STUDIES ON THE MECHANISM OF ACTION OF PLANT DERIVED POLYPHENOLIC COMPOUNDS (Resveratrol-Cu(II) induced DNA breakage in humam peripheral lymphocytes: implications for anticancer properties)

SUMMARY

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

BIOCHEMISTRY

THESIS

BY

ASFAR SOHAIL AZMI

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

2006

SUMMARY

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. 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. Gallocatechins are constituents of green tea, the consumption of which is considered to reduce the risk of various cancers such as those of bladder, prostrate, oesphagus and stomach. 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.

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 also 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 various xenobiotics, radiation etc. However, some data in literature suggests that antioxidant properties of the polyphenolic compounds may not fully account for their anticancer effects. Most of the plant polyphenols possess both antioxidant as well as prooxidant properties and reports from this laboratory 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.

As mentioned above resveratrol, (3,4',5-trihydroxy stilbene) a polyphenol belonging to the class of compounds known as stilbenes 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 resveratrol by comparing its properties with its structural analog piceatannol (3,3',4,5- tetrahydroxy stilbene) as well as the parent compound trans-stilbene. Using fluorescent and absorption studies I have shown that both resveratrol as well as piceatannol are able to bind as well as reduce copper ions. Further, these polyphenols are also able to bind to DNA. Both resveratrol as well as piceatannol were able to degrade calf thymus and supercoiled plasmid pBR322 DNA in the presence of copper ions. More significantly the rate of DNA breakage correlates with the efficiency of Cu(II) reduction and the rate of formations of hydroxyl radicals. Trans-stilbene which does not have any hydroxyl groups does not reduce Cu(II) and is also not a DNA cleaving agent. These results suggest that the number and position of hydroxyl groups on the stilbene molecule is important for the DNA cleavage efficiency.

In the second chapter of the thesis I have explored whether the resveratrol-Cu(II) system is capable of causing DNA degradation in cells such as lymphocytes. Using a cellular system of lymphocytes isolated from human peripheral blood and Alkaline single cell gel electrophoresis (Comet assay), I have confirmed that resveratrol-Cu(II) system is indeed capable of causing DNA degradation in cells such as lymphocytes. Also, trans-stilbene, which does not have any hydroxyl groups, is inactive in the lymphocyte system. Preincubation of lymphocytes with resveratrol indicates that it is capable of either traversing the cell membrane or binding to it. These results are in partial support of the hypothesis that anticancer properties of various plant derived polyphenols may involve mobilization of endogenous copper and the consequent prooxidant action.

In the third and final chapter of this thesis I have shown that a number of polyphenols with diverse chemical structures are capable of inducing DNA breakage in lymphocytes in the absence of added copper ions. 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 polyphenols induce generation of hydroxyl radicals in lymphocytes and neocuproine and hydroxyl radical quenchers inhibit such radical formation. These results are in further support of the hypothesis that anticancer mechanism of plant polyhenols involves mobilization of endogenous copper, possibly chromatin bound copper, and the consequent prooxidant action.

Some evidence suggests that polyphenolic compounds such as tannins and resveratrol are able to traverse cell membranes and may enter the cytoplasmic or nuclear space. Resveratrol is sufficiently hydrophobic and has been shown to be present in such tissues as heart, liver and kidney. 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. The question of bioavailibility of polyphenols in mammalian system also needs to be addressed. Some relatively recent work with resveratrol indicates that it may have a relatively low bioavailibility due to its biotransformation and rapid elimination. For example it has been reported that in the case of rabbits the half-life of resveratrol in plasma after i.v administration of 20 mg/kg b.w was about 14 minutes and the highest concentration of resveratrol in plasma was reached within the first five minutes (2.6 ± 1 μM) after receiving 20 mg res/kg.b.w orally. Nevertheless these authors further report that 5 μM resveratrol completely inhibited the growth of B-16 M murine melanoma cells. In this context we may mention that the minimum concentration of resveratrol tested by us in the presence of added copper ions for DNA breakage in lymphocytes was 10 μM. However, as mentioned the minimum concentration of resveratrol required for DNA breakage in lymphocytes is between 100-200μM. Because of higher intracellular copper levels it may be predicted that such concentrations of resveratrol 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. 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. Based on the work presented in this thesis I we would like to propose that mobilization of endogenous copper ions by polyphenols and the consequent prooxidant action could be one of the important mechanisms for 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.

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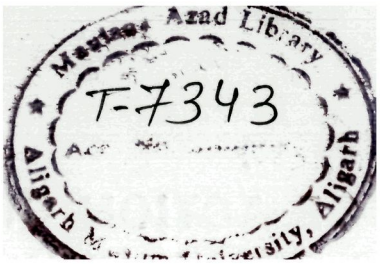
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Dated.22nd March 2006

Certificate

This is to certify that the work presented in this thesis has been carried out by **Mr Asfar Sohail Azmi** 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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Asfar Sohail Azmi

(Asfar Sohail Azmi)

To my
Mother

"LET FOOD BE THY MEDICINE AND THY MEDICINE BE THY FOOD"

(Hippocrates 460-377 BC)

CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

SUMMARY

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INTRODUCTION

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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 behavior 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 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 et al., 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 et al., 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., 1988). 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 et 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 (Phenyl alanine 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 et al., 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. Addition of cyclic esters at the side chains produces hydroxy coumarins and chromones and various condensation reactions with malonyl residues produce xanthones, stilbenes and flavonoids.

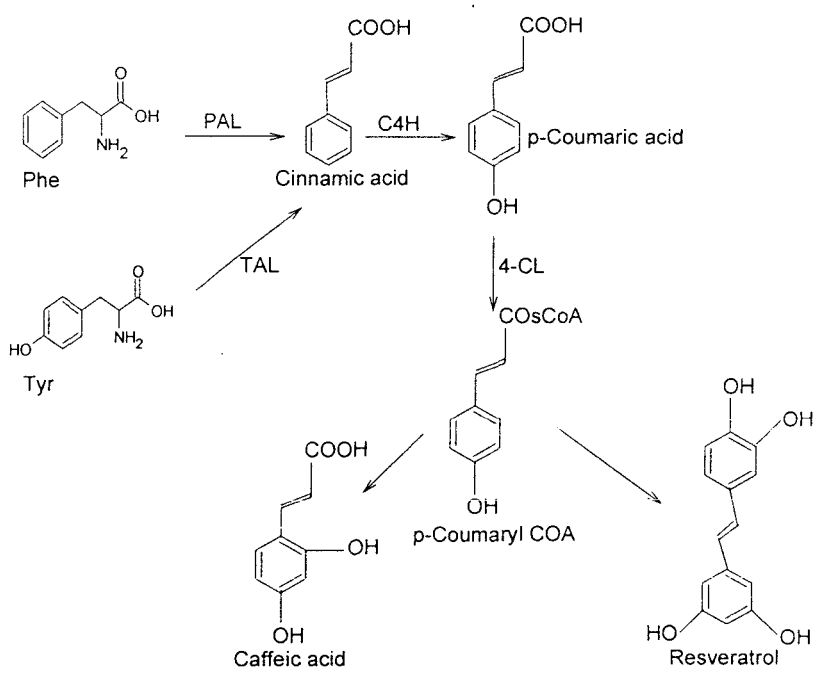


Fig 1. Biosynthesis of plant polyphenols

Anticancer Properties of plant polyphenols:

Numerous studies have reported flavonoid mediated antiproliferative effects against both human and rodent ovarian, leukemic, intestinal, lung, breast and bladder cancer cells. For example, quercetin (10μM) 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., 1997). Curcumin is cytostatic in several hormone dependant (MCF-7 and T-47D) and independent (SK-BR3, BT-20 and MDA-231) 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 HL60 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 cells and 9L gliosarcoma cell

growth, but are less effective in transformed human CCl 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 W138 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 (MNU) 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 and 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 inhibit 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 (50-100μM) significantly reduce DNA strand breakage and oxidized pyrimidine levels in H_2O_2-stressed lymphocytes (Duthie et al., 1997a, 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 activities 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 IAI and IA2 (IC_{50} < 1μM), while galangin is a selective inhibitor of P450 IA2 (Zhai et al., 1998). The ability of flavonoids to inhibit P450 IA 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 (Kanazawa 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 biases in recall and reporting of intake patterns.

Resveratrol:

Resveratrol, a potent member of antifungal phytoalexins was first isolated from the roots of the oriental medicinal plant *Polygonum capsidatum* (Ko-Jo- Kon in Japanese) (Nonomura, 1963). The observation that resveratrol was one of the major active ingredients of folk plant, known for its remedial effects against a host of disease states (Nonomura et al., 1963; Kubo et al., 1981) and that it was synthesized in response to fungal infection in grape vines (Vitis vinefera) (Langcake and Pryce, 1976) provided the early impetus for the interest in unraveling the biological properties of this

compound. Since the first reported detection of resveratrol in grape vines in 1976 (Siemann and Creasy, 1992) most of the work is focused on resveratrol in grape vines. This is mainly due to the fact that compounds found in grape vines were implicated in epidemiological data demonstrating an inverse correlation between red wine consumption and incidence of cardiovascular disease-the "French Paradox" (Kopp, 1998). Consequently the early research on resveratrol was centered on its effect on metabolic pathways regulating cardiovascular biology, such as lipid metabolism and platelet function. However since the reported chemopreventive activity of resveratrol in a murine model of carcinogenesis (Jang et al., 1997) recent investigations have been directed at understanding the molecular mechanism(s) of its diverse biological effects. As a result, the positive or negative effects of resveratrol on some important physiological pathways have been proposed as possible mechanisms for its observed cancer chemopreventive, cardioprotective and neuroprotective activities. These include suppression of cellular proliferation via inhibition of key steps in the signal transduction pathways and cyclin-dependant kinases (CDKs), promotion of cellular differentiation, scavenging/suppression of intracellular reactive oxygen intermediates, induction of apoptotic cell death through activation of mitochondria-dependant or independent pathways, anti-inflammatory activity via down regulation of pro-inflammatory cytokines and inhibition of androgen receptor function and estrogenic activity (Pervaiz, 2003).

Biosynthesis in plants:

Resveratrol is produced by a restricted number of plants (about 31 genera). It is not normally present in large amounts, and it is produced in response to stress (Langcake & Pryce, 1977; Dixon et al., 2001). Resveratrol is toxic to plant pathogens, but some parasites such as fungi overcome this toxicity through the action of membrane proteins (ABC transporters) that transport the compound out of the cellular compartments (Nakaune et al., 2002).

Overproduction of stress response molecules in plants triggers a hypersensitivity reaction that can be counteracted (Heath, 2000).

Natural Source of resveratrol and other stilbenes: Resveratrol and the analogue piceatannol and pterostilbene are found in several edible natural products such as grapes (Vitis spp.) peanuts (Arachis spp) (Sanders et al., 2000), berries (blue berries, cranberries and lingo berries, all Vaccinium spp. (Rimando et al., 2004) and in Eucalyptus spp (Hathway, 1962) mulberry (Monis spp) and red sandalwood (Pterocarpus spp. a major source of pterostilbene) (Rimando et al., 2004). Extracts from roots, heartwood bark and leaves of most of these plants are commonly used in traditional oriental medicine. The content of resveratrol in different sources varies widely, depending on factors such as cultivars, climate, fungi infections, UV exposure and wine making procedures.

Reveratrol in diet, absorption, transport, metabolism and excretion: The absorption and transport of resveratrol have been studied in several models: isolated rat intestine (Andlauer et al., 2000; Kuhnle et al., 2000), rats and mice after oral administration (Bertelli et al., 1998; Soleas et al., 2001; Asensi et al., 2002; Vitrae et al., 2003) human colon carcinoma Caco-2 cell line (Kaldas et al., 2003), human hepatocytes (Lancon et al., 2004) and healthy human subjects (Goldberg et al., 2003).

Absorption: Experiments with isolated rat intestine perfused in vitro with resveratrol-containing buffer showed that jejunum and to a lesser extent ileum are involved in the absorption of resveratrol (Kuhnle et al., 2000). In vitro studies showed that resveratrol is metabolized also by human intestinal cells. The human colonic adenocarcinoma cell line Caco-2 treated in vitro with resveratrol exhibited initially a dose-dependent increasing rate of apical to basolateral transport, until this reached a plateau with the maximal transport that is not concentration dependant (Kaldas et al., 2002). Resveratrol is present in dietary products as cis-and trans resveratrol but mainly in glycosylated forms named piceids (3-O-β-D glucosides). Plant

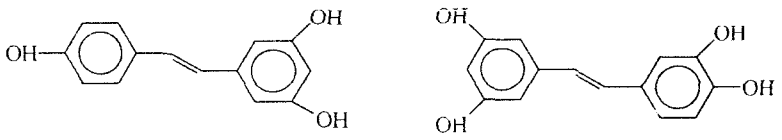
pathogens as well as the human digestive tract contain enzymes that oxidize polyphenols. However, glycosylation inhibits enzymatic oxidation of resveratrol, thereby preserving its biological activity and increasing its stability and bioavailability. (Regev et al., 2003; Krasnow et al., 2004). Since intestinal cells are only able to absorb aglycone resveratrol, the absorption process requires glycosidases (Day et al., 1998). Therefore the relative amounts of aglycone and glycosylated resveratrol in foods may modulate the absorption rate.

Transport in tissue: Conversion of resveratrol into hydrophilic conjugates may facilitate its entry in the blood stream, its diffusion throughout the body and most importantly its excretion. Shortly after oral intake, resveratrol was found in colon, whereas its tissue distribution required a few hours. In liver, resveratrol accumulated up to a concentration comparable to that which exerts biological effects in in vitro assays (micro molar range) (Vitrae et al., 2003). The uptake and metabolism of resveratrol by human liver have been studied in the in vitro models. Human hepatocytes exhibited an initially increasing rate of uptake (minutes) then the rate remained stable (hours) (Lancon et al., 2004).

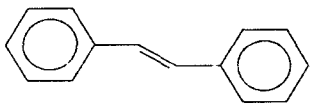
Metabolism and excretion of resveratrol: In animal model, renal excretion of resveratrol started within hours after intake and increased throughout the next 12-24 hrs. (Vitrae et al., 2003). The presence in urine of little native (aglycone) resveratrol but high amounts of its conjugates indicates that metabolism of this compound is essential for excretion (Marrier et al., 2003). In kidney, resveratrol was present mainly in native form, whereas in urine the majority of the compound was present in the conjugated from (Vitrae et al., 2003). In humans, excretion was time dependent and also dependant on the concentration of resveratrol (aglycone and conjugates) present in plasma, but there was no direct correlation between excreted and introduced amounts. This observation from Meng et al (2004) suggests that while small amounts of resveratrol are rapidly metabolized and eliminated,

retention and accumulation of the compound in tissue occurs only over a certain dose of intake, thus becoming potentially available for cellular uptake and intracellular signaling.

Figure 2. Structures of resveratrol, piceatannol and trans-stilbene



Resveratrol **Piceatannol**



Trans-stilbene

Conjugation with glycosidic or sulfatidic group is probably aimed at promoting excretion, since conjugates are not intracellularly active (Yu et al., 2003). Flavonoids such as quercetin inhibit the glucoronidation of resveratrol and therefore may increase its bioavailability (De-Santi, 2000). This observation may partly explain why low concentrations of dietary polyphenols have synergistic effects (Manzocco et al., 2002). Many questions regarding resveratrol metabolism and excretion remain unanswered. It is still to be classified if resveratrol conjugates are transported to tissues or if they are only targeted for excretion. Moreover, since liver uptake has only been studied in vitro using the native aglycone form of resveratrol, it is not known whether hepatocytes also absorb the conjugated form of resveratrol from the blood stream or if these conjugates are targeted for excretion.

Anticancer and antitumor activity of resveratrol:

Antitumor agents impair procarcinogen metabolic activation and interaction with cellular targets (DNA and proteins). They inhibit cancer development by blocking tumor cell transformation and proliferation and by inducing tumor cell death (Surh, 2003). Among food derived molecules that have been screened for their ability to inhibit or reverse such cellular processes, resveratrol is particularly interesting because it effects a broad range of intracellular mediators involved in initiation, promotion and progression of cancer (Jang et al., 1997; Kelloff et al., 2000 and Gusman et al., 2001). Since Jang et al (1997) showed in vivo antitumor potential of resveratrol, many studies revealed a variety of resveratrol intracellular targets whose modulation give rise to overlapping responses that lead to growth arrest and death. Table 1 summarizes some of the anticancer studies of resveratrol. The efficacy of this molecule is still debated because of the multiplicity of affected targets and contradictory effects related to dose and time of treatment and the cellular phenotype. The specific molecular setting determines the response to resveratrol treatment.

Resveratrol inhibits potentially carcinogenic reactive oxygen species production through antioxidant action: a defect in cells inherent ability to counteract the production of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) results in their abnormal accumulation, a state commonly referred as "oxidative stress". Exposure of cellular biomolecules (lipids, proteins and nucleic acid) to ROS results in their oxidative modifications with deleterious effects (Packer, 1992). Through its antioxidant and inhibitory action on lipid peroxidation relatively high concentrations of resveratrol neutralizes the cytotoxic effects of ROS. The antioxidant activity of resveratrol has been shown to inhibit proliferation of hepatic stellate cells (Kawada et al., 1998), a major player in liver fibrosis. Furthermore, resveratrol significantly blocks

phorbol ester induced intracellular reactive oxygen production (Martinez and Moreno, 2000). As phorbol esters are tumor promoters this inhibitory activity of resveratrol could directly inhibit the promotion of carcinogenesis (Cerrutti, 1985). However, relatively recent work provides an interesting insight into the effect of this compound on

Table 1.

Summary of resveratrol cancer studies:

CELL TYPE	CONCLUSIONS	REFERENCES
Leukemia (B lymphocytes)	Inhibition of proliferation, induction of apoptosis	Billard et al., 2002
Leukemia (HL-60)	Induction of apoptosis, inhibition of cell growth	Dorrie et al., 2001 Kang et al., 2003
Leukemia (Adult T-cell leukemia)	Apoptosis induction	Hayashibara et al., 2002
Leukemia (THP-1)	Apoptosis induction, inhibition of cell growth	Tsan et al., 2000; Pendurthi et al., 2002
Breast (MDA-MB-231)	Apoptosis induction, inhibition of cell proliferation and growth	Mgbonyebi et al., 1998; Scarlatti et al., 2003
Breast (MCF-7)	Apoptosis induction, growth inhibition	Mgbonyebi et al., 1998; Serroro et al., 2001; Lu et al., 1996
Colon (HCT-116)	Apoptosis induction	Mahyar-Roemer et al., 2001; Wolter et al., 2001
Colon (Caco-2)	Apoptosis induction	Wolter et al., 2002
Colon (F344 rat model)	Inhibits colon carcinogenesis	Tessitore et al., 2000
Prostate (LnCap)	Growth inhibition	Hseih et al., 2000; Stewart et al., 2003
Prostate (DU-145)	Apoptosis induction, growth induction	Lin et al., 2002; Kampa et al., 2000
Prostate (PC-3)	Apoptosis induction, growth inhibition	Stewart et al., 2003
Liver (HepG-2)	Growth inhibition, decrease in hepatocyte growth factor-induced HepG2 cell invasion	De Ledingham et al., 2001
Liver (Fao rat model)	Cell cycle arrest, proliferation inhibition	Delmas et al., 2000
Melanoma (A431)	Apoptosis induction	Ahmad et al., 2001; Adhami et al., 2001
Melanoma (A375)	Apoptosis induction	Niles et al., 2003
Melanoma (SK-Mel-28)	Apoptosis induction	Niles et al 2003; Larrosa et al., 2004
Ovarian (PA-1)	Apoptosis induction	Yang et al., 2003
Endometria	Proliferation inhibition	Bhat et al., 2001

intracellular redox state where both antioxidants as well prooxidant activity of resveratrol is observed. These activities were found to be dependant upon the concentrations of resveratrol as well as cell type (Ahmad et al., 2003 and Ahmad et al., 2004)

Resveratrol a phytoestrogen with agonistic and antagonistic properties: Loss of estrogen and androgen production in aging leads to deregulated functioning of tissues and organs. Moreover improperly balanced hormonal stimulation may favour cell proliferation and senescence. Consequently, hormone-dependant tumors (breast and prostrate, but also others such as colon and lung) may be prevented by daily intake of appropriate amounts of selective estrogen receptor modulators. The polyphenol resveratrol is considered a dietary phytoestrogen with potent beneficial effects on both estrogen receptor expressing and non-expressing human tumors. The chemical structure of resveratrol is similar to that of synthetic estrogen diethylstilbesterol. Resveratrol belongs to the type I class of estrogen (Levenson et al., 2003). It binds estrogen receptors in the low micro molar range with an affinity lower than that of estradiol. Therefore, it behaves as a weak competitor. This affinity makes resveratrol a superagonist in activating hormone receptor-mediated gene transcription (Gehm et al., 1997; Levenson et al., 2003). Aside from superagonism resveratrol also exerts an antiestrogen action by triggering parallel pathways that inhibit estrogen induced cellular outcomes such as proliferation, tumoral transformation and progression (Jang et al., 1997; Mgbonyebi et al., 1998 and Bhat et al., 2001).

Resveratrol a modulator of Phase I and Phase II enzymes: Cancer initiation occurs as a consequence of multiple events. The combined effects of stimulatory factors (e.g. hormones, cytokines), stress mediators (inflammatory oxygen radicals) and exogenous aggressions (viruses, radiation and xenobiotic compounds) can affect the control of cellular proliferation and lead to tumoral transformation of tissues. Xenobiotics (carcinogens and drugs) are often lipophilic substances that easily enter the

cells. In order to protect from their toxicity, cells render them more hydrophilic and extractable through a process called biotransformation. This involves oxidation by phase-I enzymes and conjugation with polar groups by phase II enzymes. The oxidative phase involves membrane bound enzymes (e.g. cytochrome P-450 monooxygenases CYP) that introduce molecular oxygen atom into substrates (Donato et al., 2003 and Marchand et al., 2004). The second phase is carried out by transferases that add hydrophilic groups or change the redox state of the molecule (e.g. glutathione-s-transferases, UDP-glucoronosyltransferases, sulfotransferases, NAD(P)H quinone oxidoreductases). Chemopreventive strategies include the inhibition of Phase I enzymes responsible for activating xenobiotics and the induction of Phase II enzymes that conjugate these active compounds to endogenous lignin's (e.g. glutathione). Resveratrol being an exogenous lipophilic compound can cross plasma membrane, be subjected to cellular metabolism and it possibly interacts with phase I enzymes. Resveratrol has been shown to inhibit human recombinant CYP 450 in vitro (Yu et al., 2003). Moreover, it also inhibited CYP450 activity from human liver microsomes (Mikstakca et al., 2002 and Ciolino et al., 1999).

Resveratrol a modulator of nitric oxide synthase: Nitric oxide synthase (NOS) is a heme containing monooxygenase. An inducible form of NOS (iNOS) activated at the transcriptional level during inflammation, provides high concentrations of nitric oxide. Nitric oxide (NO) at higher levels may induce apoptosis (by generating toxic reactive intermediates) (Kroneke et al., 1998 and Vieira et al., 2003) or inhibit it (Radisavljevic et al., 2004 an Ohshima et al., 2003) depending on the intracellular redox state. The promoter region of the iNOS is controlled by the transcription factors such as NF-κB, AP1, CREB and STATs. The expression of iNOS is often associated with the induction of tumoral markers such as cyclooxygenases 2 (COX-2), vascular adhesion molecule (VCAMs) and intracellular adhesion molecules (ICAM) (Xia et al., 2001). Finally, iNOS is upregulated in cancer cells in relation to tumor progression (Aktan, 2004; Roman et al., 2002).

Resveratrol has been shown to inhibit NO production and iNOS expression in cancer cells while it also induced apoptosis in human B cell malignancies or lymphocytic leukemia (Roman et al., 2002). Similarly, in lipopolysaccharide (LPS)-stimulated macrophages, resveratrol reduced iNOS mRNA transcription and cytosolic protein levels possibly by blocking phosphorylation of I-κB and NF-κB (Tsai et al., 1999). Although there is conflicting evidence regarding the effect of resveratrol on NO formation, it appears to have antitumoral action via the inhibition of angiogenesis or the induction of cell cycle arrest and apoptosis in cancer cells.

Resveratrol a modulator of COX: COXs (cyclooxygenases) are enzymes that catalyze the first committed steps in prostraglandin (PG) biosynthesis. Prostaglandins are known stimulators of cell proliferation and angiogenesis, and suppression of immune surveillance (Wang et al., 2004). Cyclooxygenases also possess a hydroperoxide activity. This activity leads to the generation of tyrosyl radicals that are involved in the bioactivation of promutagens and also Phase I enzymes (Rouzer et al., 2003). Resveratrol noncompetitively inhibits both COX and hydroperoxide activities of COX1 in vitro (Jang et al., 1997 and Szewezuk et al., 2004). This dual effect of resveratrol is unique since classical nonsteroidal anti-inflammatory drugs (NSAIDs) only effect COX activity (Vane, 1971 and Wu et al., 2003). Further resveratrol also inhibits human recombinant COX2 in in vitro assays (Subbaramaniah et al., 1998). Resveratrol discriminates between the two COX isoforms, being a poor inhibitor of COX2 hydroperoxide activity (Jang et al., 1997 and Szewezuk et al., 2004). The two COX isoforms contribute to the formation of thromboxane A2 (TXA2) and PG12 from PGH2 (McAdam et al., 1999) and resveratrol, with its selective action, may produce different effects according to tissue expression and function of the isoforms. The current available evidence indicates that resveratrol behaves as a tumor-promoting antagonist by triggering intracellular pathways that oppose the unbalanced production of COX.

Resveratrol induced growth arrest and apoptosis in cancer cells: Cancer cells escape from cell cycle control and from G0/G1 terminal differentiation. Resveratrol was found in a variety of cell models to arrest cellular proliferation, mostly in an irreversible way, leading to apoptosis. This effect has been traced to resveratrol's ability to modulate the activity of many key mediators of cell cycle and survival. A number of studies have reported that a variety of human cancer cells treated with resveratrol at micromolar concentrations for 12-24 hrs, arrested the proliferation of cell cycle in the G1/S boundary (Ahmad et al., 2001), in the S phase (Banerjee et al., 2002; Regione et al., 1998 and Fulda et al., 2004) or, less frequently in the G2/M phase (Liang et al., 2003 and Carbo et al., 1999). Cell sensitivity to apoptotic agents may change throughout cell cycle phases (Pucci et al., 2000) therefore, the ability of resveratrol to arrest cancer cells in the S phase, which is the most vulnerable, may strongly increase its chemopreventive potential (Fulda et al., 2004). Moreover, many authors have reported that induction of cell cycle arrest by resveratrol was followed by apoptotic cell death (Ahmad et al., 2001; Estrov et al., 2003; Pozo-Guisado et al., 2002; Adhami et al., 2001). A number of protein targets of resveratrol have been identified. Among these, pivotal roles in cell cycle progression, either in normal or in stressed conditions are exerted by p53 and p21. In a variety of cellular models resveratrol strongly upregulated p53 and p21 imposing a checkpoint on G1/S transition (Orallo et al., 2002; Mnjoyan et al., 2003; Pozo-Guisado et al., 2002). Narayana et al (2003) have demonstrated that resveratrol activated transcription of a whole set of p53-responsive genes (e.g. p21, p300/CBP, Apaf1 and BAK) related to cell cycle arrest and apoptosis, while it down regulated tumor-associated antigens (e.g. PSA, NF-κB/p56 and Bcl2). Further it has also been shown that resveratrol activated p53 and p21 and induced cell cycle arrest in proliferating serum-stimulated cells but not in quiescent cells (Mnjoyan et al., 2003). This observation is in agreement with other studies reporting that resveratrol was differentially toxic to tongue squamous carcinoma cells than gingival

fibroblasts (Babich et al., 2000) and that it was toxic to leukemic cells but not to normal hematopoietic progenitors (Gautam et al., 2000) or peripheral blood lymphocytes (Clement et al., 1998).

Another protein 'survivin' which directly inhibits apoptosis, and whose expression is frequently high in cancer cells. Survivin correlates with resistance to chemotherapy (Altieri et al., 2003 and Derveraux et al., 1999). Survivin gene contains a cyclin-dependant element and depression of this element allows expression of survivin at transition phase G1/S: survivin levels remain high during mitosis since it participates in chromosome assembly at the mitotic spindle (Lens et al., 2003). Resveratrol decreased survivin levels by enhancing its degradation as well as reducing its transcription: this was associated with decreased proliferation and sensitization to chemotherapy (Hayashibara et al., 2002). Conversely other authors assessed that resvratrol did not alter survivin expression rather it downregulated other IAPs (cIAP1 and 2) related to apoptosis induction in a concentration dependant manner (Park et al., 2001).

Genomic regulation of cell cycle and apoptosis: NF κB is a transcription factor involved in titration of balance between proliferation and apoptotic stress response. The NF-κB family includes several proteins that after release from cytosolic inhibitors (I-κB) via phosphorylation translocate to the nucleus. Dimeric transcriptional factors are activated by serine phosphorylation due to PKA, MAPKs and PKCς (Duran et al., 2003 and Vermeulen et al., 2003) and by acetylation by different acetylases (including the CREB binding protein P-300) (Zhong et al., 2002). Acetylation is a dynamic process that is reversed by deacetylases. Deacetylation reduces NF-κB transcriptional potential and increases its affinity towards cytoslic inhibitor I-κB (Zhong et al., 2002). Resveratrol was shown to inhibit NF-κB activation; this activation was associated with an antiproliferative action and with the induction of cell death (Estrov et al., 2003; Kim et al., 2003 and Narayanan et al., 2003). NF-κB controls the transcription of a variety of

Table2:
MOLECULAR TARGETS OF RESVERATROL

Activity	Target
Transcriptional Regulation	↓ AR ↓ AP-1 ↓ NF-κB ↓ β-catenin
Cell Cycle	↓ Rb ↓ Cyclin A, B1, D ↑ Cdk2 ↑ p21 ↑ p27
Apoptosis and Growth	↑ p53 ↓ TNF ↑ Fasl ↓ IL-1β ↑ Bas ↑ Adenyl cyclase ↓ Bcl-2 ↓ EGF ↓ IL-6 ↓ Survivin ↑ Caspase ↑ Ceramide
Invasion	↓ Cox-2 ↓ iNOS ↓ VCAM ↓ ICAM-1 ↓ IGF-1R ↓ VEGF ↓ Tissue Factor
Cytokines and Kinases	↑ NAG-1 ↑ TGF-β ↓ PC-GF ↓ PKC ↓ Syk ↓ PKD ↓ Erk1/2 ↓ CKII
Miscellaneous	↓ DNA polymerase (I, III) ↓ CYP1A1 ↓ Ribonucleotide reductase ↓ Tubulin polymerization

genes, including tumor promoting COX2, iNOS, matrix metalloproteases (MMP9) and endothelial adhesion molecules (Chen et al., 2004). In addition, dietary administration of resveratrol in DMBA induced tumor bearing rats reduced growth and decreased transcription of NF-κB and of its regulated genes COX2 and MMP9 in tumor tissues (Banerjee et al., 2002). Resveratrol was shown to inhibit NF-κB, AP1 and their target genes regulating the activity of the upstream MAPKs. Resveratrol down regulated TNFα induction of NF-κB by blocking JNK and MEK activation (Manna et al., 2000). The potential of resveratrol as an antiangiogenic molecule can be related to its inhibition of NF-κB activation. In TNFα stimulated endothelial cells, resveratrol impaired NF-κB activation only after prolonged and not acute treatment (Pellagatta et al., 2003). These observations suggest that cellular response to resveratrol involves gene transcription mediated promotion of parallel or overlapping and potentiating cascades of events. By modulating upstream tyrosine and serine kinases, resveratrol may regulate the activation of transcriptional factors directed to clusters of genes responsible for inducing cell cycle arrest and eventually apoptosis. Table 2 summarizes the molecular targets of resveratrol.

Effect of resveratrol on tumoral versus normal cells: A few reports have offered an interesting perspective on resveratrol actions on normal versus malignant cells. In one study the IC 50 of resveratrol for proliferation inhibition varied almost two fold: 34μM in leukemia and 59 μM in hematopoietic cells. Moreover, in a colony formation assay performed after a pulse of resveratrol (80μM for 20 hrs), there was a significant difference in the ability to proliferate between hematopoietic progenitors and leukemia cells. Human fibroblasts transformed with SV 40 virus (W138VA) were sensitive to resveratrol modulation of pro versus anti-apoptotic genes where as normal fibroblasts were not (Lu et al., 2001). Not only in leukemia but also in breast cancer, resveratrol induced apoptosis via CD95- dependant caspase. Both tumors constitutively expressed CD95 and resveratrol

promoted C95 ligand expression, which acted as an autocrine apoptosis inducer. Interestingly, peripheral blood lymphocytes, although CD95-positive, did not respond to resveratrol by ligand expression and therefore were insensitive to the compounds cytotoxicity (Clement et al., 1998). A slight difference in sensitivity to resveratrol has been reported between tongue squamous carcinoma cells and normal gingival fibroblasts (Babich et al., 2000), possibly related to the lower proliferation rate (therefore, higher resistance) of normal cells versus immortalized or highly proliferating tumoral cells. These promising results contrast with those of other studies in which resveratrol exhibited similar effects on normal and neoplastic cells. Comparable cytostatic and cytotoxic potentials have been observed in breast cancer cell lines and in immortalized mammary epithelial cells (Mgbonyebi et al., 1998).

Prooxidant properties of resveratrol and generation of ROS in the presence of Cu(II): DNA damage caused by reactive oxygen species such as H_2O_2, singlet oxygen and hydroxyl radicals has been implicated in mutagenesis, oncogenesis and aging (Ames et al., 1995). Oxidative lesions in DNA include base modifications, sugar damage, strand breaks and abasic sites. Because of the potential damaging nature of ROS, the human body has a number of antioxidant defence mechanisms that include enzymes such as superoxide dismutase, and catalase: copper and iron storage and transport proteins and both water soluble and lipid soluble molecular antioxidants. Additionally, experimental and epidemiological studies have shown that the micronutrients present in food can act as antimitotic agents, implicated in cancer initiation, promotion and progression, or mortality (Ames et al., 1995). Every antioxidant, including vitamin antioxidants is infact a redox (reduction-oxidation) agent, protecting against free radicals in some circumstances and promoting free radical generation in others (Herbert, 1996). Studies have revealed prooxidant effects of antioxidant vitamins such as vitamin E (Burkitt and Milne, 1996) and vitamin C (Podmore et al., 1998), under certain circumstances. Moreover, earlier studies in our

laboratory have established that several classes of plant derived polyphenolic compounds such as flavonoids (Rahman et al., 1990), tannins (Khan and Hadi, 1998), curcumin (Ahsan and Hadi, 1998) and capsaicins (Singh et al., 2001) are themselves capable of causing oxidative DNA breakage either alone or in the presence of transition metal ions. Similarly resveratrol is widely believed to be an antioxidant; there is evidence in literature to support its prooxidant properties, for instance the report on the concentration dependant induction of DNA strand breaks in ϕX 174 plasmid DNA by resveratrol (Win et al., 2002). Interestingly, this group was investigating the protective effect of resveratrol against H_2O_2/Cu(II)-induced strand breaks in plasmid, but rather observed an increase in strand breaks. Additionally our previous study (Ahmad et al., 2000) as well as reports from other labs have established DNA cleavage activity of resveratrol in the presence of metal ions (Fukahara and Miyata, 1998; Subramaniam et al., 2004). In flavonoids it has been proposed that a ternary complex of the drug, DNA and Cu(II) is formed which generates oxygen radicals in situ via Cu (I) (Rahman et al., 1990). Resveratrol is also capable of binding to copper ions (Ahmad et al., 2000) and DNA and forms a DNA-resveratrol-Cu(II) ternary complex. The concentration of copper in blood is about 16 μM (Sagripanti et al., 1991). Further diet-derived copper enters the liver preferentially in the monovalent Cu(I) form (Dijstra et al., 1991). Interestingly, this ion has been found at higher concentrations in breast tumors as compared with the normal breast tissue (Linder, 1991). Further, it has also been shown that about 20% of cellular copper is present in the nucleus (Agarwal et al., 1989), associated more specifically with DNA bases particularly guanine (Kagawa et al., 1991).

Piceatannol:

Piceatannol (trans-3, 4, 3', 5-tetrahydroxystilbene) is a naturally occurring stilbene present in sugarcane, berries, peanuts, red wines and skins of grapes (Rimando et al., 2004; Brinker and Seigler, 1991; Cantos et al., 2003).

Piceatannol was first isolated and characterized from *Euphorbia lagasce* in 1984. It is synthesized in response to fungal attack, ultraviolet exposure and microbial infection (Bavaresco et al., 1999). Recent research has shown piceatannol to be a metabolite of resveratrol via cytochrome P-450 1A2 and 1B1 enzymes. Research using human lymphoblast expressed cytochrome P450 1B1 microsomes has also shown trans-resveratrol to be biotransformed into piceatannol along with two other as of yet unidentified tetrahydroxylated stilbene compounds (Potter et al., 2002). These data has led investigators to postulate that resveratrol may acts as a pro-drug for production of piceatannol and other stilbenes.

Piceatannol is an antagonist of the aryl-hydrocarbon (Ah) receptor (Tutel'Yan et al., 2003), a potent tyrosine kinase inhibitor (Burke, 1994; Thakkar et al., 1993; Zou et al., 2004), an inhibitor of mast cell degranulation, bronchial constriction, bronchial edema and anaphylaxis (Kogat et al., 2002; Ulanova et al., 2005 and Luskova et al., 2004) and a potent inhibitor of COX-2 (Murais et al., 2004). Considerable research has been generated demonstrating that resveratrol is a potent anti-oxidant across a variety of assays. Due to the structural similarities between piceatannol and resveratrol, it has been hypothesized that piceatannol also possesses potent anti-oxidant activity. One study determined piceatannol to be significantly more potent in inhibiting Cu^{2+} induced lipid peroxidation in low-density proteins compared to resveratrol (Waffo-Teguo et al., 1998). Moreover, these investigators found piceatannol to be equally active in scavenging 1,1 diphenyl-2-picryl hydrazyl (DPPH), a stable free radical often employed in anti-oxidant assays compared to resveratrol. Further investigations revealed piceatannol to be a potent superoxide scavenger, suggesting a possible role in cardioprotection following ischemia (Huang et al., 2001). Other studies demonstrated the ability of piceatannol to inhibit carcinogen-induced preneoplastic lesion formation in a mouse mammary gland model. Piceatannol and resveratrol both significantly inhibited lesion formation, inhibiting 89-90% of lesions formed compared to a control group

(Lee et al., 1998). Recent research has shown piceatannol to be a more effective scavenger of nitric oxide and hydrogen peroxide compared to resveratrol. Piceatannol also exhibited highly selective cytotoxicity towards activated microglial cells that were secreting high concentrations of nitric oxide. The study also examined the ability of piceatannol and resveratrol to inhibit inducible nitric oxide synthase (iNOS) enzyme activity and determined that both stilbenes had little inhibitory effect. Further, research has also shown piceatannol to be equal to and in many cases, surpasses the antioxidant capacity of resveratrol. Many reporters have hypothesized that the addition of a hydroxyl group of piceatannol makes it a more reactive and is therefore a more potent molecule (Zhong and Ramirez, 2000). Nevertheless work presented in this thesis also finds piceatannol to be a more efficient DNA cleavage agent both in isolated plasmid pBR322 DNA as well as cellular DNA (Azmi et al., 2005; Azmi et al., 2006).

SCOPE OF THE WORK

SCOPE OF THE WORK

The health benefits of plant-derived polyphenols including resveratrol, curcumin, capsaicins, tannins and flavonoids have been attributed to their antioxidant effects. There is evidence in literature suggesting that antioxidant activity of such polyphenols may not fully account for their chemopreventive effects. This is because every antioxidant including resveratrol is a redox (oxidation-reduction) agent, protecting against ROS generation in some cases and promoting radical generation in others (Herbert, 1996). Therefore, it is plausible that other mechanisms may also be responsible for such varied pharmacological properties. Studies in this laboratory have shown that plant polyphenols behave as prooxidants in the presence of copper ions catalyzing DNA breakage through generation of reactive oxygen species (Ahmad et al., 1992; Ahsan & Hadi, 1998; Bhat & Hadi, 1994; Singh et al., 2001; Ahmad et al., 2000; Azam et al., 2004 and 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 the major metal ion present in the nucleus and it is also implicated in tumorigenesis and angiogenesis (Chevion et al., 2003). Also, serum, tissue and cellular copper levels are elevated in numerous malignancies (Linder, 1991; Margalioth et al., 1983). It is well known that red wine is a good source of resveratrol. Additionally wines are also good sources of copper (Carno, 1988; Darret et al., 1986). It has also been shown that programmed cell death induced by resveratrol in THP-1 human monocytic leukemia cells is independent of Fas signaling pathway (Tsan et al., 2000) suggesting pathways other than 'classical'.

There is extensive data in literature that points to a prooxidant rather than an 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 hypothesis according to which plant polyphenols mobilize endogenous copper leading to cytotoxic action through generation of reactive oxygen species (Hadi et al., 2000). Based on the above hypothesis, in the work presented here I have tried to elucidate the mechanism of action of plant derived polyphenolic compounds specially the stilbene resveratrol. 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 resveratrol-Cu(II) system is indeed capable of causing DNA degradation in cells such as lymphocytes. Further, DNA degradation of lymphocytes is inhibited by scavengers of reactive oxygen and neocuproine a Cu(I) specific sequestering agent. These findings demonstrate that resveratrol-Cu(II) system for DNA breakage is physiologically feasible and could be of biological significance. Experiments also show that polyphenols alone (in the absence of added Cu(II)) are also capable of lymphocyte DNA breakage and that such breakage is mediated through mobilization of endogenous copper. These results are in further support of our hypothesis that anticancer mechanisms of plant polyphenols involve mobilization of endogenous copper, possible chromatin bound copper and the consequent prooxidant action (Azmi et al., 2005; Azmi et al., 2006).

MATERIALS & 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
Deoxyribonucleic acid (Calf Thymus Type I)	Sigma Chemical Co., USA
Diphenylamine	BDH, India
Ethidium Bromide (EtBr)	Sigma Chemical Co., USA
Ethylenediaminetetraaceticacid (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 seed, 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	Isolated and purified in the lab according to the procedure of Sambrook et al., 1989
Superoxide dismutase	Sigma Chemical Co, USA
Thiourea	E. Merck, Germany
Tris(hydroxy methyl)aminomethane	Fluka AG, (Switzerland)
Triton X-100	BDH, India

* All other chemicals were commercial products of analytical grade.

METHODS

Reaction of resveratrol, piceatannol and trans-stilbene with calf thymus DNA and digestion with single strand specific nuclease:

Resveratrol, piceatannol and trans-stilbene were 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 (0.5ml) contained 10 mM Tris-HCl (pH 7.5), 500 μg of calf thymus DNA and varying amounts of stilbenes, 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 40 mM Tris-HCl (pH 7.5), 1mM 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 minutes 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, calorimetrically, 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 × 10^{-4} mM EDTA) and incubations with resveratrol were carried out in sterile tubes and sterile conditions and details of the treatment are given in legend to figures.

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

Reaction mixture (30 µl) contained 10 mM Tris-HCl (pH 7.5), 0.5 µg 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 study for stilbene-Cu(II) interaction:

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

Stoichiometric titration of Cu(I) production by stilbenes:

The amount of Cu(I) produced in the stilbene-Cu(II) reaction mixture was determined by titration with neocuproine. Stilbenes (25 µM) in 10 mM Tris-

HCl (pH 7.5) were 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 minutes of incubation at room temprature.

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 polyphenols. 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 stilbenes:

In order to compare the hydroxyl radical production by increasing concentrations of resveratrol, piceatannol and trans-stilbene 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.

Fluorescence studies:

The fluorescence studies were performed on a Shimadzu spectrofluorophotometer RF-5000 (Japan) equipped with a plotter and a calculator. Resveratrol was excited at its absorption maxima (λmax) of 308 nm (Jeandet et al., 1997) while piceatannol was excited at 326 nm. Emission spectra were recorded in the range shown in figures.

Determination of reducing power of stilbenes:

The reducing power of the three stilbenes was determined according to the method of Oyaizu (1986). Resveratrol, piceatannol and trans stilbene were

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 6,000 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.

RBC hemolysis by resveratrol:

The percent hemolysis following incubation of RBC with resveratrol in the absence and presence of 50 μM Cu(II) was measured by recording the absorbance of the hemolysate at 415 nm as described by Yoshida et al (1994). For reference, RBC were treated with distilled water and hemolysate was read at 415 nm to obtain 100 % hemolysis.

Isolation of lymphocytes:

Heparinized blood samples (2ml) 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 lymphocyte with stilbenes and other polyphenols:

Lymphocytes (1 x 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 resveratrol 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 (1989) with slight modifications. Fully frosted microscopic slides precoated with 1.0 % normal melting agarose at about 50 ^{0}C (dissolved in Ca^{++} and Mg^{++} 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 (75µl) 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 x100 added just prior to use for a minimum of 1 hour at 4 ^{0}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 ^{0}C in a field strength of 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, U.K) attached to a

Olympus (CX41) fluorescent microscope and a COHU 4910 (equipped with a 510-560 nm excitation and 590 nm barrier filters) integrated CC camera. Comets were scored at 100 x magnification. Images from 50 cells (25 from each replicate slide) were analyzed. The parameter taken to assess lymphocytes DNA damage was tail length (migration of DNA from the nucleus, μm) and was automatically generated by Komet 5.5 image analysis system.

Determination of TBARS and H_2O_2:

Thiobarbituric acid reactive substance was determined according to the method of Ramanathan (1994). A cell suspension ($1x10^5$/ml) was incubated with different polyphenols (0-400 μM) at 37 ^{0}C for 1 hour 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 (thiobarbituric acid) in boiling water bath for 10 min. The absorbance was read at 532 nm. H_2O_2 was determined in the incubation medium by FOX assay as described by Long et al (2000). The reaction mixture was the same as mentioned in lymphocyte treatment but without cells. After incubation for 2 hrs at 37 ^{0}C an aliquot of 100 μl was analyzed for H_2O_2 formation.

Statistical Analysis:

The statistical analysis was performed as described by Tice et al (2000) and is expressed as ± SEM 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 BREAKAGE BY RESVERATROL AND ITS ANALOGUE PICEATANNOL IN THE PRESENCE OF COPPER IONS

RESULTS-I

RESULTS

Stoichiometry of Cu(II) reduction by resveratrol, piceatannol and trans-stilbene:

Polyphenols are known to reduce Cu(II) to Cu(I) as well as reduce molecular oxygen to superoxide anion (Rahman et al., 1992). The superoxide thus formed spontaneously gives rise to H_2O_2 which in the presence of Cu(I) generates the hydroxyl radical (Fenton type Reaction). We have therefore compared the stoichiometry of Cu(II) reduction by resveratrol, piceatannol and the parent compound trans-stilbene. In the experiment shown in figure 3 increasing concentrations of Cu(II) were added to fixed concentrations of resveratrol, piceatannol and trans stilbene (25 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)/ the three stilbenes. However no clear maximum, where absorption plateaued, was obtained suggesting a possible recycling of copper ions in the reaction.

Formation of polyphenol-Cu(II) complex:

As Cu(II) is reduced by resveratrol and piceatannol, the possibility for the formation of a stilbene-Cu(II) complex was examined. This was carried out by recording the absorption spectra of resveratrol and piceatannol with increasing concentrations of Cu(II). The results given in Figure 4 and 5 show that the addition of Cu(II) to resveratrol/piceatannol results in a shift in the λmax of resveratrol/piceatannol with a peak emerging at a lower wavelength presumably representing on or more oxidized species of each of the two stilbenes. The structure of these oxidized products is not ascertained. In this connection it may be of interest to mention that it has been shown earlier that the oxidized products of resveratrol, generated by reducing Cu(I) are possibly also capable of reducing recycled Cu(II) to Cu(I) (Ahmad et al., 2000)

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

Resveratrol, and piceatannol in the presence of Cu(II) were 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 6 gives the dose response curve of such a reaction. Control experiments (data not shown) established that heat denatured DNA underwent 100 % hydrolysis following the treatment with nuclease. In the presence of Cu(II) (50 μM), increasing concentrations of resveratrol and piceatannol resulted in progressive increase in nuclease sensitive sites in DNA, while trans-stilbene did not show any activity.

Thermal Melting profile of DNA in the presence of resveratrol:

When S1 nuclease was used to determine the thermal melting profile of DNA in the presence resveratrol (Fig 7), a slight increase in melting temprature (78 °C versus 75 °C) was seen. At 75 °C and below S_1 nuclease susceptible hydrolysis was consistently found to be greater in the absence of resveratrol. The experiment suggests the possibility of resveratrol binding to DNA through non-covalent interactions.

Cleavage of plasmid pBR322 DNA by resveratrol, piceatannol and trans stilbene:

In order to understand the chemical basis of DNA breakage by resveratrol-Cu(II) system, I have compared the relative DNA cleavage efficacy of resveratrol, piceatannol and the parent compound trans-stilbene in plasmid pBR322 DNA. In the results given in Figure 8 both resveratrol and piceatannol cause conversion of supercoiled plasmid molecules into linear molecules. However, piceatannol also gives rise to smaller sized heterogeneous fragments as indicated by a smear on the gel. Thus,

piceatannol is a more efficient DNA cleaving agent than resveratrol. Trans-stilbene, which does not have any hydroxyl group, is not a cleaving agent. These results demonstrate that the presence of hydroxyl groups is essential for DNA cleavage. Further, the efficiency of cleavage increases with the number of hydroxyl groups.

Generation of oxygen radicals by stilbenes:

Superoxide production: the production of superoxide anion was determined by the method of Nakayama et al (1983), which involves reduction of NBT by stilbenes to a formazan. The time dependant generation of superoxide anion by 100 μM resveratrol, piceatannol and trans-stilbene, as evidenced by the increase in absorbance at 560 nm is shown in Fig 9. 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 stilbenes*:** 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 cleaving agent (Rahman et al., 1989). Therefore, the capacity of resveratrol, piceatannol and the parent compound trans-stilbene to generate hydroxyl radical in the presence of Cu(II) was compared. The assay is based on the fact degradation of DNA by hydroxyl radicals results in the release of TBA (Thiobarbituric acid) reactive material, which forms coloured adduct with TBA at 532 nm (Quinlan and Gutteridge, 1987). Results of Fig 10 clearly show that increasing concentrations of compounds led to a progressive increase in the formation of hydroxyl radicals. However at all the concentrations tested the formation of TBA reactive material was greater in the case of piceatannol followed by resveratrol while, trans-stilbene was ineffective in this reaction.

Figure 3. Detection of stoichiometry of stilbene and Cu(II) interaction.

Reaction mixture contained 10 mM Tris-HCl (pH 7.5) and fixed concentrations of stilbenes with increasing concentrations of Cu(II) (shown as molar ratios of Cu(II)/stilbenes). Absorbance was recorded after incubating the reaction mixture at room temperature for 10 minutes. (○) piceatannol; (●) resveratrol and (▼) trans-stilbene. All points represent triplicates and mean values have been plotted. Error bars denote SEM of three independent experiments.

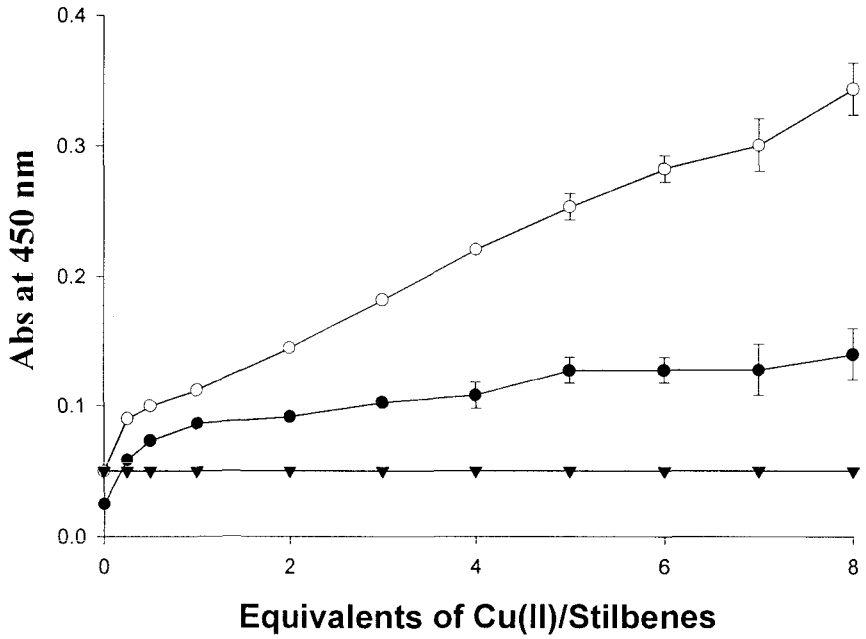

0.4
0.3
0.2
0.1
0.0
Abs at 450 nm
0
2
4
6
8
Equivalents of Cu(II)/Stilbenes

Figure 4. Detection of resveratrol-Cu(II) complex:

The 3 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 μM resveratrol and increasing amounts of Cu(II). The spectra were recorded after addition of components indicated.

(Trace 1) resveratrol alone

(Trace 2) resveratrol + 50 μM Cu(II)

(Trace 3) resveratrol + 300 μM Cu(II)

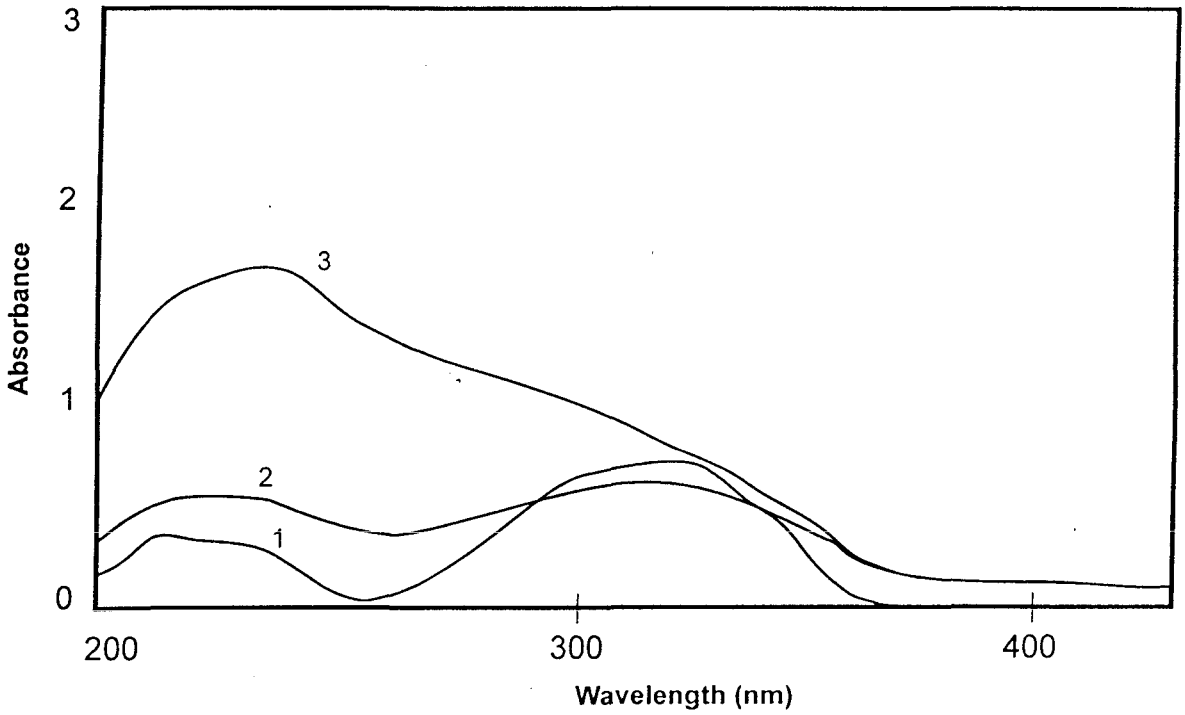

3
2
1
0
Absorbance
3
2
1
200
300
400
Wavelength (nm)

Figure 5. Detection of piceatannol-Cu(II) complex:

The 3 ml reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 μM piceatannol and increasing amounts of Cu(II). The spectra were recorded after addition of components indicated.

(Trace 1) piceatannol alone

(Trace 2) piceatannol + 50 μM Cu(II)

(Trace 3) piceatannol + 300 μM Cu(II)

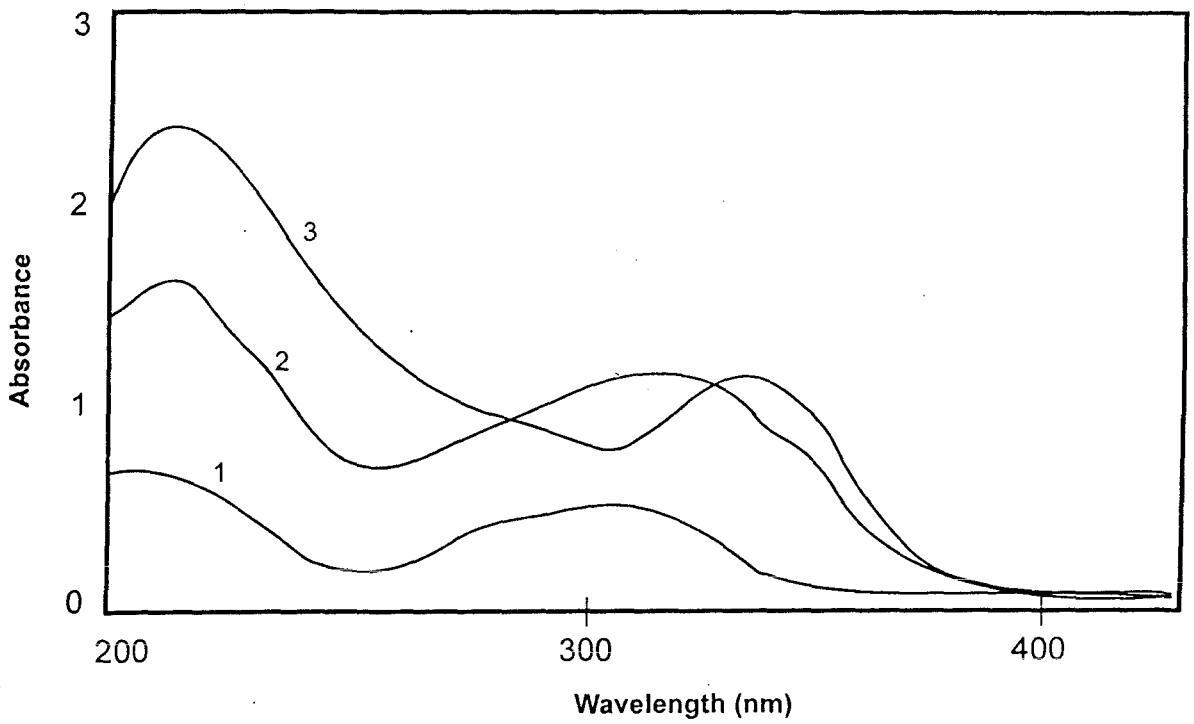

3
2
1
0
Absorbance
3
2
1
200
300
400
Wavelength (nm)

Figure 6. Degradation of calf thymus DNA by resveratrol, piceatannol and trans-stilbene 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 resveratrol, piceatannol and trans-stilbene and Cu(II) (50 μM) in a total reaction volume of 0.5 ml containing 10 mM Tris-HCl (pH 7.5). (●) Piceatannol; (○) resveratrol and (▼) trans-stilbene. Single strand specific digestion was performed as described in "Methods". All points represent triplicates and mean values have been plotted. Error bars denote SEM of three independent experiments.

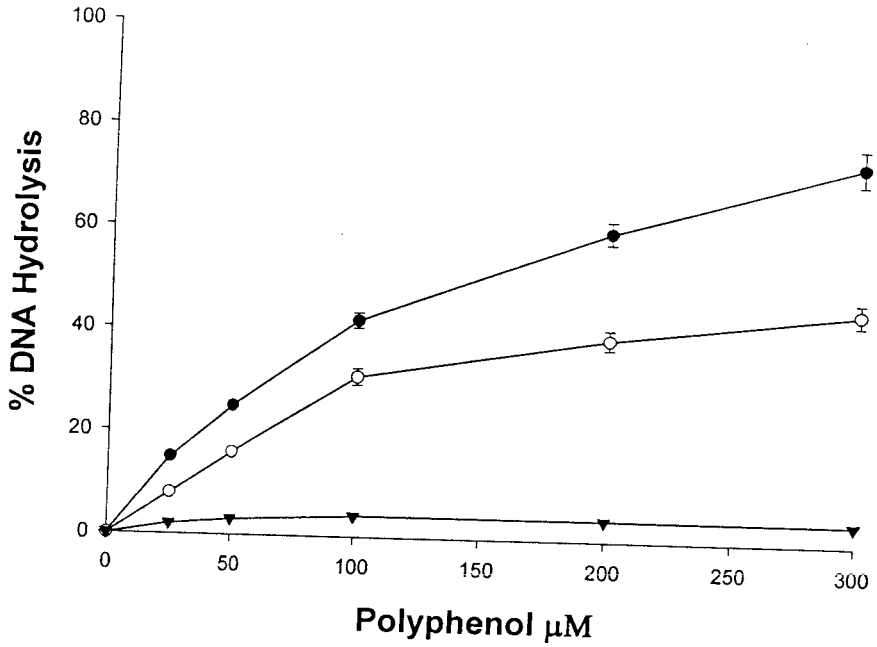

% DNA Hydrolysis
100
80
60
40
20
0
0
50
100
150
200
250
300
Polyphenol μM

Figure 7. Thermal melting profile of resveratrol treated calf thymus DNA:

Calf thymus DNA in TNE (0.01 M Tris-HCl pH 7.4, 0.01 M NaCl and 20 × 10^{-4} mM EDTA) was treated with indicated concentrations of resveratrol. The treated solution was dialyzed against 0.01 M TNE to remove unbound resveratrol. 500 μg aliquots of native as well as treated DNA were incubated for 8 minutes at the desired temperature and immediately quenched in ice for 2 minutes. S_1 digestion was performed as described in 'Methods'.

[●] Untreated calf thymus DNA

[○] DNA + 250 μM resveratrol

[▼] DNA + 500 μM resveratrol

[Δ] DNA + 1mM resveratrol

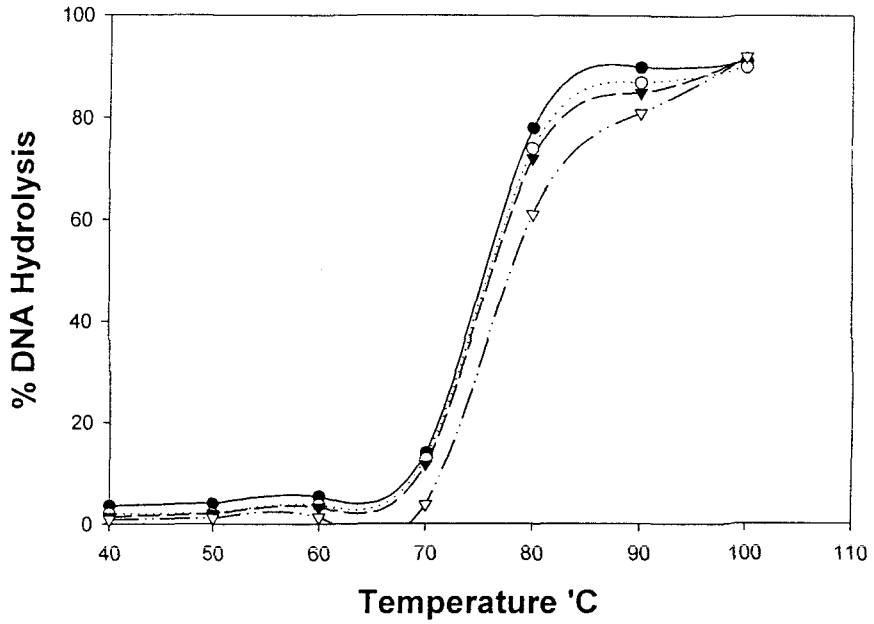
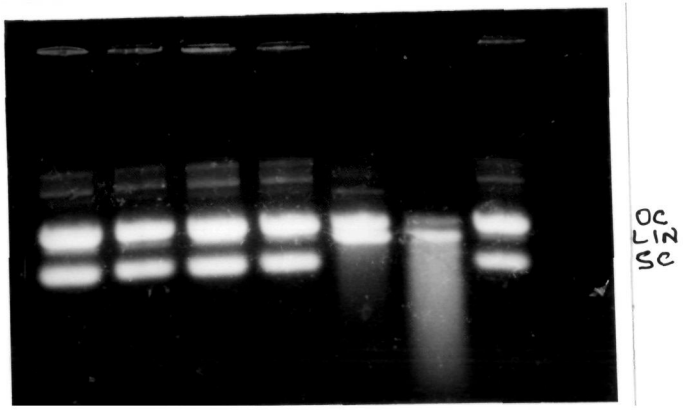

100
80
60
40
20
0
% DNA Hydrolysis
40
50
60
70
80
90
100
110
Temperature 'C

Figure 8. **Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 DNA after treatment with resveratrol, piceatannol and trans-stilbene 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 the three stilbenes and Cu(II). Incubation was carried out at 37 °C for 1 hour.

Lane 1, DNA alone; lanes 2-4, DNA + resveratrol, piceatannol and trans-stilbene (50 µM); lanes 5-7, DNA + resveratrol, piceatannol and trans-stilbene (50 µM) + Cu(II) (20 µM); SC, supercoiled DNA; OC, open circular; LIN, linear molecules.


OC
LIN
SC

Formation of complexes involving calf thymus DNA and stilbenes:

Figure 11 and 12 shows the effect of addition of increasing base molar ratios of calf thymus DNA on the fluorescence emission of resveratrol and piceatannol. Such an addition resulted in a dose-dependant quenching of the fluorescence. There was however, no shift in the λmax emission suggesting a simple mode of binding of DNA and the polyphenols. The control, native DNA alone when excited at the same wavelength (308 nm or 326 nm) did not interfere with the emission spectrum of polyphenol alone/polyphenol + DNA, thus confirming the binding results.

Binding of copper ions to stilbenes:

Binding of copper ions to resveratrol and piceatannol was studied by the effect of increasing Cu(II) molar ratios on the fluorescence emission spectra of the two compounds. The results shown in Figure 13 and 14 clearly indicate the binding as addition of Cu(II) causes quenching of the polyphenol fluorescence. These results support the result of absorption studies shown in Fig 4 and 5 where formation of polyphenol-copper was demonstrated.

Reducing power of stilbenes:

In a Fenton type reaction, Fe^{2+} or Cu^{2+} 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, activities of resveratrol, piceatannol and trans-stilbene on the reduction of Fe^{3+} to Fe^{2+} were measured as shown in Fig 15 Resveratrol exhibited a greater reducing power than piceatannol at all the concentrations tested, while trans-stilbene is not a reducing agent.

RBC hemolysis by resveratrol:

Figure 16 shows the effect of increasing concentrations of resveratrol (0-200 μM) alone or resveratrol in the presence of Cu(II) 50 μM on RBC membrane integrity. Resveratrol alone caused hemolysis upto a maximum of 20 % while in the presence of Cu(II) upto 80 % hemolysis was observed. The results indicate that resveratrol-Cu(II) reaction causes damage to cell membranes. This is also suggestive of the possibility that resveratrol alone or the resveratrol-Cu(II) complex may enter the cellular space through damaged cell membranes.

Figure 9. Photogeneration of superoxide anion by resveratrol, piceatannol and trans-stilbene on illumination under fluorescent light as a function of time:

The concentration of stilbenes were 100 μM. Details of the reaction are given in "Methods". The samples were placed 10 cm from the light source. (○) Piceatannol; (●) resveratrol; and (▼) trans-stilbene. All values reported are means of three independent experiments. Error bars represent standard error of mean.

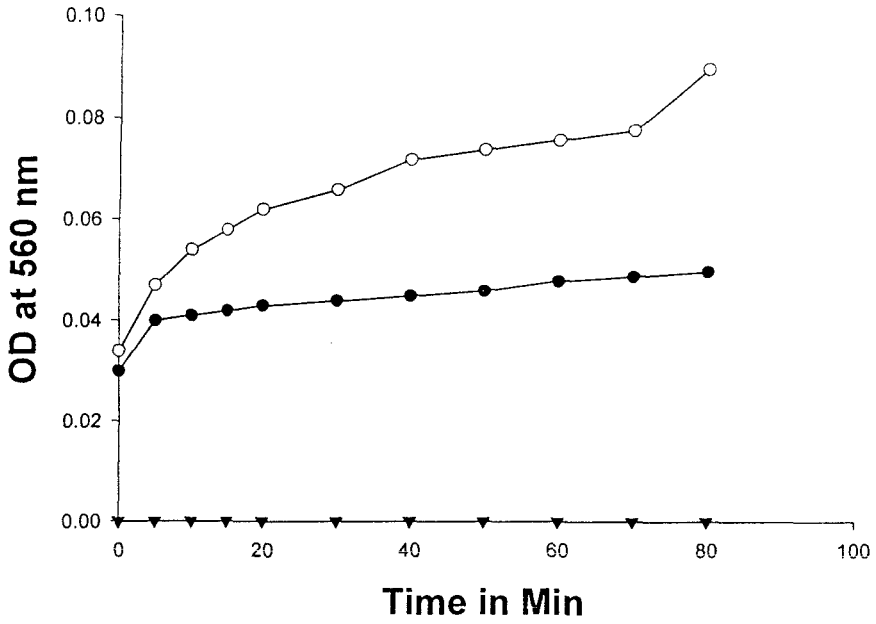

0.10
0.08
0.06
0.04
0.02
0.00
OD at 560 nm
0
20
40
60
80
100
Time in Min

Figure 10. Hydroxyl generation by resveratrol, piceatannol and trans-stilbene:

Reaction mixture (0.5 ml) contained 100 μg calf thymus DNA as substrate, 50 μM Cu(II) and indicated concentrations of the three stilbenes. The reaction mixture was incubated at 37 °C for 30 minutes. (○) Piceatannol; (●) resveratrol; and (▼) trans-stilbene. 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.

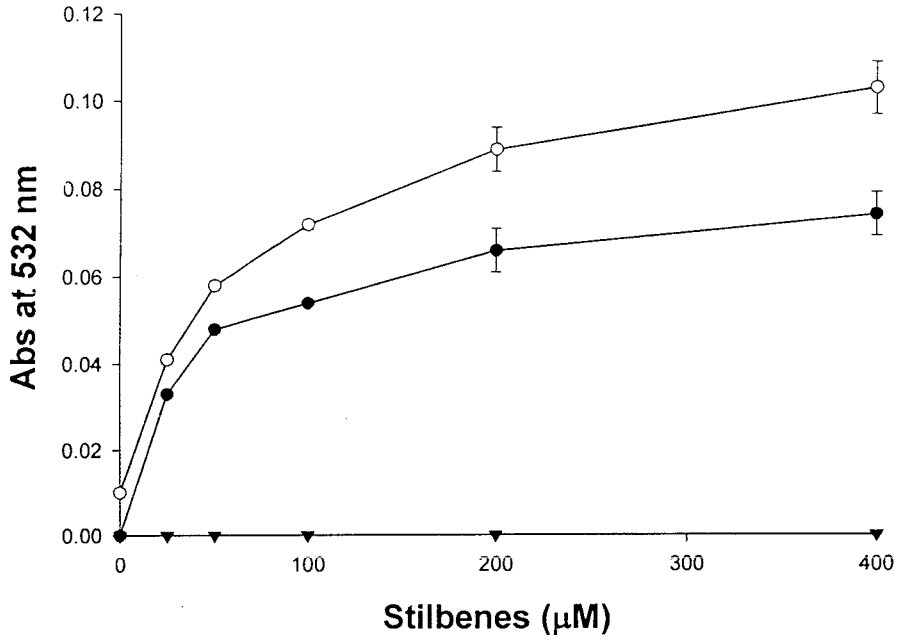

0.12
0.10
0.08
0.06
0.04
0.02
0.00
Abs at 532 nm
0
100
200
300
400
Stilbenes (μM)

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

Resveratrol (in Tris-HCl, pH 7.5) was excited at its λ_{max} (absorption) of 308 nm and the emission spectra were recorded between 420-500 nm.

[—] Resveratrol alone (25μM)

[···] Resveratrol: DNA base pair molar ratio (1:1)

[---] Resveratrol: DNA base pair molar ratio (1:2)

[-·-·] Resveratrol: DNA base pair molar ratio (1:4)

[— —] Resveratrol: DNA base pair molar ratio (1:6)

[.–.] Resveratrol: DNA base pair molar ratio (1:8)

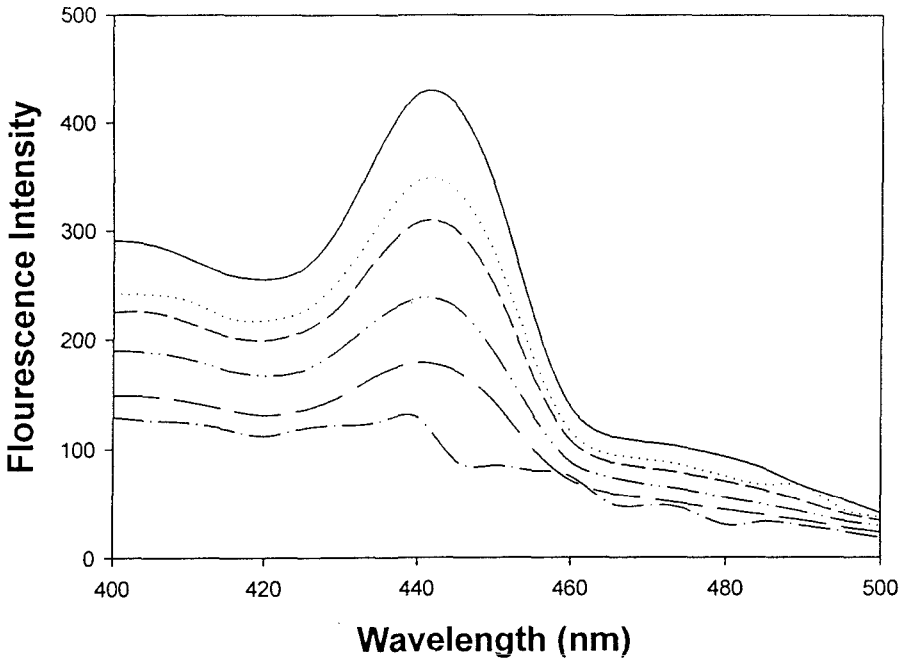

Flourescence Intensity
500
400
300
200
100
0
400
420
440
460
480
500
Wavelength (nm)

Figure 12. Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of piceatannol:

Piceatannol (in Tris-HCl, pH 7.5) was excited at its λ_{max} (absorption) of 326 nm and the emission spectra were recorded between 420-500 nm.

[—] Piceatannol alone (25μM)

[··] Piceatannol: DNA base pair molar ratio (1:1)

[---] Piceatannol: DNA base pair molar ratio (1:2)

[-·-·-] Piceatannol: DNA base pair molar ratio (1:4)

[— —] Piceatannol: DNA base pair molar ratio (1:6)

[.–.] Piceatannol: DNA base pair molar ratio (1:8)

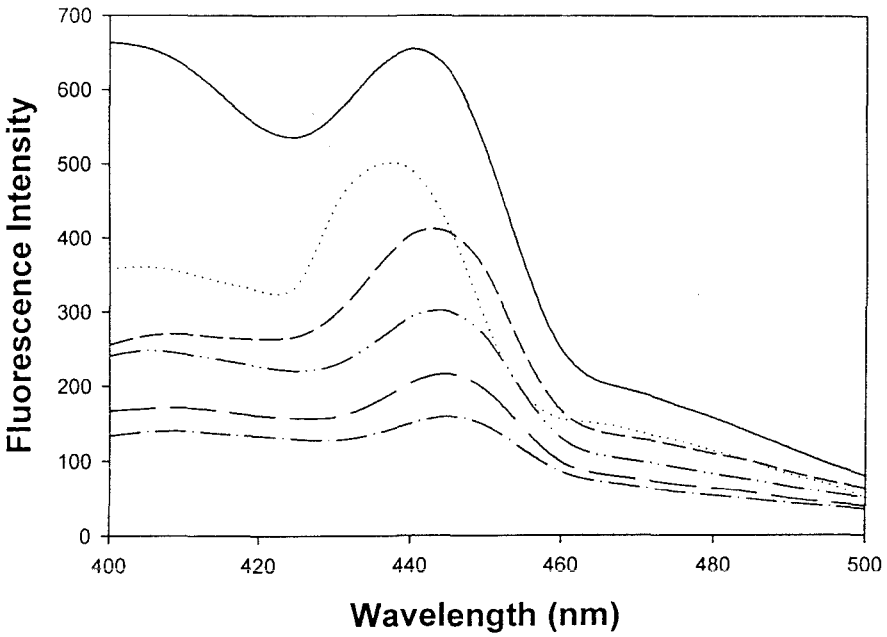

Fluorescence Intensity
700
600
500
400
300
200
100
0
400
420
440
460
480
500
Wavelength (nm)

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

Resveratrol (in Tris-HCl, pH 7.5) was excited at its λ_{max} (absorption) of 308 nm and the emission spectra were recorded between 400-500 nm.

[—] Resveratrol alone (25μM)

[··] Resveratrol: Cu(II) molar ratio (1:1)

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

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

[— —] Resveratrol: Cu(II) molar ratio (1:6)

[- -] Resveratrol: Cu(II) molar ration (1:8)

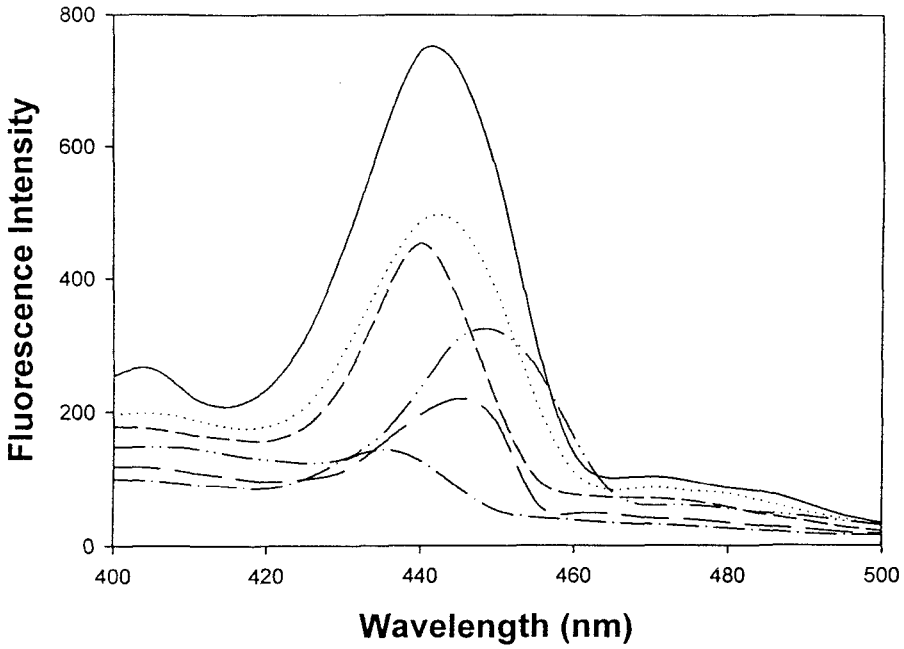

800
600
400
200
0
400
420
440
460
480
500
Fluorescence Intensity
Wavelength (nm)

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

Piceatannol (in Tris-HCl, pH 7.5) was excited at its λ_{max} (absorption) of 326 nm and the emission spectra were recorded between 420-500 nm.

| —| Piceatannol alone (25μM)

| ··| Piceatannol: Cu(II) molar ratio (1:1)

|---| Piceatannol: Cu(II) molar ratio (1:2)

|- ·-| Piceatannol: Cu(II) molar ratio (1:4)

|——| Piceatannol: Cu(II) molar ratio (1:6)

|-.-.| Piceatannol : Cu(II) molar ratio (1:8)

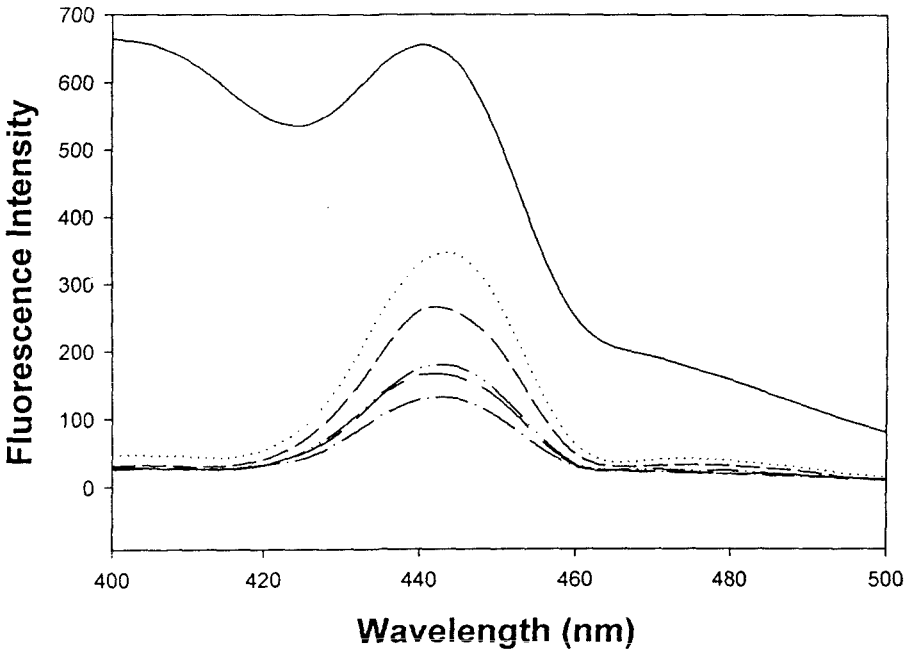

Fluorescence Intensity
700
600
500
400
300
200
100
0
400
420
440
460
480
500
Wavelength (nm)

Figure 15. Detection of reducing power of resveratrol, piceatannol and trans-stilbene:

Reaction mixture (5 ml) contained 0.2 M potassium phosphate buffer (pH 6.6), increasing concentrations of resveratrol, piceatannol and trans-stilbene (0-300 μM), 1 % potassium ferricyanide and distilled water. The reaction mixture was incubated at 50 °C for 20 minutes and processed further as described in "Methods". (●) Resveratrol; (○) piceatannol and (▼) trans-stilbene.

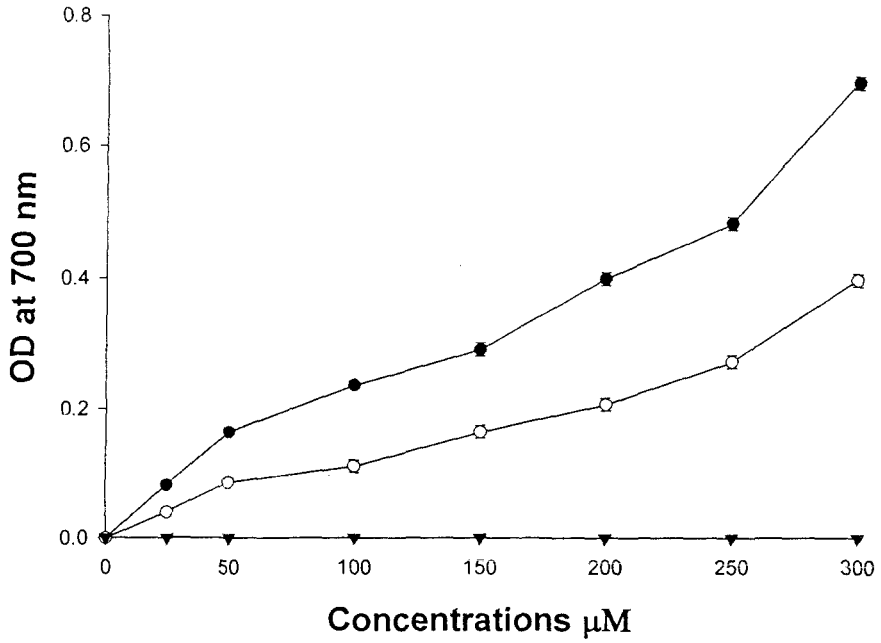
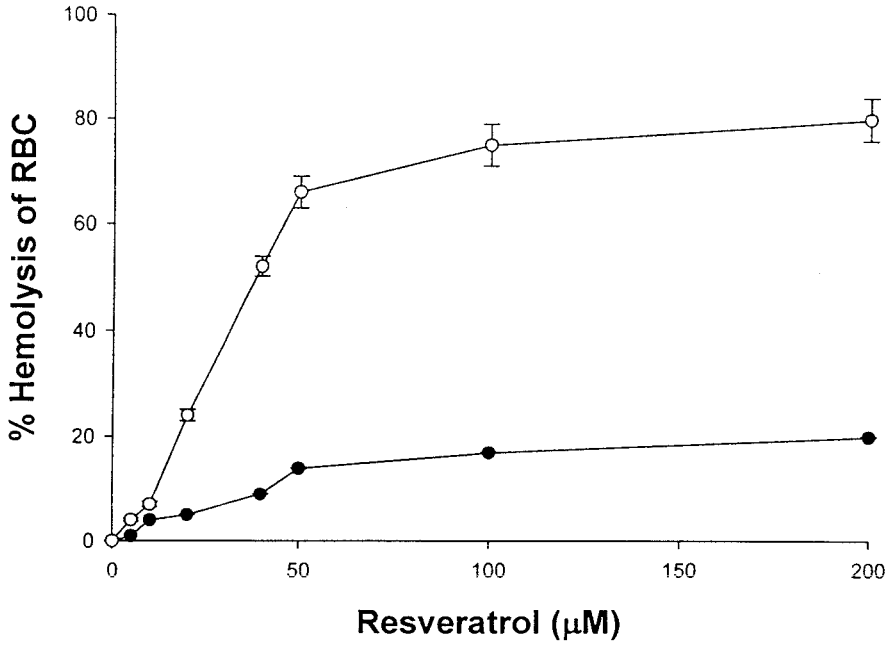

0.8
0.6
0.4
0.2
0.0
OD at 700 nm
0
50
100
150
200
250
300
Concentrations μM

Figure 16. Detection of RBC hemolysis by resveratrol in the absence and presence of Cu(II):

Reaction mixture (3 ml) contained 1ml RBC suitably diluted in PBS, 10 mM Tris-HCl (pH 7.5) and increasing concentrations of resveratrol (0-200 μM). Reaction mixture was incubated at 37 °C for 1 hr. (●) resveratrol alone; (○) resveratrol + Cu(II) 50 μM.


100
80
60
40
20
0
% Hemolysis of RBC
0
50
100
150
200
Resveratrol (μM)

DISCUSSIONS-I

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DISCUSSION

The major conclusion of the experiments described in this chapter are: (i) resveratrol and its analogue piceatannol are able to induce strand scission in calf thymus DNA and plasmid DNA, (ii) they form a complex with both DNA as well as Cu(II) and possibly in the presence of all three a ternary complex of stilbene-Cu(II)-DNA is formed. In the process 'oxidized species' of the stilbenes are generated which also appear to be capable of reducing Cu(II), (iii) redox cycling of copper leads to the generation of various reactive oxygen species, particularly the hydroxyl radical which serve as the proximal DNA cleaving agent, and (iv) the position and number of hydroxyl groups play an important role in the DNA cleavage efficacy of the stilbenes.

These results place the stilbenes 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 dependant degradation of DNA by 4-(9-acridinylamino) methanesulphone-m-anisidine (Wong et al., 1984), 1,10-phenanthroline (Gutterige and Halliwell, 1982), bleomycin (Ehrenfeld et al., 1987), adriamycin (Eliot et al., 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 these studies it was shown that a ternary complex of the drug, DNA and Cu(II) is formed which generates oxygen radical *in situ* via Cu(I). The results presented here show that resveratrol as well as its structural analogue piceatannol are 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 stilbene-Cu(II) mediated

DNA cleavage. Further, the results with stilbene analogues piceatannol and trans-stilbene (Figure 3 and 8) indicate that the DNA cleavage property of the stilbenes is dependent on the presence of hydroxyl groups. Trans-stilbene, which does not have any hydroxyl group, is ineffective in the DNA cleavage reaction while, the DNA cleavage efficacy increases with the increase in the number of hydroxyl groups with piceatannol being the most efficient among the three. Some researchers have suggested that resveratrol acts as a pro-drug and gets converted into a more potent antitumor agent piceatannol in vivo (Potter et al., 2002).

In addition to chemoprevention of cancer, both resveratrol and piceatannol have also been shown to possess apoptotic activity in human tumor cells (Bertelli et al., 1999; Clement et al., 1998; Duthie et al., 2001; Surh et al., 1999; Tsan et al., 2000; Katherine et al., 2006; Roupe et al., 2004). Resveratrol can be absorbed from dietary material as shown by its presence in plasma, heart, liver and kidney upon ingestion of red wine in rats (Bertelli et al., 1996). Further, it is well established that trans-resveratrol is biotransformed to piceatannol via cytochrome P450 1A2 and 1B1 enzymes (Piver et al., 2004). Also, it is 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 (Gutterige, 1984). Loosely bound copper has been defined by Gutterige (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 stilbenes. 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). Since resveratrol as well piceatannol have been detected in tissues such as kidney and liver, it is reasonable to assume that these stilbenes are transported across cell membranes because of their lipophilic nature. It may

be mentioned that resveratrol has been shown to bind to lipoproteins in plasma (Belguendouz et al., 1998). Further, both resveratrol and piceatannol may be rendered more lipophilic as a complex with copper. It may be further mentioned that apoptotic activity of the antioxidant 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 the 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 cellular copper levels are significantly elevated in a number of malignancies (Yoshida et al., 1993; Nazuwalis et al., 2004; Ebadi and Swanson, 1998) Irrespective of the physiological significance of our results it is clear that stilbenes have implications for development of novel antitumor and cancer chemopreventive agents.

CHAPTER II

Resvertrol-Cu(II) induced DNA breakage in human peripheral lymphocytes

RESULTS-II

RESULTS

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 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. In order to standardize Comet Assay an experiment was performed with H_2O_2. Photographs of comets (100-x) observed after treatment of lymphocytes with increasing concentrations of H_2O_2 are shown in figure 17. As can be seen untreated cells are not damaged and without any significant tail. However, with increasing concentrations of H_2O_2 a progressive increase in the length of comet tails is observed. In figure 18 the results of the same experiment are plotted as comet tail length (μm) as a function of increasing H_2O_2 concentrations.

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

Increasing concentrations of resveratrol (10-100 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. Resveratrol alone at any of the concentrations tested did not damage the lymphocyte DNA whereas, on addition of Cu(II) DNA damage to varying degrees was observed. Figure 19 shows photographs of comets (100-X) observed with varying concentrations of resveratrol in the absence and presence of copper. Untreated lymphocyte controls were similar to resveratrol alone or Cu(II) alone without any

significant DNA breakage. The corresponding tail length, tail DNA and tail moment are plotted as a function of resveratrol concentration in figure 20. The results clearly establish that resveratrol-Cu(II) system is capable of DNA breakage in lymphocytes. A similar experiment with increasing concentrations of Cu(II) (5-100 μM) was also performed. As can be seen from figure 21 an increasing degree of DNA damage with increasing concentrations of Cu(II) is observed.

Comparative study of lymphocyte DNA breakage by stilbenes:

In order to understand the chemical basis of DNA breakage by resveratrol-Cu(II) system, I have compared the relative cleavage efficiency of resveratrol, piceatannol and the parent compound trans-stilbene for DNA breakage in lymphocytes. Figure 22 shows comet tail lengths obtained as a function of increasing concentrations of the three stilbenes (0-50 μM). Similar to results of figure 8 (with plasmid DNA), piceatannol shows the formation of largest tail lengths followed by resveratrol and minimal tail formation for trans-stilbene. Thus, piceatannol is a more efficient DNA cleaving agent than resveratrol. Trans-stilbene, which does not have any hydroxyl group, is not a cleaving agent. These results demonstrate that the presence of hydroxyl groups is essential for the DNA cleavage. Further, the efficiency of cleavage increases with the number of hydroxyl groups.

Figure 17: Standardization of Comet Assay with H_2O_2:

Reaction mixture (1ml) contained 1×10^5 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 minutes and processed further for Comet Assay as described in 'Methods'.

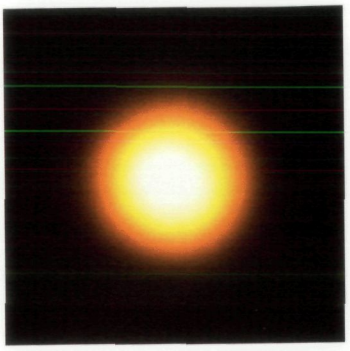
Photographs of comet (100-x) seen on incubating lymphocytes with varying concentrations of H_2O_2.

(A) Untreated lymphocytes

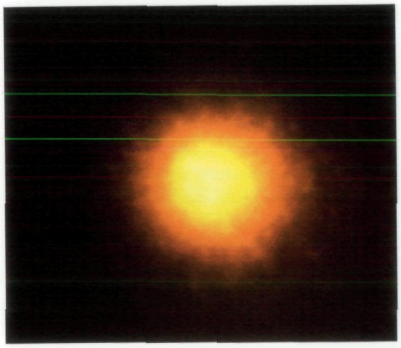
(B) Lymphocytes + 10 µM H_2O_2

(C) Lymphocytes + 20 µM H_2O_2

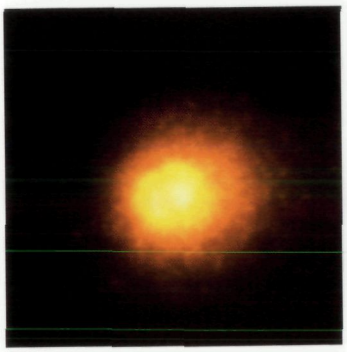
(D) Lymphocytes + 50 µM H_2O_2



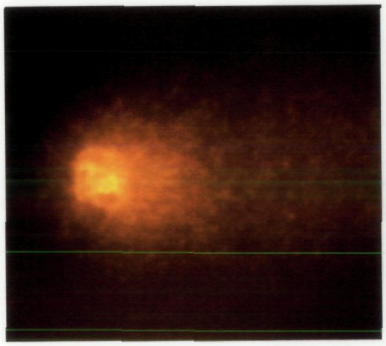
(A)



(B)



(C)



(D)

Figure 18: Standardization of Comet Assay with H_2O_2:

Reaction mixture (1ml) contained 1×10^5 cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} Free and increasing concentrations of H_2O_2 (0-50 µM). The reaction mixture was incubated at 37 °C for 30 minutes and processed further for Comet Assay as described in 'Methods'.

All points represent mean of three independent experiments. Error Bars denote SEM.

P value < 0.05 and significant when compared to control.

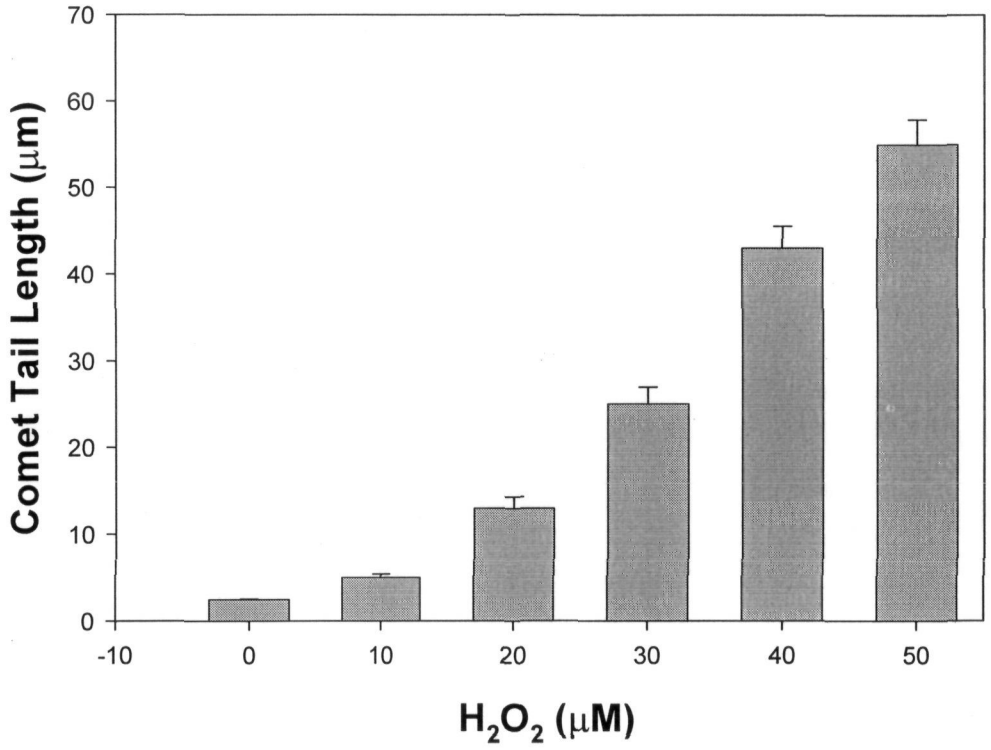

Comet Tail Length (μm)
70
60
50
40
30
20
10
0
-10
0
10
20
30
40
50
H2O2 (μM)

Figure 19: DNA breakage by resveratrol-Cu(II) in human peripheral lymphocytes as measured by Comet Assay:

Reaction mixture (1 ml) contained 1×10^5 cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} Free, increasing concentrations of resveratrol (0-50 µM) and Cu(II) 20 µM. The reaction mixture was incubated for 30 min at 37 °C. After the incubation the cells were processed further for Comet Assay as described in 'Methods'.

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

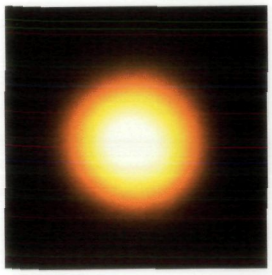
[A] Untreated lymphocytes

[B] Lymphocytes + Resveratrol 10 µM + Cu(II) 20 µM.

[C] Lymphocytes + Resveratrol 20 µM + Cu(II) 20 µM.

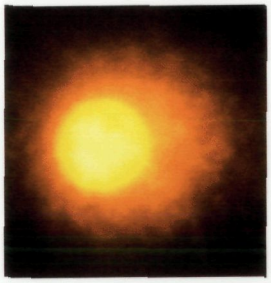
[D] Lymphocytes + Resveratrol 50 µM + Cu(II) 20 µM.

[E-H] Lymphocytes + Resveratrol (10-50 µM) in the absence of Cu(II).

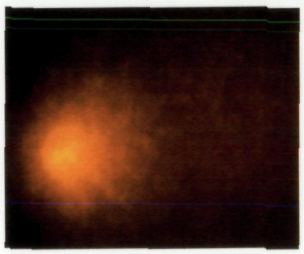


(A)

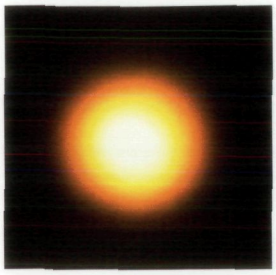
(B)



(C)

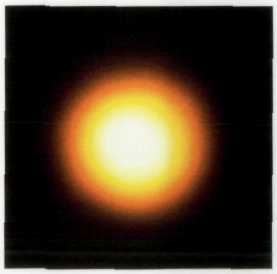


(D)

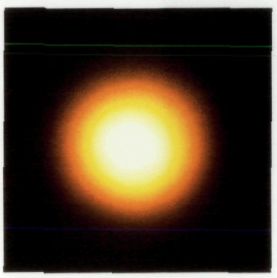


(E)

(F)



(G)



(H)

Figure 20: DNA breakage by resveratrol-Cu(II) in human peripheral lymphocytes as measured by Comet Assay:

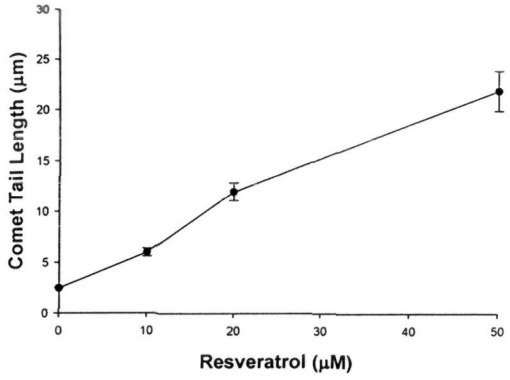
Reaction mixture (1 ml) contained 1×10^5 cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} Free, increasing concentrations of resveratrol (0-50 µM) and Cu(II) 20 µM. The reaction mixture was incubated for 30 min at 37 °C. After the incubation the cells were processed further for Comet Assay as described in 'Methods'.

[A] Comet Tail length (µm) plotted as a function of increasing concentrations of resveratrol (0-50 µM) in the presence of 20 µM Cu(II).

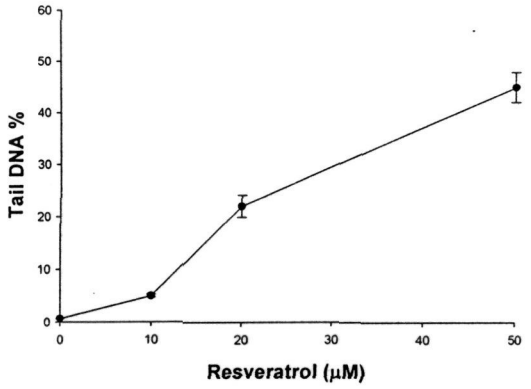
[B] Comet Tail DNA (%) plotted as a function of increasing concentrations of resveratrol (0-50 µM) in the presence of 20 µM Cu(II).

[C] Tail Moment (Arbitrary Units) plotted as a function of increasing concentrations of resveratrol (0-50 µM) in the presence of 20 µM Cu(II).

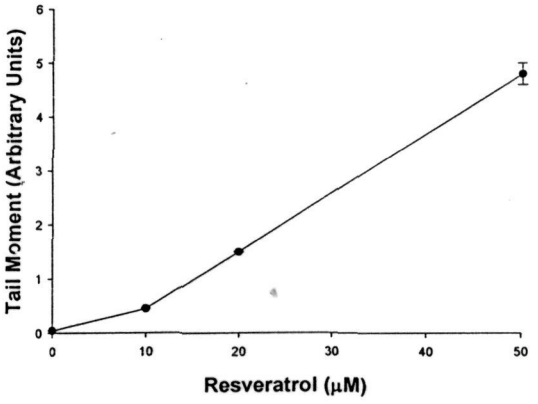
Values reported are ± SEM of three independent experiments. P value < 0.05 and significant when compared to control.


Comet Tail Length (μm)
0
5
10
15
20
25
30
0
10
20
30
40
50
Resveratrol (μM)

(A)


Tail DNA %
0
10
20
30
40
50
60
0
10
20
30
40
50
Resveratrol (μM)

(B)


Tail Moment (Arbitrary Units)
0
1
2
3
4
5
6
0
10
20
30
40
50
Resveratrol (μM)

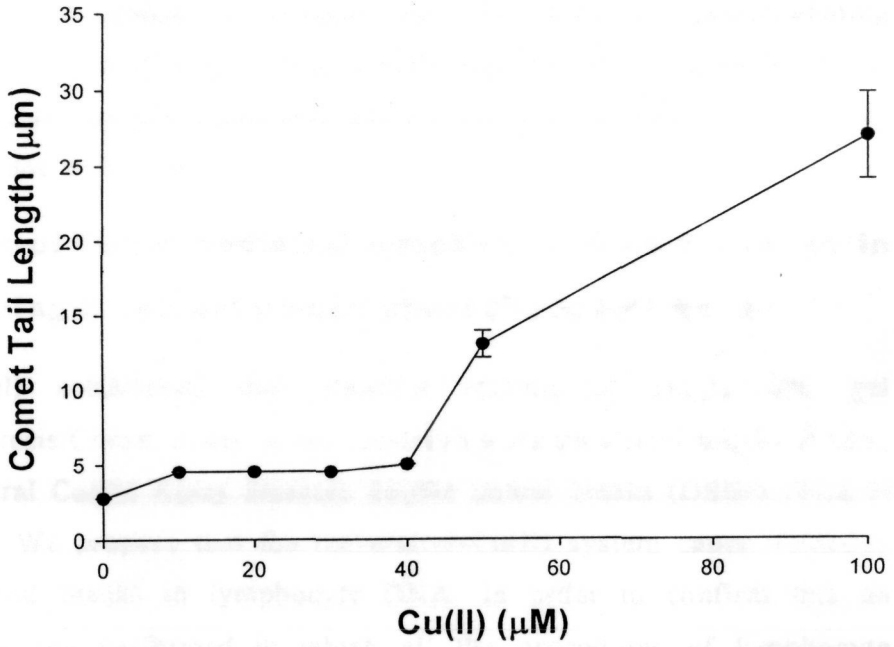
(C)

Figure 21. DNA breakage by increasing concentrations of Cu(II) in human peripheral lymphocytes:

Reaction mixture contained 1×10^5 cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} Free and increasing concentrations of Cu(II) (0-100 µM). The reaction mixtures were incubated at 37 °C for 30 minutes and the cells were processed further for Comet Assay as described in 'Methods'.

Tail length of comets plotted as a function of increasing Cu (II) concentrations (0-100 µM).

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


Comet Tail Length (μm)
35
30
25
20
15
10
5
0
0
20
40
60
80
100
Cu(II) (μM)

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

Previously it has been shown that resveratrol-Cu(II) mediated degradation of DNA and inactivation of bacteriophage λ is inhibited to significant degrees by various scavengers of reactive oxygen species (Ahmad et al., 2000). Table 3 gives the results of an experiment where the effect of sodium azide, potassium iodide, thiourea, neocuproine, superoxide dismutase and catalase were tested. 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. Neocuproine is a Cu(I) specific sequestering agent, which as expected also inhibited DNA breakage. Results therefore, suggest that the chemically induced DNA breakage in vitro and lymphocyte DNA breakage by resveratrol-Cu(II) system are most likely the result of the same mechanism.

Figure 23. Effect of pre-incubating the lymphocytes with increasing concentrations of resveratrol on DNA breakage:

Isolated cells suspended in RPMI 1640 were pre-incubated with the indicated concentrations of resveratrol for 30 minutes at 37 °C. After pelleting the lymphocytes were washed twice with PBS Ca^{2+} and Mg^{2+} Free twice before re-suspension in RPMI 1640 and further incubation for 30 minutes in the presence of 20 μM Cu(II).

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

Figure 22. A comparison of the DNA breakage in human lymphocytes by resveratrol, piceatannol and trans-stilbene:

Reaction mixture (1ml) contained 1 $\times 10^5$ cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} free increasing concentrations of the three stilbenes (0-50 µM) and Cu(II) 20 µM. the reaction mixture was incubated at 37 °C for 30 minute and the cells were processed further for Comet Assay as described in 'Methods'. (●) Resveratrol; (■) piceatannol and (▼) trans-stilbene.

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

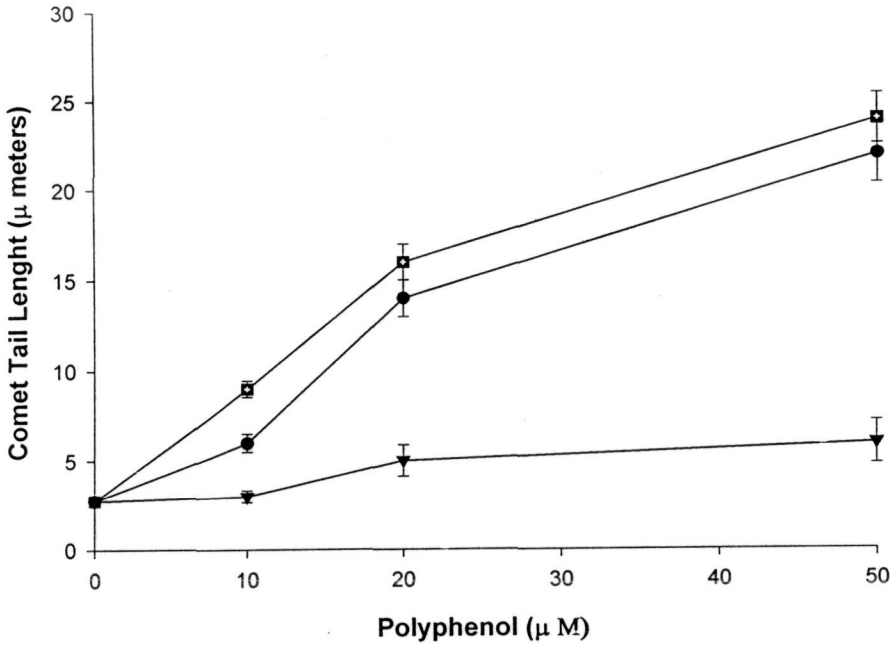

Comet Tail Lenght (μ meters)
30
25
20
15
10
5
0
0
10
20
30
40
50
Polyphenol (μ M)

Figure 23. Effect of pre-incubating the lymphocytes with increasing concentrations of resveratrol on DNA breakage:

Isolated cells suspended in RPMI 1640 were pre-incubated with the indicated concentrations of resveratrol for 30 minutes at 37 °C. After pelleting the lymphocytes were washed twice with PBS Ca^{2+} and Mg^{2+} Free twice before re-suspension in RPMI 1640 and further incubation for 30 minutes in the presence of 20 μM Cu(II).

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

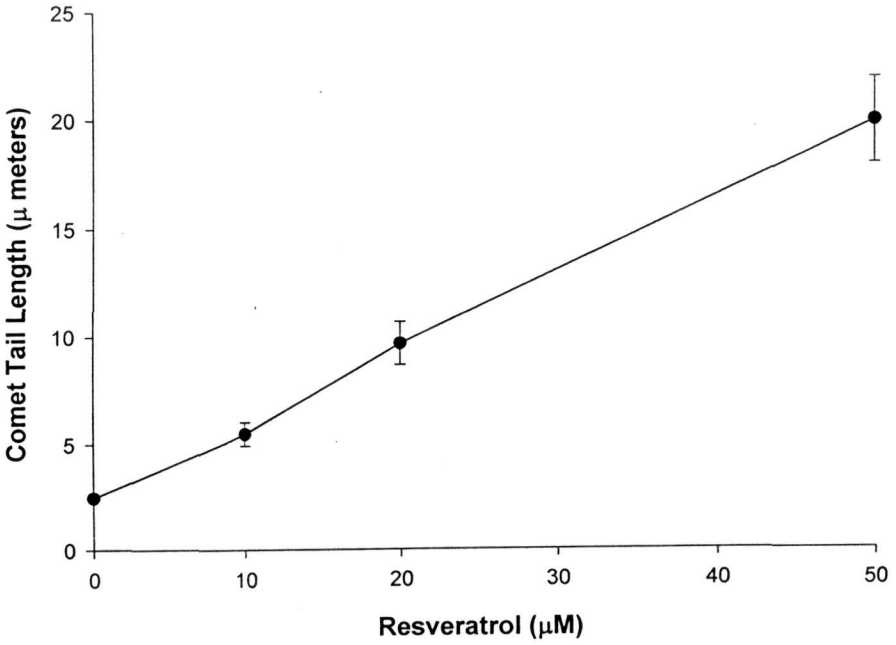

Comet Tail Length (μ meters)
25
20
15
10
5
0
0
10
20
30
40
50
Resveratrol (μM)

Figure 24: Effect of pre-incubating the lymphocytes with increasing concentrations of Cu(II) on DNA breakage:

Isolated cells suspended in RPMI 1640 were pre-incubated with the indicated concentrations of Cu(II) for 30 minutes at 37 °C. After pelleting the lymphocytes were washed twice with PBS Ca^{2+} and Mg^{2+} Free twice before re-suspension in RPMI 1640 and further incubation for 30 minutes in the presence of 50 μM resveratrol.

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

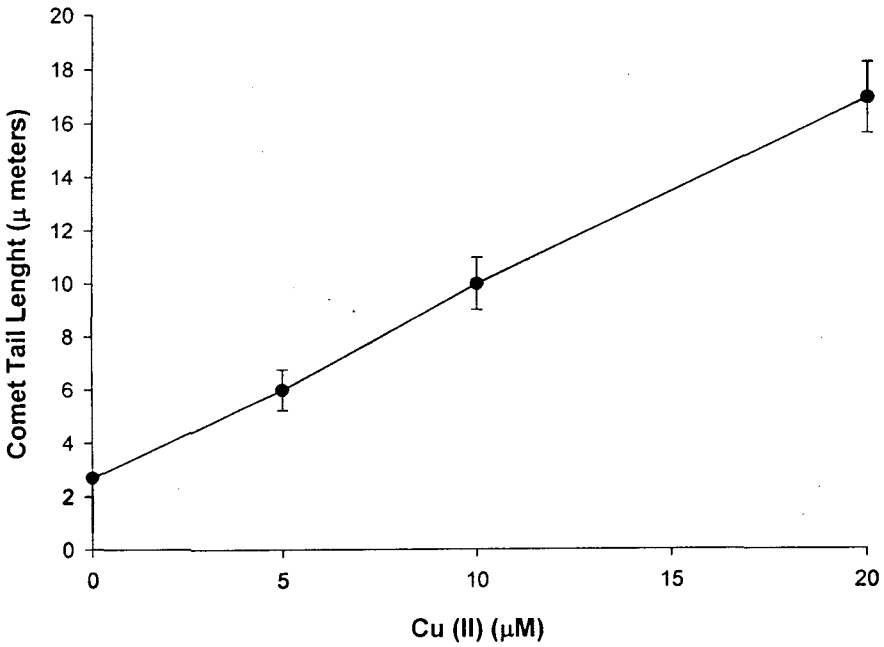

Comet Tail Lenght (μ meters)
20
18
16
14
12
10
8
6
4
2
0
0
5
10
15
20
Cu (II) (μM)

Figure 25. Effect of resveratrol-Cu(II) on isolated human peripheral lymphocytes in neutral version of Comet Assay:

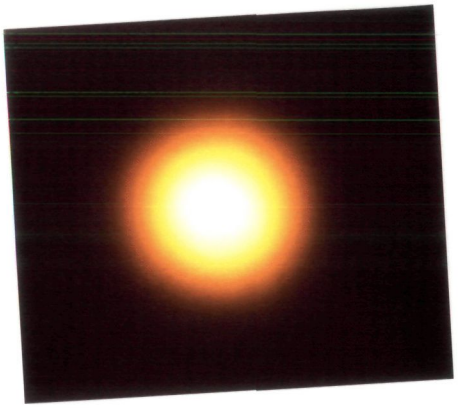
Reaction mixture (1ml) contained 1×10^5 cells, increasing concentrations of resveratrol (10-50 μM) and 20 μM Cu(II). Comet Assay was performed essentially according to the procedure in 'Methods' except electrophoresis where neutral buffer (Tris-HCl 0.4 M pH 7.5) was used.

[A] Untreated Cells

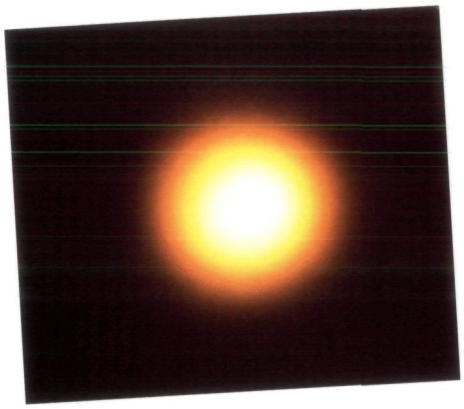
[B] Cells + 10 μM Resveratrol + 20 μM Cu(II)

[C] Cells + 20 μM Resveratrol + 20 μM Cu(II)

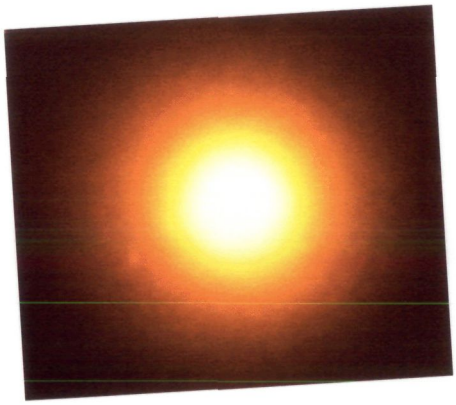
[D] Cells + 50 μM Resveratrol + 20 μM Cu(II)



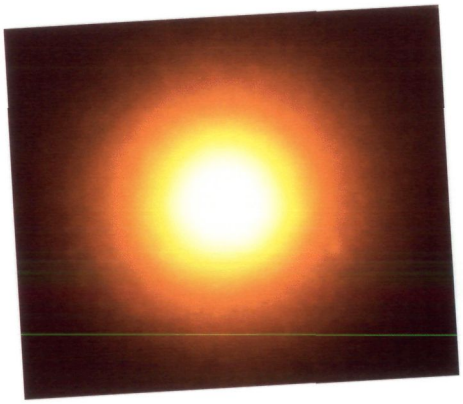
(A)



(B)



(C)



(D)

Table 3
Effect of scavengers of active oxygen species on lymphocyte DNA breakage by resveratrol–Cu(II).

Treatment	Tail length (μm)	% Inhibition
Untreated (#)	2.50 ± 0.15	-
Resveratrol(50μM) + Cu(II) (20μM)	22.88 ± 2.01*	0
+ Sodium azide (1mM)	4.22 ± 0.38*	81.55
+ Potassium iodide (1mM)	8.77 ± 0.85*	61.66
+ Thiourea (1mM)	6.03 ± 0.55*	73.64
+ Ascorbate (1mM)	7.92 ± 0.78*	65.38
+ Glutathione (1mM)	10.28 ± 1.11*	55.38
+ Neocuproine (1mM)	10.27 ± 1.03*	55.11
+ Superoxide dismutase (100μg/ml)	7.65 ± 0.73*	66.56
+Catalase (100μg/ml)	6.62 ± 0.59*	71.06

*** $p<0.05$ by comparison with control(#). Data represents median ± SEM of three experiments.**

DISCUSSION-II

THESIS

DISCUSSION

The major conclusions of the experiments performed in this chapter are (i) resveratrol-Cu(II) system is capable of oxidative DNA cleavage in whole cells, (ii) the cellular DNA damage by resveratrol-Cu(II) involves reactive oxygen species and Cu(I), (iii) similar to results of chapter 1 the cellular DNA cleavage by this system depends on the presence of hydroxyl groups and further the relative cleavage efficacy increases with increase in the number of hydroxyl 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). Further, we have previously shown 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). It is to be noted that piceatannol which is a tetra-hydroxy derivative of resveratrol is also a potent inducer of apoptosis in human SK-Mel-28 melanoma cells (Larrosa et al., 2004). Indeed it has been shown that resveratrol is converted to piceatannol by cytochrome P-450 enzyme CYP1B1 from human lymphoblast microsomes (Potter et al., 2002). Recently Dong (2003) has shown that penta-hydroxy derivative of resveratrol was more effective as an inhibitor of EGF induced cell transformation as compared with resveratrol. Based on our own observation and those of others, we have proposed a mechanism for the cytotoxic action of plant polyphenolics 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 physiologically feasible reaction and may be of biological significance. Our idea is strengthened by a number of other observations mentioned earlier (Hadi et al., 2000). More significantly 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 pathway differs between pharmacological cell death and physiological induction of cell death (Smets et al., 1994). Cu^{2+} and Zn^{2+} are the major metal ions present in the nucleus (Bryan, 1979) and serum (Ebadi and Swanson, 1988) and tissue (Yoshida et al., 1993) 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 property which is the hallmark of cells undergoing apoptosis (Burkitt et al., 1996). It has been recently shown that the polyphenol curcumin mediated apoptosis in HL-60 cells is closely related to the increase in the concentrations of reactive oxygen species possibly generated through the reduction of transition metals in cells (Yoshino et al., 2004). Thus, it is possible that cellular DNA fragmentation by plant polyphenolics that involves mobilization of intra-cellular and extra-cellular copper could be one of the mechanisms involved in the chemopreventive properties of these compounds.

CHAPTER III

Plant polyphenols mobilize endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage

RESULTS-III

RESULTS

DNA breakage by resveratrol in lymphocytes as measured by Comet Assay:

Increasing concentrations of resveratrol (50, 100, 200 μM) were tested for DNA breakage in isolated lymphocytes using the Comet Assay. Photographs of Comets seen on treatment with these concentrations are shown in Fig 26 At 50 and 100 μM concentrations resveratrol did not damage the lymphocyte DNA to any significant extent whereas at 200 μM concentration a Comet with a tail indicative of DNA breakage was observed. In Fig 27. the tail lengths obtained from the same experiment are plotted as a function of resveratrol concentration. The results clearly establish that resveratrol alone is capable of DNA breakage in lymphocytes. However, the minimum concentration required for such breakage (100-200 μM) is considerably greater than when resveratrol (10 μM) is used along with Cu(II) as was shown previously (Chapter II, Figure 19-20).

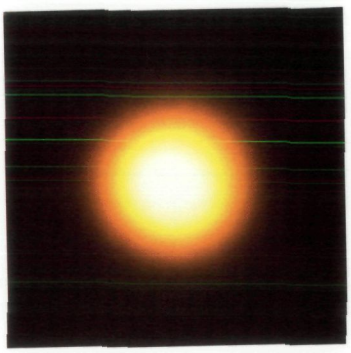
Effect of Neocuproine, a Cu(I) specific sequestering agent, on resveratrol induced DNA breakage in lymphocytes:

In the previous chapter (Table 3) we had shown that the resveratrol-Cu(II) mediated degradation of lymphocyte DNA is inhibited by neocuproine which is a Cu(I) specific chelating agent and is membrane permeable (Barbouti et al., 2001). Fig 28 gives the results of an experiment where three progressively increasing concentrations of neocuproine were tested on resveratrol induced DNA breakage in lymphocytes. A progressive decrease in the tail length as a function of increasing neocuproine concentration was seen. From the results we can conclude that the DNA breakage by the polyphenol involves endogenous copper ions and that Cu(I) is an intermediate in the pathway that leads to DNA breakage.

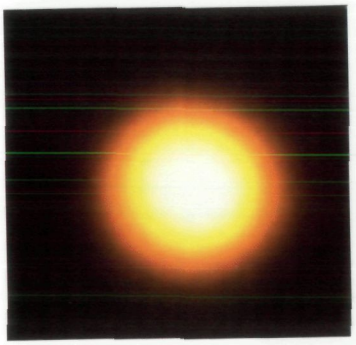
Figure 26: DNA breakage by resveratrol in lymphocytes as measured by Comet Assay:

Reaction mixture (1ml) contained 1×10^5 cells, 400 µl RPMI, PBS Ca^{2+} and Mg^{2+} free and indicated concentrations of resveratrol. The reaction mixture was incubated at 37 °C for 2 hrs and the cells were further processed for Comet Assay as described in 'Methods'

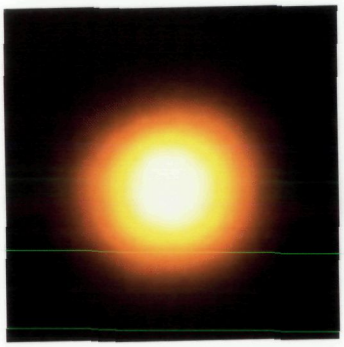
Single cell gel electrophoresis of human peripheral lymphocytes showing Comets (100-X) after treatment with different concentrations of resveratrol, (A) untreated, (B) resveratrol (50 µM), (C) resveratrol (100 µM) and (D) resveratrol (200 µM).



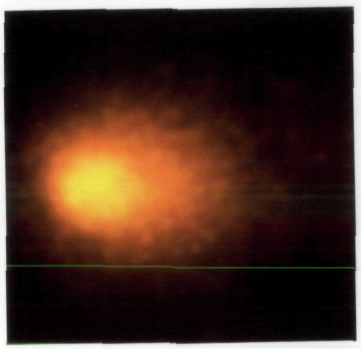
(A)



(B)



(C)



(D)

Figure 27. Effect of increasing concentrations of resveratrol on DNA breakage in lymphocytes.

Reaction mixture (1ml) contained 1×10^5 cells, 400 µl RPMI, PBS Ca^{2+} and Mg^{2+} free and indicated concentrations of resveratrol. The reaction mixture was incubated at 37 °C for 2 hrs and the cells were further processed 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.

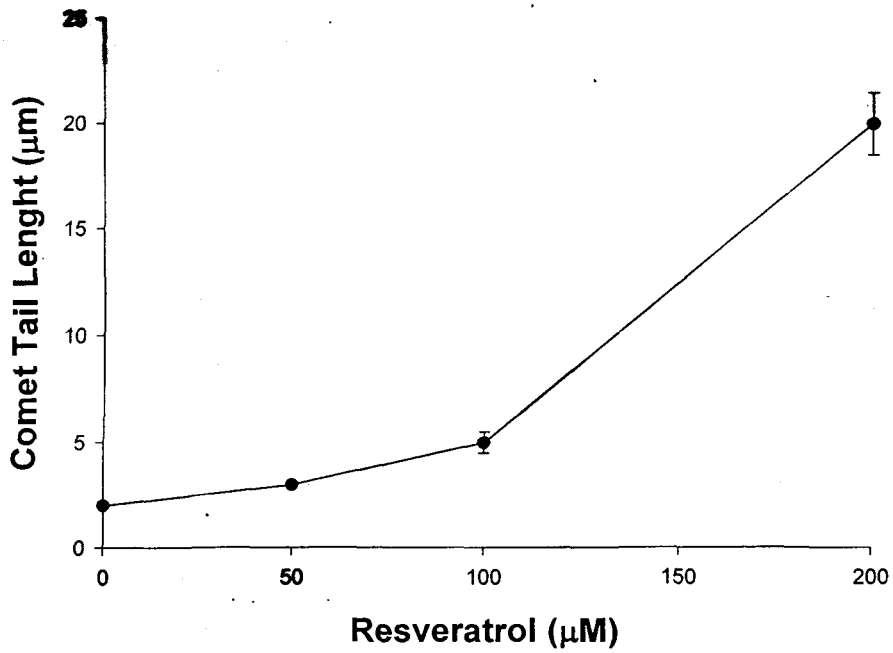
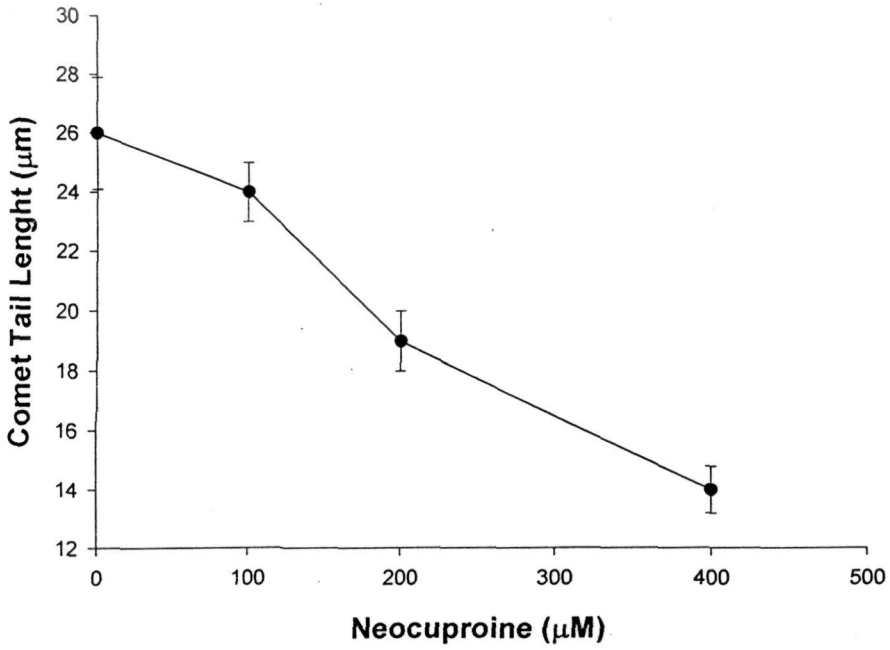

Comet Tail Lenght (μm)
0
5
10
15
20
0
50
100
150
200
Resveratrol (μM)

Figure 28: Effect of increasing concentrations of neocuproine on resveratrol induced DNA breakage in human lymphocytes:

Reaction mixture (1ml) contained 1×10^5 cells, 400 µl RPMI, PBS Ca^{2+} and Mg^{2+} free, 200 µM resveratrol and indicated concentrations of neocuproine. The reaction mixture was incubated at 37 °C for 2 hrs and the cells were further processed 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.


30
28
26
24
22
20
18
16
14
12
Comet Tail Lenght (μm)
0
100
200
300
400
500
Neocuproine (μM)

Effect of active oxygen scavengers on resveratrol induced DNA breakage in lymphocytes:

In the previous chapter (Table 3) we have shown that resveratrol-Cu(II) mediated degradation of lymphocyte DNA is inhibited to significant degrees by various scavengers of reactive oxygen species. Table 4 gives the results of an experiment where three such scavengers have been tested, namely: superoxide dismutase (SOD) and catalase which remove superoxide, and H_2O_2 respectively and thiourea which is a scavenger of several reactive oxygen species. All three cause significant inhibition of DNA breakage as evidenced by decreased tail lengths. We conclude 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. These results along with the results of Table 3 suggest that resveratrol-Cu(II) induced lymphocyte DNA breakage and DNA breakage by resveratrol alone are likely the result of the same mechanism.

Comparison of lymphocyte DNA breakage by various polyphenols:

According to our hypothesis (Hadi et al., 2000) mobilization of endogenous copper and the consequent degradation of cellular DNA is a general mechanism for anticancer properties of plant polyphenols. However, depending on the structure of the molecule there would be differences of efficiency between various polyphenols. I have therefore compared the lymphocyte DNA breakage efficiency of various polyphenols with different structures. Fig 29 shows Comet tail lengths obtained using increasing concentrations of epigallocatechin-3-gallate (EGCG) which is considered to be the most effective apoptosis inducing polyphenol present in green tea (Ahmad et al., 1992; Azam et al., 2004), resveratrol and piceatannol whose parent compound trans stilbene which does not have any hydroxyl group and gallic acid which is a structural constituent of tannins (Khan & Hadi., 1998).

Syringic acid is a derivative of gallic acid where two of the hydroxyl groups are present as methoxy groups. It is seen that except trans stilbene and syringic acid all the other polyphenols are able to induce DNA breakage up to various degrees. It is worth mentioning that trans stilbene and syringic acid are also ineffective as DNA cleaving agent in vitro (Khan & Hadi, 1998; Azmi et al., 2005) or lymphocyte DNA breakage in the presence of Cu(II) (Azmi et al., 2005).

Determination of TBARS as a measure of oxidative stress in lymphocytes by resveratrol and gallic acid in the presence of neocuproine and thiourea:

As mentioned above we presume that lymphocyte DNA breakage is the result of the generation 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 (Halliwell & 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 resveratrol and gallic acid. The effect of preincubating the cells with neocuproine and thiourea was also studied. Results given in Fig 30 (a) and (b) show that there is a dose dependant 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 both resveratrol as well as gallic acid. 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 29 Comparison of various polyphenols on the induction of DNA breakage in human lymphocytes.

Reaction mixture (1ml) contained 1×10^5 cells, 400 µl RPMI, PBS Ca^{2+} and Mg^{2+} free and indicated concentrations of different polyphenols. The reaction mixture was incubated at 37 °C for 2 hrs and the cells were further processed for Comet Assay as described in 'Methods'

(■) gallic acid, (●) EGCG, (▼) piceatannol, (○) resveratrol, (Δ) trans stilbene, (□) syringic acid. Values reported are ±SEM of three independent experiments.

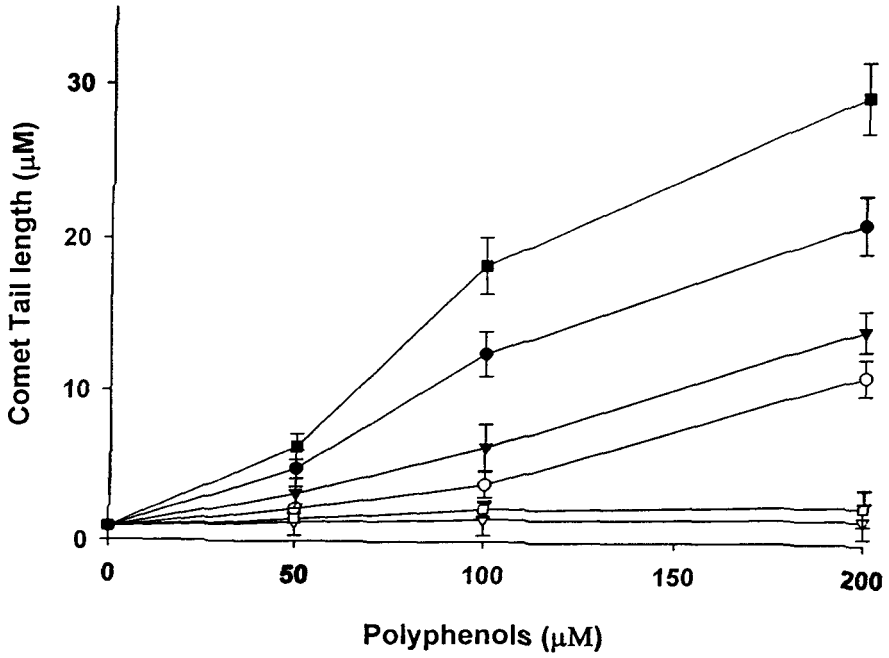

Comet Tail length (μM)
30
20
10
0
0
50
100
150
200
Polyphenols (μM)

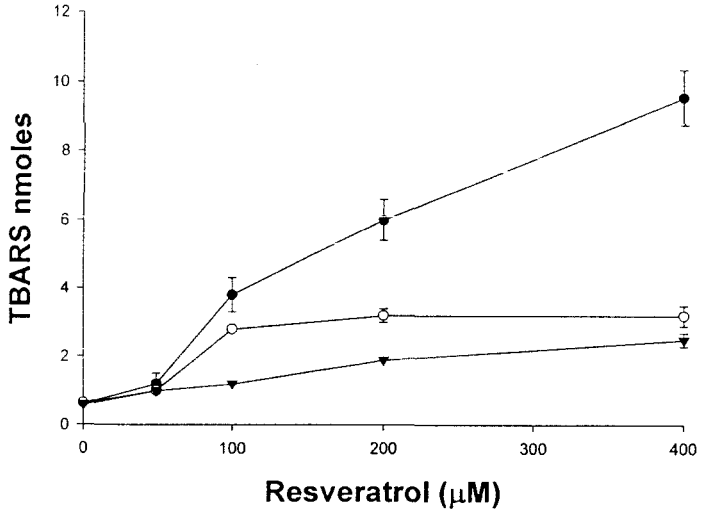
Table 4: Effect of scavengers of active oxygen species on resveratrol induced lymphocyte DNA breakage.

Dose	Tail Length (μm)	% Inhibition
Untreated	1.22 ± 0.08 #	-
Resveratrol 200 μM	20.84 ± 1.28	-
+SOD 100μg/ml	7.05 ± 0.25 *	66%
+ Catalase 100 μg/ml	8.86 ± 0.29 *	57%
+Thiourea 1mM	11.93 ± 1.01*	45%

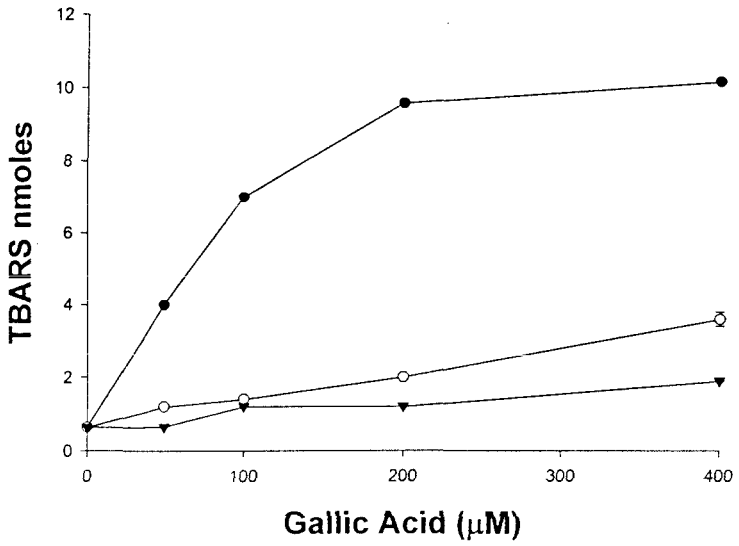
* p value < 0.05 when compared to control #. All values represent SEM of three independent experiments.

Figure 30. Effect of pre-incubation of lymphocytes with neocuproine and thiourea on TBARS generated by increasing concentrations of resveratrol (a) and gallic acid (b).

(a) Resveratrol alone (•), resveratrol + neocuproine (1mM) (○), resveratrol + thiourea (1mM) (▼). **(b): gallic acid alone** (•), gallic acid + neocuproine (1mM) (○), gallic acid + thiourea (1mM) (▼). The isolated cells ($1x10^5$) suspended in RPMI 1640 were preincubated with the indicated 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 incubation for 1 hr in the presence of increasing polyphenol concentrations. Viability of lymphocytes after preincubation with neocuproine and thiourea was more than 90 %. Values reported are ±SEM of three independent experiments.


TBARS nmoles
12
10
8
6
4
2
0
0
100
200
300
400
Resveratrol (μM)

(a)


TBARS nmoles
12
10
8
6
4
2
0
0
100
200
300
400
Gallic Acid (μM)

(b)

DISCUSSION-III

DISCUSSION

As already mentioned, over the last several years, our laboratory has extensively characterized a DNA cleavage reaction mediated by a number of polyphenols in the presence of copper ions (Rahman et al., 1989; Khan & Hadi, 1998; Ahsan et al., 1998 Ahmad et al., 2000; Azam et al., 2004). Subsequently, using human peripheral lymphocytes and Comet assay I have 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). Based on our own observations and those of others in literature we have proposed a mechanism for the cytotoxic action of plant polyphenolics against cancer cells that involves mobilization of endogenous copper and the consequent generation of reactive oxygen species particularly the hydroxyl radical (Hadi et al., 2000). These other observations in literature include the fact that copper is the major metal ion present in the nucleus (Bryan, 1987; Ebadi & Swanson, 1998) 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).

Thus, we may suggest that the conclusion of the present study is that polyphenols possessing anticancer or apoptosis inducing activity are able to mobilize endogenous copper ions possibly the copper bound to chromatin. It is realized that the above results do not categorically prove that lymphocyte DNA degradation described above involves mobilization of 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 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 & Lamelli, 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 extracellular production of reactive oxygen species that could account for lymphocyte DNA breakage. However, this does not seem to be the case in our system since we have previously shown that no lymphocyte DNA breakage is observed on preincubating the cells with resveratrol 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) (Chapter 2 Figure 23). Further we could not detect any H_2O_2 formation on incubating resveratrol (upto a concentration of 300 μM) in RPMI medium (results not shown) A comparison of the properties of the complexes formed between plant

polyphenols and Cu(II) and Fe(III) should indicate which of these metals can lead to DNA fragmentation in nucleus when complexed. Not much is known about the properties of such complexes. However, considerable information is available about 1,10 ortho-phenanthroline chelation of copper and iron ions. Burkitt et al (1996) cited several reasons why Cu(II) rather than Fe(III) may be responsible for ortho-phenanthroline stimulated internucleosomal nuclear DNA fragmentation in isolated nuclei. For example, the cumulative affinity constants for chelation of various metal ions by ortho-phenanthroline are in the order $Cu^{2+} \approx Fe^{2+} > Zn^{2+} > Fe^{3+}$, the complex formed between copper and ortho-phenanthroline has a redox potential (E^0 for Cu^{2+}/Cu^{+} = 0.17 V) that favours redox cycling, whereas that for Fe^{3+}/Fe^{2+} is 1.1 V, presumable because of stabilization in the ferrous state.

Some evidence suggests that polyphenolic compounds such as tannins and resveratrol are able to traverse cells membranes and may enter the cytoplasmic or nuclear space. Resveratrol is sufficiently hydrophobic and has been shown to be present in such tissues as heart, liver and kidney (Bertelli et al., 2000). A model for the entry and interaction of phenols with chromatin–associated copper has been described (Li & 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., 1988). The question of bioavailibility of polyphenols in mammalian system also needs to be addressed. Some relatively recent work with resveratrol indicates that it may have a relatively low bioavailibility due to its biotransformation and rapid elimination. For example Asensi et al (2003) have reported that in the case of rabbits the half-life of resveratrol in plasma after i.v administration of 20 mg/kg b.w was about 14 minutes and the highest concentration of resveratrol in plasma was reached within the first five minutes (2.6 ± 1 μM) after receiving 20 mg res/kg.b.w orally. Nevertheless these authors further report that 5 μM resveratrol completely

inhibited the growth of B-16 M murine melanoma cells. In this context we may mention that the minimum concentration of resveratrol tested by us in the presence of added copper ions for DNA breakage in lymphocytes was 10 μM (Azmi et al., 2005). However, as shown in the above results the minimum concentration of resveratrol required for DNA breakage in lymphocytes is between 100-200μM. Because of higher intracellular copper levels it may be predicted that such concentrations of resveratrol 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, 1995). 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 (Asensi et al., 2003). Based on our work we would like to propose that mobilization of endogenous copper ions by polyphenols and the consequent prooxidant action could be one of the important mechanisms for 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.

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Resveratrol–Cu(II) induced DNA breakage in human peripheral lymphocytes: Implications for anticancer properties

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Abstract **Resveratrol (3,4′,5-trihydroxy stilbene), a plant derived polyphenol found in mulberries, grapes and red wine 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. Using a cellular system of lymphocytes isolated from human peripheral blood and Comet assay, we have confirmed that resveratrol–Cu(II) system is indeed capable of causing DNA degradation in cells such as lymphocytes. Also, *trans*-stilbene, which does not have any hydroxyl groups, is inactive in the lymphocyte system. Pre-incubation of lymphocytes with resveratrol indicates that it is capable of either traversing the cell membrane or binding to it. Our results are in partial support of our hypothesis that anticancer properties of various plant derived polyphenols may involve mobilization of endogenous copper and the consequent prooxidant action.**

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Keywords: Resveratrol; Comet assay; Cu(II) reduction; Reactive oxygen species

1. Introduction

Resveratrol (3,4′,5-trihydroxy stilbene) belongs to a class of compounds known as phytoalexins and has been isolated from several spermatophytes of which grapevine peanuts and pines are the prime representatives. Studies on resveratrol have gained momentum in the last few years because of the phenomenon of 'French Paradox', which refers to the paradoxical finding that the incidence of coronary heart disease in the population of southern France is relatively low inspite of high intake of saturated fats in the diet. This phenomenon has been correlated with a higher intake of red wine in this region with resveratrol being identified as the major constituent responsible for this effect.

Resveratrol has been reported to have a variety of anti-inflammatory [1], anti-platelet [2], anti-mutagenic [3] effects and has been shown to be an agonist for the estrogen receptor [4], a property relevant to its reported cardiovascular protective properties. It has been found to confer resistance to plants against fungal infections [5] and inhibit DNA polymerase [6] and ribonucleotide reductase [7]. Frankel et al. [8] have demonstrated the inhibition of LDL oxidation by resveratrol and Belguendouz et al. [9] have attributed this activity to chelate copper ions. Resveratrol has been implicated as cancer chemopreventive agent capable of inhibiting all the three stages of chemically induced carcinogenesis namely tumor initiation, promotion and progression [1]. In agreement with the findings that cancer chemopreventive agents can induce apoptosis [10–12], resveratrol has been shown to induce apoptosis in human tumor cells [13,14].

Earlier studies in our laboratory have established that several antioxidant polyphenolic compounds of plant origin such as flavonoids [15], tannins [16] and curcumins [17] are themselves capable of inducing oxidative DNA damage either alone or in the presence of certain transition metal ions especially Cu(II). Further, it was also pointed out that several properties of these compounds such as binding to DNA and its degradation are similar to those of known anticancer drugs such as bleomycin, adriamycin and 4′-(9-acrdinylamino) methanesulphone-*m*-anisidine (mAMSA) [18–20]. It has been shown that the polyphenolic resveratrol is also capable of strand breakage in DNA in the presence of copper ions [21]. Previously we have shown that resveratrol catalyzes the reduction of Cu(II) to Cu(I), which is accompanied by the formation of oxidized product(s) of resveratrol, which in turn also appear to catalyze the reduction of Cu(II). Further, strand scission by the resveratrol–Cu(II) system was found to be biologically active as assayed by bacteriophage inactivation [22]. Using a cellular system of lymphocytes isolated from human peripheral blood and alkaline single cell gel electrophoresis (Comet assay), we have confirmed that resveratrol–Cu(II) system is indeed capable of causing DNA degradation in cells such as lymphocytes. Further, the DNA degradation of lymphocytes is inhibited by scavengers of reactive oxygen and neocuproine, a Cu(I) specific sequestering agent. Also, similar to the in vitro results, *trans*-stilbene which does not have any hydroxyl groups is inactive in the lymphocyte system. These findings demonstrate that the resveratrol–Cu(II) system for DNA breakage is physiologically feasible and could be of biological significance.

2. Materials and methods

2.1. Materials

Resveratrol, piceatannol, *trans*-stilbene, neocuproine, superoxide dismutase (SOD), agarose, low melting point agarose (LMPA), RPMI 1640, Triton 100×, Trypan blue, Histopaque 1077 and phosphate buffered saline (PBS) Ca^{++} and Mg^{++} free were purchased from Sigma

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(St. Louis, USA). Supercoiled plasmid pBR322 DNA was prepared according to standard methods [23]. All other chemicals were of analytical grade. Resveratrol was dissolved in 3 mM cold 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, resveratrol remained in solution. The volumes of stock solution 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 were obtained by venepuncture and diluted suitably in Ca^{++} and Mg^{++} free PBS. Lymphocytes were isolated from blood using Histopaque 1077 (Sigma), the cells ($\approx 2 \times 10^5$) were 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 [24]. The viability of the cells was found to be greater than 93%.

2.4. Treatment of lymphocytes

Lymphocytes were exposed to different concentrations of resveratrol, piceatannol and *trans*-stilbene in the absence and presence of various concentrations of $CuCl_2$ in a total reaction volume of 1 ml. Incubation was performed at 37 °C for 30 min or as specified. In some experiments, lymphocytes were pre-incubated either with 50 μM resveratrol or 20 μM $CuCl_2$. In another set of 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 and processed further for Comet assay.

2.5. Comet assay

Comet assay was performed under alkaline conditions essentially according to the procedure of Singh et al. [25] with slight modifications. Fully frosted microscopic slides precoated with 1.0% normal melting agarose at about 50 °C (dissolved in Ca^{++} and Mg^{++} 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 100× added just prior to use for a minimum of 1 h at 4 °C. After lysis DNA was allowed to unwind for 30 min in alkaline electrophoretic solution consisting of 300 mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was performed at 4 °C in a field strength of 0.7 V/cm and 300 mA current. The slides were then neutralized with cold 0.4 M Tris, pH 7.5, stained with 75 μl 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 a Olympus (CX41) fluorescent microscope and a COHU 4910 (equipped with a 510–560 nm excitation and 590 nm barrier filters) integrated CC camera. Comets were scored at 100 × magnification. Images from 50 cells (25 from each replicate slide) were analyzed. The parameter taken to assess lymphocytes DNA damage was tail length (migration of DNA from the nucleus, μm) and was automatically generated by Komet 5.5 image analysis system.

2.6. Statistics

The statistical analysis was performed as described by Tice et al. [26] and is expressed as ±S.E.M. of three 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 and discussion

3.1. DNA breakage induced by resveratrol–Cu(II) in lymphocytes as measured by Comet assay

The structures of resveratrol, the structurally similar piceatannol and the parent compound *trans*-stilbene used in this study are given in Fig. 1. Increasing concentrations of resveratrol (10–100 μM) either alone or in the presence of 20 μM $CuCl_2$ were tested for DNA breakage in isolated lymphocytes using the Comet assay. Resveratrol alone, at any of the concentrations tested did not damage the lymphocyte DNA whereas on addition of Cu(II) DNA damage to varying degrees was observed (results not shown). Photographs of Comets seen on treatment with 50 μM resveratrol alone (a), 20 μM Cu(II) alone (b) and in the presence of both these additions (c) are shown in Fig. 2. Untreated lymphocyte controls were similar to resveratrol alone or Cu(II) alone (results not shown). The results clearly establish that resveratrol–Cu(II) system is capable of DNA breakage in lymphocytes. A similar experiment with increasing concentrations of Cu(II) (5–20 μM) at

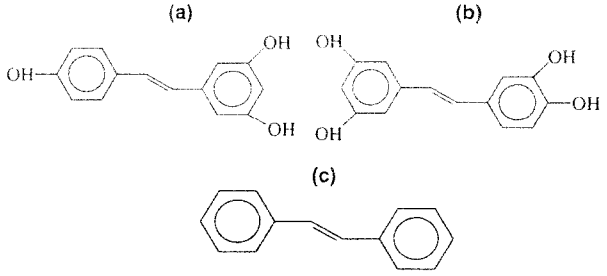

Fig. 1. Chemical structures of (a) resveratrol, (b) piceatannol and (c) *trans*-stilbene.

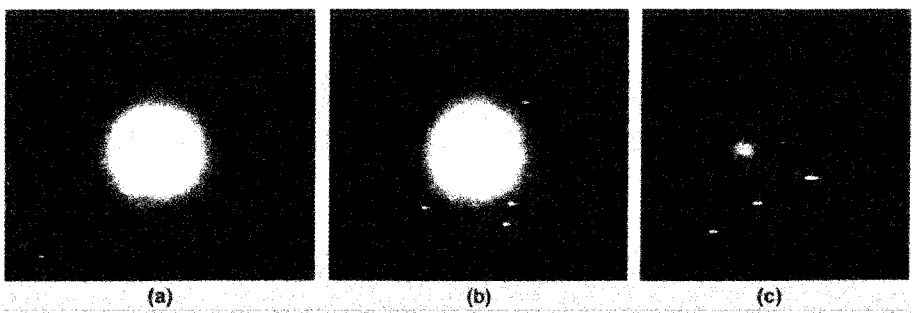

Fig. 2. Single cell gel electrophoresis of human peripheral lymphocytes showing comet (100×) after treatment with resveratrol alone (50 μM) (a), Cu(II) alone (20 μM) (b) and with resveratrol (50 μM) and Cu(II) (20 μM) (c).

Table 1
Effect of scavengers of active oxygen species on lymphocyte DNA breakage by resveratrol Cu(II)

Treatment	Tail length (μm)	Inhibition (%)
Untreated (#)	2.50 ± 0.15	
Resveratrol (50 μM) + Cu(II) (20 μM)	22.88 ± 2.01*	0
+ Sodium azide (1 mM)	4.22 ± 0.38*	81.55
+ Potassium iodide (1 mM)	8.77 ± 0.85*	61.66
+ Thiourea (1 mM)	6.03 ± 0.55*	73.64
+ Ascorbate (1 mM)	7.92 ± 0.78*	65.38
+ Glutathione (1 mM)	10.28 ± 1.11*	55.38
+ Neocuproine (1 mM)	10.27 ± 1.03*	55.11
+ Superoxide dismutase (100 μg/ml)	7.65 ± 0.75*	66.56
+ Catalase (100 μg/ml)	6.62 ± 0.59*	71.06

*$P < 0.05$ by comparison with control (#). Data represent median ± S.E.M. of three independent experiments.

fixed concentrations of resveratrol (50 μM) was also carried out. An increasing degree of DNA damage with increasing Cu(II) concentrations was seen (results not shown).

3.2. Effect of active oxygen scavengers on resveratrol–Cu(II) induced DNA damage in lymphocytes

In a previous study [22], we had shown that the resveratrol-Cu(II) mediated degradation of DNA and inactivation of bacteriophage λ is inhibited to significant degrees by various scavengers of reactive oxygen species. Table 1 gives the results of an experiment where the effect of sodium azide, potassium iodide, thiourea, neocuoproine, superoxide dismutase (SOD) and catalase were tested. 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. Neocuproine is a Cu(I) specific sequestering agent which as expected also inhibits its DNA breakage. The results suggest that the chemically induced DNA breakage observed in vitro and lymphocyte DNA damage by resveratrol–Cu(II) system are most likely the result of the same mechanism. However, it is realized that direct confirmation of the result requires additional work.

3.3. Cleavage of plasmid pBR322 DNA by resveratrol, piceatannol and trans-stilbene

In order to understand the chemical basis of DNA breakage by resveratrol–Cu(II) system, we have compared the relative DNA cleavage efficiency of resveratrol, piceatannol, and the parent compound *trans*-stilbene in plasmid pBR322 DNA. In the results given in Fig. 3, both resveratrol and piceatannol caused conversion of supercoiled plasmid molecules into linear molecules. However, piceatannol also gives rise to smaller sized heterogeneous fragments as indicated by the formation of a smear on the gel. Thus, piceatannol is a more efficient DNA cleaving agent than resveratrol. *Trans*-stilbene, which does not have any hydroxyl group, is not a cleaving agent. These results demonstrate that the presence of hydroxyl groups is essential for DNA cleavage. Further, the efficiency of cleavage increases with the number of hydroxyl groups. A similar comparison of the three (using the same concentrations, i.e., 50 μM) for DNA breakage in lymphocytes was also performed. The results are expressed as tail lengths of the Comets in Fig. 4. In agreement with the results of Fig. 3, piceatannol shows the formation of largest tail followed by resveratrol and minimal tail formation in the case of *trans*-stilbene.

3.4. Effect of pre-incubation of lymphocytes with resveratrol or Cu(II) on resveratrol–Cu(II) mediated DNA breakage

Lymphocyte DNA breakage was also studied after pre-incubating the cells with resveratrol after which the cells were washed twice with PBS and incubated further in the presence of $CuCl_2$. The results given in Fig. 5, show that with increasing concentration of resveratrol a progressive increase in DNA breakage as indicated by increased tail length of Comets is observed. A similar experiment was also done by pre-incubation with $CuCl_2$ and similar results were observed (Fig. 6). These results indicate that both resveratrol and Cu(II) are either able to enter the cells or bind to the cell membrane.

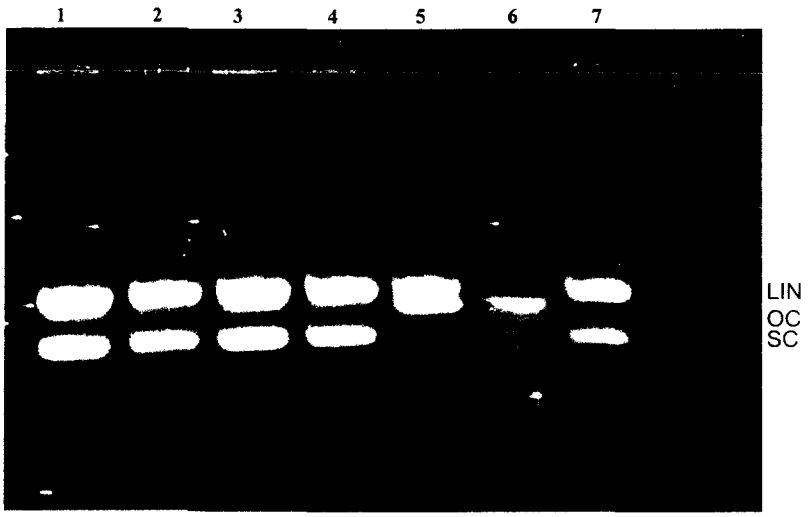

Fig. 3. Degradation of pBR322 plasmid DNA by resveratrol, piceatannol and *trans*-stilbene in the absence and presence of Cu(II). Lane 1, DNA alone; lanes 2–4, DNA + resveratrol, piceatannol and *trans*-stilbene (50 μM); lanes 5–7, DNA + resveratrol, piceatannol and *trans*-stilbene (50 μM) + Cu(II) (20 μM); SC, supercoiled DNA; OC, nicked circular; LIN, linear molecules.

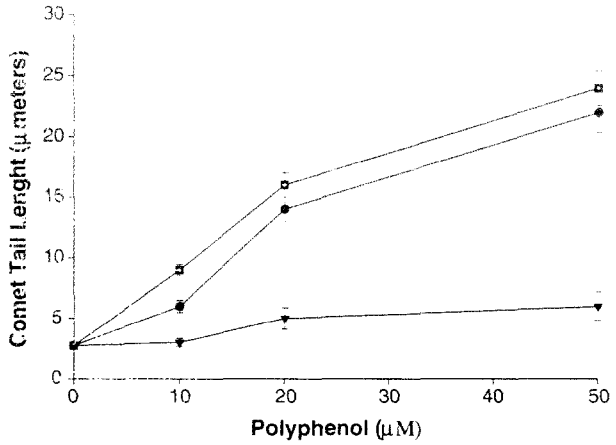

Fig. 4. A comparison of the DNA breakage in human lymphocytes by resveratrol (●), piceatannol (■) and *trans*-stilbene (▼) in the presence of Cu(II). The concentration of Cu(II) was 20 μM. Values reported are ±S.E.M. of three independent experiments.

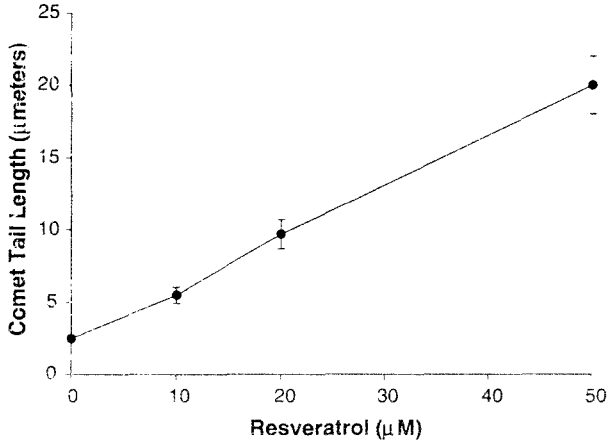

Fig. 5. Effect of pre-incubating the lymphocytes with increasing concentrations of resveratrol on DNA breakage. The isolated cells suspended in RPMI 1640 were pre-incubated with the indicated concentrations of resveratrol for 30 min at 37 °C. After pelleting the cells were washed with PBS twice before resuspension in RPMI and further incubation for 30 min in the presence of 20 μM Cu(II). Values reported are ±S.E.M. of three independent experiments.

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 polyphenols may not fully account for their anticancer effects [27]. Further, we have previously shown 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) [28]. Interestingly, modification of phenolic hydroxyl groups such as that resulting in the formation of syringic acid abolishes the apoptosis inducing capacity of gallic acid [29]. It is to be noted that piceatannol which is the tetra-hydroxy derivative of resveratrol (Fig. 1) is also a potent inducer of apoptosis in human SK-Mel-28 Melanoma cells [30]. Indeed, it has been shown that resveratrol is converted to piceatannol by cytochrome P450 enzyme CYP1B1 from human lymphoblast microsomes [31]. Recently, Dong

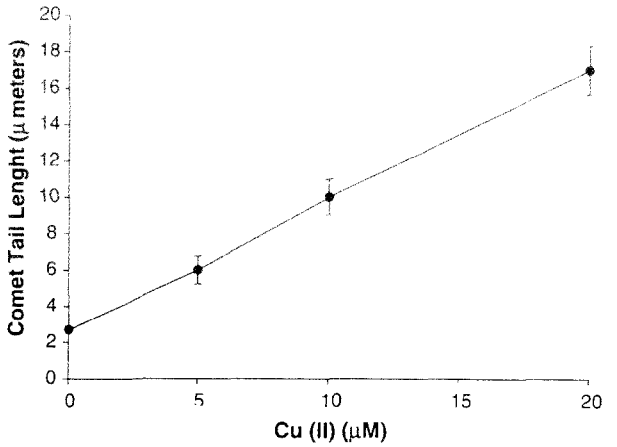

Fig. 6. Effect of pre-incubating the lymphocytes with increasing concentrations of Cu(II) on DNA breakage. The conditions of pre-treatment and further treatment with 50 μM resveratrol were the same as given in Fig. 5. Values reported are ±S.E.M. of three independent experiments.

[12] has shown that a penta hydroxyl synthetic derivative of resveratrol was more effective as an inhibitor of EGF induced cell transformation as compared with resveratrol. Based on our own observations and those of others, we have proposed a mechanism for the cytotoxic action of plant polyphenolics against cancer cells that involves mobilization of endogenous copper and the consequent prooxidant action [32]. 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 mentioned earlier [32]. More significantly 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 pathway differs between pharmacological cell death and physiological induction of cell death [33]. Cu^{++} and Zn^{++} are the major metal ions present in the nucleus [34], serum [35] and tissue [36] 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 property which is the hallmark of cells undergoing apoptosis [37]. It has recently been shown that the polyphenol curcumin mediated apoptosis of HL60 cells is closely related to the increase in the concentrations of reactive oxygen species possibly generated through the reduction of transition metals in cells [38]. Thus, it is possible that cellular DNA fragmentation by plant polyphenolics that involves mobilization of intra-cellular and extra-cellular copper could be one of the mechanisms involved in the chemopreventive properties of these compounds.

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Plant polyphenols mobilize endogenous copper in human peripheral lymphocytes leading to oxidative DNA breakage: A putative mechanism for anticancer properties

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Abstract **Plant polyphenols are important components of human diet and a number of them are considered to possess chemopreventive and therapeutic properties against cancer. They are recognized as naturally occurring antioxidants but also act as prooxidants catalyzing DNA degradation in the presence of transition metal ions such as copper. Using human peripheral lymphocytes and Comet assay we have previously confirmed that resveratrol–Cu(II) is indeed capable of causing DNA degradation in cells. In this paper we show that the polyphenols alone (in the absence of added copper) are also capable of causing DNA breakage in cells. Incubation of lymphocytes with neocuproine inhibited the DNA degradation confirming that Cu(I) is an intermediate in the DNA cleavage reaction. Further, we have also shown that polyphenols generate oxidative stress in lymphocytes which is inhibited by scavengers of reactive oxygen species and neocuproine. These results are in further support of our hypothesis that anticancer mechanism of plant polyphenols involves mobilization of endogenous copper, possibly chromatin bound copper, and the consequent prooxidant action.**

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Keywords: Plant polyphenols; Comet assay; Endogenous copper; Prooxidant action

1. Introduction

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 [1]. 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 [2–5]. Gallocatechins are constituents of green tea, the consumption of which is considered to reduce the risk of various cancers such as those of bladder, prostrate, oesophagus and stomach [5]. 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 [3–5].

Studies in our laboratory have shown that flavonoids [6], tannic acid and its structural constituent gallic acid [7], curcumin [8], gallocatechins [9] and resveratrol [10] 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 [11]. It is also 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 various 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 [12,13]. Most of the plant polyphenols possess both antioxidant as well as prooxidant properties [4,6] and we have earlier proposed that the prooxidant action of polyphenolics may be an important mechanism of their anticancer and apoptosis inducing properties [13]. 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 resveratrol in the presence of Cu(II) is indeed capable of causing DNA degradation in cells. Further, such DNA degradation in lymphocytes is inhibited by scavengers of reactive oxygen and neocuproine, a Cu(I) specific sequestering agent, indicating that the DNA breakage is caused by reactive oxygen species generated through the reduction of Cu(II)–Cu(I) by the polyphenols [14].

As a further confirmation of our hypothesis, in this paper we show that several plant polyphenols alone (in the absence of added Cu(II)) are also capable of lymphocyte DNA degradation and that such degradation is mediated through mobilization of endogenous copper ions.

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2. Materials and methods

2.1. Materials

Resveratrol, piceatannol, *trans*-stilbene, gallic acid, syringic acid, neocuproine, superoxide dismutase (SOD), agarose, low melting point agarose (LMPA), RPMI 1640, Triton X-100, Trypan blue, Histopaque 1077 and phosphate buffered saline (PBS) Ca^{++} and Mg^{++} free were purchased from Sigma, (St. Louis, USA). All other chemicals were of analytical grade. Resveratrol, piceatannol and *trans*-stilbene were dissolved in 3 mM NaOH before use as a stock of 1 mM solution. All other polyphenols were dissolved in water. Upon addition to reaction mixtures, in the presence of buffers mentioned and at the concentrations used, all the polyphenols used remained in solution. The volumes of stock 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 were obtained by venepuncture and diluted suitably in Ca^{++} and Mg^{++} 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 [15].

2.4. Lymphocyte treatment

Lymphocytes (1×10^5 cells) were exposed to different concentrations of polyphenols in a total reaction volume of 1 ml (400 μl RPMI, PBS Ca^{2+} and Mg^{2+} free and indicated concentrations of polyphenols). Incubation was performed at 37 °C for 2 h or as specified. In some experiments, scavengers of active oxygen were added at the final concentrations indicated. After the incubation, the mixture was centrifuged at 4000 rpm, the supernatant was discarded and pelleted lymphocytes were resuspended in 100 μl of PBS (Ca^{++} and Mg^{++} free) and processed further for Comet assay.

2.5. Comet assay

Comet assay was performed under alkaline conditions essentially according to the procedure of Singh et al. [16] and is described by us in detail previously [14].

2.6. Statistics

The statistical analysis was performed as described by Tice et al. [17] and is expressed as ±S.E.M. of three 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.

2.7. Determination of TBARS and H_2O_2

Thiobarbituric acid reactive substance was determined according to the method of Ramanathan et al. [18]. A cell suspension (1×10^5/ml) was incubated with different polyphenols (0–400 μM) at 37 °C for 1 h 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^{++} and Mg^{++} 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 (thiobarbituric acid) in boiling water bath for 10 min. The absorbance was read at 532 nm. H_2O_2 was determined in the incubation medium by FOX assay as described by Long et al. [19]. The reaction mixture was the same as mentioned in 2.4 above but without cells. After incubation for 2 h at 37 °C an aliquot of 100 μl was analyzed for H_2O_2 formation.

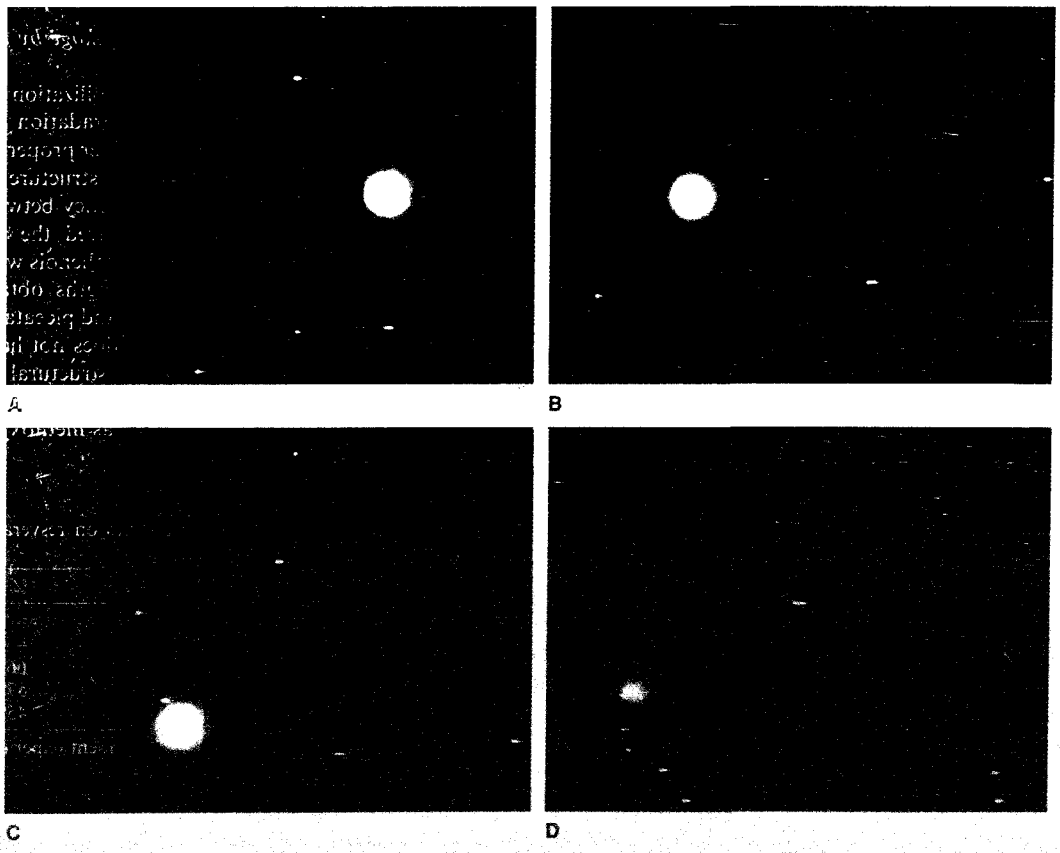

Fig. 1. Single cell gel electrophoresis of human peripheral lymphocytes showing Comets (100X) after treatment with different concentrations of resveratrol. (A) untreated, (B) resveratrol (50 μM), (C) resveratrol (100 μM) and (D) resveratrol (200 μM).

3. Results

3.1. DNA breakage by resveratrol in lymphocytes as measured by Comet assay

Increasing concentrations of resveratrol (50, 100, 200 μM) were tested for DNA breakage in isolated lymphocytes using the Comet assay. Photographs of Comets seen on treatment with these concentrations are shown in Fig 1. At 50 and 100 μM concentrations resveratrol did not damage the lymphocyte DNA to any significant extent whereas at 200 μM concentration a Comet with a tail indicative of DNA breakage was observed. In Fig. 2. the tail lengths obtained from the same experiment are plotted as a function of resveratrol concentration. The results clearly establish that resveratrol alone is capable of DNA breakage in lymphocytes. However, the minimum concentration required for such breakage (100–200 μM) is considerably greater than when resveratrol (10 μM) is used along with Cu(II) as was shown in our previous publication [14].

3.2. Effect of neocuproine, a Cu(I) specific sequestering agent, on resveratrol induced DNA breakage in lymphocytes

In the previous study [14] we had shown that the resveratrol–Cu(II) mediated degradation of lymphocyte DNA is inhibited by neocuproine which is a Cu(I) specific chelating agent and is membrane permeable [20]. Fig. 3 gives the results of an experiment where three progressively increasing concentrations of neocuproine were tested on resveratrol induced DNA breakage in lymphocytes. A progressive decrease in the tail length as a function of increasing neocuproine concentration was seen. From the results we can conclude that the DNA breakage by the polyphenol involves endogenous copper ions and that Cu(I) is an intermediate in the pathway that leads to DNA breakage.

3.3. Effect of active oxygen scavengers on resveratrol induced DNA breakage in lymphocytes

We have previously shown [14] that resveratrol–Cu(II) mediated degradation of lymphocyte DNA is inhibited to significant degrees by various scavengers of reactive oxygen species. Table 1 gives the results of an experiment where three such scavengers have been tested, namely: superoxide dismutase (SOD) and catalase which remove superoxide, and H_2O_2, respectively, and thiourea which is a scavenger of several reactive oxygen species. All three cause significant inhibition of DNA breakage as evidenced by decreased tail lengths. We conclude 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. These results along with the results of Fig. 4 suggest that resveratrol–Cu(II) induced lymphocyte DNA breakage and DNA breakage by resveratrol alone are likely the result of the same mechanism.

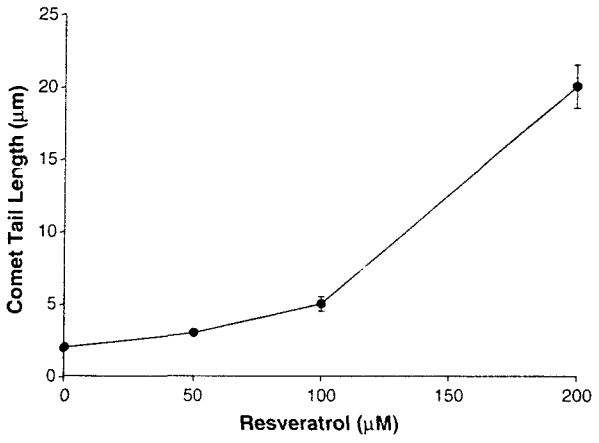

Fig. 2. Effect of increasing concentrations of resveratrol on DNA breakage in lymphocytes. The viability of cells after incubation was found to be greater than 93%. Values reported are ±S.E.M. of three independent experiments.

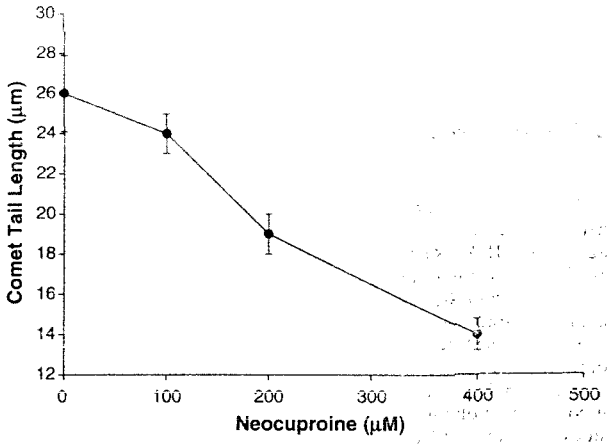

Fig. 3. Effect of increasing concentrations of neocuproine on resveratrol induced DNA breakage in human lymphocytes. Values reported are ±S.E.M. of three independent experiments.

3.4. Comparison of lymphocyte DNA breakage by various polyphenols

According to our hypothesis [13] mobilization of endogenous copper and the consequent degradation of cellular DNA is a general mechanism for anticancer properties of plant polyphenols. However, depending on the structure of the molecule there would be differences of efficiency between various polyphenols. We have therefore compared the lymphocyte DNA breakage efficiency of various polyphenols with different structures. Fig. 4 shows Comet tail lengths obtained using increasing concentrations of resveratrol and piceatannol whose parent compound *trans*-stilbene which does not have any hydroxyl group and gallic acid which is a structural constituent of tannins [7]. Syringic acid is a derivative of gallic acid where two of the hydroxyl groups are present as methoxy groups. It

Table 1
Effect of scavengers of active oxygen species on resveratrol induced lymphocyte DNA breakage

Dose	Tail length (μm)	% Inhibition
Untreated	1.22 ± 0.08 [a]	–
Resveratrol 200 μM	20.84 ± 1.28	–
+SOD 100 μg/ml	7.05 ± 0.25*	66%
+Catalase 100 μg/ml	8.86 ± 0.29*	57%
+Thiourea 1 mM	11.93 ± 1.01*	45%

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

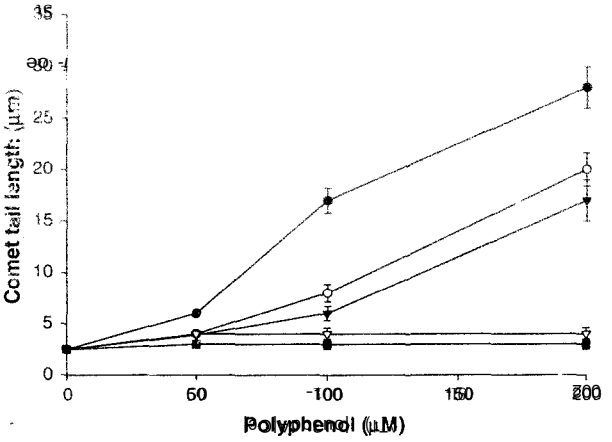

Fig. 1. Comparison of various polyphenols on the induction of DNA breakage in human lymphocytes, (●) gallic acid, (○) piceatannol, (▼) resveratrol, (△) *trans*-stilbene, (■) syringic acid. The viability of cells after treatment was found to be greater than 90%. Values reported are ± S.E.M. of three independent experiments.

is seen that except *trans*-stilbene and syringic acid all the other polyphenols are able to induce DNA breakage up to various degrees. It is worth mentioning that *trans*-stilbene and syringic acid are also ineffective as DNA cleaving agent in vitro [9] or lymphocyte DNA breakage in the presence of Cu(II) [14].

3.3. Determination of TBARS as a measure of oxidative stress in lymphocytes by resveratrol and gallic acid in the presence of neocuproine and thiourea

As mentioned above we presume that lymphocyte DNA breakage is the result of the generation 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 [31,32]. We have therefore determined the formation of TBA reactive substance (TBARS) as a measure of oxidative stress in lymphocytes with increasing concentrations of resveratrol and gallic acid. The effect of preincubating the cells with neocuproine and thiourea was also studied. Results given in Fig. 3(A) and (B) show that there is a dose dependant 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 both resveratrol as well as gallic acid. These results indicate that both DNA breakage and oxidative stress in cells is inhibited by Cu(I) oxidation 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).

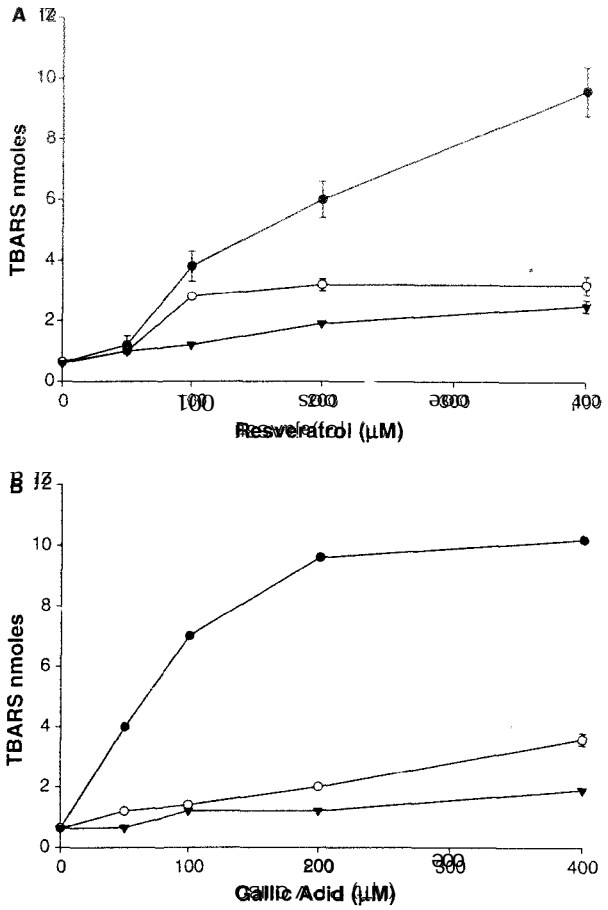

Fig. 3. Effect of lymphocyte incubation with neocuproine and thiourea on TBARS generated as increasing concentrations of resveratrol (A) and gallic acid (B). Resveratrol (●), resveratrol + neocuproine (1 mM) (○), resveratrol + thiourea (1 mM) (▼), resveratrol + neocuproine (1 mM) + thiourea (1 mM) (▽). (B) Gallic acid alone (●), gallic acid + neocuproine (1 mM) (○), gallic acid + thiourea (1 mM) (▼). The isolated cells (1×10^7) suspended in RPMI 1640 were preincubated with the indicated 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 incubation for 1 h in the presence of increasing polyphenol concentrations. Viability of lymphocytes after preincubation with neocuproine and thiourea was more than 90%. Values reported are ± S.E.M. of three independent experiments.

4. Discussion

As already mentioned, over the last several years, we have extensively characterised a DNA cleavage reaction mediated by a number of polyphenols in the presence of copper ions [6–9]. Subsequently, using human peripheral lymphocytes and Comet assay we confirmed that the polyphenol resveratrol in the presence of Cu(II) is indeed capable of DNA degradation in a cellular system [14]. Based on our own observations and those of others in the literature we have proposed a mechanism for the cytotoxic action of plant polyphenolics against cancer cells that involves mobilization of endogenous copper and the consequent generation of reactive oxygen species particularly the hydroxyl radical [33]. Those other observations in the literature include the fact that copper is the major metal ion present in the nucleus [23]. Further it has been shown that serum [24] and tissue [25,26] 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 characteristic of cells undergoing apoptosis [27]. 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 [28].

Thus, we suggest that the conclusion of the present study is that polyphenols possessing anticancer or apoptosis inducing activity are able to mobilize endogenous copper ions possibly the copper bound to chromatin. It is realized that the above results do not categorically prove that lymphocyte DNA degradation described above involves mobilization of 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 [29]. The location of 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 [30]. 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) [31]. Polyphenols are known to reduce molecular oxygen to superoxide anion, an event that may occur even outside the cell, leading to the formation of H_2O_2 [6]. Superoxide can also be formed by reoxidation of Cu(I)–Cu(II) in the ternary complex [31]. Chromatin bound copper is understood to be present in the reduced form (Cu(I)) [32] 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 can enter cells causing damage to various macromolecules [19,33,34]. This may lead to extracellular production of reactive oxygen species that could account for lymphocytes DNA breakage. However, this does not appear to be the case in our system since we have previously shown that no lymphocyte DNA breakage is observed on preincubating the cells with resveratrol alone up to a concentration of 50 μM. DNA breakage could only be seen after incubating the pre-treated cells further in the presence of Cu(II) [14]. Further, we could not detect any H_2O_2 formation on incubating resveratrol (up to a concentration of 300 μM) in RPMI medium (results not shown).

Evidence suggests that polyphenolic compounds such as tannins and resveratrol are able to traverse cell membranes and may enter the cytoplasmic or nuclear space. Resveratrol is sufficiently hydrophobic and has been shown to be present in tissues such as heart, liver and kidney [35]. The question of bioavailability of polyphenols in mammalian systems also needs to be addressed. Relatively recent work by Asensi et al. [36] with resveratrol indicates that it may have a relatively low bioavailability due to its biotransformation and rapid elimination. It was reported that the highest concentration of resveratrol in plasma was reached within the first 5 min (2.6 ± 1 μM) after receiving 20 mg res/kg.b.w orally [36]. Nevertheless these authors further report that 5 μM resveratrol completely inhibited the growth of B-16 M murine melanoma cells. Because of higher intracellular copper levels it may be predicted that such concentrations of resveratrol 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 [37]. 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 [36]. Based on our work we would like to propose that mobilization of endogenous copper ions by polyphenols and the consequent prooxidant action could be one of the important mechanisms for 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.

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