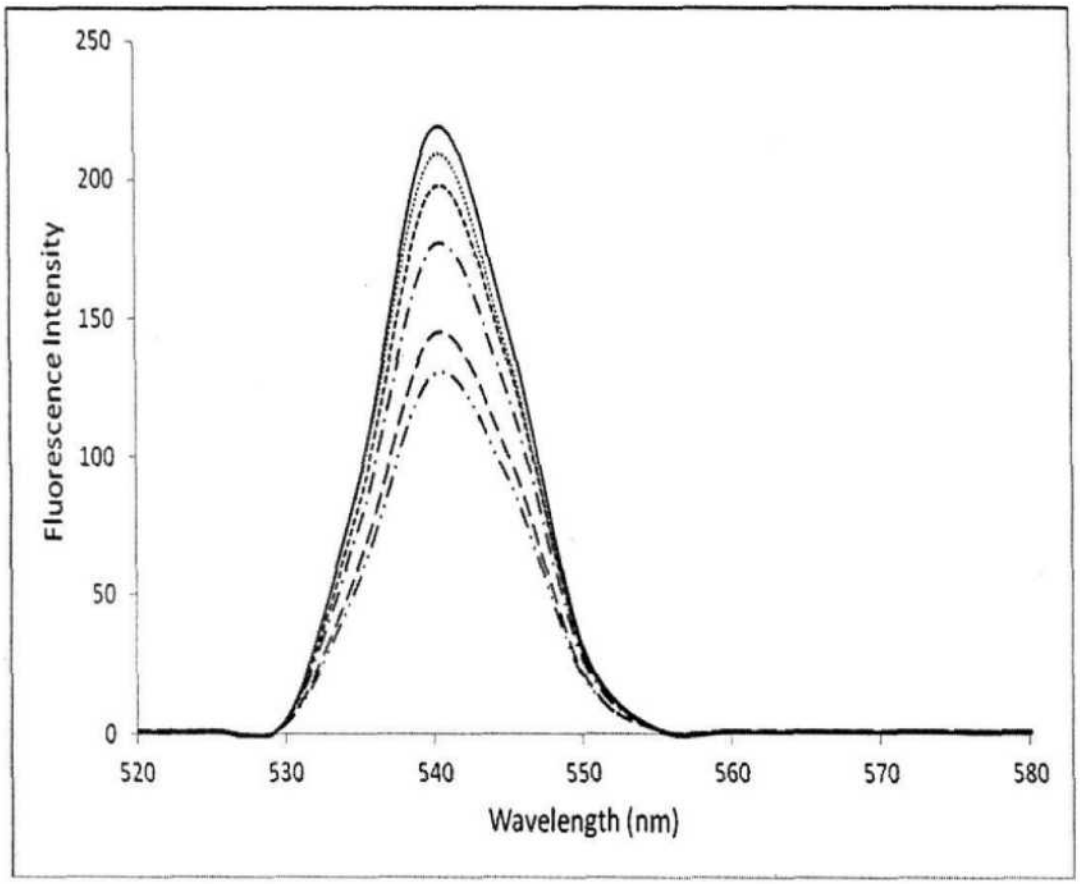
[_ _ _] Apigenin: Cu(II) molar ratio (1:2)

[— · —] Apigenin: Cu(II) molar ratio (1:4)

[__ __] Apigenin: Cu(II) molar ratio (1:6)

[_ .. _] Apigenin: Cu(II) molar ratio (1:8)

Figure 14: Effect of increasing concentration of Cu(II) on the fluorescence emission spectra of chrysin



Chrysin (in 10 mM Tris-HCl, pH 7.5) was excited at its λ_{max} (absorption) of 273 nm and the emission spectra were recorded between 520-580 nm.

[__] Chrysin alone (30 μM)

[.....] Chrysin: Cu(II) molar ratio (1:1)

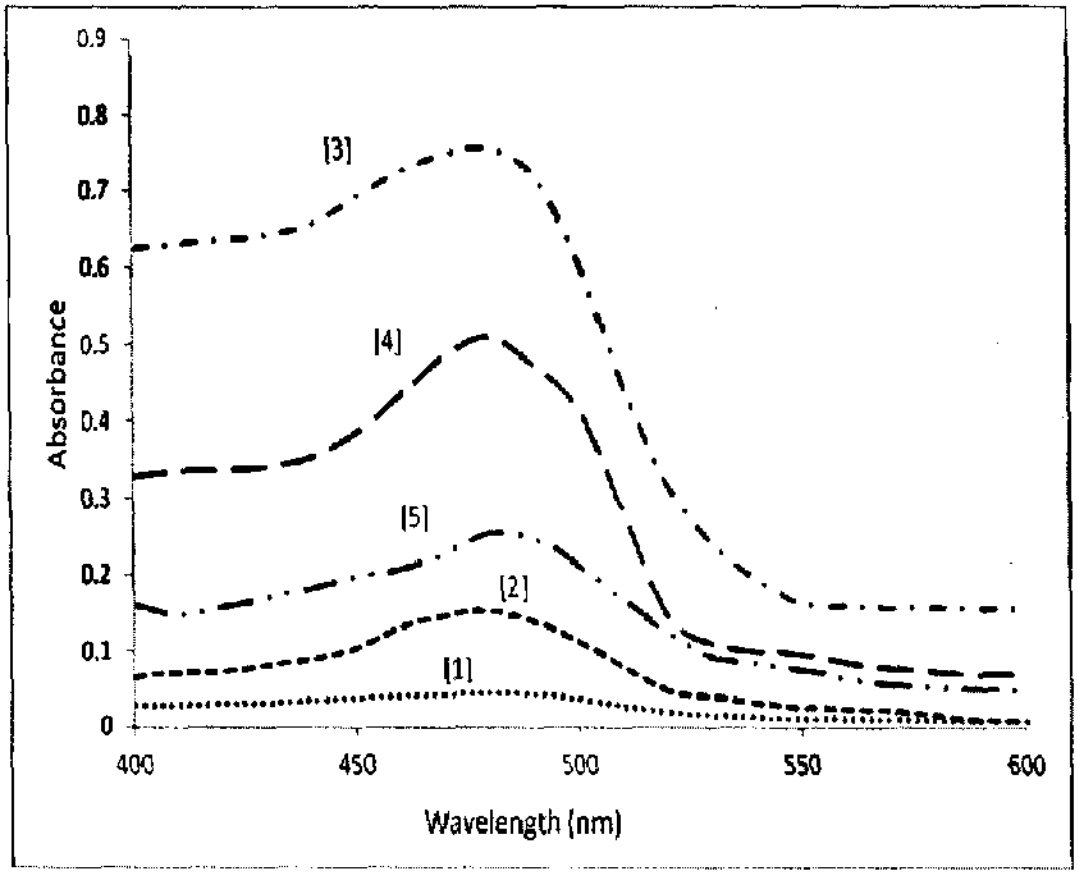
[_ _ _] Chrysin: Cu(II) molar ratio (1:2)

[— · —] Chrysin: Cu(II) molar ratio (1:4)

[__ __] Chrysin: Cu(II) molar ratio (1:6)

[_ .. _] Chrysin: Cu(II) molar ratio (1:8)

Figure 15: Detection of flavone induced Cu(I) production by Bathocuproine



Reaction mixture (3.0 ml) contained 3 mM Tris-HCl (pH 7.5) along with 300 µM bathocuproine and indicated concentrations of the following:

[1] Bathocuproine + 100 µM Cu(II)

[2] Bathocuproine + 100 µM Cu(I)

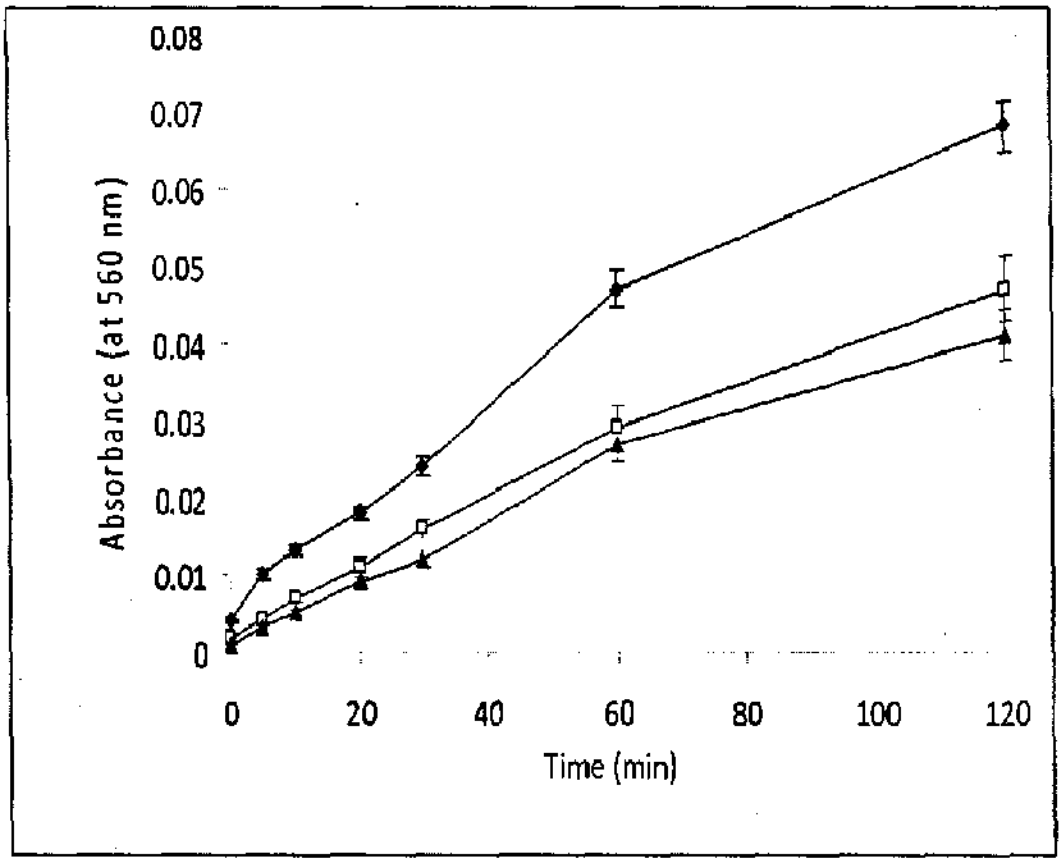
[3] Bathocuproine + 50 µM luteolin + 100 µM Cu(II)

[4] Bathocuproine + 50 µM apigenin + 100 µM Cu(II)

[5] Bathocuproine + 50 µM chrysin + 100 µM Cu(II)

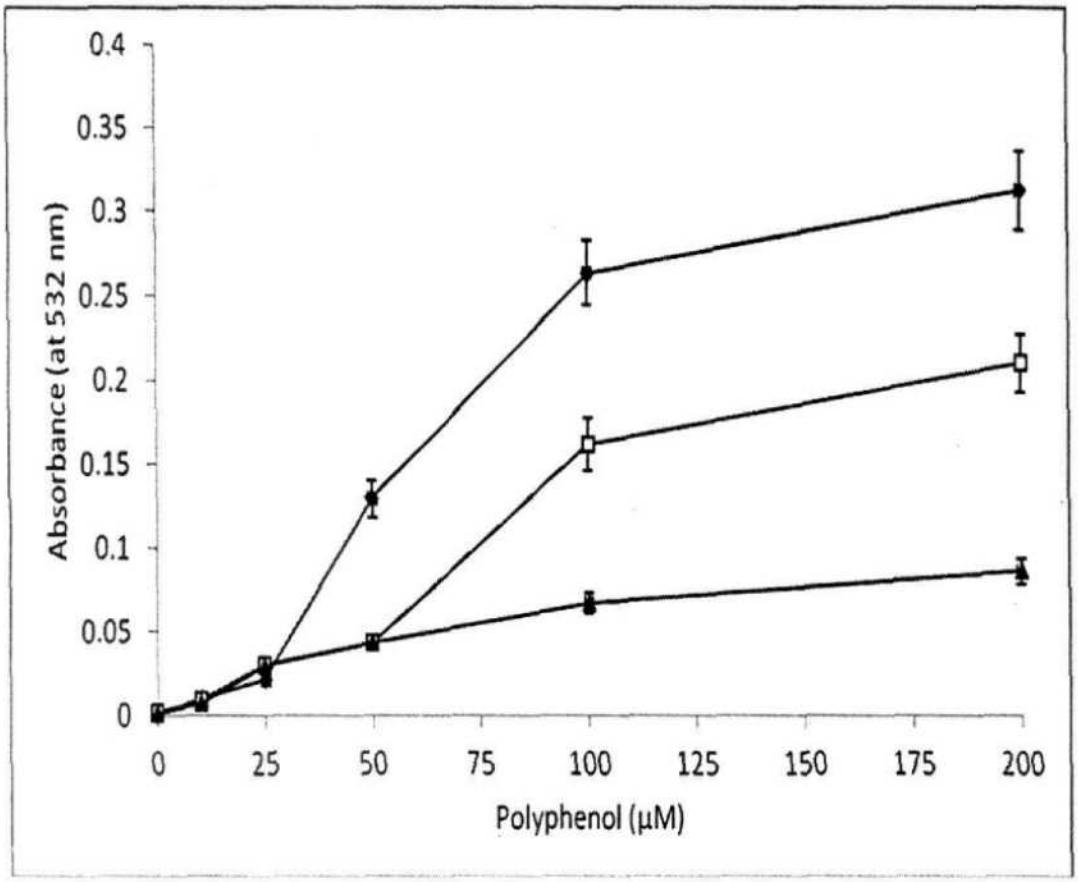
The Bathocuproine alone or bathocuproine in the presence of respective compounds did not interfere with the Bathocuproine-Cu(I) complex peak at 480 nm (not shown).

Figure 16: Photogeneration of superoxide anion by luteolin/ apigenin/ chrysin on illumination under fluorescent light as a function of time



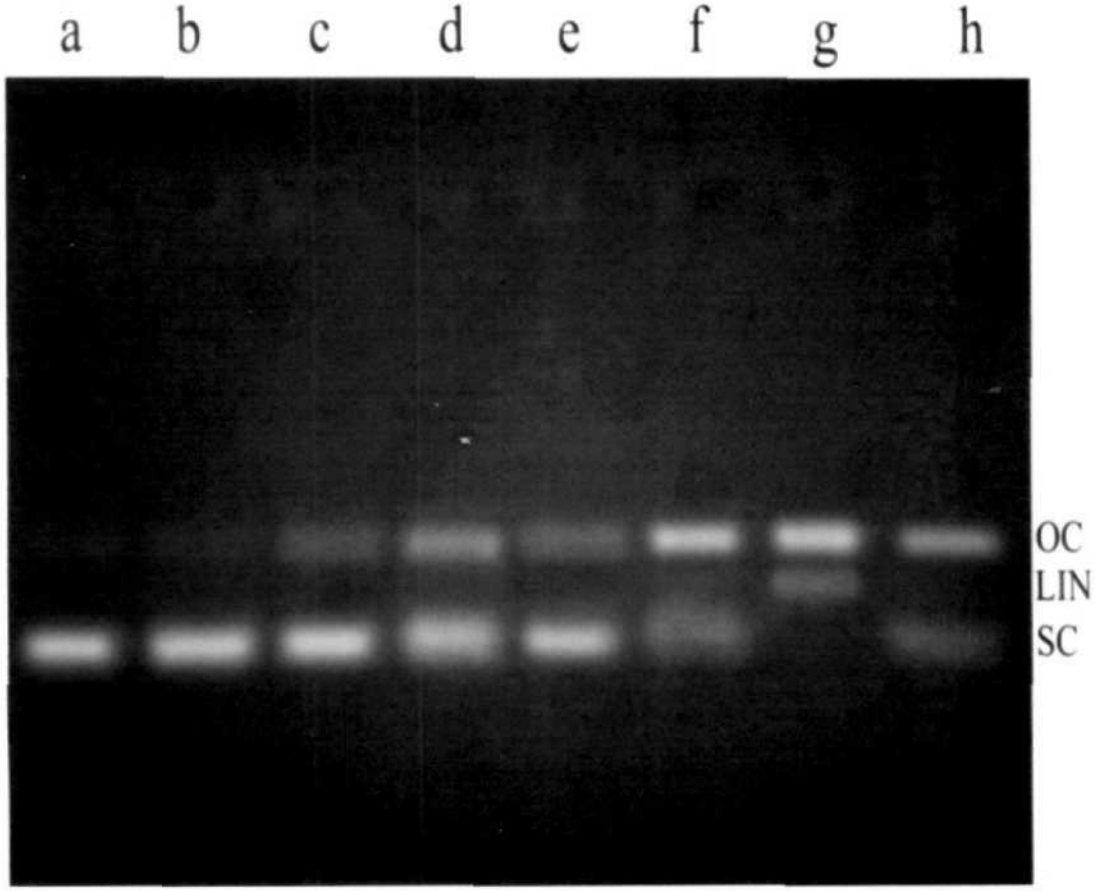
Reaction mixture contained 50 mM phosphate buffer (pH 7.5) and 100 µM of luteolin (♦), apigenin (□) and chrysin (▲). The samples were placed at a distance of 10 cm from the light source. All values reported are means of three independent experiments. Error bars represent standard error of mean.

Figure 17: Hydroxyl radical generation by luteolin/ apigenin/ chrysin



Reaction mixture (0.5 ml) contained 100 µg calf thymus DNA as substrate, 50 µM Cu(II) and indicated concentrations of luteolin (♦), apigenin (□) and chrysin (▲). The reaction mixture was incubated at 37°C for 30 min. Hydroxyl radical formation was measured by determining the TBA reactive material. All values reported are means of three independent experiments. Error bars represent standard error of mean.

Figure 18: Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 plasmid DNA after treatment with apigenin, luteolin and chrysin in the absence and presence of copper



The reaction mixture (30µl) contained 0.50 µg pBR322 DNA, 10 mM Tris-HCl (pH 7.5), indicated concentrations of the three flavones and Cu(II). Incubation was carried out at 37°C for 2 hour

Lane a: DNA alone; *Lane b*: DNA + Cu(II) 100 µM; *Lane c*: DNA + Apigenin (150µM); *Lane d*: DNA + Luteolin (150µM); *Lane e*: DNA + Chrysin (150µM) *Lane f*: DNA + Apigenin (150µM) + Cu(II) 100 µM; *Lane g*: DNA + Luteolin (150µM) + Cu(II) 100 µM; Lane h: DNA + Chrysin (150µM) + Cu(II) 100µM.

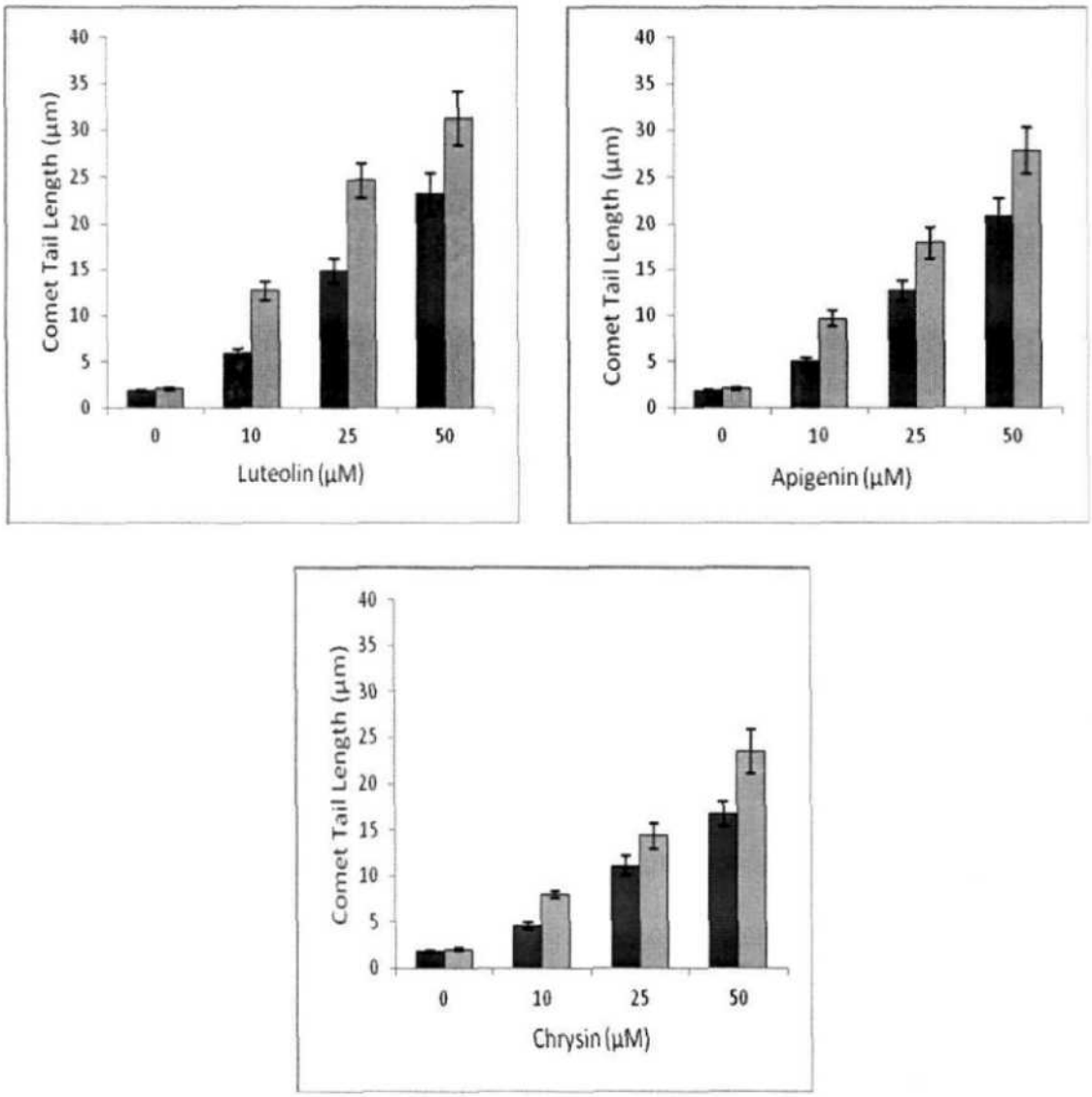
SC=Supercoiled DNA; OC= Open circular DNA; LIN=Linear DNA

Table IV: Degradation of calf thymus DNA by the flavones in the presence of Cu(II) as measured by the degree of single strand specific S_1-nuclease digestion

Flavone	Concentration (μM)	% DNA Hydrolysed	
		Without Cu(II)	With Cu(II)
APIGENIN	50	3.72 ± 0.24	15.44 ± 1.48
	100	5.45 ± 0.51	22.24 ± 2.12
	200	8.12 ± 0.67	32.62 ± 2.63
	300	10.57 ± 0.86	41.58 ± 3.44
LUTEOLIN	50	3.67 ± 0.23	17.54 ± 1.58
	100	7.3 ± 0.51	23.47 ± 1.75
	200	11.24 ± 1.03	37.71 ± 3.13
	300	13.59 ± 1.32	49.3 ± 3.46
CHRYSIN	50	2.48 ± 0.15	12.75 ± 0.96
	100	5.64 ± 0.52	17.89 ± 1.56
	200	7.61 ± 0.67	26.21 ± 2.58
	300	9.38 ± 0.77	34.1 ± 3.11

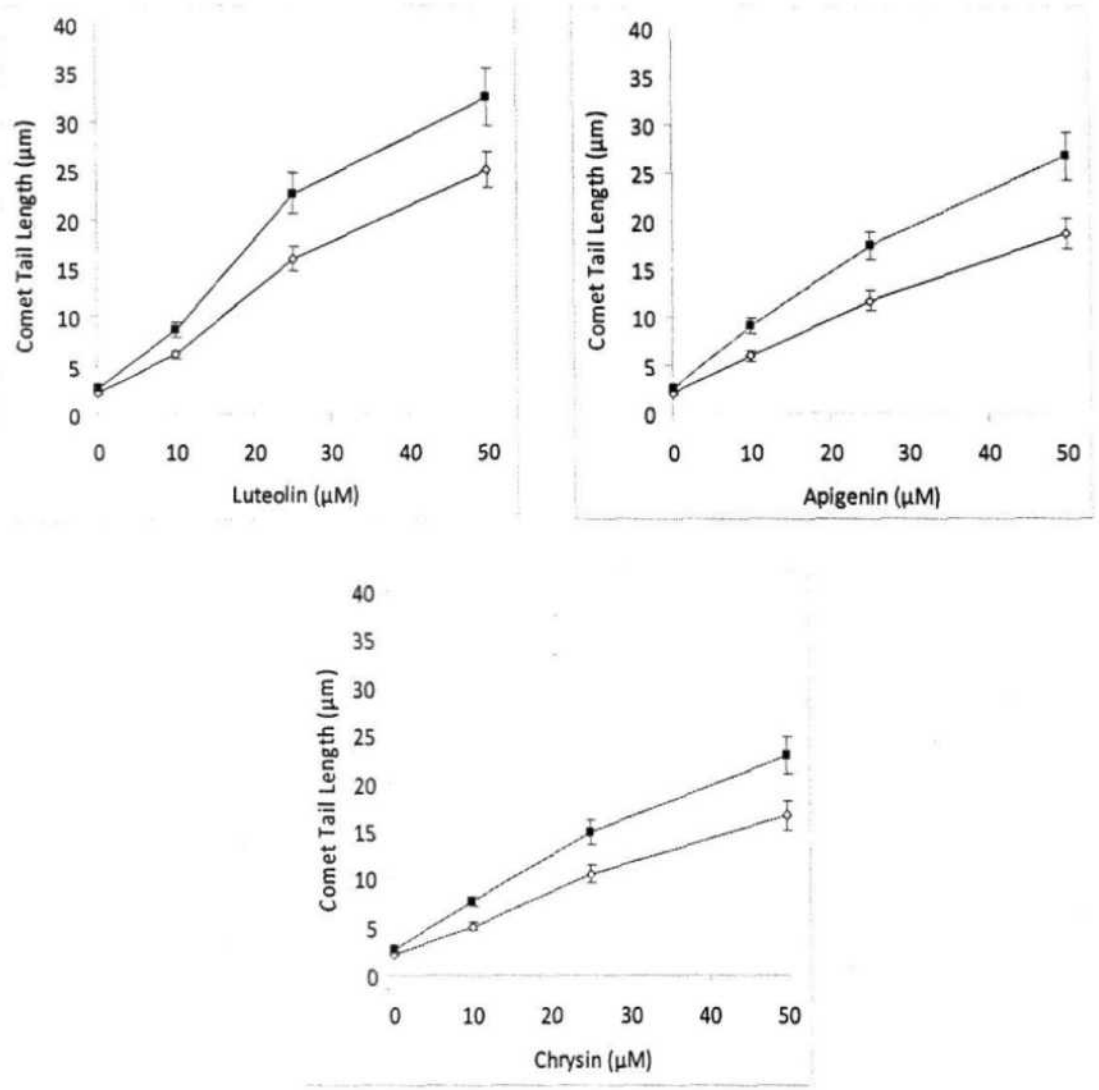
Reaction mixture (0.5 ml) containing 10 mM Tris-HCl (pH 7.5) and 500 μg calf thymus DNA was incubated at 37°C with indicated concentrations of respective polyphenol alone or polyphenol with Cu(II) (50 μM). All values represent mean ± SEM of three independent experiments.

Figure 19: DNA breakage by flavones in human peripheral lymphocytes in the absence and presence of Cu(II)



Reaction mixture (1.0 ml) contained 1 x10^5 cells, RPMI 400 μl, PBS Ca^{2+} and Mg^{2+} free, increasing concentrations of respective polyphenol (0-50 μM) and 50 μM Cu(II). The reaction mixture was incubated for 1 hr at 37°C. After incubation the cells were processed further for Comet Assay. Comet tail length (μ metres) plotted as a function of increasing concentrations of polyphenol (0-50 μM) in the absence (■) and presence (■) of 50 μM Cu(II). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.

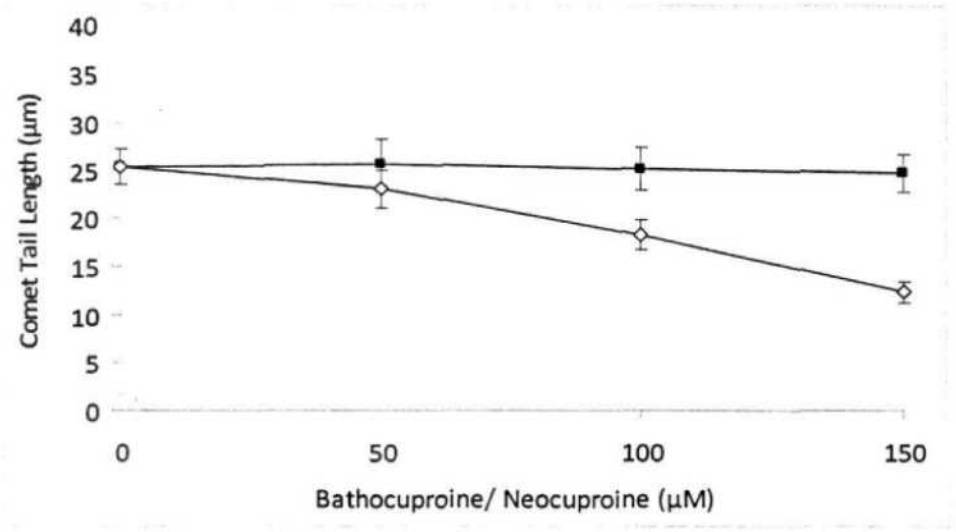
Figure 20: Comparison of DNA breakage by polyphenols in intact lymphocytes and permeabilized lymphocytes as measured by Comet assay



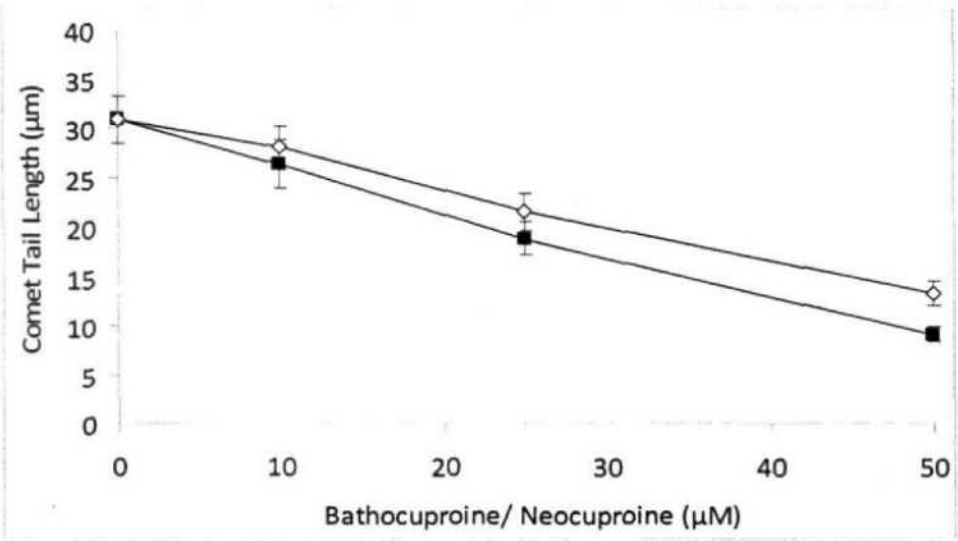
Intact lymphocytes / Permeabilized lymphocytes were incubated with the reaction mixture (2.0 ml) containing PBS Ca^{2+} and Mg^{2+} free (intact cells) / 0.4 M phosphate buffer (pH 7.5) (permeabilized cells) and indicated concentrations of respective polyphenol (0-50 µM) at 37°C for 1 hr / 30 min respectively and processed further for Comet Assay as described in 'Methods'. Comet tail length (µ metres) plotted as a function of increasing concentrations of polyphenol (0-50 µM) in intact lymphocytes (◊) and permeabilized lymphocytes (■). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control (in the absence of polyphenol).

Figure 21: Effect of neocuproine/ bathocuproine on luteolin-induced DNA breakage in intact lymphocytes (A) and permeabilized lymphocytes (B)

(A)



(B)



Lymphocytes were incubated with the reaction mixture (2.0 ml) containing luteolin (50 µM) and indicated concentrations of neocuproine / bathocuproine at 37°C for 1 hr (intact cells)/ 30 min (permeabilized cells) and processed further for Comet Assay. Comet tail length (µ metres) plotted as a function of increasing concentrations of neocuproine (◊) and bathocuproine (■) in intact lymphocytes (A) and permeabilized lymphocytes (B). Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.

DISCUSSION-I

Most of the pharmacological properties of plant polyphenols are considered to reflect their ability to scavenge endogenously generated oxygen radicals or those free radicals formed by various xenobiotics, radiation etc. However, some data in the literature suggests that the antioxidant properties of the polyphenolic compounds may not fully account for their chemopreventive effects (Gali et al., 1992; Hadi et al., 2000). Although most plant polyphenols are considered to have a physiological role as antioxidants, they may also exhibit prooxidant properties in the presence of transition metals such as copper (Ahmad et al., 1992; Inoue et al., 1994). The results presented in this chapter lead to the conclusions that all the three flavones tested, namely luteolin, apigenin and chrysin, (i) are able to interact with DNA as well as Cu(II) and possibly form a ternary complex of DNA-Cu(II)-flavone; (ii) are able to reduce Cu(II) to generate Cu(I); (iii) cause redox cycling of copper leading to the generation of various reactive oxygen species, particularly the hydroxyl radical, (iv) are able to induce strand scission in plasmid DNA and calf thymus DNA in the presence of copper ions; (v) show a similar copper dependent activation leading to enhanced DNA degradation in a cellular system of human peripheral lymphocytes; and (vi) mobilize nuclear, possibly chromatin bound copper in the DNA breakage reaction. These observations suggest that such a prooxidant mechanism of DNA breakage involving flavone-Cu(II) system is physiologically feasible and could be of biological significance.

These results place the flavones luteolin, apigenin and chrysin among the other classes of plant derived polyphenolic antioxidants such as isoflavones (Ullah et al., 2009), anthocyanidins (Hanif et al., 2008), stilbene (Azmi et al., 2006), catechins (Azam et al., 2004), curcumin (Ahsan and Hadi, 1998) and tannins (Bhat and Hadi, 1994), which also exhibit prooxidant DNA damaging properties in the presence of copper ions. Previous studies on polyphenols

from this laboratory have shown that a ternary complex of DNA, Cu(II) and polyphenol is formed which generates oxygen radicals in situ via Cu(I) (Rahman et al., 1989). The results presented here demonstrate that flavones are also capable of binding to DNA as well as copper and thus it would be reasonable to assume that a similar mechanism operates in the case of flavone-Cu(II) mediated DNA cleavage.

Interestingly, certain properties of plant derived polyphenolic compounds such as binding and cleavage of DNA and the generation of ROS in the presence of transition metal ions (Rahman et al., 1990) are similar to those of some known anticancer drugs (Ehrenfeld et al., 1987). Metal ion dependent degradation of DNA by 1,10-phenanthroline (Gutteridge and Halliwell, 1982), bleomycin (Ehrenfeld et al., 1987), adriamycin (Eliot et al., 1984; Haidle and McKinney, 1985) as well as flavonoids (Rahman et al., 1989; Ahmad et al., 1992) are based on mechanisms involving reactive oxygen radicals. The generation of hydroxyl radicals in the proximity of DNA is well established as a cause of strand scission. It is generally recognized that such reaction with DNA is preceded by the association of a ligand with DNA followed by the formation of hydroxyl radicals at that site. Among oxygen radicals the hydroxyl radical is most electrophilic with high reactivity and therefore possesses a small diffusion radius. Thus, in order to cleave DNA it must be produced in the vicinity of DNA (Pryor, 1988). Moreover, the location of the redox-active metal is of utmost importance because the hydroxyl radical, due to its extreme reactivity, interacts exclusively in the vicinity of the bound metal (Chevion, 1988). Fe^{3+} and Cu^{2+} are the most redox-active of the various metal ions in living cells. However, it is copper that is known to be present in the nucleus as a normal component of chromatin, being associated with guanine bases (Kagawa et al., 1991). Such endogenous copper can be mobilized by chemical agents such as 1,10-phenanthroline to cause internucleosomal DNA

fragmentation (Burkitt et al., 1996). Wolfe et al. (1994) have proposed that a copper mediated Fenton reaction, generating site-specific hydroxyl radicals, is capable of inducing apoptosis in thymocytes. Conceivably, direct interaction of flavone compounds apigenin, luteolin and chysin with the DNA bound copper ions in a ternary complex and localized generation of non-diffusible hydroxyl radical is a likely mechanism involved in the flavone-Cu(II) induced DNA breakage.

Also, it is evident from the results presented in this chapter that the efficiency of DNA cleavage by the flavones tested is in the order - luetolin> apigenin> chrysin, and correlates with their relative abilities to cause copper reduction and ROS generation. A possible explanation for this is provided by the structural difference between these molecules (Figure 22).

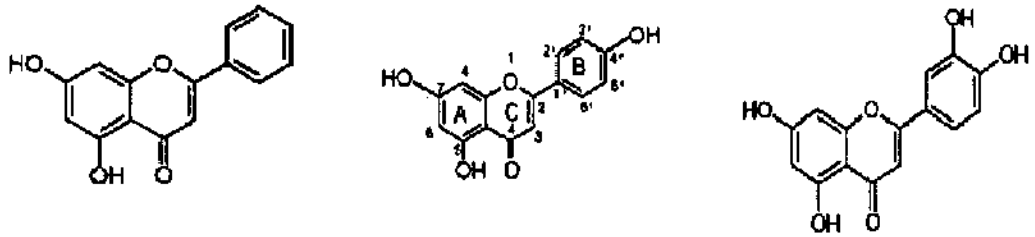


Figure 22. Structures of the three flavones used in our studies.

In a previous publication (Jain et al., 1999), this laboratory had studied the reactivities of flavonoids with different hydroxyl substituents for the cleavage of DNA in the presence of Cu(II). One of the structural feature that was identified was that Cu(II) complexes formed by 3-4, 4-5 and ortho substituents can generate species that are capable of attacking DNA. The above results are consistent with this finding where luteolin, in addition to 4-5 hydroxyl substituents, also possesses two ortho hydroxyl groups in ring B. Apigenin possesses only one hydroxyl group in the B ring and possibly this accounts for

its intermediate DNA cleavage efficiency among the three flavones tested. The present findings are further supported by the results of some other studies (Cao et al., 1997; Kozics et al. 2011) which have shown that the antioxidant and copper mediated prooxidant activities of flavonoids have a certain correlation with the number and arrangement of hydroxyl groups in their molecular structures.

Furthermore, the observation that bathocuproine, which is a membrane impermeable copper chelator, inhibits flavone-induced DNA breakage only in permeabilized cells and not in intact cells whereas its membrane permeable analogue neocuproine causes such an inhibition in both intact as well as permeabilized cells, strongly indicates that flavone-induced cellular DNA breakage involves mobilization of nuclear copper. Thus, similar to earlier findings in this laboratory, flavones also exhibit a copper dependent prooxidant action leading to oxidative breakage of cellular DNA. Therefore, it is likely that flavones belong to the class of polyphenolic compounds whose prooxidant action is possibly responsible for their apoptosis induction and anticancer activities.

Chapter - II

Oral administration of copper to rats leads to increased cellular DNA degradation by plant polyphenols

RESULTS-II

Oral administration of cupric chloride to rats leads to elevated copper levels:

When rats in the test group were orally administered copper in the form of cupric chloride in a single dose of 30 mg/Kg b.w., a clear elevation in the intracellular copper status of lymphocytes was observed. A similar increase in the plasma copper concentrations was also found. As shown in Table V, the mean copper levels were highest in the plasma as well as lymphocytes isolated 12 hours after oral administration of copper to the rats. Beyond this time, the levels of copper declined and returned to almost control values at 36 hours post-administration.

Polyphenols induced DNA breakage in lymphocytes isolated from copper-administered rats after different time intervals:

Lymphocytes isolated at different time points following copper administration to rats were treated *in vitro* with EGCG and genistein at a concentration of 50 μM. Breakage in cellular DNA was measured by alkaline single cell gel electrophoresis (comet assay). As can be seen in figure 23, both EGCG and genistein induced significantly greater DNA degradation in cells isolated at 12 hours after copper dosing to rats. Such increased DNA breakage in isolated rat lymphocytes correlates with the data shown in Table V, where the maximum elevation in copper levels was seen in lymphocytes isolated after 12 hours of copper administration. Therefore in all subsequent experiments, lymphocytes were isolated from rats 12 hours after oral administration of copper.

Oral administration of different copper doses to rats and polyphenol-induced DNA breakage in isolated lymphocytes:

Four different doses of cupric chloride ranging from 5 to 30 mg/Kg b.w. were orally administered to rats, which were sacrificed 12 hours later for isolation of plasma and lymphocytes. Table VI shows a progressive increase in the levels

of copper attained in plasma and lymphocytes of such rats, as a function of the administered dose. Further, as shown in figure 24, an *in vitro* treatment of lymphocytes, isolated from rats which were given different copper doses, with EGCG and genistein led to cellular DNA degradation that was commensurate to their copper levels (Table VI). This also suggests that the 30 mg/Kg b.w. dose which was initially selected for oral administration to rats was sufficient to cause significant copper overload and appreciable DNA breakage in rat lymphocytes, as compared to other lower doses. Increasing concentration of copper also leads to an increase in cellular DNA breakage. However, such breakage is considerably enhanced in the presence of polyphenols (see Dicussion-II).

Cellular DNA breakage induced by various polyphenols in lymphocytes isolated from rats with copper overload:

When isolated rat lymphocytes were treated with different polyphenols (Table VII), an invariably greater extent of DNA breakage was observed in the lymphocytes of copper-administered rats as compared to that of control group rats. Thus it is indicated that the elevated copper levels in copper-administered rats contributes to increased cellular DNA degradation. Further, the differences in the rate of DNA breakage by the three polyphenols tested is possibly due to differential cell membrane permeability and copper reducing efficiency of the polyphenols (Ahmad et al. 1992).

In another experiment, the effect of increasing concentrations of polyphenols were tested on cellular DNA breakage in lymphocytes isolated from copper-administered rats. As shown in figure 25, isolated rat lymphocytes treated with increasing concentrations of EGCG exhibit a progressive increase in DNA degradation. However, such increment in DNA breakage was more pronounced in lymphocytes of copper-administered rats at every concentration of EGCG tested. Similar results were obtained when increasing

concentrations of genistein and resveratrol were used to treat isolated rat lymphocytes, as shown in figure 26 and figure 27 respectively. In previous publications we have established the mobilization of endogenous copper ions by plant polyphenols using various metal ion chelators (Shamim et al., 2008; Ullah et al., 2009). The results given in figures 25-27 lend further support to the involvement of endogenous copper ions in polyphenol mediated cellular DNA degradation.

Effect of metal-specific sequestering agents on the polyphenol-induced DNA breakage in isolated rat lymphocytes with copper overload:

In the experiment shown in figure 28, we have used various metal-specific chelators, which selectively bind to copper, iron and zinc, to study their effect on polyphenol EGCG-induced DNA degradation in lymphocytes isolated from rats administered copper orally. As shown above and as expected the cellular DNA breakage was considerably greater in lymphocytes with a copper overload. Four different metal chelators were used, namely neocuproine and bathocuproine (copper sequestering agents); desferroxamine mesylate (iron chelator) and histidine (which binds zinc) at concentrations of 25, 50 and 100 μM. It was seen that only in the case of neocuproine, there was a progressive decrease in comet tail lengths. Bathocuproine, which is also a copper chelator, along with desferroxamine mesylate and histidine was ineffective. Bathocuproine is impermeable to cell membrane and we have earlier shown that when isolated cell nuclei were treated with polyphenols, bathocuproine was able to inhibit the cellular DNA breakage (Shamim et al., 2008). This explains the non-inhibition of DNA breakage by bathocuproine observed in the above experiment. Similar results were obtained when genistein (Figure 29) and resveratrol (Figure 30) were used instead of EGCG as test polyphenols. The result indicate that the polyphenol-induced cellular DNA breakage in the control group as well as copper-administered group of rats is due to a similar

mechanism involving mobilization of endogenous copper ions. Moreover, it also rules out the involvement of any other metal ion, such as iron or zinc, in the reaction leading to DNA breakage.

Effect of scavengers of active oxygen species on polyphenol-induced DNA breakage in isolated rat lymphocytes with copper overload:

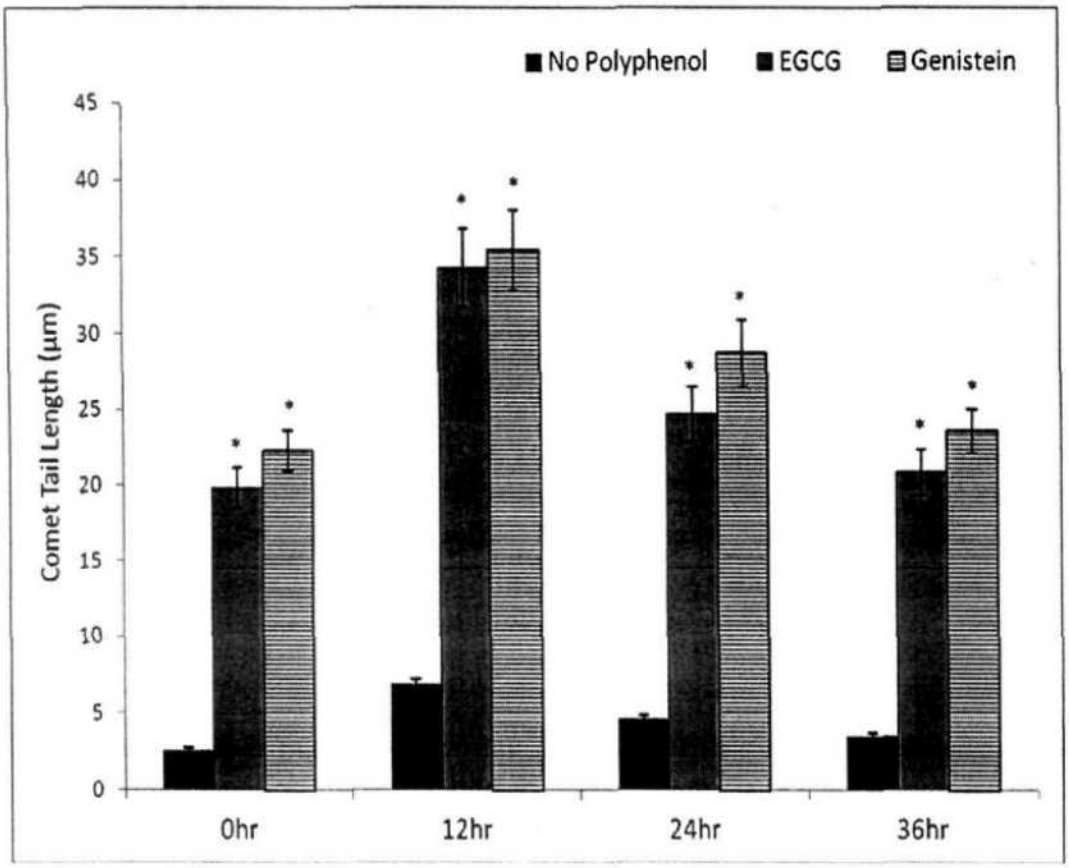
Table VIII gives the results of an experiment where scavengers of various ROS, namely superoxide dismutase, catalase and thiourea, were tested for their effect on EGCG-induced DNA breakage in lymphocytes isolated from copper-administered rats using comet assay. As shown in Table IX, similar effects of ROS scavengers on resveratrol-induced DNA degradation were also observed. Catalase and SOD remove H_2O_2 and superoxide, respectively, while thiourea scavenges hydroxyl radicals. All the three ROS scavengers caused significant inhibition of DNA breakage as evidenced by decreased tail lengths of comets in both the control as well as copper-administered group. In earlier reports (Azmi et al., 2006; Ullah et al., 2009), we have proposed that polyphenol induced cellular DNA degradation is the result of the formation of ROS such as the hydroxyl radicals. Further, due to the site specific nature of the reaction of hydroxyl radicals with DNA, it is difficult for any trapping molecule to intercept them completely (Czene et al., 1997). This possibly accounts for the fact that complete inhibition of DNA degradation was not observed even at relatively high concentration of thiourea (1 mM) and catalase (100 µg/ml). The results are in further support of the indication that irrespective of whether the lymphocytes are from the normal control rats or from the copper-administered ones, a similar mechanism of polyphenol mediated oxidative DNA degradation is involved.

Table V: Copper concentrations in plasma and lymphocytes of rats after different intervals of copper administration

Time interval between copper dosing and lymphocyte isolation	Mean Copper conc. in plasma (µg/ml ± SD)	Mean copper level in lymphocytes (µg/10^6 cells ± SD)
0 hr	1.46 ± 0.09	8.96 ± 0.69
12 hrs	5.11 ± 0.34	31.28 ± 2.55
24 hrs	3.34 ± 0.21	18.64 ± 1.36
36 hrs	1.79 ± 0.05	10.56 ± 0.86

Rats were gavaged in groups of 5 with 30 mg/Kg b.w. $CuCl_2$ in water and sacrificed at intervals indicated for isolation of plasma and lymphocytes.

Figure 23: Polyphenol induced DNA breakage in lymphocytes isolated from copper-administered rats at different time points



The rats were gavaged with 30 mg/kg b.w. $CuCl_2$ and sacrificed after the indicated periods. The isolated lymphocytes were then treated with the polyphenols (50 µM) for one hour and subsequently subjected to comet assay. The tail lengths of comets were determined as given in materials and methods.

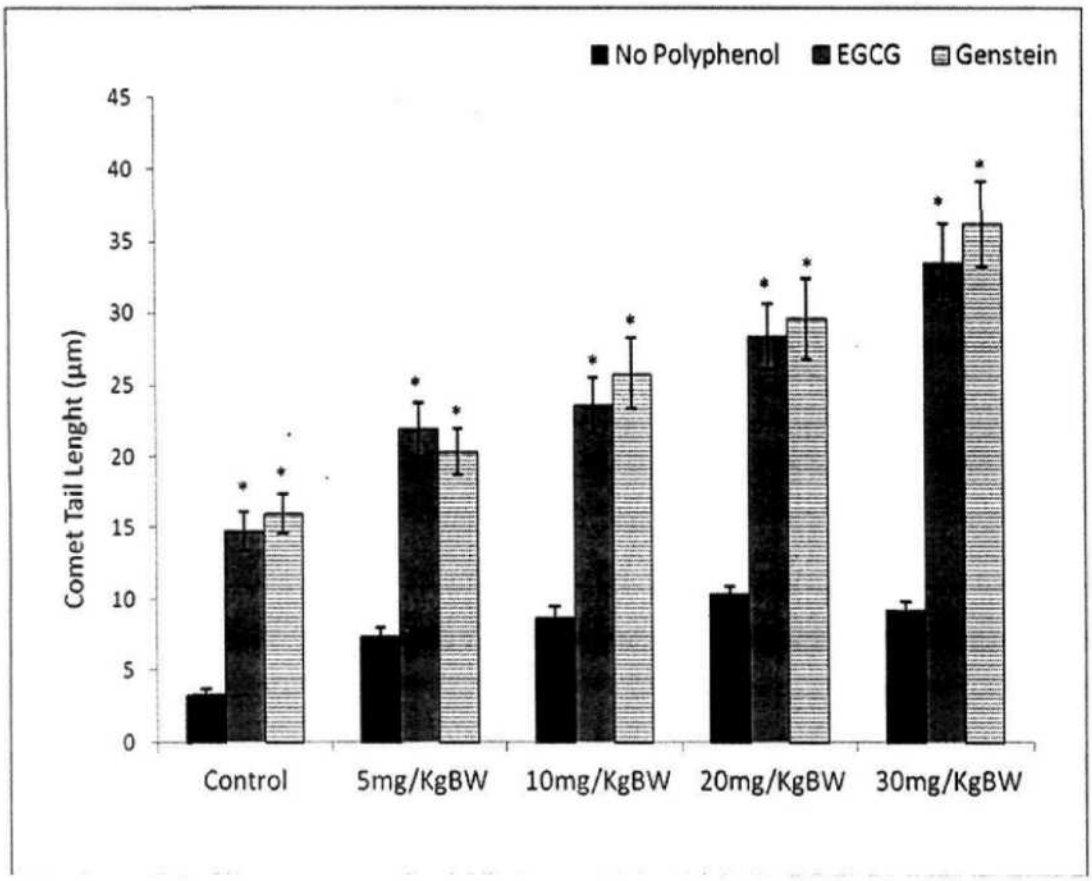
* Values are found to be significant when compared with control (no polyphenol) at $P < 0.01$. All groups comprised of 5 animals each.

Table VI: Copper concentrations in plasma and lymphocytes of rats administered different doses of copper

Copper dose (mg/Kg b.w)	Mean Copper conc. in plasma (µg/ml ± SD)	Mean copper level in lymphocytes (µg/10^6 cells ± SD)
0	1.37 ± 0.11	8.59 ± 0.73
5	1.92 ± 0.34	11.17 ± 0.85
10	2.54 ± 0.2	16.84 ± 1.71
20	3.66 ± 0.25	21.65 ± 1.96
30	5.42 ± 0.61	29.59 ± 3.03

Rats were gavaged in groups of 5 with $CuCl_2$ in different doses and sacrificed 12 hours post-administration for isolation of plasma and lymphocytes

Figure 24: Polyphenol induced DNA breakage in lymphocytes isolated from rats administered different doses of copper



The rats were sacrificed 12 hours after oral administration of $CuCl_2$. The isolated lymphocytes were then treated with the polyphenols (50 µM) for one hour and subsequently subjected to comet assay. The tail lengths of comets were determined as given in materials and methods.

* Values are found to be significant when compared with control (no polyphenol) at $P < 0.01$. All groups comprised of 5 animals each.

Table VII: Cellular DNA breakage induced by different polyphenols in lymphocytes isolated from copper-administered rats as analyzed by comet assay

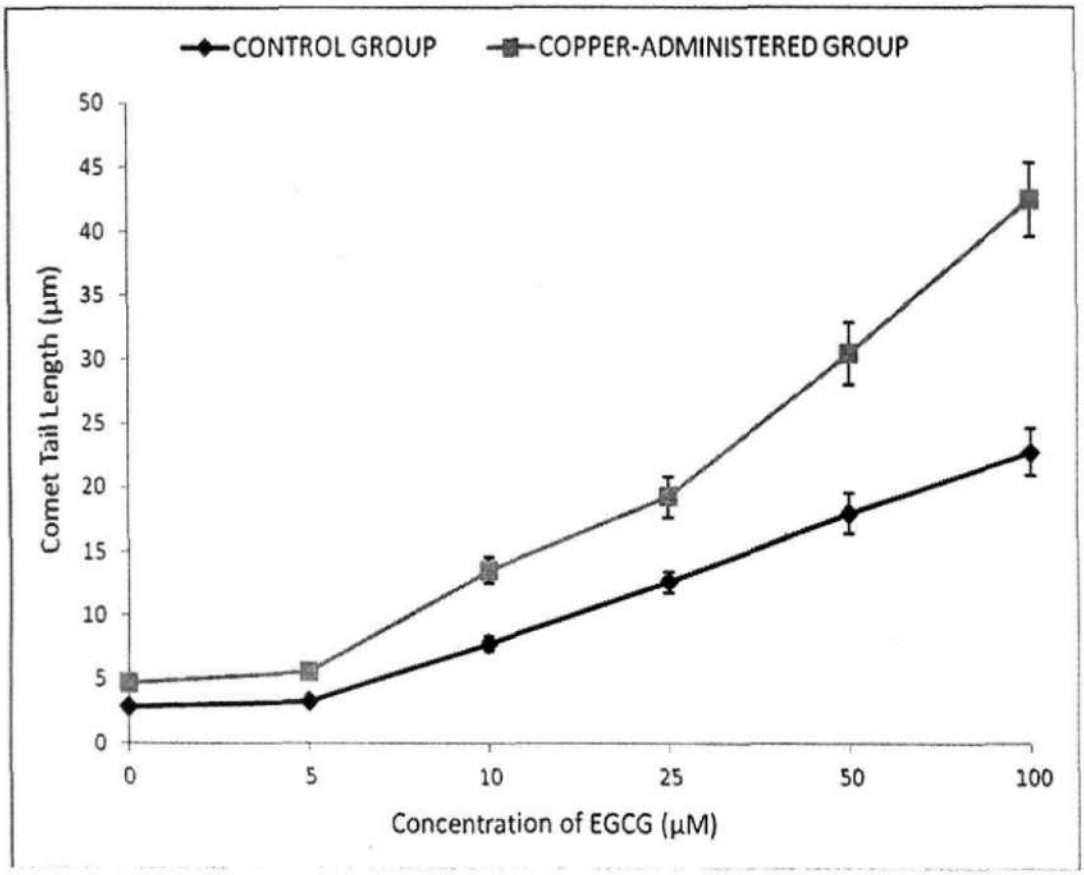
Treatment	Control Group Tail Length (µm)	Copper Administered Group Tail Length (µm)
Untreated (Control)	3.47 ± 0.22$^{\#}$	6.12 ± 0.47†
Resveratrol	16.62 ± 0.93*	25.72 ± 1.59**
Genistein	24.55 ± 1.64*	36.21 ± 2.34**
EGCG	22.36 ± 1.86*	31.8 ± 1.97**

Lymphocytes from control rats (gavaged drinking water) and copper overloaded rats (gavaged 30 mg/Kg b.w. $CuCl_2$) were treated with the mentioned polyphenols at a concentration of 50 µM for one hour at 37ºC.

Values reported are Mean ± S.E.M. Both groups had 5 animals each.

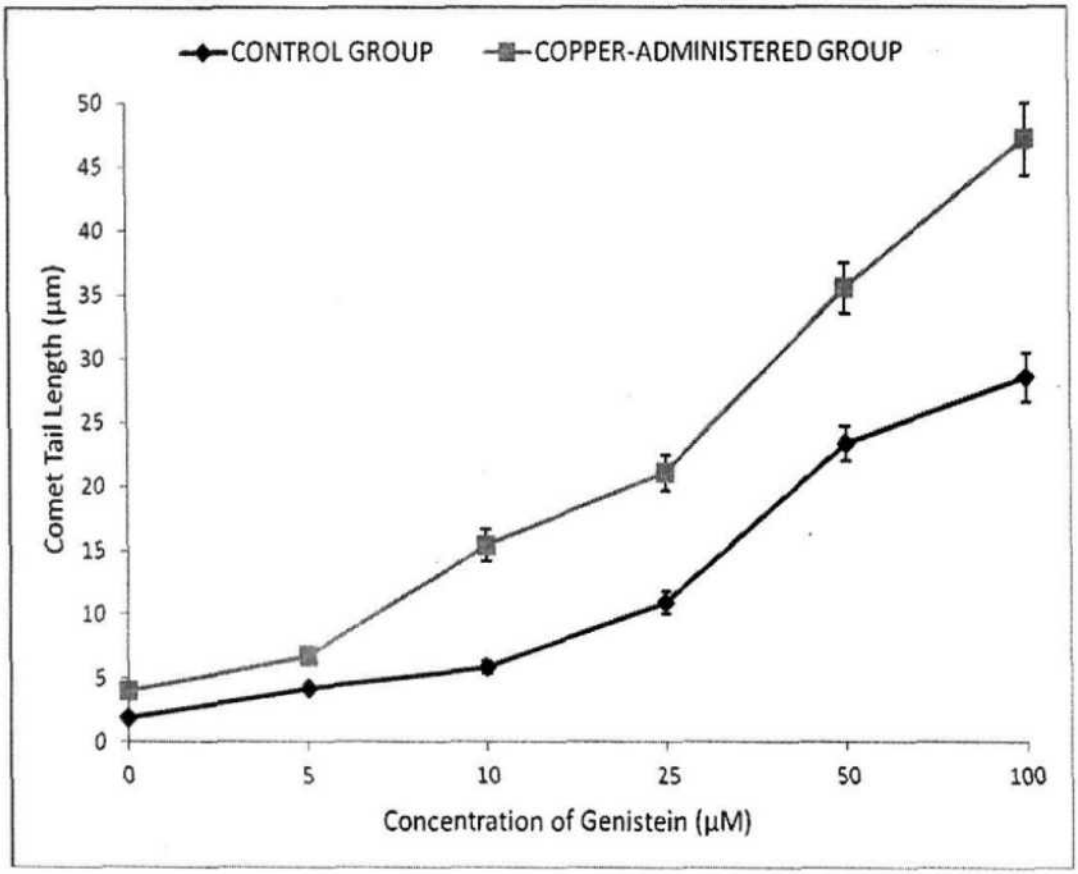
*Values are significant when compared with control # at $P < 0.05$. **Values are significant when compared with control † at $P < 0.05$. Mean values of the *control group were compared with **treated group and found to be significant at $P < 0.05$

Figure 25: Cellular DNA breakage induced by polyphenol EGCG at different concentrations in lymphocytes isolated from copper-administered rats



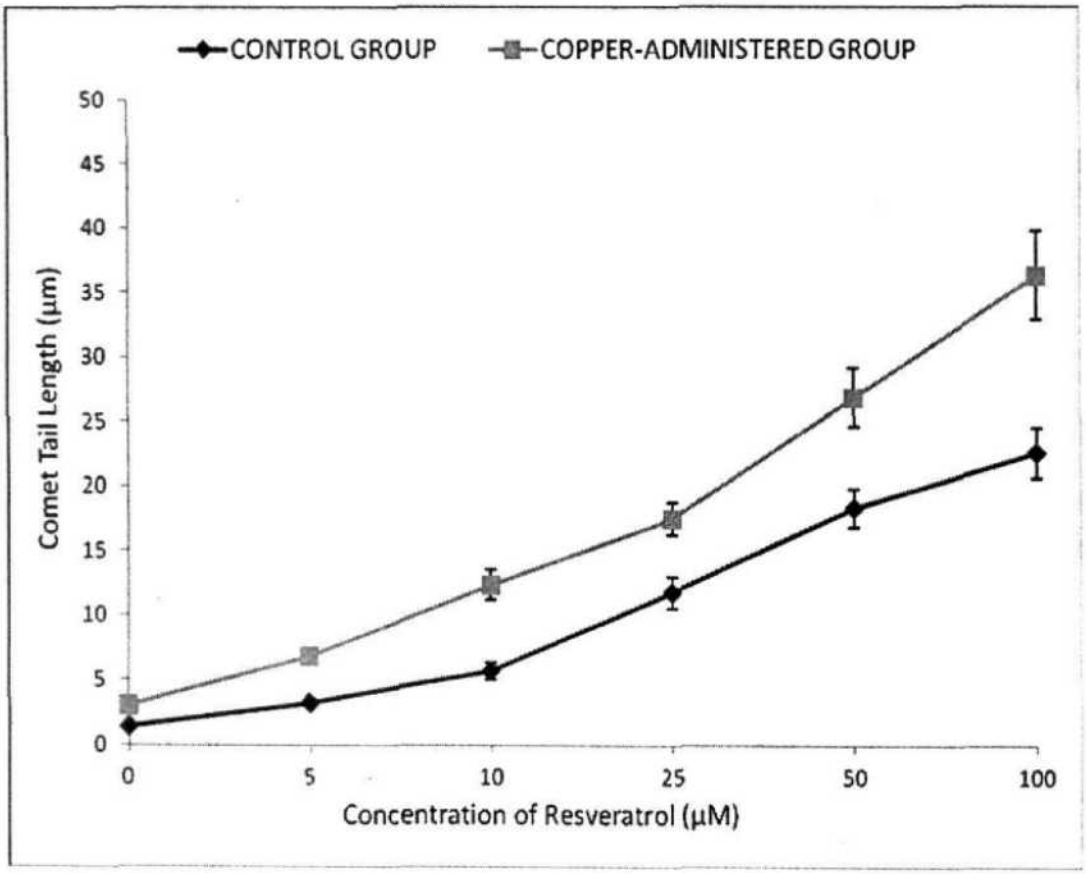
Cellular DNA breakage induced by different concentrations of EGCG in lymphocytes isolated from Control (gavaged drinking water) and copper-administered rats (gavaged 30 mg/Kg b.w. $CuCl_2$). Isolated lymphocytes were treated with increasing concentrations of EGCG as indicated in the figure for one hour.

Figure 26: Cellular DNA breakage induced by polyphenol genistein at different concentrations in lymphocytes isolated from copper-administered rats



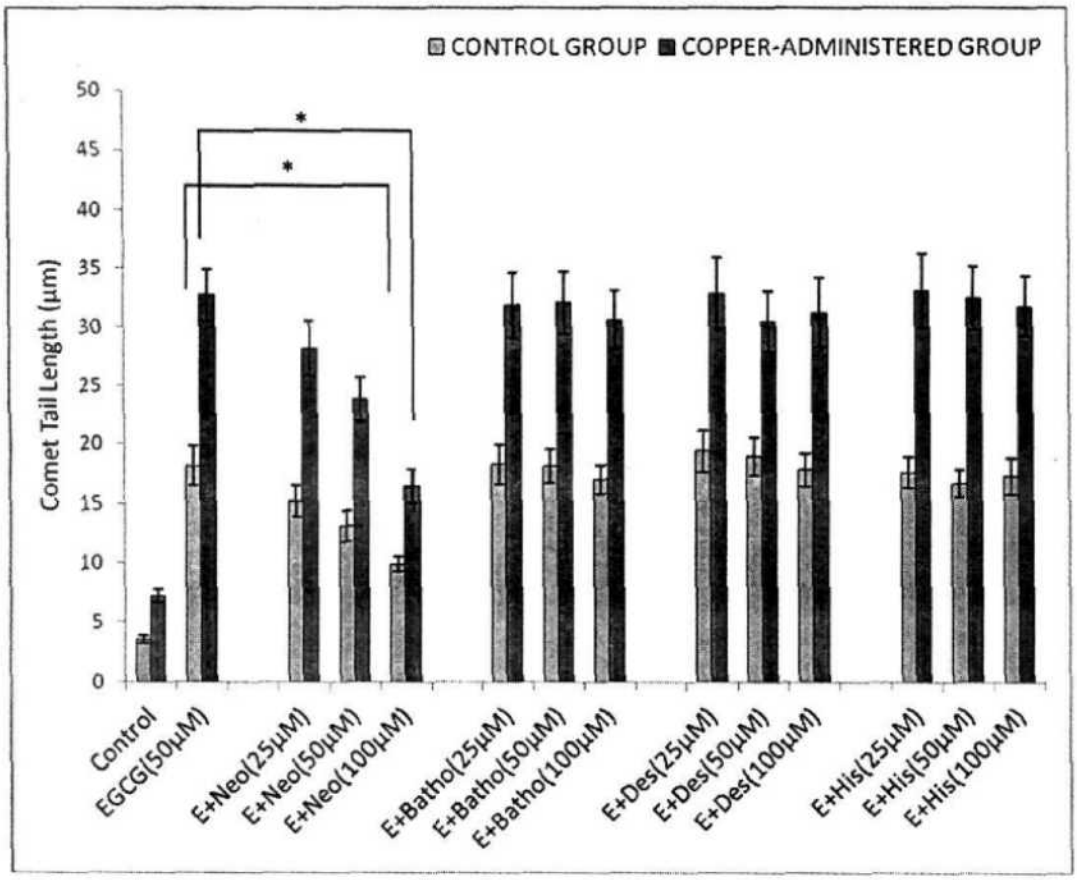
Cellular DNA breakage induced by different concentrations of genistein in lymphocytes isolated from Control (gavaged drinking water) and copper-administered rats (gavaged 30 mg/Kg b.w. $CuCl_2$). Isolated lymphocytes were treated with increasing concentrations of Genistein as indicated in the figure for one hour

Figure 27: Cellular DNA breakage induced by resveratrol at different concentrations in lymphocytes isolated from copper-administered rats



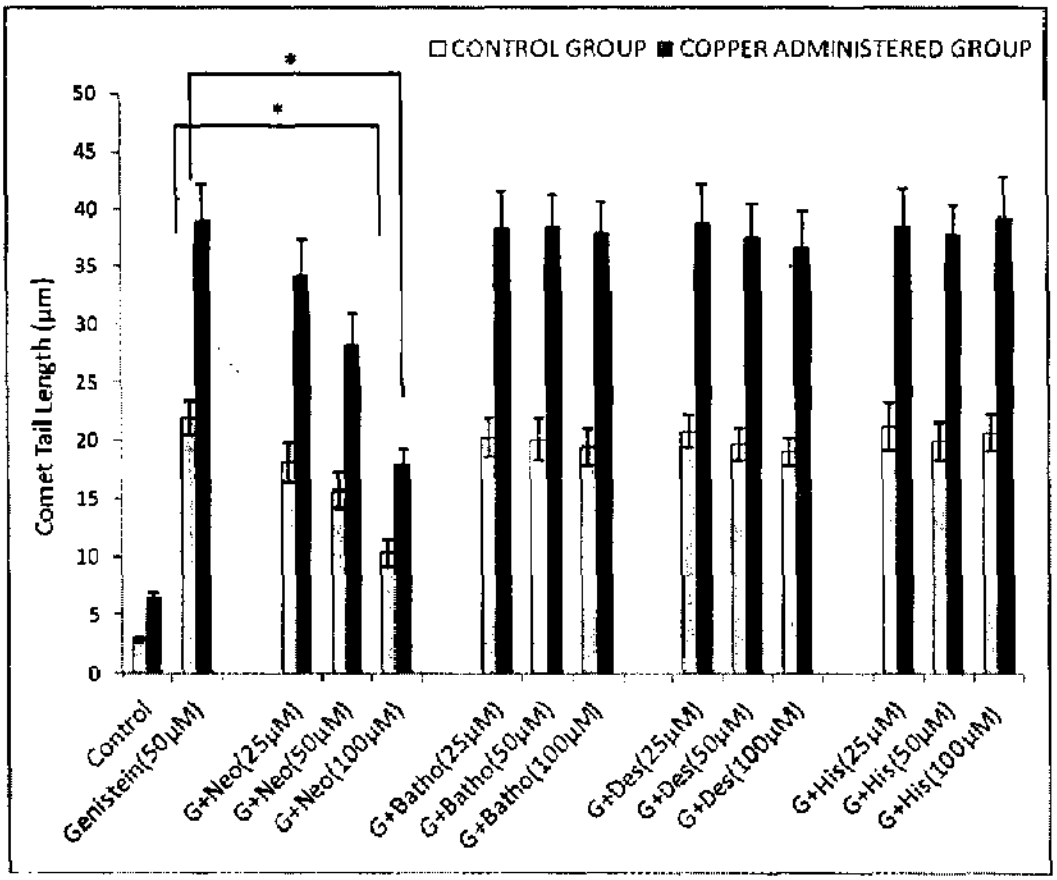
Cellular DNA breakage induced by different concentrations of resveratrol in lymphocytes isolated from Control (gavaged drinking water) and copper-administered rats (gavaged 30 mg/Kg b.w. $CuCl_2$). Isolated lymphocytes were treated with increasing concentrations of Resveratrol as indicated in the figure for one hour.

Figure 28: Effect of preincubating the lymphocytes isolated from copper-administered rats with metal chelators on cellular DNA breakage induced by EGCG



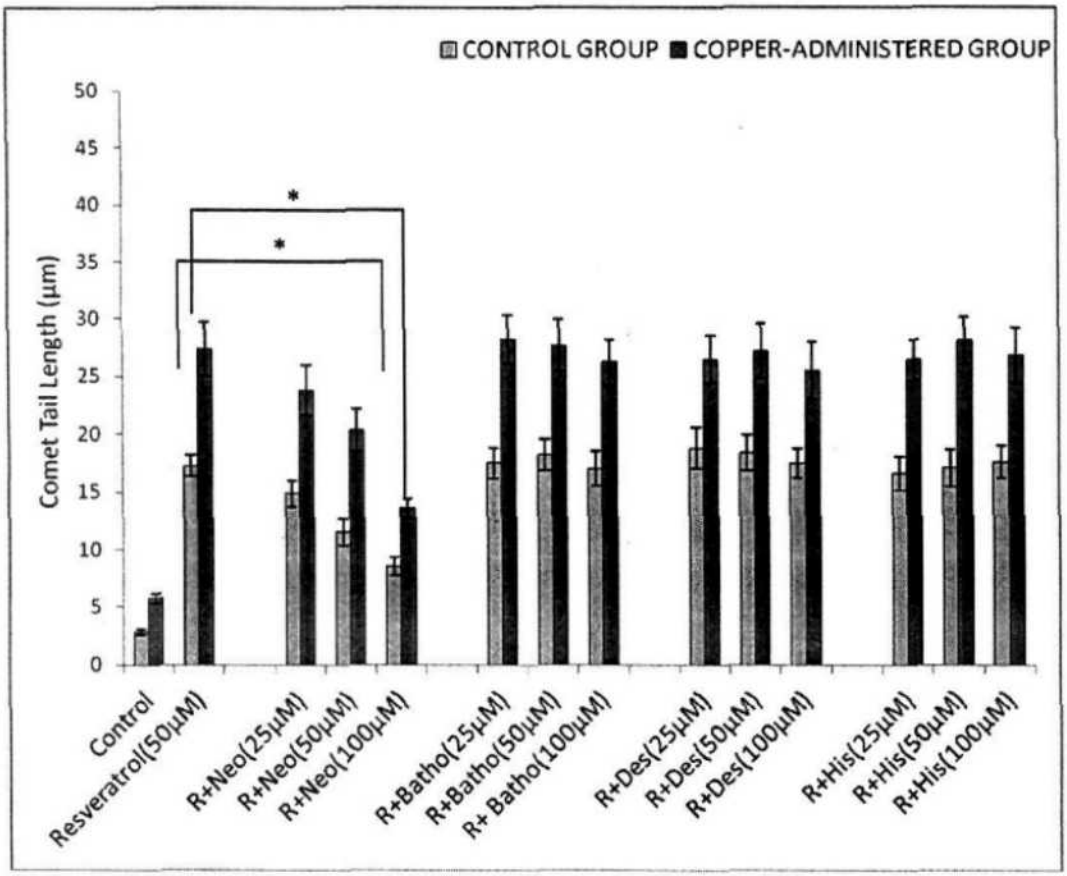
The lymphocytes were preincubated at 37ºC for 30 min and subsequently treated with EGCG (50 µM) and subjected to comet assay as described in methods. The three different concentrations of neocuproine, bathocuproine, desferroxamine mesylate and histidine as indicated were used for preincubation. * $P < 0.01$

Figure 29: Effect of preincubating the lymphocytes isolated from copper-administered rats with metal chelators on cellular DNA breakage induced by genistein



The lymphocytes were preincubated at 37ºC for 30 min and subsequently treated with genistein (50 µM) and subjected to comet assay as described in methods. The three different concentrations of neocuproine, bathocuproine, desferroxamine mesylate and histidine as indicated were used for preincubation. * $P < 0.01$

Figure 30: Effect of preincubating the lymphocytes isolated from copper-administered rats with metal chelators on cellular DNA breakage induced by resveratrol



The lymphocytes were preincubated at 37ºC for 30 min and subsequently treated with resveratrol (50 µM) and subjected to comet assay as described in methods. The three different concentrations of neocuproine, bathocuproine, desferroxamine mesylate and histidine as indicated were used for preincubation. * $P < 0.01$

Table VIII: Effect of scavengers of ROS on EGCG-induced DNA breakage in lymphocytes isolated from copper-administered rats

Lymphocyte Treatment	Tail length (μm)	% Inhibition
Control (Untreated)	2.41 ± 0.17#	-
+ EGCG (50 μM)	20.04 ± 0.81*	-
+ EGCG + SOD (100 μg/ml)	9.39 ± 0.36*	60.41
+ EGCG + Catalase (100 μg/ml)	10.96 ± 0.53*	51.5
+ EGCG + Thiourea (1 mM)	12.52 ± 0.78*	42.65
Copper-administered (Untreated)	5.15 ± 0.30†	-
+ EGCG (50 μM)	34.73 ± 1.35**	-
+ EGCG + SOD (100 μg/ml)	13.38 ± 0.69**	72.17
+ EGCG + Catalase (100 μg/ml)	11.67 ± 0.74**	77.96
+ EGCG + Thiourea (1 mM)	15.66 ± 1.09**	64.47

Rats were orally administered $CuCl_2$ (30 mg/kg b.w.) and lymphocytes were isolated 12 hours later. Each group comprised of 5 rats.

* Mean values were significant when compared with control #, $P < 0.05$; ** mean values were significant when compared with control †, $P < 0.05$.

Table IX: Effect of scavengers of ROS on resveratrol-induced DNA breakage in lymphocytes isolated from copper-administered rats

Treatment	Tail length (μm)	% Inhibition
Control (Untreated)	2.41 ± 0.17#	-
Resveratrol (50 μM)	18.17 ± 0.96*	-
Res + SOD (100 μg/ml)	9.08 ± 0.22*	57.68
Res + Catalase (100 μg/ml)	11.62 ± 0.85*	41.56
Res + Thiourea (1 mM)	12.20 ± 0.79*	37.88
Copper-administered (Untreated)	5.15 ± 0.30†	-
+ Resveratrol (50 μM)	26.90 ± 1.22**	-
+ Res + SOD (100 μg/ml)	13.27 ± 1.07**	62.66
+ Res + Catalase (100 μg/ml)	10.86 ± 0.58**	73.75
+ Res + Thiourea (1 mM)	12.38 ± 0.89**	66.76

Rats were orally administered $CuCl_2$ (30 mg/kg b.w.) and lymphocytes were isolated 12 hours later. Each group comprised of 5 rats.

* Mean values were significant when compared with control #, $P < 0.05$; ** mean values were significant when compared with control †, $P < 0.05$.

DISCUSSION-II

The role of copper has been extensively studied in the etiology and growth of tumors (Goodman et al., 2004; Brewer, 2005). Such studies were based on reports that copper distribution is altered in tumor bearing mice, rats and humans (Semczuk and Pomykalski, 1973; Apelgot et al., 1986). Gupte and Mumper (2008) have reviewed several studies which indicate that both serum and tumor copper levels are significantly elevated in cancer patients compared to healthy subjects. Moreover, there are a number of studies that have focused on determining the levels of four important biological trace elements, namely copper, iron, zinc and selenium, in cancer patients. These studies showed that while iron, zinc and selenium concentrations were significantly lower in cancer patients, the copper levels were almost always found to be significantly elevated (up to two to three folds) compared to age-matched samples from normal tissue (Kuo et al., 2002; Zuo et al., 2006). However, the reason for an increased copper concentration in tumors is not clearly known.

We have earlier proposed that an important anticancer mechanism of plant-derived polyphenolic compounds could be the mobilization of endogenous copper ions and the consequent prooxidant action (Hadi et al., 2000). This is based on several lines of indirect evidence in literature and our own studies (Hadi et al., 2007). Using intact lymphocytes and isolated nuclei from these cells, we have established that plant polyphenols are able to mobilize chromatin bound copper leading to redox cycling of copper ions (Shamim et al., 2008). In the presence of molecular oxygen such a reaction leads to the formation of ROS such as hydroxyl radical, causing DNA cleavage. Further, we have suggested that the preferential cytotoxicity of plant polyphenols towards cancer cells is explained by the observation made several years earlier which showed that serum (Margalioth et al., 1987; Ebadi and Swanson, 1998), tissue (Yoshida et al., 1993) and intracellular copper levels in cancer cells (Ebara et

al., 2000) are significantly increased in various malignancies. Indeed, such levels have been described as a sensitive index of disease activity of several hematologic and non-hematologic malignancies (Pizzolo et al., 1978).

Since cancer cells contain elevated levels of copper, they may be more subject to electron transfer with polyphenols (Zheng et al., 2006) to generate ROS. In normal cells, there exists a balance between the free radical generation and the antioxidant system (Devi et al., 2000). However, it has been clearly documented that tumor cells are under persistent oxidative stress and have an altered antioxidant defense (Powis and Baker, 1997; Pervaiz and Clement, 2004) and thus any further ROS stress in these malignant cells, surpassing a threshold level, could result in apoptosis (Gupte and Mumper, 2008). These observations suggest that neoplastic cells may be more vulnerable to oxidative stress as they function with a heightened basal level of ROS owing to increased rate of growth and metabolism (Kong et al., 2000). Thus, in cancer cells, a further enhanced exposure to ROS, generated through the redox cycling of intracellular copper by polyphenols, can overwhelm the cells antioxidant capacity, leading to irreversible damage and apoptosis. On the other hand, normal non-malignant cells can better tolerate such an action due to their low basal ROS output and normal metabolic regulation, as also normal copper redox status. Hence, a disparity in the redox states of cancer cells and normal cells may provide a molecular basis for selective killing of cancer cells by the use of agents like polyphenols, that can cause further ROS insults to the malignant cells. Therefore, we propose that this accounts for the preferential cytotoxicity of plant polyphenols toward cancer cells. Indeed we have recently shown that the polyphenol genistein mediated apoptotic cell death and cell proliferation inhibition in MDA-MB-231 and MDA-MB-468 breast cancer cell lines is inhibited by the copper chelator neocuproine, whereas iron and zinc chelators have little effect (Ullah et al., 2011).

In order to further substantiate our hypothesis, through the studies presented in this chapter, we have attempted to elevate copper levels in lymphocytes by administering cupric chloride to rats to a range similar to what has been reported in leukemic cancer patients (Carpentieri et al., 1986) [31.28±2.55 µg Cu/10^6 cells (Table V) Vs 52±16 µg Cu/10^6 cells (in leukemia patients)]. It must be mentioned that copper itself is known to be cytotoxic and leads to cellular DNA cleavage. However as shown in the results the levels of copper achieved in rat lymphocytes on cupric chloride administration does not lead to a significant increase in comet tail lengths as compared to the untreated controls (Figure 23). More importantly, the addition of polyphenols (EGCG and genistein) causes several fold increase in comet tail lengths, indicating that the contribution of copper alone to cellular DNA breakage is insignificant. Thus the above results taken together lead to the conclusion that the elevated copper levels in lymphocytes, such as those found in cancer patients could be an important factor in the anticancer mechanism of plant polyphenols.

Since the hypothesis proposed by our group (Hadi et al., 2000), involving mobilization of endogenous copper ions leading to prooxidant cellular DNA breakage by plant polyphenols, we have made considerable progress in substantiating our idea. The various achievements made in this context have been listed in the "Scope of the work presented' section. In the present chapter, we have shown that oral administration of copper to rats leads to elevated levels of copper in plasma and in lymphocytes and that this contributes to increased lymphocyte cellular DNA degradation by polyphenols. This further supports the role of elevated copper levels in cancer tissue and cells in the anticancer mechanism of plant polyphenols.

Chapter - III

Plant Polyphenols induce cell death in human cancer cells in culture through mobilization of intracellular copper and ROS generation

RESULTS-III

Apigenin and luteolin inhibit growth and induce apoptosis in different types of cancer cells:

In order to examine cancer cell growth inhibition by flavones, besides normal breast epithelial cells (MCF10A), cells from three different cancer cell lines, namely MDA-MB-468 (breast), BxPC-3 (pancreatic) and PC3 (prostate), were subjected to treatment with varying concentrations of apigenin and luteolin in a MTT assay. As can be seen from the results given in figure 31, both apigenin and luteolin caused a clear concentration dependent inhibition of growth in all the three types of human cancer cells used. However, normal epithelial MCF10A cells were found to be relatively resistant to this growth inhibition by both the flavones at the same concentrations. Further, the induction of apoptosis in these cancer cell lines by apigenin and luteolin was assayed by Histone/DNA ELISA. This analysis indicates that the cytotoxicity induced by apigenin and luteolin was mainly due to apoptosis, which increased with the concentration of the compounds (Figure 32). In these results luteolin was found to be more effective as a growth inhibitor as well as apoptosis inducer of the cancer cell lines used. These results are in agreement with other studies that have shown apigenin and luteolin to possess anticancer activity against various other cancer cell lines (Yin et al., 1999; Kilani-Jaziri et al., 2012).

Apigenin and luteolin induced antiproliferation and apoptosis in cancer cells is inhibited by copper chelator but not by iron and zinc chelators:

In the previous chapter, it has been shown that membrane permeable copper chelator neocuproine is able to inhibit the polyphenol induced oxidative breakage of cellular DNA (Chapter II), suggesting the involvement of endogenous copper in the process. The results shown in figure 33, indicate that only copper chelator neocuproine was able to protect the MDA-MB-468,

BxPC-3 and PC3 cancer cells to a significant extent against the growth inhibitory action of apigenin and luteolin. On the other hand, desferroxamine and histidine (iron and zinc chelarors, respectively) failed to show such effect to any significant degree, except in case of PC3 cells where desferroxamine and histidine also showed some protective effect on luteolin-induced growth inhibition. However, this was still less than that with neocuproine. Further the effect of various metal chelators was also tested against the apigenin/luteolin-induced apoptosis. As given in figure 34, copper chelator neocuproine showed a protective effect to a significant degree whereas such protection was not observed to an appreciable extent when either iron or zinc chelator was used, thus confirming the conclusion that anticancer mechanism of the flavones apigenin and luteolin involves mobilization of endogenous copper.

Apigenin and luteolin limit the cancer cell proliferation in a clonogenic assay:

Figure 35 gives the result of a clonogenic assay that was performed to support the above findings which demonstrated that the antiproliferative activity of apigenin and luteolin in cancer cells involve endogenous copper (Figure 33). Clonogenic or colony formation assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. The assay is designed to assess a cell's ability to grow unattached to a surface and it is a method of choice to determine cell's reproductive death after treatment with a cytotoxic agent. As shown in figure 35, treatment of breast, pancreatic and prostate cancer cells with apigenin and luteolin resulted in the reduction of anchorage independent colonies. However, the presence of copper chelator neocuproine nullifies the effect of these flavones leading to cancer cell survival as the number of colonies were found to be closer to the number of colonies in the control (in the absence of apigenin/luteolin).

Apoptosis of cancer cells induced by apigenin and luteolin is mediated by ROS:

As shown in chapter II, the DNA breakage induced by polyphenols in lymphocytes is mediated by the generation of ROS. In order to verify the results in cancer cell lines, the effect of various scavengers of ROS such as catalase, thiourea and SOD on apigenin and luteolin induced apoptosis in three different types of cancer cells was studied. As can be seen from Table X, all three ROS scavengers caused moderate to considerable suppression of apigenin/luteolin-induced apoptotic activity in the various cancer cell lines tested. This reaffirms the role of ROS as effectors of polyphenol-induced apoptosis. In other words, this itself establishes that the anticancer action of polyphenolic compounds involves a prooxidant pathway leading to cell death.

Anticancer action of EGCG and resveratrol also involves mobilization of endogenous copper and ROS generation:

In order to extend the above findings with some other polyphenolic compounds, EGCG and resveratrol were tested for their anticancer action against cancer cells and the role of endogenous copper and ROS in such an action was examined. Both EGCG and resveratrol showed growth inhibitory effects in two different prostate cancer cell lines - PC3 and C42B (Figure 36). Such an antiproliferative effect of these polyphenols was prevented to a significant extent when PC3 cancer cells were incubated with copper specific chelator neocuproine, while iron and zinc specific chelators failed to do so (Figure 37). Moreover, as shown in figure 38, PC3 cells supplemented with neocuproine were found to be protected from the EGCG/resveratrol-induced reduction in anchorage-independent colonies in a clonogenic assay. Further, different scavengers of ROS were able to cause significant inhibition in the EGCG/resveratrol-induced apoptosis of PC3 cancer cells (Table XI). All these

results taken together, suggest that the anticancer and apoptosis-inducing action of EGCG and resveratrol, similar to that of the flavones luteolin and apigenin, also involves the mobilization of endogenous copper and consequent prooxidant effect leading to cell death. Therefore, it may be concluded that this mechanism of anticancer action is a mechanism common to several polyphenolic compounds with diverse chemical structures.

Supplementation with copper sensitizes normal breast epithelial cells to antiproliferative action of polyphenols:

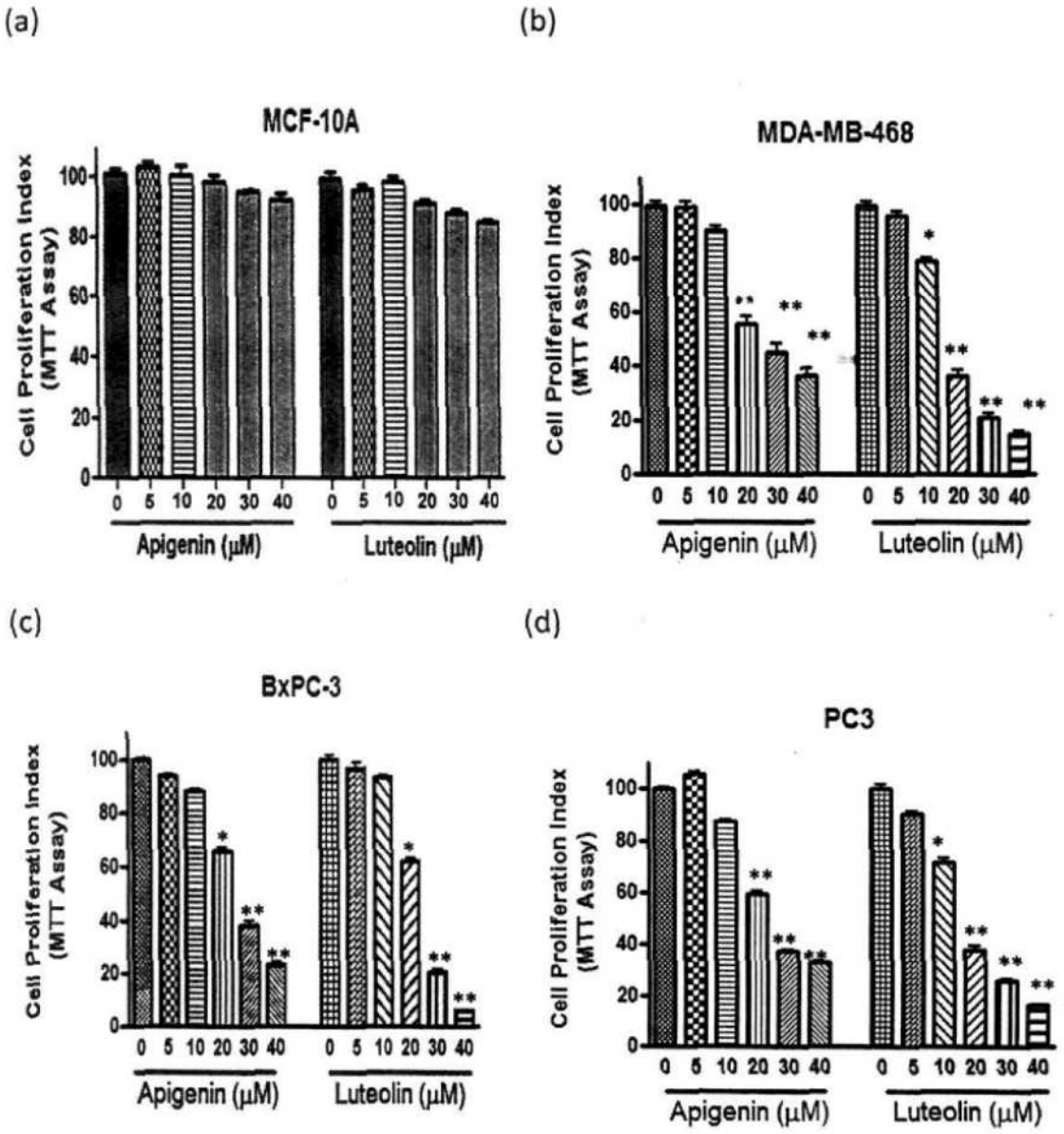
Normal breast epithelial cells, MCF-10A, were cultured in media supplemented with 25 μM copper. When such copper supplemented cells (MCF-10A+Cu) were treated with various polyphenolic compounds, a decline in cell proliferation was observed, which was significant in comparison to non-copper-supplemented MCF-10A cells (Figure 39 A). This indicates that supplementation with exogenous copper leads to sensitization of normal epithelial cells to polyphenol-induced cell growth inhibition. Furthermore, as shown in the blot in figure 39(B), copper supplemented MCF-10A+Cu cells have an increased expression of copper transporter (Ctr1) protein. Therefore, there appears a certain correlation between membrane bound Ctr1 expression and the sensitization to cell growth inhibition by polyphenols in normal cells cultured with copper.

Polyphenol-induced ROS generation is inhibited by copper chelator neocuproine as visualized by confocal microscopy:

Using Image-iT LIVE Green ROS detection kit (Molecular Probes) and confocal microscopy, ROS generation induced by the polyphenol luteolin in breast cancer cells, was detected. M0A-MB-231 cells treated with luteolin (75 μM) showed production of ROS (green fluorescence) in and around the DPAI stained cell nuclei as shown in figure 40. This ROS generation was, however,

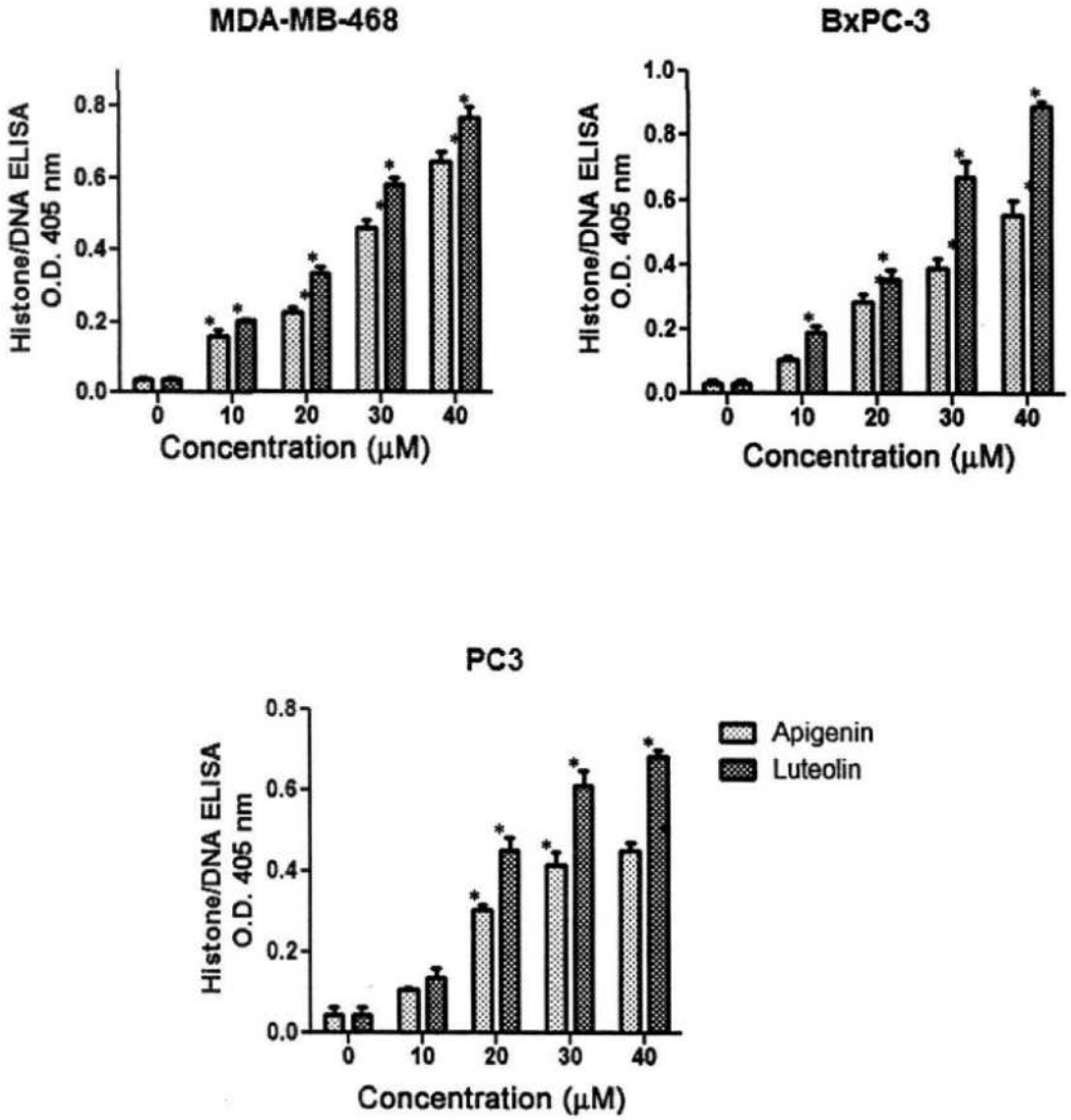
significantly reduced in cells that were incubated with luteolin in the presence of copper-specific chelator neocuproine. Therefore, this observation further confirms our proposition that it is the mobilization of endogenous copper by polyphenols that leads to ROS formation in the cell.

Figure 31: Effect of apigenin and luteolin on cell proliferation in normal breast epithelial cells and various cancer cell lines as detected by MTT assay



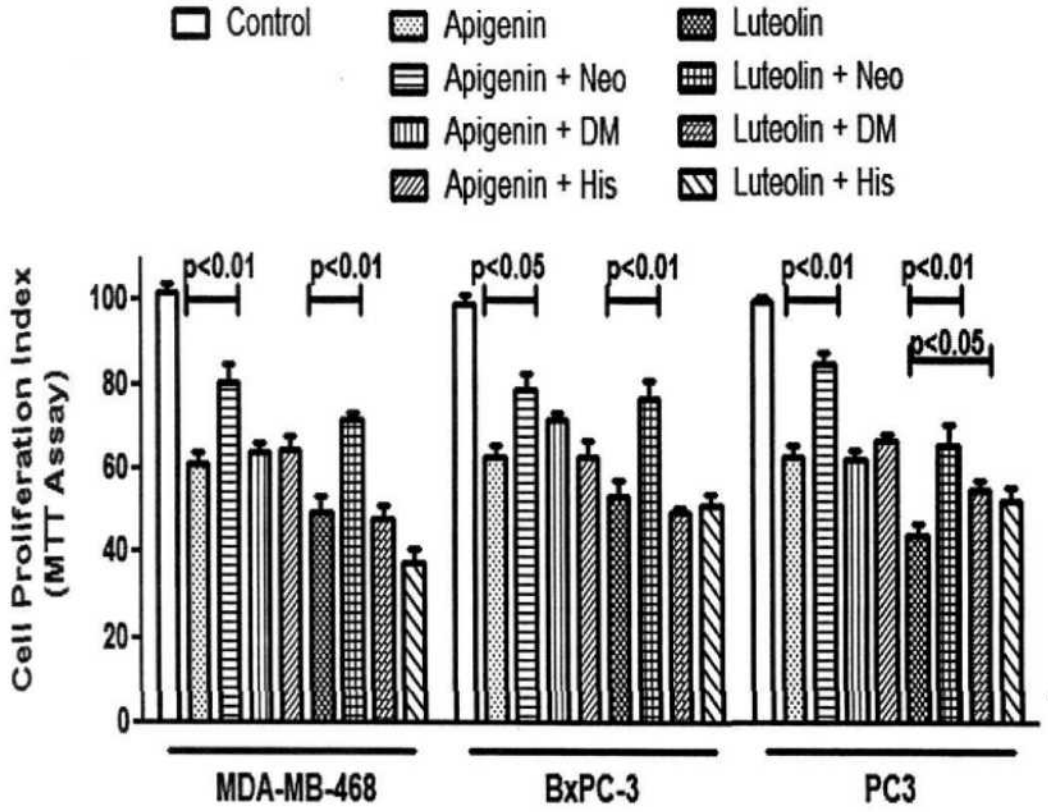
Cells from (a) MCF10A (normal breast cell line) and (b) MDA-MB-468, (c) BxPC-3, (d) PC3 cancer cell lines were incubated with indicated concentrations of apigenin and luteolin for 72 hours. The effect on cell proliferation was detected by performing MTT assay as described in 'Methods'. All results are expressed as percentage of control ±S.E of triplicate determinations. *$p < 0.05$ and **$p < 0.01$ when compared to respective untreated control.

Figure 32: Apoptosis induction by apigenin and luteolin in different cancer cell lines



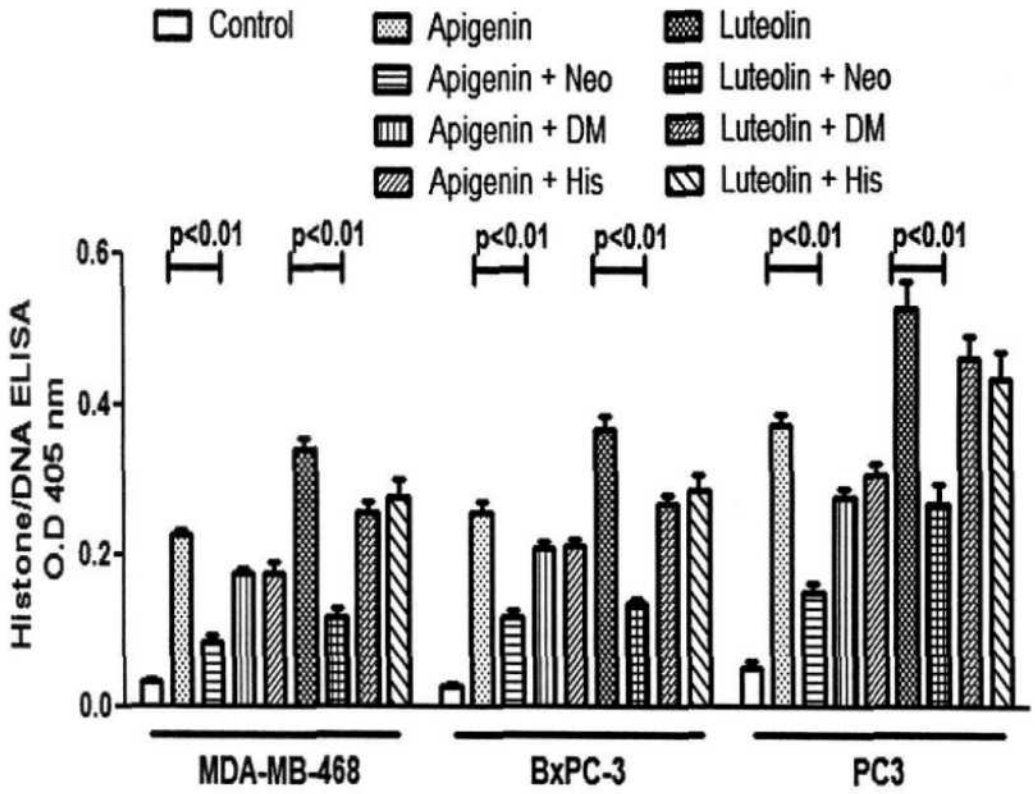
The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in cells from different cancer cell lines after incubation for 72 hours with increasing concentrations of apigenin and luteolin as indicated in the figure and described in 'Methods'. Values reported are ±S.E of three independent experiments. *p value < 0.01 when compared to control.

Figure 33: Effect of various metal-specific chelators on the antiproliferative activity of apigenin and luteolin in three different cancer cell lines



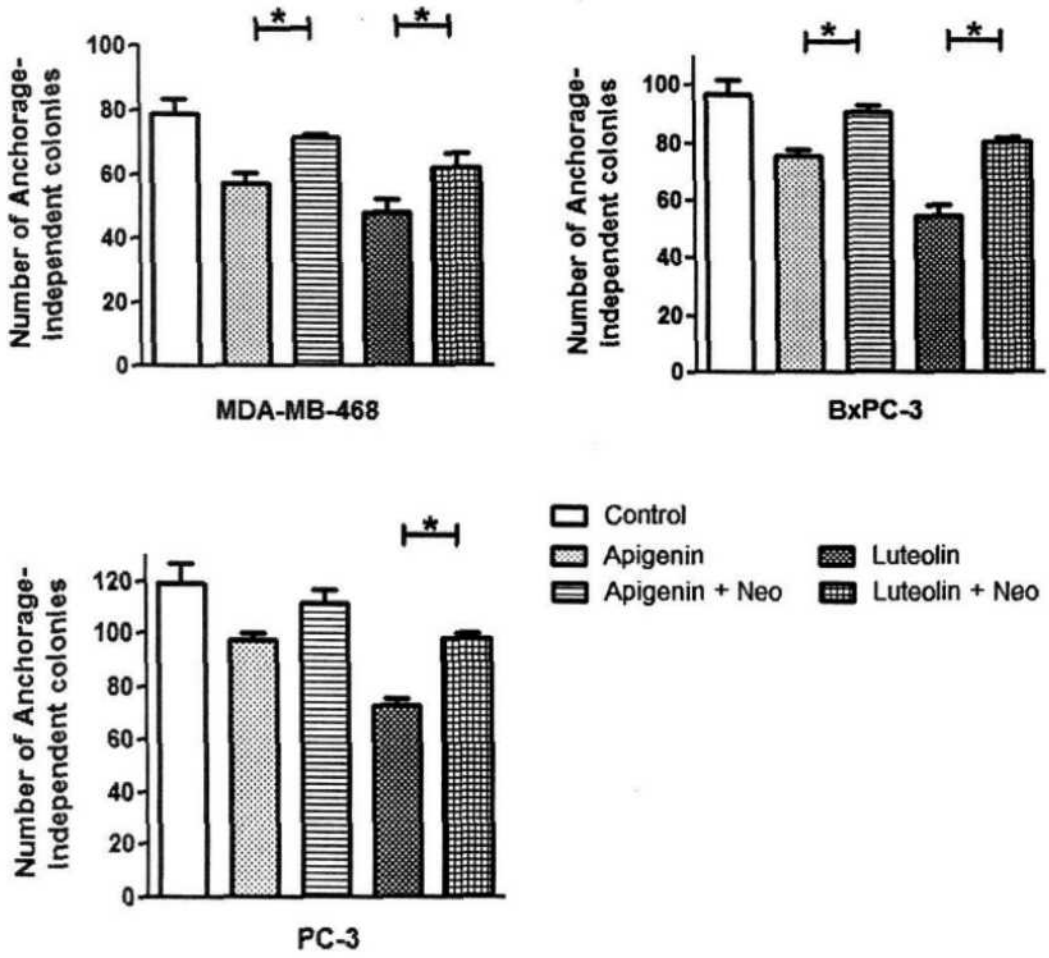
MDA-MB-468, BxPC-3 and PC3 cancer cells were treated with 20 μM of apigenin/ luteolin either alone or in the presence of copper chelator neocuproine (Neo), iron chelator desferrioxamine mesylate (DM) or zinc chelator histidine (His) as indicated in the figure. The concentration of metal chelators used was 50 μM. MTT assay was performed after 72 hours of treatment as described in 'Methods'. Values reported are ±S.E of three independent experiments.

Figure 34: Effect of different metal chelators on apoptosis induction by apigenin and luteolin in three different cancer cell lines



The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in MDA-MB-468, BxPC-3 and PC3 cancer cells treated with apigenin/ luteolin (20 μM) in the absence and presence of copper chelator neocuproine (Neo), iron chelator desferrioxamine mesylate (DM) or zinc chelator histidine (His) as indicated in the figure and described in 'Methods'. The concentration of metal chelators used was 50 μM and time of incubation was 72 hours. Values reported are ±S.E of three independent experiments.

Figure 35: Effect of copper chelator neocuproine on the clonogenic potential of cancer cells treated with apigenin and luteolin in a clonogenic assay.



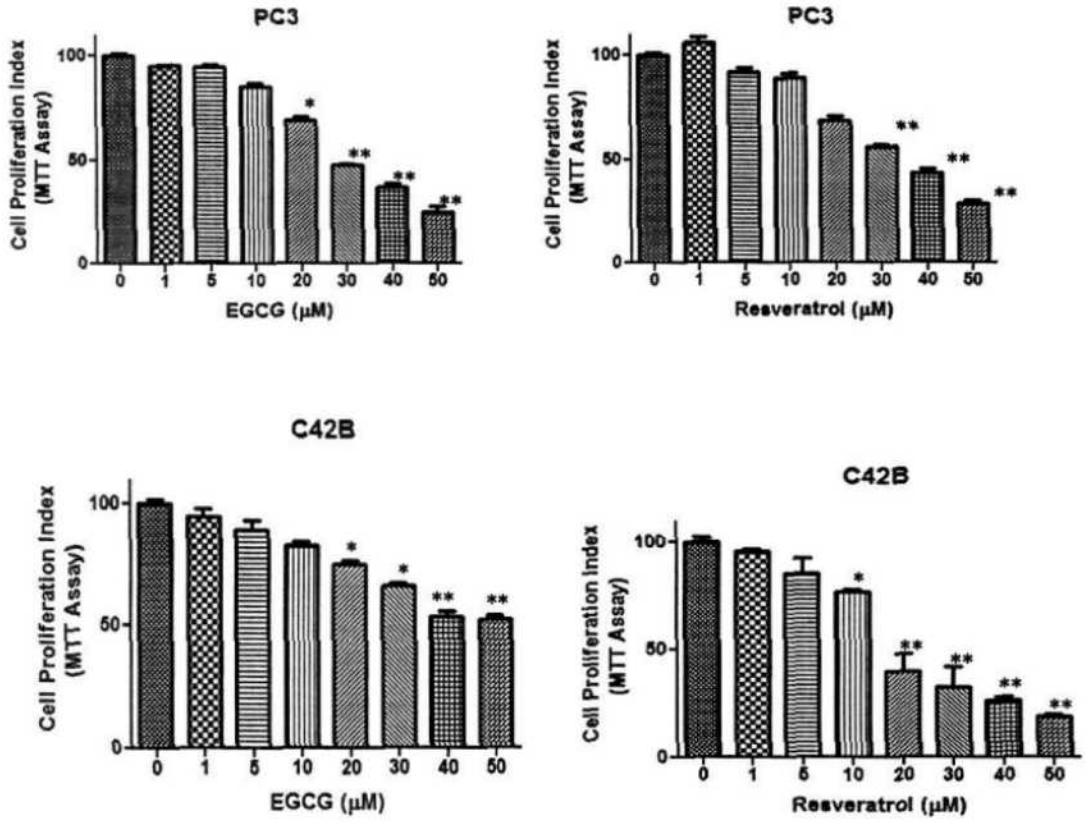
Breast, pancreatic and prostate cancer cells (3 x 10^4) were plated in 24-well plates as described in 'Methods'. Cultures were supplemented with apigenin/ luteolin at a concentration of 20 μM with or without 50 μM of copper chelator (Neo) as indicated in the figure. After appropriate culture time (22 days), colonies (>50 cells) were counted. Experiments were carried out in quadruplicate and mean values are reported. *$p < 0.05$

Table X: Effect of ROS scavengers on apigenin and luteolin-induced apoptotic activity in three different cancer cell lines

Cell Lines		Apoptosis (folds)	% Inhibition of apoptosis
MDA-MB-468	Untreated	-	
	Apigenin	2.7	
	+TU	2.01	25.56
	+SOD	1.46	45.93
	+Cat	1.87	30.74
	Luteolin	3.9	
	+TU	1.87	52.05
	+SOD	2.56	34.36
	+Cat	2.23	42.82
BxPC-3	Untreated	-	
	Apigenin	3.2	
	+TU	1.99	37.81
	+SOD	2.33	27.19
	+Cat	2.68	16.26
	Luteolin	4.4	
	+TU	1.73	60.68
	+SOD	2.67	39.32
	+Cat	2.19	50.23
PC3	Untreated	-	
	Apigenin	4.8	
	+TU	2.41	49.79
	+SOD	2.76	42.5
	+Cat	3.13	34.79
	Luteolin	5.5	
	+TU	2.5	54.54
	+SOD	2.9	47.27
	+Cat	3.12	43.27

Along with apigenin and luteolin at a concentration of 20 µM, cancer cells were incubated with various ROS scavengers, namely TU, 700 mM Thiourea; SOD, 100 mg/mL superoxide dismutase; and Cat, 100 mg/mL catalase. Effect on apoptosis was assessed using Histone/DNA ELISA as described in 'Methods'. 'Apoptosis (folds)' is the fold increase in apoptosis relative to untreated control at $p < 0.05$.

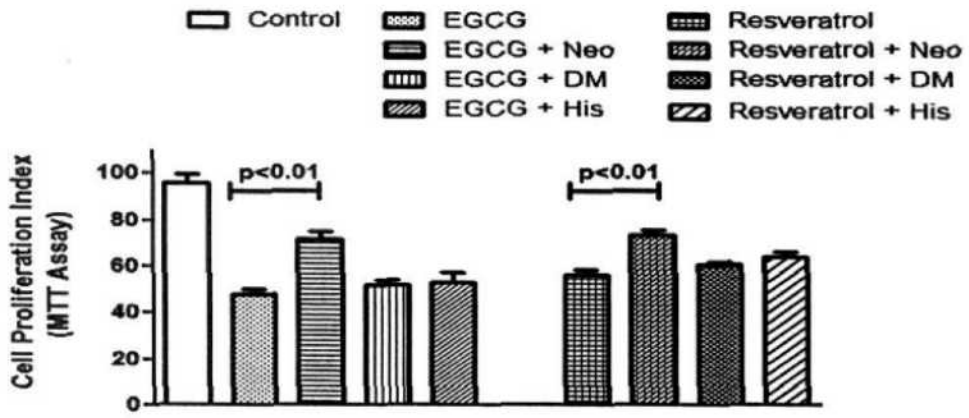
Figure 36: Effect of EGCG and resveratrol on cell proliferation of two different prostate cancer cell lines as detected by MTT assay



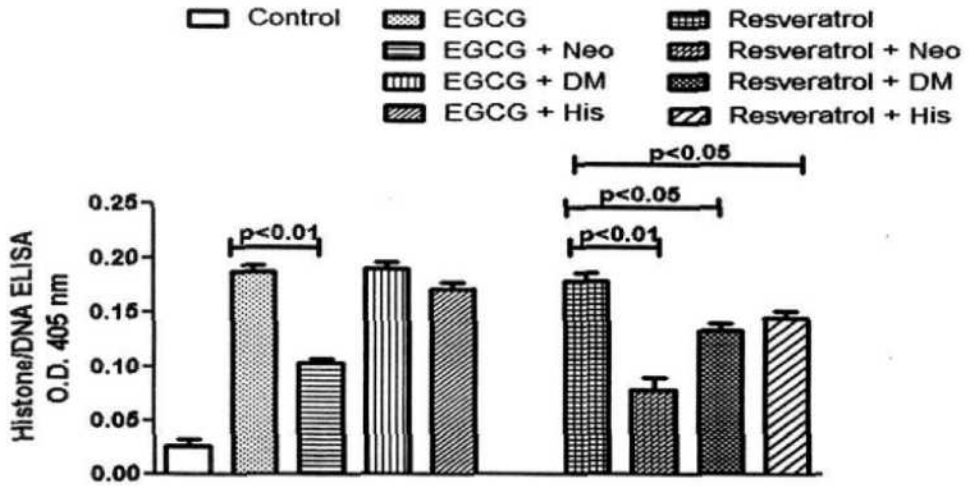
Prostate cancer cell lines PC3 and C42B were treated with increasing concentrations of EGCG/ resveratrol for 72 hours. The effect on cell proliferation was detected by performing MTT assay as described in 'Methods'. All results are expressed as percentage of control ±S.E of triplicate determinations from three independent experiments. *p < 0.05 and **p < 0.01 when compared to respective untreated control (0 μM polyphenol).

Figure 37: Effect of various metal chelators on (A) antiproliferative activity and (B) apoptosis induction by EGCG and resveratrol in PC3 prostate cancer cell line

(A)

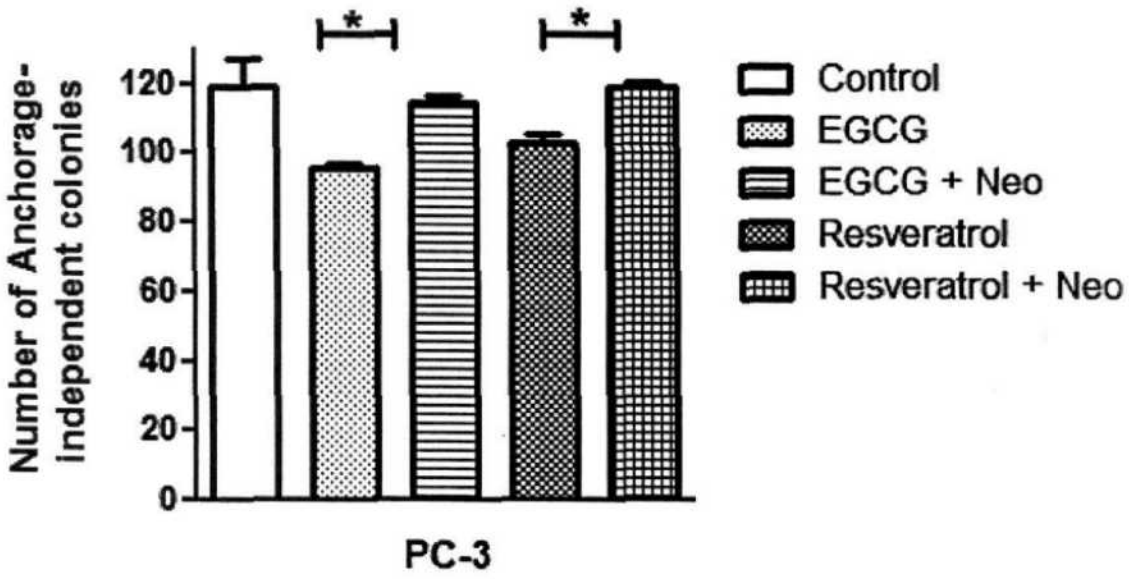


(B)



PC3 prostate cancer cells were treated with EGCG/ resveratrol (30 μM) in the absence and presence of copper chelator neocuproine (Neo), iron chelator desferrioxamine mesylate (DM) or zinc chelator histidine (His) as indicated in the figure. MTT assay and Histone/DNA ELISA were used to analyze (A) cell proliferation and (B) apoptosis induction, respectively as described in 'Methods'. The concentration of metal chelators used was 50 μM and time of incubation was 72 hours. Values reported are ±S.E of three independent experiments.

Figure 38: Effect of copper chelator neocuproine on the clonogenic potential of PC3 prostate cancer cells treated with EGCG and resveratrol in a clonogenic assay



PC3 cancer cells (3×10^4) were plated in 24-well plates as described in 'Methods'. Cultures were supplemented with EGCG/ resveratrol at a concentration of 30 µM with or without 50 µM of copper chelator (Neo) as indicated in the figure. After appropriate culture time (22 days), colonies (>50 cells) were counted. Experiments were carried out in quadruplicate and mean values are reported. *$p < 0.05$

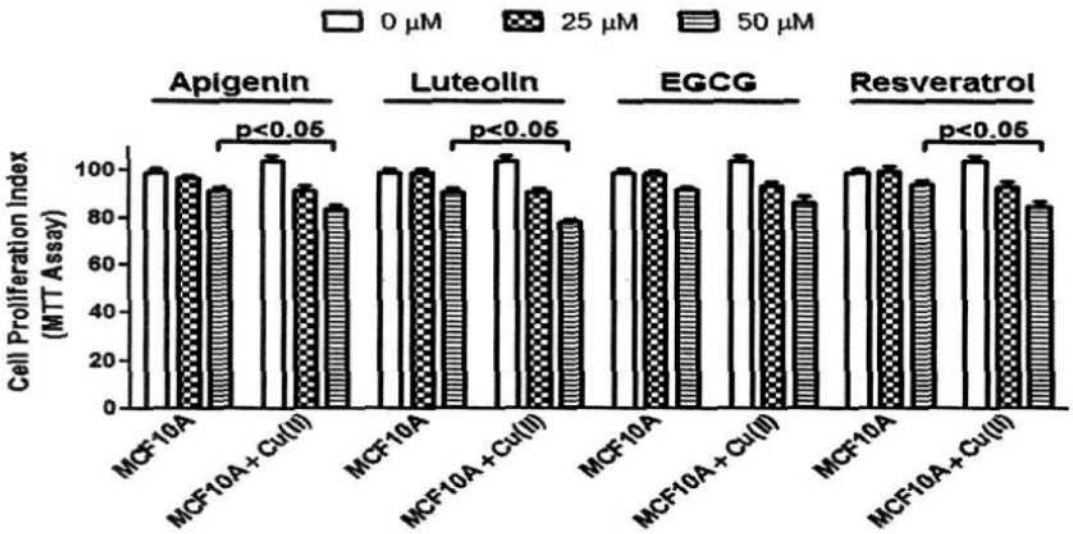
Table XI: Effect of ROS scavengers on EGCG and resveratrol induced apoptotic activity in PC3 cancer cells

	Apoptosis (folds)	% Inhibition of apoptosis
Untreated	-	
EGCG	2.3	
+TU	1.05	54.35
+SOD	1.39	39.57
+Cat	1.45	36.96
Resveratrol	2.1	
+TU	1.49	29.05
+SOD	1.48	29.52
+Cat	1.1	47.62

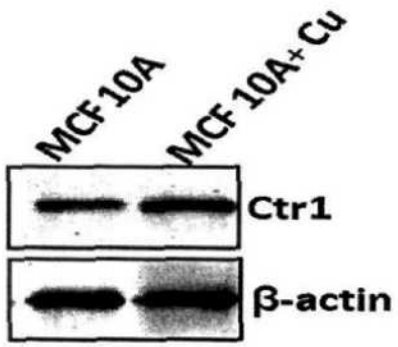
Along with of EGCG and resveratrol at a concentration of 30 μM, cancer cells were incubated with various ROS scavengers, namely TU, 700 mM Thiourea; SOD, 100 mg/mL superoxide dismutase; and Cat, 100 mg/mL catalase. Effect on apoptosis was assessed using Histone/DNA ELISA as described in 'Methods'. 'Apoptosis (folds)' is the fold increase in apoptosis relative to untreated control at $p < 0.05$.

Figure 39: (A) Effect of apigenin, luteolin, EGCG and resveratrol on inhibition of cell proliferation in MCF10A (normal breast epithelial cells) and MCF10A cells cultured in media supplemented with Cu(II) (MCF10A+Cu). (B) Effect of Cu(II) supplementation on Ctr1 expression in breast epithelial cells

(A)



(B)



Both MCF10A and MCF10A+Cu (normal cells cultured in a medium containing 25 μM $CuCl_2$) were subjected to treatment with various polyphenolic compounds for 72 hours at two different concentrations as indicated in the figure. Cell proliferation was subsequently estimated by MTT assay as described in the 'Methods' (A). Expression of Ctr1 protein in copper enriched MCF10A cells was seen by Western blot analysis. β-actin was used as protein loading control for the blot (B).

Figure 40: Effect of copper chelator on ROS generation in MDA-MB-231 cancer cells treated with luteolin

Control Luteolin alone Luteolin + Neocuproine

Using Image-iT LIVE Green ROS Detection Kit (Molecular Probes, Life Technologies, USA), ROS generation was detected in MDA-MB-231 breast cancer cells treated with luteolin (75 μM) for 1 hour with or without copper chelator neocuproine (50 μM). Cells were visualized under confocal microscope after proper staining with Hoechst 33342.

DISCUSSION-III

Based on the studies using human peripheral lymphocytes and rat lymphocytes (with copper overload), in the previous chapters it has been shown that polyphenols are able to cause cellular DNA breakage and that such a DNA breakage mechanism involves mobilization of endogenous copper ions and consequent DNA degradation through the generation of ROS. Studies in the present chapter demonstrate that a similar copper-dependent prooxidant pathway is also instrumental in the polyphenol-induced growth inhibition and apoptosis in cancer cells. Here we show that such cell growth inhibition and apoptosis induction by polyphenols in cancer cells could be reversed by copper specific sequestering agent neocuproine to a significant extent whereas iron and zinc chelators are relatively ineffective, thus confirming the role of endogenous copper in the cytotoxic action of polyphenols against cancer cells. Therefore, this mechanism of mobilization of endogenous copper ions could be one of the important mechanisms for the cytotoxic action of plant polyphenols against cancer cells and is possibly a common mechanism for all plant polyphenols. In fact, similar results obtained with four different polyphenolic compounds in this study, namely apigenin, luteolin, EGCG and resveratrol, strengthen this idea.

It is also found that while cancer cells undergo growth inhibition upon treatment with polyphenols, their non-transformed counterparts remain relatively resistant. Interestingly, these normal breast epithelial MCF10A cells have earlier been shown to possess no detectable copper (Daniel et al., 2005), which may explain their resistance to polyphenols apigenin and luteolin-induced growth inhibition as observed here (Figure 31). It was earlier suggested (Hadi et al., 2007) that this preferential cytotoxicity of plant polyphenols towards cancer cells is explained by the observation made several

years earlier which showed that copper levels in cancer cells are significantly elevated in various malignancies.

Further, copper enrichment culturing of non-transformed breast MCF10A cells, done to simulate the *in vivo* copper status of cancer cells, leads to sensitization of such cells to polyphenol-induced growth inhibition and an enhanced expression of membrane bound copper transporter Ctr1. This finding assumes significance in view of the fact that copper transporters are overexpressed in malignant cells, which aid the uptake and accumulation of excess of copper in the cells (Peng et al., 2006). It thus implies that copper-enriched MCF10A cells expressing higher Ctr1 accumulated copper and the ensuing high copper redox status resulted in the cells becoming susceptible to greater growth inhibition by polyphenols.

It may be mentioned that significantly high levels of copper have been found in many types of human cancers (Kuo et al., 2002; Gupte and Mumper, 2008). The reason for an increased copper concentration in tumors is not clearly understood. However, copper might be required for the expression of ceruloplasmin, the major copper binding protein that is also elevated in cancer cells (Hrgovcic et al., 1973; Nayak et al., 2003) and has been proposed to be an endogenous angiogenic stimulator (Brewer, 2005). Thus, because of higher intracellular copper levels in cancer cells, it may be predicted that the cytotoxic concentrations of polyphenols required would be lower in these cells as compared to normal cells. Such lower cytotoxic concentrations of polyphenols against cancer cells have been demonstrated by several authors (Chen et al., 1998; Lu et al., 2000) as well as in this chapter (Figure 31)

As shown previously, the results in this chapter further confirm that plant polyphenol-induced generation of ROS involves the mobilization of intracellular, possibly chromatin bound copper as demonstrated by confocal

microscopy of polyphenol treated cancer cells. Therefore cancer cells, having elevated levels of copper, may be more subject to electron transfer with polyphenols (Zheng at al., 2006)leadingto generation of ROS which contributes to the consequent prooxidant cell death.Thus the mechanism proposed by us would be an alternative, non-enzymatic and copper-dependent pathway for the cytotoxic action of these compounds that are capable of mobilizing and reducing endogenous copper. As such this would be independent of Fas and mitochondria mediated programmed cell death. Several studies have indicated that apoptosis induction by several polyphenols and other anti-cancer agents is independent of caspases and mitochondria (Piwocka et al., 1999; Leist and Jaattela, 2001) and is accompanied by an increase in the intracellular levels of ROS (Yoshino et al., 2004; Heiss et al., 2007; Noda et al., 2007). This is also consistent with our hypothesis where it is proposed that plant polyphenols mobilize chromatin-bound copper which is redox cycled and which in turn leads to the formation of ROS.

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LIST OF PUBLICATIONS

1) **Khan HY,** Zubair H, Ullah MF, Ahmad A, Hadi SM. Oral administration of copper to rats leads to increased lymphocyte cellular DNA degradation by dietary polyphenols: implications for a cancer preventive mechanism. *Biometals*, 2011; 24(6): 1169-1178.

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Oral administration of copper to rats leads to increased lymphocyte cellular DNA degradation by dietary polyphenols: implications for a cancer preventive mechanism

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Abstract To account for the observed anticancer properties of plant polyphenols, we have earlier proposed a mechanism which involves the mobilization of endogenous copper ions by polyphenols leading to the generation of reactive oxygen species (ROS) that serve as proximal DNA cleaving agents and lead to cell death. Over the last decade we have proceeded to validate our hypothesis with considerable success. As a further confirmation of our hypothesis, in this paper we first show that oral administration of copper to rats leads to elevated copper levels in lymphocytes. When such lymphocytes with a copper overload were isolated and treated with polyphenols EGCG, genistein and resveratrol, an increased level of DNA breakage was observed. Further, preincubation of lymphocytes having elevated copper levels with the membrane permeable copper chelator neocuproine, resulted in inhibition of polyphenol induced DNA degradation. However, membrane impermeable chelator of copper bathocuproine, as well as iron and zinc chelators were ineffective in causing such inhibition in DNA breakage, confirming the involvement of endogenous copper in polyphenol induced cellular DNA degradation. It is well established that serum and tissue concentrations of copper are greatly increased in various malignancies. In view of this fact, the present results further confirm our earlier findings and strengthen our hypothesis that an important anticancer mechanism of plant polyphenols could be the mobilization of intracellular copper leading to ROS-mediated cellular DNA breakage. In this context, it may be noted that cancer cells are under considerable oxidative stress and increasing such stress to cytotoxic levels could be a successful anticancer approach.

Keywords Cancer chemoprevention · Copper · EGCG · Genistein · Polyphenols · Prooxidant DNA breakage

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Introduction

Epidemiological studies have suggested that human consumption of fruits, vegetables and beverages such as green tea and red wine is associated with reduced risk of cardiovascular disease and certain types of

cancers (Vainio and Weiderpress 2006; Park and Surh 2004). Plant polyphenols are important components of human diet and a number of them are considered to possess chemopreventive and therapeutic properties against cancer. Various classes of polyphenols are found in plants such as flavonoids, gallocatechins, tannins, curcuminoids, stilbenes such as resveratrol and anthocyanidins such as delphinidin. Of particular interest is the observation that the green tea polyphenol EGCG was found to induce internucleosomal DNA fragmentation in cancer cell lines such as human epidermoid carcinoma cells, human carcinoma keratinocytes, human prostate carcinoma cells and mouse lymphoma cells. However, such DNA fragmentation was not observed in normal human epidermal keratinocytes (Ahmed et al. 1997). Likewise, gallic acid showed cytotoxicity for a number of tumour cell lines but primary cultured rat hepatocytes and macrophages were found to be refractory to the cytotoxic effect (Inoue et al. 1994). Similar studies have shown that soy isoflavone genistein and red wine polyphenol resveratrol are able to induce apoptotic cell death in various cancer cell lines but not in normal cells (Chang et al. 2008; Clement et al. 1998). Moreover, Moiseeva et al. (2007) have reported that physiological concentrations of dietary phytochemicals including genistein results in reduced growth and induction of apoptosis in cancer cells.

Earlier studies in our laboratory have shown that flavonoids (Ahmad et al. 1992), tannic acid and its structural constituent gallic acid (Khan and Hadi 1998), curcumin (Ahsan and Hadi 1998), gallocatechins (Malik et al. 2003) and resveratrol (Ahmad et al. 2000) cause oxidative strand breakage in DNA either alone or in the presence of transition metal ions such as copper. Copper is an important metal ion present in chromatin and is closely associated with DNA bases, particularly guanine (Kagawa et al. 1991). It is also one of the most redox active of the various metal ions present in cells. Most of the copper present in human plasma is associated with ceruloplasmin, which has six tightly held copper atoms and a seventh, easily mobilized one (Swain and Gutteridge 1995). In a study by Satoh et al. (1997) copper was found to enhance the apoptosis-inducing activity of polyphenols, whereas iron was inhibitory. Although iron is considerably more abundant in biological systems, the major ions in the nucleus are copper and zinc (Bryan 1979).

Most of the plant polyphenols possess both antioxidant as well as prooxidant properties (Inoue et al. 1994; Ahmad et al. 1992) and we have earlier proposed that the prooxidant action of polyphenols may be an important mechanism of their anticancer and apoptosis inducing properties (Hadi et al. 2000). Some interesting studies have in fact suggested that increasing reactive oxygen species (ROS) generation over an established threshold by lowering antioxidant defenses may contribute to selective killing of cancer cells (Schumacker 2006; Trachootham et al. 2006). Such a mechanism for the cytotoxic action of polyphenolic compounds against cancer cells would involve mobilization of endogenous copper ions, possibly chromatin bound copper and the consequent prooxidant action.

According to our hypothesis, the preferential cytotoxicity of polyphenols toward cancer cells is explained by the fact that copper levels are significantly elevated in cancer cells. Indeed it has been shown that serum (Ebadi and Swanson 1998) and tissue (Yoshida et al. 1993; Nasulewis et al. 2004) concentrations of copper are greatly increased in various malignancies. As a further confirmation of our hypothesis, in this paper we show that oral administration of copper to rats leads to elevated copper levels in plasma as well as in lymphocytes. Further, when such lymphocytes are treated by various polyphenols, an increased level of cellular DNA degradation is observed.

Materials and methods

Chemicals

EGCG, genistein, resveratrol, neocuproine, bathocuproine, catalase, superoxide dismutase (SOD), agarose, low melting point agarose (LMPA), Histopaque 1077, RPMI 1640, phosphate buffered saline (PBS) Ca^{2+} and Mg^{2+} free, Triton X-100 and Trypan blue were purchased from Sigma (St. Louis, USA). All other chemicals used were of analytical grade. EGCG and resveratrol were dissolved in 3.0 mM cold NaOH before use as fresh stocks of 1.0 mM. Fresh solution of genistein was prepared as a stock of 1.0 mM in absolute methanol. Upon addition to reaction mixtures, in the presence of buffers mentioned and at the concentrations used, all the polyphenols used remained in solution. The volumes of stock solution

added did not lead to any appreciable change in the pH of reaction mixtures.

Animals

Adult male Wistar rats weighing between 190 and 220 g were used in the study. The rats were housed in isopropelyene cages and acclimatized for a period of 1 week to laboratory conditions (23 ± 2°C and 60% humidity). They received a commercial standard diet (Ashirwad Industries, Chandigarh, India) and water ad libitum. After acclimatization, the rats were randomly divided into two equal groups and henceforth, identified as control and test groups. All the animal studies were carried out in compliance with the international practices for animal use according to the guidelines of Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

Cupric chloride was administered orally to rats from the test group in order to raise their intracellular status of copper. Rats from the control group were gavaged with only drinking water. Twelve hours post-gavaging, blood was drawn from control as well as copper-administered rats by cardiac puncture and kept in heparinised tubes.

Isolation of lymphocytes

Heparinized blood samples (2.0 ml) from both untreated rats and their copper-overloaded counterparts were diluted suitably in Ca^{2+} and Mg^{2+} free PBS. Lymphocytes were then isolated from the blood using Histopaque 1077 (Sigma) and the isolated cells (2×10^6) were subsequently suspended in RPMI 1640.

Viability assessment of lymphocytes

The lymphocytes were checked for their viability before the start and after the end of the reaction using Trypan Blue Exclusion test (Pool-Zoble et al. 1993). The viability of the cells was found to be almost 94%.

Measurement of copper in plasma and lymphocytes

3.9 ml of nitric acid (2.5%) was added to aliquots of 100 µl plasma/lymphocytes and vortexed. The solutions were then kept at 37°C for 5 h with regular shaking. The mixture was centrifuged at 3000 rpm for 5 min. Copper was measured in the clear supernatant by means of flame atomic absorption spectrophotometry (FAAS) (Varian Spectra 200 FS, Varian Inc, California, USA) (hollow cathode lamp, Flame type: Air acetylene; replicate 3; wavelength 324.8 nm) as described (US EPA 1994). Plasma and cellular copper levels of each animal were measured and a mean value was determined for the whole group.

Treatment of rat lymphocytes with polyphenols

Isolated lymphocytes were exposed to specified concentrations of polyphenols EGCG, genistein and resveratrol in a total reaction volume of 1.0 ml. Incubation was performed at 37°C for 1 h. In some experiments, lymphocytes were pre-incubated with various concentrations of different metal chelators prior to being treated with polyphenols. In another set of experiments, scavengers of ROS were added to the reaction mixture containing polyphenol at the final concentrations indicated. After incubation, the reaction mixture was centrifuged at 4000 rpm, the supernatant was discarded and pelleted lymphocytes were resuspended in 100 µl of PBS and processed further for Comet assay.

Evaluation of DNA breakage by comet assay

Comet assay was performed under alkaline conditions essentially according to the procedure of Singh et al. (1998) with slight modifications. Fully frosted microscopic slides precoated with 1.0% normal melting agarose at about 50°C (dissolved in Ca^{2+} and Mg^{2+} free PBS) were used. Around 10,000 cells were mixed with 75 µl of 2.0% LMPA to form a cell suspension and pipetted over the first layer and covered immediately by a coverslip. The agarose layer was allowed to solidify by placing the slides on a flat tray and keeping it on ice for 10 min. The coverslips were removed and a third layer of 0.5% LMPA (75 µl) was pipetted and coverslips placed over it and kept on ice for 5 min for proper solidification of layer. The coverslips were removed and the slides were immersed in cold lysing solution containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, and 1% Triton X-100 added just prior to

use for a minimum of 1 h at 4°C. After lysis DNA was allowed to unwind for 30 min in alkaline electrophoretic solution consisting of 300 mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was performed at 4°C in a field strength of 0.7 V/cm and 300 mA current. The slides were then neutralized with cold 0.4 M Tris, pH 7.5, stained with 75 µl Ethidium Bromide (20 µg/ml) and covered with a coverslip. The slides were placed in a humidified chamber to prevent drying of the gel and analyzed the same day. Slides were scored using an image analysis system (Komet 5.5, Kinetic Imaging, Liverpool, UK) attached to a Olympus (CX41) fluorescent microscope and a COHU 4910 (equipped with a 510–560 nm excitation and 590 nm barrier filters) integrated CC camera. Comets were scored at ×100 magnification. Images from 50 cells (25 from each replicate slide) were analyzed. The parameter taken to assess lymphocytes DNA damage was tail length (migration of DNA from the nucleus, µm) and was automatically generated by Komet 5.5 image analysis system.

Statistics

The statistical analysis was performed as described by Tice et al. (2000) and is expressed as mean ± SEM. A student's *t*-test was used to examine statistically significant differences. Analysis of variance was performed using ANOVA. P values <0.05 were considered statistically significant.

Results

Oral administration of cupric chloride to rats leads to elevated copper levels

When rats in the test group were orally administered copper in the form of cupric chloride in a dose of 30 mg/Kg b.w., a clear elevation in the intracellular copper status of lymphocytes was observed. A similar increase in the plasma copper concentrations was also found. As shown in Table 1, the mean copper levels were highest in the plasma as well as lymphocytes isolated 12 h after oral administration of copper to the rats. Beyond this time, the levels of copper declined and returned to almost control values at 36 h post-administration.

Table 1 Copper concentrations in plasma and lymphocytes of rats after different intervals of copper administration

Time interval between copper dosing and lymphocyte isolation (h)	Mean copper conc. in plasma (µg/ml ± SD)	Mean copper level in lymphocytes (µg/10^6 cells ± SD)
0	1.46 ± 0.09	8.96 ± 0.69
12	5.11 ± 0.34	31.28 ± 2.55
24	3.34 ± 0.21	18.64 ± 1.36
36	1.79 ± 0.05	10.56 ± 0.86

Rats were gavaged in groups of five with 30 mg/Kg b.w. $CuCl_2$ in water and sacrificed at intervals indicated for isolation of plasma and lymphocytes

Cellular DNA breakage induced by polyphenols in lymphocytes isolated from rats with copper overload

Lymphocytes isolated at different time points following copper administration to rats were treated in vitro with EGCG and genistein at a concentration of 50 µM. Breakage in cellular DNA was measured by alkaline single cell gel electrophoresis (comet assay). As can be seen in Fig. 1, both EGCG and genistein induced significantly greater DNA degradation in

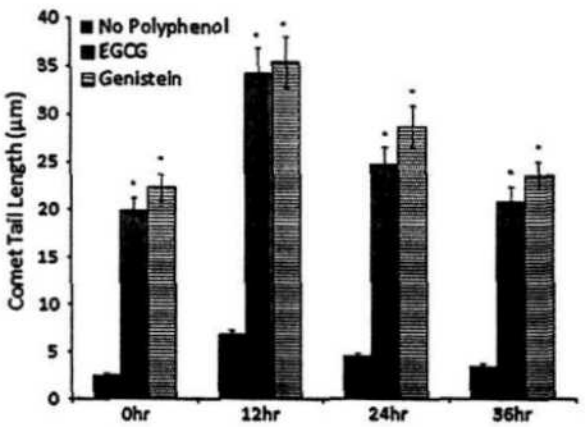

Fig. 1 Polyphenol-induced DNA breakage in lymphocytes isolated from copper-administered rats at different time points. The rats were gavaged with 30 mg/kg b.w. $CuCl_2$ and sacrificed after the indicated periods. The isolated lymphocytes were then treated with the polyphenols (50 µM) for 1 h and subsequently subjected to comet assay. The tail lengths of comets were determined as given in "Materials and methods" section. Values are found to be significant when compared with control (no polyphenol) at $P < 0.01$. Both groups comprised of five animals each

Table 2 Cellular DNA damage induced by different polyphenols in lymphocytes isolated from copper-administered rats as analysed by comet assay

Treatment	Control group comet tail length (μm)	Copper administered group comet tail length (μm)
Untreated (Control)	3.47 ± 0.22#	6.12 ± 0.47†
Resveratrol	16.62 ± 0.93*	25.72 ± 1.59**
Genistein	24.55 ± 1.64*	36.21 ± 2.34**
EGCG	22.36 ± 1.86*	31.8 ± 1.97**

Lymphocytes from control rats (gavaged drinking water) and copper overloaded rats (gavaged 30 mg/Kg b.w. $CuCl_2$) were treated with the mentioned polyphenols at a concentration of 50 μM for 1 h at 37°C. Values reported are Mean ± SEM. Both groups had five animals each

* Values are significant when compared with control # at $P < 0.05$; ** values are significant when compared with control † at $P < 0.05$. Mean values of the *control group were compared with **treated group and found to be significant at $P < 0.05$

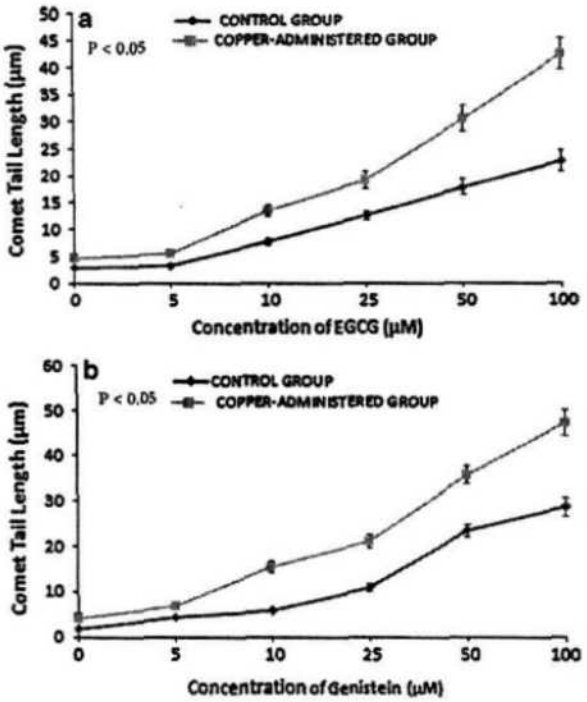

Fig. 2 Cellular DNA breakage induced by different concentrations of polyphenols EGCG (**a**) and genistein (**b**) in lymphocytes isolated from Control (gavaged drinking water) and copper-administered rats (gavaged 30 mg/Kg b.w. $CuCl_2$). Isolated lymphocytes were treated with increasing concentrations of EGCG/Genstein as indicated in the figure for 1 h

cells isolated at 12 h after copper dosing to rats. Such increased DNA breakage in isolated rat lymphocytes correlates with the data shown in Table 1, where the maximum elevation in copper levels was seen in lymphocytes isolated after 12 h of copper administration.

In all subsequent experiments, lymphocytes were isolated from rats 12 h after oral administration of copper. When isolated rat lymphocytes were treated with different polyphenols (Table 2), an invariably greater extent of DNA breakage was observed in the lymphocytes of copper-administered rats as compared to that of control group rats. Thus it is indicated that the elevated copper levels in copper-administered rats contributes to increased cellular DNA degradation. Further, the differences in the rate of DNA breakage by the three polyphenols tested is possibly due to differential cell membrane permeability and copper reducing efficiency of the polyphenols (Ahmad et al. 1992).

In another experiment, the effect of increasing concentration of polyphenols was tested on cellular DNA breakage in lymphocytes isolated from copper-administered rats. As shown in Fig. 2a, isolated rat lymphocytes treated with increasing concentrations of EGCG exhibit a progressive increase in DNA degradation. However, such increment in DNA breakage was more pronounced in lymphocytes of copper-administered rats at every concentration of EGCG tested. Similar results were obtained when increasing concentrations of genistein were used to treat isolated rat lymphocytes, as shown in Fig. 2b. In previous publications we have established the mobilization of endogenous copper ions by using various metal ion chelators (Shamim et al. 2008; Ullah et al. 2009). The results given in Fig. 2a and b lend further support to the involvement of endogenous copper ions in polyphenol mediated cellular DNA degradation.

Effect of metal-specific sequestering agents on the polyphenol-induced DNA breakage in isolated rat lymphocytes with copper overload

In the experiment shown in Fig. 3, we have used various metal-specific chelators, which selectively bind to copper, iron and zinc, to study their effect on polyphenol EGCG-induced DNA degradation in lymphocytes isolated from rats administered copper

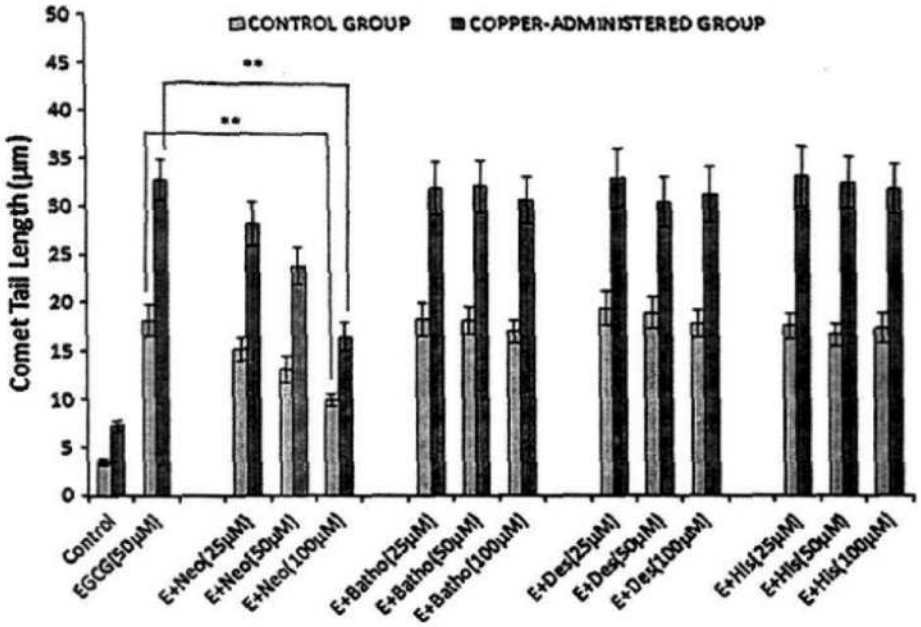

Fig. 3 Effect of preincubating the lymphocytes isolated from copper-administered rats with metal chelators on cellular DNA breakage induced by EGCG. The lymphocytes were preincubated at 37°C for 30 min and subsequently treated with EGCG and subjected to comet assay as described in methods. The three different concentrations of neocuproine, bathocuproine, desferroxamine mesylate and histidine as indicated were used for preincubation. The concentration of EGCG used was 50 μM

orally. As shown above and as expected the cellular DNA breakage was considerably greater in lymphocytes with a copper overload. Four different metal chelators were used, namely neocuproine and bathocuproine (copper sequestering agents); desferroxamine mesylate (iron chelator) and histidine (which binds zinc) at concentrations of 25, 50 and 100 μM. It was seen that only in the case of neocuproine, there was a progressive decrease in comet tail lengths. Bathocuproine, which is also a copper chelator, along with desferroxamine mesylate and histidine was ineffective. Bathocuproine is impermeable to cell membrane and we have earlier shown that when isolated cell nuclei were treated with polyphenols, bathocuproine was able to inhibit the cellular DNA breakage (Shamim et al. 2008). This explains the non-inhibition of DNA breakage by bathocuproine observed in the above experiment. Similar results were obtained (data not shown) when genistein and resveratrol were used instead of EGCG as test polyphenols. The result indicate that the polyphenol-induced cellular DNA breakage in the control

Table 3 Effect of scavengers of ROS on EGCG-induced DNA breakage in lymphocytes isolated from copper-administered rats

Lymphocyte treatment	Comet tail length (μm)	% Inhibition
Control (untreated)	2.41 ± 0.17#	–
+ EGCG (50 μM)	20.04 ± 0.81*	–
+ EGCG + SOD (100 μg/ml)	9.39 ± 0.36*	60.41
+ EGCG + Catalase (100 μg/ml)	10.96 ± 0.53*	51.5
+ EGCG + Thiourea (1 mM)	12.52 ± 0.78*	42.65
Copper-administered (Untreated)	5.15 ± 0.30†	–
+ EGCG (50 μM)	34.73 ± 1.35**	–
+ EGCG + SOD (100 μg/ml)	13.38 ± 0.69**	72.17
+ EGCG + Catalase (100 μg/ml)	11.67 ± 0.74**	77.96
+ EGCG + Thiourea (1 mM)	15.66 ± 1.09**	64.47

Rats were orally administered $CuCl_2$ (30 mg/kg b.w.) and lymphocytes were isolated 12 h later. Each group comprised of five rats

* Mean values were significant when compared with control # $P < 0.05$; ** mean values were significant when compared with control † $P < 0.05$

group as well as copper-administered group of rats is due to a similar mechanism involving mobilization of endogenous copper ions. Moreover, it also rules out the involvement of any other metal ion, such as iron or zinc, in the reaction leading to DNA breakage.

Effect of scavengers of active oxygen species on polyphenol-induced DNA breakage in isolated rat lymphocytes with copper overload

Table 3 gives the results of an experiment where scavengers of various ROS, namely superoxide dismutase, catalase and thiourea, were tested for their effect on EGCG-induced DNA breakage in lymphocytes isolated from copper-administered rats using comet assay. Catalase and SOD remove H_2O_2 and superoxide, respectively, while thiourea scavenges hydroxyl radicals. All the three ROS scavengers caused significant inhibition of DNA breakage as evidenced by decreased tail lengths of comets in both the control as well as copper-administered group. In earlier reports (Ullah et al. 2009; Azmi et al. 2006), we have proposed that polyphenol induced cellular DNA degradation is the result of the formation of ROS such as the hydroxyl radicals. Further, due to the site specific nature of the reaction of hydroxyl radicals with DNA, it is difficult for any trapping molecule to intercept them completely (Czene et al. 1997). This possibly accounts for the fact that complete inhibition of DNA degradation was not observed even at relatively high concentration of thiourea (1 mM) and catalase (100 μg/ml). The results are in further support of the indication that irrespective of whether the lymphocytes are from the normal control rats or from the copper-administered ones, a similar mechanism of polyphenol mediated oxidative DNA degradation is involved.

Discussion

The role of copper has been extensively studied in the etiology and growth of tumors (Brewer 2005; Goodman et al. 2004). Such studies were based on reports that copper distribution is altered in tumor bearing mice, rats and humans (Apelgot et al. 1986; Semczuk and Pomykalski 1973). Gupte and Mumper (2008) have reviewed several studies which indicate that both serum and tumor copper levels are significantly elevated in cancer patients compared to healthy subjects. Moreover, there are a number of studies that have focused on determining the levels of four important biological trace elements, namely copper, iron, zinc and selenium, in cancer patients. These studies showed that while iron, zinc and selenium concentrations were significantly lower in cancer patients, the copper levels were almost always found to be significantly elevated (up to two to three folds) compared to age-matched samples from normal tissue (Kuo et al. 2002; Zuo et al. 2006). However, the reason for an increased copper concentration in tumors is not clearly known.

We have earlier proposed that an important anticancer mechanism of plant-derived polyphenolic compounds could be the mobilization of endogenous copper ions and the consequent prooxidant action (Hadi et al. 2000). This is based on several lines of indirect evidence in literature and our own studies (Hadi et al. 2007). Using intact lymphocytes and isolated nuclei from these cells, we have established that plant polyphenols are able to mobilize chromatin bound copper leading to redox cycling of copper ions (Shamim et al. 2008). In the presence of molecular oxygen such a reaction leads to the formation of ROS such as hydroxyl radical, causing DNA cleavage. Further, we have suggested that the preferential cytotoxicity of plant polyphenols towards cancer cells is explained by the observation made several years earlier which showed that serum (Ebadi and Swanson 1998; Margalioth et al. 1987), tissue (Yoshida et al. 1993) and intracellular copper levels in cancer cells (Ebara et al. 2000) are significantly increased in various malignancies. Indeed, such levels have been described as a sensitive index of disease activity of several hematologic and non-hematologic malignancies (Pizzolo et al. 1978).

Since cancer cells contain elevated levels of copper, they may be more subject to electron transfer with polyphenols (Zheng et al. 2006) to generate ROS. In normal cells, there exists a balance between the free radical generation and the antioxidant system (Devi et al. 2000). However, it has been clearly documented that tumor cells are under persistent oxidative stress and have an altered antioxidant defense (Powis and Baker 1997; Pervaiz and Clement 2004) and thus further ROS stress in these malignant cells, surpassing a threshold level, could result in apoptosis (Gupte and Mumper 2008). These

observations suggest that neoplastic cells may be more vulnerable to oxidative stress as they function with a heightened basal level of ROS owing to increased rate of growth and metabolism (Kong et al. 2000). Thus, in cancer cells, a further enhanced exposure to ROS, generated through the redox cycling of intracellular copper by polyphenols, can overwhelm the cells antioxidant capacity, leading to irreversible damage and apoptosis. On the other hand, normal non-malignant cells can better tolerate such an action due to their low basal ROS output and normal metabolic regulation, as also normal copper redox status. Hence, a disparity in the redox states of cancer cells and normal cells may provide a molecular basis for selective killing of cancer cells by the use of agents, like polyphenols, that can cause further ROS insults to the malignant cells. Therefore, we propose that this accounts for the preferential cytotoxicity of plant polyphenols toward cancer cells. Indeed we have recently shown that the polyphenol genistein mediated apoptotic cell death and cell proliferation inhibition in MDA MB 231 and MDA MB 468 breast cancer cell lines is inhibited by the copper chelator neocuproine, whereas iron and zinc chelators have little effect (Ullah et al. 2011).

In order to further substantiate our hypothesis, in this paper we have attempted to elevate copper levels in lymphocytes by administering cupric chloride to rats to a range similar to what has been reported in leukemic cancer patients (Carpentieri et al. 1986) [31.28 ± 2.55 μg $Cu/10^6$ cells (Table 1) vs. 52 ± 16 μg $Cu/10^6$ cells (in leukemia patients)]. It must be mentioned that copper itself is known to be cytotoxic and leads to cellular DNA cleavage. However as shown in the results the levels of copper achieved in lymphocytes on cupric chloride administration does not lead to a significant increase in comet tail lengths as compared to the untreated controls (Fig. 1). More importantly, the addition of polyphenols (EGCG and genistein) causes several fold increase in comet tail lengths, indicating that the contribution of copper alone to cellular DNA breakage is insignificant. Thus the above results taken together lead to the conclusion that the elevated copper levels in lymphocytes, such as those found in cancer patients could be an important factor in the anticancer mechanism of plant polyphenols.

In summary and in confirmation of our hypothesis, the following milestones have been achieved: (i) an in vitro reaction between plant polyphenols, Cu(II) and DNA leading to DNA cleavage has been characterised. Most polyphenols are capable of causing DNA breakage in this reaction (Ahsan and Hadi 1998; Ahmad et al. 2000; Rahman et al. 1989; Azam et al. 2004); (ii) as a further step we have shown that polyphenol-Cu(II) system is indeed capable of causing DNA degradation in a cellular system and this reaction could be of biological significance (Azmi et al. 2005); (iii) we have also shown that polyphenols are capable of mobilizing endogenous copper ions from cells leading to cellular DNA breakage (Azmi et al. 2006); (iv) we have further demonstrated that in the above oxidative cellular DNA breakage nuclear copper is mobilized (Shamim et al. 2008); (v) we have shown that polyphenol induced growth inhibition in breast cancer cell lines is inhibited by copper chelator to a significant extent whereas iron and zinc chelators are relatively ineffective (Ullah et al. 2011); (vi) finally, in this paper we have shown that oral administration of copper to rats leads to increased lymphocyte cellular DNA degradation by polyphenols. This substantiates the role of elevated copper levels in cancer tissue and cells in the anticancer mechanism of plant polyphenols.

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Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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