BIOPRODUCTION OF A PLANT DERIVED COMPOUND PLUMBAGIN (2, METHYL, 5-HYDROXY 1-4 NAPHTHOQUINONE) AND THE STUDY OF ITS PRO-OXIDANT ACTIVITY *IN VIVO/IN VITRO*

(Production of plumbagin from hairy root cultures of *Plumbago zeylanica* & degradation of DNA and cell killing through a pro-oxidant mechanism)

SUMMARY

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

BIOCHEMISTRY

BY

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2008

SUMMARY

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A cursory survey of the local flora and available literature indicated that *Plumbago zeylanica* belonging to the family Plumbaginaceae, a perennial subscandent shrub indigenous to Southeast Asia, is endowed with unique medicinal properties. The pharmacological importance of this perennial shrub lies in its ability to produce a naphthoquinone called plumbagin, mainly in its roots. Plumbagin (5-hydroxy-2-methyl,1-4 naphthoquinone) shows a broad range of pharmaceutical activities such as anticancer, cardiotonic, radiomodifying, leishmanicidal, antimicrobial, antifertile, antimalarial, antimutagenic and insecticidal properties. These properties of plumbagin have enhanced the demand of this medicinal plant for its roots. Previous experiments to obtain this quinone *in vitro* showed that plumbagin was best synthesized and accumulated in *Plumbago zeylanica*. In vitro production of plumbagin from *Plumbago zeylanica* is receiving more attention because conventional propagation of this plant is rather difficult and insufficient to meet the growing demand owing to the poor germination of seeds and death of young seedlings under natural conditions. Very few reports on cultivation, breeding and improvement programmes and *in vitro* studies of *P.zeylanica* are available despite its commercial importance. Furthermore, as the medicinally active constituents of *P.zeylanica* are generally obtained from the root tissues and since plants grow quite slowly and takes long time until the roots are suitable for use, it is advisable to search for alternative sources of plumbagin. Evaluation and exploitation of the *Agrobacterium rhizogenes* mediated hairy root cultures of this important plant species may result in elevated levels of plumbagin production. In wide range of medicinal plants, hairy root cultures have already proven to be an efficient alternative production system for root derived phytochemicals of consistent quality within much shorter time than is commonly expected from *in vivo* grown plants.

In view of this in the present study we have standardized an efficient protocol for induction and establishment of hairy root cultures leading to the bioproduction of bioactive molecule plumbagin in *P.zeylanica*. This study reports (i) a rapid and reproducible method for *in vitro* organogenesis in *P.zeylanica* from nodal explants and (ii) the successful induction and establishment of *A.rhizogenes* mediated hairy root cultures with higher potential for *in vitro* plumbagin production. This may not only provide an alternative production system but may also indirectly facilitate conservation of this important medicinal plant species by sparing its indiscriminate uprooting.

As mentioned above, *Plumbago zeylanica* is endowed with unique medicinal properties, of which anticancer property has been the subject of considerable interest. Plumbagin present in *Plumbago zeylanica* has been shown to exert anticancer and anti proliferative activities in animal models as well as in cells in culture. Even though there are reports of plumbagin having antitumor activity, there are only very few studies on the mechanism of cell death induced by plumbagin in human cancer cells and no unifying mechanism encompassing all of these data exists. So elucidation of the mechanism(s) by which plumbagin induces its anticarcinogenic and antiproliferative effect is necessary to provide a solid foundation for its use as an agent for anticancer prevention strategies. Earlier studies have established that several compounds of plant origin such as flavanoids, tannins, curcumin and the stilbene-resveratrol are themselves capable of inducing oxidative DNA damage either alone or in the presence of certain transition metal ions especially 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. Studies have also shown that metals, particularly copper; are capable of mediating the activation of several compounds, such as benzoyl peroxide, quercetin and dietary flavanoids by a redox mechanism leading to the formation of reactive oxygen and other organic radicals. It

was also previously shown that copper can induce the oxidation of 1,4 HQ, a benzene metabolite, producing 1,4BQ and H_2O_2 through a semiquinone intermediate. More interestingly, earlier reports have established that copper ion has been found at higher concentrations in various tumors as compared with the normal cells. These data in literature suggest that pro-oxidant action of these plant derived compounds 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 pro-oxidant action.

Plumbagin due to the presence of quinone moiety has been reported to generate reactive oxygen species and these ROS have been implicated to play a role in the apoptosis process. In view of this, we therefore decided to investigate the DNA damage induced by copper mediated activation of plumbagin. Infact our study is the first to investigate the role of a metal ion (Copper) in the observed cytotoxic action of plumbagin. In the second chapter of this thesis, we for the first time demonstrated that in the presence of micromolar concentrations of copper, DNA strand breaks was induced by plumbagin in plasmid pBR322 and calf thymus DNA. In addition, We have explored whether the plumbagin-Cu(II) system is capable of causing 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), we have confirmed that plumbagin-Cu(II) system is indeed capable of causing DNA degradation is cells such as lymphocytes. Preincubation of lymphocytes with plumbagin indicates that it is capable of either traversing the cell membrane or binding to it. Addition of active oxygen scavengers and neocuproine (a Cu(I) specific sequestering agent) inhibited the DNA degradation of lymphocytes indicating that a Cu(II) /Cu(I) redox cycle and ROS generation are the two major determinants involved in the observed DNA damage. These results are in partial support of the hypothesis that anticancer properties of various plant derived

compounds may involve mobilization of endogenous copper and the subsequent pro-oxidant action. Another major inference during this study is that, plumbagin alone at any of the concentrations tested did not elicit any damage in plasmid as well as lymphocyte DNA, which further support the fact that plumbagin exhibits selectivity for malignant cells.

In the third and final chapter of this thesis, we established the cytotoxicity of plumbagin using two different cell lines. As mentioned above plumbagin is thought to be a redox cycling compound that generates reactive oxygen species, which can damage various biomolecules. Of particular interest is the observation from earlier reports that plumbagin induces apoptotic cell death in various cell lines but not in normal cells. Results obtained in the second chapter of this thesis further support the above fact. Therefore plumbagin can be developed as an anticancer drug. So in this study our aim was to determine whether plumbagin inhibits the growth of human epidermoid carcinoma (A431) and Dalton's lymphoma ascites (DLA) cells through the induction of apoptosis. Because plumbagin induced cell death may be due to the production of ROS, we also studied the effect of plumbagin after pretreating the cells with free radical scavengers such as SOD, catalase, thiourea and also neocuproine, a Cu(I) specific sequestering agent. To our knowledge this study provide the first evidence that plumbagin induces effective cell growth inhibition by inducing apoptosis in A-431 & DLA cells. Incubation of cancer cells with scavengers of reactive oxygen species and neocuproine inhibited the cytotoxic action of plumbagin which proves that generation of ROS and Cu(I) are the critical mediators in plumbagin-induced cell growth inhibition. These results corroborate the results obtained in the second chapter of this thesis and throws light on the fact that plumbagin-Cu(II) mediated DNA breakage is physiologically feasible and could be one of the mechanisms for its antitumor action and these properties of plumbagin could be further explored for the development of anticancer agents with higher therapeutic index, especially for skin cancer.

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Certificate

This is to certify that the research work presented in the thesis entitled **"Bioproduction of a plant derived compound plumbagin (2, methyl, 5-hydroxy 1-4 naphthoquinone) and the study of its pro-oxidant activity *in vivo/in vitro*"** submitted to the Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh, in fulfillment for the award of the degree of **Doctor of Philosophy** embodies the original research work carried out by **Mr. Nazeem. S.,** M.Sc. (Biochemistry) under our supervision and has not been submitted in part or full for the award of any degree or diploma of this or any other University. It is further certified that he has fulfilled all the requirements for the degree, regarding the nature and prescribed period of work.

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Dedicated

to

My Parents & Grand Father (Late)
whose blessings, love & noble ideas
have always been guiding light to
my career.....

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Nazeem S.

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INTRODUCTION

INTRODUCTION

MEDICINAL PLANTS-IMPORTANCE AND SCOPE

Herbs are staging a comeback and herbal 'renaissance' is happening all over the globe. The herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human environment. Although herbs had been priced for their medicinal, flavoring and aromatic qualities for centuries, the synthetic products of the modern age surpassed their importance, for a while. However, the blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security. Not only, that plant-derived drug offers a stable market world wide, but also plants continue to be an important source for new drugs. Traditional systems of medicine continue to be widely practiced on many accounts. Population rise, inadequate supply of drugs, prohibitive cost of treatments, side effects of several allopathic drugs and development of resistance to currently used drugs for infectious diseases have led to increased emphasis on the use of plant materials as a source of medicines for a wide variety of human ailments. Global estimates indicate that 80% of about 4 billion population cannot afford the products of the Western Pharmaceutical Industry and have to rely upon the use of traditional medicines which are mainly derived from plants (Farnsworth et al., 1991). This fact is well documented in the inventory of medicinal plants, listing over 20,000 species. In spite of the overwhelming influences and our dependence on modern medicine and tremendous advances in synthetic drugs, over three-quarters of the world population still relies mainly on plants and plant extracts for health care. In many of the developing countries, the use of plant drugs is

increasing because modern life saving drugs are beyond the reach of three quarters of the third world's population although many such countries spend 40-50% of their total wealth on drugs and health care. As a part of the strategy to reduce the financial burden on developing countries, it is obvious that an increased use of plant drugs will be followed in the future.

The age-old traditional values attached with the various forest types and the varieties of forest products (i.e., medicinal plants) have gained tremendous importance in the present century (Stein et al., 2004; Kala et al., 2004). Furthermore, the cosmetic industries are increasingly using natural ingredients in their products, and these natural ingredients include extracts of several medicinal plants (Kit, 2003). More than 30% of the entire plant species, at one time or other, were used for medicinal purposes. The trend is evident from greater increase in annual growth of herbal medicine in comparison to pharmaceutical products. The domestic market of Indian system of medicine and Homeopathy (ISM & H) is of the order of 4,000 crore (US $ 800 million) of which Ayurveda drug market alone is about 3,500 crore (US $ 700 million), the value correlated to a quantitative consumption level of 128,000 tonnes. India's total export earnings from crude drugs, herbal extracts and finished products stands at Rs 800 crore (US $ 160 million). Globally, these plant derived medicines, essential oil and products are worth about Rs 360,000 crore (US $ 72 trillion). World wide, which include global business of medicinal herbal material of Rs 300,000 crore (US $ 60 trillion) (The Hindu Survey of Indian Agriculture, 2006). It has been estimated that in developed countries such as United States, plant drugs constitute as much as 25% of the total drugs, while in fast developing countries such as China and India, the contribution is as much as 80%. Moreover India and China are two of the largest countries in Asia, which have the richest arrays of registered and

relatively well-known medicinal plants (Raven et al., 1998). Since the Indian subcontinent is well known for its diversity of forest products and the age-old healthcare traditions, there is an urgent need to establish these traditional values in both the national and international perspectives realizing the ongoing developmental trends in traditional knowledge. Apart from health care, medicinal plants are mainly the alternate income-generating source of underprivileged communities (Myers et al., 1991; Lacuna-Richman et al., 2002). Of the 2,50,000 higher plant species on earth, more than 80,000 are medicinal. India is one of the world's 12 biodiversity centers with the presence of over 45000 different plant species. India's diversity is unmatched due to the presence of 16 different agro-climatic zones, 10 vegetation zones, 25 biotic provinces and 426 biomes (habitats of specific species). Of these, about 15000-20000 plants have good medicinal value. However, only 7000-7500 species are used for their medicinal values by traditional communities (Shiva, 1996). This proportion of medicinal plants is the highest proportion of plants known for their medical purposes in any country of the world for the existing flora of that respective country (Samant et al. 1998; Shiva, 1996; Schippmann et al., 2002) (**Table 1**). Ayurveda, the oldest medical system in Indian sub-continent, has alone reported approximately 2000 medicinal plant species, followed by Siddha and Unani (Anonymous 2004, 2005) (**Table 2**).

The Charak Samhita, an age-old written document on herbal therapy, reports on the production of 340 herbal drugs and their indigenous uses (Prajapati, 2003). Currently, approximately 25% of drugs are derived from plants, and many others are synthetic analogues built on prototype compounds isolated from plant species in modern pharmacopoeia (Rao et al., 2004). Over 25% of the new drugs approved in the last 30 years are based on molecule of plant

origin, and about 50% of the top selling chemicals derive from knowledge on plant secondary metabolism (Terryn et al., 2006). Seven plant derived compounds with anticancer activity have received FDA approval for clinical use. Taxol/paclitaxel (*Taxus brevifolia*), vinblastin and vincristin (*Catharanthus roseus*), topotecan and irinotecan (*Camptotheca acuminata*) and etoposide and temposide (*Podophyllum sp*). Since 1986, the National Cancer Institute has screened over 40,000 plant samples and five chemicals showing significant activity against AIDS have been isolated. Due to their complex structures and in spite of extensive efforts to develop partial or total chemical syntheses, isolation of such plant derived compounds from their natural sources remains the only viable option, with very few exceptions. In cases where such syntheses were developed, these constitute more of an intellectual academic success rather than a viable economic production process (Galera et al., 2007). The continuous increase in human population is one of the causes for concern in meeting the daily requirements of food and medicine as the economy and livelihoods of human societies living in developing countries primarily depend on plant products. This phenomenon is leading to continuous erosion of forest and the forest products (Samal et al., 2004), thus making challenge to meet the requirements as well as to conserve useful bio-resources. More and more species are being gradually added in the *Materia Medica*; however, the standards of their purity and correct identification do not keep pace with the process of expansion (Kaul, 1997). The market prices for medicinal plants and derived materials provide only a limited insight into the workings of the market, and not on the precise information of profits, supply and demand.

Table 1. DISTRIBUTION OF MEDICINAL PLANTS

Country or Region	Total number of native species in flora	No of Medicinal plant species reported	% of Medicinal plants	Source
World	297000	52885	10	Schippmann et al., 2002
India	17000	7500	44	Shiva, 1996
Indian Himalayas	8000	1748	22	Samant et al., 1998

*Kala *et al. Journal of Ethnobiology and Ethnomedicine*, 2006

Table 2. THE STATUS OF VARIOUS MEDICAL SYSTEMS IN INDIA

	MEDICAL SYSTEMS				
Characteristics	Ayurveda	Siddha	Unani	Tibetan	Homeopathy
Medicinal plants known	2000	1121	751	337	482
Licensed pharmacies	8533	384	462	-	613
Hospitals	753	276	74	-	223
Dispensaries	15193	444	1193	-	5634
Registered practitioners	438721	17560	43578	-	217460
Under graduate college	219	6	37	-	178
Post graduate college	57	3	8	-	31

Modified after Anonymous 2004, 2005.

*Kala *et al. Journal of Ethnobiology and Ethnomedicine*, 2006

More than 95% of the 400 plant species used in preparing medicine by various industries are harvested from wild populations in India (Uniyal, 2007). Harvesting medicinal plants for commercial use, coupled with the destructive harvest of underground parts of slow reproducing, slow growing and habitat-specific species, are the crucial factors in meeting the goal of sustainability (Kala et al., 2005; Ghimire et al., 2005). Furthermore, rising demand with shrinking habitats may lead to the local extinction of many medicinal plant species. The continuous exploitation of several medicinal plant species from the wild (Kala et al., 2003) and substantial loss of their habitats during past 15 years have resulted in population decline of many high value medicinal plant species (FAO, 2003). The primary threats to medicinal plants are those that affect any kind of biodiversity used by humans (Sundriyal et al., 1995; Rao et al., 2004). The weakening of customary laws, which have regulated the use of natural resources, are among the causes of threatening the medicinal plant species (Ghimire et al., 2005; Kala et al., 2005). There are many other potential causes of rarity in medicinal plant species, such as habitat specificity, narrow range of distribution, land use disturbances, introduction of non-natives, habitat alteration, climatic changes, heavy livestock grazing, explosion of human population, fragmentation and degradation of population, population bottleneck, and genetic drift (Kala et al., 2000; Weekley, 2001; Oostermeijer et al., 2003; Kala et al., 2005). Additionally, natural enemies (i.e., pathogens, herbivores, and seed predators) could substantially limit the abundance of rare medicinal plant species in any given area (Bevill et al., 1999; Dhyani and Kala, 2005).

An estimated 4,000 to 10,000 species of medicinal plants face potential local, national, regional or global extinction, with subsequent serious consequences for livelihoods, economies and health care systems (Hamilton et al., 2004). About 50% of the tropical

forests, the treasure house of plant and animal diversity have already been destroyed. In India, forest cover is disappearing at an annual rate 1.5mha/yr. What is left at present is only 8% as against a mandatory 33% of the geographical area. *The Red Data Book of India* has 427 entries of endangered species of which 28 are considered extinct, 124 endangered, 81vulnerable, 100 rare and 34 insufficiently known species (Thomas, 1997). To encounter over exploitation of natural resources and consequent threat to biodiversity, sustainable practices have been recommended and several world wide organizations have formulated specific guidelines for collection and sustainable cultivation of medicinal plants (Klingestein et al., 2006).

For developing the 'herbal industries', India possesses a rich diversity of medicinal plant species across the various forest types along an altitudinal gradient (as discussed in the use and diversity of medicinal plants). Such a high diversity of medicinal plants would be helpful for further scientific research on exploring their medical efficacy, value addition, and use in curing various old and new diseases (Kala et al., 2004). India has already established a reputation as a low-cost manufacturer of high quality generic drugs in the global market (Mashelkar et al., 2005). Recently the US Government has established the "Office of Alternative Medicine" at the National Institute of Health at Bethesda and its support to alternative medicine includes basic and applied research in traditional systems of medicines such as Chinese, Ayurvedic, etc. with a view to assess the possible integration of effective treatments with modern medicines.

Plants synthesize and preserve a variety of biochemical products, many of which are extractable and used as chemical feed stocks or as raw material for various scientific investigations. Many secondary metabolites of plant are commercially important and find use in a number of pharmaceutical compounds. However, a

sustained supply of the source material often becomes difficult due to the factors like environmental changes, cultural practices, diverse geographical distribution, labour cost, selection of the superior plant stock and over exploitation by pharmaceutical industry. Plants, especially used in Ayurveda can provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and /or reduced toxicity. The small fraction of flowering plants that have so far been investigated have yielded about 120 therapeutic agents of known structure from about 90 species of plants. Some of the useful plant drugs include vinblastine, vincristine, taxol, podophyllotoxin, camptothecin, digitoxigenin, gitoxigenin, digoxigenin, tubocurarine, morphine, codeine, aspirin, atropine, pilocarpine, capscicine, allicin, curcumin, artemesinin and ephedrine among others. In some cases, the crude extract of medicinal plants may be used as medicaments. On the other hand, the isolation and identification of the active principles and elucidation of the mechanism of action of a drug is of paramount importance. Hence, works in both mixture of traditional medicine and single active compounds are very important. Where the active molecule cannot be synthesized economically, the product must be obtained from the cultivation of plant material. About 121 (45 tropical and 76 subtropical) major plant drugs have been identified for which no synthetic one is currently available. The scientific study of traditional medicines, derivation of drugs through bioprospecting and systematic conservation of the concerned medicinal plants are thus of great importance.

MEDICINAL IMPORTANCE OF *PLUMBAGO ZEYLANICA* L.

A cursory survey of the local flora and available literature indicated that *Plumbago zeylanica* belonging to the family Plumbaginaceae, a perennial subscandent shrub indigenous to Southeast Asia, is endowed with unique medicinal properties (**Fig 1**).

Roots and root barks of this plant are the most frequently used plant parts (Anonymous 1989) which have traditionally been used for the treatment of various ailments, such as dyspepsia, piles, diarrhea, skin diseases, leprosy, and rheumatism (Kirtikar and Basu, 1993). Roots are also reported to posses antibacterial, antifungal, abortifacient (Didry et al., 1994; Uma Devi et al., 1999), and vescicant diuretic properties (Anonymous 1989) and further may be used as a substitute for Cantharides (Chetia and Handique 2000). The pharmacological importance of this perennial shrub lies in its ability to produce a naphthoquinone called plumbagin (Modi, 1961) mainly in its roots. Plumbagin (5-hydroxy-2-methyl,1-4 naphthoquinone) shows a broad range of pharmaceutical activities such as anticancer (Krishnaswami and Purushothaman, 1980; Parimala and Sachdanandam, 1993; Naresh et al., 1996; Singh et al., 1997; Sugie et al., 1998; Hazra et al., 2002), cardiotonic (Itoigawa et al., 1991) radiomodifying (Uma Devi et al., 1999) leishmanicidal (Kayser et al., 2000), antimicrobial (Didry et al., 1994; Wang et al., 2005), antifertile (Bhargava, 1984), antimalarial (Likhitwitayawuid et al., 1998), antimutagenic and insecticidal (Kubo et al., 1983) properties. These properties of plumbagin have enhanced the demand of this medicinal plant for its roots. Previous experiments to obtain this quinone *in vitro* showed that plumbagin was best synthesized and accumulated in the cell suspension cultures of *P.zeylanica* (Heble et al., 1974). More than 32 patents involving plumbagin were obtained in the United States and many of these patents involve polymer scale prevention agents (US Patent and Trademark Office 1999). Due to the presence of natural naphthoquinone, *P.zeylanica* is much sought after in western countries as *Chlorophytum borivilianum* for saponin content (Chaplot et al., 2005). *In vitro* production of plumbagin from *P.zeylanica* is receiving more attention because conventional propagation of this plant is rather difficult and insufficient to meet

the growing demand (Anonymous, 1989). Propagation through seed is unreliable due to poor seed quality, erratic germination and seedling mortality as under natural field conditions. Extensive and destructive harvesting of plants by the pharmaceutical industries for procurement of naturally occurring secondary metabolites (plumbagin) from the plant and insufficient attempts to either allow its replenishment or its cultivation have led to the depletion of the natural plant population. Very few reports on cultivation, breeding and improvement programmes and *in vitro* studies of *P.zeylanica* are available despite its commercial importance. So the intervention of modern biotechnological approaches may be necessary.

Reports on plant regeneration from callus cultures of *P.zeylancia* (Rout et al., 1999) and clonal multiplication of *P.indica* (Gradner et al., 1991; Chetia and Handique, 2000) *P.rosea* (Satheesh Kumar and Bhavanandan, 1988; Binoy Jose et al., 2007) through various culture systems leading to large scale production of plants had been published earlier. Establishment of an efficient and reproducible organogenic system in such important medicinal plant species is an important pre-requisite not only for basic research and commercial exploitation of such important medicinal plant species but also shows significant promise for any future genetic transformation studies. Recently, a thoughtful approach has been made in this direction involving *P.zeylanica* (Selvakumar et al., 2001) which although fulfills the aim of conservation of this species, but leaves space for further improvement in terms of the rate of multiplication and relative time requirement. Furthermore, as the medicinally active constituents of *P.zeylanica* are generally obtained from the root tissues and since plants grow quite slowly and takes long time until the roots are suitable for use (Kitanov and Pashankov, 1994), it is advisable to search for alternative sources of plumbagin. Evaluation and exploitation of the *Agrobacterium rhizogenes*

mediated hairy root cultures of this important plant species may result in elevated levels of plumbagin production. In wide range of medicinal plants, hairy root cultures have already proven to be an efficient alternative production system for root derived phytochemicals of consistent quality within much shorter time than is commonly expected from *in vivo* grown plants (Tepfer, 1989; Wysokinska and Chimel, 1997; Banerjee et al., 1998; Canto-Canche and Loyola-Vargas, 1999; Flores et al., 1999; Shanks and Morgan, 1999).

So the present study was undertaken in an attempt to standardize an efficient protocol for induction and establishment of hairy root cultures leading to the bioproduction of bioactive molecule plumbagin in *P.zeylanica*. This may not only provide an alternative production system but may also indirectly facilitate conservation of this important medicinal plant species by sparing its indiscriminate uprooting. This study reports (i) a rapid and reproducible method for in vitro organogenesis in *P.zeylanica* from nodal explants and (ii) the successful induction and establishment of *A.rhizogenes* mediated hairy root cultures with higher potential for *in vitro* plumbagin production.

TISSUE CULTURE

In-vitro propagation of plants holds tremendous potential for the production of high-quality plant-based medicines (Murch et al., 2000). This can be achieved through different methods including micropropagation. Micropropagation or tissue culture is basically defined as *in vitro* growth of plantlets from any part of the plants in suitable nutritive culture medium of both organic and inorganic substances at a specific proportion. The application of tissue culture for large- scale plant production meant for commercial purposes is

well demonstrated in the case of several agri-crops and horticulture species. Many varieties have grown remarkably well *in vitro*, allowing various kinds of experimental manipulations. In many instances, the objectives of the investigations have been more than fulfilled and many solutions have been found to overcome the problems of plant growth, differentiation and morphogenesis. Micropropagation has many advantages over conventional methods of vegetative propagation, which suffer from several limitations (Nehra and Kartha, 1994). With micropropagation, the multiplication rate is greatly increased. It also permits the production of pathogen-free material. Micropropagation of various plant species, including many medicinal plants, has been reported (Murashige et al., 1978; Withers and Anderson, 1986; Skirvin et al., 1990). Propagation from existing meristems yields plants that are genetically identical with the donor plants (Hu and Wang, 1983). Numerous factors are reported to influence the success of *in-vitro* propagation of different medicinal plants (Hussey et al., 1980; Hu and Wang, 1983; Bhagyalakshmi and Singh, 1988; Short and Roberts, 1991). The most outstanding premiums offered by aseptic propagation (tissue culture) technology over the conventional methods are;

- Unlike the conventional methods of plant propagation, micro propagation of even temperate species may be carried out through out the year.
- In a relatively short time and space large number of plants can be produced, starting from single explant.
- Tissue cultured plants are generally free from fungal and bacterial diseases. Virus eradication and maintenance of plants in a virus-free-state are also readily achieved in tissue culture.

BIOPRODUCTION OF SECONDARY METABOLITES

Secondary metabolites are of low molecular weight compounds and they are often restricted to special plant families or even genera. They are not important for the primary metabolism of the plant, but in many cases of great importance for the plant to survive in its environment. Secondary metabolites contain a wide range of chemical compounds, which perform a variety of functions and including pharmaceuticals, flavors, fragrance, colours and insecticides. These compounds belong to a group collectively known as secondary products or secondary metabolites that are found only in specific organisms, or groups of organisms.

Secondary metabolites have high economical and pharmacological importance and the industries are deeply interested in large variety of chemicals being produced by plants due to their high efficacy and low toxicity. Higher plants contain a variety of substances, which are useful medicines, food additives, perfumes etc. A wide variety of plant secondary metabolites have value as pharmaceuticals, food colors, flavors and fragrances. Plant pharmaceuticals include taxol, camptothecin, genistein, codeine, morphine, quinine, shikonin, ajmalacine and serpentine. Food product examples are anthocyanins, saffron, vanilla, mint and a variety of fruit and vegetable flavours. However, decreased plant resources increased labor cost and other problems in obtaining these high-value substances from natural plants have pointed towards the use of plant cell cultures for production of the products. Because, plant cell culture is not affected by changes in such environmental conditions such as climate or natural degradation, improved production may be available in any place or season. Therefore, studies on the production of useful metabolite by plant cells and organ cultures (root and hairy roots) have been carried out in an increasing scale since the end of the 1950's and early 1960's in

the U.S, Canada and Europe. Their results stimulated more recent studies on the industrial applications of this technology in many countries.

Since plant cells can be cultivated in various fermentors in a similar way to microbial fermentations, many industrial companies in Japan have tried to apply this technology for commercial production of useful compounds. Since the feasibility of producing useful compounds in plant cell cultures was first recognized in 1970s, a number of researchers in both academic and industrial circles have been struggling to apply the great biosynthetic potential of plant cells for commercial production. However, despite more than 20 years of effort, the compounds that are available now or are about to be produced commercially are very limited. Notable examples in relation to commercial production through cell suspension cultures are shikonin from *Lithospermum erythrorhizon* (Fujita *et al.*, 1981) and ginsenosides from *Panax ginseng* (Ken Hibino and Ushiyama, 1999). The production cost of metabolites is one of the problems because of the low productivity of cultured plant cells. In order to decrease the cost, increase of production efficiency per cell is an essential factor, which means that higher amounts of products must be produced as quickly as possible. Industrial application of plant cell culture has been limited by drawbacks including slow growth rates; low product yields, and poorly understood metabolic regulation. Plant cell growth rates and levels of secondary metabolites produced are strongly influenced by key effectors variables such as carbon source, nitrogen source, hormones, precursor compounds and pH. However, efforts to increase production levels typically rely on trial-and-error studies. Because of the large number of potential effector combinations, genetic instability of the differentiated plant cells and slow growth rates, screening studies in shake flasks or petri dishes can be very labor intensive and time consuming. In addition genetic instability of

plant cell suspension cultures during cultivation is yet another problem compounding the production of active metabolites. Therefore some works have favored the development of organ cultures as a superior system for the production of target compounds (Banarjee et al., 1998).

GENETIC TRANSFORMATION AND HAIRY ROOT CULTURE

The recent advances and developments in plant genetics and recombinant DNA technology have helped to improve and boost research into secondary metabolite biosynthesis. A major line of research has been to identify enzymes of a metabolic pathway and then manipulate these enzymes to provide better control of that pathway. Transformation is currently used for genetic manipulation of more than 120 species of at least 35 families, including the major economic crops, vegetables, ornamental, medicinal, fruit, tree and pasture plants, using *Agrobacterium* mediated or direct transformation methods (Birch et al., 1997). However, *Agrobacterium*-mediated transformation offers several advantages over direct gene transfer methodologies (particle bombardment, electroporation, etc), such as the possibility to transfer only one or few copies of DNA fragments carrying the genes of interest at higher efficiencies with lower cost and the transfer of very large DNA fragments with minimal rearrangement (Gheysen et al., 1998; Hansen and Wright, 1999; Shibata and Liu, 2000). The gram-negative soil bacteria, *A.tumefaciens,* and the related species, *A.rhizogenes* are causal agents of the plant diseases crown gall tumor and hairy root, respectively. These species, which belong to the *Rhizobiaceae* (Kersters and Ley, 1984), are natural engineers that are able to transform or modify, mainly dicotyledonous plants, although there are reports on the infection of monocotyledonous plants (Tepfer et al., 1990; Hiei et al., 1994; Ishida et al., 1996).

Virulent strains of *A.tumefaciens* and *A.rhizogenes* contain a large megaplasmid (more than 200 kb) which plays a key role in tumor induction and for this reason it was named Ti plasmid, or Ri in the case of *A.rhizogenes.* During infection the T-DNA, a mobile segment of Ti or Ri plasmid, is transferred to the plant cell nucleus and integrated into the plant chromosome. *A.tumefaciens* transfers the T-DNA into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease (Chilton et al., 1982; Binns and Thomashow, 1988). T-DNA contains two types of genes: the genes encoding for the synthesis of opines, the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation.

Pioneering work in the early part of this century showed that a soil bacterium was responsible for a neoplastic outgrowth of fine roots at the site of infection. *A.rhizogenes* was subsequently identified as being the etiological agent of the disease (Ricker et al., 1930; Hilderbrand, 1934). *A.rhizogenes* has been used regularly for gene transfer in many dicotyledonous plants (Tepfer et al., 1990). Plant infection with this bacterium induces the formation of proliferative multibranched adventitious roots at the site of infection; the so-called 'hairy roots' (Chilton et al., 1982). This infection is followed by the transfer of a portion of DNA i.e. T-DNA, known as the root inducing plasmid (Ri-plasmid), to the plant cell chromosomal DNA. Species of *Agrobacterium are* plant pathogens, capable of interacting genetically with the susceptible plants including the neoplastic diseases (Hooykaas, 1984). More than 450 species of many different genera and families are known to be susceptible to the infection by *A.rhizogenes* (Porter, 1991) and the opines produced by infected plant tissues, act as a nutritive substrate for *A.rhizogenes* in the rhizosphere. A number of separate isolates of

A.rhizogenes have resulted in strains being classified according to opine type, with commonly used strains being of the agropine, mannopine or cucumopine types. Comparative mapping and DNA: DNA hybridization experiments indicated that all strains have highly conserved "core" DNA, which is essential for hairy root formation (Filetici et al., 1987).

The research is going for the application of plant transformation and genetic modification using *A. rhizogenes*, in order to boost production of those secondary metabolites, which are naturally synthesized in the roots of the mother plant. Transformed hairy roots mimic the biochemical machinery present and active in the normal roots, and in many instances transformed hairy roots display higher product yields. Biosynthesis of secondary metabolites in transformed roots is genetically controlled as explained by Hamill and Rhodes (1988). It is strongly influenced by nutritional environmental factors (Payne et al., 1988). Fujita et al (1981) reported that composition of the culture medium affects both the growth and production of secondary metabolites. A major line of research has been to identify enzymes of a metabolic pathway and then manipulate these enzymes to provide better control of that large-scale bioreactor system.

Genetic transformation has been reported for various medicinal plants. Genetic transformation would be a powerful tool for enhancing the productivity of novel secondary metabolites of limited yield. Hairy roots, transformed with *A.rhizogenes*, have been found to be suitable for the production of secondary metabolites because of their stable and high productivity in hormone-free culture conditions. A number of plant species including many medicinal plants have been successfully transformed with *A.rhizogenes* (Naina et al., 1989; Yun et al., 1992; Cucu et al., 2002; Coroch et al., 2002). Thus, these

transformed hairy roots have great potential as a commercially viable source of secondary metabolites. These roots can be cultured in hormone-free medium and there are several examples of enhanced accumulation of secondary products, relative to non-transformed tissue (Flores et al., 1986; Jung et al., 1987; Yoshikawa and Furuya, 1987). Hairy roots grow rapidly, show plagiotropic growth, and are highly branched on phytohormone-free medium. The transformed root is highly differentiated and can cause stable and extensive production of secondary metabolites, whereas other plant cell cultures have a strong tendency to be genetically and biochemically unstable and often synthesize very low levels of useful secondary metabolites (Rhodes et al., 1990; Merkli et al., 1997; Kittipongpatana et al., 1998). Most importantly, *A.rhizogenes* can transfer T-DNA from binary vectors and enable the production of transgenic plants containing foreign genes carried on a second plasmid. This property has been used to produce transgenic plants (Tepfer et al., 1984; Christey et al., 1992). The biotechnological application of hairy root cultures is promising for a number of reasons:

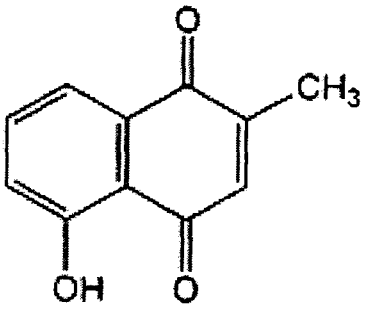
- Stable, high level production.
- Fast auxin-independent growth, and
- The suitability for adaptation to fermentor systems.

It is, therefore one of the most feasible techniques from an industrial point of view. Similar to cell cultures, root and hairy cultures have been identified as a potential source for the production of root specific bioactive compounds (Flores, 1987; 1992; Hamill et al., 1993; Banerjee et al., 1995). In terms of industrial application as biological catalysts, root cultures have been shown to display stable long term product profiles which will reduce the amount of selection required to maintain high producing culture lines. In addition to metabolic stability, roots have also shown extended

physical stability under culture conditions e.g. root cultures of *Lithospermum erythrorhizon have* been successfully maintained in a production state as far as 220 days (shimomura *et al.*, 1991). Shi and Kintzios (2003) have reported genetic transformation of *Pueraria phaseoloides* with *A.rhizogenes* and puerarin production in hairy roots. The content of puerarin in hairy roots reached a level of 1.2 mg/ g dry weight and was 1.067 times the content in the roots of untransformed plants. Thus these transformed hairy roots have great potential as a commercially viable source of secondary metabolites.

MEDICINAL IMPORTANCE OF PLUMBAGIN AND ITS ANTICANCER PROPERTY

Quinones represent the largest class of quinoid compounds, which are widely distributed in nature. Many quinones have been identified to have various biological activities, including anticancer activity (Prasad et al., 1981; Thompson et al., 1987; Noto et al., 1989; Nutter et al., 1991). Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone) a naturally occurring yellow pigment, is found in the plants of the *Plumbaginaceae, Droseraceae, Ancestrocladaceae, and Dioncophyllaceae* families (Sandur et al., 2006).



Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone)

Plant parts from the above families have been used in India, China, and other Asian countries for the treatment of rheumatoid arthritis, dysmenorrheal, contrusion of extremities and cancer. The root of *P.zeylanica* (also called chitrak) a major source of plumbagin, has been

used in the Indian medicine since the period of charaka, from 750 BC, as an antiatherogenic, cardiotonic, hepatoprotective and neuroprotective agent (Padhye and Kulakarni, 1973; Tilak et al., 2004). The active principle, plumbagin was first isolated in 1829 (D' Astafort et al., 1829). Plumbagin is also present along with a series of other structurally related naphthoquinones in the roots, leaves, barks and wood of *Juglans regia* (English walnut, Persian walnut, and California walnut), *Juglans Cinerea* (butternut and white Walnut) and *Juglans nigra* (black walnut) (Hedin et al., 1980; Binder et al., 1989). Preparations derived from black walnut have been used as hair dyes, and skin colorants in addition to being applied topically for the treatment of acne, inflammatory diseases, ring worm, and fungal, bacterial and viral infections (Inbaraj and Chignell, 2004).

Plumbagin present in *P.zeylanica* and *P.rosea* has been prescribed for cancer in the "Siddha" system of medicine (Mudaliar, 1969). Plumbagin isolated from the root of *P.zeylanica* has been shown to exert anticancer and anti proliferative activities in animal models as well as in cells in culture (Krishnaswami and Purushothaman, 1980; Parimala and Sachdanandam, 1996; Naresh et al., 1996; Singh et al., 1997; Hazra et al., 2002). Sugie et al (1998) have shown that plumbagin significantly inhibited azoxymethane induced intestinal carcinogenesis in rats, suggesting its chemopreventive activity. Plumbagin has also been shown to induce S-G_2/M cell cycle arrest through the induction of p21 (an inhibitor of cyclin-dependent kinase (Jaiswal et al., 2002). It has been found to inhibit the growth of Raji, Calu-1, HeLa and Wish cell lines (Lin et al., 2003). An earlier report showed that plumbagin has a chemotherapeutic potential as an anticancer agent in ovarian cancer cells with the mutated BRCA1 gene (Srinivas et al., 2004). Earlier studies also showed that plumbagin induces apoptosis in human lung cancer A549 cells through JNK/P^{53} pathways (Hsu et al., 2006) and caused autophagic cell death in

breast cancer by P13K/AKT inhibition (Kuo et al., 2006). Plumbagin has been reported to inhibit the activity of Topoisomerase II (Fuji et al., 1992). Plumbagin due to the presence of quinone moiety has been reported to generate ROS (Inbaraj and Chignell, 2004) and ROS has been implicated to play a role in the apoptosis process (Srinivas et al, 2004). In a very recent report plumbagin was shown to induce cell cycle arrest and apoptosis through reactive oxygen species in human melanoma A375.S2 cells (Wang et al., 2007). In addition to all these facts plumbagin does not exert an apoptotic effect on normal cells and therefore can be developed as an anticancer drug (Hsu et al., 2006, Sandur et al., 2006; Kawiak et al., 2007). **Table.3** summarizes some of the anticancer studies of plumbagin.

Traditional medicine, although claimed to be safe and efficacious, in most cases neither the chemical entity nor the molecular mechanism of action are well defined. Even though there are reports of plumbagin having antitumor activity, there are only very few studies on the mechanism of cell death induced by plumbagin in human cancer cells and no unifying mechanism encompassing all of these data exist. So elucidation of the mechanism(s) by which plumbagin induces its anticarcinogenic and antiproliferative effect is necessary to provide a solid foundation for its use as an agent for anticancer prevention strategies. It is well known that most of the currently used anticancer agents induce apoptosis in tumor cells. The role of reactive oxygen species in mediating the apoptotic process is being increasingly recognized. A variety of anticancer agents induce apoptosis through the generation of ROS (Mizutani et al., 2002; Chang et al., 2005; Kim et al., 2005). Recent reports indicate that cell death triggered by ROS is not only a result of cumulative oxidative damage but can be a consequence of a more subtle role of ROS, that of regulatory molecules (Junn et al., 2000). ROS are capable of modifying the structure and function of proteins in defined ways and a variety of

proteins, including transcription factors, protein tyrosine phosphatases and molecular chaperons are regulated through the response to the redox status in the cell (Barford, 2004). All aerobic organisms are subject to physiologic oxidant stress as a consequence of aerobic metabolism. Aerobic respiration coupled to the generation of ATP leads to the formation of the superoxide anion radical ($O_2^{-\cdot}$). Superoxide anion radicals can then form other reactive oxygen species such as hydrogen peroxide H_2O_2 and the highly reactive hydroxyl radical ($OH^{\cdot}$) (Feig, 1994; Schumaker et al., 2006). ROS normally exist in all aerobic cells in balance with biochemical antioxidants, which convert ROS into water (Kondo et al., 2006; Ham et al., 2006). Oxidative stress occurs when this critical balance is disrupted because of excess ROS production and/or antioxidant depletion (Kondo et al., 2006; Ham et al., 2006). Evidence is accumulating which indicates that many chemotherapeutic agents may be selectively toxic to tumor cells because they increase oxidant stress and enhance these already stressed cells beyond their limit (Kim et al., 2005; Moungjaroen et al., 2006).

Earlier studies have established that several Compounds of plant origin such as flavanoids, tannins, curcumin and the stilbene-resveratrol are themselves capable of inducing oxidative DNA damage either alone or in the presence of certain transition metal ions especially copper (Ahmad et al., 1992; Bhat and Hadi, 1994; Ahsan et al., 1998; Azmi et al., 2005). Studies have also shown that metals, particularly copper; are capable of mediating the activation of several compounds, such as benzoyl peroxide, quercetin and dietary flavanoids by a redox mechanism leading to the formation of reactive oxygen and other organic radicals (Swauger et al.,1991; Ahmad et al., 1992). It was also previously shown that copper can induce the oxidation of 1,4 HQ, a benzene metabolite, producing 1,4BQ and H_2O_2 through a semiquinone intermediate (Li and Trush, 1993). Many

clinically important antitumor drugs contain quinone nucleus (Driscoll et al 1974). The antitumor and cytotoxic effects of quinoid drugs are thought to be mediated through their one electron reduction to semiquinone radicals (Baucher et al., 1978).

Copper is an essential trace element which is distributed throughout the body (Linder, 1991). Besides forming the essential redox active centre in a variety of metalloproteins, Copper has also been found in the nucleus and to be closely associated with the chromosomes and DNA bases, particularly guanine (Kagawa et al., 1991). DNA associated copper has been suggested to be involved in maintaining normal chromosome structure and in gene regulatory processes (Furst et al., 1989; Prutz et al., 1990; Thiele et al., 1992). 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 is the nucleus (Agarwal et al., 1989) associated more specifically with DNA bases particularly guanine (Kagawa et al., 1991). Since copper is closely associated with DNA and 1,4-naphthoquinones are thought to induce oxidative damage as a consequence of their ability to undergo redox cycling (Munday et al., 1994), with the generation of reactive oxygen species, we therefore decided to investigate the DNA damage induced by copper mediated activation of plumbagin using calf thymus DNA, plasmid pBR322 DNA and also using a cellular system of lymphocytes isolated from human peripheral blood. Infact our study is the first to investigate the role of a metal ion (Copper) in the observed cytotoxic action of plumbagin.

Table 3. PLUMBAGIN AS CHEMOTHERAPEUTIC AGENT- SUMMARY

Tumor Cell Line	Results/Conclusions	References
Lymphocytic leukemia (P388)	Inhibition of cell growth	Krishnaswamy and Purushothaman, 1980
MC- induced Fibrosarcomas	Cell growth inhibition	Krishnaswamy and Purushothaman, 1980
BALB/c 3T3 H-ras cells	Inhibition of cell growth	Fuji et al., 1992
Dalton's Ascetic Lymphoma	Inhibition of cell growth	Kavimani et al., 1996
Ehrlisch ascites tumors and Sarcoma-180	Apoptosis induction, growth inhibition	Naresh et al., 1996
Ehrlich ascites tumor	Inhibition of cell growth	Sing and Udupa,1997
Melanoma (B16F1)	Inhibition of proliferation	Kini et al., 1997
Azoxymethane induced intestinal carcinogenesis	Decrease incidence & multiplicity of tumors	Sugie et al., 1998
Mouse Leukemia L5178Y	Inhibition of cell growth	Suzuki et al., 1998
Raji, Calu-1, HeLa & Wish tumor cells	Inhibition of cell growth	Lin et al., 2002
HaCaT keratinocytes	Decreased viability	Inbaraj & Chignell, 2003
HeLa-GFP cells	Apoptosis induction	Montoya et al., 2004
Ovarian cancer cells(BG-1)	Inhibition of cell proliferation	Srinivas et al., 2004
Human embryonic kidney cells(HEK 293) & brain tumor cells (LN229)	Inhibition of cell growth	Chang et al., 2004
Breast cancer cells(MCF-7) & Bowes cells (Melanoma)	Apoptosis induction and Growth inhibition	Nguyen et al., 2004
Cervical cancer cells ME-180	Apoptosis induction	Srinivas et al., 2004
Human chronic myeloid leukemia(KBM-5) and Lung adenocarcinoma (H1299)	Inhibition of cell growth	Sandur et al., 2006
Promyelocytic leukemia (HL-60)	Apoptosis induction	Kawiak et al., 2006
Human non small cell lung cancer cells(A 549)	Apoptosis induction	Hsu et al., 2006
Human melanoma (A375.S2 cells)	Apoptosis induction and growth inhibition	Wang et al., 2007
Cervical cancer cells C33A	Apoptosis induction	Nair et al., 2008

APOPTOSIS INDUCING PROPERTY OF PLUMBAGIN

The term programmed cell death was introduced in 1964, proposing that cell-death during development is not of accidental nature but follows a sequence of controlled steps leading to locally and temporally defined self-destruction (Lockshin, 1964). The apoptotic mode of cell death is an active and defined process that plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions. It should be stressed that apoptosis is a well-defined and possibly the most frequent form of programmed cell death, but that other, non-apoptotic types of cell death also might be of biological significance (Leist, 2001).

Properties of carcinogenic agents (chemical agents as well as radiations) are the growth-inhibition power and the ability to induce cell death. These properties are widely used in anticancer chemo and radiotherapies. By contrast, the mechanisms relating cell death and cancer induction have received scarce attention. In general the cell death induced by radiations and carcinogenic agents has been hastily ascribed to toxicity, even if toxic compounds produce a different response. Given the great progress in basic cancer research, it should be wise to reconsider the relationship between cell death and cancer development by using emerging technologies to create new conceptual paradigms.

In the last decade, basic cancer research has produced remarkable advances in our understanding of cancer biology and cancer genetics. Among the most important of these advances is the realization that apoptosis and the genes that control it have a profound effect on the malignant phenotype. For example, it is now clear that some oncogenic mutations disrupt apoptosis, leading to

tumor initiation, progression or metastasis. Conversely, compelling evidence indicates that other oncogenic changes promote apoptosis, thereby producing selective pressure to override apoptosis during multistage carcinogenesis. The life span of both normal and cancer cells within a living system are regarded to be substantially affected by rate of apoptosis. In addition, apoptosis is a discrete way of cell death, different from necrotic cell death and is regarded as an ideal way of cell elimination. In recent years, many cancer chemotherapeutic agents have been shown to induce apoptosis (John et al., 1992; Fesus et al., 1995; Samaha et al., 1997) and conversely, several tumor promoters have also been shown to inhibit apoptosis (Wright et al., 1994). There are pro-apoptotic genes (e.g. Caspase-3, 8, 9) and anti-apoptotic genes (e.g. Bcl-2, Bcl-xL, Bcl-w) within a cell and the expression of each group of genes are tightly regulated by a complex mechanism. A central theme in much of cancer research today is the ability of tumor cells to resist apoptosis in response to triggers that typically induce cell cycle arrest or death in their untransformed counterparts.

Plumbagin is thought to be a redox cycling compound that generates superoxide, a reactive species that can damage various biomolecules (Imlay and Fridovich, 1992). There is an emerging evidence that plumbagin induce oxidative damage as a consequence of their ability to undergo redox cycling [Munday et al., 1994], with the generation of reactive oxygen species, which mediates apoptosis in cancer cells (Jaiswal et al., 2002; Montoya et al., 2004; Srinivas et al., 2004; Hsu et al., 2006; Sandur et al., 2006; Kawiak et al., 2007; Wang et al., 2007; Nair et al., 2008; Powolny and Singh, 2008). In addition to all these facts, recent studies have also established that plumbagin does not exert an apoptotic effect on normal cells and therefore can be developed as an anticancer drug (Hsu et al., 1998; Sandur et al., 2006; Kawiak et al., 2007). So in this study we also

investigate the cytotoxic action of plumbagin in two cancer cell lines and the aim of this study was to determine whether plumbagin induces cell death in cancer cell lines (A431 and DLA), exhibit any biochemical characteristics of apoptosis and to check whether free radical scavengers such as SOD, catalase, thiourea and also neocuproine (a Cu(I) specific sequestering agent) can reverse the cytotoxic effects of plumbagin, in order to further establish the mechanism of cell death induced by plumbagin.

CHAPTER-I

Bioproduction, Isolation, Identification and Characterization of Plumbagin from *Plumbago zeylanica* L.

MATERIALS & METHODS-I

MATERIALS & METHODS-I

Plant material

2-3 weeks old young shoots of *Plumbago zeylanica* L. (**Fig 1**) collected from the field grown plants were served as the source of explants. A voucher specimen has been deposited at the Institute herbarium (TBGT), under registration number 26390.

Medium Used

Organic and inorganic salts of Murashige and Skoog (MS) (1962) agar (0.6% w/v) medium supplemented with different concentrations of hormones (BAP, NAA and IAA) were used for the complete experiments.

Glassware and Chemicals

All the glassware used in the experiments were made of borosilicate glass (Borosil India Ltd., Bombay). They were cleaned with chromic acid (potassium dichromate in concentrated sulphuric acid) or 1% Teepol (Glaxo India Ltd, Bombay) before washing in tap water and thoroughly rinsing with glass distilled water. After draining the water on a drain board, the vessels were dried at 180^0C for at least 8 h in a temperature controlled hot air oven before use. The chemicals used were from different sources. All the salts used for media preparation and silica gel were supplied by Merck. Vitamins and plant growth regulators were obtained from Sigma Chemical Co. USA. All other chemicals were commercial products of analytical grade.

Fig 1. Field grown plant of *Plumbago zeylanica* L.

Surface Sterilization

The MS media used for the micropropagation is properly closed with a suitable bacteria proof closure and the vial is autoclaved at 1.5 Kg/cm^{-2} (121^0C) for 20 min from the time the medium reaches the required temperature. Petri dishes, conical flasks, culture tubes, chemicals etc were sterilized together with the medium. The laminar airflow cabinet is sterilized using UV light. The instruments used for aseptic manipulation such as forceps and scalpels were sterilized by dipping in 90% ethanol followed by flaming and cooling. The plant materials were washed in commercial detergent Teepol (Glaxo India Pvt.Ltd. Mumbai), treated with antifungal reagent (sterlique) for 7-10 min and then by running tap water. The explants were transferred to the laminar airflow and immersed in 0.1% (w/v) $HgCl_2$ for 5 min followed by several rinses in sterile distilled water.

Culture Initiation

Young tender twigs (2-3 week old) collected from plants that were grown in the field were served as the source of explants. Defoliated young shoot cuttings of 4-5 cm lengths were washed first under running tap water (30 min) and treated with 0.2% (v/v) Teepol for 10 min followed by repeated rinsing with distilled water. Then it was rinsed with sterile distilled water and further sterilization was done under aseptic conditions in a Laminar Airflow Hood (Klenzaids, Bombay). Explants were surface sterilized with 50% (v/v) ethanol (1 min) and followed by 0.01% (w/v) $HgCl_2$ (3 min). Finally, the explants were washed thoroughly (three-five times) with sterilized distilled water. After trimming both cut ends, explants of 1.0-1.5 cm long with single node were vertically implanted into the medium consisted of full strength inorganic and organic salts of Murashige and Skoog (MS) (1962) solid medium supplemented with different plant

growth regulators (PGRs). The medium was solidified with 0.6% (w/v) agar. For all the treatments, MS medium supplemented with 3% sucrose and varied concentrations and combinations of BAP (6-benzylaminopurine) 0.5-3.5 mgl^{-1}, or permutative combinations of BAP and 0.1-2.0 mgl^{-1} IAA (Indole-3-acetic acid) were used and pH of the medium was adjusted to 5.8 using 1N NaOH/IN HCl before adding agar 0.6% (w/v) (Merck, India). After dissolving the agar by boiling, the medium was dispensed in 15 ml aliquots into 20x150 mm culture tubes and autoclaved at 121^0C and 1.5 kg/cm^2 for 18 min. All the cultures were maintained under 12 h cool white fluorescent tubes (Phillips, India) (30-35 μEm^2s^{-1}) at 26 ± 2^0C. For each treatment twenty replicates were used and repeated all the experiments at least twice.

Shoot Multiplication and Elongation

For shoot multiplication, shoot buds of 1.0-2.5 cm initiated upon the nodal explants were dissected out and transferred to MS solid medium supplemented with 3% sucrose and different concentrations of BAP alone (0.5-3.5 mgl^{-1}) and combinations of BAP (1.0-3.5 mgl^{-1}) plus 0.1-2.0 mgl^{-1} œ-naphthalene acetic acid (NAA) or 1.0-3.5 mgl^{-1} BAP plus 0.1-2.0 mgl^{-1} Indole-3-acetic acid (IAA).

After 3 weeks of subculture, clusters of shoots multiplied in media supplemented with BAP were divided and clumps of 2-3 shoots of 0.5-1.5 cm length were transferred to basal solid MS media supplemented with reduced level of BAP 0.5-1.5 mg^{-1} for shoot elongation. After 2 weeks of shoot elongation, 3.0-4.5 cm long shoots were used for hairy root induction using wild strains of *Agrobacterium rhizogenes*.

Induction of Hairy Root Cultures

Bacterial Strains

Three different strains *of A.rhizogenes* viz, **A4, TR105 and 15834,** procured from Prof. Pauline M Doran of University of South Wales, USA were used for infection to induce hairy root formation in *P.zeylanica.* The bacterial strains were grown on yeast extract mannitol (YEM) medium at 25±2°C **(Fig 2).**

Bacterial Culture Medium

Yeast Extract Mannitol (YEM) medium was used for developing the bacterial cultures. Chemicals and yeast extract were weighed and dissolved in distilled water. After making up the medium to the desired volume, the pH of the medium was adjusted to 7.0 using IN NaOH/IN HCl before adding 1.5% (w/v) agar. The medium was autoclaved at 1.5 Kg/cm^{-2} (121^0C) for 20 min. The autoclaved medium was poured to the disposable plastic petri-dishes for bacterial plating.

Subculture of Bacteria

Bacteria from the master plates were carefully transferred to YEM broth in the 100 ml conical flasks and kept the cultures on a Gyratory shaker (G-10) (New Brunswick Scientific Co; USA) for overnight incubation at 25^0C and after that a loop full of cultured broth was taken and streaked on separate petri dishes containing YEM agar (1.5% w/v) medium and again incubated overnight at 25^0C. These bacterial cultures were used for inducing hairy roots on aseptic shoots of *P.zeylanica.*

Pre-incubation Media

Agar (Merck, India) at a concentration of 1.0% (w/v) was added to 100 ml distilled water, melted and poured 20-25 ml in fine bottles and autoclaved at 1.5 kg cm^2 pressure for 20 min at 121^{o}C.

Methodology

Aseptic shoots (3.0-4.5 cm long) of *P.zeylanica* already developed were transferred to the pre-incubation media and kept for 2 days before bacterial infection. Scalpel method (Ooms *et al.*, 1985) was employed for inducing hairy roots on aseptic shoots. Overnight grown bacterial cultures were introduced into shoots by making small wounds using sterile scalpel blade and shoots wounded without bacterial infection were kept as controls. In each experiment at least six shoots were used for bacterial infection and six as control.

Decontamination of Hairy Roots

Hairy roots produced on the aseptic shoot cultures were carefully removed one by one and transferred to MS solid medium added with 500 mgl^{-1} antibiotic (Streptomycin) for 10-15 days. Two culture passages at an interval of 10 days were done to remove the bacteria from the hairy roots.

Establishment of Hairy Root Culture

After two-culture passages at 10-day intervals of incubation in antibiotic medium, the decontaminated hairy roots were transferred to MS solid medium supplemented with 3% sucrose and kept under complete dark. After 3-4 weeks, these roots were transferred to MS basal liquid medium for establishing the root culture under continuous agitation on a Gyratory shaker at 80 rpm.

Growth of Roots

An initial inoculum (~150 mg fwt) of decontaminated established fresh hairy roots were chopped and transferred to 250 ml conical flask filled with 60 ml MS basal medium devoid of agar and the cultures were kept on a G-10 Gyratory shaker at 80 rpm under complete darkness. Growth of roots was studied periodically at an interval of 5 days up to 40 days. Fresh weight (fwt) and dry weight (dwt) measurements were determined. Freshly harvested roots were subjected to complete drain of media using filter paper and measured the fresh weight using a top loading electronic balance. Dry weight (dwt) determination was done after drying the roots at room temperature for 48 h. Growth index of the hairy root cultures was determined at two week intervals from 2 weeks to 10 weeks of culture following the formula,

$$\text{Growth index (G.I)} = \frac{\text{Final fresh weight-Initial fresh weight}}{\text{Initial fresh weight}}$$

Isolation, Identification and Characterization of Plumbagin

Extraction of Plumbagin

Fresh hairy roots of *P.zeylanica* were harvested after different periods of culture, dried under room temperature and powdered. Samples of 1 or 2 g powder were extracted with chloroform for 8 h at room temperature until all the pigments were leached out of the samples. The chloroform extract was filtered through Whatman No.1 filter paper and the filtrate was concentrated under vacuum in Heildoph Rotavapor. The concentrated samples were used for chromatographic analysis.

Thin Layer Chromatography (TLC)

The crude plumbagin extracts from the dry hairy roots of *P.zeylanica* was analyzed by TLC using the method of Heble et al (1974). TLC plates were prepared using silica gel G-60 (Hi-Media Laboratories, Bombay) as the absorbent. A slurry of silica gel was prepared by mixing silica gel with distilled water in 1:2 (w/v) ratio and spread on 5 mm thick glass plates (20x20 cm) with the help of a TLC applicator which was adjusted to a thickness of 1.0 mm. The plates were dried at room temperature and activated at 100^0C for 30 min in a thermoregulated hot air oven before use. Benzene was used as the solvent to run TLC. Rectangular glass chromatography chambers (30 x 20 x 25 cm) were lined with filter paper to provide a saturated atmosphere. The chromatographic run was carried out at room temperature.

Standard plumbagin (Sigma Chemical Co., USA) and concentrated samples in 0.5 ml of chloroform extract were applied on TLC plates, 2 cm from the bottom of the plates using separate microsyringes. When the spots were thoroughly dried, the plates were placed in the chromatographic chambers and sealed tightly with the lid. The solvent was allowed to run until its front reached the top of the plate. The chromatograms were then removed, the solvent front was marked and allowed to dry. The co-chromatographed authentic sample of plumbagin was used to detect the presence of plumbagin in the sample lane on the TLC plate.

Purification and Quantification of Plumbagin

The bright yellow fluorescent band corresponding to the Rf value 0.67 of standard plumbagin was scraped using thin spatula and eluted with chloroform. After the removal of the sedimenting silica gel by

centrifugation, the final volume of the supernatant was adjusted to 10-20 ml depending on the intensity of yellow colour. The amount of plumbagin in the chloroform solution was determined by measuring absorbances in the UV (254 nm) and visible range (415 nm), using a Shimadzu 2100 UV/VIS spectrophotometer. The standard curve was drawn using standard plumbagin of various concentrations, which were run in the TLC and eluted as above. In each separate experiment, a known quantity was also run along with the sample to check the reproducibility of the extraction. The recovery was always more than 99 percent. Analar grade chloroform was used as the blank. On drying at room temperature, plumbagin present in chloroform solution eluted from TLC plates got crystallized were used for biochemical studies in the later part of this study. Additional evidences for the identity and purity of the compound were obtained from HPLC analysis.

High Pressure Liquid Chromatography (HPLC)

HPLC analysis was carried out on a Gilson 321 liquid chromatography system equipped with UV-absorbance detector (156 C) and injector as described by Crouch et al (1990). Kromasil C-18 column (245 mm x 4.6 mm) was used for the plumbagin analysis. The solvent system consisted of 80% methanol and aqueous 0.2 M acetic acid, the pH of acetic acid being adjusted to 3.5 with trimethylamine. The solvent was degassed before use and was prepared freshly for each experiment.

Preparation of Sample

Crystals of authentic plumbagin and TLC-eluted plumbagin from the root extracts were dissolved in absolute methanol, vortexed for 3 min, and was stored in a stoppered vial.

Identification of Plumbagin

Aliquot of suitably diluted sample in methanol (10 or 15 µl) was injected into the column to find the retention time. A linear gradient of methanol: aqueous buffer (80:20) over 20 min was used for fractionation and the eluates were monitored for absorbance maximum at 254 nm. Plumbagin present in the sample was identified by comparison of the retention time of the unknown peak with that of the standard.

Statistical Analysis

Data was subjected to analysis of variance (ANOVA) using a completely random design (CRB) and means were compared by Duncan's multiple range LSD test at 5% significance level.

RESULTS-I

RESULTS-I

Micropropagation of *Plumbago zeylanica* L.

Approximately 56% of the young nodal explants remained contamination free during culture initiation and efforts to reduce the percentage of contamination without affecting the viability and morphogenetic response of the explants by changing the surface sterilization procedures did not yield encouraging results. Shoot bud formation from the nodal explants was delayed up to 3 weeks in basal medium. In media supplemented with cytokinins, 95% of the contamination free explants readily responded with bud initiation in 5 days **(Fig 3)**

Among the individual concentrations of the cytokinins and combinations of cytokinins and IAA tested, BAP at 3.0 mgl^{-1} induced maximum number of shoots (3.5±0.5) with a length of 0.87 ± 0.14 cm in 95% of the contamination free nodal explants in 3 weeks (**Fig 4**). Higher concentrations of BAP (>3.0 mgl^{-1}) induced increased callusing and relatively less number of bud formation. The explants in medium containing BAP and IAA at varied concentration were produced shoot buds in a period of 10 days. Though the number of shoot buds in medium with BAP and IAA was slightly more compared to BAP alone medium, the shoots so proliferated were short (0.5-1.0 cm). Along with shoot bud initiation profuse callusing was also noticed in cultures grown in medium containing BAP (2.0-3.5 mgl^{-1}) and IAA (1.0-2.0 mgl^{-1}) after 20 days of culture.

Individual shoots of (1.5-2.0 cm) initiated on nodal segments in BAP medium after 3 weeks were dissected out and subcultured in MS agar medium supplemented with BAP alone (0.5-3.5 mgl^{-1}), BAP

(1.0-3.5 mgl^{-1}) and IAA (0.1-2.0 mgl^{-1}) or BAP (1.0-3.5 mgl^{-1}) and NAA (0.1-2.0 mgl^{-1}) were proliferated further in 2 weeks. Shoot bud proliferation was mostly from the base of shoots. Number of shoot buds/shoot was more in media containing combinations of BAP and NAA or BAP and IAA and it was observed that shoot proliferation was associated with callusing. Shoots subcultured to media containing only BAP, significant increase in multiplication of shoots occurred and an aggregate number of 7.41 ± 2.18 shoots was obtained in media supplemented with 2.0-3.0 mgl^{-1} BAP in a period of 4 weeks. It was interesting to note that shoot proliferation was continuous and after a period of 6-8 weeks, numerous shoot buds were obtained and each shoot had an average length of 1.5 cm. During the period of incubation in the same medium, there was slight callusing along with shoot proliferation. Due to callusing, isolation of shoots became difficult and elongation of shoots in the medium found to be restricted even the cultures were maintained for 8-10 months. For shoot elongation, shoots multiplied in the medium were carefully taken out and isolated individual shoots were transferred to MS basal solid medium supplemented with reduced concentration of BAP (< 0.5-1.5 mgl^{-1}). Shoot bud elongation was noticed after 2-3 weeks in medium containing 0.5 mgl^{-1} BAP and elongation of shoots was also observed in medium devoid of plant hormones. There was no significant difference in shoot elongation noted in basal and media containing reduced concentrations of BAP. The shoots so subjected to elongation were grown to 3.0-4.5 cm long after 5 weeks in the same medium (**Fig 5**). The aseptic shoots of 3.0-4.5 cm long derived from the nodal explants were used for the induction and establishment of hairy root cultures.

Induction and Establishment of Hairy Root Cultures

After 7 days of infection, small protuberances emerged at the wounded sites of aseptic shoots infected with bacteria and subsequently these structures differentiated into white cottony hairy roots in another period of 15 days (**Fig 6**). Hairy root formation was not observed in the control. From each incision, an average of 5-6 roots (2.0-3.0 cm) were emerged after 20 days of infection. Among the bacterial strains tested, best root formation was obtained on shoots infected with A4 strain in a period of 20 days. Whereas in the shoots infected with other two strains, root formation was delayed upto 20-30 days and frequency of rooting was also less (**Table 4**). Hairy root formation due to the integration of bacterial DNA was confirmed by comparing the root formation between control and infected shoots (**Fig 7**).

After two culture passages at an interval of 10 days in antibiotic medium, the roots transferred to MS basal solid medium supplemented with 3% sucrose under complete dark were started further elongation and lateral branching in 3-5 days. After 3-4 weeks, white cottony roots spreaded throughout the medium and these were taken out and transferred to 250 ml flask containing 60 ml MS basal liquid medium and kept under continuous agitation on a Gyratory shaker at 80 rpm for establishing the root culture (**Fig 8**). When compared to solid medium lateral root formation in liquid medium was slightly faster. After 25 days hairy roots established in MS basal liquid medium were chopped and transferred 150 mg fwt roots to 250 ml conical flask containing 60 ml MS liquid medium and kept the culture on G-10 gyratory shaker at 80 rpm for the detailed study on the growth and production of plumbagin over a period of 40 days.

Growth and biomass production of hairy root studied in detail at 5 days interval recorded a progressive growth of roots over a period of 40 days. It was noted that root growth was maximum in 30 days and beyond that roots became necrotic and declined further growth as evidenced from the growth index (GI) of the culture (**Fig 9**). After 35 days, the colour of the roots became brownish dark and ceased further growth. During the growth of the roots, plumbagin level in the roots was also determined at a concentration of 0.91% g dwt. It was noticed that plumbagin concentration in the hairy roots during the growth revealed parallel to the root biomass production. Plumbagin concentration in the roots remains same per unit weight and the results showed that plumbagin synthesis in hairy roots was more than the reported concentration (0.9% g dwt) of the compound in the tuberous roots of the field grown plant. Identity of the compound was confirmed by TLC, HPLC and UV spectral determinations.

Isolation, Characterization and Identification of Plumbagin

The crude chloroform extract subjected to TLC analysis revealed the presence of plumbagin having the same Rf value (0.67) as that of the standard sample (**Fig 10**). The bright yellow spot corresponding to authentic sample was scraped and the compound was eluted with chloroform. The compound eluted and purified with preparative TLC showed absorbance maximum at 415 nm and the values agreed well with the plumbagin standard (**Fig 11**). The identity of the compound as plumbagin was further confirmed by the identical retention time (8.30 min) of both the sample and standard as evident from HPLC elution profile (**Fig 12**).

Fig 2. Overnight grown bacterial culture (*A.rhizogenes*) on agar plate.

Fig 3. Single axillary shoot differentiated from a nodal explant after 5 days of culture in MS solid medium supplemented with 3% sucrose and 3.0 mg^{-1} BAP.

Fig 4. Multiple shoots formed on nodal explants in MS solid medium supplemented with 3.0 mg^{-1} BAP after 3 weeks.

Fig 5. Elongated shoots in MS solid medium containing 3.0 mg^{-1} BAP after 20 days.

Fig 6. Hairy roots initiated on aseptic shoots of *P.zelanica* after 15 days of Infection with *A.rhizogenes* (A4).

Fig 7. Aseptic shoot without bacterial infection (control) after 15 days.

Fig 8. Hairy roots established in MS basal liquid medium after 35 days of growth.

Fig 10. TLC of plumbagin (A) Plumbagin isolated from fresh hairy root cultures of *P.zeylanica* and (B) Authentic plumbagin

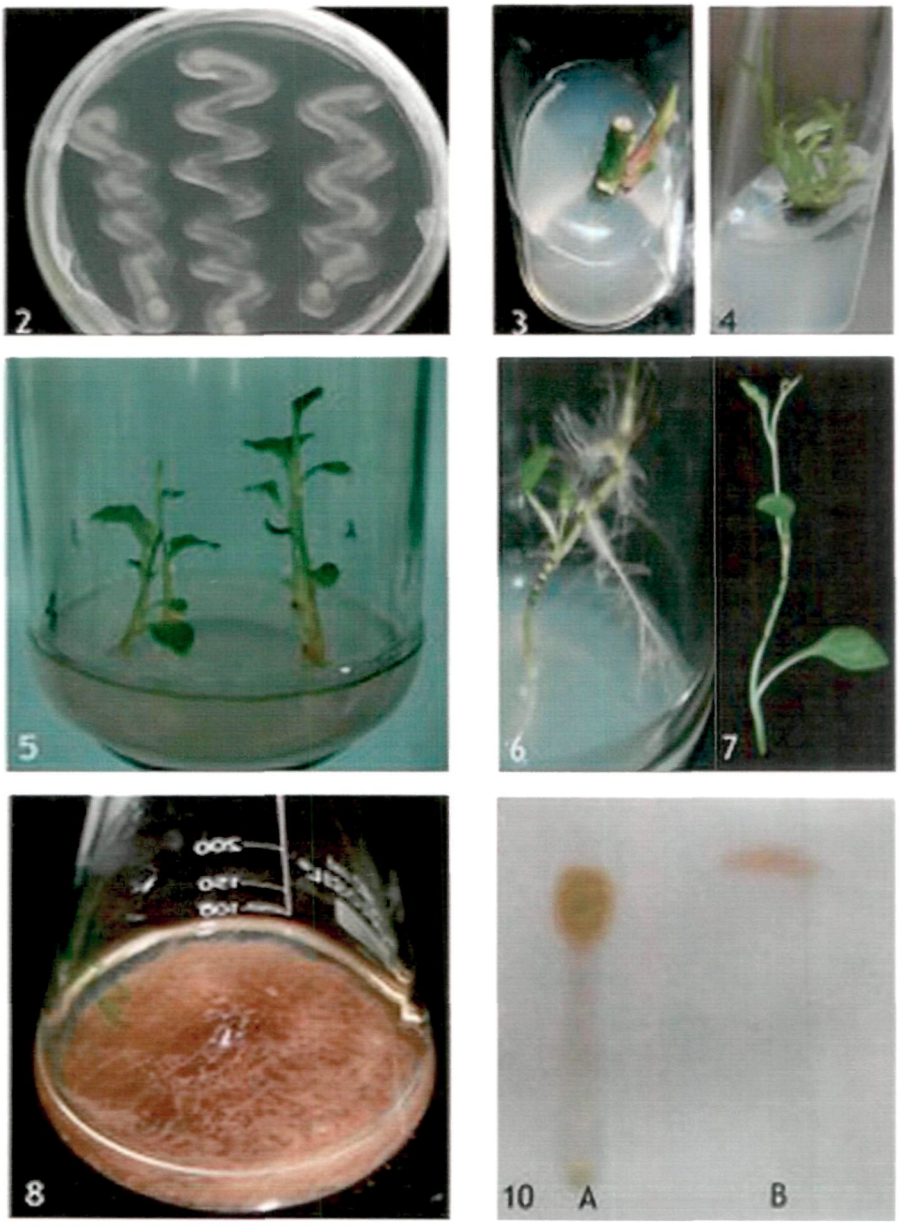

2
3
4
5
6
7
8
10
A
B

Fig 9. Growth of hairy root cultures over a period of 40 days

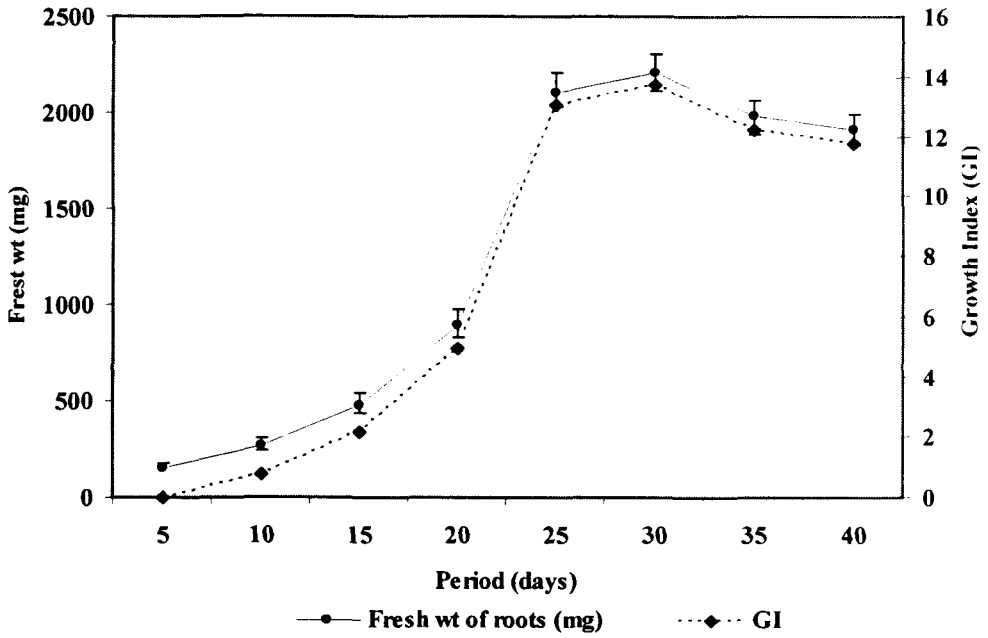
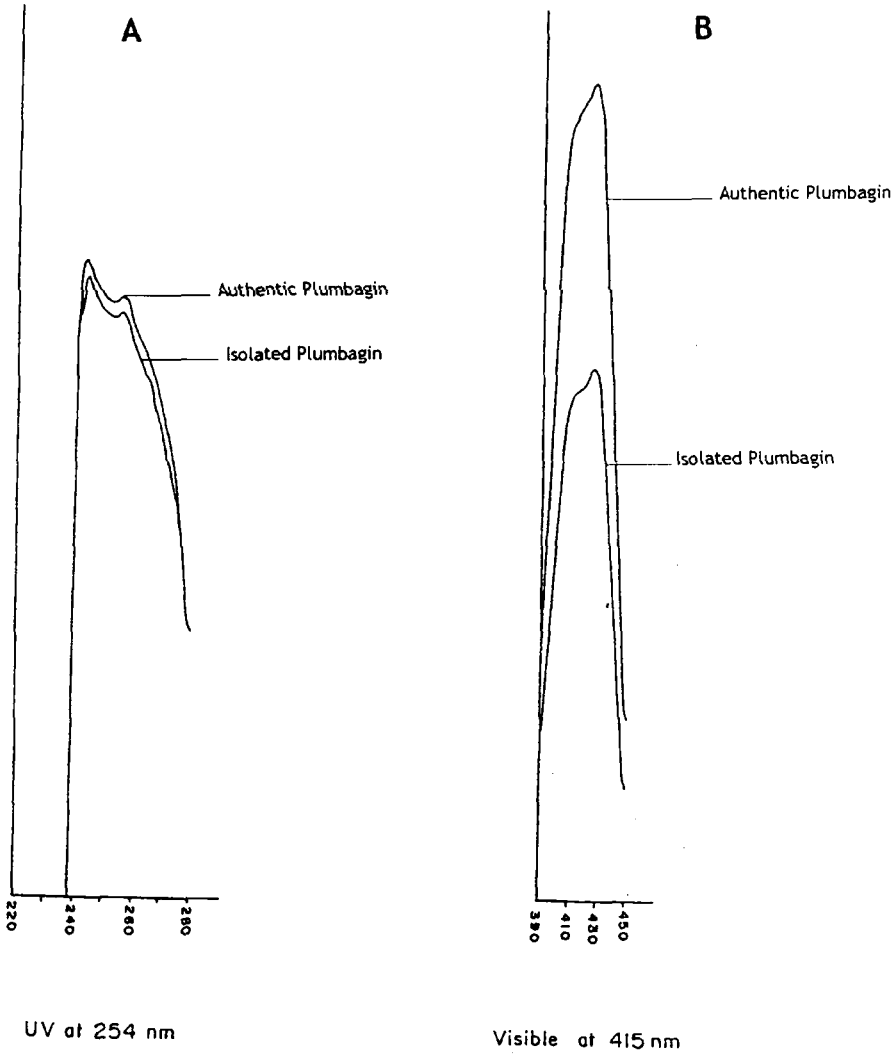


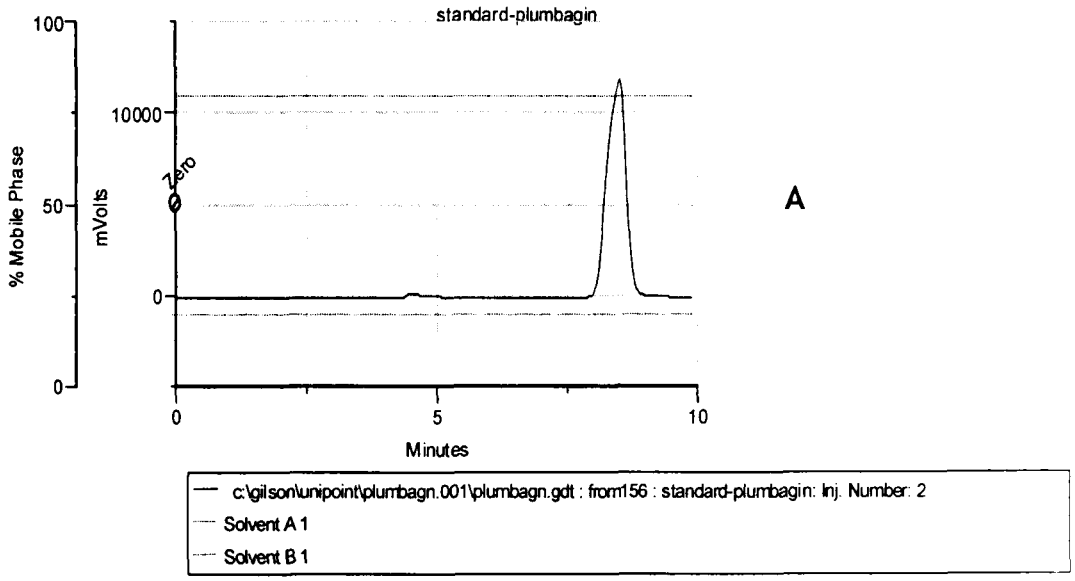
Fig 11. UV and Visible Spectrum of plumbagin

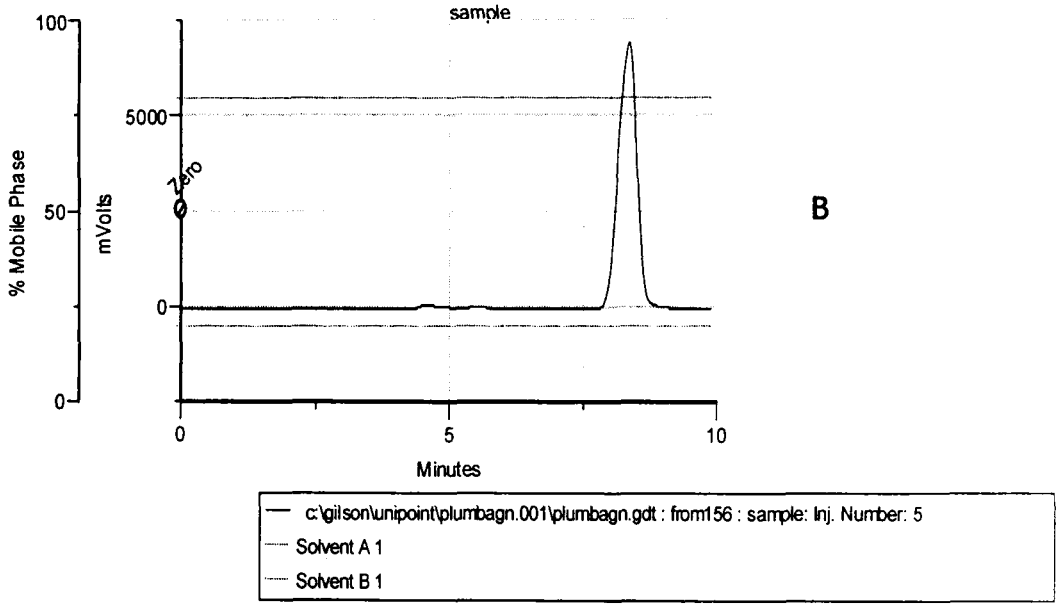


[A] UV spectrum of plumbagin isolated from fresh hairy root cultures of *P.zeylanica* along with authentic plumbagin.

[B] Visible spectrum of plumbagin isolated from fresh hairy root cultures of *P.zeylanica* along with authentic plumbagin.

Fig 12. HPLC Profile of Plumbagin





[A] HPLC traces of authentic plumbagin

[B] HPLC traces of plumbagin isolated from fresh hairy root cultures of *P.zeylanica*

Table 4. Comparative Performance of Different Bacterial Strains on Hairy Root Formation in Aseptic Shoot Cultures of *Plumbago zeylanica* L.

Agrobacterium strain	Infection frequency *(%)*	Number of hairy roots (or tumors)	Root length (cm)
A4	66	5.3±1.4	2.7±0.4
TR105	41	3.7±1.1	2.2±0.3
15834	28	2.7±1.2	1.8±0.2

DISCUSSION-I

DISCUSSION-1

Plants are a tremendous source for the discovery of novel products of pharmaceutical importance for drug development. Seventy five percent of the world population is inclined to use alternative or complementary medicine, especially herbals on account of high cost, non-availability and side effects of modern medicine. Many of the drugs sold today are simple synthetic modifications or copies of the naturally obtained substances. The emerging commercial importance of plant-derived secondary metabolites has in recent years resulted in a great interest in secondary metabolism, particularly in the possibility of altering the production of bioactive plant metabolites by means of tissue culture technology. Plant cell culture technologies were introduced at the end of the 1960's as a possible tool for both studying and producing plant secondary metabolites. Different strategies, using an *in vitro* system, have been extensively studied to improve the production of plant chemicals.

The objective of this study was to develop a viable protocol for establishing a hairy root culture system for the production of a bioactive molecule, plumbagin. Transformed hairy roots are induced *in vitro* using a soil bacterium *Agrobacterium rhizogenes* on different plant parts under aseptic condition. The common explants used for hairy root induction are roots, leaves, cotyledons, seedlings etc. Among the different organs/explants, most commonly used ones are aseptic shoots. In *P.zeylanica*, *in vitro* regeneration and multiplication of plants for field cultivation was already published by several authors (Rout et al., 1999; Selvakumar et al., 2001; Verma et al., 2002; Chaplot et al., 2006).

There are both physical and chemical factors influence the successful *in vitro* propagation of different plants including medicinal taxa (Hussey et al., 1980; Bhagyalakshmi and Singh, 1988; Short, 1991). In every plant tissue culture experiment, contamination is a major problem, which is effectively overcome by using different decontaminants including 0.01-0.5% w/v $HgCl_2$, 70% C_2H_5OH, 5-15% v/v sterilique etc. Similarly, type and age of explants, time given for treating the explants with surface sterilants are also reported to have very important role. Contamination rate (44 %) recorded in the present investigation is reasonably high when compared to the previous report of Rout et al (1999) and in many other reported systems, which might be due to either the systemic growth of fungal hyphae in the tissue or due to the problem of the explants collected from the field grown mother plant.

Initial responses of nodal explants in MS medium irrespective of the hormonal regimes are similar to the published report of Rout et al (1999), Verma et al (2002) and also in *P.rosea* by Binoy Jose et al (2007). Induction of shoot buds in a very short period of inoculation is not uncommon and is presumed to be the meristematic ability of the cells in the resident axillary meristem as reported earlier in *Adhatoda beddomei* (Sudha and Seeni, 1994). All the combinations of BAP and IAA promoted less number of shoot multiplication than the individual concentrations of BAP. The difference in shoot multiplication obtained in MS medium containing BAP alone is not in consonance with the previous report of Rout et al (1999) where a combination of BAP and IAA was used for initiating the shoots, which might be related to either genotypic difference or age of the explants used. Rate of shoot multiplication continued in the same medium during subculturing in the present study presumed to be owing to the retrospective effect of endogenous cytokinin (BAP). However, shoot elongation was restricted which confirmed the inhibitory influence of

cytokinin on shoot elongation as reported in other systems. (Bermudez et al., 2002). The present study revealed the amenability of *P.zeylanica* on *in vitro* culture experiments and the direct shoot multiplication protocol can be commercially exploited to multiply plants more rapidly within a shorter time period, and also can be used for developing *in vitro* strategies for the conservation and sustainable use of this medicinal plant.

Hairy root culture technology has been employed in several medicinal taxa for the extraction of high-value, low-volume bioactive compounds. It has tremendous potential for producing high- value products including therapeutics. However, large- scale cultivation of such hairy- root cultures is still not very prevalent. For hairy root induction, different strains of *Agrobacterium rhizogenes* have been successfully used in many dicotyledons plant species (Banarjee et al., 1998). Since the first report of successful gene transfer in 1983, *Agrobacterium* vectors have been the most widely used means of stable genetic transformation. There are host-range limitations to the utility of *Agrobacterium* for genetic transformation (Godwin et al., 1992). Recent evidence has indicated that the host-range is dependent on the protocol used to infect plant tissues with *Agrobacterium.* Modifications of co-cultivation conditions can lead to successful gene transfer to species once thought beyond the host-range of *Agrobacterium,* including a number of monocots (Hernalsteens et al., 1984; Schafer et al., 1987; Hansen, 2000). Hence, when defining the host-range of *Agrobacterium,* distinction must be made between host-range for pathogenicity (oncogenicity) and host-range for transformation. Important factors in determining the ability of *Agrobacterium* to transfer the T-DNA to the plant genome include genetic, physiological and physical components of the bacterium × plant interaction.

P.zeylanica was evaluated for its response to inoculation with wild strains of *A.rhizogenes* viz. A4, TR105 and 15834. A protocol for induction and establishment of *A.rhizogenes*-mediated hairy root cultures of *P.zeylanica* was developed through optimization of the most suitable bacterial strain. The present study demonstrated that genetic transformation by wild type of *A. rhizogenes* is a promising approach in induction and establishment of hairy roots in *P.zeylanica*. Scalpel method (Ooms et al., 1985) was employed for the infection of bacterium into the explants as employed successfully in many medicinal species which is different from the method (co-cultivation) employed in *P.zeylanica* earlier (Verma et al., 2002). Strain specificity of the bacterium and frequency of transformation events were analyzed. Significant differences in inducing roots on shoots observed between bacterial strains are common and reported extensively in many cases (Momcilovic et al., 2001). The best response in terms of transformation ability was obtained with *A.rhizogenes* strain A_4, followed by TR105 and 15834 obviously indicating the virulence as well as host specificity of the bacterial strains as reported earlier (Verma et al., 2002). The number of roots produced at the site of infection after a specific time span of *in vitro* culture (7-8 weeks) has been used as measure of virulence. The efficiency of transformation is known to differ with different bacterial strains. Certain phenolic compounds, e.g. acetosyringone, œ-hydroxy acetosyringone, etc are known to induce transcription of *vir* region. Plant species differ in their temporal competence for transformation. Acetosyringone has been known to enhance transformation efficiency due to activation of *vir* genes in *A.tumefaciens* (Gelvin, 2000). However, *Plumbago* species even without the addition of stimulators like acetosyringone itself responds to *Agrobacterium* mediated hairy root production to a significant level as in many dicotyledons species.

The infection of *in vitro* raised shoots of *P.zeylanica* with the A4 strain resulted in the emergence of hairy roots at 66% relative transformation frequency. Generally, agropine type *Agrobacterium* strains have high-virulent pathotypes, which might be the reason for the use of these strains in most studies for hairy root induction (Momcilovic et al., 2001). Excision and subculturing of the emerging hairy root clones in full strength MS medium with 3% sucrose exhibited reasonably good growth. Growth index analysis of the hairy root cultures revealed a gradual increase in fresh weight up to 4 weeks of culture at which a maximum 13.74 fold increase in biomass could be recorded and the synthesis of plumbagin paralleled the growth and biomass production of roots is in consonance with several reports. Biomass production of hairy roots in the present study is differed from the previous report (21 fold) of Verma et al (2002), where MS medium was fortified with 4% sucrose in the place of 3% sucrose used in our study. However, the concentration of plumbagin in the hairy roots recorded in the present study is significantly higher (0.91% g dwt) than the concentration of plumbagin accumulated in the hairy roots reported earlier (Verma et al., 2002; Wei et al., 2006) and this enhanced production of plumbagin might be due to the genotypic difference of the explant used or due to the culture conditions employed in the present investigation. The production of plumbagin in the hairy root cultures highlights the importance of the present study for the commercial prospects of the root culture system.

One advantage of the hairy root induction protocol developed in the present study is that the yield potentials of the hairy root cultures, in terms of plumbagin content superseded that of all other reports (Verma et al., 2002; Wei et al., 2006). In short the present study demonstrates the successful establishment, maintenance and growth of hairy root cultures of *P.zeylanica* with desired phyto-molecule

production potential, which can serve as an effective substitute to tuberous roots of the field grown plants. The production of this compound can further be optimized in order to make the production commercially viable, which might, subsequently help to spare indiscriminate up-rooting and exploitation of this commercially important, endangered medicinal plant species. The plumbagin isolated and purified was used to perform experiments in the later stages of this work.

CHAPTER-II

Plumbagin-Cu(II) Induced Oxidative DNA Breakage in Plasmid pBR322 DNA & Human Preripheral Lymphocytes: Implications for its Anticancer Properties

MATERIALS & METHODS-II

MATERIALS & METHODS-II

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
Nitro blue 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
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	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
DMSO	Rankem, India

* All other chemicals were commercial products of analytical grade.

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

Reaction mixtures (0.5ml) contained 10 mM Tris- HCl (pH 7.5), 500 µg of calf thymus DNA and varying amounts of plumbagin, 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 h. The reaction was stopped by adding 0.2 ml bovine serum albumin (10 mg/ml) and 1.0 ml of 14% perchloric acid (chilled). The tubes were immediately transferred to 0°C for 45 min before centrifugation at 2500 rpm for 10 min at room temperature to remove undigested DNA and precipitated protein. Acid soluble deoxyribonucleotides were determined in the supernatant, calorimetrically, using the diphenylamine method (Schneider, 1957). To a 1.0 ml aliquot, 2.0 ml diphenyl reagent (freshly prepared by dissolving 1 g 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 and the intensity of blue colour was read at 600 nm. The melting profile of DNA using S1 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 $20x10^{-4}$ mM EDTA) and incubations with plumbagin 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 plumbagin 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.

Detection of Cu(II) reduction by plumbagin

The selective sequestering agent neocuproine was employed to detect the reduction of Cu(II) to Cu(I) by recording the formation of neocuproine- Cu(I) complex which absorbs maximally at 450 nm. The reaction mixuture (3.0 ml) contaned 10 mM Tris (pH 7.5), fixed concentration of Cu(II) (100 µM), 300 µM neocuproine and varying concentration of plumbagin (50 and 100 µM). The reaction was started by adding Cu(II) and the spectra was recorded immediately.

Stoichiometric titration of Cu(I) production by plumbagin

The amount of Cu(I) produced in the plumbagin-Cu(II) reaction mixture was determined by titration with neocuproine. Plumbagin (50 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 min of incubation at room temperature.

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

Superoxide was detected by the reduction of nitroblue tetrazolium (NBT) essentially as described by Nakayama et al (1983). A typical assay mixture contained 100 mM potassium phosphate buffer, pH (7.5), 1 mM NBT, 1 mM EDTA and 0.6% Triton X-100 in a total volume of 3 ml. The reaction was started by the addition of plumbagin (50 µM). 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 plumbagin

In order to study the hydroxyl radical production by increasing concentrations of plumbagin in the presence of 20 µM Cu(II), the method of Quinlan and Gutteridge (1987) was followed. Calf thymus DNA (100 µg) was used as a substrate and the malondialdehyde generated from deoxyribose radicals was assayed by recording the absorbance at 532 nm.

Fluorescence studies

The fluorescence studies were performed on a Shimadzu spectrofluorophotometer RF-5000 (Japan) equipped with the plotter and a calculator. Plumbagin was excited at its absorption max of 415nm. Emission spectra were recorded in the range shown in figures.

Isolation of lymphocytes

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

Treatment of lymphocytes with plumbagin & Cu(II)

Lymphocytes ($1x10^5$ cells) were exposed to different concentrations of plumbagin 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 plumbagin). In some experiments lymphocytes were pre-incubated with 50 µM plumbagin or 20 µM Cu(II). On another set of experiments, scavengers of reactive oxygen were added at the final concentrations indicated. Incubation was performed at 37°C for indicated time periods. After the incubation, the reaction mixture was centrifuged at 4000 rpm, the supernatant was discarded and pelleted lymphocytes were resuspended in 100 µl PBS and processed further for Comet assay.

Viability assessment of lymphocytes

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

Alkaline single cell gel electrophoresis/Comet assay

Comet assay was performed under alkaline conditions essentially according to the procedure of Singh et al (1989) with slight modifications. Fully frosted microscopic slides precoated with 1.0% normal melting agarose at about 50°C (dissolved in Ca++ 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 x 100 added just prior to use for a minimum of 1 h at 4°C. After lysis DNA was allowed to unwind for 30 min in alkaline electrophoretic solution consisting of 300 mM NaOH,1mM EDTA, pH>13. Electrophoresis was performed at 4°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, UK) attached to an Olympus (C X 41) fluorescent microscope and a COHU 4910 (equipped with a 510-560 nm excitation and 590nm 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.

Statistical Analysis

The statistical analysis was performed as described by Tice et al (2000) and is expressed as $\pm$ 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.

RESULTS-II

RESULTS-II

Reduction of Cu(II) by plumbagin

The production of Cu(I), formed as a result of reduction of Cu(II) by plumbagin, was analyzed using neocuproine which is a sensitive Cu(I) sequestering agent that binds specifically to the reduced form of copper Cu(I) but not to the oxidized form [Cu(II)]. The Cu(I) - neocuproine complex formed exhibits an absorption maximum at 450 nm. Under the experimental conditions employed, Cu(II)-plumbagin combination did not interfere with this maximum, where as plumbagin at 100 μM and 50 μM concentrations react to generate Cu(I) which complexes with neocuproine to give a peak appearing at 450 nm **(Fig. 13)**. The implication of this finding is that Cu(II) is reduced by plumbagin in the complex to generate Cu(I).

Stoichiometry of Cu(II) reduction by plumbagin

Plant 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 studied the stoichiometry of Cu(II) reduction by plumbagin. In the experiment shown in **Fig 14**, increasing concentrations of Cu(II) were added to fixed concentrations of plumbagin (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)/ plumbagin. Fig 13 shows a clear increase in absorbance corresponding to increasing molar ratios of Cu(II).

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

Plumbagin 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. **Fig 15** 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) (20 μM), increasing concentrations of plumbagin resulted in progressive increase in nuclease sensitive sites in DNA.

Cleavage of plasmid pBR322 DNA by plumbagin

In order to understand the chemical basis of DNA breakage by plumbagin-Cu(II) system, the DNA cleavage efficacy of plumbagin in plasmid pBR322 DNA was studied. **Fig 16** shows the ethidium bromide stained banding pattern of pBR322 DNA treated with increasing concentrations of plumbagin (lanes 2, 3 and 4 respectively). As can be seen, at the lower concentration of plumbagin alone, approximately equal intensity of the supercoiled DNA bands are present. In presence of 20 μM concentration of Cu(II), plumbagin induced extensive DNA strand breaks as indicated by the disappearance of the supercoiled (SC) form and the formation of open circular (OC) forms (Lanes 5-7).

Generation of oxygen radicals by plumbagin

Superoxide production: the production of superoxide anion was determined by the method of Nakayama et al (1983), which involves reduction of NBT by plumbagin to a formazan. The time independent generation of superoxide anion by 50 μM of plumbagin, as evidenced

by the increase in absorbance at 560 nm is shown in **Fig 17**. 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 plumbagin: 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 plumbagin to generate hydroxyl radical in the presence of Cu(II) was studied. The assay is based on the fact that degradation of DNA by hydroxyl radicals results in the release of TBA (Thiobarbituric acid) reactive material, which forms coloured adduct with TBA at 532nm (Quinlan and Gutteridge, 1987). Results given in **Fig 18** clearly show that increasing concentrations of plumbagin led to a progressive increase in the formation of hydroxyl radicals.

Binding of copper ions to plumbagin

Binding of copper ions to plumbagin was studied by the effect of increasing Cu(II) molar ratios on the fluorescence emission spectra of plumbagin. The result shown in **Fig 19** clearly indicates the binding, as addition of Cu(II) causes quenching of the plumbagin fluorescence. These results support the result of absorption studies shown in **Fig 14** where formation of plumbagin-copper complex was demonstrated.

Formation of complexes involving calf thymus DNA and plumbagin

Fig 20 shows the effect of addition of increasing base molar ratios of calf thymus DNA on the fluorescence emission spectra of plumbagin. Such an addition resulted in a dose-dependent quenching of the fluorescence. There was however, no shift in the λ-max emission suggesting a simple mode of binding of DNA and the plumbagin.

The control, native DNA alone when exited at the same wavelength (308 or 326) did not interfere with the emission spectrum of plumbagin alone/ plumbagin + DNA, thus confirming the binding results.

Standardization of alkaline single cell gel electrophoresis (Comet assay)

Alkaline single cell gel electrophoresis is a 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 **Fig 21**. 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 was observed. In **Fig 22** 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 plumbagin-Cu(II) in lymphocytes as measured by Comet assay

Increasing concentrations of plumbagin (10-50 μM) either alone or in the presence of 20 μM Cu(II) was tested for DNA breakage in isolated human peripheral lymphocytes using the Comet assay. Plumbagin 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. **Fig 23** shows photographs of comets (100-X) observed with varying concentrations of plumbagin in the absence and presence of copper. Untreated lymphocyte controls were similar to plumbagin 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 plumbagin concentration in **Fig 24**. The results clearly establish that plumbagin-Cu(II) system is capable of DNA breakage in lymphocytes. A similar experiment with increasing concentrations of Cu(II) (5-20 μM) was also performed. As can be seen from **Fig 25** an increasing degree of DNA damage with increasing concentrations of Cu(II) was observed. The corresponding tail length is shown in **Fig 26.**

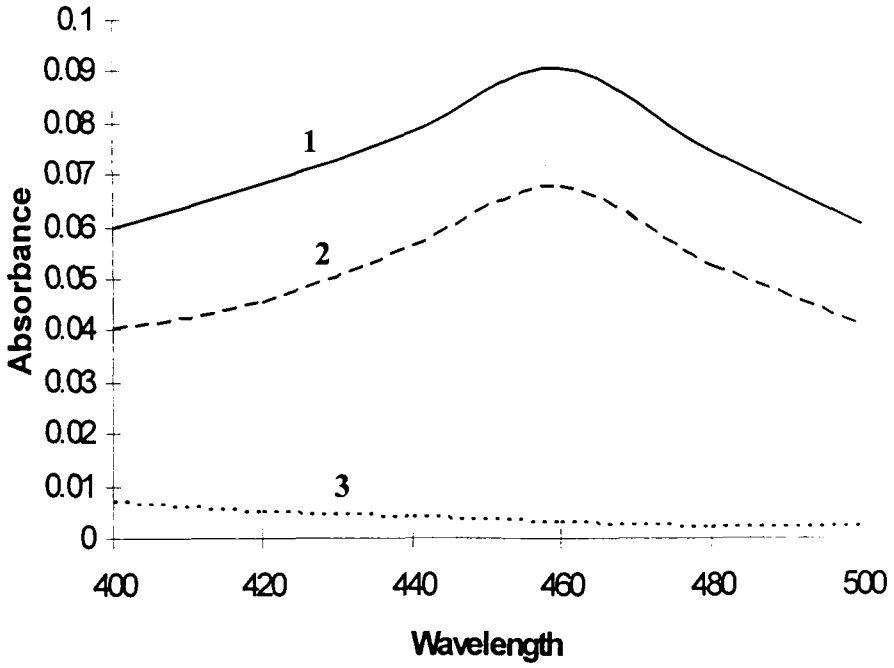
Effect of pre-incubating the lymphocytes with plumbagin or Cu(II) on plumbagin-Cu(II) mediated DNA breakage

Lymphocyte DNA breakage was also studied after pre-incubating the cells with plumbagin after which the cells were washed twice with PBS and incubated further in the presence of $CuCl_2$. The photographs of comets (100-X) in **Fig 27** clearly shows that with increasing concentrations of plumbagin a progressive increase in DNA breakage was observed. The corresponding comet tail length as indicated with increased tail length of comets is shown in **Fig 28.** A similar experiment was also done after pre-incubation with $CuCl_2$ and similar results were observed (**Fig 29** and **Fig 30**). Untreated lymphocyte controls were similar to those of treatment with plumbagin alone or Cu(II) alone. These results indicate that both plumbagin and Cu(II) are either able to enter the cells or bind to the cell membrane and moreover these results are in further support of our initial findings that plumbagin-Cu(II) system is capable of DNA breakage in lymphocytes.

Effect of active oxygen scavengers and neocuproine on plumbagin-Cu(II) induced DNA breakage in lymphocytes

The effect of free radical scavenging agents (sodium azide, potassium iodide, thiourea, superoxide dismutase & catalase) and neocuproine, Cu(I) specific sequestering agent on plumbagin induced DNA breakage were studied. 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 (**Table 5**) we may 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 cleaving agents. Neocuproine is a Cu(I) specific sequestering agent, which as expected also inhibited DNA breakage indicating that a copper redox cycle and reactive oxygen generation are two major determinants involved in the observed DNA damage. Results therefore, suggest that the chemically induced DNA breakage *in vitro* and lymphocyte DNA breakage by plumbagin-Cu(II) system are most likely the result of the same mechanism.

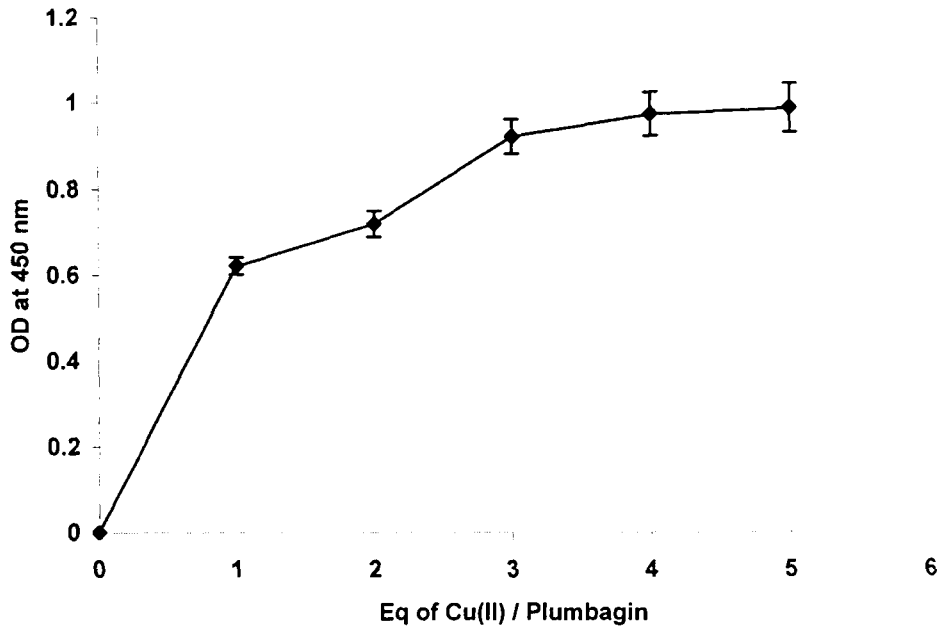
Fig 13. Reduction of Cu(II) by plumbagin



The 3 ml reaction mixture contained 10 mM Tris (pH 7.5), plumbagin, 300 μM neocuproine and 100 Cu(II). The spectra were recorded after addition of components indicated.

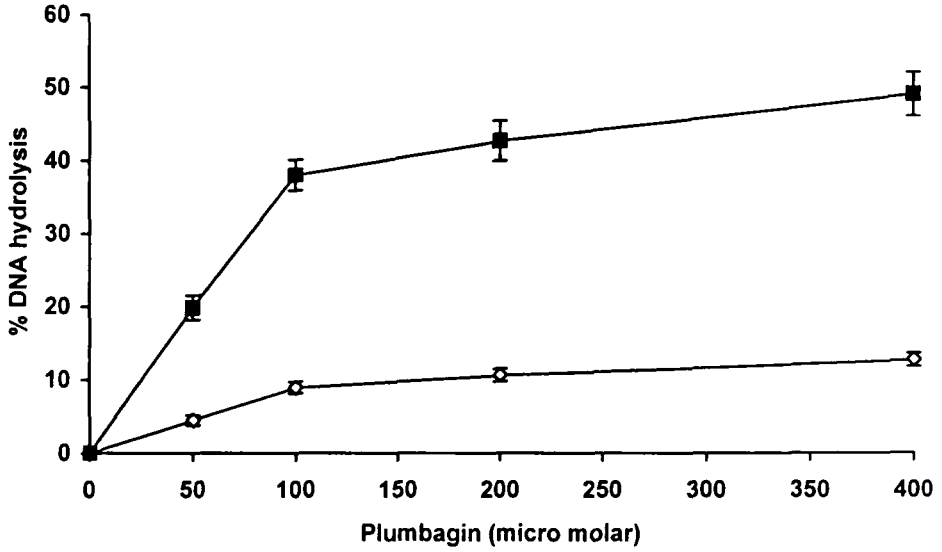
(Trace 1) Plumbagin (100 μM) + Cu(II) (100 μM) + Neocuproine (300μM)
(Trace 2) Plumbagin (50 μM) + Cu(II) (100 μM) + Neocuproine (300μM)
(Trace 3) Plumbagin (100 μM) + Cu(II) (100 μM)

Fig 14. Detection of stoichiometry of plumbagin and Cu(II) interaction



Reaction mixture contained 10 mM Tris HCl (pH 7.5), fixed concentration of plumbagin (25 μM) with increasing concentration of Cu(II) (shown as molar ratios of Cu(II)/plumbagin) and neocuproine (300 μM) Absorbance was recorded after incubating the reaction mixture at room temperature for 30 min. All points represent triplicates and mean values have been plotted. Error bars denote ±SEM of three independent experiments.

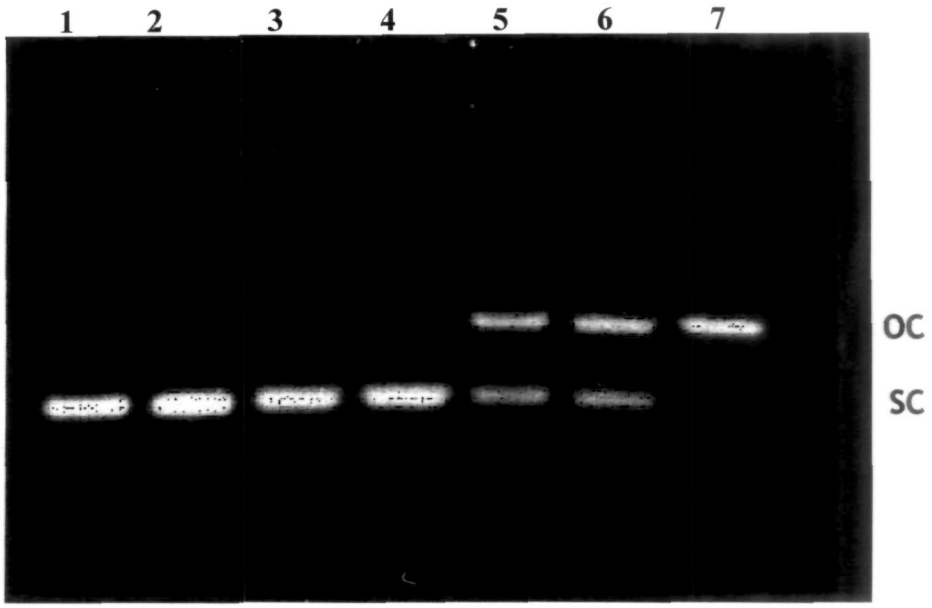
Fig 15. Degradation of calf thymus DNA by plumbagin in the presence of Cu(II) as measured by the degree of single strand specific nuclease digestion



500 µg Calf thymus DNA was incubated at 37^{0}C with indicated concentration of plumbagin and Cu(II) (20 µM) in a total reaction volume of 0.5 ml containing 10 mM Tris-HCl pH (7.5). Single strand specific digestion was performed as described in 'Materials & Methods'. All points represent triplicates and mean values have been plotted. Error bars denote ±SEM of three independent experiments.

- o Plumbagin alone
- ▪ Plumbagin + Cu(II)

Fig 16. Agarose gel electrophoretic pattern of ethidium bromide stained pBR322 DNA after treatment with plumbagin in the absence and presence of Cu(II)

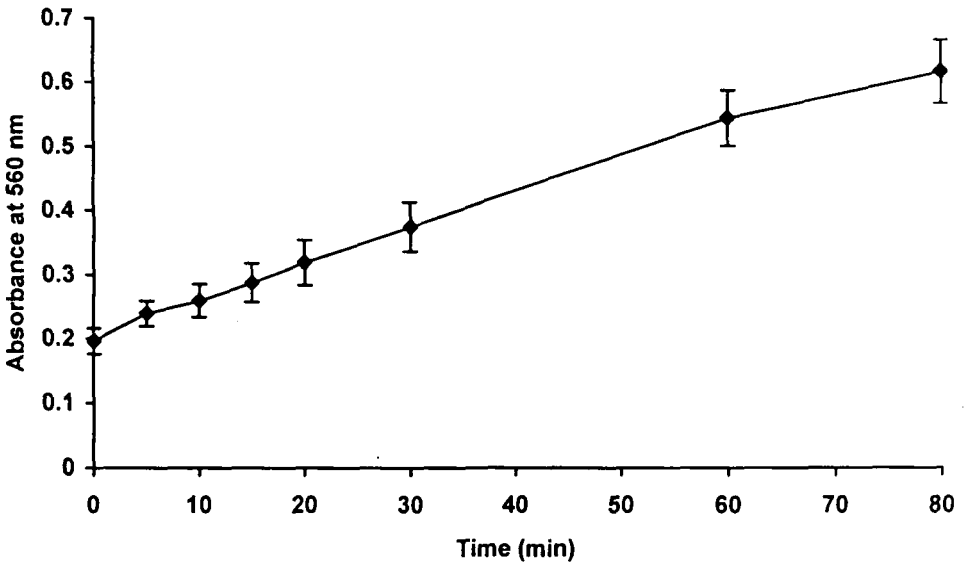


The reaction mixtures (30 µl) contained 0.50 µg pBR322 DNA, 10 mM Tris-HCl (pH 7.5), indicated concentrations of plumbagin and Cu(II). Incubation was carried out at 37^0c for 30 min.

Lane 1 DNA alone;
Lane 2 DNA + Plumbagin (50 µM);
Lane 3 DNA + Plumbagin (100 µM);
Lane 4 DNA + Plumbagin (200 µM);
Lane 5 DNA + Plumbagin (50 µM) + Cu(II) (20 µM)
Lane 6 DNA + Plumbagin (100 µM) + Cu(II) (20 µM)
Lane 7 DNA + Plumbagin (200 µM) + Cu(II) (20 µM)

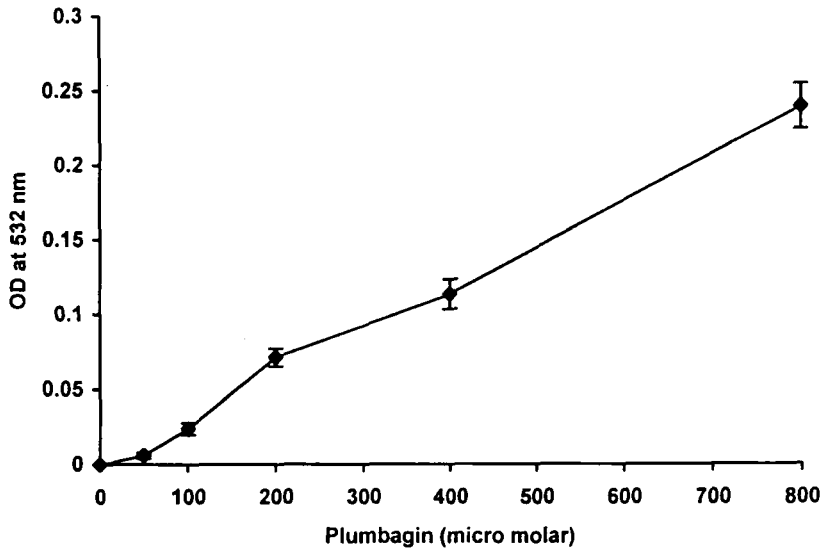
SC - super coiled DNA; OC - Open circular DNA

Fig 17. Photogeneration of superoxide anion by plumbagin on illumination under fluorescent light as a function of time



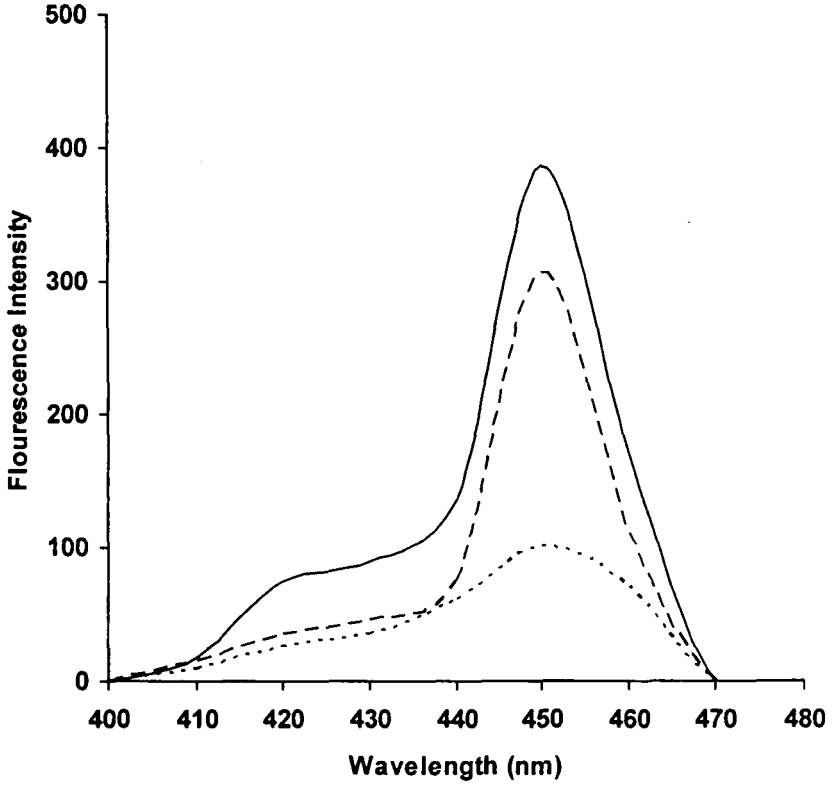
The concentration of plumbagin was 50 μM. The sample was placed 10 cm from the light source. Details are given in 'Materials and Methods'. All values reported are means of three independent experiments. Error bars represent standard error of mean.

Fig 18. Hydroxyl radical generation by plumbagin



Reaction mixture (0.5 ml) contained 100 µg calf thymus DNA as substrate, 20 µM Cu(II) and indicated concentrations of plumbagin. The reaction mixture was incubated at 37°C for 30 min. Hydroxyl radical formation was measured by determining the TBA reactive material as described in 'Materials & Methods'. All values reported are means of three independent experiments. Error bars represent standard error of mean.

Fig 19. Effect of increasing concentration of Cu(II) on the fluorescence emission spectra of plumbagin



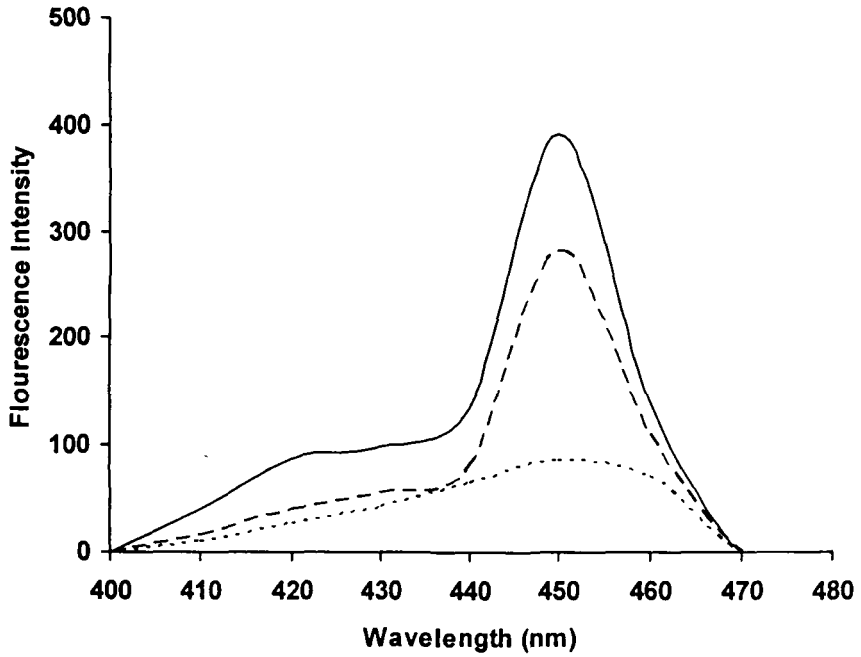
Plumbagin (in Tris-HCl, pH 7.5) was excited at its λ-max (absorption) of 415 nm and the emission spectra were recorded between 400-480 nm.

[—] Plumbagin alone

[– –] Plumbagin + Cu(II) molar ratio 1:2

[····] Plumbagin + Cu(II) molar ratio 1:5

Fig 20. Effect of increasing native DNA base pair molar ratios on the fluorescence emission spectra of plumbagin



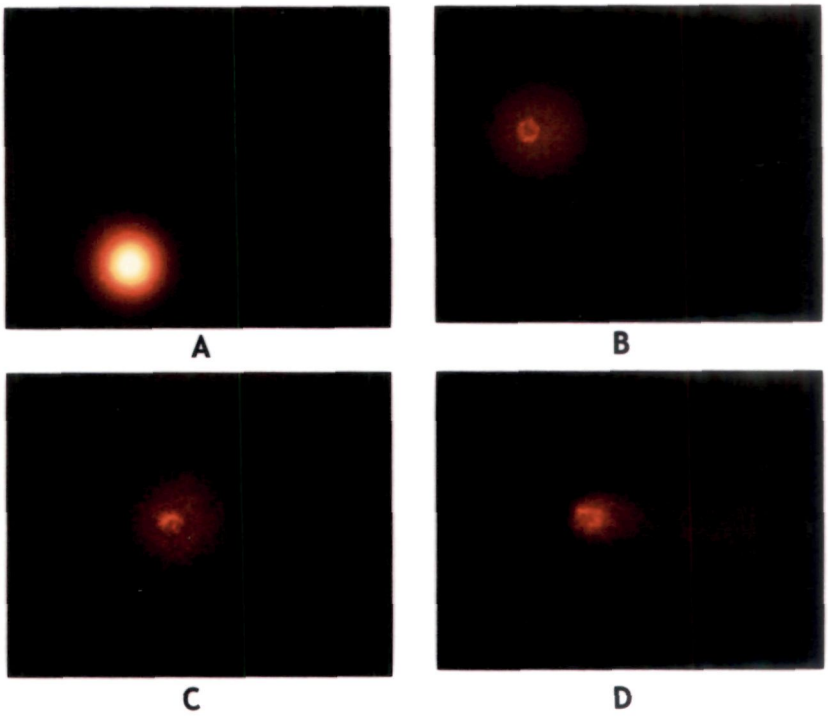
Plumbagin (in Tris-HCl, pH 7.5) was exited at its λ-max (absorption) of 415 nm and the emission spectra were recorded between 400-480 nm.

[—] Plumbagin alone

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

[····] Plumbagin + DNA base pair molar ratio (1:5)

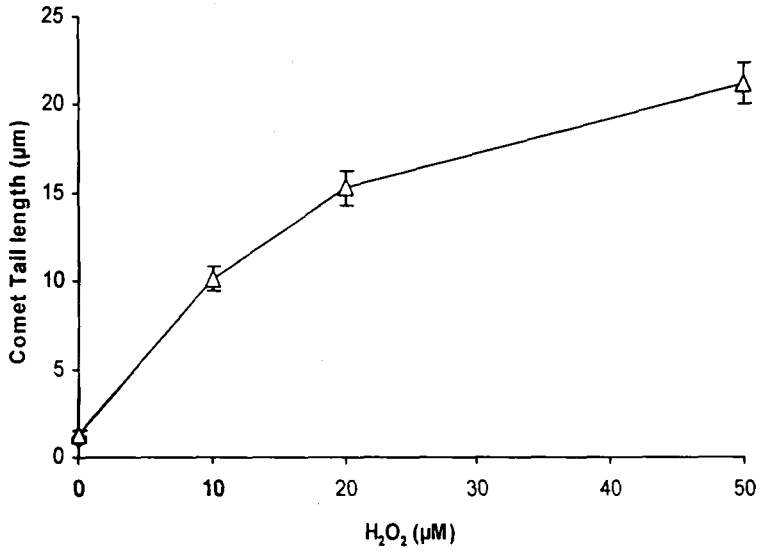
Fig 21. Standardization of Comet assay with H_2O_2



Photographs of comet (100 x) seen on incubating lymphocytes with varying concentrations of H_2O_2. Reaction mixture (1 ml) contained $1x10^5$ cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} free and indicated concentrations of H_2O_2 (10-50 µM). The reaction mixture was incubated at 37°C for 30 min and processed further for comet assay as described in 'Materials & Methods'.

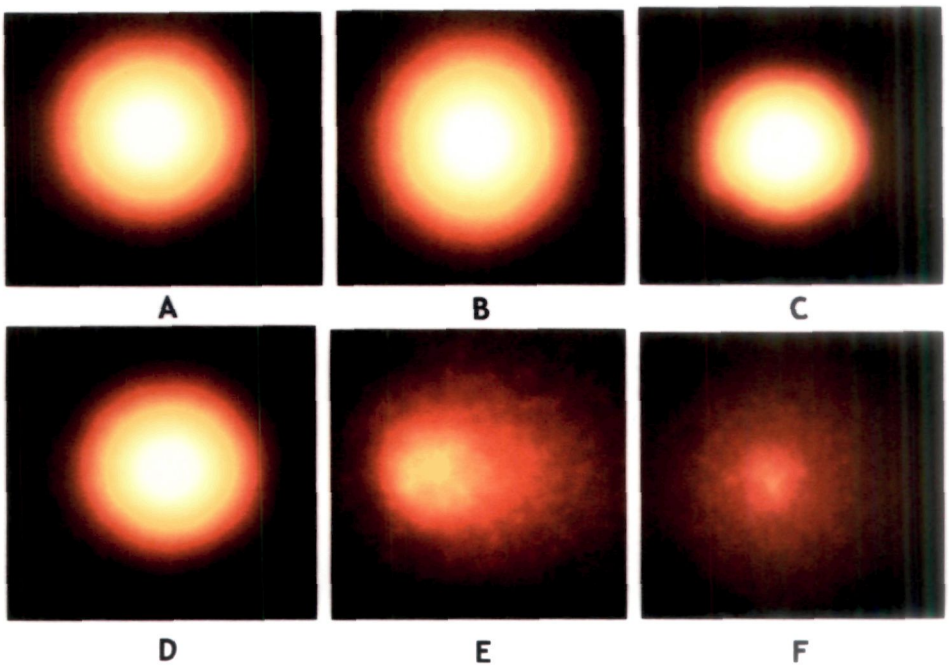
[A] Untreated Lymphocytes
[B] Lymphocytes + 10 µM H_2O_2
[C] Lymphocytes + 20 µM H_2O_2
[D] Lymphocytes + 50 µM H_2O_2

Fig 22. Tail length of comets plotted as a function of increasing concentrations of H_2O_2



Reaction mixture (1 ml) contained 1x 10^5 cells, RPMI 400 µl, PBS Ca^{2+} and Mg^{2+} free and indicated concentrations of H_2O_2 (10-50 µM). The reaction mixture was incubated at 37°C for 30 min and processed further for comet assay as described in 'Materials & Methods'. All points represent mean of three independent experiments. Error Bars denote SEM. *P* value <0.05 and significant when compared to control.

Fig 23. DNA breakage by plumbagin-Cu(II) system in human peripheral lymphocytes as measured by Comet assay. (Effect of increasing concentrations of plumbagin (10-50 μM) in the absence and presence of fixed concentration of Cu(II) (20 μM) on DNA breakage in human peripheral lymphocytes)



Photograph of Comet (100-X) obtained after treatment of lymphocytes with increasing concentrations of plumbagin and fixed concentration of Cu(II). Reaction mixture (1ml) contained 1x10 ^{5} cells, RPMI 400 μl, PBS ca^{2+} and mg^{2+} free, plumbagin (10-50 μM) and Cu(II) (20 μM). The reaction mixture was incubated for 30 min at 37^{0}C. After the incubation the cells were processed further for Comet assay as described in 'Materials & Methods'.

[A] Untreated lymphocytes

[B] Lymphocytes + Plumbagin (10 μM)

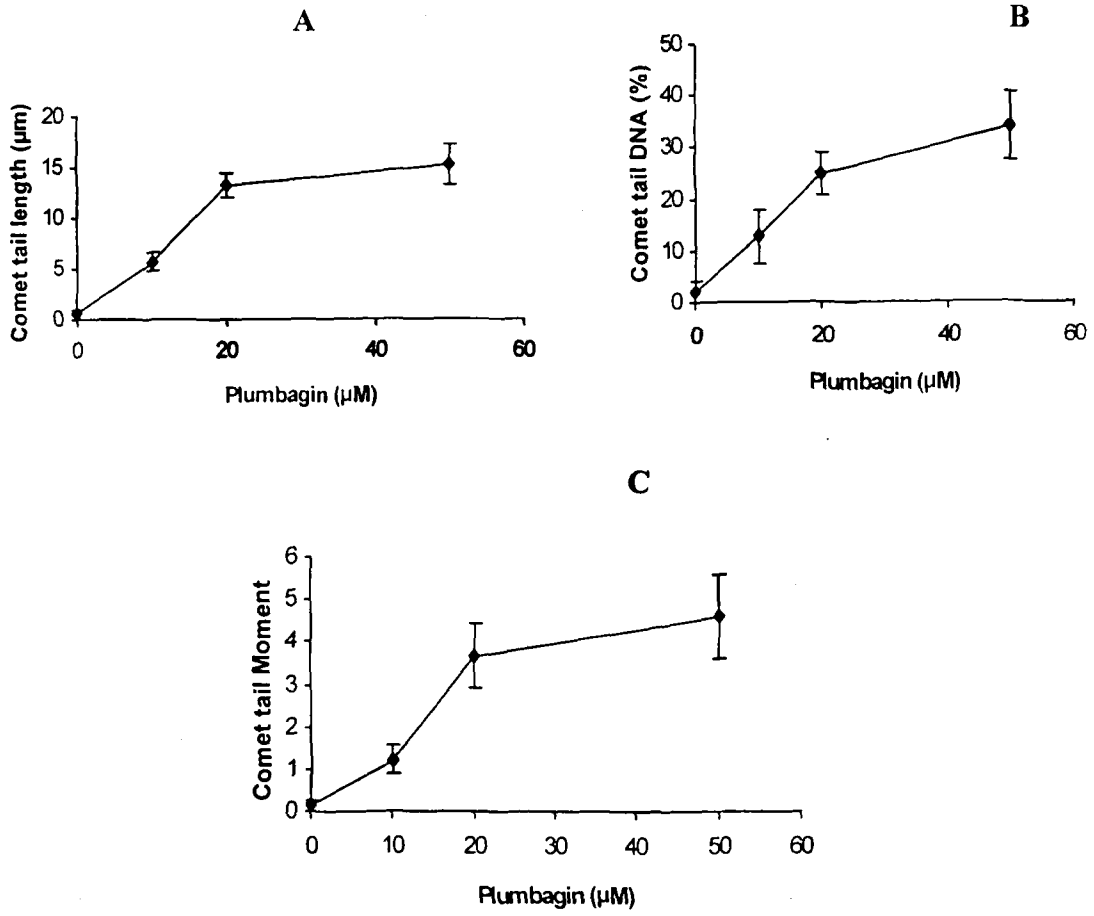
[C] Lymphocytes + Plumbagin (20 μM)

[D] Lymphocytes + Plumbagin (50 μM)

[E] Lymphocytes + Plumbagin (20 μM) + Cu(II) (20 μM)

[F] Lymphocytes + Plumbagin (50 μM) + Cu(II) (20 μM)

Fig 24. DNA breakage by plumbagin-Cu(II) system in human peripheral lymphocytes as measured by Comet assay



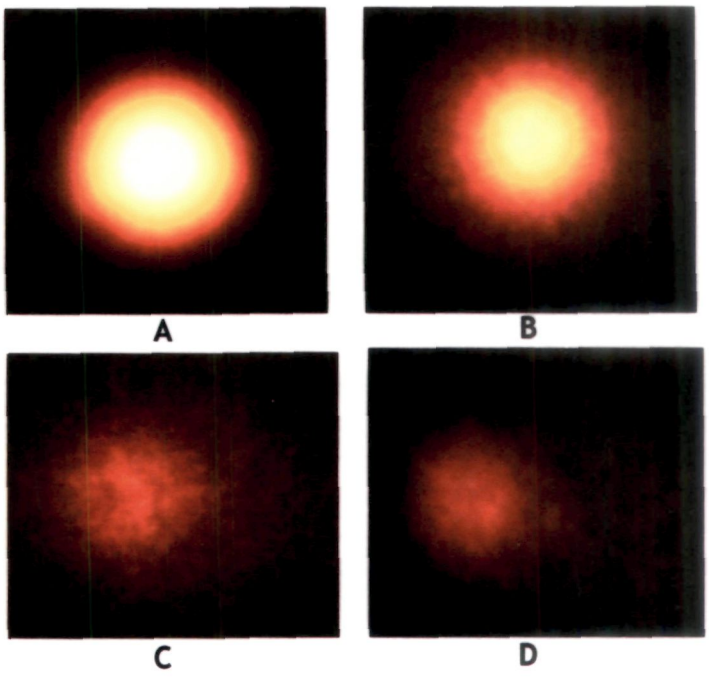
[A] Comet tail length (µm) plotted as a function of increasing concentrations of plumbagin (10-50 µM) is the presence of 20 µM Cu(II).

[B] Comet Tail DNA (%) plotted as a function of increasing concentrations of plumbagin (10-50 µM) in the presence of 20 µM Cu(II).

[C] Tail Moment (Arbitrary Units) plotted as a function of increasing concentrations of plumbagin (10-50 µM) is 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.

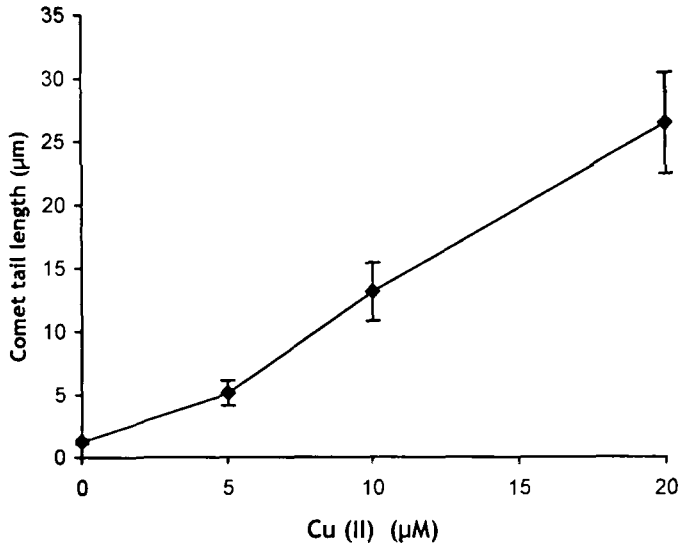
Fig 25. Effect of increasing concentrations of Cu(II) (5-20 μM) and fixed concentration of plumbagin (50 μM) on DNA breakage in human peripheral lymphocytes



Photographs of comets (100-X) obtained after treatment of lymphocytes with increasing concentrations of Cu(II) and fixed concentration of plumbagin. Reaction mixture contained $1x10^5$ cells, RPMI 400 μl, PBS Ca^{2+} and Mg^{2+} free and Cu(II) (5-20 μM). The reaction mixtures were incubated at 37^0C for 30 min and the Cells were processed further for comet assay as described in 'Materials & Methods'.

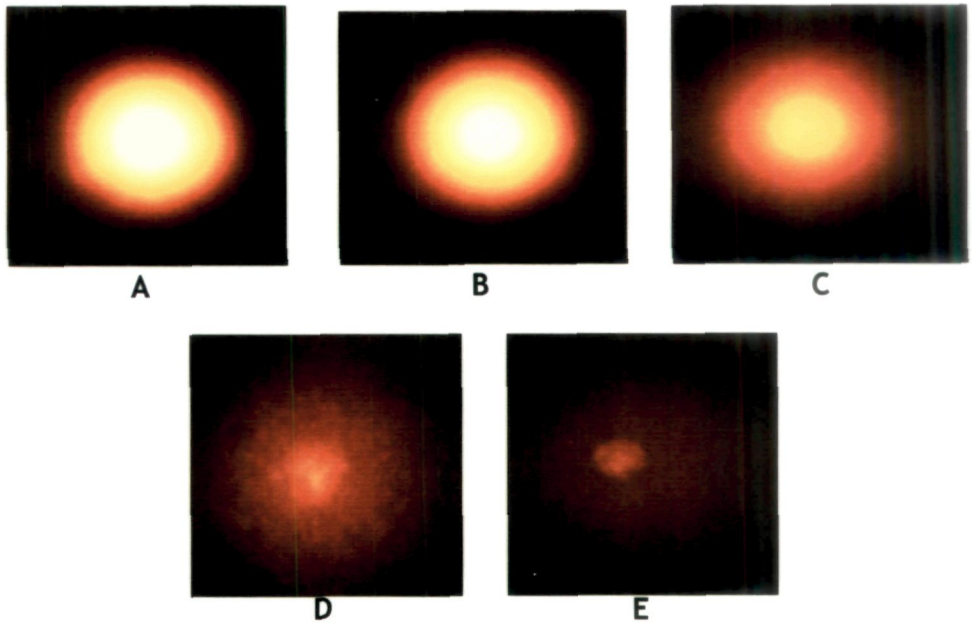
[A] Untreated lymphocytes
[B] Lymphocytes + Plumbagin 50 μM + Cu(II) 5 μM
[C] Lymphocytes + Plumbagin 50 μM + Cu(II) 10 μM
[D] Lymphocytes + Plumbagin 50 μM + Cu(II) 20 μM

Fig 26. Comet tail length (μm) plotted as a function of increasing concentrations of Cu(II) (5-20 μM) and fixed concentration of plumbagin (50 μM) on DNA breakage in human peripheral lymphocytes



Tail length of comets plotted as a function of increasing concentrations of Cu(II) (5-20 μM) and fixed concentration of plumbagin (50 μM). Reaction mixture contained $1x10^5$ cells, RPMI 400 μl, PBS Ca^{2+} and Mg^{2+} free and Cu(II) (5-20 μM). The reaction mixtures were incubated at 37^0C for 30 min and the cells were processed further for comet assay as described in 'Materials & Methods'. Values reported are ±SEM of three independent experiments. *P* value <0.05 and significant when compared to control.

Fig 27. Effect of pre-incubating the lymphocytes with increasing concentrations of plumbagin (10-50 μM) on plumbagin-Cu(II) induced DNA breakage



Photographs of comets (100-X) obtained after pre-incubation of lymphocytes with increasing concentrations of plumbagin. Isolated cells suspended in RPMI 1640 were pre-incubated with the indicated concentrations of plumbagin for 30 min at 37^0C. After pelleting, the lymphocytes were washed twice with PBS Ca^{2+} and Mg^{2+} free before re-suspension in RPMI 1640 and further incubation for 30 min in the presence of 20 μM Cu(II).

[A] Untreated lymphocytes

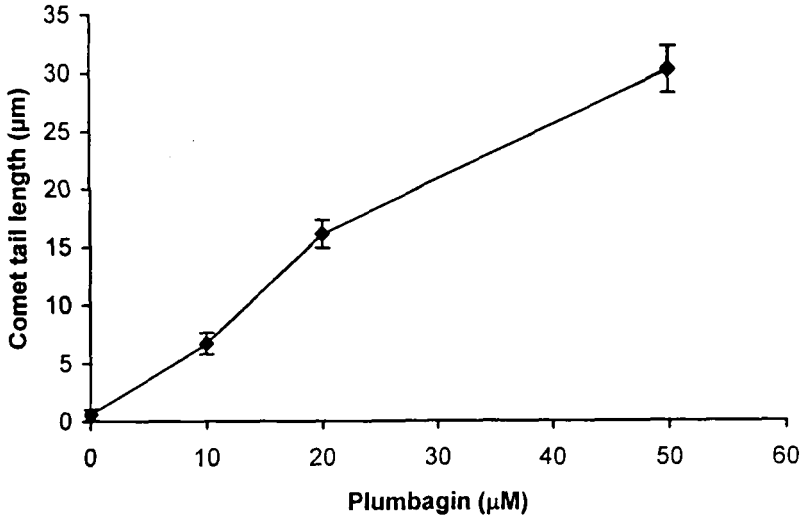
[B] Lymphocytes + Plumbagin alone (50 μM)

[C] Lymphocytes + Plumbagin 10 μM + Cu(II) 20 μM

[D] Lymphocytes + Plumbagin 20 μM + Cu(II) 20 μM

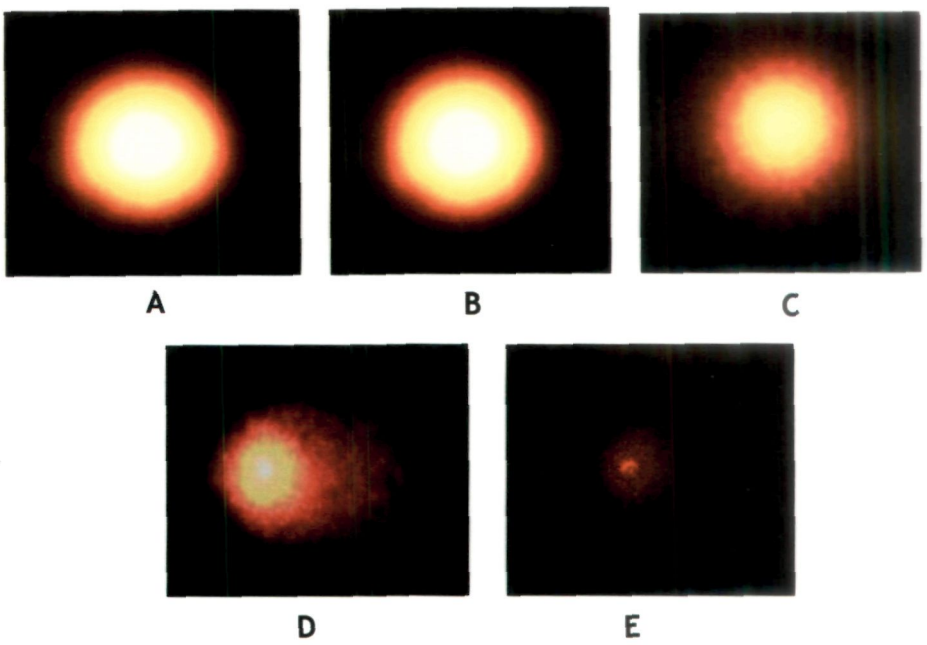
[E] Lymphocytes + Plumbagin 50 μM + Cu(II) 20 μM

Fig 28. Comet tail length (μm) plotted as a function of pre-incubating the lymphocytes with increasing concentrations of plumbagin on plumbagin-Cu(II) induced DNA breakage



Comet tail length (μm) plotted as a function of pre-incubation of lymphocytes with increasing concentrations of plumbagin. Isolated cells suspended in RPMI 1640 were pre-incubated with the indicated concentrations of plumbagin for 30 min at 37^0C. After pelleting the lymphocytes were washed twice with PBS Ca^{2+} and Mg^{2+} free before re-suspension in RPMI 1640 and further incubation for 30 min in the presence of 20 μM Cu(II). Values reported are $\pm$SEM of three independent experiments. *P* value <0.05 and significant when compared to control.

Fig 29. Effect of pre-incubating the lymphocytes with increasing concentrations of Cu(II) (5-20 μM) on plumbagin-Cu(II) induced DNA breakage



Photographs of comets (100-X) obtained after pre-incubation of lymphocytes with increasing concentrations of Cu(II). Isolated cells suspended in RPMI 1640 were pre-incubated with the indicated concentrations of Cu(II) for 30 min at 37^0C. After pelleting, the lymphocytes were washed twice with PBS Ca^{2+} and Mg^{2+} free before re-suspension in RPMI 1640 and further incubation for 30 min in the presence of 50 μM plumbagin.

[A] Untreated lymphocytes

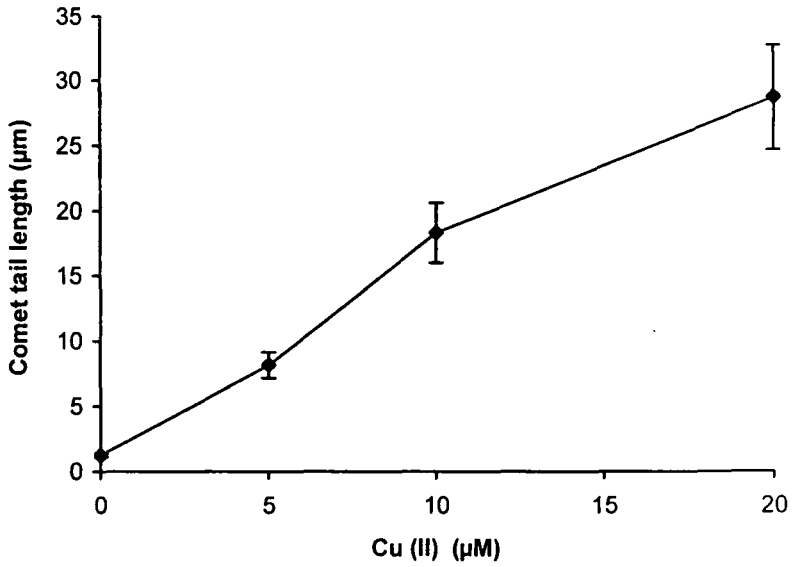
[B] Lymphocytes + Cu(II) alone (20 μM)

[C] Lymphocytes + Cu(II) 5 μM + Plumbagin 50 μM

[D] Lymphocytes + Cu(II) 10 μM + Plumbagin 50 μM

[E] Lymphocytes + Cu(II) 20 μM + Plumbagin 50 μM

Fig 30. **Comet tail length (μm) plotted as a function of pre-incubating the lymphocytes with increasing concentrations of Cu(II) on plumbagin-Cu(II) induced DNA breakage**



Comet tail length (μm) plotted as a function of pre-incubation of lymphocytes with increasing concentrations of Cu(II). Isolated cells suspended in RPMI 1640 were pre-incubated with the indicated concentrations of Cu(II) for 30 min at 37^0C. After pelleting, the lymphocytes were washed twice with PBS Ca^{2+} and Mg^{2+} free before re-suspension in RPMI 1640 and further incubation for 30 min in the presence of 50 μM plumbagin. Values reported are ±SEM of three independent experiments. *P* value <0.05 and significant when compared to control.

Table 5. Effect of scavengers of reactive oxygen species and neocuproine on plumbagin-Cu(II) induced DNA breakage in lymphocytes

Treatment	Tail Length (μm)	% Inhibition
Untreated	2.7 ± 0.11	-
Plumbagin 50 μM +Cu(II) 20 μM #	29.5 ± 2.25	-
+ Thiourea (1mM)	7.9 ±0.98 *	73.2 %
+ Sodium Azide (1mM)	15.1 ± 1.5 *	48.8%
+ Potassium Iodide (1mM)	10.6 ± 1.3 *	64.0 %
+ SOD (100 μg/ml)	17.5 ± 1.4 *	40.6 %
+ Catalase (100 μg/ml)	9.6 ± 1.0 *	67.4 %
+ Neocuproine (1mM)	11.1 ± 0.9*	62.3 %

* $P < 0.05$ by comparison with treated control (#). Data represent mean ±SEM of three independent experiments

DISCUSSION-II

DISCUSSION-II

The present studies lead to the following major conclusions.

1. Plumbagin in the presence of micromolar concentrations of Cu(II) causes DNA strand breaks in human peripheral lymphocytes as well as calf thymus and plasmid DNA.

2. Redox cycling of copper leads to the generation of various reactive oxygen species, particularly the hydroxyl radical which serves as the proximal DNA cleaving agent

3. The observed DNA damage could be inhibited by catalase and neocuproine, a Cu(I) specific sequestering agent, indicating that a copper redox cycle and H_2O_2 generation are two major determinants involved in the observed DNA damage. Therefore, the reactive oxygen species generated from the further interaction between copper and H_2O_2 may be responsible for the DNA damage mediated by copper-induced activation of plumbagin.

These results place plumbagin in a class of plant derived compounds such as flavanoids (Ahmad et al., 1992), tannins (Bhat and Hadi, 1994), catechins (Azam et al., 2004) and stilbene-resveratrol (Azmi et al., 2005), which also exhibit pro-oxidant 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-ansidine (Whong et al.,1984),1,10-phenanthroline (Gutteridge and Halliwell, 1982),

bleomycin (Ehrenfeld et al., 1987), adriamycin (Eliot et al., 1984; Haidle and McKinney, 1985) as well as flavanoids and stilbenes (Ahmad et al., 1992; Rahman et al., 1989; Azmi et al., 2005) are based on mechanisms involving oxygen derived radicals. The results presented here show that plumbagin is capable of binding to DNA as well as copper and thus it would be reasonable to assume that a similar mechanism operates in the case of plumbagin-Cu(II) mediated DNA cleavage.

Copper is a normal component of chromatin and is required for maintaining chromosome structure and gene regulatory processes (Furst and Hamer 1989; Prutz et al., 1990). Such copper can be mobilized by metal chelating agents such as 1,10 phenanthroline leading to DNA breakage (Burkit et al., 1996). It is thus conceivable that similar to 1,10 phenanthroline plumbagin is also capable of mobilizing endogenous copper, both from chromatin as well as copper binding proteins especially ceruloplasmin, causing oxidative DNA damage. This would account for the observed genotoxic/cytotoxic properties of plumbagin in human peripheral lymphocytes. Earlier studies have established that Copper can induce the oxidation of 1,4-HQ, a benzene metabolite, producing 1,4-BQ and H_2O_2 through a semiquinone intermediate (Li and Trush, 1993). Indeed Li and Trush have proposed similar model of reactive oxygen species mediated oxidative DNA damage by a number of phenolic compounds to account for their genotoxic/cytotoxic effects and also in recent reports, plumbagin was demonstrated to induce apoptosis of human cervical cancer cells (ME-180 cell lines) and HaCaT keratinocytes through the generation of ROS (Inbaraj and Chignell, 2003; Srinivas et al., 2004). In this study we first assessed the role of Cu(II)/Cu(I) redox cycle and reactive oxygen generation in plumbagin-Cu(II) induced DNA damage. It was clear from our results that plumbagin induced extensive DNA strand breaks in plasmid pBR322 DNA, as indicated by the

disappearance of the supercoiled (SC) form and the formation of open circular (OC) forms of DNA. It was also evident from the result that plumbagin or Cu(II) alone elicited no damage to DNA. Further using a cellular system of lymphocytes isolated from human peripheral blood and alkaline single cell gel electrophoresis (Comet assay) we confirmed that plumbagin-Cu(II) system is indeed capable of causing DNA degradation in cells such as lymphocytes. Further, the DNA degradation of lymphocytes was inhibited by scavengers of reactive oxygen species and neocuproine, a Cu(I) specific sequestering agent, indicating that a copper redox cycle and reactive oxygen generation are two major determinants involved in the observed DNA damage (Table 5).

Thus, the major conclusion of the present study is that the possible mechanism by which plumbagin exhibits anticancer properties may involve mobilization of endogenous copper and the consequent pro-oxidant action as mentioned earlier and this mechanism infact is physiologically feasible and may be of biological significance. It is also realized that these 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 precede 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 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). Moreover the generation of hydroxyl radicals from the reaction of Cu(II) complexes with biological reductants such as ascorbic acid, glutathione, acetylcysteine and hydroquinone was already established by spin trapping experiments using electron spin resonance (ESR) (Ueda et al., 1998). Indeed it was earlier proved that flavanoids are able to form a ternary complex with DNA and Cu(II), where Cu(II) is reduced to Cu(I) (Rahman et al.,1989). Superoxide can also be formed by reoxidation of Cu(I) to Cu(II) in the ternary complex (Rahman et al., 1989). Chromatin bound copper is understood to be present in the reduced form (Cu(I)) (Lewis and Lamelli, 1982) and thus would be available for reoxidation to Cu(II) by H_2O_2 in the Fenton type reaction and binding to phenolic compounds and recycling.

These other observations in literature include the fact that copper along with zinc is the major metal ion present in the nucleus (Bryan, 1979). The concentration of copper in various tissue ranges from 10 to >100 μM with 20% found in the nucleus (Linder, 1991). Further, serum (Ebadi and Swason, 1998), tissue (Yoshida et al., 1993) and cellular (Carpentieri, 1986) 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 (Burkit et al., 1996). Further, it has also been proposed that most clinically used anticancer drugs can activate the 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).

In view of the results described above and other observations (Hadi et al., 2000; Rietgens et al., 2002) we would like to propose that anticancer and apoptosis inducing property of plumbagin involves its

ability to mobilize endogenous copper ions, possibly the copper bound to chromatin. Since copper is capable of mediating activation of a variety of phenolic compounds as mentioned earlier (Li and Trush, 1994), it is reasonable to propose that the DNA associated copper in cells may have the potential to activate plumbagin via a copper-redox reaction, producing reactive oxygen and electrophilic plumbagin intermediates (semiquinone intermediate). The interaction of plumbagin with DNA associated copper may finally result in oxidative DNA damage and this might contribute to the cytotoxicity induced by plumbagin. In short, our results throws light into the fact that plumbagin have implications for development of novel antitumor and cancer chemopreventive agents. So with the help of diversity oriented synthesis and other combinatorial synthetic methodologies, derivatized compounds of plumbagin can be synthesized and screened for potential cytotoxicity, to find out more potential lead compound which may have therapeutic applications in the treatment of cancer.

CHAPTER-III

Cytotoxic and Morphological Studies of Plumbagin on Human Epidermoid Carcinoma (A431) and Mouse Dalton's Lymphoma Ascites (DLA) Cell Lines

MATERIALS & METHODS-III

MATERIALS & METHODS-III

Cell Lines

Human epidermoid carcinoma (A431) and Mouse Dalton's Lymphoma Ascites (DLA) cell lines were procured from National Center for Cell Science (Pune, India) and maintained at Laboratory of Tumor Immunology and Functional Genomics, Regional Cancer Centre (Thiruvananthapuram, India). The cells were maintained in monolayer culture at 37°C and 5% CO_2 in DMEM supplemented with 10% FCS, 100U/ml of penicillin, 100 µg/ml of streptomycin.

Chemicals	Source
DMEM	Sigma Chemical Co., USA
Fetal Calf Serum	Sigma Chemical Co., USA.
MTS (non radioactive cell proliferation assay kit)	Promega, Madison, WI, USA
Streptomycin and penicillin	Gibco BRL, Gaithersburg, MD, USA
Trypan blue	Sigma-Aldrich Co., USA
Ethidium Bromide	Sigma Chemical Co., USA
Acridine Orange	Sigma Chemical Co., USA
Trypsin	Sigma Chemical Co., USA
Sodium chloride	Qualigens, India
KCl	Qualigens, India
Na_2HPO4	Qualigens, India
KH_2PO4	Qualigens, India

* All other chemicals were commercial products of analytical grade.

Cell cultures from frozen cells were established as follows:

- Placed 10 ml of growth medium in a 15 ml conical tube.
- Thawed the frozen cryovial of cells within 40-60 sec by gentle agitation in a 37°C water bath.
- Removed the cryovial from the water bath and decontaminated the cryovial by immersing it in 70% (v/v) ethanol (at room temperature).
- Transferred the thawed cell suspension to the conical tube containing 10 ml of growth medium.
- Collected the cells by centrifugation at 200×g for 5 min at room temperature.
- Removed the growth medium by aspiration.
- Resuspended the cells in the conical tube in 5 ml of fresh growth medium.
- Added 10 ml of growth medium to a 75 cm^2 tissue culture flask and transferred the 5 ml of cell suspension to the same tissue culture flask. Placed the cells in a 37°C incubator at 5% CO_2.
- Monitored cell density daily. Cells were passaged when the culture was at 50% confluency.

Preparation of cell liquid nitrogen stock

- When growing cells for the production of liquid nitrogen stock, cultures were maintained at 50% confluence.

- Collected cells from a healthy, log-phase culture. Removed the culture medium by aspiration. Trypsinized cells for 1-3 min in 1.5 ml of Trypsin-EDTA solution.

- Diluted the cells with 8.5 ml of growth medium. The serum in the medium inactivates the trypsin. Transferred the suspension to a 15 ml conical tube, and then collected the cells by centrifugation at 600×*g* for 5 min at room temperature.

- Removed the medium by aspiration. Resuspended the cell pellet in a minimal volume of growth medium (containing 10% fetal bovine serum). Counted the cells present in an aliquot of the resuspension using a hemocytometer.

- Diluted the cell suspension to 1×10^6 cells/ml in freezing medium, and then dispensed 1 ml aliquots of the suspension into 2 ml cryovials.

- Frozen the cell aliquots gradually by placing the vials in a shell freezer and then placed in a -80°C freezer overnight.

- Transferred the vials of frozen cells to liquid nitrogen for long-term storage.

Passaging of cells

- Removed the growth medium by aspiration. Washed cells once with 10 ml of phosphate-buffered saline.

- Trypsinized cells for 1-3 min in 1.5 ml of Trypsin-EDTA solution.

- Diluted the cells with 8.5 ml of growth medium to inactivate the trypsin.

- Transferred 1 ml of the cell suspension to a fresh 75 cm^2 tissue culture flask and added 9 ml fresh growth medium. Placed the cells in a 37°C incubator at 5% CO_2.

- Monitored cell-density daily.

Determination of cytotoxicity of plumbagin in A431 cells using MTS assay

Inhibition of cell proliferation by plumbagin was measured by MTS assay. MTS assay uses the soluble tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), and it is versatile and offers several

advantages over MTT and other cytotoxicity assays due to the solubility of the MTS formazan product in culture medium (Cory et al., 1991). MTS is chemically reduced by cells into formazan, which is soluble in culture medium. The measurement of the absorbance of the formazan can be carried out using 96 well microplates at 490 nm. The assay measures dehydrogenase enzyme activity found in metabolically active cells. Since the production of formazan is proportional to the number of living cells; the intensity of the produced color is a good indication of the viability of the cells.

A431 cells (5000 cells / well), control 1 (medium only) and control 2 (medium + cells) were seeded in triplicates in 96 well microtitre plates and incubated for attachment at 37°C in 5% CO_2 incubator for 12 h. After 12 h, 100 µl of different concentrations of plumbagin (1 µM to 500 µM) were added to each well excluding the control wells. The plates were then incubated at 37°C in 5% CO_2 incubator for 24-48 h. 20 µL of MTS/PMS solution was added and incubated in dark for another 4 h and absorbances were recorded at 490 nm using ELISA plate reader. Graph was plotted with percentage cell cytotoxicity on Y-axis and concentration of plumbagin (µM) on X- axis. Percentage cytotoxicity and IC_{50} was determined. Percentage cell viability was calculated using the formula of James Kumi-Diaka et al (2004). Percentage cell viability = (AT-AB)/(AC-AB)-100; where AT, AB and AC are the absorbances of test, blank and control, respectively.

Development of Lymphoma

Dalton's lymphoma cell lines were maintained in the inbred BALB/c mice. The lymphoma cells collected from the peritoneum in cold PBS in aseptic conditions were pelleted after centrifugation. The cells were counted in a hemocytometer. Each experimental mouse received $1x10^6$ cells, intraperitoneally. The lymphoma was allowed to grow *in vivo* for about 14 days. At the end of the experimental period the animals were sacrificed.

Determination of cytotoxicity of plumbagin in DLA cells using MTS assay

DLA cells (5000 cells / well), control 1 (medium only) and control 2 (medium + cells) were seeded in triplicates in 96 well microtitre plates and incubated at 37°C in 5% CO_2 for 6 h. After 6 h, 100 µl of different concentrations of plumbagin (concentration ranging from 1 µM to 100 µM) were added to each well excluding the control wells. The plates were then incubated at 37°C in 5% CO_2 incubator for 24-48 h. 20 µL of MTS/PMS solution was added and incubated in dark for another 4 h and absorbances were recorded at 490 nm using ELISA plate reader. Graph was plotted with percentage cell cytotoxicity on Y-axis and concentration of plumbagin (µM) on X-axis. Percentage cytotoxicity and IC_{50} was determined.

Deducing cell morphology by Acridine Orange/Ethidium Bromide dual stain (Apoptosis analysis)

Drug induced apoptosis and necrosis were determined morphologically after labelling with acridine orange and ethidium bromide (Duke and Cohen, 1992). Acridine orange/Ethidium bromide staining is used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. Cells are viewed under a fluorescence microscope and counted to quantify apoptosis. Acridine orange (AO) is a nucleic acid selective fluorescent cationic dye useful for cell cycle determination. It is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions. When bound to DNA, it is very similar spectrally to fluorescein. Ethidium bromide intercalates and stains DNA, providing a fluorescent red-orange stain. Although it will not stain healthy cells, it can be used to identify cells that are in the final stages of apoptosis, such cells have much more permeable membranes. Consequently, ethidium bromide is often used as a marker for apoptosis in cell populations and to locate bands of DNA in

gel electrophoresis. The stain may also be used in conjunction with acridine orange (AO) in viable cell counting. This EB/AO combined stain causes live cells to fluoresce green whilst apoptotic cells retain the distinctive red-orange fluorescence.

The role of plumbagin in inducing apoptosis in A431 cells was evaluated in this study. Plumbagin at 25 μM could induce apoptosis in A431 cells as indicated by morphological changes characteristic of apoptosis. A431 cells (5000 cells in 100 μl) were cultured with 100 μl of 25 μM plumbagin at 37°C in 5% CO_2 incubator for 12-48 h. Then the medium was removed and 1 μl of 100 μg/ml of acridine orange with 100 μg/ml of ethidium bromide was added to cells. After a few min, the cells were immediately washed with PBS once and viewed under fluorescent microscope with a suitable filter. Live cells were determined by the uptake of acridine orange (green fluorescence) and exclusion of ethidium bromide (red fluorescence) stain. Live and dead apoptotic cells were identified by perinuclear condensation of chromatin stained by acridine orange or ethidium bromide, respectively, and by the formation of apoptotic bodies. Necrotic cells were identified by uniform labelling of the cells with ethidium bromide.

Determination of ROS generation and Cu(II)/Cu(I) redox cycling

To investigate the mechanisms leading to loss of cell proliferation by plumbagin and also to demonstrate whether ROS generation and Cu(II)/Cu(I) redox cycling are involved in plumbagin induced cell death in cancer cells, the free radical scavenging agents (SOD, catalase & thiourea) and a Cu(I) specific sequestering agent (neocuproine) were used in this study. The A431 and DLA cells (5000 cells / well) seeded in 96-well plate were pretreated with SOD,

catalase, thiourea and neocuproine for 1.5 h and subsequently treated with plumbagin (25 μM & 175 μM respectively). One set of cells were treated with plumbagin only (25 μM for A-431 & 175 μM for DLA cells). The plates were then incubated at 37°C in 5% CO_2 incubator for 24 h. 20 μL of MTS/PMS solution was added and incubated in dark for another 4 h and absorbances were recorded at 490 nm using ELISA plate reader.

Statistics

The statistical analysis was performed as described by Tice et al (2000) and is expressed as $\pm$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.

RESULTS-III

RESULTS-III

Plumbagin inhibits cell proliferation in A431 and DLA Cells

To investigate the potential cell proliferative inhibition activity of plumbagin, we examined the percentage cell death induced by plumbagin in both A-431 and DLA cells. Exposure of A-431 and DLA cells to various concentrations of plumbagin (1-500 μM) for 48 h inhibited the growth of both the cell lines in a dose and time dependent manner **(Figures 31 & 32)**.

The IC_{50} values of plumbagin were 25 μM and 175 μM for A-431 and DLA cells respectively. These IC_{50} concentrations of plumbagin were used in further experiments. Plumbagin was found to be more potent in inducing cell death in human epidermoid carcinoma A431 cells (IC_{50} 25 μM) compared to DLA cells as is evident from the present data. This inference is of great significance, as plumbagin has been shown to exert antiproliferative effect on skin cancer in various reports (Melo et al., 1974; Wang et al., 2007). This study supports the role of plumbagin as a promising and potential lead compound against skin cancer.

Plumbagin induces morphological alterations (Characteristics of apoptosis) in human epidermoid carcinoma (A431) cells

Apoptotic cells were identified on the basis of morphological features that included contracted cell bodies, condensed, uniformly circumscribed and densely stained chromatin, or membrane- bound apoptotic bodies containing one or more nuclear fragments (Shanker, 2000).

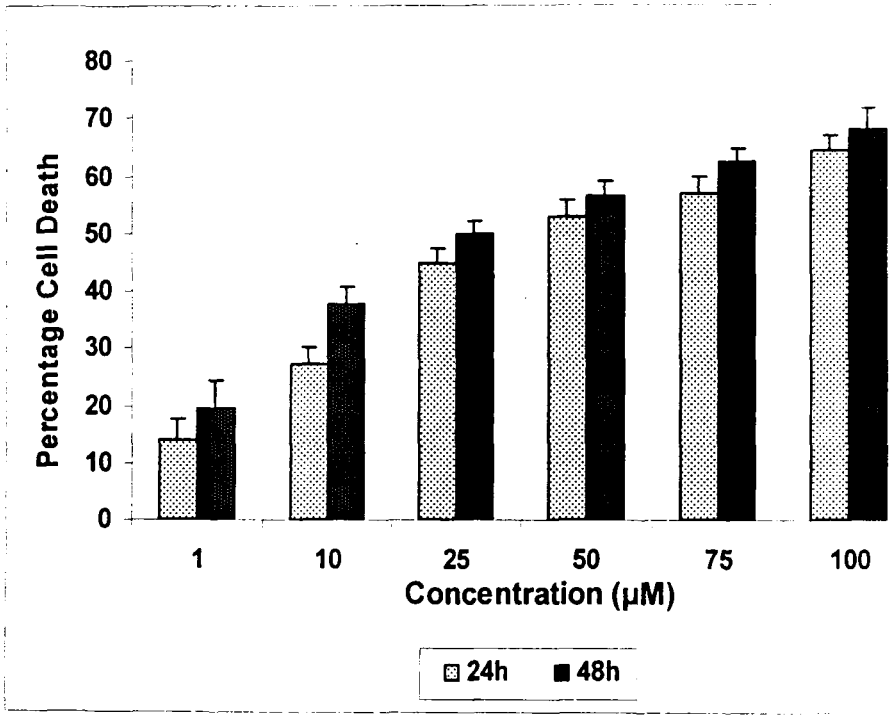
The phenotypic characteristics of plumbagin treated cells were evaluated by microscopic inspection of overall morphology. Untreated A431 cells after 12, 18 and 24 h showed green fluorescence representing 100% viability **(Fig 33A)**. A431 cells treated with 25 μM

of plumbagin showed varying degrees of evident apoptosis in a time dependent manner. Nuclear and cytoplasmic condensation with blebbing of the plasma membrane, and formation of apoptotic bodies (Orange-red fluorescence) were prominent at the end of 12, 24 and 48 h, the latter being severe (**Figures 33B, 33C and 33D**). However, some cells showed necrosis (red fluorescence) as the membrane integrity was lost due to cell rupture releasing noxious cellular contents. The results obtained in figure 33B, 33C and 33D clearly establish that the phenomenon of cytotoxicity shown by plumbagin in cancer cells as obtained by MTS assay may be due to apoptosis as confirmed by morphological evaluation of cells by Acridine Orange/Ethidium bromide dual staining.

Free radical scavengers and neocuproine shows preventive effect on plumbagin induced apoptotic response in A431 and DLA cells

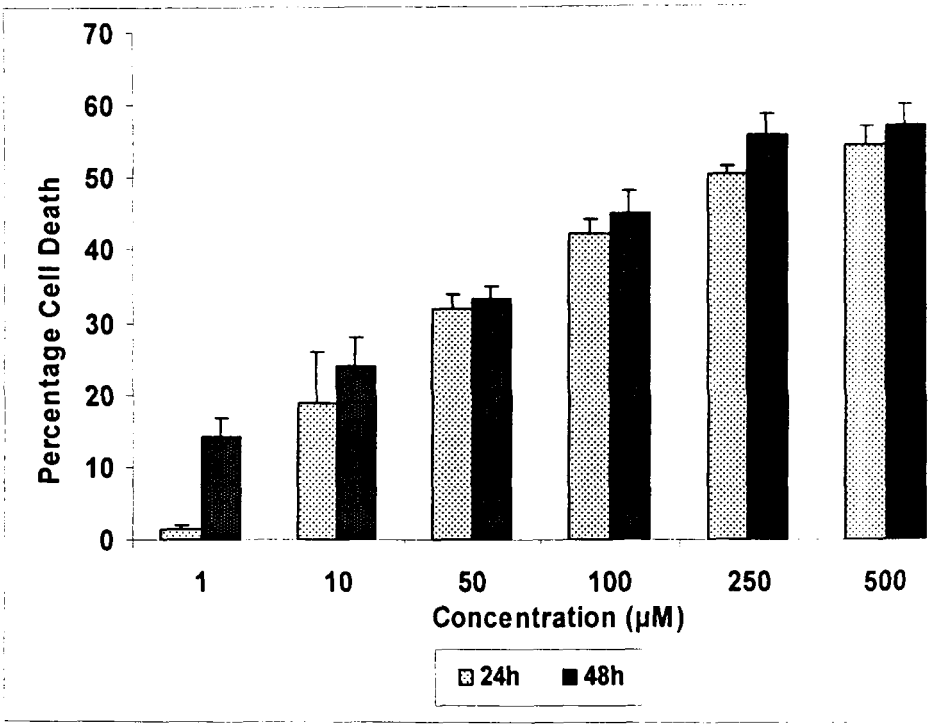
Free radical producing activities have been described as biological effects of plumbagin. In the absence of plumbagin, free radical scavengers and neocuproine did not show any appreciable toxicity to the cells at the given concentration for the entire time period. Therefore, prior treatment of the cells with SOD, catalase, thiourea and neocuproine for 1.5 h followed by plumbagin treatment at indicated concentrations for 24 h was performed. The results shown in **Figures 34 & 35** clearly indicate that plumbagin induced growth inhibition of A431 and DLA cells could be inhibited by ROS scavengers and also neocuproine to a significant level suggesting the involvement of ROS. Inhibition of plumbagin induced cell death by neocuproine (a Cu(I) specific sequestering agent) confirms the role of copper in the cytotoxic reaction. These results strongly suggest that anticancer action of plumbagin may possibly be due to the mobilization of endogenous copper ions and the subsequent pro-oxidant action as described earlier.

Fig 31. Analysis of cell death in plumbagin treated A431 cells by MTS assay



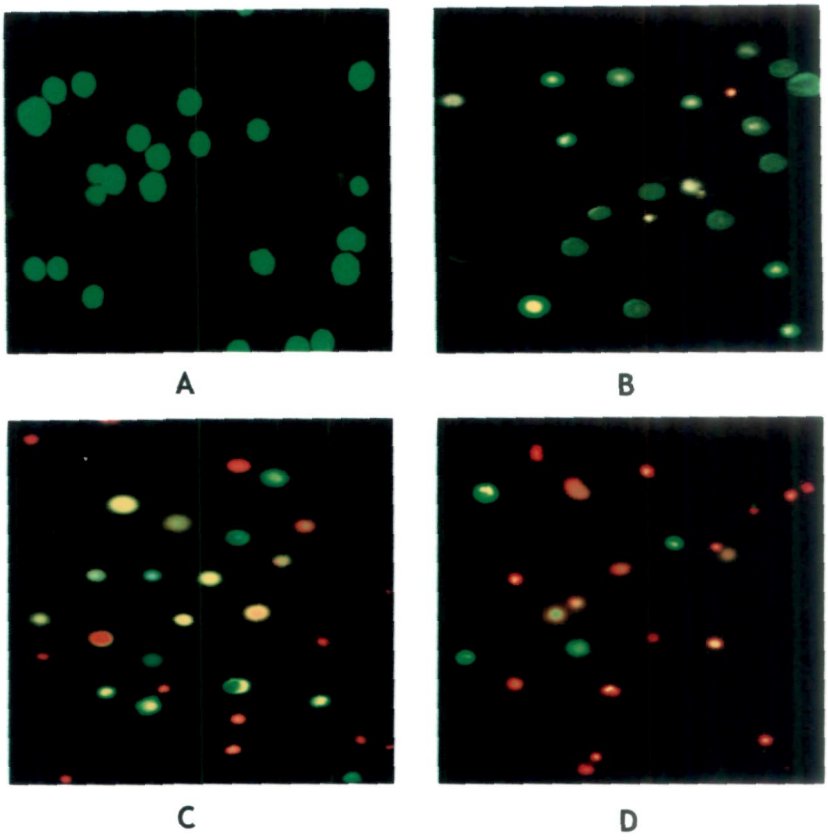
A431 cells (5000 cells/ well) treated with varying concentrations of plumbagin (1-500 μM) after 24 h as obtained by MTS assay. Percentage cell death was assessed by MTS assay with triplicate samples as described in 'Materials & Methods'. All results are expressed as the mean percentage of control ±SEM of triplicate determinations from three independent experiments. *P* value <0.05 and significant when compared to control.

Fig 32. Analysis of cell death in plumbagin treated DLA cells by MTS assay



DLA cells (5000 cells/ well) treated with varying concentrations of plumbagin (1-500 µM) after 24 h as obtained by MTS assay. Percentage cell death was assessed by MTS assay with triplicate samples as described in 'Materials & Methods'. All results are expressed as the mean percentage of control ±SEM of triplicate determinations from three independent experiments. *P* value <0.05 and significant when compared to control.

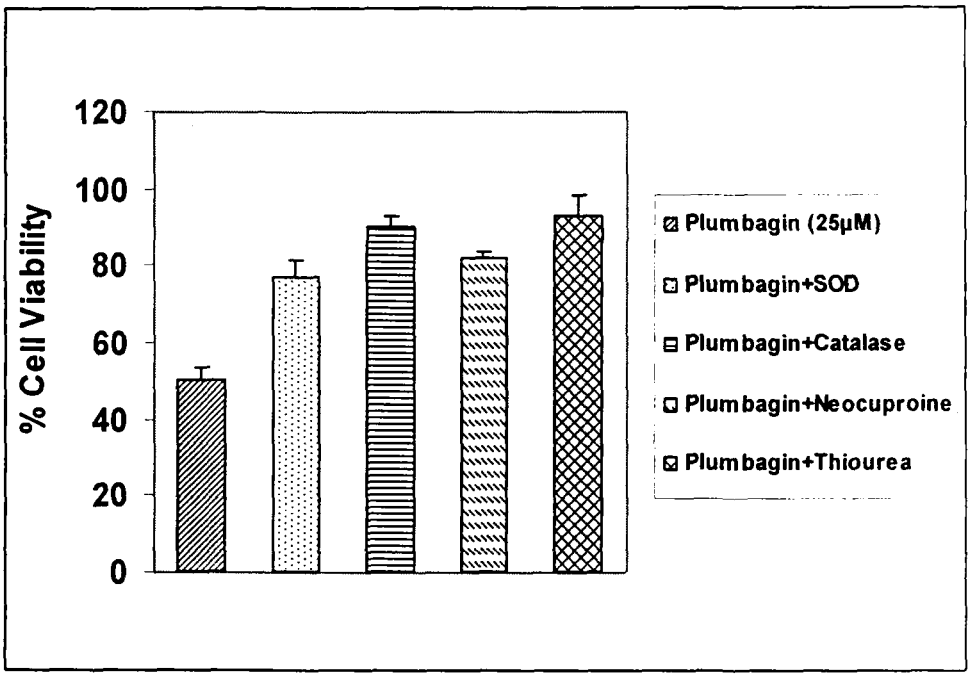
Fig 33. Acridine orange/Ethidium bromide dual staining of A431 cells (Apoptosis analysis)



Changes in nuclear morphology of A431 cells induced by plumbagin. A431 cells (5000 cells/well) were seeded in 12-well plates and then treated with or without plumbagin (25 μM) for different time periods. After washing with PBS, the cells were stained with a mixture of acridine orange/ethidium bromide mixture. The cells were viewed under inverted fluorescent microscope and photographed as described in 'Materials & Methods'.

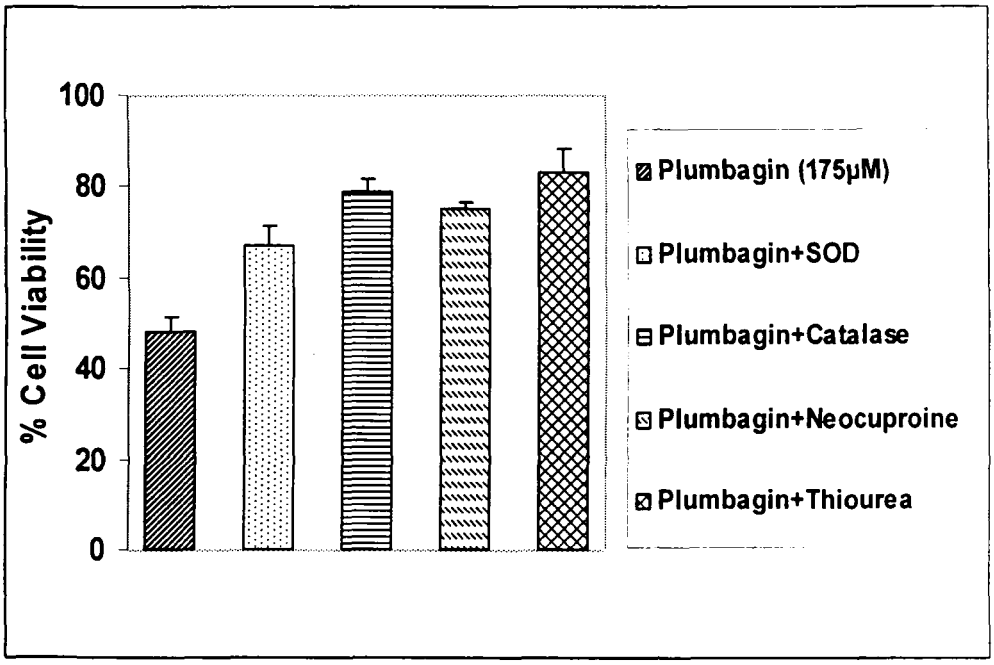
[A] Control (Untreated cells) (Green fluorescence)
[B] Cells treated with plumbagin after 12 h
[C] Cells treated with plumbagin after 24 h
[D] Cells treated with plumbagin after 48 h

Fig 34. Effects of free radical scavengers and neocuproine on plumbagin induced cell death in A431 cells



A431 cells were plated into 96-well plate for 24 h and then treated with SOD (100 μg/ml), catalase (100 μg/ml), thiourea (1 mM) and neocuproine (1 mM) for 1.5 h followed by plumbagin (25 μM) treatment for a further 12 h. MTS was added to the medium for additional 3 h. The cell viability was expressed as percentage over the control. Each value is presented as the mean ±SEM of triplicate determinations from three independent experiments. $P<0.001$, the mean percentage viability was significantly higher from the corresponding plumbagin treated groups as analyzed by Student's *t*-test.

Fig 35. **Effects of free radical scavengers and neocuproine (a Cu(I) specific sequestering agent) on plumbagin induced cell death in DLA cells**



DLA cells were plated into 96-well plate for 24 h and then treated with SOD (100 µg/ml), catalase (100 µg/ml), thiourea (1 mM) and neocuproine (1 mM) for 1.5 h followed by plumbagin (175 µM) treatment for a further 12 h. MTS was added to the medium for additional 3 h. The cell viability was expressed as percentage over the control. Each value is presented as the mean ±SEM of triplicate determinations from three independent experiments. $P<0.001$, the mean percentage viability was significantly higher from the corresponding plumbagin treated groups as analyzed by Student's *t*-test.

DISCUSSION-III

DISCUSSION-III

Prevention is undeniably the sensible maneuver towards the ultimate goal of cancer control (Suffness and Pezzuto, 1991). Several methods exist for the treatment of cancer in modern medicine. These include chemotherapy, radiotherapy and surgery. Chemotherapy is now considered as the most effective method of cancer treatment. Intervention with chemopreventive agents at the early stage in carcinogenesis is theoretically more rational than attempting to eradicate fully developed tumors with chemotherapeutic drugs. However, most cancer chemotherapeutants severly affect the host normal cells (Mascarenhas, 1994). Hence, the use of natural products now has been contemplated of exceptional value in the control of cancer and its eradication program (Suffness and Pezzuto, 1991).

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a quinonoid constituent isolated from the roots of *Plumbago zeylanica L.*, has been proven to possess antitumor activity in both *in vitro* and *in vivo* studies. It has been found to inhibit the growth of Raji, Calu-1, HeLa and Wish cell lines (Lin et al., 2003) and has been reported to cause a regression of fibrosarcomas in Wistar rats (Krishnaswamy and Purushothaman, 1980). Plumbagin has been reported to induce G2/M phase arrest in non-small cell lung cancer A549 cells through the induction of p21 (Hsu et al., 2006). Plumbagin also exhibit an inhibitory effect on carcinogenesis in the intestines, causes cytogenic and cell cycle changes in mouse Ehrlich ascites carcinoma and also possess antiproliferation activity in human cervical cells (Singh and Udupa, 1997; Sugie et al., 1998; Srinivas et al., 2005). Moreover recent studies indicate that plumbagin can down regulate the expression of NF-κB regulated gene products involved in the cell proliferation and antiapoptosis. Inhibition of the NF-κB activation

pathway by plumbagin was found to increase the apoptotic activity of TNF and paclitaxel (Sandur et al., 2006). Plumbagin has been reported to inhibit the activity of Topoisomerase II (Fuji et al., 1992). In a very recent report plumbagin was shown to induce cell cycle arrest and apoptosis through reactive oxygen species in human melanoma A375.S2 cells (Wang et al., 2007). In addition to all these facts recent studies has also established that plumbagin does not exert any apoptotic effect on normal cells and therefore can be developed as an anticancer drug (Hsu et al., 2006; Sandur et al., 2006; Kawiak et al., 2007).

Quinones can generate ROS through one electron transfer, and can also react with thiols through Michael addition (Thompson et al., 1987). The production of ROS has been suggested to play a key role in the DNA damage, and may effect the cytotoxic effects of menadione (a structurally related quinone), in cultured human cells (Noto et al., 1989; Chang et al., 1991). Because of their high reactivity, ROS affect various cellular molecules such as fatty acids, carbohydrates, proteins and nucleic acids. An excess of ROS may lead to cell death when their level overwhelms the cellular antioxidant capacity (Mason and Chignell, 1981; Cerruti et al., 1985; McCord et al., 1993). Plumbagin due to the presence of a quinone moiety, has been reported to generate ROS and ROS have been implicated to play a role in the apoptosis process of plumbagin (Srinivas et al, 2004). Further it was also reported that the cytotoxicity of plumbagin is mainly the result of redox cycling and H_2O_2 production (Inbaraj and Chignell, 2004). However the mechanism by which plumbagin generates ROS was not fully established.

In the second chapter of this study we for the first time, confirmed using plasmid DNA, calf thymus DNA and also in a cellular system of lymphocytes isolated from human peripheral blood, the role of metal

ion (copper) in the observed cytotoxic action of plumbagin, which possibly involves mobilization of endogenous copper ions and the subsequent pro-oxidant action. The objective of this part of my study was to determine whether plumbagin inhibits the growth of human epidermoid carcinoma (A431) and Dalton's Lymphoma Ascites (DLA) cell lines through the same mechanism which we proposed earlier (Chapter-II). Infact this study is the first to investigate the anticancer effect of plumbagin in human epidermoid carcinoma cells. In this chapter, we demonstrate that plumbagin is able to inhibit the growth of human epidermoid carcinoma A431 cells and also mouse DLA cells. Anti-tumor agents, which can modulate apoptosis, may be able to affect the steady state of cell populations that are helpful in the management and therapy of cancer. Results of the present study suggested that plumbagin could induce tumor cell death through the induction of apoptosis. Most of the cytotoxic anti-cancer drugs in current use have been shown to induce apoptosis in susceptible cells. It has been established that apoptotic cells display DNA fragmentation at internucleosomal sites followed by morphological changes and loss of membrane integrity (Christopher et al., 1992; Daniel et al., 1993).

The major conclusion of this study is that both A431 and DLA cells are highly sensitive to growth inhibition and apoptosis induction by plumbagin and the mechanism of cell death induced by plumbagin is possibly via the same mechanism which we proposed earlier. In both the cell lines used the cytotoxic action of plumbagin is through the induction of apoptosis, as is evident from the present data. Moreover, we could conclude that the induction of apoptosis by plumbagin is through the generation of ROS as is evident from the inhibition upon addition of ROS scavengers like SOD, catalase and thiourea. Further, inhibition of cytotoxicity of plumbagin upon addition of neocuproine, a Cu(I) specific sequestering agent suggest the possible involvement of copper ions also in the observed cytotoxic action of plumbagin.

It was also interesting to note that in this study, plumbagin shows a more significant cytotoxicity in human epidermoid carcinoma (A431) cells (IC_{50} 25 μM) compared to its cytotoxic action on DLA cells. A very recent report established that ROS accumulation contributes to plumbagin-induced cell death in human melanoma cells (Wang et al., 2007). Skin cancer is the third most common human malignancy. Increase of the incidence of malignant skin cancer is rising at an alarming rate (Miller and Mihm, 2006; Gray-Schopfer, 2007). Epithelial squamous carcinoma is one of the most common types of malignant tumors that affect the integumentary system, accounting for approximately 25% of all skin cancer and unlike basal cell skin carcinoma this form of cancer can metastasize and therefore it is important to get early treatment. Conventional therapies like radiation therapy and surgery treatments may often causes side effects. Common topical medication available for the treatment of squamous skin carcinoma include Imiquimod, a cream which acts as an immune response modifier but reported serious side effects including local skin reactions and severe auto-immune and neuro-immune effects. So novel therapies are needed to reduce the effect of the increasing incidence of human skin carcinoma.

The results obtained in this chapter of my study indicate that human epidermoid carcinoma cells are highly sensitive to growth inhibition and apoptosis induction by plumbagin. More importantly, based on our own observation and that of many others that the plumbagin does not exhibit any significant toxicity in normal cells (Hsu et al., 2006; Sandur et al., 2006; Kawiak et al., 2007), we suggest that plumbagin possesses selectivity between normal and cancer cells. Therefore, these properties of plumbagin could be further explored for the development of anticancer agents with higher therapeutic index, especially for skin cancer.

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LIST OF PUBLICATIONS

Nazeem, S., Azmi, A.S., Hanif, S. and Kumar, K.S. Reactive oxygen-dependent DNA damage resulting from the oxidation of plumbagin by a copper-redox cycle mechanism: implications for its anticancer properties. *Austral-Asian Journal of Cancer*. 2008; **2**, 65-72.

Nazeem, S., Azmi, A.S., Hanif, S. and Kumar, K.S. Plumbagin Induces reactive oxygen species through a copper-redox cycle mechanism which mediates apoptosis in human skin carcinoma A-431 cells: A potent topical remedy for skin cancer. *Mutation Research*. (Communicated)

Reactive Oxygen-Dependent DNA Damage Resulting from the Oxidation of Plumbagin by a Copper-redox Cycle Mechanism: Implications for its Anticancer Properties

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ABSTRACT

Plumbagin, a naphthoquinone derived from the medicinal plant Plumbago zeylanica has been shown to exert anticancer and antiproliferative activities in animal models as well as cells in culture. This study is the first to investigate the role of a metal ion (Copper) in the observed cytotoxic action of plumbagin. Recently copper has been shown to be capable of mediating the action of several phenolic compounds producing reactive oxygen and other radicals. Since copper exist in the nucleus and is closely associated with chromosomes and DNA bases, in this study we have investigated whether the activation of plumbagin by copper can induce strand breaks in double stranded DNA. In the presence of micromolar concentrations of copper, DNA strand break was induced by plumbagin in plasmid pBR322. Further using a cellular system of lymphocytes isolated from human peripheral blood and Comet assay, we have confirmed that plumbagin-Cu(II) system is indeed capable of causing DNA degradation in cells such as lymphocytes. Addition of active oxygen scavengers and neocuproine inhibited the DNA cleavage reaction suggesting the role of reactive oxygen species and Cu(I). These results indicate that the plumbagin-Cu(II) mediated DNA breakage is physiologically feasible and could be one of the mechanisms for its antitumor action.

Key words: Plumbagin; Comet assay; Copper reduction; Reactive oxygen species; DNA cleavage;

1. INTRODUCTION

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) derived from the roots of *Plumbago zeylanica*, has been shown to exert anticarcinogenic or cytotoxic effects on animals and cancer cells.[1-5] Sugie et al[6] have shown that plumbagin significantly exhibited azoxymethane induced intestinal carcinogenesis in rats, suggesting its chemopreventive activity.[6] Plumbagin has also been shown to induce S-G2/M cell cycle arrest through the induction of P21 (an inhibitor of cyclin dependant kinase).[7] An earlier report showed that plumbagin has a chemotherapeutic potential as an anticancer agent in ovarian cancer cells with the mutated BRCA1 gene.[8] The cytotoxic action of plumbagin in keratinocytes and cervical cancer cells was found to be due to a change in the redox status of the cell.[9, 10] In embryonic kidney and brain tumor cells, plumbagin inhibited the enzyme NAD(P)H oxidase,[11] linked with anticarcinogenic[1-6] and atherosclerotic effects.[12] In addition, plumbagin does not exert an apoptotic effect on normal cells and therefore can be developed as an anticancer drug.[13, 14, 15]

Even though several mechanisms have been proposed, the exact mechanism(s) by which plumbagin exerts anticancer effect remains unexplored. Elucidation of the exact mechanism by which plumbagin induces its anticarcinogenic and antiproliferative effects is necessary to provide a solid foundation for its use as an agent for prevention strategies. This study is the first to investigate the role of a metal ion (copper) in the observed cytotoxic action of plumbagin.

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Earlier studies have established that several compounds of plant origin such as flavanoids,[16] tannins,[17] curcumins,[18] and the stilbene -resveratrol[19] are themselves capable of inducing oxidative DNA damage either alone or in the presence of certain transition metal ions especially copper. It has also shown that several polyphenols of plant origin can induce oxidative DNA breakage in cells such as lymphocytes.[20] Further, it was 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-acridynlamino)-menthanesulaphone-*m*-anisidine (mAMSA).[21-23] It was previously shown that copper can induce the oxidation of 1,4 HQ, a benzene metabolite, producing 1,4 –BQ and H_2O_2 through a semiquinone intermediate.[24] Copper is an essential trace element which is distributed through out the body.[25] Besides forming the essential redox active center in a variety of metalloprotiens,[25] copper has also been found in nucleus and to be closely associated with chromosomes and DNA bases, particularly guanine.[26-31] DNA associated copper has been suggested to be involved in maintaining normal chromosome structure and in gene regulatory processes.[32-34] Since copper is closely associated with DNA, we therefore decided to investigate the DNA damage induced by copper mediated activation of plumbagin.

In this paper using Comet assay we show that plumbagin is capable of causing oxidative DNA breakage in human peripheral lymphocytes in the presence of copper ions. Further plumbagin-Cu(II) system could also cleave plasmid DNA and that this breakage is mediated by reactive oxygen species, especially the hydroxyl radical. These results indicate that the plumbagin-Cu(II) mediated DNA breakage reaction is physiologically feasible and could be one of the mechanisms for its antitumor action.

2. MATERIAL AND METHODS

2.1 Materials

Plumbagin used in the experiments was isolated from the hairy root cultures established in our laboratory from the medicinal plant *Plumbago zeylanica*. The purity and identity of plumbagin were fully established by chromatographic methods, UV, IR, NMR and HPLC. The sample of plumbagin was identical with an authenticated sample. Stock concentrations of drugs were prepared in dimethyl sulphoxide. Agarose was obtained from Bio-Rad laboratories (USA). Supercoiled plasmid pBR322 DNA was prepared according to standard methods.[35] All other chemicals were of analytical grade.

2.2 Methods

2.2.1 Reaction with plasmid pBR322 DNA

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

2.2.2. Assay of Active Oxygen Species

Superoxide anion was detected by the reduction of nitroblue tetrazolium (NBT) essentially as described by Nakayama et al (1983).[36] A typical assay mixture contained 100mM potassium phosphate buffer, pH (7.5), 1mM NBT, 1mM EDTA and 0.6% Triton X-100 in a total volume of 3 ml. After mixing, absorbance was recorded at 560 nm against a blank, which did not contain the compound, at different time intervals.

In order to study the hydroxyl radical production by increasing concentrations of plumbagin (50-800 µm) in the presence of 20 µm Cu(II), the method of Quinlan and Gutteridge[37] was followed. 2mM deoxyribose was used as a substrate and the malondialdehyde generated from deoxyribose radicals was assayed as described earlier.[38]

2.2.3. 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), the cells ($\approx 2x10^5$) were suspended in RPMI 1640.

2.2.4. 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.[39] The viability of the cells was found to be greater than 93%.

2.2.5. Treatment of lymphocytes

Lymphocytes were exposed to different concentrations of plumbagin 10µM-50µM in the absence and presence of various concentration of $CuCl_2$. In Some experiments,

lymphocytes were pre-incubated either with 50μM plumbagin or 20μm Cu(II). 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.

2.2.6. Comet assay

Comet assay was performed under alkaline conditions essentially according to the procedure of Singh et al. [40] with slight modifications. Fully frosted microscope 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 from 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 cover- slips were removed and 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 slides were immersed in cold lysing solution containing 2.5M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, and 1% Triton 100-X added just prior to use for minimum of 1 h at 4°C. After lysis DNA was allowed to unwind for 30 min in alkaline electrophoretic solution consisting of 300mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was performed at 4 °C in 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 an Olympus (CX41) fluorescent microscope and a COHU 4910 (equipped with a 510-560 nm excitation and 590 nm barrier filters) integrated CC camera, Comets were scored at 100X magnification. Image 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.

2.2.7. Statistics

The statistical analysis was performed as described by Tice et al.[41] 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

3.1. Cleavage of plasmid pBR322 DNA by plumbagin in the absence and presence of Cu(II)

The structure of plumbagin is given in **Fig. 1**. In order to study the chemical basis of DNA breakage by plumbagin-Cu(II) system, the DNA cleavage efficacy of plumbagin in plasmid pBR322 DNA was studied. **Fig. 2** shows the ethidium bromide stained banding pattern of pBR322 DNA treated with increasing concentrations of plumbagin (lanes 2, 3 and 4 respectively). As can be seen, at the lower concentration of plumbagin alone, approximately equal

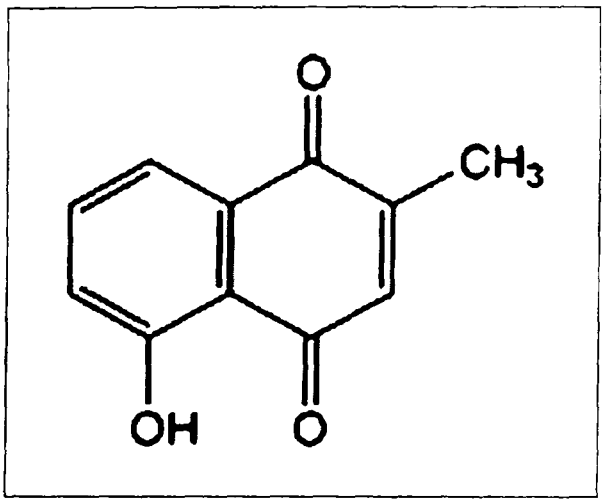

Figure 1. Structure of plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone)

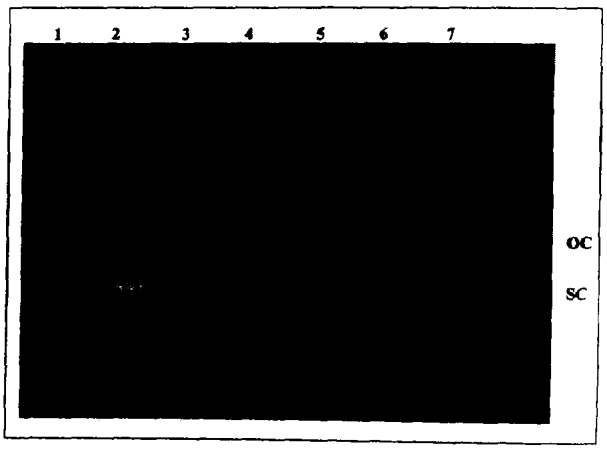

Figure 2. Degradation of plasmid pBR322 DNA by plumbagin in the absence and presence of Cu(II) lane 1, Native DNA alone; lanes 2-4, DNA + plumbagin (50-200 μM); Lane 5-7, DNA + plumbagin (50-200 μM) + Cu(II) (20 μM); SC, supercoiled DNA; OC, open circular DNA.

intensity of the supercoiled DNA bands are present. In presence of 20μM concentration of Cu(II) plumbagin induced extensive DNA strand breaks as indicated by the disappearance of the supercoiled (SC) form and the formation of open circular (OC) forms (Lanes 5-7).

3.2 Production of active oxygen species by plumbagin

It is known that generation of the $O_2^{\cdot}$ anion may lead to the formation of H_2O_2. The addition of a second electron to the $O_2^{\cdot}$ anion gives the peroxide ion ($2O_2^{\cdot}$), which has no unpaired electron and is not a radical. However, at neutral pH the peroxide ion is immediately protonated to give hydrogen peroxide (H_2O_2). Alternatively, in aqueous solution the superoxide anion undergoes dismutation to form H_2O_2 and O_2 (Halliwell and Gutteridge) (1984).[42] The rate of generation of superoxide anion by plumbagin was studied. The increase in absorption at 560 nm was observed on reduction of NBT by superoxide anion. From **Fig 3**, it is evident that the production of superoxide radical increases with the increasing time of incubation. The capacity of plumbagin to generate hydroxyl radicals in the presence of Cu(II) was also studied **Fig 4**. This assay uses deoxyribose as a substrate.[37] Hydroxyl radicals react with deoxyribose to form a free radical intermediate that decomposes to form an aldehyde, which in turn gives an adduct with thiobarbituric acid (TBA). As observed increasing concentrations of plumbagin lead to a progressively increased formation of the hydroxyl radicals.

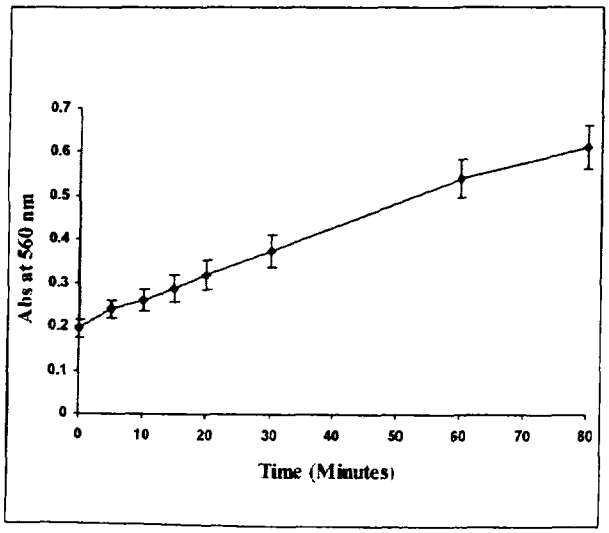

Figure 3. Photogeneration of superoxide anion by plumbagin. The concentration of plumbagin was 50 μM. The sample was placed 10 cm from the light source. Details are given in Materials and Methods. Values

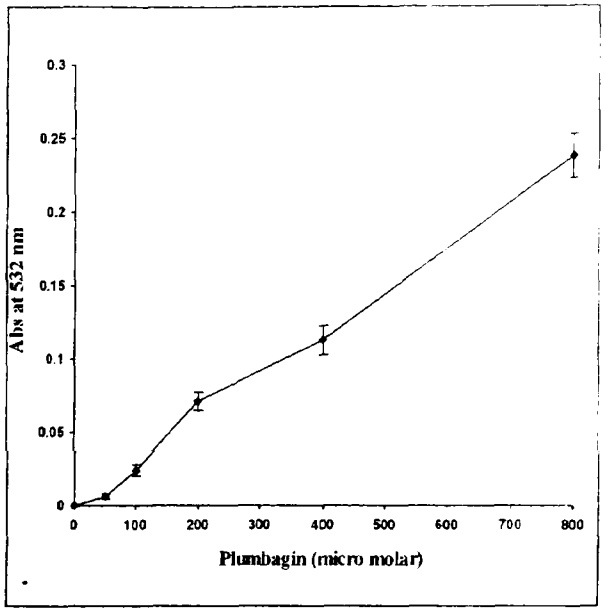

Figure 4. Formation of hydroxyl radicals as a function of plumbagin concentration in the presence of Cu(II). The reaction mixtures were as described in Materials and Methods containing 20 μM Cu(II) and increasing concentrations of plumbagin (50-800 μM). The incubation was at 37 °C for 30 minutes. Values reported are ±SEM of three independent experiments.

3.3 DNA breakage induced by Plumbagin-Cu(II) in lymphocytes as measured by comet assay

Increasing concentrations of plumbagin (10-50 μM) either alone or in the presence of 20 μM Cu(II) was tested for DNA breakage in isolated lymphocytes using the Comet assay. Plumbagin alone at any of the concentrations tested did not damage lymphocyte DNA whereas on additions of Cu(II) DNA damage to varying degrees was observed. Photographs of Comets seen on treatment with 50μM plumbagin alone (a), 20 μM Cu(II) alone (b) and in the presence of both these additions (c) are shown in **Fig. 5.**

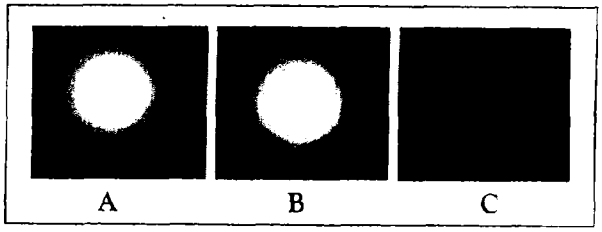

Figure 5. Single Cell gel electrophoresis of human peripheral lymphocytes showing Comets (100x) after treatment with plumbagin alone (50 μM) (a), Cu(II) alone (20 μM) (b) and with plumbagin (50 μM) and Cu (II) (20 μM) (c).

Untreated lymphocyte controls were similar to plumbagin alone or Cu(II) alone. The results clearly establish that plumbagin-Cu(II) system is capable of DNA breakage in lymphocytes. The corresponding tail length (μm) is shown in **Fig 6.** A similar experiment with increasing concentrations of Cu(II) (5-20 μM) at fixed concentrations of plumbagin (50 μM) was also carried out. An increasing degree of DNA damage with increasing Cu(II) concentration was seen (results not shown).

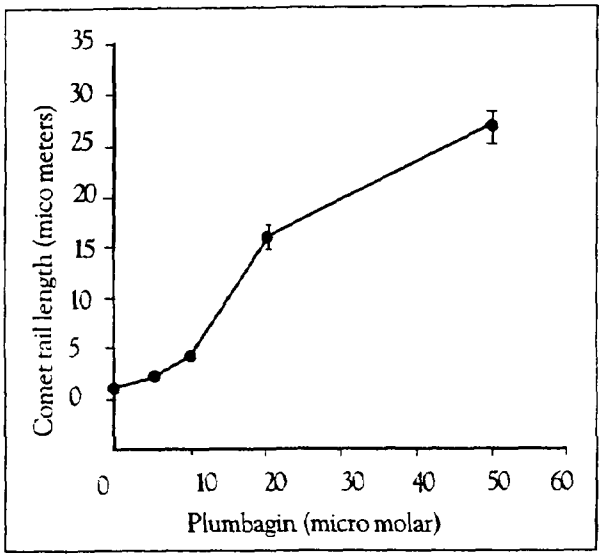

Figure 6. DNA breakage by plumbagin-Cu(II) system in human peripheral lymphocytes as measured by Comet assay. Comet tail length (μm) plotted as a function of increasing concentrations of plumbagin (10-50 μM) in the presence of 20 μM Cu(II). Values reported are ±SEM of three independent experiments.

3.4. Effect of scavengers of reactive oxygen species and neocuproine, a Cu(I) specific sequestering agent on plumbagin Cu(II) induced DNA breakage in lymphocytes

Table 1 gives the results of an experiment where the effect of SOD, catalase, sodium azide, thiourea, potassium iodide and neocuproine on lymphocyte DNA damage was 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 radical. From the data we may 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 cleaving agents. Neocuproine is a Cu(I) specific sequestering agent which as expected also inhibits DNA breakage, indicating that a copper redox cycle and reactive oxygen generation are two major determinants involved in the observed DNA damage. However, it is realized that direct confirmation of the result requires additional work.

4. DISCUSSION

The present studies lead to the following major conclusions.

(i) Plumbagin in the presence of micromolar concentrations of Cu(II) causes DNA strand breaks in human peripheral lymphocytes as well as plasmid DNA.

(ii) The observed DNA damage could be inhibited by catalase and neocuproine, a Cu(I) specific sequestering agent, indicating that a copper redox cycle and H_2O_2 generation are two major determinants involved in the

Table.1. Effect of scavengers of reactive oxygen species and neocuproine on plumbagin-Cu(II) induced DNA breakage in lymphocytes.

Treatment	Tail Length (μm)	% Inhibition
Untreated #	2.7 ±0.11	-
Plumbagin 50 μM +Cu(II) 20 μM	29.5 ± 2.25 *	-
+ Thiourea (1mM)	7.9 ±0.98 *	73.2%
+ Sodium Azide (1mM)	15.1 ± 1.5 *	48.8%
+ Potassium Iodide (1mM)	10.6 ± 1.3 *	64.0%
+ SOD (100 μg/ml)	17.5 ± 1.4 *	40.6%
+ Catalase (100 μg/ml)	9.6 ± 1.0 *	67.4%
+ Neocuproine 300 (μM)	11.1 ± 0.9 *	62.3%

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

observed DNA damage. Therefore, the reactive oxygen species generated from the further interaction between copper and H_2O_2 may be responsible for the DNA damage mediated by copper-induced activation of plumbagin.

Copper is a normal component of chromatin and is required for maintaining chromosome structure and gene regulatory processes.[32-33] Such copper can be mobilized by metal chelating agents such as 1,10 phenanthroline leading to DNA breakage.[43] It is thus conceivable that similar to 1,10 phenanthroline plumbagin is also capable of mobilizing endogenous copper, both from chromatin as well as copper binding proteins especially ceruloplasmin, causing oxidative DNA damage. This would account for the observed genotoxic/ cytotoxic properties of plumbagin in human peripheral lymphocytes. Indeed Li and Trush[44] have proposed a similar model of reactive oxygen species mediated oxidative DNA damage by a number of phenolic compounds to account for their genotoxic/cytotoxic effects and also in a recent report, plumbagin was demonstrated to induce apoptosis of human cervical cancer cells (ME-180 cell lines) through the generation of ROS.[9]

In this study we first assessed the role of Cu(II)/Cu(I) redox cycle and reactive oxygen generation in plumbagin-Cu(II) induced DNA damage .As shown in Fig 2, in the presence of 20 μM Cu(II), plumbagin induced extensive DNA strand breaks as indicated by the disappearance of the supercoiled (SC) form and the formation of open circular(OC) forms of DNA. It was confirmed from the result that plumbagin or Cu(II) alone elicited no damage to DNA. Further using a cellular system of lymphocytes isolated from human peripheral blood and alkaline single cell gel electrophoresis (Comet assay) we confirmed that plumbagin–Cu(II) system is indeed capable of causing DNA degradation in cells such as lymphocytes. Further, the DNA degradation of lymphocytes was inhibited by scavengers of reactive oxygen species and neocuproine, a Cu(I) specific sequestering agent, indicating that a copper redox cycle and reactive oxygen generation are two major determinants involved in the observed DNA damage (Table1).

Thus, the major conclusion of the present study is that the possible mechanism by which plumbagin exhibits anticancer properties may involve mobilization of endogenous copper and the consequent pro-oxidant action as mentioned earlier and this mechanism infact is physiologically feasible and may be of biological significance, as observed in comet assay. It has recently been shown that 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 and also our idea is strengthened by number of other observations.[45-46] Cu^{++} and Zn^{++} are the major metal ions present in the nucleus[47] Serum[48] and tissue[49] 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 that is the hallmark of cells undergoing apoptosis.[43] Since copper is capable of mediating activation of a variety of phenolic compounds as mentioned earlier,[24,44] it is reasonable to propose that the DNA associated copper in cells may have the potential to activate plumbagin via a copper-redox reaction, producing reactive oxygen and electrophilic plumbagin intermediates (semiquinone intermediate). The interaction of plumbagin with DNA associated copper may finally result in oxidative DNA damage and this might contribute to the cytotoxicity induced by plumbagin. In short, our result strongly suggests that plumbagin is an excellent lead compound in anticancer drug discovery process. So with the help of diversity oriented synthesis and other combinatorial synthetic methodologies, derivatized compounds of plumbagin can be synthesized and screened for potential cytotoxicity, to find out more potential lead compound which may have therapeutic applications in the treatment of cancer.

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