MECHANISM OF PHYSIOLOGICAL ACTION OF PLANT DERIVED POLYPHENOLS

(The plant polyphenol delphinidin mobilizes endogenous copper leading to oxidative DNA breakage: Implications for anticancer properties)

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. **Sarmad Hanif** 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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This work is a consequence of several *resonances* of seen and unseen kinds. The 3^{plus} years spent (or gained ?!) in this department during my PhD remains an unforgettable piece of memory in the matrix of my mind. Many events, many meetings, many "encounters" that happened added up to the chapter of my "lif(v)e experiences", and today standing at the threshold of my academic career, I can very aptly say "Patience and Perseverence have a magical effect before which difficulties disappear and obstacles vanish".

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Sarmad Hanif

Dedicated

to

My Family

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SUMMARY

SUMMARY

Epidemiological studies have suggested that human consumption of fruits and vegetables is associated with a reduced risk of cardiovascular disease and certain types of cancers. Many fruits, vegetables and nuts are known to be a rich source of polyphenolic compounds such as flavonoids, tannins, curcuminoids, gallocatechins, stilbenes and anthocyanidins. Plant polyphenols possess a wide range of pharmacological properties, the mechanisms of which have been the subject of considerable interest. They are recognized as naturally occurring antioxidants and have been implicated as anticancer compounds. In recent years several reports have documented that plant polyphenolics such as curcumin (from the spice turmeric), resveratrol (from red grapes and red wine), epigallocatechin-3-gallate (EGCG) (from green tea) and delphinidin (from pomegranate juice) induce apoptosis in various cancer cell lines. Of particular interest is the observation that a number of these polyphenols including EGCG, gallic acid and resveratrol induce apoptotic cell death in various cell lines but not in normal cells.

Earlier studies in this laboratory have shown that flavonoids, tannic acid and its structural constituent gallic acid, curcumin, gallocatechins and resveratrol 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. It is one of the most redox active of the various metal ions present in cells. 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, radiation etc. However, some data in the literature suggests that antioxidant properties of the polyphenolic compounds may not fully account for their

chemopreventive effects. Most of the plant polyphenols possess both antioxidant as well as prooxidant properties and we have earlier proposed that the prooxidant action of polyphenolics may be an important mechanism of their anticancer and apoptosis inducing properties. Such a mechanism for the cytotoxic action of these compounds against cancer cells would involve mobilization of endogenous copper ions and the consequent prooxidant action.

Delphinidin, a polyphenol belonging to the class of compounds known as anthocyanidins is considered to possess chemopreventive properties against cancer. It is recognized as a naturally occurring antioxidant but also catalyzes oxidative DNA degradation *in vitro* in the presence of transition metal ions such as copper. In view of this, I have examined the oxidative DNA cleavage mechanism of delphinidin *in vitro*. Using fluorescence and absorption studies, I have shown that delphinidin is able to bind as well as reduce copper ions. Further, delphinidin is able to bind to DNA as well. Delphinidin is also capable of degrading calf thymus and supercoiled plasmid pBR322 DNA in the presence of copper ions. These results suggest that delphinidin is an efficient DNA cleaving agent in the presence of copper ions.

In the second chapter, using a cellular system of lymphocytes isolated from human peripheral blood and alkaline single cell gel electrophoresis (Comet Assay), I have confirmed that delphinidin is capable of mobilizing endogenous copper ions from lymphocytes, which in turn leads to the degradation of cellular DNA. Preincubation of lymphocytes with delphinidin indicates that it is capable of either traversing the cell membrane or binding to it. Incubation of lymphocytes with neocuproine inhibited the DNA degradation confirming that Cu(I) is an intermediate in the DNA cleavage reaction. Further, I have also shown that delphinidin induces the generation

of hydroxyl radicals in lymphocytes and neocuproine and hydroxyl radical quenchers inhibit such radical formation.

In the third and final chapter of this thesis, using the lysed version of Comet Assay, I have further confirmed that delphinidin is able to mobilize nuclear copper from lymphocyte nuclei. Also, it was shown that delphinidin induced DNA breakage in lymphocyte nuclei is inhibited by various scavengers of reactive oxygen species (ROS) suggesting that the same mechanism involving ROS and Cu(I) is responsible for DNA breakage irrespective of whether intact lymphocytes or lymphocyte nuclei are used. 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 of their anticancer and chemopreventive properties. Indeed such a common mechanism would better explain the anticancer effects of polyphenols with diverse chemical structures as also the preferential cytotoxicity towards cancer cells.

INTRODUCTION

INTRODUCTION

Dietary constituents and prevention of disease :

Food provides not only essential nutrients needed for life but also other bioactive compounds for health promotion and disease prevention. Pervious epidemiological studies have consistently shown that diet plays crucial role in the protection of chronic diseases (Temple, 2000; Willett, 1994). Consumption of fruits and vegetables as well as grains, has been strongly associated with reduced risk of cardiovascular disease, cancer, diabetes, Alzheimers disease, cataracts and age related functional decline (Temple, 2000; Willett, 1994; Willett, 1995). Heart disease, cancer and stroke are the top three causes of death in most industrialized countries. It is estimated that one third of all cancer deaths can be avoided through appropriate dietary modifications (Willett, 1995; Doll and Peto, 1981). This convincing evidence suggests that a change in dietary behaviour such as increasing consumption of fruits, vegetables and grains is a practical strategy for significantly reducing the incidence of chronic diseases.

The biologically active chemicals found in fruits, vegetables and grains are termed as phytochemicals and have been linked to the reduction in the risk of major chronic disease. It is estimated that more than 5000 phytochemicals have been identified but a large percentage still remains unknown (Shahidi et al., 1995) and they need to be identified before their health benefits are fully understood. However, more and more convincing evidence suggests that the benefits of phytochemicals in fruits and vegetables may be even greater than is currently understood because oxidative stress induced by free radicals is involved in the etiology of a wide range of chronic disease (Ames et al., 1991). Cells in humans and other organisms are constantly exposed to a variety of oxidizing agents some of which are necessary for life. These agents may

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be present in air, food or water or they may be produced by metabolic activities within the cells. The key factor is to maintain a balance between oxidants and antioxidants to sustain optimal physiological conditions within the body. Overproduction of oxidant can cause an imbalance leading to oxidative stress, especially in chronic bacterial, viral and parasitic infections (Liu and Hotckiss, 1995). Oxidative stress can cause damage to large biomolecules such as proteins, lipids and DNA resulting in an increased risk for cancer and cardiovascular disease (Ames et al., 1991; Liu and Hotckiss, 1995; Ames et al., 1993). To prevent or slow down the oxidative stress induced by free radicals, sufficient amounts of antioxidants need to be consumed. Fruits and vegetables contain a wide variety of secondary metabolites that possess antioxidant properties. These include polyphenols and carotenoids that may help protect cellular systems from oxidative damage and also lower the risk of chronic disease. There has been considerable scientific evidence, both epidemiological and experimental accumulated in the past three decades indicating that modification in life style including diet, can have a major effect on the risks of numerous cancers (Martinez and Giavanucci, 1997). Of particular relevance is the consistent cancer protective effect reported for individuals consuming increased quantities of fruits and vegetables compared to those with low intakes. The cancer inhibitory action by a variety of human nutrients derived from plants as well as of non-nutritive plant derived constituents (phytochemicals) has been confirmed in different animal tumor models (Dragsted et al., 1993; Pezzuto, 1996) and has led to an increased emphasis on cancer prevention strategies in which these dietary factors are utilized. There have been two major diet related prevention strategies that have been involved in combating cancer, i.e. cancer chemoprevention and dietary prevention with appreciable overlap existing between them. Generally, cancer chemoprevention is recognized as the pharmacological

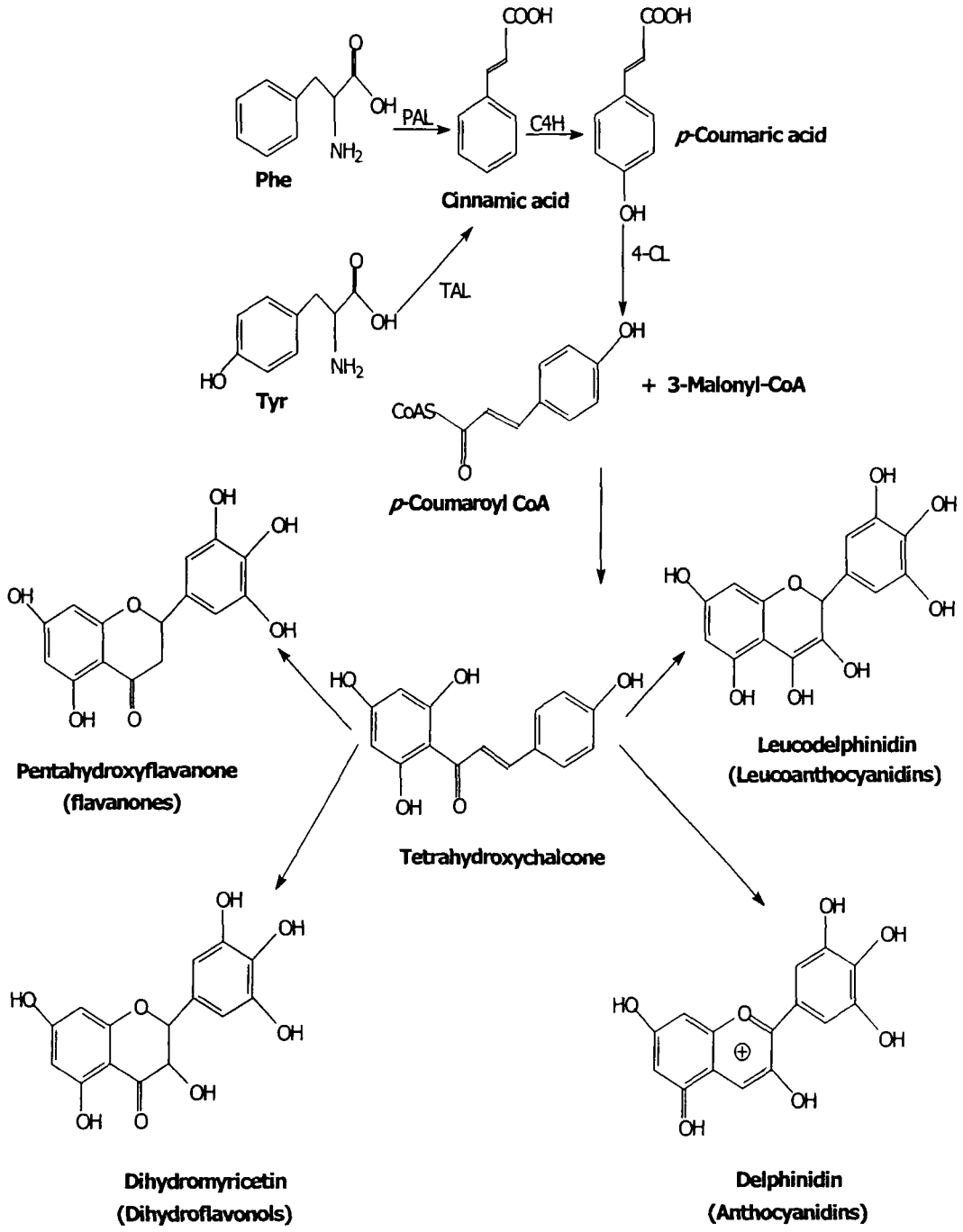
intervention with synthetic or naturally occurring chemicals to prevent, inhibit or reverse carcinogenesis or prevent development of invasive cancer (Kelloff and Boone, 1996; Kelloff et al., 1997; Mayne and Lipman, 1997; Sporn, 1991). On the other hand dietary prevention is recognized as the changes in food consumption pattern necessary to decrease cancer development (Goodman, 1997; Schatzkin and Kelloff, 1995). Plant derived polyphenolic compounds such as flavonoids, tannins, curcumin and the stilbene resveratrol possess a wide range of pharmacological properties, the mechanisms of which have been the subject of considerable interest. They are recognized as naturally occurring antioxidants and have been implicated as anticancer compounds (Mukhtar et al., 1998). In recent years, several reports have documented that plant polyphenolics, including curcumin, resveratrol and gallocatechins such as gallic acid, epigallocatechin, epicatechin-3-gallate and epigallocatechin-3-gallate (EGCG) induce apoptosis in various cancer cell lines (Jaruga et al., 1998, Clement et al., 1998; Inoue et al., 1994). Gallocatechins are constituents of green tea, the consumption of which is considered to reduce the risk of various cancers such as those of bladder, prostate, esophagus and stomach (Ahmad et al., 1997). Resveratrol is present in human dietary material such as peanuts, grapes, mulberries and beverages such as red wine. Of particular interest is the observation that a number of these polyphenols including epigallocatechin-3-gallate, gallic acid and resveratrol induce apoptotic cell death in various cancer cell lines but not in normal cells (Inoue et al., 1994; Ahmad el al., 1997; Clement et al., 1998).

Biosynthesis of plant polyphenols :

Polyphenolic compounds are produced as secondary metabolites in higher plants. These compounds fulfill a vast array of important

functions in plants, being involved in development and interactions with the environment (Croteu et al., 2000). For example stilbenes and coumarins serve to defend pathogen attacks, flavonoids act as UV irradiation protectents while isoflavone serve as flower pigments.

The majority of polyphenolic compounds produced by plants are synthesized by a highly branched phenylpropenoid pathway. The initial compound is cinnamic acid, which arises from phenylalanine by the action of Pal (Phenylalanine 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. Tetrahydroxychalcone provides the precursor for all classes of flavonoids, which include the flavones, flavonols, flavan-diols, flavan-4-ols, proanthocyanidins (condensed tannins), isoflavonoids and anthocyanins.



PAL, Phenyl-ammonia lyase; C4H, Cinnamate-4-hydroxylase; TAL, Tyrosine- ammonia lyase; 4CL, 4-coumaryl lyase.

Figure. 1 Biosynthesis of plant polyphenols

Anticancer Properties of plant polyphenols :

Numerous studies have reported flavonoid mediated antiproliferatlve effects against both human and rodent ovarian, leukemic, intestinal, lung, breast and bladder cancer cells. For example, quercetin (10 μM) strongly suppresses transformed OVCA 433 human ovarian cancer cell growth. Moreover, quercetin inhibits normal proliferation in cultured primary ovarian adenocarcinoma tumor cells (Scambia et al., 1994 a , b). At low concentrations, quercetin inhibits DNA synthesis (IC_{50} 10 μM) and growth (IC_{50} 7.7 μM) in HL60 human promyelocytic leukemia cells (Uddin & Chawdhury, 1995; Kang & Liang, 1997). The citrus flavonoid tangeretin suppresses HL60 proliferation (measured as tritiated thymidine incorporation into DNA) even more strongly, with an IC_{50} of 0.17 μM (Hirano et al., 1995) while genistein is inhibitory at concentrations similar to conventional anticancer drugs such as deoxorubicin and methotrexate (Hirano et al., 1994). Genistein, kaempferol and quercetin inhibit the proliferation of human colon cancer cells Caco-2 and HT29 (Agullo et al., 1994; Kuo, 1996) while naringenin and catechin do not (Kuo et al., 1996). Curcumin is cytostatic in several hormone dependant (MCF-7 and T-47D) and independent (SK-BR3, BT-20 and MDA-23I) breast tumor cell lines (Mehta et al., 1997) while 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). Quercetin inhibits tritiated thymidine uptake and proliferation in several non-small-cell lung carcinoma cell lines and reduces bromodeoxyuridine incorporation in primary lung tumor slices (Caltagirone et al., 1997). Quercetin also inhibits ML-3 murine hepatoma cell growth (Chi et al., 1997).

Very few studies have investigated the cytostatic ability of flavonoids

both in malignant cells and in their untransformed counterparts although several polyphenols, most notably genistein, while showing considerable growth inhibition in HL-60 cells had little or no effect on mitogen-induced blastogenesis in normal human peripheral blood lymphocytes (Hirano et al., 1994). Similarly, tritiated thymidine uptake is inhibited in HL60 cells following exposure to tangeretin, but is unchanged in normal lymphocytes (Hirano et al., 1995). The polyhydroxylated flavonoid quercetin and taxifolin and the polymethoxylated flavonoids nobiletin and tangeretin inhibit HTB 43 squamous cell carcinoma and 9L gliosarcoma cell growth but are less effective in transformed human CCI embryonic fibroblast cells (Kandeswami et al., 1992). While these studies appear to suggest that the flavonoids display a tumor-specific action, it should be noted that comparisons were not made on cells derived from the same tissue. In an elegant study by Chen et al. (1998), epigallocatechin gallate (EGCG), the major polyphenol present in green tea, inhibited colorectal cancer and breast cancer growth more than in their respective normal counterparts. Similarly, EGCG reduced W138 human lung fibroblast cell growth only weakly compared to their virally transformed (VA) counterparts. The IC_{50} value of EGCG was 120 μM in WI38 cells compared with only 10 μM in W138VA cells. Conversely, the flavonoids quercetin and genistein are equally toxic towards colonic cancer cells and non-transformed intestinal crypt cells (Kuo, 1996).

In addition to cell culture studies, the capacity of certain dietary polyphenols to protect against both chemically induced or spontaneous formation of tumors in animals is well established. For example, quercetin administered to rats in combination with dimethyl-benz-(a)-anthracene (DMBA) or N-nitrosomethylurea (NMU) reduces the incidence and multiplicity of mammary tumor by 30 % and 50 %,

respectively (Verma et al., 1988). Quercetin and luteolin (10 g/Kg diet) decreases 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/Kgb.w) also increases the survival and reduces the tumor burden of mice (Balb/c) 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).

Several studies have described a protective effect of tea polyphenols against carcinogenesis. Rats fed on a diet containing 10 g green tea catechins/kgb.w have a considerably reduced mortality (7 % reduced mortality) from mammary tumors following DMBA treatment compared with rats given carcinogen alone (66 %) (Hirose et al., 1994). Similarly hamster fed on green tea polyphenols display fewer hyperplastic pancreatic duct lesions after treatment with N-nitrosobis (2-oxopropyl) amine (Majima et al., 1998). In a comprehensive study, Yang et al (1998) described the ability of both green and black tea infusions to inhibits N-nitrosodiethyl-amine-induced lung carcinogenesis in A/J mice.

There are several suggested mechanisms by which polyphenols exert anticancer effects:

Antioxidant effects: Carcinogenesis is a multi-stage process of genetic change affecting proto-oncogenes or tumor suppressor genes in a single cell or a clone of cells. Such genetic alterations may be initiated by increased and persistent damage to DNA causing permanent alterations in the genetic message when the cell replicates its DNA and divides. Reactive O and N species are potential carcinogens as they can

directly and indirectly induce structural alterations in DNA by oxidation, methylation, depurination and deamination reaction. The ability of certain polyphenols to inhibit oxidative DNA damage is well documented. For example, luteolin, kaempferol, quercetin and myricetin at relatively low concentrations (50-100 μM) significantly reduce DNA strand breakage and oxidized pyrimidine levels in H_2O_2-stressed lymphocytes (Duthie et al., I997a, b; Noroozi et al., 1998). Similarly, tea polyphenols decrease the incidence of hydroxyl radical-generated chromatid breaks in lymphocytes exposed to fluorescent light irradiation (Parshad et al., 1998). The number and positioning of the hydroxyl groups in the flavonoid structure appear important to the antioxidant and cytoprotective potential of the compound. There are also many studies with Caco-2 cells, which are generally accepted as a good model for normal human colonocytes, which indicate a cytoprotective ability of flavonoids against oxidative DNA damage (Raeissi et al., 1997; Ricchi et al., 1997; Venturi et al., 1997; Duthie & Dobson, 1999).

Ex-vivo studies also suggest that the antioxidant potential of polyphenols may be anticarcinogenic. For example, the ability of plasma to inhibit oxygen free radical induced DNA damage to lymphocytes was increased by 20 % 1 hour after consumption of 300 ml wine (Fenech et al., 1997). Moreover, indices of oxidized DNA in bladder mucosal cells of smokers inversely correlate with the level of phenolics measured in their urine (Malaveille et al., 1998).

Modulation of enzyme activation associated with carcinogen activation and detoxification: One of the mechanisms by which polyphenols may exert their anticarcinogenic effect is by modulating the enzyme systems that metabolize carcinogens or pro-carcinogens to

genotoxins. In this way, the activation of the carcinogen may be inhibited, or it may be converted to a less reactive compound before it reacts with DNA and initiates carcinogenesis. The cytochrome P-450 superfamily of enzymes metabolizes a large number of procarcinogens to reactive intermediates, which bind covalently to DNA and can induce malignant transformation. The activity of some P-450s are either induced or inhibited by flavonoids. For example, naringenin and tangeretin are potent inhibitors of microsomal 7-ethoxyresorufin-O-deethylase (EROD) activity, which is a marker substrate for P-450 IA (Obermeier et al., 1995). Similarly, quercetin inhibits EROD activity (IC_{50} < 1 μM) in microsomes from human hepatoma HepG2 cells (Musonda et al., 1997). Pentoxyresorufin-O-dealkylase (PROD) activity is also decreased, indicating ability of the flavonoids to inhibit P450 2B activity. Tangeretin inhibits nifedepine oxidase, (P450 3A) in human liver microsomes (Obermeier et al., 1995). Flavone and several hydroxylated derivatives (3-OH-, 5-OH-, 7-OH- and 3, 7-dihydroxyflavone) are potent inhibitors of cDNA expressed human P450s IA1 and IA2 (IC_{50} < 1 μM), while galangin is a selective inhibitor of P450 IA2 (Zhai et al., 1998). The ability of flavonoids to inhibit P450 1A is directly related to their antimutagenic properties. Several flavones, including apigenin and luteolin and flavonols such as kaemferol, quercetin and myricetin, reduce the mutagenicity of the food-derived heterocyclic amine 3-amino-1-methyl-5H-Pyrido [4, 3-b] indole (Trp-P-2) in the Ames test (Salmonella typhimurium TA 98). Trp-P-2 is metabolized by P450 IA to the ultimate mutagen N-hydroxy-Trp-P-2 that binds to the DNA molecule and initiates carcinogenesis (Kanagawa et al., 1998).

Therefore, the effect of flavonoids on xenobiotic metabolizing enzyme is complex and highly dependent on a number of factors including the

chemical structure of the flavonoid, the species under investigation and the model system being employed. Despite the considerable experimental evidence that certain polyphenols have potent anti-carcinogenic activity, epidemiological support is contradictory. For example, some ecological, cohort and case-control studies suggest that tea consumption lowers the risk of developing cancer whereas other investigations have failed to find such associations or have even indicated procarcinogenic effects (Blot et al., 1996). In addition, no correlation was observed between estimated flavonoid intake (determined in 1985) and cancer incidence (P = 0.54) and mortality (P = 0.51) at all sites after a 5-year period in 738 elderly Dutch men (65-84 years; Hertog et al., 1994). The inconclusive nature of the epidemiological studies may reflect a lack of information on the duration and amount of polyphenol intake, inadequate control of confounding and potential basis in recall and reporting of intake patterns.

Anthocyanins :

Anthocyanins are dietary polyphenolic phytochemicals which belong to the flavonoid family. They are natural pigments and contribute to the brilliant colors of berries, cherries, pomegranate and other fruits and vegetables. Anthocyanins are stable under acidic conditions but are unstable and rapidly broken down under neutral conditions. Anthocyanins are implicated in many biological processes (Kong et al., 2003) that may impact positively on health. These pigments may reduce the risk of coronary heart disease through modulation of arterial vasomotion (Colantuoni et al., 1991), inhibition of platelet aggregation (Morazzoni et al., 1990) or endothelial protection (Youdim et al., 2002). In addition, anthocyanins could exert anticarcinogenic activities *in vitro* (Fimognari et al., 2004), reduce inflammatory insult (Youdim et al., 2002) and also modulate immune response (Wang & Mazza, 2002). Anthocyanins,

accounting to several hundreds in total, are glycosides of glucose, rhamnose, galactose or other monosaccharides and combinations thereof. The difference between individual anthocyanins are related to the number of hydroxyl groups, the nature and number of sugars and the position of these attachments (Kong et al., 2003). The aglycons of anthocyanins which consists of diphenylpropane-based polyphenolic ring structures are known as anthocyanidins. Only a few kinds of anthocyanidins are known (Mazza and Miniati, 1993). The most common anthocyanidins present in higher plants are cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin and are distributed in nature by 50, 12, 12, 12, 7 and 7 % respectively. The pigmented leaves, fruits and flowers contain primarily the glycosides of cyanidin, delphinidin and pelargondin with cyanidin being the most abundant (Kong et al., 2003). Among fruits, pomegranate is an interesting rich source of anthocyanins and other phenolic compounds. Pomegranate (*Punica granatum*, Punicaceae), native to Persia, is an edible fruit cultivated in Mediterranean countries, Afghanistan, India, China, Japan, Russia, and some parts of the United States. Pomegranate has been extensively used as a folk medicine by many cultures (Langley, 2000). Pomegranate has featured prominently in all major religions of the world. Pomegranates appear in the coats of arms of several British medical societies (Langley, 2000). The pomegranate is a symbol of life, longevity, health, femininity, fecundity, knowledge, morality, immortality and spirituality, if not divinity (Mahdihassan, 1984). In Ayurvedic medicine the pomegranate is considered "a pharmacy unto itself," and the bark and roots are believed to have anthelmintic and vermifuge properties (Naovi et al., 1991), the peels a powerful astringent and cure for diarrhea and oral aphthae, and the juice a "refrigerant" (Arseculeratne et al., 1985) and "blood tonic" (Lad and Frawley, 1986). In India (Nagaraju and Rao, 1990), Tunisia (Boukef et al., 1982), and Guatemala (Caceres et al., 1987), dried pomegranate peels are decocted in water and employed both internally and externally for numerous problems demanding astringents and/or germicides, especially for

aphthae, diarrhea and ulcers. Mixtures of pomegranate seed, juice and peel products paradoxically have been reported to not only prevent abortion (Ramirez et al., 1988) but also conception (Gujral et al., 1960; Jochle, 1971; Zhan, 1995). In Unani medicine, a Middle Eastern traditional medical system that later took root in India (Izhar, 1989), pomegranate flowers serve as a remedy for diabetes mellitus (Saxena and Vikram, 2004). Modern uses of pomegranate derived products now include treatment of acquired immune deficiency syndrome (AIDS) (Lee and Watson, 1998), in addition to use for cosmetic beautification (Kawamada and Shimada, 2002; Moayadi, 2004) and enhancement (Curry, 2004), hormone replacement therapy (Lansky, 2000), resolution of allergic symptoms (Watanabe and Hatakoshi, 2002), cardiovascular protection (Shiraishi et al., 2002; Aviram and Dornfeld, 2003), oral hygiene (Kim and Kim, 2002), ophthalmic ointment (Bruijn et al., 2003), weight loss soap (Guojian, 1995), and as an adjunct therapy to increase bioavailability of radioactive dyes during diagnostic imaging (Il'iasov, 1975; Amorim et al., 2003).

Biosynthesis of Delphinidin in plants :

An outline of delphinidin biosynthesis is given in Figure-1. Delphinidin (an anthocyanidin) is a polyoxygenated derivative of 2-phenyl–benzopyrylium cation, which consists of three six membered rings as shown in figure-2. Delphinidin is derived from a branch of the flavonoid pathway, for which chalcone synthase (CHS) provides the first committed step by condensing one molecule of p-coumaroyl-CoA with three molecules of malonyl-CoA to produce tetrahydroxychalcone (a chalcone). CHS belongs to the family of polyketide synthases and the structure of this enzyme has been solved (Austin & Noel, 2003; Ferrer et al., 1999). Chalcones which provide a central branchpoint in the flavonoid pathway, serve as subtrates for the biosynthesis of flavanones, dihydroflavonols, leucoanthocyanidins and anthocyanidins (Grotewold, 2006). Among fruits and beverages, berries (bilberries and

blackberries) (Talavera et al., 2003), pomegranate (Noda et al., 2002) and red wine are considered to be the richest sources of anthocyanins. The daily intake of anthocyanins in humans has been estimated to be about 180-215 mg / day (Kuhnau, 1976).

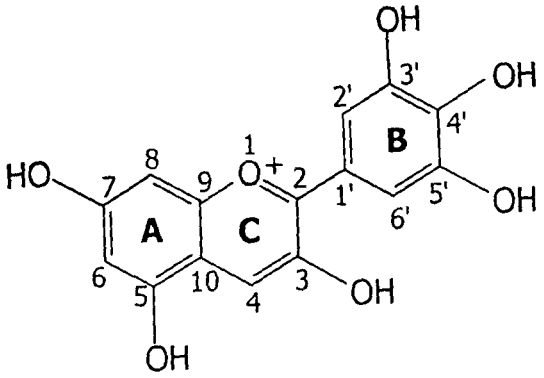


Figure-2 : Chemical Structure of Delphinidin

Absorption, Metabolism and Bioavailability of Delphinidin :

The anthocyanidin, delphinidin and its glycosidic derivatives like delphinidin-3-glucopyranoside (Dp3G) are easily absorbed in the intact form after oral administration in rats and humans (Matsumoto et al., 2001). Even after 4 hour, the plasma concentration of Dp3G was estimated to be about 30 nmol/l (Ichiyanagi et al., 2004). It is generally accepted that orally administered anthocyanins are absorbed from the stomach because anthocyanins are stable as the flavylium cation form in acidic media like gastric juice and this better explains the ability of anthocyanins to permeate the gastric mucosa and their fast plasma appearance in rats and humans. Anthocyanin glycosides are both bulky and polar compounds and therefore carrier-mediated mechanisms of absorption from gastro-intestinal tract are likely to be involved. Recently it has been shown that an organic anion carrier, bilitranslocase, expressed in the gastric epithelium, could be involved in the absorption of anthocyanins at the gastric level (Passamonti et al ., 2003). Reports also suggest that the efficient

absorption of anthocyanins from the small intestine involves an interaction with the intestinal sodium-dependent glucose transporter SGLT1. Furthermore, anthocyanins are quickly metabolized and excreted into bile and urine as intact glycosides as well as methylated forms and glucuronidated derivatives (Talavera et al., 2004). The bioavailability of delphinidin and cyanidin has been estimated to be about 60 % in healthy volunteers after consumption of red wine (Frank et al., 2003).

Delphinidin as an antioxidant :

The anthocyanidin, delphinidin has recently gained interest because of the relatively high potential intake in humans and their broad spectrum of potentially positive health effects such as reduction of lipid peroxidation and LDL oxidation to potential therapeutic effects acting as antineoplastic, radiation-protective, vasotonic, vasoprotective, anti-inflammatory and chemoprotective agent (Rice-Evans et al., 1995, 1996; Lapidot et al., 1999). These biological activities of delphinidin are closely related to their potent antioxidant properties. The role of delphinidin as an antioxidant is well described in doxorubicin induced cardiotoxicity in H9c2 cardiomyocytes (Choi et al., 2007). Delphinidin also exerts strong antioxidant effects and inhibits UVB-mediated lipid peroxidation in HaCaT keratinocytes (Afaq et al., 2007). Among the various anthocyanidins, delphinidin has been shown to possess potent free radical scavenging activity. Reports suggest that the presence of three free hydroxyl groups on the B-ring of delphinidin could be responsible for the above mentioned effect of delphinidin. Glycosylation and O-methylation on the free phenolic groups decreases the antioxidant potential of delphinidin (Rahman et al., 2006).

Anticancer properties of pomegranate juice and delphinidin :

Pomegranate fruits are widely consumed in fresh and beverage forms as juice. Table-1 describes the chemopreventive efficacy of anthocyanidins present in

pomegranate. Recently there have been numerous reports on the *in vitro* and *in vivo* anticancer properties of pomegranate. The activity of pomegranate against specific molecular targets associated with carcinogenesis was evaluated in CD-1 mouse model of chemical carcinogenesis. Skin cancer was initiated in these animals using DMBA followed by biweekly applications of another tumor promoting agent, 12-O-tetradecanoyl phorbol-13-acetate (TPA). Topical application of pomegranate fruit extract (PFE) prior to TPA treatment inhibited TPA mediated increase in skin edema and hyperplasia, epidermal ornithine decarboxylase (ODC) activity and protein expression of ODC and COX-2 as well as activation of MAPK and NF-κB pathways. Both tumor incidence and tumor multiplicity were greatly reduced in pomegranate treated CD-1 mouse models (Syed et al., 2007). It has also been shown that PFE treatment of human prostate cancer PC-3 cells resulted in a dose dependent inhibition of cell growth / cell viability and induction of apoptosis. PFE treatment of PC-3 cells resulted in induction of proapoptotic proteins and induction of cell cycle regulatory molecules. Oral administration of PFE to athymic nude mice implanted with androgen sensitive CWR22Ru1 cells resulted in a significant inhibition in tumor growth concomitant with a significant decrease in serum prostate specific antigen (PSA) levels (Khan et al., 2008). *In vitro* studies by the same authors also suggest the antiproliferative effect of pomegranate juice on human breast cancer cells. In murine mammary gland organ culture, pomegranate juice effected 47 % inhibition of cancerous lesion formation induced by the carcinogen DMBA. Further studies also show that the treatment with PFE was found to result in a decrease in the viability of human lung carcinoma A549 cells. PFE treatment of A549 cells also resulted in dose-dependent arrest of cells in G_0-G_1 phase of the cell cycle, induction of cell cycle regulatory proteins, inhibition of MAPK and NF-kB pathways, and inhibition of NF-kB DNA-binding activity. Oral administration of PFE to athymic nude mice implanted with A549 cells resulted in a significant inhibition in tumor growth. The chemopreventive effect of PFE was also

studied on lung tumorigenesis induced by benzo(a)pyrene [B(a)P] and N-nitroso-tris-chloroethylurea (NTCU) in A/J mice. Mice exposed to B(a)P and NTCU and treated with PFE had statistically significant lower lung tumor multiplicities than mice treated with carcinogens only (Khan et al., 2008). Reports also suggest pomegranate juice as an antiproliferative and apoptosis inducing agent against HT-29 colon cancer cells (Adam et al., 2006). Thus, initial research suggests that the pomegranate fruit and its associated pytochemicals may possess a strong potential for development as a chemopreventive and possibly as a therapeutic agent against various human cancers (Syed et al., 2007).

Table-1 : Chemopreventive efficacy of anthocyanidins

Anthocyanidins	Cell line / fraction	Effect	Reference
Delphinidin	JB6	↓ TPA-induced AP-1 activation	Hou et al. 2004a
		↓ JNK / ERK phosphorylation	Hou et al. 2004a
	A431	↓ EGFR tyrosine kinase activity	Meiers et al. 2001
		↓ Elk-1 activation	Meiers et al. 2001
	HT29	↓ EGFR tyrosine kinase activity	Marko et al. 2004
		↑ PDE4 inhibition	Marko et al. 2004
	CaCo-2	G_2/M phase arrest	Lazze et al. 2004
		↑ Apoptosis	Lazze et al. 2004
	HeLaS3	G_2/M phase arrest	Lazze et al. 2004
		↑Apoptosis	Lazze et al. 2004
	Human embryonic fibroblasts	↑Apoptosis	Lazze et al. 2004
		S-Phase arrest	Lazze et al. 2004
	HL60	↑ Apoptosis	Hou et al. 2003
		Caspase 3 activation	Hou et al. 2003
		↑ JNK phosphorylation	Hou et al. 2003
	RAW264	↓ LPS-induced 1kBdegradation	Hou et al. 2004b
		↓ LPS-induced NFkB activation	Hou et al. 2004b
		↓ LPS-induced Cox 2 expression	Hou et al. 2004b
	Rat seminal vesicle	↓ Cox-1 activity	Seeram et al. 2003
	Insect cell lystate	↓ Cox-2 activity	Seeram et al. 2003
Cyanidin	JB6	↓ TPA-induced AP-1 activation	Hou et al. 2004a
	HL-60	↑ Apoptosis	Hou et al. 2003
	Normal human fibroblasts	GI phase arrest	Lazze et al 2004

	A431	↓ EGFR tyrosine kinase activity	Meiers et al. 2001
		↓ Elk-1 activation	Meiers et al. 2001
	HT29	↓ EGFR tyrosine kinase activity	Marko et al 2004
		↑ PDE4 inhibition	Marko et al. 2004
	U937[a]	Arrest of G_2/M Phase	Hyun et al. 2004
		Apoptosis	Hyun et al. 2004
	Rat seminal vesicle	↓ Cox-1 activity	Seeram et al 2001
	Rat seminal vesicle	↓ Cox-1 activity	Seeram et al 2003
	Insect cell lysate	↓ Cox-1 activity	Seeram et al 2001
	Insect cell lysate	↓ Cox-1 activity	Seeram et al 2003
	Rat liver microsomes	Antioxidant	Tsuda et al. 1994
	Liposomes	Antioxidant	Tsuda et al. 1994
	Rabbit erythrocyte membranes	Antioxidant	Tsuda et al. 1994
Malvidin	U937[a]	Arrest of G_2/M phase	Hyun et al. 2004
		↑ Apoptosis	Hyun et al. 2004
	A431	↓ Elk-1 activation	Meiers et al 2001
	JB6	Weak inhibition of AP-1 activity	Hou et al. 2004a
	HT29	↓ EGFR tyrosine kinase activity	Marko et al. 2004
		PDE4 inhibition	Marko et al. 2004
	Rat seminal vesicle	↓ Cox-1 activity	Seeram et al. 2003
	Insect cell lysate	↓ Cox-2 activity	Seeram et al. 2003
Petunidin	JB6	↓ TPA-induced AP-1 activation	Hou et al. 2004a
	HL60	↑ Apoptosis	Hou et al. 2003
Peonidin	HT29	↓ EGFR tyrosine kinase activity	Marko et al. 2004
		↑ PDE4 inhibition	Marko et al. 2004
	Rat seminal vesicle	↓ Cox-1 activity	Seeram et al. 2003
	Insect cell lysate	↓ Cox-2 activity	Seeram et al. 2003
Pelargonidin	JB6	Weak inhibition of AP-1 activity	Hou et al. 2004a
	HT29	↓ EGFR tyrosine kinase activity	Marko et al. 2004
		PDE4 inhibition	Marko et al. 2004
	Rat seminal vesicle	↓ Cox-1 activity	Seeram et al. 2003
	Insect cell lysate	↓ Cox-2 activity	Seeram et al. 2003

From: Cooke et al., 2005

Delphinidin, the major anthocyanidin in pomegranate juice has also been studied extensively as an anticancer chemotherapeutic agent. Delphinidin has been shown to possess chemopreventive effect on UVB-mediated responses in human immortalized HaCaT keratinocytes under *in vitro* situation and SKH-1 hairless mouse model *in vivo* situation. Studies suggest that 10 μM dose of delphinidin provided substantial protection against UVB-mediated decrease in cell viability. Using immunocytochemical staining, UVB radiation induced oxidative damage in DNA (resulting in the formation of the adduct of 8-OHdG) was analysed and was found that the treatment of cells with delphinidin resulted in a significant protection against UVB-mediated formation of 8-OHdG. Protective effect of delphinidin in HaCaT cells against UVB-mediated apoptosis via modulation of BcL-2 family members was also shown. The *in vivo* part of the same study describes that the topical application of delphinidin to SKH-1 hairless mouse skin inhibited UVB-mediated apoptosis and the markers of DNA damage like cyclobutane pyrimidine dimers (CPDs) and 8OHdG (Afaq et al., 2007). Delphinidin can also be considered as a promising antiproliferative agent. It has been shown that delphinidin inhibits both serum- and vascular endothelium growth factor (VEGF)-induced proliferation of bovine aortic endothelial cells (BAECs), through cell cycle progression arrest in G_0/G_1 phase. The antiproliferative effect was independent of the nitric oxide (NO) pathway but was triggered by the activation of ERK-1/-2 pathway. Authors have also reported alterations of the mitogenic signal transduction reflected by an inhibition of Ras expression, an over-expression of caveolin-1, an induction of $p21^{WAF1/Cip1}$ expression and finally the down-regulation of cyclin D1. Altogether, the antiproliferative effects of delphinidin represent potential mechanisms for prevention of cardiovascular diseases and cancer, in which endothelial cell proliferation favors atherosclerotic plaque development and/or fragility and tumor metastasis (Martin et al., 2003).

The molecular mechanism involved in the anticarcinogenic activity of delphinidin have been extensively studied. Delphinidin has been shown to

inhibit TPA-induced activator protein-1 (AP-1) transcriptional activity and cell transformation in JB6 mouse epidermal cell line (Hou et al., 2004a). Structure activity studies indicated that the ortho-dihydroxyphenyl structure on the B-ring of delphinidin seems essential for the inhibitory action because pelargonidin, peonidin and malvidin, having no such ortho-dihydroxyphenyl structure, failed to show the inhibitory effects in both AP-1 activity and cell transformation. The results from signal transduction analysis indicated that delphinidin blocked extracellular signal regulated kinase (ERK) phosphorylation at early times and c-jun N-terminal kinase (JNK) phosphorylation at later times, but not p38 phosphorylation at any time (Hou et al., 2004a). Moreover, delphinidin blocked the phosphorylation of MAPK/ERK kinase (MEK, an ERK kinase), SAPK/ERK kinase (SEK, a JNK kinase), and c-Jun (a phosphorylation target of ERK and JNK). The data suggest that the inhibition of TPA-induced AP-1 activity and cell transformation by delphinidin involves the blockage of ERK and JNK signaling cascades. Furthermore, a greater inhibition was observed in combinations of superoxide dismutase (SOD) with anthocyanidins that have the ortho-dihydroxyphenyl structure on the B-ring. Multiplicative model analysis suggested that this greater inhibition between SOD and delphinidin is synergistic not additive (Hou et al., 2004a). Thus the inhibitory effects of anthocyanidins on AP-1 activation and cell transformation would be due in part to their potent scavenging activity for superoxide radicals and in part to MAPK blockage. SOD has been shown to selectively inhibit the TPA-induced activation of protein kinase Epsilon and to prevent subsequent activation of JNK2 in response to TPA, thereby delaying AP-1 activation and inhibiting mouse skin tumor promotion. Thus, the signaling pathways blocked by delphinidin or SOD may differ in part although both are considered to be important in the cancer prevention activity of anthocyanidins (Hou et al., 2004b). Delphinidin, cyanidin and petunidin induced apoptosis of HL-60 cells detected by morphological changes and by DNA fragmentation, whereas pelargonidin, peonidin and malvidin showed no

induction of apoptosis (Hou et al., 2003). The anthocyanidin glycosides (anthocyanins) extracted from bilberry such as delphinidin glycosides and cyanidin glycosides also induced apoptosis in HL-60 cells (Katsube et al., 2003). Structure-activity studies indicated that the potency of apoptosis induction of anthocyanidins is associated with the number of hydroxyl groups at the B-ring and the ortho-dihydroxyphenyl structure at the B-ring appears essential for apoptosis actions (Hou et al., 2003). It is noteworthy that anthocyanidins increased the levels of hydrogen peroxide in HL-60 cells with a structure activity relationship that depends on the number of hydroxyl groups at the B-ring (Hou et al., 2003) and appears in the order of delphinidin > cyanidin, petunidin > pelargonidin, peonidin and malvidin.

Mechanistic analysis indicates that the apoptosis induction by delphinidin may involve an oxidation/JNK-mediated caspase pathway. Delphinidin treatment increased the levels of intracellular ROS, which may be a sensor to activate JNK. Concomitant with the apoptosis, JNK pathway activation such as JNK phosphorylation, c-jun gene expression and caspase-3 activation was observed in delphinidin-treated cells (Hou et al., 2003). Antioxidants such as N-acetyl-L-cysteine (NAC) and catalase effectively blocked delphinidin-induced JNK phosphorylation, caspase 3 activation and DNA fragmentation (Hou et al., 2003). Thus, delphinidin may trigger an apoptotic death program in HL-60 cells through an oxidative stress-mediated JNK signaling cascades. Authors suggest the prooxidant role of delphinidin for the above mentioned apoptotic induction in HL-60 cells (Hou et al., 2003).

SCOPE OF THE WORK PRESENTED

SCOPE OF THE WORK PRESENTED

The health benefits of plant derived polyphenols including delphinidin, caffeic acid, resveratrol, curcumin, capsaicins, tannins and flavonoids have been attributed to their antioxidant effects. However, 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 plausible that other mechanisms may be responsible for such varied pharmacological properties. Most antioxidants of plant origin are redox (reduction-oxidation) agents, protecting against ROS generation 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 reactive oxygen species (Ahmad et al., 1992; Bhat & Hadi, 1994; Ahsan & Hadi, 1998; Ahmad et al., 2000; Azam et al., 2004; Ahmad et al., 2005). 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). Copper is a major metal ion present in the nucleus and is also implicated in tumorigenesis and angiogenesis (Chevion, 1988). Also, serum, tissue and cellular copper levels are significantly elevated in a number of malignancies (Linder, 1991). Pomegranate and red wine are known good sources of delphinidin. Induction of apoptosis by delphinidin has been shown in human promyelocytic leukemia HL-60 cells (Hou et al., 2003).

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 we have proposed a mechanism according to which plant polyphenols mobilize

endogenous copper leading to cytotoxic action through the generation of reactive oxygen species (Hadi et al., 2000). Based on the above hypothesis, in the work presented here, I have attempted to elucidate the mechanism of action of plant derived polyphenolic compounds specially the anthocyanidin, delphinidin. In this thesis, using a cellular system of human peripheral lymphocytes isolated from human blood and alkaline single cell gel electrophoresis (Comet Assay), I have confirmed that delphinidin-Cu(II) system is indeed capable of causing DNA degradation in cells such as lymphocytes. Further, DNA degradation of lymphocytes is inhibited by the scavengers of reactive oxygen and neocuproine which is a Cu(I) specific sequestering agent. These findings demonstrate that delphinidin-Cu(II) system for DNA breakage is physiologically feasible and could be of biological significance. Experiments also show that delphinidin alone (in the absence of added Cu(II)) is also capable of DNA breakage in intact lymphocytes as well as in lymphocyte nuclei and that such breakage is mediated through mobilization of endogenous copper. These results are in further support of the hypothesis that anticancer mechanisms of plant polyphenols may involve mobilization of endogenous copper, possibly nuclear copper and the consequent prooxidant action (Hanif et al., 2008).

MATERIALS AND METHODS

MATERIALS

Chemicals	Source
Agarose	Sigma Chemical Co., USA
Ammonium persulphate	Sigma Chemical Co., USA
Bovine Serum Albumin	Sigma Chemical Co., USA
Catalase	Sigma Chemical Co., USA
Delphinidin	Extra Synthese (Genay) France
Deoxyribonucleic acid (Calf Thymus Type I)	Sigma Chemical Co., USA
Diphenylamine	BDH, India
Ethidium Bromide (EtBr)	Sigma Chemical Co., USA
Ethylenediaminetetraacetic acid (EDTA)	Qualigens, India
Low melting point agarose	Sigma Chemical Co., USA
Neocuproine	Sigma Chemical Co., USA
Nitroblue Tetrazolium (NBT)	Sisco Research Lab, India
Phosphate Buffered Saline Ca^{2+} and Mg^{2+} free	Sigma Chemical Co., USA
RPMI 1640 media	Sigma Chemical Co., USA
Trans-resveratrol	Sigma Chemical Co., USA
Single strand specific nuclease	Isolated and purified from germinating pea seeds, in the lab according to the procedure of Wani & Hadi, 1979
Sodium Azide	E. Merck, Germany
Sodium Benzoate	E. Merck, Germany
Supercoiled plasmid pBR322 DNA	Genei, India
Superoxide dismutase	Sigma Chemical Co., USA

Thiourea	E. Merck, Germany
Tris(hydroxyl methyl)aminomethane	Fluka AG, Switzerland
Triton X – 100	BDH, India

* All other chemicals were commercial products of analytical grade.

METHODS

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

Delphinidin was dissolved in 3mM NaOH before use as stock of 1mM. All other polyphenols were dissolved in water. Upon addition to reaction mixtures, in the presence of buffers and at the concentrations used, all the polyphenols remained in solution. The volumes of the stock solution added did not lead to any appreciable change in the pH of the reaction mixtures. Reaction mixtures (0.5 ml) contained 10 mM Tris-HCl (pH 7.5), 500 µg of calf thymus DNA and varying amounts of delphinidin and cupric chloride. All solutions were sterilized before use. Incubation was performed at 37 °C for specified time periods. Single strand specific digestion was performed as described by Wani and Hadi (1979). 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 40mM Tris-HCl (pH 7.5), 1 mM Magnesium Chloride, water and enzyme. The reaction mixture was incubated at 37 °C for 2 hrs. 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. The melting profile of DNA using S_1 nuclease was determined essentially as described by Case and Baker (1975). DNA solution was prepared in TNE (0.01 M Tris-HCl

pH 7.4, 0.01 M Nacl and 20 x 10^{-4} mM EDTA) and incubations with delphinidin was carried out in sterile tubes and sterile conditions. Details of the treatment are given in legends to figures.

Treatment of pBR322 DNA with delphinidin 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 time periods specified in legends. 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 gels. The gels were stained with ethidium bromide (0.5 µg/ml), viewed and photographed on a transilluminator.

Absorption studies :

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

Spectrophotometric detection of Cu(II) reduction by delphinidin :

The selective sequestering agent neocuproine was employed to detect reduction of Cu(II) to Cu(I) by recording the formation of neocuproine–Cu(I) complex which absorbs maximally at 450 nm. The reaction mixture (3.0 ml) contained 3 mM Tris–HCl (pH 7.5), fixed concentration (100 µM) of Cu(II), 300 µM neocuproine and varying concentrations (50-100 µM) of delphinidin. The reaction was started by adding Cu(II) and the spectra were recorded immediately afterwards.

Stoichiometric titration of Cu(I) production by delphinidin :

The amount of Cu(I) produced in the delphinidin-Cu(II) reaction mixture was determined by titration with neocuproine. Delphinidin (50 µM) in 10 mM Tris-

HCl (pH 7.5) was mixed with varying concentrations of Cu(II) ($CuCl_2$) and 300 μM neocuproine in a total reaction volume of 3 ml. Absorbance was recorded at 450 nm after 10 min of incubation at room temperature.

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

Superoxide 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 8.0), 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 delphinidin. 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 delphinidin

Inorder to compare the hydroxyl radical production by increasing concentrations of delphinidin in the presence of 50 μM Cu(II), the method of Quinlan and Gutteridge (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. Delphinidin was excited at its absorption maxima (λmax) of 548 nm. Emission spectra were recorded in the range shown in figures.

Determination of reducing power of delphinidin :

The reducing power of delphinidin was determined according to the method of Oyaizu (1986). Delphinidin was mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1 % potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then an equal volume of 1 % trichloroacetic acid was added to the mixture, which was then centrifuged at 6000 rpm for 10 min. The upper layer of the solution was mixed with distilled water and 0.1 % $FeCl_3$ with a ratio of 1 : 1 : 2, and the absorbance at 700 nm was measured. Increased absorbance of the reaction mixture indicated increased reducing power.

Isolation of lymphocytes :

Heparinized blood samples (2 ml) from healthy donors were 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.

Treatment of lymphocytes with delphinidin and other polyphenols :

Lymphocytes (1×10^5 cells) were exposed to different concentrations of polyphenols in the absence and presence of indicated concentrations of Cu(II) in a total reaction volume of 1 ml (400 µl RPMI, PBS Ca^{2+} and Mg^{2+} free and indicated concentrations of polyphenols). In some experiments lymphocytes were pre-incubated with 50 µM delphinidin or 20 µM Cu(II). In another set of experiments, scavengers of reactive oxygen were added at the final concentrations indicated. Incubation was performed at 37 °C for indicated time periods. After the incubation, the reaction mixture was centrifuged at 4000 rpm, the supernatant was discarded and pelleted lymphocytes were resuspended in 100 µl PBS and processed further for Comet Assay.

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 %.

Alkaline single cell gel electrophoresis/Comet Assay :

Comet Assay was performed under alkaline conditions essentially according to the procedure of Singh et al., (1989) 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 1.0 % LMPA to form a cell suspension and pipetted over the first layer and covered immediately by a coverslip. The slides were placed on a flat tray and kept on ice for 10 min to solidify the agarose. The coverslips were removed and a third layer of 0.5 % LMPA (75 µl) was pipetted and coverslips placed over it and allowed to solidify on ice for 5 min. 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 hr 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 0.7 volts/cm and 300 mA current (for neutral Comet Assay 0.4 M Tris-HCl pH 7.5 was used as electrophoretic buffer). The slides were then neutralized with cold 0.4 M Tris, pH 7.5, stained with 75 µl EtBr (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 an 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 x magnification. Images

from 50 cells (25 from each replicate slide) were analyzed. The parameter taken to access lymphocyte DNA damage was tail length (migration of DNA from the nucleus, μm) and was automatically generated by Komet 5.5 image analysis system.

Determination of TBARS in lymphocytes:

Thiobarbituric acid reactive substance was determined according to the method of Ramanathan et al., (1994). A cell suspension (1 x 10^5/ml) was incubated with delphinidin (0-400 μM) at 37 °C for 1 hr and then centrifuged at 1000 rpm. In some experiments the cells were pre-incubated with fixed concentrations of neocuproine and thiourea. The cell pellet was washed twice with phosphate buffered saline (Ca^{2+} and Mg^{2+} free) and suspended in 0.1 N NaOH. This cell suspension (1.4 ml) was further treated with 10 % TCA and 0.6 M TBA (2-thiobarbituric acid) in boiling water bath for 10 min. The absorbance was read at 532 nm.

Lysed Version of Comet Assay :

Lysed version of Comet Assay was performed as described by Kasamatsu et al (1996) with some modifications. Lymphocytes isolated from 2 ml blood were diluted to the count of $2x10^5$ cells/2ml and suspended in RPMI 1640. Approximately 10,000 of these cells were mixed with 75 μl of prewarmed LMPA in PBS and immediately applied to frosted microscopic slide layered with 75 μl of 1% normal melting agarose in PBS. The slides were allowed to gel at 4 °C for 10 min. Lysis of cells was then performed by submerging the slides in a tank containing lysis solution in the absence of light for 1 h at 4 °C. The use of a tank instead of a coplin jar allowed simultaneous processing of a number of slides. The lysis solution (pH 10) consisted of 2.5 M NaCl, 0.1M EDTA, 10 mM Tris and 1% Triton x-100 added just prior to use. After lysis, slides were transferred to another tank containing 0.4 M Phosphate buffer (pH

7.5) for 10 min. Each slide was then transferred to a rectangular dish (8cmx3cmx5mm) which contained a reaction mixture of polyphenols and other additions as mentioned in various legends to figures and tables. The slides with the reaction mixture was incubated at 37 °C for indicated time periods. In another set of experiments, scavengers of reactive oxygen were added at the final concentrations indicated. The slides were then washed twice by placing in 0.4 M phosphate buffer pH 7.5 for 5 min at room temperature. DNA unwinding and expression of alkali labile sites was done by leaving the slides in the high pH electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH > 13 prepared in PBS) at 4 °C for 30 min. Subsequently, the electrophoresis, neutralization and staining of the slides was carried out as described above. Comet images were observed at 100X magnification with a fluorescence microscope (Olympus CX41) and COHU 4910 (equipped with a 510-560 nm excitation and 590 nm barrier filters) integrated CC camera. 50 images were randomly selected from each sample and their lengths (diameter of the nucleus plus migrated DNA) were measured on the screen as automatically generated by Komet 5.5 image analysis system of Kinetic Imaging, Liverpool, UK.

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

Detection of H_2O_2 generation in the incubation medium of nuclei.

The ferrous oxidation-xylenol orange (FOX) assay was adapted as described by Long et al (2000) to detect and quantify the generation of H_2O_2 in the incubation medium (phosphate buffer 0.4 M, pH 7.5) by delphinidin. The simplified reaction sequence involves the oxidation of ferrous (Fe^{2+}) to ferric

(Fe^{3+}) ions by H_2O_2 with the subsequent binding of the Fe^{3+} ion to the ferric sensitive dye xylenol orange, yielding an orange to purple complex, which is measured at 560 nm. The reaction mixture contained delphinidin and phosphate buffer (incubation medium used in the treatment of nuclei). After incubation for 2 h at 37°C, an aliquot of **100** µl was analyzed for H_2O_2 formation.

Isolation of nuclei from lymphocytes and determination of TBARS in nuclei :

Nuclei were isolated from lymphocytes by following the procedure given in Qiagen Genomic DNA Handbook, 2001. 3ml of lymphocyte suspension (2 x 10^7 cells) was added to an equal volume of ice cold cell lysis buffer (1.28 M Sucrose, 40 mM Tris-HCl pH 7.5, 20 mM $Mgcl_2$, 4 % Triton X-100) and 3 volumes of ice cold distilled water. The suspension was mixed by inverting the tubes several times and incubated for 10 min on ice. The buffer lyses the cells but stabilizes and preserves the nuclei. The lysed cells were then centrifuged at 4 °C for 15 min at 1300 x g. The nuclear pellet thus obtained was resuspended in 1.5 ml of nuclei suspension buffer consisting of 50 mM Tris-HCl pH 7.5, 20 % Glycerol, 5 mM $Mgcl_2$ and 0.1 mM EDTA .

Thiobarbituric-acid-reactive substance (TBARS) was determined according to the method of Ramanathan et al (1994) with slight modifications. 200 µl of the nuclei suspension was incubated with delphinidin (0-200 µM) at 37 °C in a total volume of 3 ml for 1 hr. The reaction was stopped by the addition of 30 µl of 5 N NaOH. In some experiments nuclei were preincubated with a fixed concentration of neocuproine and thiourea. To 1.5 ml of the reaction mixture was added 0.5 ml of 10 % TCA and 0.5 ml of 0.6 M TBA (2-thiobarbituric acid) and the mixture incubated in a boiling water bath for 10 min. The absorbance was read at 532 nm and converted into nmoles of TBA reactive substance using the molar extinction coefficient.

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.

CHAPTER-I

DNA Binding and its degradation by delphinidin in the presence of copper ions

RESULTS-I

RESULTS-I

Reduction of Cu (II) by delphinidin:

The production of Cu(I), formed as a result of reduction of Cu(II) by delphinidin, was analyzed using neocuproine which is a selective Cu(I) sequestering agent that binds specifically to the reduced form of copper (Cu(I)) but not to the oxidized form (Cu(II)) (Simpson et al., 1992). The Cu (I)-neocuproine complex formed exhibits an absorption maximum at 450 nm. Under the experimental conditions employed, neither Cu(II)-neocuproine nor delphinidin-Cu(II) combinations interfere with this maximum, whereas delphinidin (100μM and at 50μM concentrations) react to generate Cu(I) which complexes with neocuproine to give a peak appearing at 450 nm (Figure 3). The implication of this finding is that Cu(II) is reduced by delphinidin in the complex to generate Cu(I).

Stoichiometry of Cu(II) reduction by delphinidin :

Polyphenols are known to reduce Cu(II) to Cu(I) as well as reduce molecular oxygen to superoxide anion (Rahman et al., 1990).The superoxide thus formed spontaneously gives rise to H_2O_2 which in the presence of Cu(I) generates hydroxyl radical (Fenton type reaction). We have therefore deduced the stoichiometry of Cu(II) reduction by delphinidin. In the experiment shown in Figure 4 increasing concentrations of Cu(II) were added to a fixed concentration of delphinidin (50 μM) in the presence of 300 μM neocuproine (a Cu(I) specific sequestering agent). The results are plotted as absorbance at 450 nm Vs equivalents of Cu(II)/delphinidin. A clear maximum starting with a Cu(II) / delphinidin molar ratio of 2 was obtained where absorption plateaued. Thus one mole of delphinidin is able to reduce two moles of Cu(II).

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

Delphinidin in the presence of Cu(II) was found to generate single strand 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. Figure 5 gives the dose response curve of such a reaction. However, delphinidin in the absence of Cu(II) did not generate single strand nuclease sensitive sites in calf thymus DNA. Control experiments (data not shown) established that heat denatured DNA underwent 100 % hydrolysis following the treatment with nuclease. As seen in Figure in the presence of Cu(II) (50 μM), increasing concentrations of delphinidin resulted in progressive increase in nuclease sensitive sites in DNA leading to increased DNA hydrolysis.

Cleavage of plasmid pBR322 DNA by delphinidin :

In Figure 6, I have tested the ability of delphinidin to cause cleavage of supercoiled plasmid pBR322 DNA in the presence of copper ions. Three different concentrations (25, 50, 100 μM) of delphinidin were tested and in the absence of copper none of these caused DNA breakage (lanes 2-4). However in the presence of Cu(II) (50 μM) conversion of supercoiled to open circles and linear molecules was seen when 100 μM delphinidin was used (lane 7). These results demonstrate that delphinidin is capable of DNA degradation in the presence of copper ions. mechanism

Generation of oxygen radicals by delphinidin :

Superoxide production *:* The production of superoxide anion was determined by the method of Nakayama et al (1983), which involves reduction of NBT by delphinidin to a formazan. The time dependent generation of superoxide anion by 100 μM delphinidin, as evidenced by the increase in absorbance at 560 nm is shown in figure 7. The fact that NBT

was genuinely assaying superoxide was confirmed by SOD (100 μg/ml) inhibiting the reaction (results not shown).

Hydroxyl radical generation by delphinidin : 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 delphinidin to generate hydroxyl radical 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 coloured adduct with TBA whose absorbance is read at 532 nm (Quinlan and Gutteridge, 1987). The result of figure 8 clearly show that increasing concentrations of delphinidin lead to a progressive increase in the formation of hydroxyl radicals.

Formation of complexes involving calf thymus DNA and delphinidin : Figure 9 shows the effect of addition of increasing molar base pair ratios of calf thymus DNA on the fluorescence emission of delphinidin. Such an addition resulted in a dose-dependent enhancement of the fluorescence. There was however, no shift in the λ_{max} emission suggesting a simple mode of binding of DNA and delphinidin. The control native DNA alone when excited at the same wavelength (548nm) did not interfere with the emission spectrum of polyphenol alone/polyphenol + DNA, thus confirming the binding results.

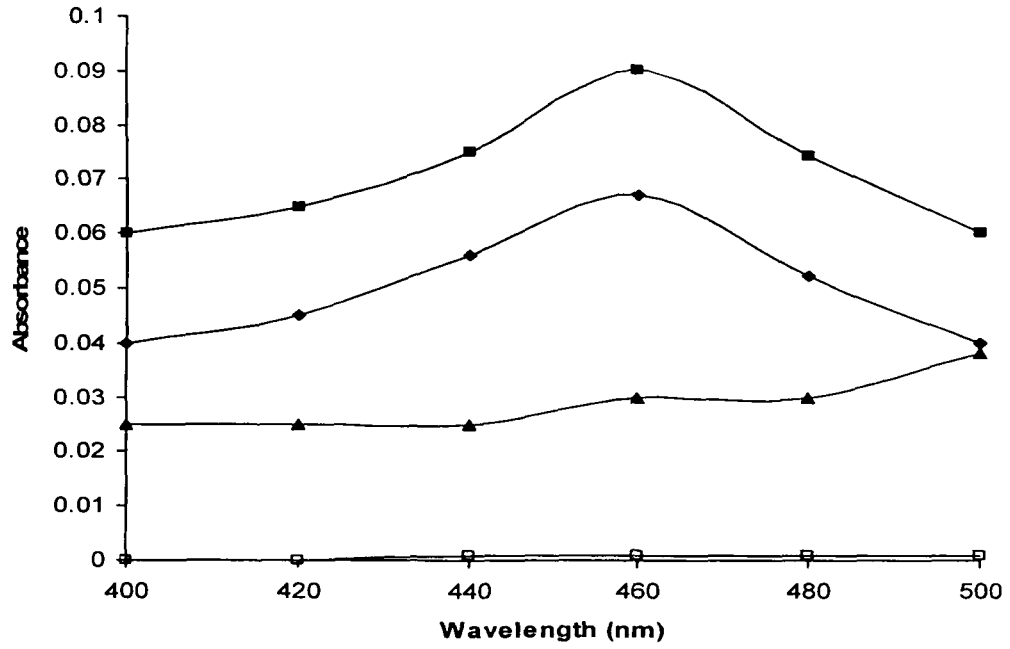
Binding of copper ions to delphinidin :

Binding of copper ions to delphinidin was studied by the effect of increasing Cu(II) molar ratios on the fluorescence emission spectra of delphinidin. The result shown in figure 10 clearly indicate the binding as addition of Cu(II) causes quenching of delphinidin fluorescence. These results support the result of absorption studies shown in figure 3 where formation of delphinidin-copper complex was demonstrated.

Reducing power of delphinidin :

In a Fenton type reaction, Fe(II) or Cu(I) reacts with H_2O_2, resulting in the production of hydroxyl radicals, which is considered to be the most reactive radical for biological macromolecules. Fe^{2+} is oxidized to Fe^{3+} in the Fenton reaction by many reductants. The resultant oxidized forms of iron can be reduced to form Fe^{2+} by polyphenols, which can enhance the generation of hydroxyl radicals. To elucidate the role of reducing power in prooxidant effects, activity of delphinidin on the reduction of Fe^{3+} to Fe^{2+} was measured as shown in figure 11. At all the concentrations tested, delphinidin was found to be an efficient reducing agent.

Figure 3. Detection of delphinidin induced Cu(I) production by neocuproine :



The 3 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), delphinidin, 300 μM neocuproine and 100 μM Cu(II).

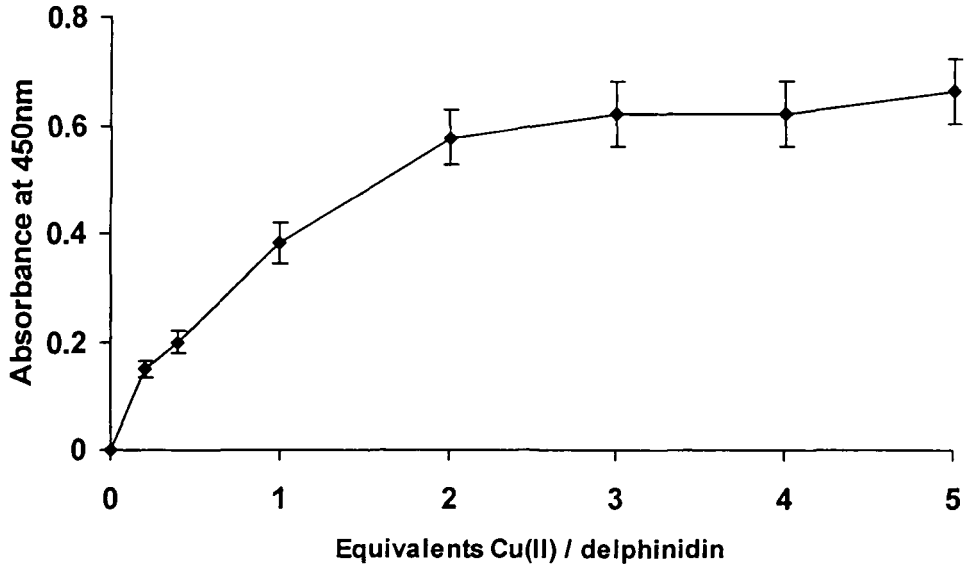
(■) delphinidin (100μM) + Cu (II) + neocuproine

(♦) delphinidin (50μM) + Cu (II) + neocuproine

(▲) Cu(II) + neocuproine

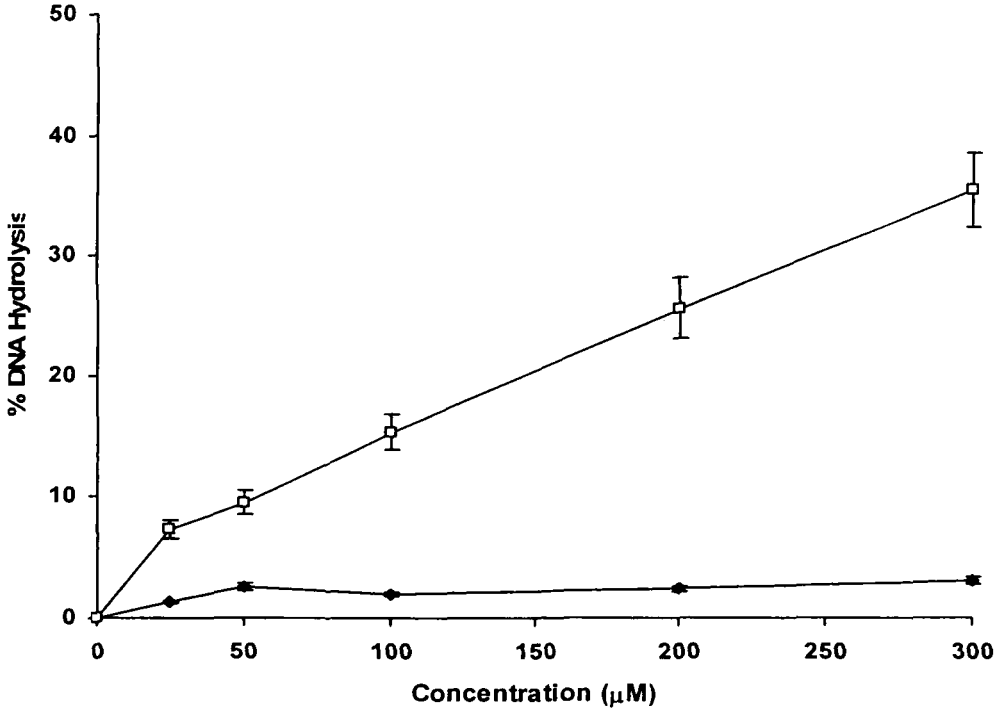
(□) delphinidin (50μM) + Cu(II)

Figure 4. Detection of stoichiometry of delphinidin and Cu(II) interaction :



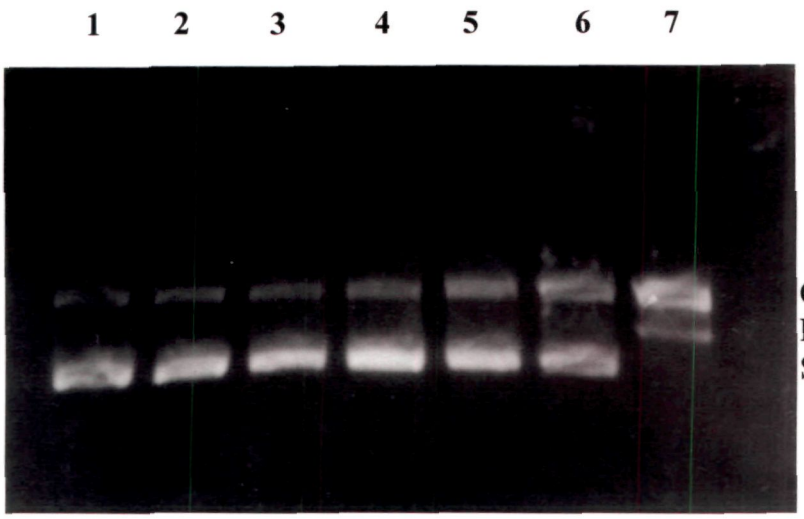
Reaction mixture contained 10 mM Tris-HCl (pH 7.5) and fixed concentration of delphinidin, with increasing concentrations of Cu(II) (shown as molar ratios of Cu(II)/delphinidin). Absorbance was recorded at 450 nm after incubating the reaction mixture at room temperature for 10 min. (♦) delphinidin. All points represent triplicates and mean values have been plotted. Error bars denote SEM of three independent experiments.

Figure 5. Degradation of calf thymus DNA by delphinidin in the presence of Cu(II) as measured by the degree of single strand specific nuclease digestion :



500 μg calf thymus DNA was incubated at 37 °C with indicated concentrations of delphinidin alone (◆) and delphinidin with Cu(II) (50 μM) (□) in a total reaction volume of 0.5 ml containing 10 mM Tris-HCl (pH 7.5). Single strand specific digestion was performed as described in "Methods". All points represent triplicates and mean values have been plotted. Error bars represent SEM of three independent experiments.

Figure 6. Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 DNA after treatment with delphinidin in the absence and presence of Cu(II) :



The reaction mixtures (30 μl) contained 0.50 μg pBR322 DNA, 10 mM Tris-HCl (ph 7.5), indicated concentrations of delphinidin and Cu(II). Incubation was carried out at 37 °C for 30 min.

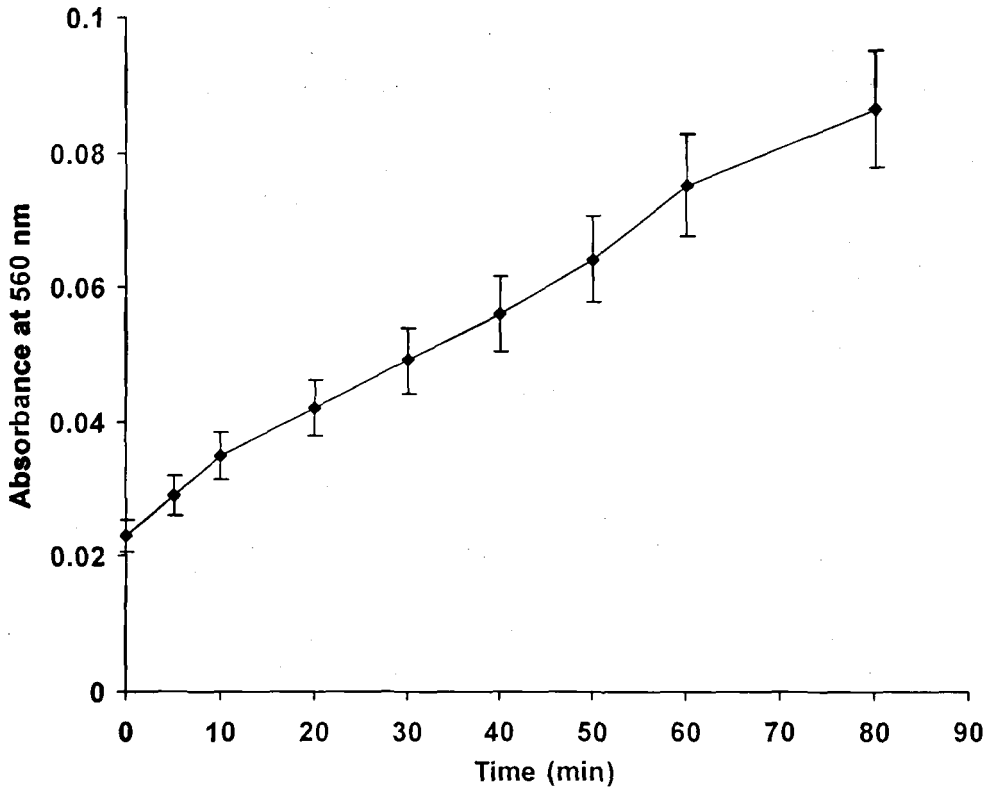
lane 1, DNA alone

lanes 2-4, DNA + delphinidin (25, 50, 100 μM)

lanes 5-7, DNA + delphinidin (25, 50, 100 μM) + Cu(II) (50μM)

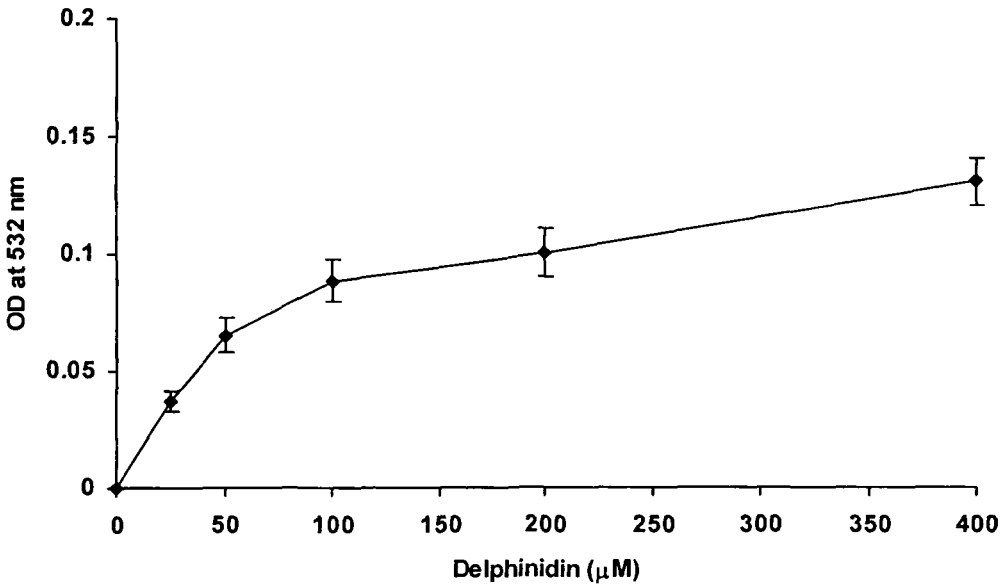
SC, supercoiled : OC, nicked circular : LIN, linear molecules

Figure 7. Photogeneration of superoxide anion by delphinidin on illumination under fluorescent light as a function of time :



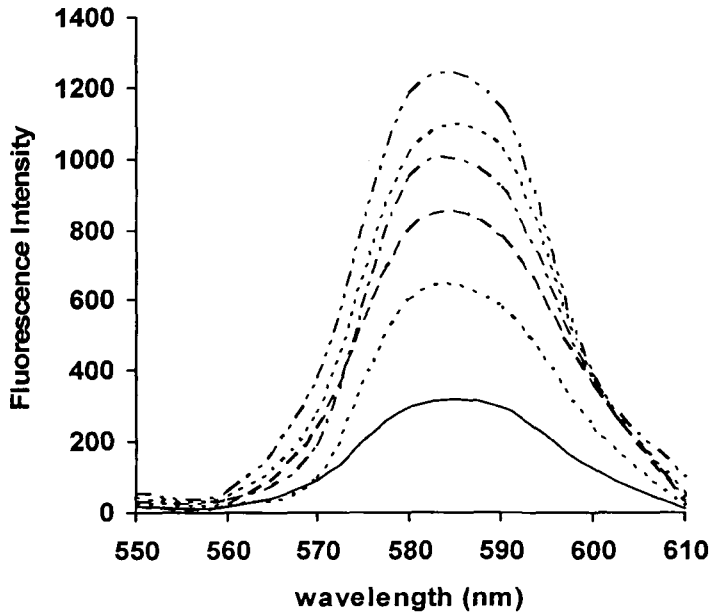
The concentration of delphinidin was 100 μM. Details of the reaction are given in "Methods". The samples were placed 10 cm from the light source. All values reported are means of three independent experiments. Error bars represent standard error of mean.

Figure 8. Hydroxyl radical generation by delphinidin :



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

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



Delphinidin (in Tris-HCl, pH 7.5) was excited at its λ_{max} (absorbtion) of 548 nm and the emission spectra was recorded between 550-610 nm.

[–] Delphinidin alone (25 μM)

[----] Delphinidin : DNA base pair molar ratio (1:1)

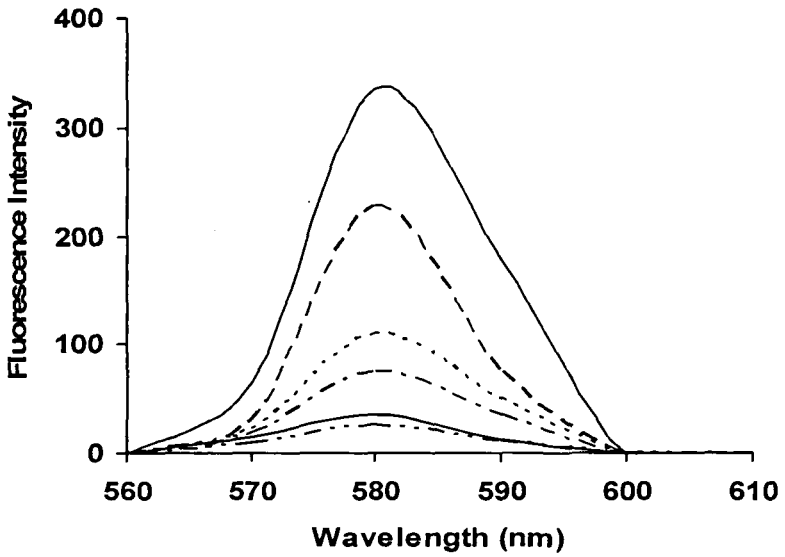
[– – –] Delphinidin : DNA base pair molar ratio (1:2)

[– · –] Delphinidin : DNA base pair molar ratio (1:4)

[-----] Delphinidin : DNA base pair molar ratio (1:6)

[– · · –] Delphinidin : DNA base pair molar ratio (1:8)

Figure 10. Effect of increasing concentration of Cu(II) on the fluorescence emission spectra of delphinidin :



Delphinidin (in Tris-HCl, pH 7.5) was excited at its λ_{max} (absorbtion) of 548 nm and the emission spectra was recorded between 560-610 nm.

[−] Delphinidin alone (25 μM)

[− −] Delphinidin : Cu(II) molar ratio (1:1)

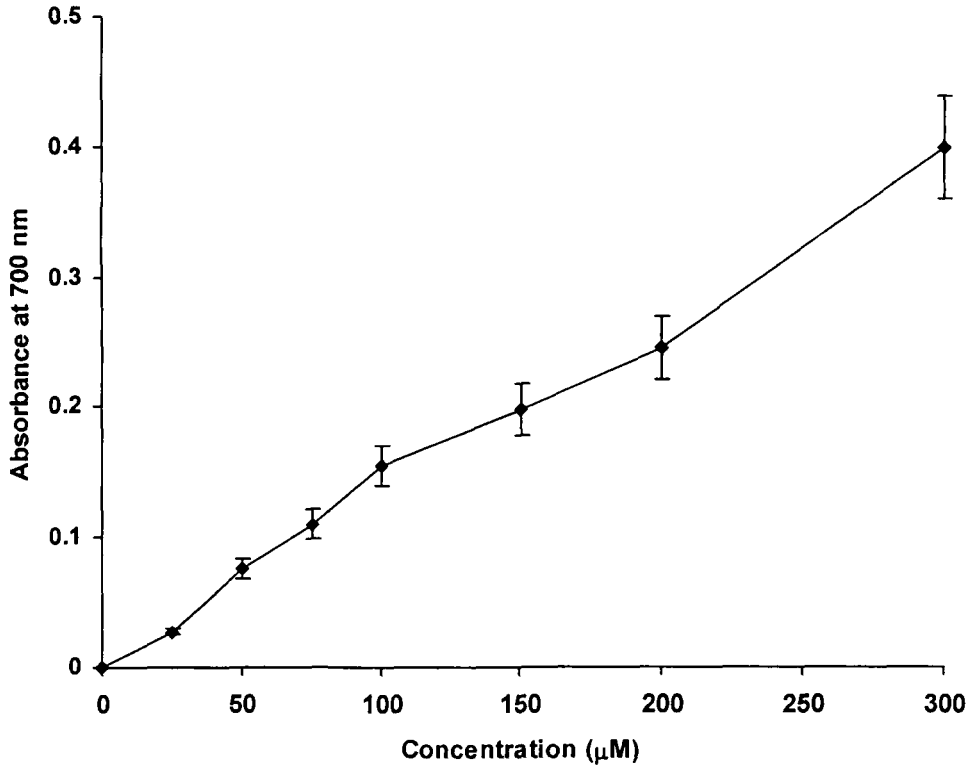
[----] Delphinidin : Cu(II) molar ratio (1:2)

[----] Delphinidin : Cu(II) molar ratio (1:4)

[−] Delphinidin : Cu(II) molar ratio (1:6)

[− · ·] Delphinidin : Cu(II) molar ratio (1:8)

Figure 11 . Detection of reducing power of delphinidin :



Reaction mixture (5 ml) contained 0.2 M potassium phosphate buffer (pH 6.6), increasing concentrations of delphinidin (0-300 μM), 1 % potassium ferricyanide and distilled water. The reaction mixture was incubated at 50 °C for 20 min and processed further as described in "Methods". All values reported are means of three independent experiments. Error bars represent standard error of mean.

DISCUSSION-I

DISCUSSION I

The major conclusion of the experiments described in this chapter are:
(i) Delphinidin is able to induce strand scission in calf thymus DNA and plasmid DNA in the presence of copper ions. (ii) It forms a complex with both DNA as well as Cu(II) and possibly in the presence of all three a ternary complex of delphinidin-Cu(II)-DNA is formed. (iii) Cu(II) is reduced by delphinidin in the complex to generate Cu(I). (iv) Redox cycling of copper leads to the generation of various reactive oxygen species, particularly the hydroxyl radical which serves as the proximal DNA cleaving agent.

These results place delphinidin in a class of plant-derived polyphenolic antioxidants such as flavonoids (Ahmad et al., 1992), tannins (Bhat and hadi, 1994), and catechins (Azam et al., 2004), which also exhibit prooxidant DNA damaging properties. The generation of oxygen radicals in the proximity of DNA is well established as a cause of strand scission (Ahmad et al., 1992; Bhat and hadi, 1994; Rahman et al., 1989). It is generally recognized that such reactions with DNA are preceded by association of ligand with DNA, followed by the production of oxygen radicals at that site (Pryor, 1988). Metal ion dependent degradation of DNA by 4-(9-acridinylamino) methanesulphone-m-anisidine (Wong et al., 1984), 1,10-phenanthroline (Gutteridge and Halliwell, 1982), bleomycin (Ehrenfeld et al., 1987), adriamycin (Eliot., 1984; Haidle and McKinney, 1985) as well as flavonoids (Ahmad et al., 1992; Rahman et al., 1989) are based on mechanisms involving oxygen-derived radicals. In our studies on flavonoids it was shown that a ternary complex of the drug, DNA and Cu(II) is formed which generates oxygen radicals insitu via Cu(I). The results presented here show that delphinidin is 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 delphinidin-Cu(II) mediated DNA cleavage.

Delphinidin is known to be a potential chemopreventive agent against cancer (Martin et al., 2003; Hou et al., 2004a). In addition to its anticancer properties, delphinidin has also been shown to induce apoptosis in HL-60 cells as detected by morphological changes and DNA fragmentation (Hou et al., 2003, Shih et al., 2005; Lazze et al., 2004). The anthocyanidin, delphinidin and its glycosidic derivatives like delphinidin-3-glucopyranoside (Dp3G) are easily absorbed in the intact form after oral administration in rats and humans (Matsumoto et al., 2001). Even after 4 hour, the plasma concentration of Dp3G was estimated to be about 30 nmol/l (Ichiyanagi et al., 2004). Cellular uptake and intracellular accumulation of anthocyanin and anthocyanidins have also been reported (Shih et al., 2005). The bioavailability of delphinidin and cyanidin has been estimated to be about 60 % in healthy volunteers after consumption of red wine (Frank et al., 2003). It is also known that red wines are a good source of copper (Carno, 1988; Darret et al., 1986). Copper is an essential constituent of many enzymes such as tyrosinase and superoxide dismutase. Normal serum contains upto 8 μM loosely bound copper and other biological fluids may also contain comparable amounts (Gutteridge, 1984). Loosely bound copper has been defined by Gutteridge (1984) as that copper which is available for binding to the chelating agent 1,10-phenanthroline. It is possible that such loosely bound copper can also be mobilized by delphinidin. Copper has also been reported to be a normal component of chromatin and such endogenous copper can be mobilized by chemical agents such as 1,10-phenanthroline to cause internucleosomal DNA fragmentation (Burkitt et al., 1996). It may be further mentioned that the apoptotic activity of the polyphenol gallic acid is abolished on modification of phenolic hydroxyl groups (required for copper chelation) (Inoue et al., 1994). This result correlates with a previous study in

this laboratory where it was shown that such a modification of gallic acid resulting in the formation of syringic acid also considerably reduces the DNA cleavage efficiency in the presence of Cu(II) (Khan and Hadi, 1998). Polyphenolic antioxidant curcumin has also been shown to induce apoptosis in human leukemia cells and such apoptosis is prevented by superoxide dismutase and catalase (Kuo et al., 1996). It would thus appear that prooxidant action of polyphenolics rather than antioxidant activity is responsible for the induction of apoptosis in these studies. One of the mechanisms could be that in tumor cells endogenous copper in serum or chromatin is relatively easily mobilized by polyphenolic compounds and similar apoptosis inducing agents. This could be likely as it has been shown that serum, tissue and intracellular copper levels are significantly elevated in various malignancies (Yoshida et al., 1993; Nazulewis et al., 2004; Ebadi and Swanson, 1988). Irrespective of the physiological significance of our results it is clear that delphinidin has implications for development as a novel antitumor and cancer chemopreventive agent.

CHAPTER-II

Delphinidin induced oxidative DNA breakage in human peripheral lymphocytes

RESULTS-II

RESULTS-II

Standardization of alkaline single cell gel electrophoresis/Comet Assay:

Alkaline single cell gel electrophoresis is a very sensitive technique for detecting DNA single strand breaks at the level of a single cell. In this technique a small number of cells are treated with the test agent, layered on glass slides and sandwiched between layers of agarose. The slides are electrophoresed in alkaline conditions, stained and viewed under a fluorescent microscope for DNA single strand breaks. The technique is called Comet Assay because of a comet like appearance of damaged cellular DNA. H_2O_2 is a known genotoxic agent and is routinely used in genotoxicity testing. Inorder to standardize Comet Assay an experiment was performed with H_2O_2. Photographs of comets (100x) observed after treatment of lymphocytes with increasing concentrations of H_2O_2 are shown in figure 12. As can be seen, untreated cells are not damaged and do not show a tail. However, with increasing concentrations of H_2O_2 a progressive increase in the length of comet tails is observed. In figure 13 the results of the same experiment are plotted as comet tail length (μm) as a function of increasing H_2O_2 concentration.

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

Increasing concentrations of delphinidin (10-50 μM) either alone or in the presence of 20 μM $CuCl_2$ was tested for DNA breakage in isolated human peripheral lymphocytes using the Comet Assay. It is seen that whereas delphinidin alone causes some breakage of cellular DNA, the degree of such breakage is considerably greater in the presence of Cu(II). Figure 14 shows photographs of comets (100x) observed with varying concentrations of delphinidin in the absence and presence of copper. Untreated lymphocyte controls were similar to delphinidin alone or Cu(II) alone without any

significant DNA breakage. The corresponding tail length is plotted as a function of delphinidin concentration in figure 15. A similar experiment with increasing concentrations of Cu(II) (5-20 μM) and fixed delphinidin concentration (50 μM) was also performed. As can be seen from figure 16 a significant DNA damage was only observed at 20 μM concentration of Cu(II). Lower concentrations of Cu(II) did not lead to any appreciable DNA damage. The results clearly establish that delphinidin-Cu(II) system is capable of DNA breakage in lymphocytes and is physiologically feasible and could be of biological significance.

Effect of pre-incubating the lymphocytes with delphinidin or Cu (II) on delphinidin-Cu(II) mediated DNA breakage :

Lymphocyte DNA breakage was also studied after pre-incubating the cells with delphinidin after which the cells were washed twice with PBS and incubated further in the presence of $CuCl_2$. The results given in figure 17 show that with increasing concentration of delphinidin a progressive increase in DNA breakage as indicated by increased tail length of comets is observed. A similar experiment was also done by pre-incubating lymphocytes with $CuCl_2$ (figure 18) and the results were similar to those observed in figure 17. These results indicate that both delphinidin and Cu(II) are either able to traverse the cell membrane or bind to it.

Effect of reactive oxygen scavengers on delphinidin-Cu(II) induced DNA breakage in lymphocytes :

It is previously established from our work that the plant polyphenol-Cu(II) mediated DNA cleavage in vitro (Rahman et al., 1989) and the inactivation of bacteriophage λ by this system is inhibited to significant degrees by various scavengers of reactive oxygen species (Ahmad et al., 2000). Table–2 gives the results of an experiment where the effect of sodium azide,

potassium iodide, thiourea, superoxide dismutase (SOD) and catalase were tested on delphinidin-Cu(II) induced DNA degradation in lymphocytes. SOD and catalase remove superoxide and H_2O_2 respectively. Sodium azide is a scavenger of singlet oxygen and potassium iodide and thiourea remove hydroxyl radicals. From the data we conclude that H_2O_2 is an essential component in the pathway that leads to the formation of reactive oxygen species, of which superoxide anion and singlet oxygen are alternate DNA damaging agents. Results therefore suggest that the chemically induced DNA breakage *in vitro* and lymphocyte DNA breakage by delphinidin-Cu(II) system are most likely the result of the same mechanism.

DNA breakage induced by delphinidin alone in lymphocytes as measured by Comet Assay :

Increasing concentrations of delphinidin (50, 100, 200 μM) were tested for DNA breakage in isolated lymphocytes using the Comet Assay. At 50 μM concentration, delphinidin causes some breakage of cellular DNA whereas at the concentrations of 100 and 200 μM comets with a clear tail indicative of significant DNA breakage are observed (figure 19). The results clearly indicate that delphinidin alone is also capable of cellular DNA breakage. However, the minimum concentration required for such breakage (100-200 μM) is considerably greater than when delphinidin (10 μM) is used along with Cu(II) as shown previously (figure 15).

Effect of Neocuproine, a Cu(I) specific sequestering agent, on DNA breakage induced by delphinidin-Cu(II) and delphinidin alone :

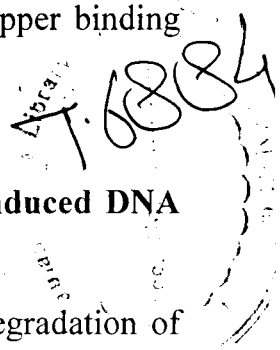
Neocuproine is a Cu(I) specific chelating agent and is membrane permeable (Barbouti et al., 2001). Figure 20 gives the results of an experiment where three progressively increasing concentrations of neocuproine were tested on

delphinidin-Cu(II) induced DNA breakage in lymphocytes. A progressive decrease in the tail length as a function of increasing neocuproine concentration was seen. These results indicate that DNA breakage by delphinidin-Cu(II) system involves the reduction of Cu(II) to Cu(I) and that Cu(I) is an intermediate in the pathway that leads to DNA breakage. Another experiment was performed where the effect of neocuproine was seen on DNA breakage induced by delphinidin alone (100 μM) in the absence of added $CuCl_2$. Similar results (figure 21) were obtained where a progressive decrease in the tail length as a function of increasing neocuproine concentration was seen. These results indicate that DNA breakage by delphinidin involves mobilization of endogenous copper ions.

A comparison of DNA breakage in lymphocytes by delphinidin with various polyphenols :

We have previously shown that the presence and position of hydroxyl groups in polyphenols particularly the presence of ortho-dihydroxy groups is important for DNA cleavage in the presence of copper ions (Jain et al., 1989; Azmi et al., 2005). Thus the parent compounds of various classes of polyphenols that do not possess any hydroxyl group are not cleaving agents. I have therefore compared the relative lymphocyte DNA breakage efficiency of several polyphenols with delphinidin. Figure 22 shows comet tail lengths obtained using increasing concentrations of various polyphenols. The results show that gallic acid (a constituent of tannic acid) is the most effective DNA cleaving agent followed by EGCG, delphinidin and resveratrol. Syringic acid, a derivative of gallic acid where two of the three hydroxyl groups are methoxylated is not a cleaving agent (result not shown). It may be suggested that the differential DNA cleavage efficiency of various polyphenols could be a function of the difference in the number and position of hydroxyl

groups, the relative cell membrane permeability as well as copper binding efficiency.



Effect of neocuproine and bathocuproine on delphinidin induced DNA breakage in lymphocytes :

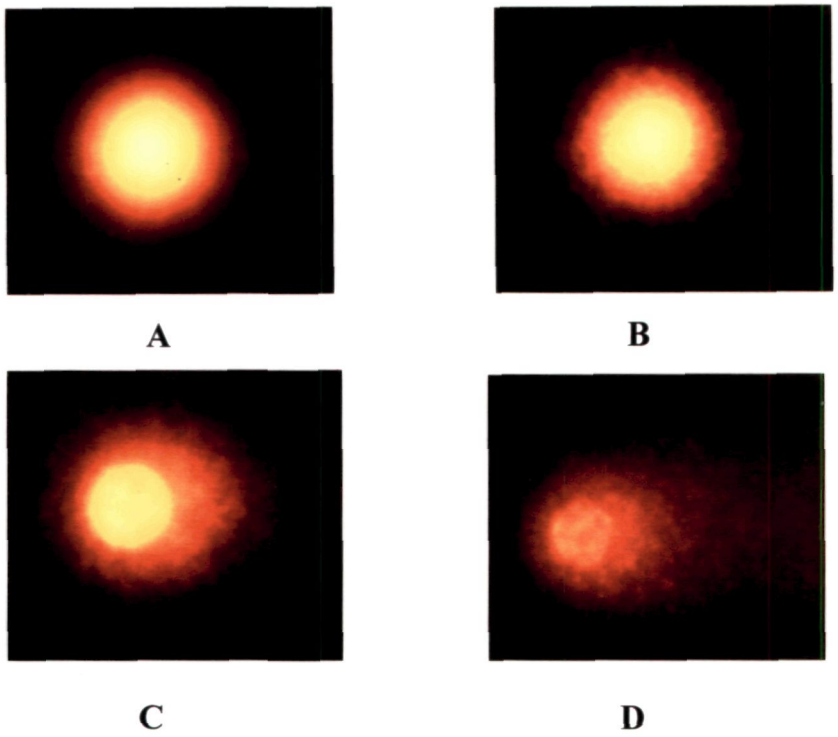
As already shown in figure 21, delphinidin mediated DNA degradation of lymphocytes is inhibited by neocuproine which is a Cu(I) specific chelating agent and is membrane permeable. In the experiment shown in Figure 23, I have also used bathocuproine disulphonate, the water-soluble membrane impermeable analog of neocuproine to show that whereas neocuproine inhibits delphinidin induced DNA breakage in lymphocytes, bathocuproine as expected, is ineffective in causing such inhibition. A progressive decrease in the tail length as a function of increasing neocuproine concentration was seen. It may be concluded that the DNA breakage by delphinidin involves endogenous copper ions and that Cu(I) is an intermediate in the pathway that leads to DNA breakage.

Determination of TBARS as a measure of oxidative stress induced in lymphocytes by delphinidin in the presence of neocuproine and thiourea:

As mentioned above we presume that lymphocyte DNA breakage is the result of hydroxyl radicals and other reactive oxygen species in situ. Oxygen radical damage to deoxyribose or DNA is considered to give rise to TBA reactive material (Quinlan & Gutterige, 1987). We have therefore determined the formation of TBA reactive substance (TBARS) as a measure of oxidative stress in lymphocytes with increasing concentrations of delphinidin. The effect of preincubating the cells with neocuproine and thiourea was also studied. Results given in Figure 24 show that there is a dose dependent increase in the formation of TBA reactive substance in

lymphocytes. However, when cells were preincubated with neocuproine and thiourea there was a considerable decrease in the rate of formation of TBA reactive substance by delphinidin. These results indicate that both DNA breakage and oxidative stress in cells is inhibited by Cu(I) chelation and scavenging of reactive oxygen. Thus it can be safely concluded that the formation of reactive oxygen species by polyphenols in lymphocytes involves their interaction with intracellular copper as well as its reduction to Cu(I).

Figure 12 . Standardization of Comet Assay with H_2O_2 :



A B C D

Reaction mixture (1 ml) contained 1 x10^5 cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} free and indicated concentrations of H_2O_2 (0-50 µM). The reaction mixture was incubated at 37 °C for 30 min and processed further for Comet Assay as described in "Methods".

Photographs of comets (100x) obtained on incubating lymphocytes with varying concentrations of H_2O_2.

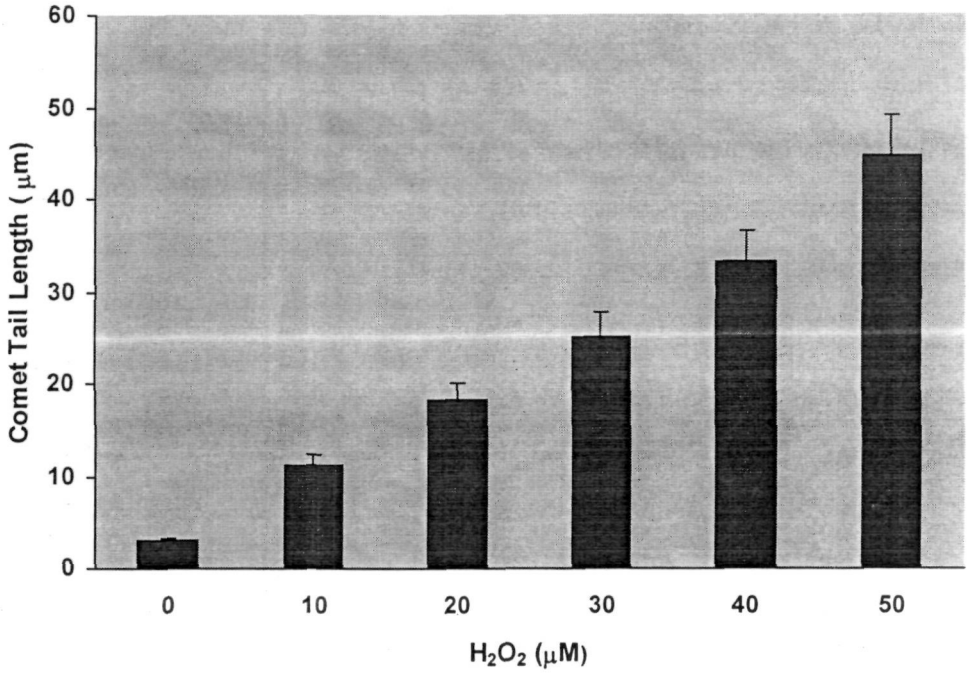
(A) Untreated lymphocytes

(B) Lymphocytes + 10 µM H_2O_2

(C) Lymphocytes + 20 µM H_2O_2

(D) Lymphocytes + 50 µM H_2O_2

Figure 13. Standardization of Comet Assay with H_2O_2 :



Reaction mixture (1 ml) contained 1 x10^5 cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} free and indicated concentrations of H_2O_2 (0-50 µM). The reaction mixture was incubated at 37 °C for 30 min and processed further for Comet Assay as described in "Methods".

Comet tail length (µ metres) plotted as a function of increasing concentrations of H_2O_2 (0-50 µM).

All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.

Figure 14 . DNA breakage by delphinidin-Cu(II) in human peripheral lymphocytes as measured by Comet Assay :

Reaction mixture (1 ml) contained 1 x10^5 cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} free increasing concentrations of delphinidin (0-50 µM) and 20 µM Cu(II). The reaction mixture was incubated for 1 hr at 37 °C. After incubation the cells were processed further for Comet Assay as described in "Methods".

Photographs of comets (100x) obtained after treatment of lymphocytes with increasing concentrations of delphinidin and fixed concentration of Cu(II).

(A, E) Untreated lymphocytes

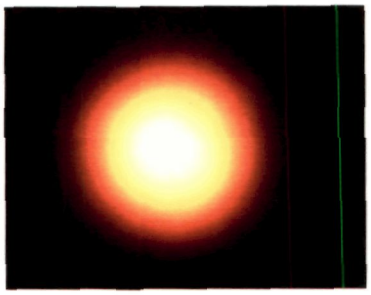
(B-D) Lymphocytes + delphinidin (10-50 µM) in the absence of Cu(II)

(F) Lymphocytes + delphinidin 10 µM + Cu(II) 20 µM

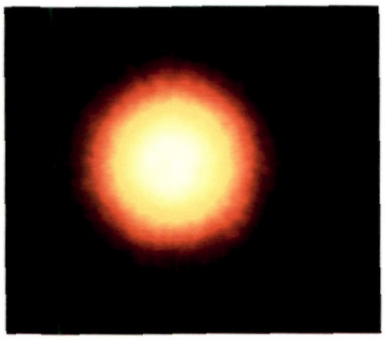
(G) Lymphocytes + delphinidin 20 µM + Cu(II) 20 µM

(H) Lymphocytes + delphinidin 50 µM + Cu(II) 20 µM

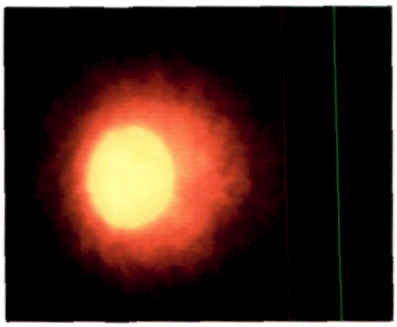
A



E



B



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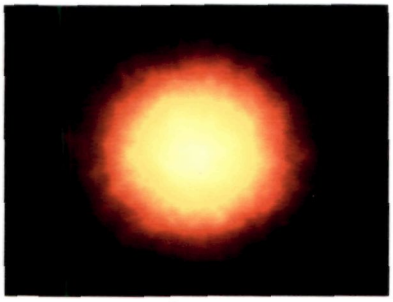

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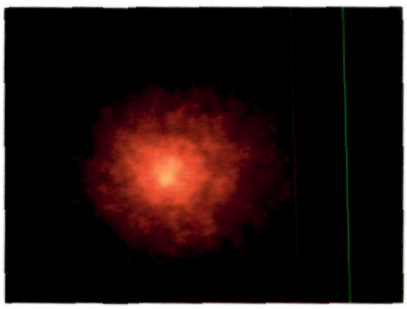

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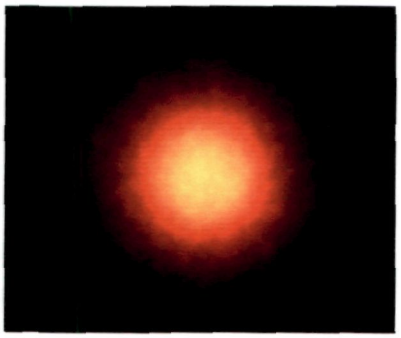

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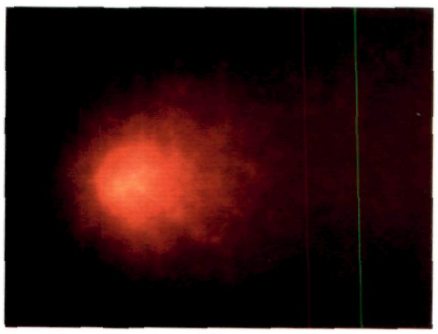
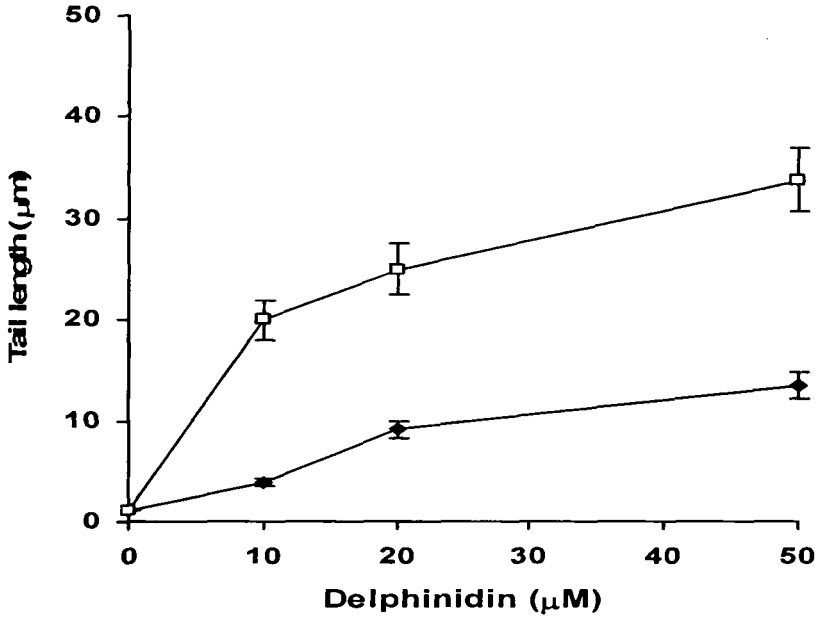


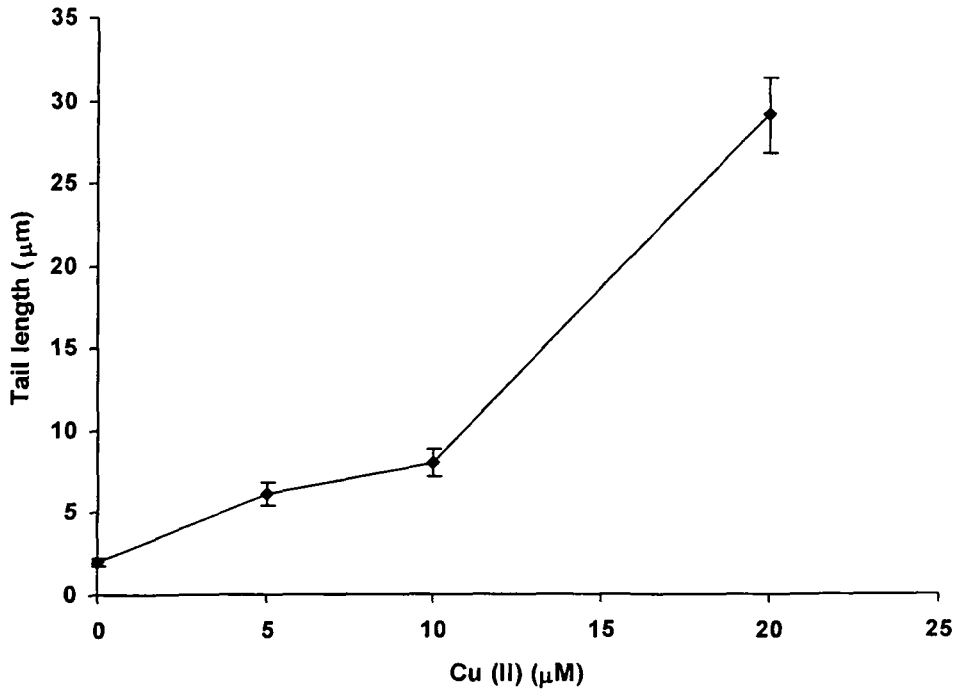
H

Figure 15. DNA breakage by delphinidin-Cu(II) in human peripheral lymphocytes with increasing delphinidin concentrations :



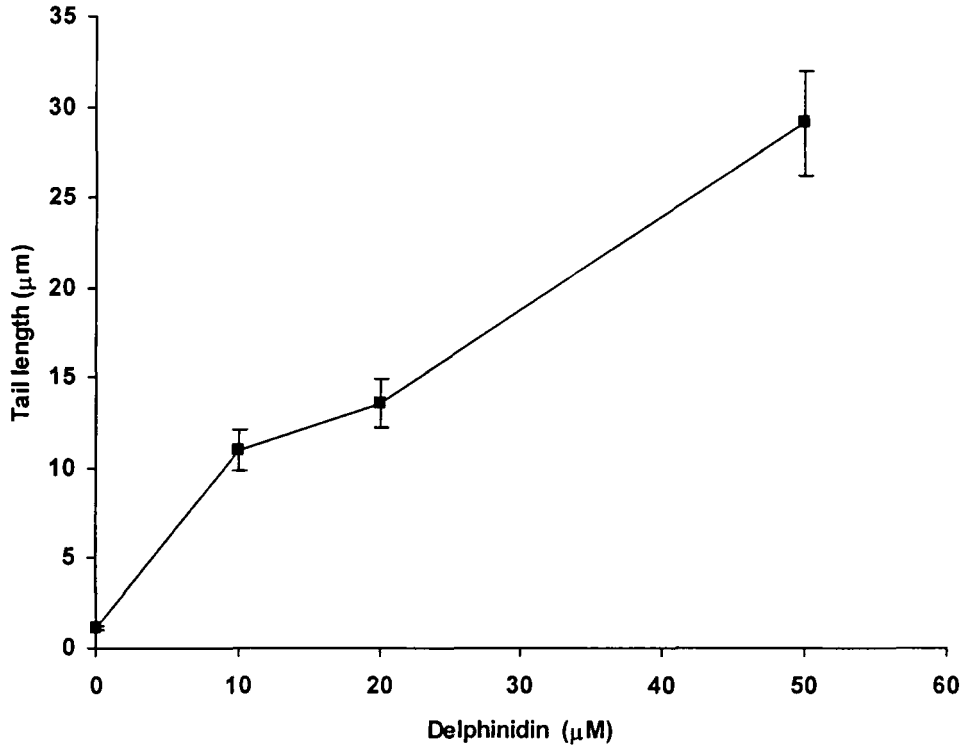
Reaction mixture (1 ml) contained 1 x10^5 cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} free increasing concentrations of delphinidin (0-50 µM) and 20 µM Cu(II). The reaction mixture was incubated for 1 hr at 37 °C. After incubation the cells were processed further for Comet Assay as described in "Methods". Comet tail length (µ metres) plotted as a function of increasing concentrations of delphinidin (0-50 µM) in the absence (♦) and presence(□) of 20 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 16. DNA breakage by delphinidin-Cu(II) in human peripheral lymphocytes with increasing Cu(II) concentrations:



Reaction mixture (1 ml) contained 1 x10^5 cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} free increasing concentrations of Cu(II) 5-20 µM and 50 µM delphinidin. The reaction mixture was incubated for 1 hr at 37 °C. After incubation the cells were processed further for Comet Assay as described in "Methods". Comet tail length (µ metres) plotted as a function of increasing concentrations of Cu(II) (0-20 µM) in the presence of 50 µM delphinidin. All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.

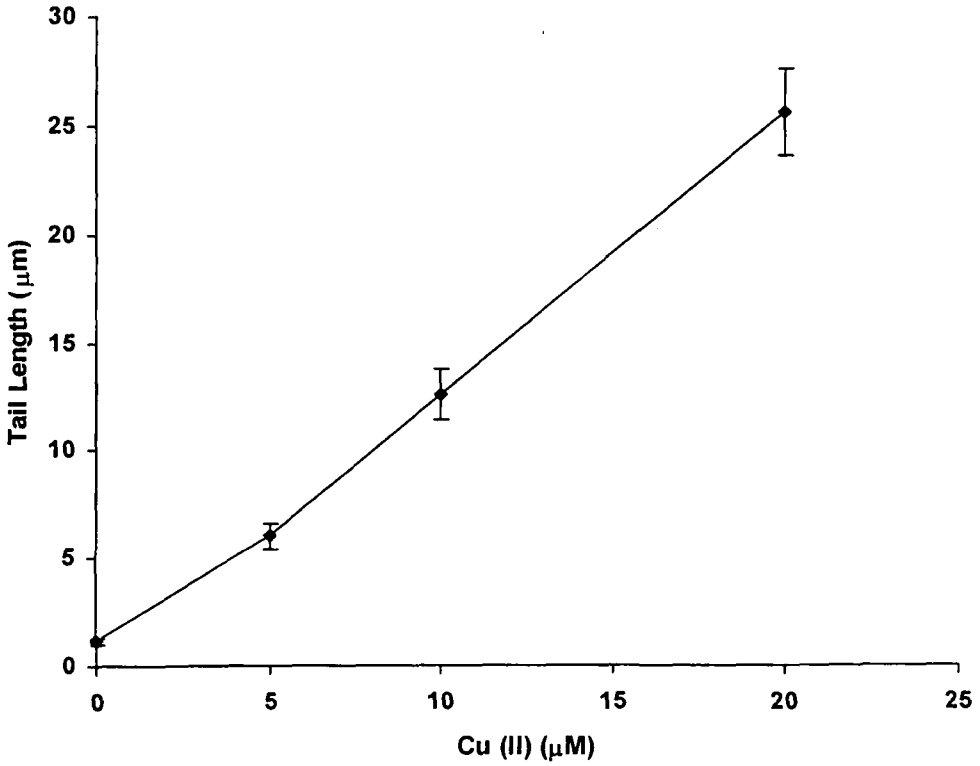
Figure 17. Effect of preincubating the lymphocytes with increasing concentrations of delphinidin on DNA breakage :



Isolated lymphocytes suspended in RPMI 1640 were preincubated with the indicated concentrations of delphinidin for 30 min at 37 °C. After pelleting, the cells were washed twice with PBS Ca^{2+} and Mg^{2+} free before resuspension in RPMI 1640 and further incubated for 1 hr in the presence of 20 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 18. Effect of preincubating the lymphocytes with increasing concentrations of Cu(II) on DNA breakage :



Isolated lymphocytes suspended in RPMI 1640 were preincubated with the indicated concentrations of Cu(II) for 30 min at 37 °C. After pelleting, the cells were washed twice with PBS Ca^{2+} and Mg^{2+} free before resuspension in RPMI 1640 and further incubated for 1 hr in the presence of 50 μM delphinidin. All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.

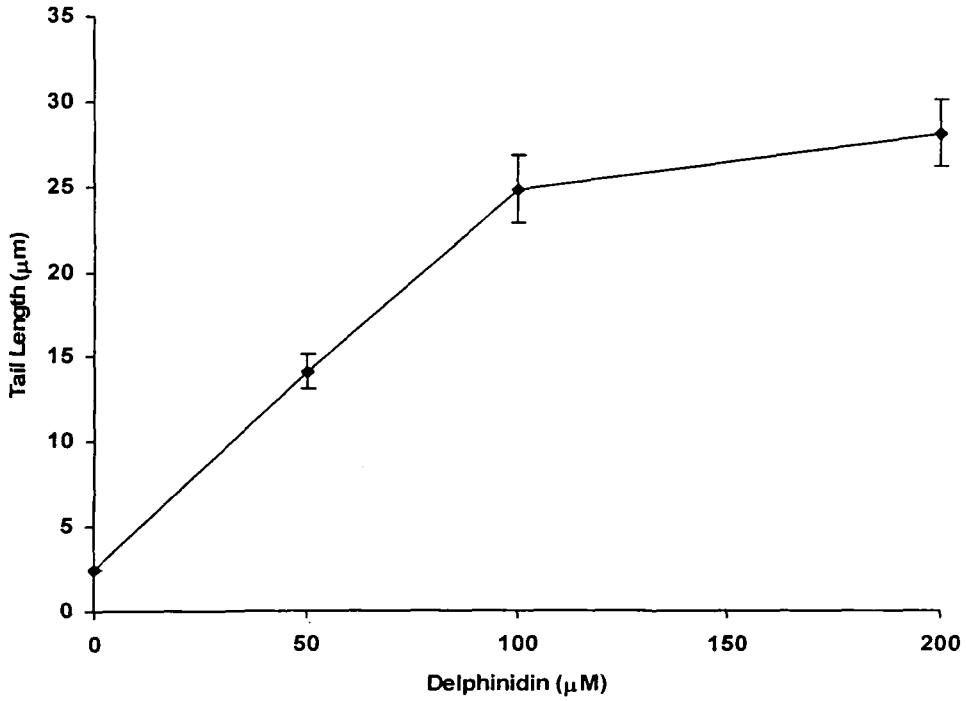
Table-2 Effect of scavengers of reactive oxygen species on delphinidin-Cu(II) induced lymphocyte DNA breakage

Treatment	**Tail length (μm)**	**% inhibition**
Control	1.5 ± 0.15	-
Delphinidin (50μM) + Cu(II) (20 μM)	34.3 ± 3[a]	0
+thiourea (1mM)	13.1 ± 1.2*	61.8
+KI (1mM)	10.5 ± 1.03*	69.3
+sodium azide (1mM)	11.5 ± 1.1*	66.4
+ SOD(100 μg/ml)	11.14 ± 1.1*	67.5
+Catalase (100μg/ml)	14.3 ± 1.4*	58.3

All values represent S.E.M. of three independent experiments.

* P-values < 0.05 when compared to control[a].

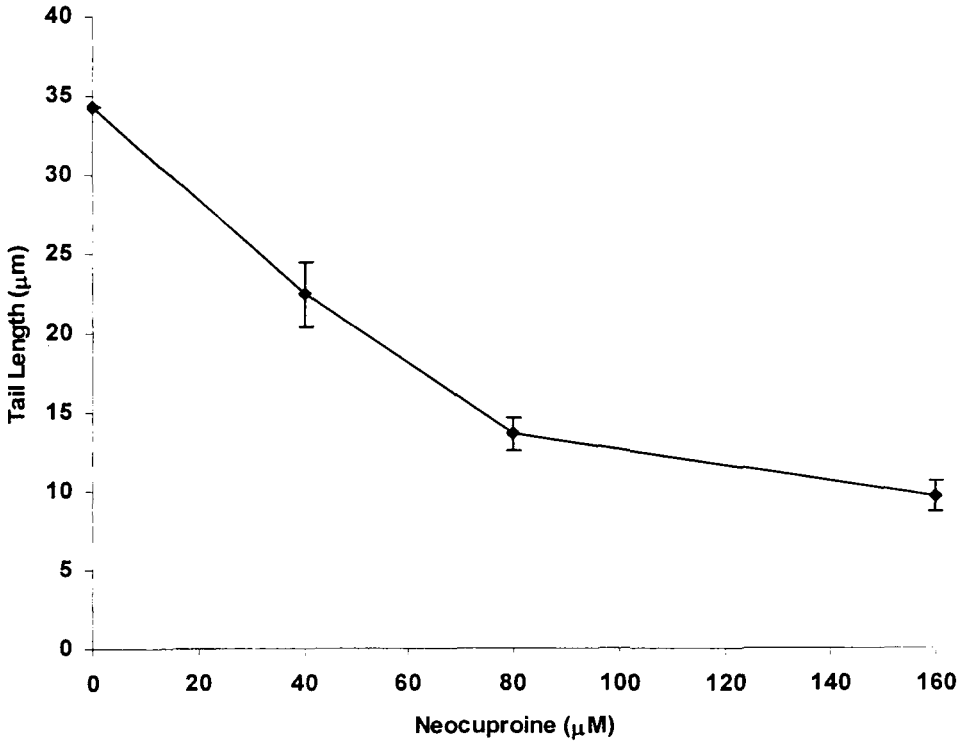
Figure 19. Effect of increasing concentrations of delphinidin alone on DNA breakage in lymphocytes :



Reaction mixture (1 ml) contained 1 x10^5 cells, 400 µl RPMI, PBS Ca^{2+} and Mg^{2+} free and indicated concentrations of delphinidin. The reaction mixture was incubated for 1 hr at 37 °C. After incubation the cells were processed further for Comet Assay as described in "Methods".

The viability of cells after incubation was found to be greater than 93 %. Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.

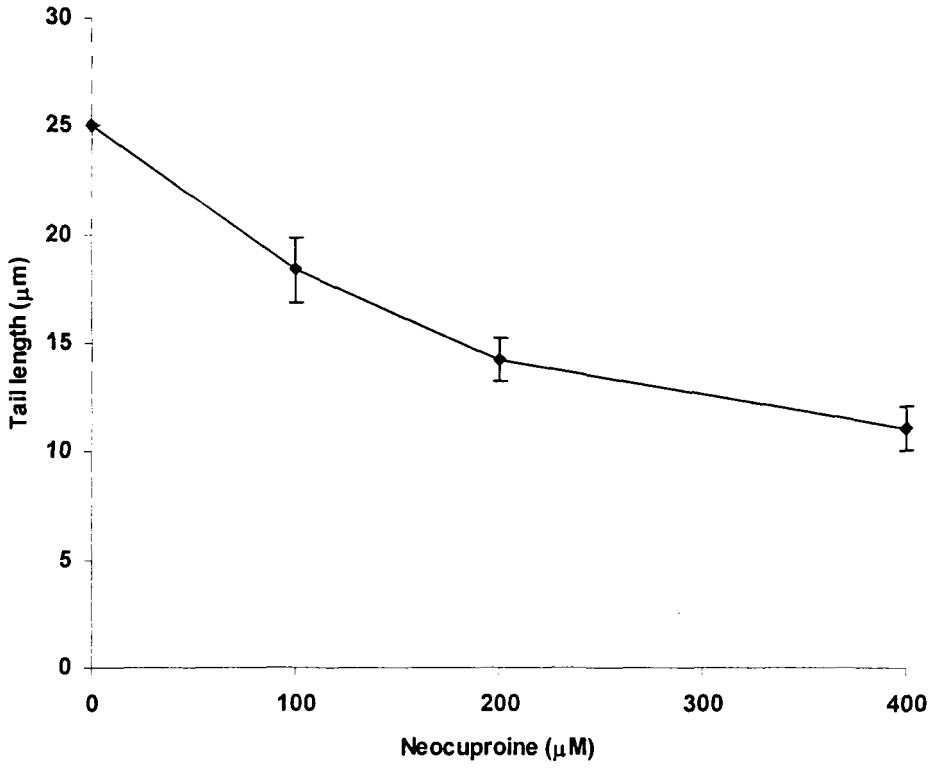
Figure 20. Effect of increasing concentrations of neocuproine on delphinidin-Cu(II) induced DNA breakage in human lymphocytes :



Reaction mixture (1 ml) contained 1 x10^5 cells, 400 μl RPMI, PBS Ca^{2+} and Mg^{2+} free, 50 μM delphinidin, 20 μM Cu(II) and indicated concentrations of neocuproine. The reaction mixture was incubated for 1 hr at 37 °C and the cells were processed further for Comet Assay as described in "Methods".

Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.

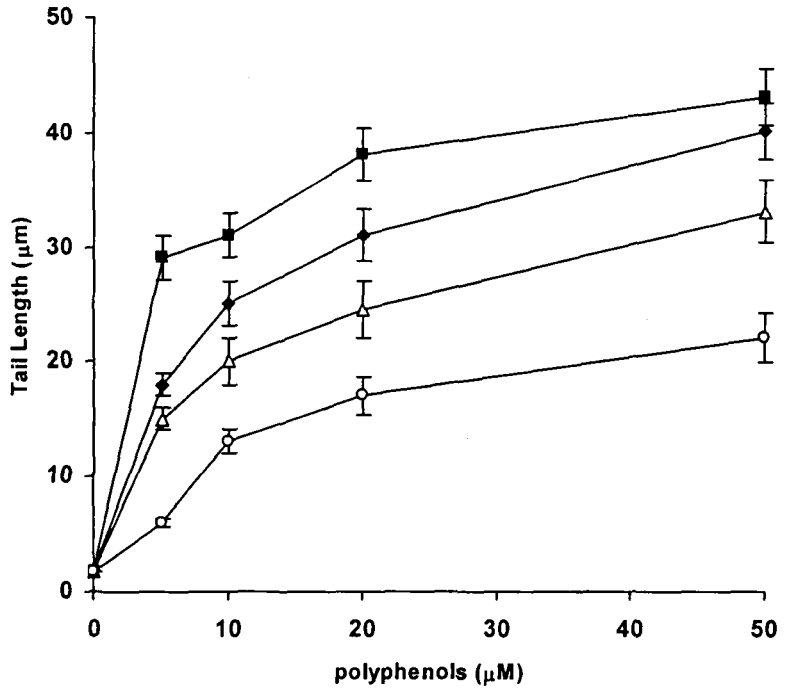
Figure 21. Effect of increasing concentrations of neocuproine on delphinidin alone induced DNA breakage in human lymphocytes :



Reaction mixture (1 ml) contained 1 x10^5 cells, 400 µl RPMI, PBS Ca^{2+} and Mg^{2+} free, 100 µM delphinidin and indicated concentrations of neocuproine. The reaction mixture was incubated for 1 hr at 37 °C and the cells were processed further for Comet Assay as described in "Methods".

Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control

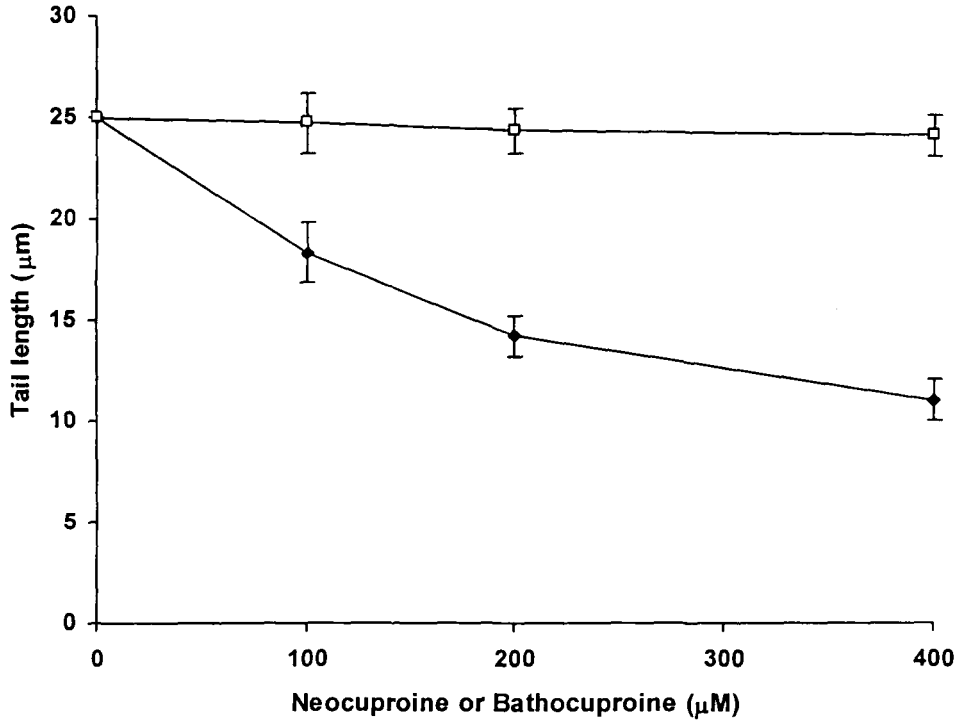
Figure 22. Comparison of various polyphenols on the induction of DNA breakage in human lymphocytes :



Reaction mixture (1 ml) contained 1 x10^5 cells, 400 μl RPMI, PBS Ca^{2+} and Mg^{2+} free, 20μM Cu(II) and indicated concentrations of different polyphenols. The reaction mixture was incubated for 1 hr at 37 °C and the cells were processed further for Comet Assay as described in "Methods".

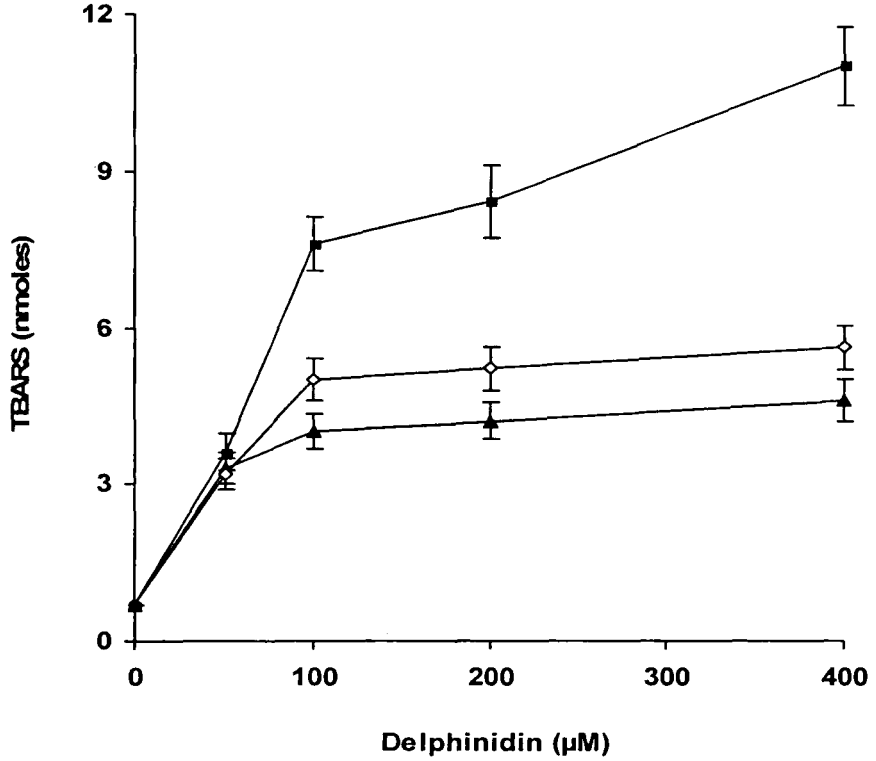
(■) gallic acid, (◆) EGCG, (△) delphinidin, (○) resveratrol. Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.

Figure 23. Effect of increasing concentrations of bathocuproine / neocuproine on delphinidin induced DNA breakage in human lymphocytes :



Reaction mixture (1 ml) contained 1 x10^5 cells, 400 µl RPMI, PBS Ca^{2+} and Mg^{2+} free, 100 µM delphinidin and indicated concentrations of neocuproine (♦) / bathocuproine (□). The reaction mixture was incubated for 1 hr at 37 °C and the cells were processed further for Comet Assay as described in "Methods". Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.

Figure 24. Effect of preincubation of lymphocytes with neocuproine and thiourea on TBARS generated by increasing concentrations of delphinidin :



The isolated cells (1 x10^5) suspended in RPMI 1640 were preincubated with the fixed concentrations of neocuproine and thiourea for 30 min at 37 °C. After pelleting, the cells were washed twice with PBS Ca^{2+} and Mg^{2+} free before resuspension in RPMI and further incubated for 1 hr in the presence of increasing concentrations of delphinidin. Delphinidin alone (■), delphinidin + neocuproine (1 mM) (◇), delphinidin + thiourea (1 mM) (▲). Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.

DISCUSSION-II

DISCUSSION - II

The major conclusions of the experiments performed in this chapter are (i) delphinidin-Cu(II) system is capable of oxidative DNA cleavage in human peripheral lymphocytes, (ii) delphinidin and Cu(II) both are able to traverse the cell membrane and enter the cells, (iii) cellular DNA damage by delphinidin-Cu(II) involves reactive oxygen species and Cu(I), (iv) delphinidin alone in the absence of added copper ions is able to cause DNA breakage, (v) the cellular DNA cleavage by this system depends on the presence of phenolic groups on the polyphenols and the relative cleavage efficacy increases with increase in the number and position of such groups.

As already mentioned several classes of plant derived antioxidant polyphenols also exhibit oxidative DNA degradation properties particularly in the presence of transition metal ions such as copper. Evidence in the literature suggests that antioxidant properties of these compounds may not fully explain their anticancer effects (Gali et al., 1992; Ahmad et al., 2000). It may be noted that the polyphenol gallic acid is highly efficient in DNA degradation as compared with syringic acid (where two of the hydroxyl groups of gallic acid are modified) (Khan and Hadi, 1998). Interestingly, modifications of phenolic hydroxyl groups such as that resulting in the formation of syringic acid abolishes the apoptosis inducing capacity of gallic acid (Inoue et al., 1994). Further, structure-activity studies with delphinidin also indicate that the number of hydroxyl groups in the B ring and the presence of orthodihydroxy phenolic groups in the B-ring of delphinidin is essential for apoptosis induction (Hou et al., 2003). The result (Fig 22) presented in this study also suggests that the DNA cleavage property of polyphenols is dependent on the presence and number of hydroxyl groups. Further, experimental evidence suggest that anthocyanidin, delphinidin is able to traverse the cell membrane and enter the cytoplasmic or nuclear

space. Cellular uptake and intracellular accumulation of anthocyanidins have already been reported (Shih et al., 2005). A model for the entry and interaction of phenols with chromatin-associated copper has also been described (Li and Trush, 1994). The ability of gallotannins to enter the cell is indicated by the observation that tannic acid prevents formation of the benz(a)pyrene-DNA adduct by inhibiting the binding of the ultimate carcinogen to target tissue DNA rather than by altering the metabolism of benz(a)pyrene (Mukhtar et al., 1998).

Based on our own observations and those of others, we have proposed a mechanism for the cytoyoxic action of plant polyphenols against cancer cells that involves mobilization of endogenous copper and the consequent prooxidant action (Hadi et al., 2000). Thus, the major conclusion of the present study is that polyphenol-Cu(II) mediated chemical cleavage of DNA is a physiologically feasible reaction and may be of biological significance. Our idea is strengthened by a number of other observations in literature. Copper is a major metal ion present in the nucleus (Bryan, 1979; Ebadi and Swanson, 1988) and serum (Yoshida et al., 1993) and tissue (Nazulewis et al., 2004) concentrations of copper are greatly increased in various malignancies. Copper ions from chromatin can be mobilized by metal chelating agents giving rise to internucleosomal DNA fragmentation, a hallmark property of cells undergoing apoptosis (Burkitt et al., 1996). Further, it has been proposed that most clinically used anticancer drugs can activate late events of apoptosis (DNA degradation and morphological changes) and the essential signaling pathways differ between pharmacological cell death and physiological induction of cell death (Smets, 1994). It is realized that the above results do not categorically prove that lymphocyte DNA degradation described above involves chromatin bound copper. However, there are a number of observations which suggest that this is indeed the case. 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 the oxygen radicals, the hydroxyl radical is the 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 the DNA (Pryor, 1988). The location of the redox-active metals is of utmost importance for the ultimate effect, because the hydroxyl radical, due to its extreme reactivity, interacts exclusively in the vicinity of the bound metal (Chevion, 1988). Indeed we have earlier shown that flavonoids are able to form a ternary complex with DNA and Cu(II) where Cu(II) is reduced to Cu(I) (Rahman et al., 1989). Polyphenols are known to reduce molecular oxygen to superoxide anion leading to the formation of H_2O_2 (Ahmad et al., 1992). Superoxide can also be formed by reoxidation of Cu(I) to Cu(II) in the ternary complex (Rahman et al., 1989). Chromatin bound copper is understood to be present in the reduced form (Cu(I)) (Lewis and Laemelli, 1982) and thus would be available for reoxidation to Cu(II) by H_2O_2 in the Fenton type reaction and binding to polyphenols and recycling. It is well known that polyphenols autooxidize in cell culture media to generate H_2O_2 and quinones that could enter cells causing damage to various macromolecules (Long et al., 2000; Halliwell, 2003; Clement et al., 2002). This may lead to extrancellular production of reactive oxygen species that could account for lymphocyte DNA breakage. However, this does not appear to be the case in our system since we have shown that no lymphocyte DNA breakage is observed on preincubating the cells with delphinidin alone upto a concentration of 50 μM. DNA breakage could only be seen after incubating the pre-treated cells further in the presence of Cu(II) (figure 17). Further, we could not detect any H_2O_2 formation on incubating delphinidin (upto a concentration of 300 μM) in RPMI medium (results not shown).

It may also be mentioned that the minimum concentration of delphinidin tested by me in the presence of copper ions for DNA breakage in lymphocytes was 10 μM. However, the minimum concentration of delphinidin alone required for DNA breakage is between 100-200 μM (Hanif et al., 2008). Because of higher intracellular copper levels it may be predicted that such concentrations of delphinidin for cytotoxic action against cancer cells would be considerably lower. Indeed it has been shown that ascorbate which also acts as a prooxidant in the presence of copper ions is cytotoxic to a leukemic cell line at a lower concentration than normal lymphocytes (Singh, 1997).

Most studies on anticancer mechanisms of plant polyphenols invoke the induction of cell cycle arrest at the S/G2 phase transition brought about by an increase in cyclins A and E and inactivation of cdc 2. Other mechanisms have also been proposed (Hou et al., 2004b). Based on our work, it is possible that cellular DNA fragmentation by plant polyphenolics that involves mobilization of intracellular (endogenous) and extracellular copper could be one of the mechanisms involved in the chemopreventive properties of these compounds (Azmi et al., 2005; Azmi et al., 2006).

CHAPTER-III

Delphinidin mobilizes nuclear copper from human lymphocytes leading to oxidative degradation of cellular DNA

RESULTS-III

RESULTS-III

Standardization of Lysed version of Comet Assay using H_2O_2 :

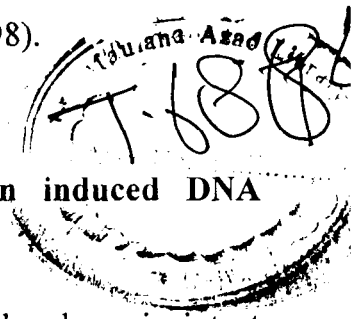
Lysed version of the comet assay has been used to study the direct interaction of various agents with cell nuclei as it eliminates the barrier presented by cell membrane and the intracellular environment (Szeto et al., 2002). Lysed version of comet assay was performed as described by Kasamatsu et al., (1996) with some modifications. In this technique, the lymphocytes (embedded in agarose on slides) were lysed and the nuclei on such slides were subsequently treated with the test agent (See "Materials and Methods"). H_2O_2 is a known genotoxic agent and is routinely used in genotoxicity testing. In order to standardize the lysed version of Comet Assay an experiment was performed with H_2O_2. Photographs of comets (100x) observed after treatment of lymphocyte nuclei with increasing concentrations of H_2O_2 are shown in figure 25. As can be seen, untreated lymphocyte nuclei were not damaged and do not show a tail. However, with increasing concentrations of H_2O_2, a progressive increase in the length of comet tails is observed. In figure 26, the results of the same experiment are plotted as comet tail length (μm) as a function of increasing H_2O_2 concentration.

A comparison of DNA breakage by delphinidin in intact lymphocytes and lymphocyte nuclei as measured by Comet Assay :

Increasing concentrations of delphinidin (10-50μM) were tested for DNA breakage in intact lymphocytes (standard version of comet assay) and compared with that observed in lymphocyte nuclei (lysed version). As can be seen from Figure 27, the rate of tail length formation is considerably greater in the case of lysed version. The result indicate that delphinidin is able to directly interact with nuclei when lysed version of comet assay is used.

Effect of Neocuproine, a Cu(I) specific sequestering agent on delphinidin induced DNA breakage in lymphocyte and lymphocyte nuclei :

Fig 28 shows an experiment where the effect of increasing neocuproine concentration on delphinidin induced DNA breakage is shown in intact lymphocytes (standard version of comet assay) and lymphocyte nuclei (lysed version of comet assay). As was seen in the previous experiment (figure 27) and as expected, considerably greater DNA breakage is observed in the lymphocyte nuclei. However neocuproine is able to inhibit the DNA degradation in intact lymphocytes as well as in the nuclei. These results indicate that neocuproine which is membrane permeable is able to enter the nuclei. It may also be noted that the nuclear pore complex is known to be permeable to small molecules (Mazzanti, 1998).



Effect of active oxygen scavengers on delphinidin induced DNA breakage in lymphocyte nuclei :

In table 2, I had shown that delphinidin induced DNA breakage in intact lymphocytes is inhibited to significant degrees by various scavengers of reactive oxygen species. Table 3, gives the results of an experiment where five scavengers (potassium iodide, sodium azide, superoxide dismutase, catalase and thiourea) were tested for their effect on delphinidin induced DNA breakage in lymphocyte nuclei using the lysed version of Comet Assay. All five caused significant inhibition of DNA breakage as evidenced by decreased tail lengths. It may be concluded that superoxide anion and H_2O_2 are essential components in the pathway that leads to the formation of hydroxyl radical and other species which would be the proximal DNA cleaving agents. Since the results are similar to those seen with whole lymphocytes, it is suggested that the same mechanism involving reactive

oxygen species is responsible for DNA breakage irrespective of whether intact lymphocytes or lymphocyte nuclei are treated with delphinidin.

A comparison of DNA breakage in lymphocyte nuclei by delphinidin with various polyphenols :

We have previously shown (Azmi et al., 2006) that the presence and position of hydroxyl groups in polyphenols particularly the presence of ortho-dihydroxy groups, is important for cellular DNA cleavage. Thus the parent compounds of various classes of polyphenols that do not possess any hydroxyl group are not cleaving agents. I have therefore compared the relative DNA breakage efficiency of several polyphenols with delphinidin in lymphocyte nuclei. Figure 29 shows comet tail lengths obtained using increasing concentrations of various polyphenols. Similar to the results obtained in figure 22, it is seen that gallic acid (a constituent of tannic acid) is the most effective DNA cleaving agent followed by EGCG, delphinidin and resveratrol. It may be suggested that, irrespective of whether intact lymphocytes or lymphocyte nuclei are treated with polyphenols, the differential DNA cleavage efficiency of various polyphenols is a function of the difference in the number and position of hydroxyl groups as well as copper binding efficiency.

Determination of TBARS as a measure of oxidative stress in lymphocyte nuclei by delphinidin in the presence of neocuproine and thiourea :

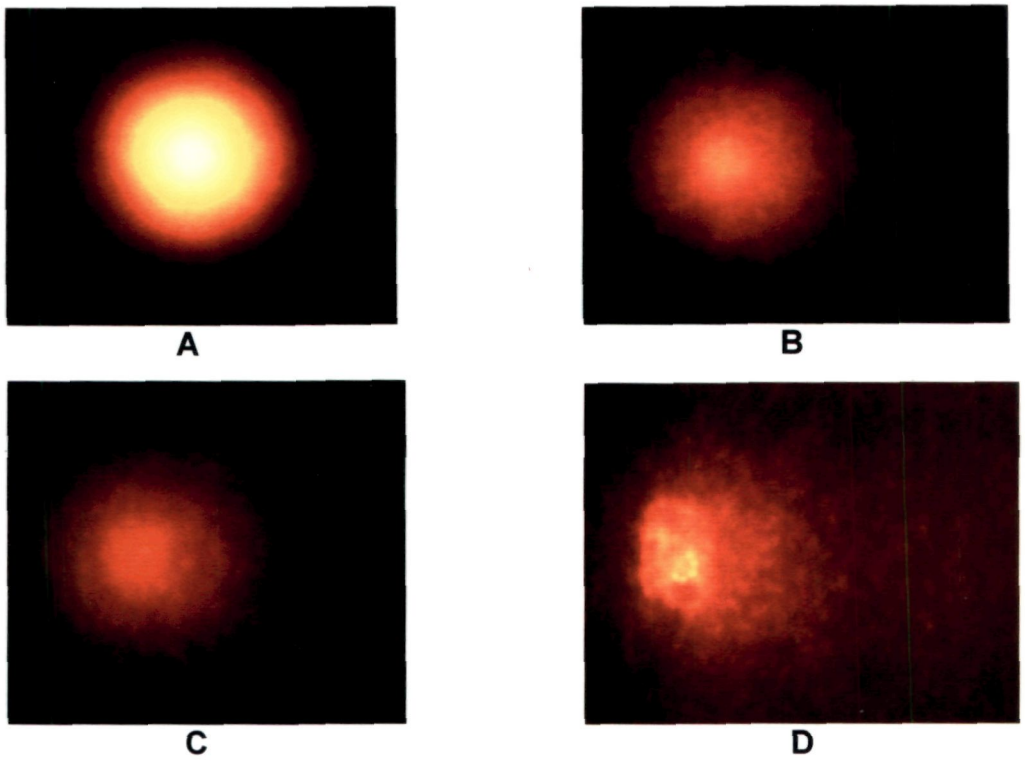
As mentioned in table-3, we presume that the DNA breakage observed in lymphocyte nuclei is the result of the generation of hydroxyl radicals and other reactive oxygen species. Oxygen radical damage to deoxyribose or DNA is considered to give rise to TBA reactive material (Smith et al., 1992; Quinlan and Guttridge, 1987). I have therefore determined the formation of TBA reactive substance (TBARS) as a measure of oxidative stress in

lymphocyte nuclei with increasing concentrations of delphinidin. The effect of preincubating the nuclei with neocuproine and thiourea was also studied. Results given in Figure 30 show a dose dependant increase in the formation of TBA reactive substance in lymphocyte nuclei by delphinidin. However, a considerable decrease in the rate of formation of TBARS was observed in the presence of neocuproine and thiourea. The results indicate that DNA breakage in nuclei is inhibited by Cu(I) chelation and scavenging of reactive oxygen. Thus it may be concluded that the formation of reactive oxygen species by delphinidin in lymphocyte nuclei involves their interaction with chromatin bound copper as well as its reduction to Cu(I).

Detection of H_2O_2 generation by delphinidin in the incubation medium of nuclei :

It is well known that polyphenols autooxidize in cell culture media to generate H_2O_2 and quinones that can enter cells/nuclei causing damage to various macromolecules (Long et al., 2000). Such extraneous production of H_2O_2 could account for DNA breakage in the present studies. I have therefore determined the formation of H_2O_2 in the suspension/incubation medium of nuclei (0.4M phosphate buffer) and also compared it with a known generator of H_2O_2 namely tannic acid (Bhat and Hadi, 1994). The results given in figure 31 show that the rate of H_2O_2 formation by delphinidin is almost negligible but that of tannic acid is quite significant. It is therefore suggested that the nuclear DNA breakage observed in our experiments is not the result of extraneous generation of H_2O_2.

Figure 25. Standardization of Lysed version of Comet Assay with H_2O_2 :



Reaction mixture (2 ml) contained 0.4 M phosphate buffer (pH 7.5) and indicated concentrations of H_2O_2 (0-20 μM). The reaction mixture was incubated at 37 °C for 30 min and processed further for lysed version of Comet Assay as described in "Methods".

Photographs of comets (100x) obtained on incubating lymphocyte nuclei with varying concentrations of H_2O_2.

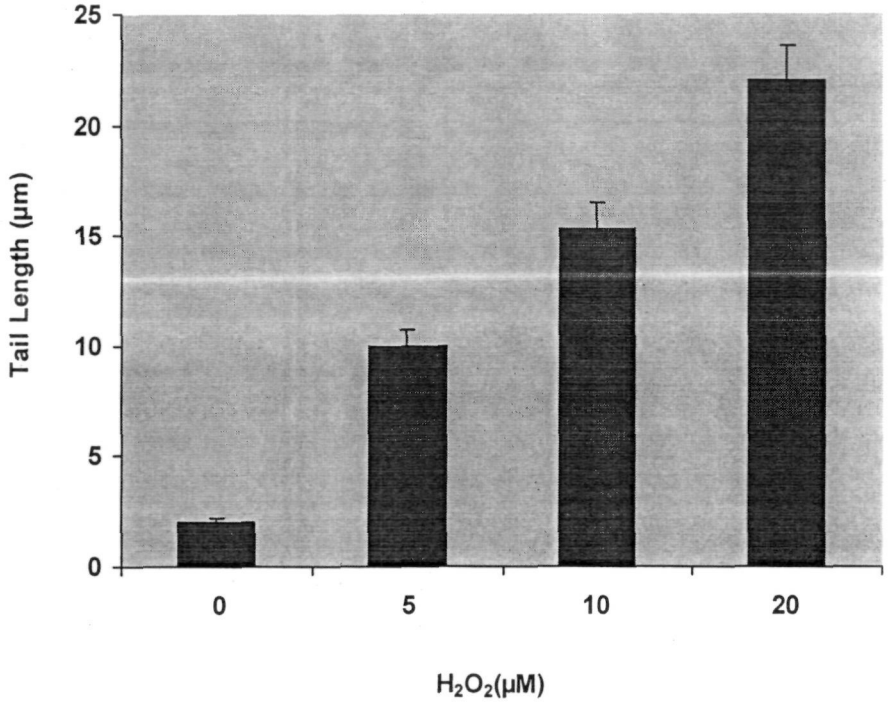
(A) Untreated lymphocytes

(B) Lymphocytes + 05 μM H_2O_2

(C) Lymphocytes + 10 μM H_2O_2

(D) Lymphocytes + 20 μM H_2O_2

Figure 26. Standardization of Lysed version of Comet Assay with H_2O_2

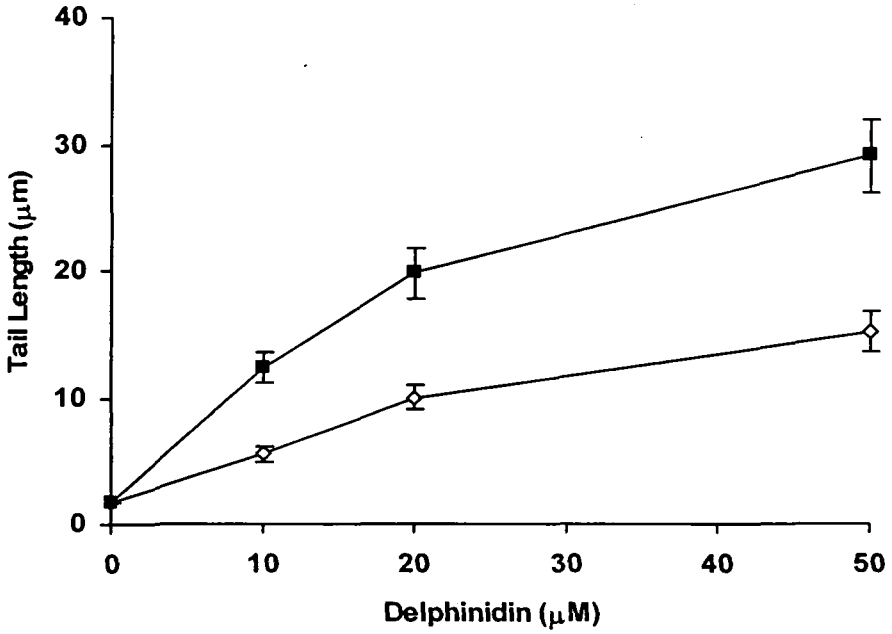


Reaction mixture (2 ml) contained 0.4 M phosphate buffer (pH 7.5) and indicated concentrations of H_2O_2 (0-20 µM). The reaction mixture was incubated at 37 °C for 30 min and processed further for lysed version of Comet Assay as described in "Methods".

Comet tail length (µ metres) plotted as a function of increasing concentrations of H_2O_2 (0-20 µM).

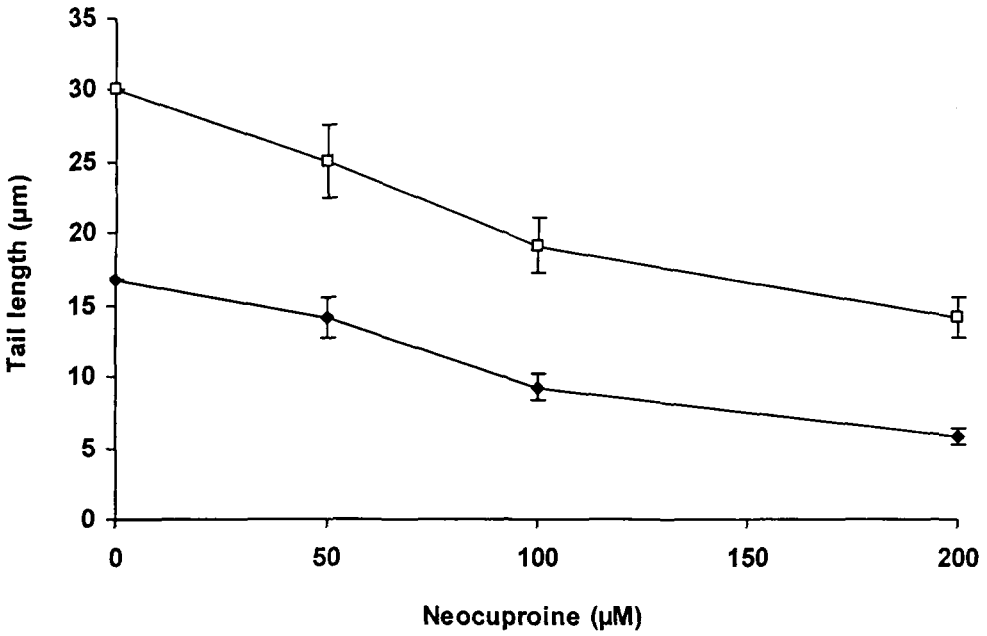
All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.

Figure 27. Comparison of DNA breakage by delphinidin in intact lymphocytes and lymphocyte nuclei as measured by Comet assay :



Reaction mixture (2 ml) contained 0.4 M phosphate buffer (pH 7.5) (lysed version) / PBS Ca^{2+} and Mg^{2+} free (standard version) and indicated concentrations of delphinidin (0-50 μM). The reaction mixture was incubated at 37 °C for 1 hr and processed further for Comet Assay as described in "Methods". Comet tail length (μ metres) plotted as a function of increasing concentrations of delphinidin (0-50 μM) in lymphocytes (◇) and lymphocyte nuclei (■). All points represent mean of three independent experiments. Error bars denote ± SEM. P value < 0.05 and significant when compared to control.

Figure 28. Effect of neocuproine on delphinidin induced DNA breakage in lymphocytes and lymphocyte nuclei as measured by Comet Assay:



Reaction mixture (2 ml) contained 0.4 M phosphate buffer (pH 7.5) (lysed version) / PBS Ca^{2+} and Mg^{2+} free (standard version), delphinidin (50 µM) and indicated concentrations of neocuproine. The reaction mixture was incubated at 37 °C for 1 hr and processed further for Comet Assay as described in "Methods". Comet tail length (µ metres) plotted as a function of increasing concentrations of neocuproine (0-200 µM) in lymphocytes (♦) and lymphocyte nuclei (□). Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.

Table - 3

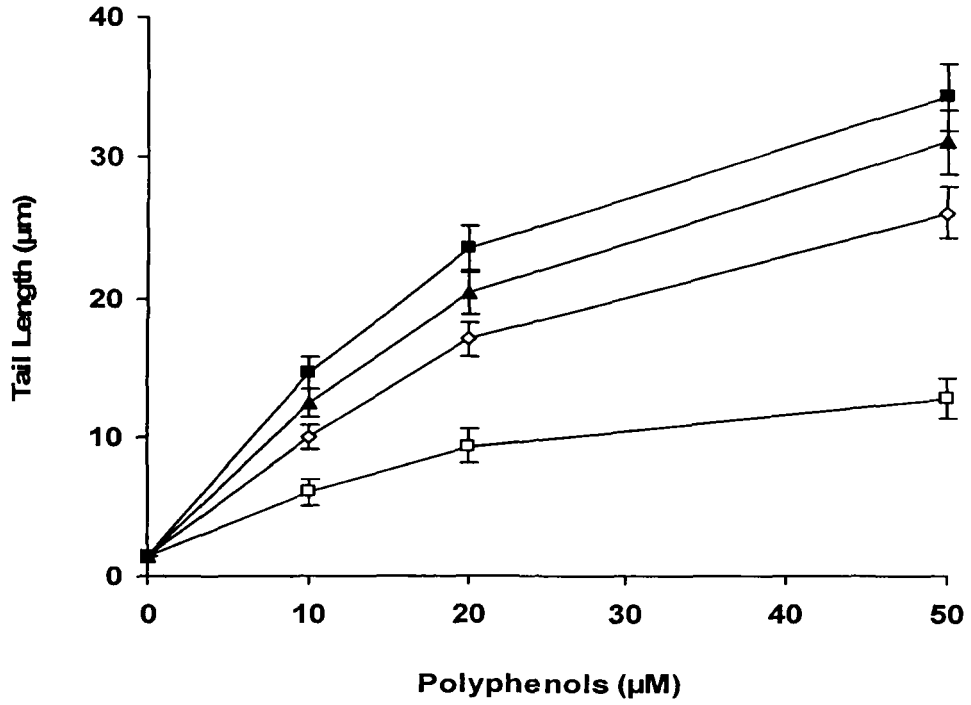
Effect of scavengers of active oxygen species on delphinidin induced DNA damage in lymphocyte nuclei .

Treatment	Tail length (μm)	% inhibition
Control	1.8 ± 0.15	-
Delphinidin (50μM)	30.1 ± 2.9[a]	0
+Thiourea (1mM)	9.8 ± 0.9*	67.4
+KI (1mM)	8.5 ± 0.78*	71.7
+sodium azide (1mM)	9.4 ± 0.88*	68.7
+ SOD(100μg/ml)	7.9 ± 0.67*	73.7
+catalase (100μg/ml)	10.0 ± 1*	66.7

All values represent S.E.M. of three independent experiments.

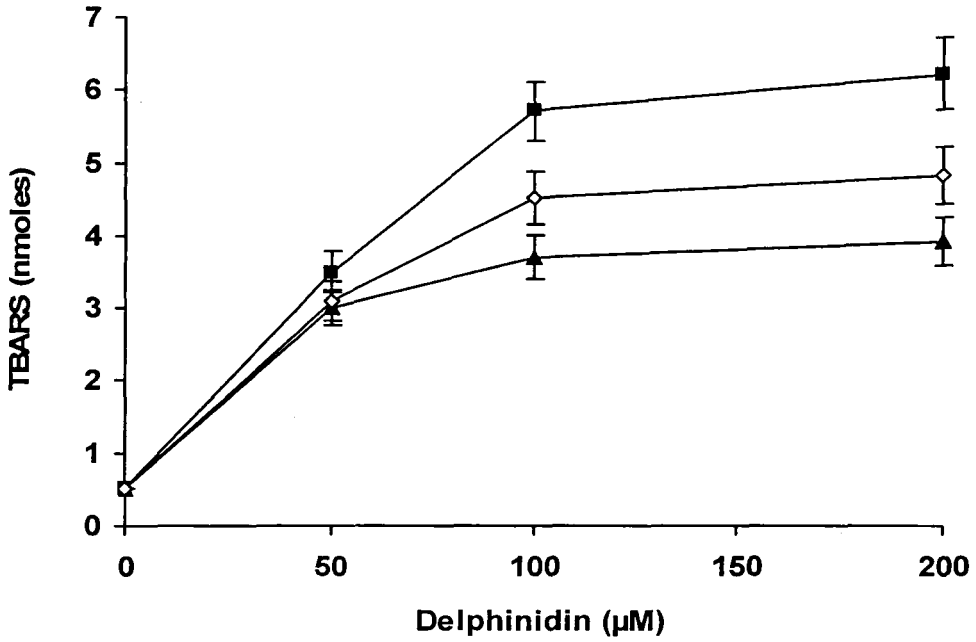
* P-values < 0.05 when compared to control[a]

Figure 29. Comparison of various polyphenols on the induction of DNA breakage in lymphocytes nuclei :



Reaction mixture (2 ml) contained 0.4 M phosphate buffer (pH 7.5) and indicated concentrations of different polyphenols. The reaction mixture was incubated at 37 °C for 1 hr and processed further for lysed version of Comet Assay as described in "Methods". (■) gallic acid, (▲) EGCG, (◇) delphinidin, (□) resveratrol. Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to untreated control.

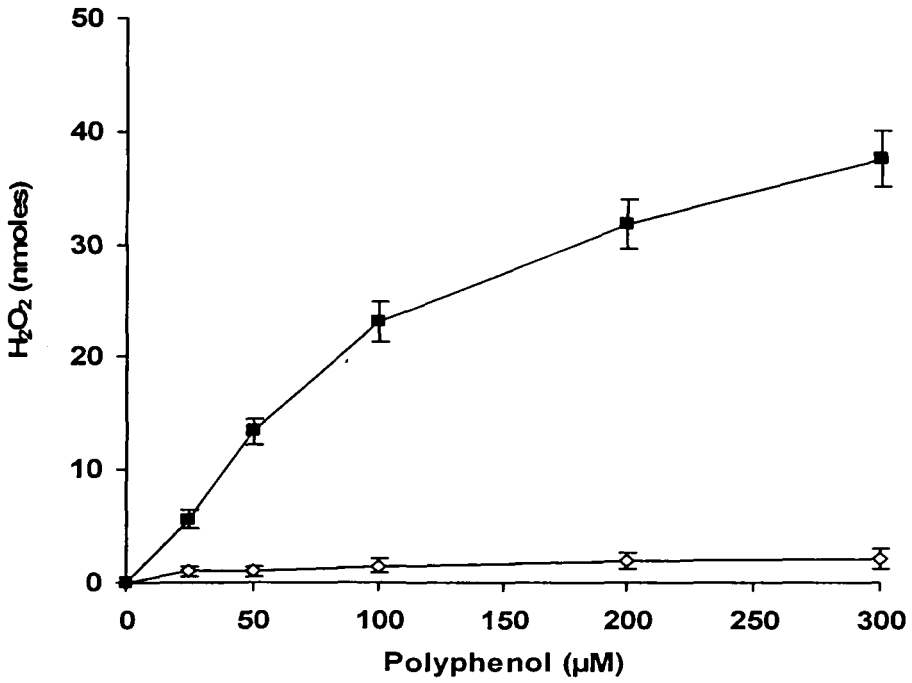
Figure 30. Effect of preincubation of lymphocyte nuclei with neocuproine and thiourea on TBARS generated by increasing concentrations of delphinidin:



The nuclei suspension was preincubated with fixed concentration of neocuproine and thiourea for 30min at 37°C after which it was further incubated for 1hr in the presence of increasing delphinidin concentration.

Delphinidin alone (■),delphinidin + neocuproine (1 mM) (◇), delphinidin + thiourea (1 mM) (▲). Values reported are ± SEM of three independent experiments.

Figure 31. Detection of delphinidin induced H_2O_2 generation in the incubation medium of lymphocyte nuclei by FOX Assay:



Reaction mixture (2 ml) contained the incubation medium (0.4 M phosphate buffer, pH 7.5) and indicated concentrations of delphinidin (◇) and tannic acid (■). After incubation for 2 hr at 37 °C, an aliquot of 200μl was analyzed for H_2O_2 formation.

Values reported are ± SEM of three independent experiments.

DISCUSSION-III

DISCUSSION III

Over the last several years, this laboratory has extensively characterized a DNA cleavage reaction mediated by a number of plant polyphenols in the presence of copper ions (Rahman et al., 1989; Khan and Hadi, 1998; Ahsan and Hadi, 1998; Ahmad et al., 2000; Azam et al., 2004). Subsequently, using human peripheral lymphocytes and Comet Assay, we have already confirmed that the polyphenol resveratrol in the presence of Cu(II) is indeed capable of DNA degradation in a cellular system (Azmi et al., 2005). Further, we have also shown that plant polyphenols alone are capable of oxidatively degrading lymphocyte DNA through mobilization of cellular copper (Azmi et al., 2006). The results of the previous chapter clearly indicated that the anthocyanidin, delphinidin, similar to various other classes of plant polyphenols is able to cause cellular DNA degradation either alone or in the presence of copper ions. Such cellular DNA breakage is also inhibited by antioxidants such as catalase, superoxide dismutase and scavengers of hydroxyl radical (Hanif et al., 2008). The major conclusions of the experiments performed in this chapter are (i) Delphinidin is capable of DNA breakage in cell nuclei possibly through mobilization of nuclear copper, (ii) such DNA breakage is caused by the generation of reactive oxygen species (ROS).

As already mentioned, 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). 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, radiation etc. However, some data in the literature suggests that antioxidant properties of the polyphenolic compounds may not fully account for their chemopreventive effects (Gali et al., 1992; Hadi et al., 2000). 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 plant polyphenols involving mobilization of endogenous copper ions could be an important mechanism of their anticancer properties (Hadi et al., 2000; Hadi et al., 2007). Further, we have suggested that the preferential cytotoxicity of plant polyphenols towards cancer cells is explained by the observation made several decades earlier which showed that serum, (Ebadi and Swanson, 1988; 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. Since cancer cells contain elevated levels of copper, they may be more subject to electron transfer with polyphenols (Zheng et al., 2006) to generate reactive oxygen species (ROS). 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 (Chen et al., 1998; Lu et al., 2000). Indeed, plant polyphenols have been shown to cause regression of tumors in animal models (Orsolic et al., 2004; Orsolic et al., 2005; Mukhtar et al., 1994; Gupta et al., 2001).

Fe^{3+} and Cu^{2+} are the most redox-active of the metal ions in living cells. Although iron is considerably more abundant in biological systems, the major ions in the nucleus are copper and zinc (Bryan, 1979). Further, a comparison of the properties of complexes formed between plant

polyphenolics and Cu^{2+} and Fe^{3+} should indicate which of these two metal ions could lead to DNA fragmentation in the nucleus when complexed. Not much is known about the properties of such complexes. However, considerable information is available about 1,10-phenanthroline (OP) chelation of copper and iron ions. Burkitt et al., (1996) cited several reasons why Cu^{2+} rather than Fe^{3+} may be responsible for OP-stimulated internucleosomal DNA fragmentation in isolated nuclei. For example, the cumulative affinity constants (β_3 in 0.1 M salt) for chelation of various metal ions by OP are in the order $Cu^{2+} \approx Fe^{2+} > Zn^{2+} > Fe^{3+}$. The complex formed between OP and Cu^{2+} has a redox potential (E^0 for Cu^{2+} / Cu^{+} = 0.17 V) that favors redox cycling, whereas that for Fe^{3+} / Fe^{2+} is 1.1 V, presumably because of stabilization in the ferrous state. Copper is also shown to be present in chromosomes as Cu^{+} ions because of stabilization in the presence of DNA. This overcomes the need for the reduction of Cu^{2+} to Cu^{+} and can directly generate the hydroxyl radicals leading to breakage of cellular DNA. According to our hypothesis, plant polyphenols possessing anticancer and apoptosis inducing properties are able to mobilize endogenous copper ions possibly the copper bound to chromatin leading to the formation of ROS such as the hydroxyl radical close to the proximity of the site of DNA cleavage (Pryor, 1988; Chevion, 1988). Essentially, this would be an alternative, non-enzymatic and copper dependent pathway for the cytotoxic action of certain anticancer agents that are capable of mobilizing and reducing endogenous copper. Indeed such a common mechanism better explains the anticancer effects of polyphenols with diverse chemical structures as also the preferential cytotoxicity towards cancer cells. 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 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; Noda et al., 2007; Heiss et al., 2007). An important component of our hypothesis is that plant polyphenols mobilize chromatin bound copper which is redox cycled and which in turn leads to the formation of ROS. This is also in concurrence with the idea that because of its extreme reactivity the hydroxyl radical must be produced in the vicinity of DNA (Pryor, 1988) in order to cause its cleavage. The results presented in this chapter show that polyphenols are able to mobilize endogenous copper ions from lymphocyte nuclei and thus are in agreement with this requirement. Thus, we 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.

The question of bioavailability of delphinidin in mammalian systems also needs to be addressed. It has been reported that delphinidin and cyanidin effectively inhibited vascular endothelial growth factor (VEGF) expression at concentrations as low as 10μM in vascular smooth muscle cells (Oak et al., 2006). It is to be noted that in our studies also 10μM delphinidin (in the absence of added copper) is able to cause significant DNA breakage in lymphocyte nuclei (Figure 27). Such concentrations are likely to be achieved in blood after red wines consumption as the concentration of cyanidin and delphinidin in red wine is about 150 μM (Nyman and Kumpulainen, 2001), and the bioavailability of cyanidin and delphinidin has been estimated to be about 60% in healthy volunteers after consumption of red wine (Frank et al., 2003).

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The anthocyanidin delphinidin mobilizes endogenous copper ions from human lymphocytes leading to oxidative degradation of cellular DNA

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ABSTRACT

Epidemiological and experimental evidence exists to suggest that pomegranate and its juice possess chemopreventive and anticancer properties. The anthocyanidin delphinidin is a major polyphenol present in pomegranates and has been shown to be responsible for these effects. Plant polyphenols are recognized as naturally occurring antioxidants but also catalyze oxidative DNA degradation of cellular DNA either alone or in the presence of transition metal ions such as copper. In this paper we show that similar to various other classes of polyphenols, delphinidin is also capable of causing oxidative degradation of cellular DNA. Lymphocytes were exposed to various concentrations of delphinidin (10, 20, 50 μM) for 1 h and the DNA breakage was assessed using single cell alkaline gel electrophoresis (Comet assay). Inhibition of DNA breakage by several scavengers of reactive oxygen species (ROS) indicated that it is caused by the formation of ROS. Incubation of lymphocytes with neocuproine (a cell membrane permeable Cu(I) chelator) inhibited DNA degradation in intact lymphocytes in a dose dependent manner. Bathocuproine, which is unable to permeate through the cell membrane, did not cause such inhibition. We have further shown that delphinidin is able to degrade DNA in cell nuclei and that such DNA degradation is also inhibited by neocuproine suggesting that nuclear copper is mobilized in this reaction. These results indicate that the generation of ROS possibly occurs through mobilization of endogenous copper ions. The results are in support of our hypothesis that the prooxidant activity of plant polyphenols may be an important mechanism for their anticancer properties.

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1. Introduction

Epidemiological studies have suggested that human consumption of fruits and vegetables is associated with a reduced risk of cardiovascular diseases and certain types of cancers (Vainio and Weiderpass, 2006; Holmes, 2006; Temple and Gladwin, 2003; Thomasset et al., 2007). Many fruits, vegetables and nuts are known to be a rich source of polyphenolic compounds such as flavonoids, tannins, curcuminoids, gallocatechins, stilbenes and anthocyanidins. Plant polyphenols possess a wide range of pharmacological properties, the mechanisms of which have been the subject of considerable interest. They are recognized as naturally occurring antioxidants and have been implicated as anti cancer compounds (Thomasset et al., 2007). In recent years several reports have documented that plant polyphenolics such as curcumin (from the spice turmeric), resveratrol (from red grapes and red wine), epigallocatechin-3-gallate (EGCG) (from green tea) and delphinidin (from pomegranate juice) induce apoptosis in various cancer cell lines (Jaruga et al., 1998; Clement et al., 1998; Inoue et al., 1994; Ahmad et al., 1997; Hou et al., 2003). Of particular interest is the observation that a number of these polyphenols including EGCG, gallic acid and resveratrol induce apoptotic cell death in various cell lines but not in normal cells (Clement et al., 1998; Inoue et al., 1994; Ahmad et al., 1997).

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 one of the most redox active of the various metal ions present in cells. 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, radiation,

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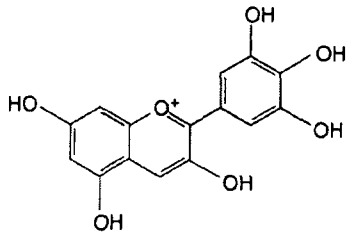

Fig. 1. Chemical structure of delphinidin.

etc. However, some data in the literature suggests that antioxidant properties of the polyphenolic compounds may not fully account for their chemopreventive effects (Gali et al., 1992; Hadi et al., 2000). 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 poly phenolics may be an important mechanism of their anticancer and apoptosis inducing properties (Hadi et al., 2000, 2007). Such a mechanism for the cytotoxic action of these compounds against cancer cells would involve mobilization of endogenous copper ions and the consequent prooxidant action. Using a cellular system of peripheral lymphocytes isolated from human blood and alkaline single cell gel electrophoresis (Comet assay), we have confirmed that plant polyphenols in the presence of Cu(II) are indeed capable of causing DNA degradation in cells (Azmi et al., 2005; Bhat et al., 2007).

Pomegranates have been used extensively in folk medicine in many cultures. The anthocyanidin, delphinidin (Fig. 1) is the major polyphenol present in pomegranate and has been shown to possess anticancer and apoptosis inducing properties (Hou et al., 2003; Bagchi et al., 2004; Syed et al., 2007). In this paper we show that delphinidin is capable of mobilizing endogenous copper ions from human peripheral lymphocytes, which in turn leads to the degradation of cellular DNA.

2. Materials and methods

2.1. Materials

Delphinidin was purchased from Extrasynthese (Genay, France), neocuproine, bathocuproine, agarose, low melting point agarose (LMPA), RPMI 1640, Triton X-100, Trypan blue, Histopaque-1077 and phosphate buffered saline (PBS) Ca^{2+} and Mg^{2+} free were purchased from Sigma (St. Louis, USA). All other chemicals were of analytical grade. Delphinidin was dissolved in 3 mM NaOH before use as a stock of 1 mM solution. Upon addition to reaction mixtures, in the presence of buffers mentioned and at the concentrations used, delphinidin remained in solution. The volumes of stock solutions added did not lead to any appreciable change in the pH of reaction mixtures.

2.2. Isolation of lymphocytes

Heparinized blood samples (2 ml) from healthy donors was obtained by venepuncture and diluted suitably in Ca^{2+} and Mg^{2+} free PBS. Lymphocytes were isolated from blood using Histopaque 1077 (Sigma), and the cells were finally suspended in RPMI 1640.

2.3. 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).

2.4. Lymphocyte treatment

Lymphocytes (1×10^5 cells) were exposed to different concentrations of delphinidin in the absence and presence of $CuCl_2$ in a total reaction volume of 1 ml. Incubation was performed at 37 °C for 1 h or as specified. In some experiments, scavengers of reactive oxygen were added at the final concentrations indicated. After the incubation, the reaction mixture was centrifuged at 4000 rpm, the supernatant was discarded and pelleted lymphocytes were resuspended in 100 μl of PBS (Ca^{2+} and Mg^{2+} free) and processed further for Comet assay (Azmi et al., 2005).

2.5. Treatment of lymphocyte nuclei with delphinidin using lysed version of comet assay

Lymphocytes embedded in agarose were lysed and subsequently treated by delphinidin and other agents. Such lysed version of the comet assay has been earlier used to study the direct interaction of various agents with cell nuclei as it eliminates the effect of cell membrane as a barrier and the intracellular environment (Szeto et al., 2002) Lysed version of comet assay was performed as described by Kasamatsu et al. (1996) with some modifications. Lymphocytes (2×10^5 cells) isolated from 2 ml blood was suspended in RPMI 1640. Approximately 10,000 of these cells were mixed with 75 μl of prewarmed LMPA in PBS and immediately applied to frosted microscopic slide layered with 75 μl of 1% standard agarose in PBS. The slides were allowed to gel at 4 °C for 10 min. Lysis of cells was then performed by submerging the slides in a tank containing lysis solution in the absence of light for 1 h at 4 °C. The use of a tank instead of a coplin jar allowed simultaneous processing of a number of slides. The lysis solution (pH 10) consisted of 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris and 1% Triton X-100 added just prior to use. After lysis, slides were transferred to another tank containing 0.4 M phosphate buffer (pH 7.5) for 10 min. It may be mentioned that chromatin core particles undergo a reversible transition with increasing pH (Libertini and Small, 1984). Possibly such a reversal of the transition may be brought about by neutralization to the physiological pH. Each slide was then transferred to a rectangular dish (8 cm × 3 cm × 5 mm) which contained a reaction mixture of delphinidin and other additions as mentioned in various legends to figures. The slides with the reaction mixture were incubated at 37 °C. The slides were then washed twice by placing in 0.4 M phosphate buffer (pH 7.5) for 5 min at room temperature. DNA unwinding and expression of alkali labile sites was done by leaving the slides in the high pH electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH > 13 prepared in PBS) at 4 °C for 30 min. Subsequently, the electrophoresis, neutralization and staining of the slides was carried out as described earlier (Azmi et al., 2005). Comet images were observed at 100× magnification with a fluorescence microscope (Olympus CX41) and COHU 4910 (equipped with a 510–560 nm excitation and 590 nm barrier filters) integrated CC camera. 50 images were randomly selected from each sample and their lengths (diameter of the nucleus plus migrated DNA) were measured on the screen as automatically generated by Komet 5.5 image analysis system of kinetic imaging, Liverpool, UK.

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

2.6. Detection of Cu(II) reduction by delphinidin

The selective sequestering agent neocuproine was employed to detect reduction of Cu(II) to Cu(I) by recording the formation of neocuproine–Cu(I) complex which absorbs maximally at 450 nm. The reaction mixture (3 ml) contained 3 mM Tris–HCl (pH 7.5), fixed concentration (100 μM) of Cu(II), 300-μM neocuproine and varying concentrations (50–100 μM) of delphinidin. The reaction was started by adding Cu(II) and the spectra were recorded immediately afterwards.

2.7. Statistics

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.

3. Results

3.1. Reduction of Cu(II) to Cu(I) by delphinidin

We have previously shown that polyphenols that oxidatively cleave DNA are able to reduce Cu(II) to Cu(I) (Rahman et al., 1989; Bhat and Hadi, 1994). In the experiment shown in Fig. 2 we have used neocuproine that sequesters Cu(I) selectively forming a complex that absorbs maximally at 450 nm. As seen in the figure under the conditions employed neither Cu(II) nor delphinidin interferes with this maximum whereas delphinidin + Cu(II) react to generate Cu(I) as shown by the peak appearing at 450 nm. The results with two different concentrations (50 and 100 μM) of delphinidin are given.

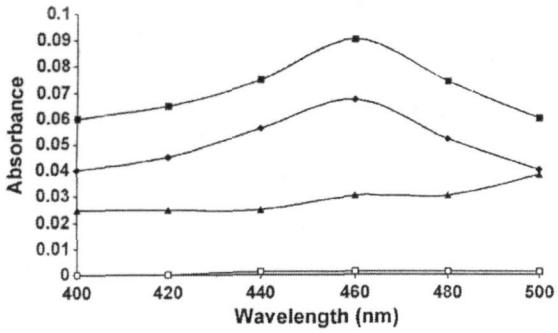

Fig. 2. Detection of delphinidin-induced Cu(I) production by neocuproine. (■) Delphinidin (100 μM) + Cu(II) + neocuproine; (♦) delphinidin (50 μM) + Cu(II) + neocuproine; (▲) Cu(II) + neocuproine; (□) delphinidin(50 μM) + Cu(II). The concentration of Cu(II) and neocuproine was 100 and 300 μM, respectively.

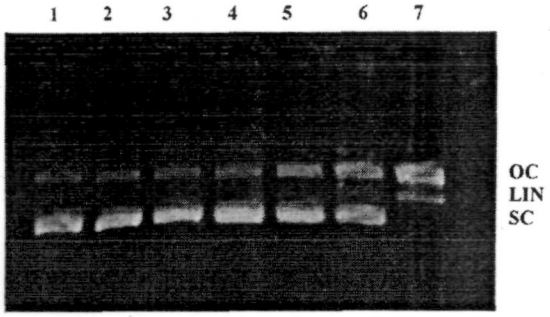

Fig. 3. Degradation of pBR322 plasmid DNA by delphinidin in the absence and presence of Cu(II). Lane 1, DNA alone; lanes 2–4, DNA + delphinidin (25, 50, 100 μM); lanes 5–7, DNA + delphinidin (25, 50, 100 μM) + Cu(II) (50 μM); SC, supercoiled; OC, nicked circular; LIN, linear molecules.

3.2. Cleavage of plasmid pBR322 DNA by delphinidin

In the experiment shown in Fig. 3 we have tested the ability of delphinidin to cause cleavage of supercoiled plasmid pBR322 DNA in the presence of copper ions. Three different concentrations (25, 50, 100 μM) of delphinidin were tested and in the absence of copper none of these caused DNA breakage (lanes 2–4). However in the presence of Cu(II) (50 μM) conversion of supercoiled to open circles and linears was seen when 100 μM delphinidin was used (lane 7). We have shown previously that Cu(II) alone upto a concentration of 150 μM does not lead to cleavage of plasmid DNA (Husain and Hadi, 1995). Thus similar to various other plant polyphenols delphinidin is also capable of DNA degradation in the presence of copper ions.

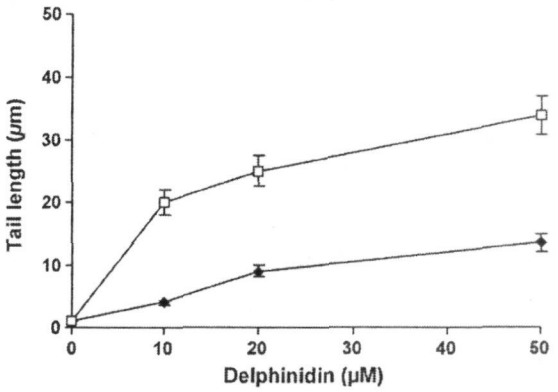

Fig. 4. Effect of increasing concentrations of delphinidin on DNA breakage in lymphocytes in the absence (●) and presence (□) of Cu(II) (20 μM). The viability of cells after incubation was found to be greater than 93%. Values reported are ±S.E.M. of three independent experiments. $P < 0.01$ by comparison with control (in the absence of delphinidin).

3.3. Delphinidin–Cu(II) mediated DNA breakage in human peripheral lymphocytes

Increasing concentrations of delphinidin (10, 20, 50 μM) either alone or in the presence of 20 μM $CuCl_2$ were tested for DNA breakage in isolated lymphocytes using the Comet assay (Fig. 4). It is seen that whereas delphinidin alone causes some breakage of cellular DNA, the degree of such breakage is considerably greater in the presence of copper ions. Cu(II) alone upto a concentration of 50 μM does not lead to any lymphocyte DNA breakage under the conditions employed (Azmi et al., 2005). These results indicate that delphinidin-Cu(II) system is physiologically feasible and could be of biological significance.

3.4. DNA breakage induced by delphinidin alone in lymphocytes as measured by Comet assay

As already mentioned in Section 1 we have proposed that one of the mechanisms for the cytotoxic action of plant polyphenols against cancer cells may involve mobilization of endogenous copper, possibly copper bound to chromatin and the consequent prooxidant action. We have therefore tested increasing concentrations of delphinidin alone for DNA breakage in isolated human lymphocytes using the comet assay. Photographs of comets as seen on treatment with these concentrations are shown in Fig. 5. At 50 μM concentration, delphinidin causes some breakage of cellular DNA whereas at the concentrations of 100 and 200 μM comets with a clear tail indicative of significant DNA breakage are observed. The results clearly indicate that delphinidin alone is capable of cellular DNA breakage.

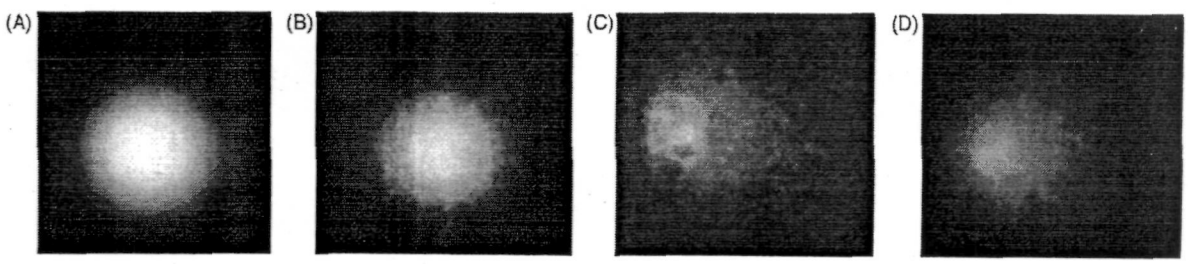

Fig. 5. Single cell gel electrophoresis of human peripheral lymphocytes showing Comets (100×) after treatment with different concentrations of delphinidin alone, (A) untreated, (B) delphinidin (50 μM), (C) delphinidin(100 μM) and (D) delphinidin (200 μM).

Table 1
Effect of scavengers of reactive oxygen species on delphinidin induced lymphocyte DNA breakage

Treatment	Tail length (m)	%Inhibition
Untreated	1.5 ± 0.15	-
Delphinidin (100 μM)	25.0 ± 2[a]	0
+Thiourea (1 mM)	10.36 ± 1.03*	58.5
+KI (1 mM)	7.8 ± 0.73*	68.8
+SOD (100 μg/ml)	8.5 ± 0.85*	66
+Catalase (100 μg/ml)	10.3 ± 1*	58.8

All values represent S.E.M. of three independent experiments. *P-values <0.05 when compared to control[a].

3.5. Effect of reactive oxygen scavengers on delphinidin induced DNA breakage in lymphocytes

It is previously established from our work that the plant polyphenol mediated DNA cleavage in vitro (Rahman et al., 1989) and the inactivation of bacteriophage λ by this system is inhibited to significant degrees by various scavengers of reactive oxygen species (Ahmad et al., 2000). Table 1 gives the results of an experiment where the effect of potassium iodide, thiourea, superoxide dismutase (SOD) and catalase were tested on delphinidin induced DNA degradation in lymphocytes. SOD and catalase remove superoxide and H_2O_2, respectively, and potassium iodide and thiourea remove hydroxyl radicals. From the data we conclude that the lymphocyte DNA degradation is the result of the formation of reactive oxygen species of which superoxide anion and the hydroxyl radical are the proximal DNA damaging agents. It is to be noted that the generation of superoxide anion may spontaneously lead to the formation of H_2O_2 which in turn causes the formation of hydroxyl radical through oxidation of reduced copper (Fenton reaction) (Badwey and Karnovsky, 1980).

3.6. Effect of neocuproine and bathocuproine on delphinidin induced DNA breakage in lymphocytes

In a previous study (Azmi et al., 2005), we have shown that resveratrol mediated DNA degradation of lymphocyte DNA is inhibited by neocuproine which is a Cu(I) specific chelating agent and is membrane permeable (Barbouti et al., 2001; Stefan et al., 1995). In the experiment shown in Fig. 6 we have also used bathocuproine disulphonate, the water-soluble membrane impermeable analog of neocuproine to show that whereas neocuproine inhibits delphinidin induced DNA breakage in lymphocytes, bathocuproine as expected, is ineffective in causing such inhibition. A progressive decrease in the tail length as a function of increasing neocuproine concentration was seen. The reason why complete inhibition of

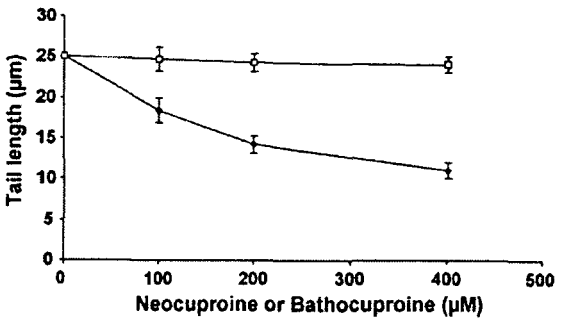

Fig. 6. Effect of increasing concentrations of bathocuproine (□) or neocuproine (●) on delphinidin (100 μM) induced DNA breakage in human peripheral lymphocytes. Values reported are ±S.E.M. of three independent experiments. $P < 0.01$ by comparison with control (in the absence of neocuproine).

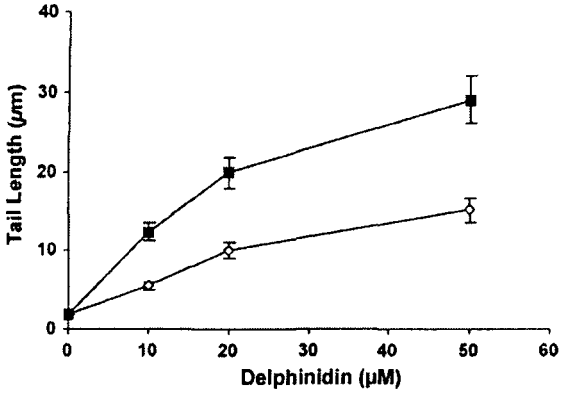

Fig. 7. A comparison of DNA breakage in intact lymphocytes (○) and lymphocyte nuclei (■) using increasing concentrations of delphinidin. Values reported are ±S.E.M. of three independent experiments. $P < 0.05$ by comparison with control (in the absence of delphinidin).

DNA breakage by neocuproine is not observed could be that neocuproine-Cu(I) complex is also able to induce strand breaks in DNA in addition to its property of inhibiting copper induced oxidative stress (Byrnes et al., 1992). Presumably at a relatively higher concentration the former property is predominant. It may be concluded that the DNA breakage by delphinidin involves endogenous copper ions and that Cu(I) is an intermediate in the pathway that leads to DNA breakage.

3.7. A comparison of DNA breakage by delphinidin in intact lymphocytes and lymphocyte nuclei as measured by Comet assay

Since in the lysed version of the comet assay, membrane and cytoplasmic barrier is eliminated (see Section 2) it would be reasonable to assume that the polyphenol is able to directly interact with the cell nuclei. Thus, considerably greater DNA breakage should be observed in the lysed version as compared with the standard version where intact lymphocytes are used. Increasing concentrations of delphinidin (10, 20, 50 μM) were tested for DNA breakage in intact lymphocytes (standard version of comet assay) and compared with that observed with lymphocyte nuclei (lysed version) (Fig. 7). It is seen that the rate of tail length formation is considerably greater in the case of lysed version suggesting that delphinidin is able to directly interact with nuclei when lysed version of comet assay is used. Similar results have also been reported by Kasamatsu et al. (1996) using H_2O_2 and Bleomycin.

3.8. Mobilization of nuclear copper

Fig. 8 shows an experiment where the effect of increasing neocuproine concentration on delphinidin induced DNA breakage is shown in intact lymphocytes (standard version of comet assay) and lymphocyte nuclei (lysed version of comet assay). As shown in the previous experiment and as expected, considerably greater DNA breakage is seen in the nuclei. However neocuproine is able to inhibit the DNA degradation in intact lymphocytes as well as in the nuclei. Thus the results indicate that neocuproine which is membrane permeable is able to enter the nuclei. It may also be noted that the nuclear pore complex is known to be permeable to small molecules (Mazzanti, 1998).

4. Discussion

The above results lead to the conclusion that similar to various other classes of plant polyphenols the anthocyanidin delphini-

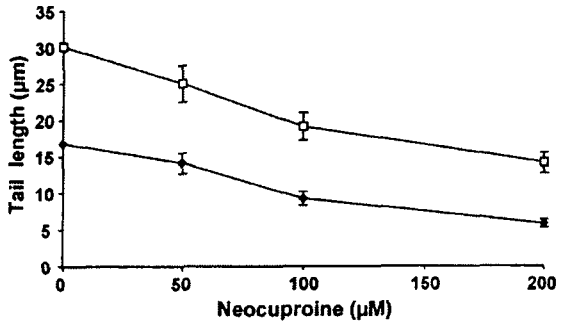

Fig. 8. Effect of increasing concentrations of neocuproine on delphinidin induced DNA breakage in lymphocytes (♦) and lymphocyte nuclei (□). Values reported are ±S.E.M. of three independent experiments. $P < 0.01$ by comparison with control (in the absence of neocuproine).

din is able to cause (i) cellular DNA degradation either alone or in the presence of copper ions (ii), such DNA breakage is caused by the generation of reactive oxygen species (ROS) and (iii) delphinidin is capable of DNA breakage in cell nuclei possibly through mobilization of nuclear copper. Pomegranate has been used for centuries in traditional medicine and is now recognized as a potential chemopreventive and anticancer agent. In animal models pomegranate juice has been shown to possess cytotoxic properties against cancers of skin, prostate, lung, breast, colon and blood. A number of proteins and transcription factors involved in the apoptosis induction pathways have been shown to be up or down regulated on ingestion of pomegranate juice (Syed et al., 2007). Delphinidin, the major anthocynanidin present in pomegranate juice has also been shown to induce apoptosis in human pro-myelocytic leukemia (HL-60) cells (Hou et al., 2003). It was shown that an oxidation mediated JNK activation pathway may be involved in delphinidin-induced apoptosis. Further, delphinidin also caused the cells to produce hydrogen peroxide and antioxidants such as N-acetylcysteine and catalase inhibited delphinidin induced JNK phosphorylation, caspase-3-activation and DNA fragmentation. Structure–activity studies indicated that the presence of orthodihydroxy phenolic groups on the B-ring of anthocyanidins appears to be essential for apoptosis induction. It may be noted that we have earlier shown that such a structural configuration of hydroxyl groups in flavonoids and tannins is also important in oxidative degradation of DNA in the presence of copper ions (Jain et al., 1999; Khan and Hadi, 1998). Similarly we have also shown that gallic acid (which has ortho dihydroxy phenolic groups) is active in lymphocyte DNA degradation whereas its modified derivative syringic acid is not. Such cellular DNA breakage is also inhibited by antioxidants such as catalase, superoxide dismutase and scavengers of hydroxyl radical (Azmi et al., 2005, 2006).

The question of bioavailability of delphinidin in mammalian systems also needs to be addressed. It has been reported that delphinidin and cyanidin effectively inhibited vascular endothelial growth factor (VEGF) expression at concentrations as low as 10 μM in vascular smooth muscle cells (Oak et al., 2006). It is to be noted that in our studies also 10 μM delphinidin (in the absence of added copper) is able to cause significant DNA breakage in lymphocyte nuclei (Fig. 7). Such concentrations are likely to be achieved in blood after red wine consumption as the concentration of cyanidin and delphinidin in red wine is about 150 μM (Nyman and Kumpulainen, 2001), and the bioavailability of cyanidin and delphinidin has been estimated to be about 60% in healthy volunteers after consumption of red wine (Frank et al., 2003).

As already mentioned we have proposed (Hadi et al., 2000, 2007) that the prooxidant action of plant polyphenols involving mobilization of endogenous copper ions could be an important mechanism of their anticancer properties. According to our hypothesis the preferential cytotoxicity of plant polyphenols towards cancer cells is explained by the observation made several decades earlier which showed that serum (Ebadi and Swanson, 1988; 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. Since cancer cells contain elevated levels of copper, they may be more subject to electron transfer with polyphenols (Zheng et al., 2006) to generate reactive oxygen species. 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 (Chen et al., 1998; Lu et al., 2000). Indeed, plant polyphenols have been shown to cause regression of tumors in animal models (Orsolic et al., 2004, 2005; Mukhtar et al., 1994; Gupta et al., 2001). Thus, plant polyphenols possessing anticancer and apoptosis inducing properties are able to mobilize endogenous copper ions possibly the copper bound to chromatin leading to the formation of ROS such as the hydroxyl radical close to the proximity of the site of DNA cleavage (Pryor, 1988; Chevion, 1988). Essentially, this would be an alternative, non-enzymatic and copper dependent pathway for the cytotoxic action of certain anticancer agents that are capable of mobilizing and reducing endogenous copper. Indeed such a common mechanism better explains the anticancer effects of polyphenols with diverse chemical structures as also the preferential cytotoxicity towards cancer cells. As such this would be independent of Fas and mitochondria mediated programmed cell death.

Fe^{3+} and Cu^{2+} are the most redox-active of the metal ions in living cells. Although iron is considerably more abundant in biological systems, the major ions in the nucleus are copper and zinc (Bryan, 1979). Further, a comparison of the properties of complexes formed between plant polyphenolics and Cu^{2+} and Fe^{3+} should indicate which of these two metal ions could lead to DNA fragmentation in the nucleus when complexed. Not much is known about the properties of such complexes. However, considerable information is available about 1,10-phenanthroline (OP) chelation of copper and iron ions. Burkitt et al. (1996) cited several reasons why Cu^{2+} rather than Fe^{3+} may be responsible for OP-stimulated internucleosomal DNA fragmentation in isolated nuclei. For example, the cumulative affinity constants (β_3 in 0.1 M salt) for chelation of various metal ions by OP are in the order $Cu^{2+} \approx Fe^{2+} > Zn^{2+} > Fe^{3+}$. The complex formed between OP and Cu^{2+} has a redox potential (E° for $Cu^{2+}/Cu^{+} = 0.17$ V) that favors redox cycling, whereas that for Fe^{3+}/Fe^{2+} is 1.1 V, presumably because of stabilization in the ferrous state.

The generation of ROS in normal cells is under tight homeostatic control (Klein and Ackerman, 2003). However, increased generation of ROS resulting in oxidative stress can be induced by a large number of factors including metals, drugs and prooxidants such as H_2O_2 resulting in the induction of apoptosis. Since in various malignancies, copper levels are known to be elevated presumably the oxidative stress is further enhanced in cancer cells. Several studies have indicated that apoptosis induction by several 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; Noda et al., 2007; Heiss et al., 2007). An important component of our hypothesis is that plant polyphenols mobilize chromatin bound copper, which is redox cycled and which in turn leads to the formation of ROS. This is also in concurrence with the idea that because of its extreme reactivity the hydroxyl radical must be produced in the vicinity of DNA (Pryor, 1988) in

order to cause its cleavage. Thus the present results which show mobilization of nuclear copper by plant polyphenols are a step in further validation of our hypothesis.

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