# EEFECT OF GREEN LEAVES USED IN TRADITIONAL FOOD PREPARATION ON DNA REPAIR

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# DECLARATION

I hereby declare that this thesis entitled **"Effect of green leaves used in traditional food preparations on DNA repair"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title, of any other university or society.

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07/04/2010                                               (2007-11-117)

**CERTIFICATE**

Certified that this thesis entitled **"Effect of green leaves used in traditional food preparations on DNA repair"** is a record of research work done independently by Ms. Arpitha, Y.R. (2007-11-117) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or assaciateship to her.

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Dedicated to My Beloved
Parents, brother and
Teachers

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| **dH₂O** | Distilled water |
| **DNA** | Deoxyribonucleic acid |
| **EDTA** | Ethylene diamine tetra acetic acid |
| **EMS** | Ethyl methanesulphonate |
| **EtBr** | Ethidium bromide |
| **FYM** | Farmyard manure |
| **g** | Gram |
| **HCl** | Hydrochloric acid |
| **KCl** | Potassium chloride |
| **LMPA** | Low point melting agarose |
| **M** | Molar |
| **mg** | Milligram |
| **ml** | millilitre |
| **mM** | Millimolar |
| **MMS** | Methyl methanesulphonate |
| **N** | Normal |
| **NaOH** | Sodium hydroxide |
| **NaCl** | Sodium chloride |
| **NMA** | Normal melting agarose |
| **PBS** | Phosphate buffered saline |
| **rpm** | Revolution per minute |
| **Tris** | Tris (hydroxyl methyl) aminomethane |
| **µg** | Microgram |
| **µl** | Microlitre |
| **µm** | Micrometre |
| **%** | Per cent |

# Introduction

# 1. INTRODUCTION

The DNA is continuously subjected to damage from exogenous agents, internal cellular processes and spontaneous decomposition. This leads to a variety of alterations in the normal DNA structure viz., single and double strand breaks, chemically modified bases, abasic sites, bulky adducts, inter and intra strand cross links and base pairing mismatches. Repair of such damage is essential to suppress the development of many diseases and to slow down the ageing process.

There are evidences from human studies that generalised malnutrition or low intakes of specific nutrients may affect DNA repair. Also, a diet rich in fruits and vegetables can play a role in preventing cancer as well as degenerative disorders related to DNA damage (Danaei et al., 2005 and Lock et al., 2005). Phytochemicals contained in vegetables and fruits, including flavonoids and other types of polyphenolic compounds, can influence the DNA repair process (He et al., 2006 and Pomerleau et al., 2006). There are reports, showing the positive effects of plant/leaf extracts in imparting disease tolerance/DNA damage repair. There are only a few studies made to understand the influence of diet or nutrient status on DNA repair processes.

*Azima tetracantha* (ashenku), *Cissampelos pareira* Linn. (malathangi), *Myxopyrum serratulum* (chaturamulla), *Pongamia pinnata* (ungu) and *Thespesia populnea* (poovarasu) are traditionally being used in traditional food preparations and karkidaka kanji and are believed to increase body immunity and general health. However, scientific validation of this has not been done. The present study was undertaken with the objective of evaluating the effect of green leaf extracts of these five plants on DNA repair, employing the Comet Assay, which is a sensitive and rapid technique for quantifying and analyzing DNA damage in individual cells. Swedish researchers Ostling and Johanson developed this technique in 1984. Singh, et al. (1988) modified this technique as the Alkaline Comet Assay.

# Review of literature

# 2. REVIEW OF LITERATURE

DNA repair is a biochemical term that defines biological processes during which alterations in the chemistry of DNA (DNA damage) are removed and the integrity of the genome is restored. DNA in the living cell is subject to radiation and intracellular physical and chemical stresses. If the genetic information encoded in the DNA is to remain uncorrupted, any structural & chemical changes must be corrected. A failure to repair DNA produces a mutation. If left unrepaired, DNA damage can lead to detrimental biological consequences in organisms, including cell death, mutations and transformation of cells to malignant cells. Therefore, DNA repair is regarded as one of the essential events in all life forms. The strategy to favour the intake of protective factors and to modulate the host defence mechanisms, is referred to as chemoprevention and is based on dietary and/or pharmacological and/or nutraceutical interventions, has already found extensive application in the field of cardiovascular disease and is encountering a growing interest in cancer prevention (De Flora et al., 2001). The study of "Effect of green leaves used in traditional food preparation on DNA repair" aims at validation of green leaves of some plant species used in karkidaka kanji preparation such as *Azima tetracantha*(ashenku), *Cissampelos pareira* Linn(malani), *Myxopyrum serratulum*(chaturamulla), *Pongamia pinnata*(ungu) and *Thespesia populnea*(poovarasu) can offer general tolerance against diseases by employing comet assay technique to know its effect on DNA damage repair and those can be used in the prophylactic strategies for preventing cancer.

During the month of June-July (karkidaka masa), there is a special preparation called karkidaka kanji, made from the leaves (fresh or dried) samples of ashenku, malani, chaturamulla, ungu, poovarasu, kurumthotti, keezharnelli, sathakuppa, cumin, fenugreek and cheroola from ancient period in Kerala. It is believed that, consumption of this preparation increases the body immunity and helps in the prevention of diseases.

Till date, no report has been published regarding scientific validation of these plant species preparations, so experiments have been done to standardise the protocol for comet assay in tomato, induction of DNA damage in tomato seedlings using chemical mutagens such as EMS (ethyl methanesulphonate) and MMS (methyl methanesulphonate) and knowing the effect of the aqueous extracts of the leaves of the plants on DNA repair, under *in vitro* conditions, using comet assay. In this chapter, literature on causes of DNA damages, DNA repair mechanisms, comet assay technique, and nutritional modulation of DNA repair has been reviewed.

## 2.1 CAUSES OF DNA DAMAGES

Genomic integrity is under constant threat in all species. These threats come in many forms viz., agents that damage DNA like certain wavelengths of radiation, example ionizing radiation such as gamma rays and x-rays, ultraviolet rays, especially the UV-C rays (~260nm) that are absorbed strongly by DNA and also the longer-wavelength UV-B that penetrates the ozone shield. The random energy deposition by ionizing radiations induces a wide array of DNA lesions. Ionizing radiations induce damage to DNA by direct ionization and through generation of hydroxyl radicals that attack DNA resulting in single-strand breaks, double-strand breaks, and oxidative damage to sugar and base residues that can be converted into strand breaks subsequently. Muller (1927), discovered that X-rays can cause mutations (as a consequence of occasional failure to properly repair damage induce by ionising radiation). It was the first experimental demonstration that environmental factors can affect genome stability. Because of induction of DNA double-strand breaks, the ionizing radiation is extremely effective in producing chromosomal aberrations leading to genomic instability (Natarajan, et al., 1986).

DNA damage caused by oxygen-derived species including free radicals is the most frequent type encountered by aerobic cells. When this type of damage occurs to DNA, it is called oxidative DNA damage and it can produce a multiplicity of modifications in DNA including base and sugar lesions, strand breaks, DNA-protein cross-links and base-free sites. Free radicals may be defined as any chemical species that are capable of existing with one or more unpaired outer shell electrons. They are extremely reactive and generally highly unstable. Reactive oxygen species (ROS) is a collective term often used by biologists to include oxygen radicals superoxide $[O_2^{\cdot-}]$, hydroxyl $[OH\cdot]$, peroxyl $[RO_2\cdot]$ and alkoxyl $[RO\cdot]$ and certain other nonradicals that are either potential oxidizing agents and/or are easily converted into radicals, such as hypochlorous acid (HOCl), ozone ($O_3$), peroxynitrite (ONOO-) singlet oxygen ($^1O_2$) and hydrogen peroxide ($H_2O_2$). Reactive nitrogen species (RNS) is a term becoming popular to encompass nitrogen dioxide radical ($NO_2\cdot$), peroxynitrite, nitrous acid ($HNO_2$) and related species, are of the greatest biological significance. They are extremely reactive and potentially damaging transient chemical species. In addition to exogenous sources of free radicals, such as ionizing radiation, tobacco smoke, pesticides, pollutants, and some medications, they are produced continuously in all cells, as metabolic byproducts by a number of intracellular systems: small cytoplasmic molecules, cytoplasmic proteins, membrane enzymes, peroxisomes, mitochondrial electron transport systems, and microsomic electron transport systems (Ames, 1989).

All cellular components, proteins, polyunsaturated fatty acids, nucleic acids and carbohydrates, are prominent biological targets of reactive oxygen species, giving rise to metabolic and cellular disturbances. ROS and RNS mediated 'spontaneous' DNA damage is thought to contribute to cancer development e.g. via mutations in the tumour suppressor gene p53 (Cerutti, 1994).

DNA damage is caused by chemicals in the environment like many hydrocarbons, including some found in cigarette smoke (benzopyrene). Plant and

microbial products, e.g. the aflatoxins produced in mouldy peanuts cause DNA damage. Aflatoxins are one of the most potent naturally occurring toxic substances, being a group of closely related mycotoxins produced by *Aspergillus flavus* and parasiticus. Aflatoxin contamination affects human and animal health as well as food supply and markets. Since the recognition that aflatoxin exposure was related to an increased risk of neoplasia, the International Cancer Research Institute has classified them as a Class I carcinogens, leading to the acceptance of very low level of contamination of foods by these toxins, being 20 ppb in grains and 0.5 ppb in milk, the Food and Drugs Administration (FDA) (Henry, et al., 1999). Chemicals used in chemotherapy, especially those used in the treatment of cancers. Spontaneous chemical changes and finally errors in DNA metabolism can also lead to a variety of alterations in the normal DNA structure like single- and double strand breaks, chemically modified bases, abasic sites, bulky adducts, inter- and intra-strand cross-links and base-pairing mismatches and have many direct and indirect effects on cells and organisms such as mutations, genetic recombination, the inhibition or alteration of cellular processes, chromosomal aberration, tumorigenesis and cell death.

## 2.2 DNA REPAIR SYSTEMS

DNA is constantly subject to damage arising either spontaneously or from a plethora of endogenous and exogenous agents. Such DNA damages, if unrepaired, leads to aberrant gene expression and is fundamental to the initiation and development of cancer, with additional implications for ageing and a wide range of diseases including diabetes, vascular disease and dementia. Although DNA damage arises frequently, with $2 \times 10^5$ damaging events occurring per cell per 24 h, mutation is rare, with only one in $10^{10}$ nucleotides becoming mutated per cell generation (Jackson and Loeb, 2001). This low incidence of mutation is largely a result of the ability of cells to perform an array of evolutionarily-conserved DNA repair mechanisms that maintain the integrity of the genome. Human cells have five complementary DNA repair systems, encoded by >150

genes and protein products. Each system detects and repairs specific types of DNA damage. The five human DNA repair systems are: direct reversal; mismatch repair; base excision repair (BER); double-strand break repair; nucleotide excision repair (NER).

## 2.2.1 Direct reversal

Direct reversal involves the direct removal of damage adducts from the DNA rather than entire damaged nucleotides. Although direct reversal systems are common in prokaryotic systems, in man only one such system exists. Alkylation of guanine at the $O^6$ position results in an $O^6$-alkylguanine lesion, which is capable of pairing with thymine and, if left unrepaired, results in a G→A transition mutation post replication. In human subjects $O^6$-alkylguanine can be repaired by $O^6$-methylguanine-DNA methyltransferase, which transfers the alkyl group to a cysteine residue within the protein, restoring the damaged guanine to its correct form.

## 2.2.2 Mismatch repair

Mismatch repair serves to repair a number of common mutagenic lesions. Misincorporation (mispairing) of bases (e.g. T/C or A/G), caused by errors in DNA replication as well as alkyl adducts, and oxidatively-damaged bases are repaired by mismatch repair. One such mismatch lesion that is commonly found arises from the deamination of cytosines to uracil or methylated cytosines to thymine, which if unrepaired cause a C→T transversion post DNA replication. Mismatch repair also serves to repair small insertions and deletions in the DNA caused when the DNA polymerase 'slips', usually in repetitive microsatellite DNA sequences. The process of mismatch repair involves twenty-six genes and proteins and their encoded proteins that act in three stages to remove and repair damage. First, the mismatched base is recognised by the MSH2/MSH6 heterodimer. The damaged strand around the lesion is unwound and removed,

involving the MLH1/PMS2 heterodimer and other proteins. This stage leaves a single-stranded gap, which is repaired by specific polymerases and DNA ligase 1 (Jiricny and Nystrom-Lahti, 2000).

### 2.2.3 Double-strand break repair

Double-strand breaks are the most cytotoxic and potentially mutagenic lesions that can afflict DNA, as they can quickly lead to chromosomal breaks or exchanges and cell death. Double-strand breaks are induced by ionising radiation, mechanical stress and calastogens (agents causing visible chromosomal damage) such as the chemotherapeutic agent cisplatin (Pfeiffer, et al., 2000). Repair of double-strand breaks may proceed through two distinct pathways: the error-free homologous recombination; the error-prone non-homologous end joining, which is crude in that it simply ligates the two ends of a double-strand break (Christmann, et al., 2003). Homologous recombination uses the homology of the sister chromatid to facilitate high-fidelity repair of double-strand breaks. Homologous recombination begins with the digestion of one strand of DNA at the break site, resulting in a single-stranded 30 overhang (Christmann et al. 2003). The RAD52 and RAD51 proteins promote the formation of nucleofilaments and facilitate interactions between the damaged strand and the undamaged DNA on the sister chromosome. Thereafter, strand exchange takes place, allowing the synthesis of new DNA across the break using the undamaged chromosome as a template.

### 2.2.4 Base excision repair

BER is responsible for the repair of numerous small mutagenic lesions that do not disrupt the DNA double helix. The most-frequently-repaired lesions are apurinic/ apryimidinic sites, in which the base is missing from the DNA backbone (Seeberg, et al., 1995). Base deamination, oxidation and alkylation, which can cause mispairing and lead to mutations post replication, are also repaired through

the BER pathway. BER can be divided into three stages: recognition of damage; removal of damaged bases creating an apurinic/apryimidinic site; and filling of apurinic/apryimidinic sites during synthesis of the new DNA. In humans, there are eleven glycosylases that are able to recognise damaged bases and cleave them from the sugar phosphate backbone, creating an apurinic/apryimidinic site (Hung, et al., 2005). For repair to continue apurinic/ apryimidinic sites must be incised to create a single-strand break, either by the glycosylase itself or by the apurinic/ apryimidinic endonuclease enzyme. Such sites (created spontaneously, by glycosylases or by apurinic/apryimidinic endonuclease) are repaired in one of two ways: (1) shortpatch repair, in which a single nucleotide in inserted into the apurinic/apryimidinic site by DNA B polymerase and DNA ligase; (2) long-patch repair, in which an additional two to thirteen nucleotides are removed and the gap repaired by polymerases and ligase.

## 2.2.5 Nucleotide excision repair (NER):

NER is a complex DNA repair pathway involving over thirty genes and proteins and is responsible for the repair of any DNA lesion that causes distortion to the DNA double helix. NER commonly repairs lesions induced by UV as well as numerous exogenous agents such as those derived from food and smoking, including 2-amino-1-methyl-6-phenylimidazo [4,5-ß] pyridine and benzo[a]pyrene diol-epoxide.

NER can be divided into two distinct pathways: transcription-coupled repair and global genomic repair. Transcription- coupled repair, repairs lesions that block the progression of RNA polymerase along actively-transcribed genes. Global genomic repair is independent of transcription and acts to repair lesions in non-transcribed regions of the genome as well as those in the non-transcribed strand of active genes. These two pathways differ only in the way DNA damage is detected, with the subsequent repair steps being identical. In transcription-coupled repair a helix-distorting lesion located in an actively-transcribed gene halts the

progression of the RNA polymerase at the site of damage. The stalling of the polymerase initiates the removal and repair of the damage, allowing transcription to continue. In global genomic repair the xeroderma pigmentosum (XP) complementation group C (XPC) protein, in association with UV excision repair, protein RAD23 homologue B, recognises and binds to the helix distortion caused by the damage rather than to the lesion itself. The initiation of transcription-coupled repair is faster than the initiation of global genomic repair, presumably because of the implications of DNA damage during mRNA synthesis (Benhamou and Sarasin, 2000).

Once the site of damage has been recognised, DNA is unwound around the lesion by two helicase enzymes XPB (unwinding 30 to 50) and XPD (unwinding 50 to 30). Both XPD and XPB are part of the basal transcription factor IIH complex, which is essential for the initiation of transcription by RNA polymerase II. This unwinding creates distinct junctions between double-stranded and single stranded DNA, which are essential for the progression of repair (Friedberg, 2001). The damaged strand of DNA is incised at these two junctions, i.e. at the 30 side of the damage by XPG protein and at the 50 side by the XPF– excision repair cross-complementing rodent repair deficiency complementation group 1 (ERCC1) complex. This step results in cleavage and subsequent removal of approximately thirty nucleotides of single-stranded DNA containing the damage. The remaining single-stranded gap is filled by DNA polymerase d or e and the new strand is joined to the existing DNA by DNA ligase.

## 2.3 NUTRITIONAL MODULATION OF DNA REPAIR

### 2.3.1 Diet and DNA repair system

To date a relatively small number of studies have investigated the influence of diet or nutrient status on DNA repair processes. The dietary antioxidant is also able to modulate DNA repair via redox-sensitive pathways that

ultimately influence nucleotide or base excision repair, or transcription-coupled repair (Cooke, et al., 1998). It has been claimed that Vitamin C has a novel function, quite apart from its direct antioxidant ability (Cooke, et al., 2001), in modifying DNA repair mechanisms; in addition to simply scavenging free radicals, this function could be responsible for its anti-cancer properties. The supplementation with the antioxidant ubiquinone-10 enhances Base excision repair (BER) activity in humans (Tomasetti, et al., 2001). Perhaps not surprisingly, malnourished Mexican children carrying an infection have been found to have a lower capacity for repair of oxidative DNA damage when compared with uninfected well-nourished children (Gonzalez, et al., 2002). Such findings indicate that undernutrition and associated ill health may impair the capacity for DNA repair, but they are not able to identify which factors, dietary or otherwise, are responsible. In a further study (Collins, et al., 2003) BER capacity, as measured using a modified comet assay, was found to be increased after supplementation of healthy human volunteers with one, two or three kiwi fruit per day, but there was no evidence of a dose–response relationship. In a cohort of 559 healthy individuals those in the lowest folate intake were reported (Wei, et al., 2003) to have an 18% reduction in NER capacity compared with those in the highest intake of folate. A significant ($P<0.001$) inverse association was found between total dietary folate intake (adjusted for total energy intake) and repair capacity in non-users of supplemental folate. The α-carotene-ß-carotene capsule dietary supplements specifically leading to an increase in DNA SSB (single strand break) repair activity. However supplementation of healthy human volunteers with carrot extract increases repair of an $H_2O_2$ damaged plasmid DNA (Astley et al. 2004)

As part of a systematic study of the effects of phytochemicals beyond antioxidation on DNA repair. Gao, et al., (2006) investigated whether naringenin (NR), a citrus flavonoid, stimulates DNA repair following oxidative damage in LNCaP human prostate cancer cells. The 8-hydroxydeoxyguanosine (8-OH-dG) to deoxyguanosine (dG) ratio was measured after cells were treated with 200

μmol/L of ferrous sulphate in serum-free medium followed by NR exposure for 24 h in growth medium. The results demonstrated that exposure to 10–80 μmol/L of NR led to a significant decrease in the ratio of 8-OH-dG to $10^6$ dG. In conclusion, the cancer-preventive effects of citrus fruits demonstrated in epidemiological studies may be due in part to stimulation of DNA repair by NR, which by stimulating BER processes may prevent mutagenic changes in prostate cancer cells.

It is not possible with any techniques currently available to define the mechanism responsible for this enhanced recovery with certainty. However, two possible interpretations are immediately obvious. The first is that increased cellular antioxidant act to enhance the DNA repair. The second is that antioxidant taken up by the cells provides a degree of protection for cellular DNA against further DNA damage being generated over the protracted time course following exposure to $H_2O_2$ and such genotoxicants.

## 2.3.2 Protection of DNA from ongoing damages

The harmful action of the free radicals can, however, is blocked by antioxidant substances which scavenge the free radicals and detoxify the organism. Antioxidant components are microconstituents present in the diet that can delay or inhibit lipid oxidation, by inhibiting the intiation or propagation of oxidizing chain reactions, and are also involved in scavenging free radicals. Food such as fruits, vegetables and grains are reported to contain a wide variety of antioxidant components. These compounds are found to be well correlated with antioxidant potential. Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancers  and neurodegenerative diseases, including Parkinson's and Alzheimer's diseases, as well as inflammation and problems caused by cell and cutaneous aging (Ames, et al., 1993).

While the enzymatic antioxidants like superoxide dismutase, glutathione peroxidase, catalase are intrinsic to the organism, the non-enzymatic components are both of intrinsic and exogenous nature. The non-enzymatic antioxidants consist of nutrient and non-nutrient compounds. Some non-nutrient antioxidants like glutathione and coenzyme Q are mainly of endogenous origin, whereas a majority of nutrient and non-nutrient types of nonenzymatic antioxidants are derived from the foods we consume. While vitamins such as vitamin A, C, E and minerals such as zinc and selenium are some of the important nutrients with antioxidant activity, there are a variety of non-nutrient substances such as carotenoids, flavanoids, phenolics, polyphenols and uric acid that are potent antioxidants. There are many more minor components in plant foods, such as sulphides, thiols, saponins, lignans and inositol, which have excellent antioxidant activity.

Carotenoids are C40 isoprenoids or tetraterpenes which are found in all photosynthesizing plants, and are located in the chloroplast. These pigments have an effective mechanism of scavenging ROS and preventing the generation of these species (Pallett and Young, 1993). Beta-carotene is a major carotenoid and precursor of vitamin A. Many studies have demonstrated high β-carotene intake is associated with lower cancer risk, including ovarian (McCann, et al., 2001), colorectal (Vecchia, et al., 2001), and breast (Ching, et al., 2002) cancer.

Flavonoids are polyphenolic compounds which are found in foods of plant origin. The antioxidant activity of flavonoids is due to the hydrogen donating property of the hydroxyl group, and the antioxidant power is related to the number and positions of hydroxyl groups (Pallett and Young, 1993). Some epidemiological studies indicate a negative correlation between the intake of dietary flavonoids and coronary heart disease (Hertog, et al., 1993), stroke and cancer.

Flavonols are members of a subclass of flavonoids, and the most representative compound of this class is quercetin, which is a well studied antioxidant as it is the major flavonol in the diet. Quercetin is an effective inhibitor of LDL oxidation and is a scavenger of superoxide anion. Increased concentration and antioxidant capacity in the blood after ingestion of quercetin-rich food (apples, onions) or beverage (tea) has been demonstrated (de Vries, et al., 1998; Morand, et al., 1998), and metabolites of quercetin in the plasma of rats have been shown to have antioxidant properties (Morand et al., 1998). The protective effect was reported at concentrations as low as 7.3 μM (Noroozi, et al., 1998). Quercitrin and quercetin- 3-glucoside, derivatives of quercetin, were also found to be protective against hydrogen peroxide (Noroozi et al., 1998) and quercitin was reported to protect DNA of lymphocyte and sperm from stress produced by Trp-P-2 (Anderson et al., 1998a and 1998b; Anderson et al., 2001). Jurkat human leukemia cells incubated with a green tea extract, which is very rich in epigallocatechin gallate, showed increased resistance to iron-induced DNA damage (Erba, et al., 1999).

Soy foods are known to be rich in isoflavones. These phytoestrogens exhibit mild oestrogen-like effects and high consumption of phytoestrogens is suggested to be protective against certain types of cancer and cardiovascular disease (Davis, et al., 1999). Supplementation with soy milk for 4 weeks was reported to decrease oxidized bases in DNA and to increase resistance to hydrogen peroxide. Soybean molasses has also been shown to protect Chinese hamster lung cells from damage induced by 2AAAF (Plewa, et al., 1998).

Phenolic compounds are widely found in the secondary products of medicinal plants, as well as in many edible plants (Hagerman, et al., 1998). The ability of phenolic compounds to serve as antioxidants has been recognized, leading to speculation about the potential benefits of ingesting phenolic- rich foods. Several studies have described the antioxidant properties of medicinal

plants, foods, and beverages which are rich in phenolic compounds (Brown and Rice-Evans, 1998; Krings & Berger, 2001).

Resveratrol (non-flavonoid phenolic compound) is a stilbene, found in grape skin and seeds, which has oestrogen-like properties. The concentration of the trans-isomer of resveratrol in red wine ranges between 0.1 and 15 mg/L (Fremont, 2000). Resveratrol is generally believed to offer cardioprotection (Wu et al., 2001). Resveratrol has been reported to protect cells from $H_2O_2$-induced apoptosis (Jang and Surh, 2001) and at 25-50 μM to protect against cigarette tobacco smoke-induced DNA damage (Sgambato et al., 2001).

Choi et al. (2002) conducted a study on several Korean medicinal plants to evaluate for free radical scavenging capacities and antioxidant activities using commonly accepted assays. They were extracted with dichloromethane, methanol or ethanol, respectively and selected for the best antioxidant results. Flavonoids, such as catechin, morin, naringenin, quercetin and rutin, were included and used as standards in this study. Each sample under assay condition showed a dose-dependent free radical scavenging effect of DPPH (1,1-diphenyl-2-picryl hydrazyl radical) and a dose-dependent inhibitory effect of xanthine oxidase and lipid peroxidation. Among plant extracts, the root bark of *Morus alba* and the leaf of *Saururus chinensis* showed stronger SC50 or ID50 values than other plant extracts. They also showed a protective effect on DNA damage caused by hydroxyl radicals generated from UV-induced photolysis of hydrogen peroxide. A rapid evaluation for antioxidants using TLC screening and DPPH staining methods demonstrated each plant extract having various free radical scavenging capacity. Stained silica layer revealed a purple background with yellow spots at the location of drops, which showed radical scavenging capacity. The intensity of the yellow color depends upon the amount and nature of radical scavenger present in the samples. This antioxidant potential corresponded with the results of DPPH spectrophotometric assay.

Bub et al. (2003) determined the effects of 2 polyphenol-rich juices (330 ml/d) supplemented for 2 weeks on bioavailability of polyphenols, markers of antioxidative and immune status, and reduction of DNA damage. Juices provided 236 mg (A) and 226 mg (B) polyphenols with glycosides (A) and epigallocatechin gallate (B) as major polyphenolic ingredients. There was no accumulation of plasma polyphenols after two weeks of juice supplementation. In contrast, plasma malondialdehyde decreased with time during juice interventions. Moreover, juice consumption also increased lymphocyte proliferative responsiveness, with no difference between the two juices. Interleukin-2 secretion by activated lymphocytes and the lytic activity of natural killer cells were significantly increased by both juices. Juice intervention had no effect on single DNA strand breaks, but significantly reduced oxidative DNA damage in lymphocytes. A time-delay was observed between the intake of fruit juice and the reduction of oxidative DNA damage and the increase in interleukin-2 secretion. Conclude that consumption of either juice enhanced antioxidant status reduced oxidative DNA damage and stimulated immune cell functions.

Wong et al. (2006) studied the antioxidant activities and total phenolic contents of 30 Chinese medicinal plants were evaluated using the ferric reducing antioxidant power assay and the Folin–Ciocalteu method, respectively. The Chinese medicinal plants were extracted by the traditional method, boiling in water and also in 80 per cent methanol. A significant and linear correlation coefficient between the antioxidant activity and the total phenolic content was found in both aqueous (R2 = 0.7917) and methanol (R2 = 0.7584) extracts. Cyanidin phenolic compounds are thus a major contributor of antioxidant activity. Comparing the extraction efficiency of the two methods, the boiling water method extracted phenolic compounds more efficiently, and antioxidant activity of the extract was higher. It was found that the Chinese medicinal plants *Rhodiola sacra* Fu, the stem of *Polygonum multiflorum* Thunb and the root of *P. multiflorum Thunb* possessed the highest antioxidant activities and thus could be potential rich sources of natural antioxidants.

Belloir et al. (2006) studied the antigenotoxic activity of several garlic organosulfur compounds (OSC) in the human hepatoma cell line HepG2, using comet assay. The OSC selected were allicin (DADSO), diallyl sulfide (DAS), diallyl disulfide (DADS), S-allyl cysteine (SAC) and allyl mercaptan (AM). To explore their potential mechanisms of action, two approaches were performed: (i) a pre-treatment protocol which allowed study of the possible modulation of drug metabolism enzymes by OSC before treatment of the cells with the genotoxic agent; (ii) a co-treatment protocol by which the ability of OSC to scavenge direct-acting compounds was assessed. Preliminary studies showed that, over the concentration range tested (5–100 μM), the studied OSC neither affected cell viability nor induced DNA damage by themselves. In the pre-treatment protocol, aflatoxin B1 genotoxicity was significantly reduced by all the OSC tested except AM. DADS was the most efficient OSC in reducing benzo(a)pyrene genotoxicity. SAC and AM significantly decreased DNA breaks in HepG2 cells treated with dimethylnitrosamine. Additionally, all the OSC studied were shown to decrease the genotoxicity of the direct acting compounds, hydrogen peroxide and methyl methanesulfonate. This study demonstrated that garlic OSC displayed antigenotoxic activity in human metabolically competent cells.

Pudina extract (*Mentha spicata Linn.*) used as flavouring in culinary preparation throughout the plains of India was examined for its DNA damage protecting activity and antioxidant potential. The n-Butanol soluble fraction (PE) derived from methanol extract of *Mentha spicata Linn* at 10 μg/ml exhibited significant protecting activity against DNA strand scission by $^.$OH on pBluescript II SK(–) DNA. This potential bioactivity of pudina extract was associated with its high polyphenolic content (Kumar and Chattopadhyay, 2007)

Ross et al. (2007) used Raspberry extracts enriched in polyphenols, but devoid of organic acids, sugars and vitamin C, were prepared by sorption to C18 solid phase extraction matrices and tested for their ability to inhibit the

proliferation of human cervical cancer (HeLa) cells in vitro. The raspberry extract reduced proliferation in a dose-dependent manner.

Azevedo (2007) conducted a study to evaluate both the antimutagenicity/antigenotoxicity and mutagenicity/genotoxicity of aqueous extract obtained from the Solanum melanogena, a possible novel source of anthocyanin, and its main purified anthocyanin extract (delphinidin), using the single cell (comet) assay and micronucleus test. Pretreatment with higher doses of the purified anthocyanin (10 and 20 mg/kg b.w.) led to a statistically significant reduction ($p < 0.05$) in the frequency of micronuclei in polychromatic erythrocytes induced by cyclophosphamide. No apparent genotoxicity and mutagenicity was found for either the anthocyanin or delphinidin extracts. Taken together, these results suggest that mice pre-treated with specific compounds present in anthocyanins (delphinidin) displayed a lower incidence of mutations induced by cyclophosphamide.

However, DNA damage is only of consequence if it cannot be adequately repaired, so that the individual's ability to perform DNA repair may be at least as important as the damage they sustain.

2.4 THE COMET ASSAY

The integrity of our DNA is crucial to health. It is accepted that DNA damage is detrimental, and then preventing DNA damage or increasing efficiency of repair is beneficial. Nutraceuticals that protect DNA from oxidant challenge or that promote DNA repair may have potential health benefits and help lower risk of age-related disease. Therefore, a sensitive and reliable laboratory tool to investigate DNA damage, protection and repair is needed to investigate the potential health benefits of nutraceuticals. The single cell electrophoresis, or 'comet', assay for DNA damage is widely used for this purpose, and was judged

to be one of the few adequate methods for assessing *in vivo* antioxidant activity and DNA effects (Griffiths, et al., 2002).

"Comet Assay" or "Single Cell Gel Electrophoresis (SCGE) assay" is now considered a very important alternative for the cytogenetic tests; it is much less labour intensive, more rapid and less expensive. The use of the Comet Assay in eco- (geno-) toxicological studies becomes more common as scientists begin to realize the importance of the genetic damage caused by pollutants.

## 2.4.1 Development of the Comet assay

Rydberg and Johanson (1978) were the first to directly quantitate DNA damage in individual cells after gamma-irradiation by lysing and embedding them in agarose on slides under mild alkali conditions to allow the partial unwinding of DNA. The cells were stained with acridine orange and the extent of DNA damage was measured by the ratio of green (indicating double-stranded DNA) to red (indicating single-stranded DNA) fluorescence. To improve the sensitivity for detecting DNA damage in isolated cells, Ostling and Johanson (1984) proposed that strand breaks would enable DNA loops to stretch out upon electrophoresis, so the microgel electrophoresis technique was developed. Electrophoresis acted to pull negatively charged damaged DNA away from the nucleoid towards the anode and resulted in characteristic images that looked like a comet with head and tail. This technique permitted the detection of double stranded DNA breaks only. The microgel method was progressively improved for sensitivity and reproducibility for detecting single-strand breaks. More complete protein lysis was accomplished, and alkaline treatment step was included before or during electrophoresis (Singh, et al., 1988; Olive, et al., 1990a). Alkali caused denaturation of the duplex DNA, and allowed the individual strands to separate and migrate independently. The name "Comet assay" was introduced and the application of the first image analysis program was described in 1990 (Olive et al., 1990b). Image analysis has become essential for objective measurement of low-dose effects, or for

distinguishing small differences among sub-populations of cells. Microscopic examination (Collins et al., 1997) remains useful for observing larger differences (e.g. screening drugs or measuring the percentage of apoptic cells).

## 2.4.2 Detection of DNA damage

While the general steps for single-cell gel electrophoresis are fairly well defined, in the past years, the Comet assay has had several modifications but the underlying principles are based on the neutral (Olive's) and alkaline (Singh's) version. Alkaline conditions (pH>13) enables detection of not only frank strand breaks but also alkali-labile sites, DNA crosslinking, and transient DNA strand breaks arising due to DNA repair processes (Singh et al., 1988). In neutral pH, only the detection of double-strand breaks is possible, since at this pH, DNA base pairing is not disrupted and thus the discontinuities in single-strand breaks cannot be detected (Mckelvey-Martin, et al., 1993). More recently, the assay was modified further to enable the detection of specific kinds of DNA damage by combining the assay with the use of a purified DNA repair enzymes, which recognize the lesions along the DNA and convert them into the DNA single-strand breaks expressed as an increase in comet DNA migration (Collins, et al., 1993, 1996, 1997). For this purpose various DNA repair enzymes are used, especially T4 endonuclease for determining the relative amounts of pyrimidine dimers (Gedik et al., 1992), UV-DNA damage endonuclease (UVDE) or uracil glycosylase (UDG), Fapy-DNA glycosylase (FPG) and endonuclease III (Endo III) recognizing oxidized bases (Collins et al., 1993). The Comet assay has technical variables affecting its sensitivity, the main ones are: the composition and pH of the lysing solution; the composition and pH of the electrophoretic buffer; and the electrophoretic conditions basically voltage, amperage and unwinding length and running time (Fairbairn et al.,1995). For example, the modification of the Comet assay described by Angelis et al., (1999) on plant systems employs various combinations of neutral and alkali pH solutions immediately prior or during electrophoresis. Exposure of DNA to high alkali prior to electrophoresis

under neutral conditions (so called A/N protocol) allows for the preferential detection of DNA SSBs. The majority of alkali labile sites become detectable when electrophoresis is performed in alkaline solution (A/A protocol). DSBs cause comet formation even under completely neutral conditions (N/N protocol).

**2.4.3 Why to use the Comet assay**

The Comet assay is an economic, fast, sensitive, reliable, and rapid method. Advantages of the Comet assay for assessing DNA damage includes: (1) damage to the DNA in individual cells is measured; (2) only small number of cells are needed to carry out the assay (< 10, 000); (3) data can be generated very quickly by visual scoring (Collins et al., 1993); (4) it is as sensitive method for detecting DNA damage than conventional cytogenetic tests in detecting low levels of exposure (Collins et al., 1996; Leroy et al., 1996; Lee and Steinnert, 2003) and (5) the assay can be performed on virtually any eukaryotic cell type. This technique can be applied to proliferating and non proliferating cells and the cells of those tissues, which are the first sites of contact with mutagenic/carcinogenic substances. What makes this assay even more valuable is the specificity for detecting genotoxicity. Like in other tests, DNA effects induced due to cytotoxicity is a big issue. Data show that cytotoxic effects can be detected (dead cells show specific kinds of comets called "clouds") and distinguished from genotoxic effects, therefore, should have no confounding effects on results (Henderson et al., 1998; Speit et al., 1998). Given its overall characteristics, this method has been widely used in several different areas. On the other hand, the Comet assay is not without shortcomings: (1) the majority of the DNA lesions detected by Comet assay can be repaired by cell before being fixed as mutations; (2) it is rate limiting and also has sample bias due to the small cell sample; (3) there is no single appropriate comet parameter capable of adequately describing the observed damage, so the interpretation of results might be difficult (Hartmann, 1999); (4) there are also wide variations in the methodologies followed during alkali treatment and electrophoresis (Kassie, et al., 2000).

**2.4.4 Applications of the Comet assay**

The major applications of the Comet assay are in the following areas: (1) genotoxicology – to evaluate *in vitro/in vivo* genotoxicity of several chemicals; (2) clinical area – to investigate the consequences of certain pathological conditions or therapeutical exposure to chemicals at the cellular level; (3) DNA repair investigations – to reflect the types of DNA lesions and the DNA repair that is taking place in the damaged cells; (4) environmental biomonitoring (aquatic, terrestrial) and (5) human biomonitoring (aging, nutrition, malnourishment, exercise) (Anderson et al., 1998; Rojas et al., 1999). The Comet assay was widely and successfully used in vertebrates, especially in mammalian cells and cells from invertebrates (Salagovic et al., 1996). Plant genetic assay systems are also excellent *in situ* environmental monitors and some studies have been published also on the use of the Comet assay in *Vicia faba* (Koppen and Verschaeve, 1996), onion (Navarrete, et al., 1997), tobacco (Gichner and Plewa, 1998), barley (Jovtchev et al., 2001) or Arabidopsis (Menke et al., 2001). Miloshev, et al., (2002) detected DNA damage in the yeast *Saccharomyces cerevisiae* and Iwahori et al. (1999) applied this assay to *Euglena gracilis.*

# Materials and methods

# 3. MATERIALS AND METHODS

The experiments to evaluate the effect of green leaves used in traditional food preparations on repair of DNA damage were carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during September 2008 to August 2009. Details regarding the experimental materials used and methodology adopted for various experiments are presented in this chapter.

## 3.1 PLANT MATERIAL USED FOR INDUCING DNA DAMAGE

Tomato (*Lycopersicon esculentum)* of variety Vellayani Vijaya was procured from the Department of Olericulture, College of Agriculture, Vellayani.

The tomato seedlings needed for the experiment were raised under natural condition, using red earth, sand and FYM in 1:1:1 ratio as seedbed. Need based plant protection measures were taken up. There was regular watering and seedlings of four leaf stage were used for the experiments. Periodic sowing at weekly interval was done to get steady continuous supply of fresh seedlings for the experiment.

## 3.2 CHEMICALS AND GLASSWARE

Plant mutagens like ethyl methanesulphonate and methyl methanesulphonate were purchased from Sisco Research Laboratories (SRL), India. Agarose like low point melting and normal melting was of molecular grade and procured from Himedia Laboratories, India.

Microscope glass cover slides of standard grade, 24-50 mm size and glass slide, pathology grade, non interleaved, cut edged, frosted on one side, 25.4 mm $\times$ 76.2 mm (1" $\times$ 3") size were purchased from Himedia Laboratories, India.

## 3.3 PREPARATION OF PLANT LEAF EXTRACTS

The plants viz. *Cissampelos sp* (Malathangi), *Azima tetracantha* (Ashenku), *Pongamia glabra* (Ungu), *Thespesia populnea* (Poovarasu) and *Myxopyrum serratulum* (Chaturamulla) were obtainded from Herbal Garden, Instructional Farm, College of Agriculture, Vellayani.

The leaves from each plant species were collected dried under shade and ground to fine powder for preparation of aqueous extract. The following two methods of extraction procedures were tried

In method 1, 1 gm of fine powder of respective plant sample, was taken in 50 ml distilled water, kept overnight on a shaker at 400 rpm and filtered using whatman filter paper. Filtrate was collected and evaporated on a water bath till it reaches 10 ml. It was used for the DNA repair assay.

In method 2, 1 gm of fine powder of respective plant sample, was taken in 50 ml distilled water, boiled for 30 minutes and filtered using Whatman filter paper. Filtrate was collected and was evaporated on a water bath till it reaches 10 ml. The extract was used for the DNA repair assay.

## 3.4 INDUCTION OF DNA DAMAGE USING CHEMICAL MUTAGENS IN TOMATO SEEDLINGS

The tomato seedlings were taken from the pot and the soil particles were carefully removed from the roots using water. The roots of the seedlings were immersed in test tubes containing 10ml of 0, 1 mM, 2 mM, 3 mM and 4 mM ethyl mathanesulphonate (EMS) and methyl methanesulphonate (MMS) in distilled water (dH2O) respectively (Gichner, 2003) and the seedlings were incubated in darkness at room temperature for 12 hours. After the treatment the seedling roots were rinsed in water. The leaves were used for isolation of nuclei from the leaves.

*Lycopersican esculentum*



*Myxopyrum serretulum*



*Cissampelos pareira*



*Azima tetracantha*



*Thespesia populneus*



*Pongamia pinnata*

**Plate 1.** Tester plant and Test plant

**3.4.1 Comet assay**

The Comet assay- SCGE (Single cell gel electrophoresis), is widely used as biomonitoring tool to assess the genotoxic and genoprotective effects of various compounds. Increased DNA migration results from the induction of DNA single-strand breaks, alkaline labile sites and incomplete excision repair sites at the time of lysis. The intensity of the fluorescence in the tail relative to the head provides information about the numbers of strand breaks. Tail length, percentage of total DNA in the tail and the tail moment all reflect DNA damage. Swedish researchers Ostling and Johanson (1984) developed this technique. Singh, et al., in (1988) later modified this technique, as the Alkaline Comet Assay.

*3.4.1.1 Isolation of nuclei from leaves*

The plant cell has a wall that cannot be removed as in the case of animal cell membrane by lysis in high concentrations of detergents and salts, and so the nuclei have to be isolated mechanically. It is extremely important to gently isolate the nuclei from the leaves. All operations were conducted under dim or yellow light. A small part of the leaf (about 2 x 1 cm) was placed in a 60 mm plastic Petri dish. Onto each leaf 200 to 300 μl of cold 0.4 M Tris buffer (Appendix I) was spread. Using a fresh razor blade, the leaf was sliced into a fringe. The petridish was kept tilted in the ice so that the nuclei would collect in the buffer.

*3.4.1.2 Slide preparation*

Microscope slides were dipped into a solution of 1 per cent normal melting point agarose (NMA) (Appendix III) prepared with water at 50ºC. The bottom of the slides were wiped to remove the agarose, placed horizontally on a level surface and dried overnight at room temperature. After the slides were prepared they are kept dry in slide boxes until their use.

Onto each slide with the dried NMA layer, 50 µl of the nuclear suspension and 50 µl of 1 per cent molten low melting point agarose (LMA) prepared out of phosphate buffer saline (PBS) (Appendix IV & II) at 40° C was added. The nuclei and the LMA were gently mixed by repeated pipetting using a cut tip and a coverslip (24 x 50 mm) placed on the mixture. The slide was placed on an iced surface for a minimum of 5 minutes after which the coverslip was removed and a final layer of 100 µl molten 0.5 per cent LMA was placed on the slide and kept on an iced surface for 5 minutes.

### 3.4.1.3 Unwinding and electrophoresis

The coverslips were removed and all of the slides are placed in tray containing cold (4°C) electrophoresis buffer (Appendix V). The nuclei were incubated for 15 minutes to allow the DNA to unwind. After unwinding the slides were placed in an electrophoresis box with the electrophoretic buffer and electrophoresis was conducted at 0.74 V/cm (25 V, 300 mA) for 30 min, at 4°C. Following electrophoresis, the slides were neutralized 3 times with 0.4 M Tris buffer, and incubated after neutralization for 15 minutes in methanol and left overnight to dry, and stored in slide boxes.

### 3.4.1.4 Staining of nuclei

The stored slides were incubated for 10 minutes in cold distilled water before staining. The slides were stained with 80 µl (20 µg/ml concentration) ethidium bromide (Appendix VI) for 5 minutes and the excess ethidium bromide was remove by dipping in ice cold water and covered with a coverslip. Slides with stained nuclei were scored within 6 hours.

### *3.4.1.5 Screening the slides*

For each slide 25 randomly chosen cells per slide were analyzed with a fluorescence microscope (Leica) with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. The images of comet slide which are focused at 20X magnification were taken with the help of CCD (charged couple device) camera which was attached to the fluorescence microscope. A computerized image analysis system, comet assay IV, developed by Perceptive instruments, UK was employed to measure DNA damage. The damage was expressed in terms of tail moment (TM) value (expressed in μm) or the per cent of DNA in the tail. Three slides were evaluated per treatment. The average tail moment value or the average per cent of tail DNA was calculated from each slide values. The computerized image analysis system measures the amount of DNA in the head and in the tail, and the length of the tail was expressed in μm. For expressing the DNA damage, the parameter per cent tail DNA or tail moment (TM) can be used. The TM value represents the per cent of tail DNA multiplied by the tail length divided by 100.

### *3.4.1.6 Statistics*

Data were analyzed statistically using AGRES software. With the slide as the unit of measure the mean tail moment values and the per cent of tail DNA were used in a one-way analysis of variance test. The significant F-value of $P \leq 0.05$, a Dunken multiple comparison versus the control group analysis was conducted. Differences between two groups are statistically evaluated by the Paired *t* -test.

**3.4.2 Experiment to check the genotoxicity of plant leaf extracts in tomato seedlings**

*3.4.2.1 Treatment condition*

The tomato seedlings were taken from the pot and the soil particles were carefully removed from the roots using water. The roots of the seedlings were immersed in test tubes containing 10 ml of leaf extract of each test plant samples obtained by method 1 and 2 separately and the seedlings were incubated in darkness at room temperature for 12 hours. After 12 hours of incubation seedlings were taken from the test tubes and the roots were rinsed in water. They were used for isolation of nuclei for comet assay.

Isolation of nuclei from leaves, slide preparation, unwinding and electrophoresis, staining, screening the slides were done by following the procedures mentioned above.

3.5 EXPERIMENTS TO TEST THE EFFECT OF GREEN LEAF EXTRACT ON DNA DAMAGE IN TOMATO SEEDLINGS

**3.5.1 Pre- Supplementation treatment**

The tomato seedlings were taken from the pot and the soil particles were carefully removed from the roots using tap water. The roots of the seedlings were immersed in test tubes containing 10 ml of leaf extracts of each plant species separately prepared by method 1 and 2 and the seedlings were incubated in darkness at room temperature for 12 hours. After 12 hours of incubation seedling were taken from the test tubes and the seedling roots were rinsed in water.

The pre-treated tomato seedlings were incubated in test tube containing 10 ml of 4 mM EMS and MMS separately for 12 hour at room temperature in darkroom. After 12 hours of incubation seedling were taken from the test tubes and the seedling roots were rinsed in water. It was used for isolation of nuclei from the leaves.

Isolation of nuclei from leaves, slide preparation, unwinding and electrophoresis, staining, screening the slides were done by following the procedures mentioned above.

### 3.5.2 Post- Supplementation treatment

The tomato seedlings were taken from the pot and the soil particles were carefully removed from the roots using water. The roots of the seedlings were immersed in test tubes containing 10 ml of 4 mM ethylmethanesulphonate (EMS) and methylmethanesulphonate (MMS) in distilled water respectively, maximum DNA damage was seen with 12h hold seedlings at this concentration. After chemical mutagen treatment the seedling roots were rinsed in water. It was used for incubate in leaf extracts of test plants to know their efficiency in repairing DNA damage.

The roots of seedlings incubated in leaf extracts (10 ml) prepared by method 1 and 2 extraction methods for 12 hours under darkness at room temperature. After treatment the roots of seedling were rinsed in water. It was used for isolation of nuclei from leaves.

Isolation of nuclei from leaves, slide preparation, unwinding and electrophoresis, staining, screening the slides were done by following the procedures mentioned above.

### 3.5.3 Co- supplementation treatment

The tomato seedlings were taken from the pot and the soil particles were carefully removed from the roots using water. The roots of the seedlings were simultaneously treated with the chemical mutagen (4 mM concentration of EMS and MMS) and plant leaf extract prepared by method 1 and 2 extraction protocol for 12 hours under darkness at room temperature. After incubation tomato seedlings were taken out from the test tubes, rinsed in water and was used for isolation of nuclei from the leaves.

Isolation of nuclei from leaves, slide preparation, unwinding and electrophoresis, staining, screening the slides were done by following the procedures mentioned above.

# Results

# 4. RESULTS

The results of the study entitled "Effect of green leaves used in traditional food preparation on DNA repair" carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during September 2007 to November 2009 are presented below.

## 4.1 INDUCTION OF DNA DAMAGE USING CHEMICAL MUTAGENS IN TOMATO SEEDLINGS

In the first set of treatment, tomato seedlings were treated with two mutagens at different concentrations to find out the extent of DNA damage caused by them using comet assay and to select the concentration which gives higher tail moment.

Tail length, percentage of total DNA in the tail, and the tail moment all reflect DNA damage. The tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail).

Tail moment value obtained when tomato seedlings were treated with ethyl methanesulphonate (EMS) ranged from 81.90 μm to 232.15 μm. Plants treated with EMS at a concentration of 4 mM recorded significantly higher tail moment value (232.15 μm), which was on par with tomato seedlings treated with EMS at 3 mM (222.12 μm). At all the concentration tried, EMS treatment induced significant DNA damage (Table 1), compared to control (81.90 μm).

In plants treated with methyl methanesulphonate (MMS) the tail moment ranged from 95.49 μm to 275.79 μm. The MMS at 4 mM concentration recorded significant higher tail moment of 275.79μm, which was followed by MMS at 3 mM (203.48 μm). All concentrations of MMS treatment in tomato seedlings

**Table 1. Effect of EMS on DNA damage in tomato seedlings**

| Concentration of EMS (mM) | Tail moment (μm) mean | Tail intensity (%) mean | Tail length (μm) mean |
|---|---|---|---|
| 1.0 | 125.50 | 50.10 | 294.52 |
| 2.0 | 183.78 | 72.86 | 418.01 |
| 3.0 | 222.12 | 83.57 | 499.57 |
| 4.0 | 232.15 | 86.10 | 538.44 |
| 0 (Control) | 81.90 | 38.06 | 217.64 |
|  |  |  |  |
| SED± | 19.349 | 4.484 | 46.524 |
| CD (0.05) | 43.112 | 9.991 | 103.663 |

**Table 2. Effect of MMS on DNA damage in tomato seedlings**

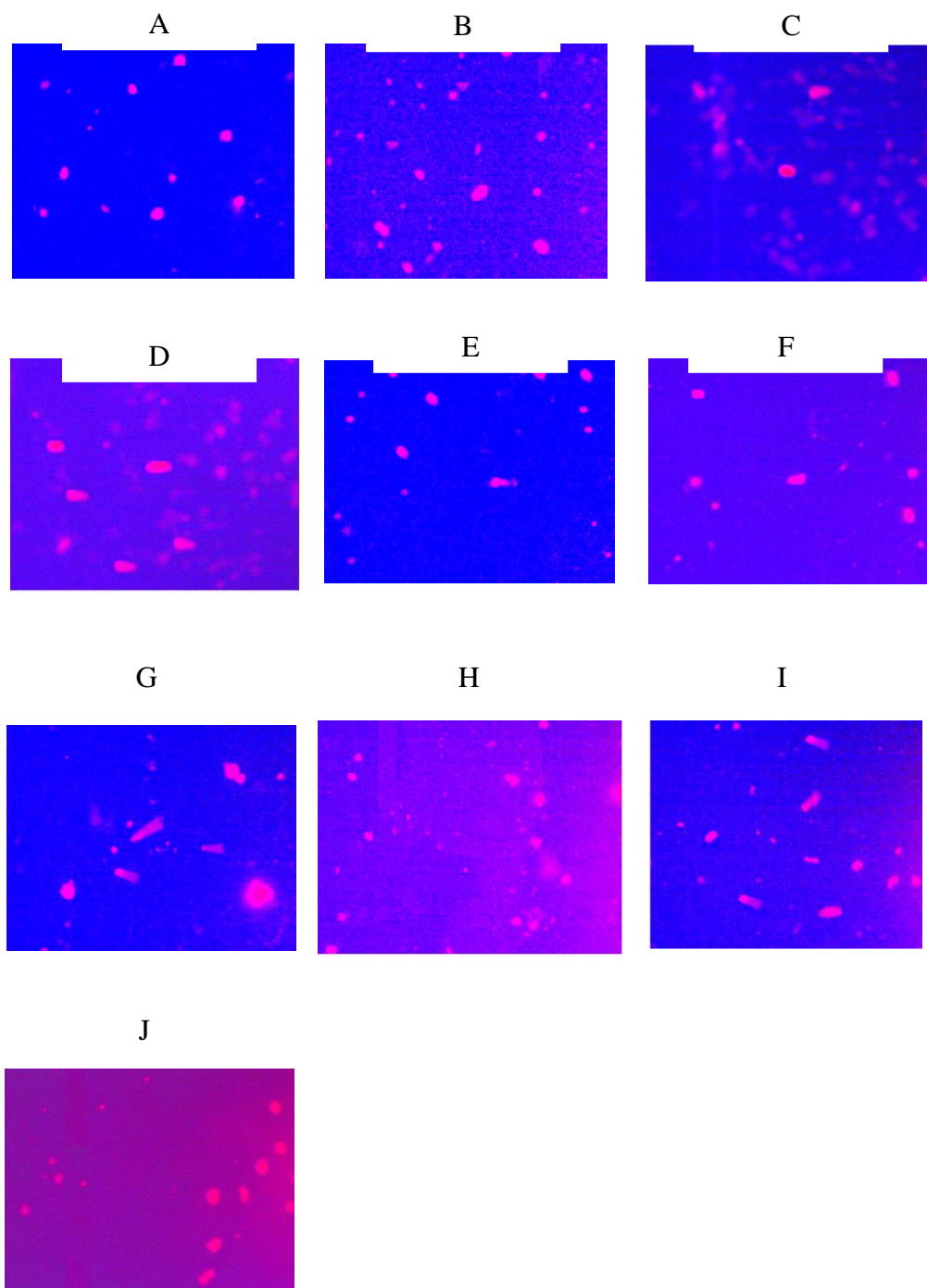| Concentration of MMS (mM) | Tail moment (μm) mean | Tail intensity (%) mean | Tail length (μm) mean |
|---|---|---|---|
| 1.0 | 169.09 | 33.804 | 234.665 |
| 2.0 | 126.66 | 45.958 | 296.996 |
| 3.0 | 203.48 | 69.947 | 436.844 |
| 4.0 | 275.79 | 95.064 | 584.194 |
| 0 (Control) | 94.49 | 38.07 | 217.64 |
|  |  |  |  |
| SED± | 20.477 | 6.7359 | 46.088 |
| CD (0.05) | 45.625 | 15.0085 | 102.691 |

**Plate 2:** Comet images of nuclei taken from tomato leaves treated with mutagens: EMS and MMS at different concentrations. A) 1mM, B) 2mM, C) 3mM, D) 4mM, E) control of EMS, F) 1mM, G) 2mM, H) 3mM, I) 4mM, J) control of MMS.

showed significantly higher DNA damage than control (Table 2), except MMS at 2 mM which was on par with control (94.49 μm).

## 4.2 EFFECT OF PLANT LEAF EXTRACT ON TOMATO DNA

In the second set of treatment, tomato seedlings were treated with aqueous plant leaf extracts prepared by method 1 and method 2 to check the genotoxicity if any, prior to their use in DNA repair experiments.

Tomato seedlings treated with plant leaf extracts, prepared by method 1, recorded tail moment values ranging from 110.43 μm to 0.03 μm. The tomato seedlings treated with various plant leaf extracts (Table 3) obtained by method1 recorded lower tail moment than the control. *Thespesia populneus* extract obtained by method 1 recorded the lower tail moment value (0.03 μm), which was followed by the values in tomato seedlings treated with *Pongamia pinnata*, *Myxopyrum serratulum* and *Azima tetracantha* extracts obtained by method 1. The highest tail moment value was observed in tomato seedling incubated in distilled water (control) (110.42 μm). It was followed by tomato seedling incubated in *Cissampelos pareira* extract obtained by method 1 (49.31 μm).

The tail moment values recorded (Table 4) in case of tomato seedlings treated with plant leaf extract, obtained by method 2 ranged from 110.43 μm to 0.03 μm.

*Thespesia populneus* extract prepared by method 2 recorded the lowest tail moment value of 0.03μm, which was followed by the extracts of *Pongamia pinnata*, *Myxopyrum serratulum*, *Cissampelos pareira* and *Azima tetracantha*. The lowest tail moment values were recorded in tomato seedlings treated with plant leaf extract obtained by method 2 than the control.

**Table 3.  Effect of plant leaf extracts prepared by method 1 on DNA damage in tomato seedlings**

| Plant leaf extracts | Tail moment (µm) mean | Tail intensity (%) mean | Tail length (µm) mean |
|---|---|---|---|
| *Azima tetracantha* | 18.28 | 20.09 | 72.76 |
| *Cissampelos pareira* | 49.31 | 29.87 | 144.58 |
| *Myxopyrum serratulum* | 2.35 | 33.64 | 20.41 |
| *Pongamia pinnata* | 0.07 | 1.31 | 51.16 |
| *Thespesia populneus* | 0.03 | 1.04 | 48.61 |
| Distilled water  (Control) | 110.43 | 48.83 | 273.88 |

**Table 4. Effect of plant leaf extracts prepared by method 2 on DNA damage in tomato seedlings**

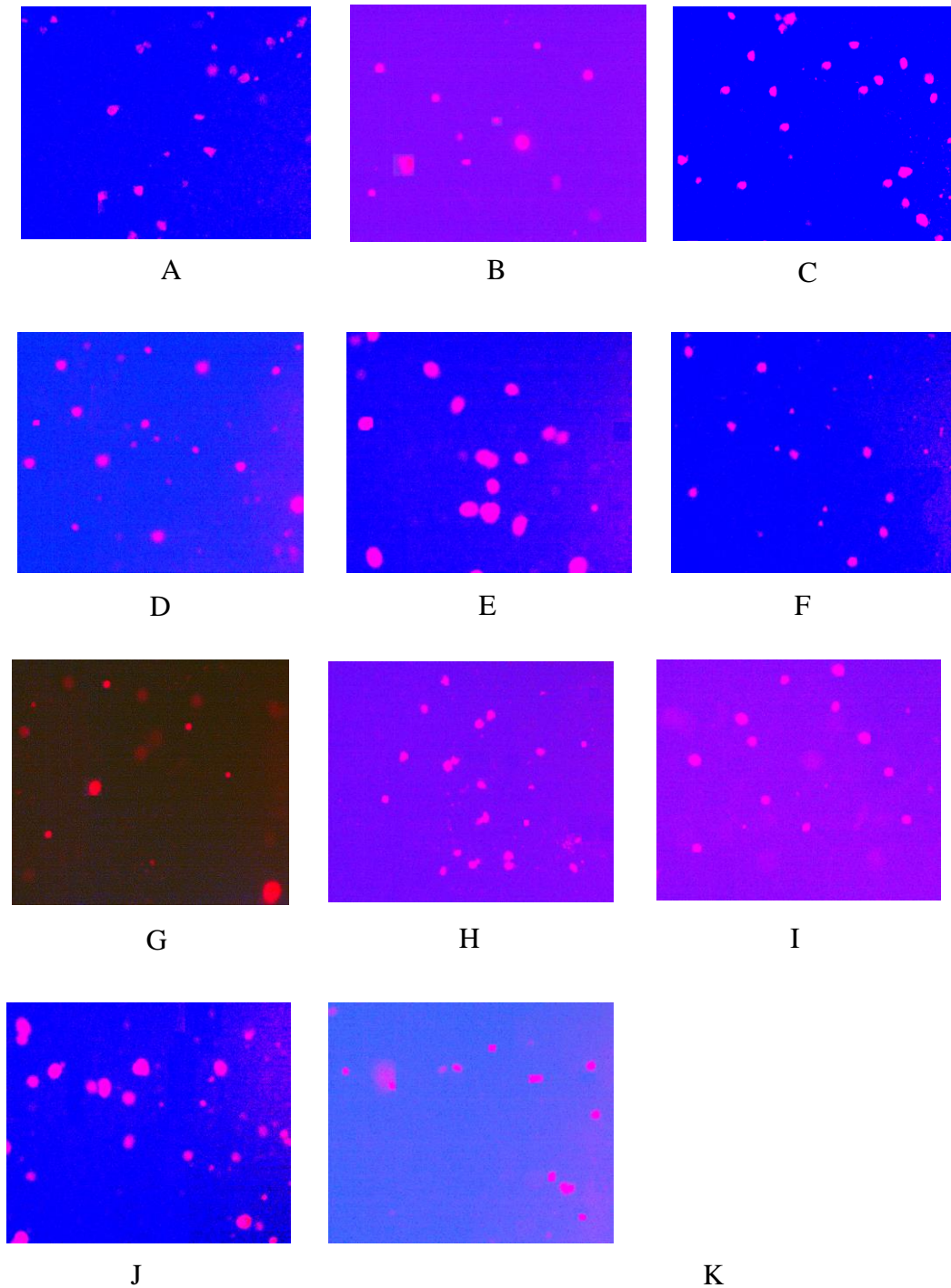| Plant leaf extracts | Tail moment (µm) mean | Tail intensity (%) mean | Tail length (µm) mean |
|---|---|---|---|
| *Azima tetracantha* | 9.63 | 15.93 | 54.74 |
| *Cissampelos pareira* | 0.10 | 1.29 | 60.47 |
| *Myxopyrum serratulum* | 0.90 | 12.35 | 52.33 |
| *Pongamia pinnata* | 0.07 | 1.22 | 59.42 |
| *Thespesia populneus* | 0.03 | 1.04 | 50.26 |
| Distilled water (Control) | 110.43 | 48.83 | 273.88 |

**Plate 3:** Comet images of nuclei taken from tomato leaves exposed to plant leaf extract (method 1 and method 2). A) *Azima tetracantha,* B) *Cissampelos pareira,* C) *Pongamia pinnata,* D)*Myxopyrum serratulum,* E) *Thespesia populneus* prepared by method 1 and F) *Azima tetracantha*, G) *Cissampelos pareira*, H) *Pongamia pinnata*, I) *Myxopyrum serratulum*, J) *Thespesia populneus* prepared by method 2, K) Control.

4.3 PRE-SUPPLEMENTATION TREATMENT

**4.3.1 Pre-treatment of tomato seedlings with plant leaf extract prepared by method 1, before EMS treatment, on DNA damage protection**

Treatment of tomato seedlings with plant leaf extracts obtained by method 1 prior to EMS treatment influenced tail moment value. The values ranged from 0.00 μm to 148.61μm. The lowest tail moment value (0.00μm) was observed in tomato seedlings pre treated with *Pongamia pinnata* extract obtained by method1, before EMS treatment, followed by the treatments with *Myxopyrum serratulum*, *Cissampelos pareira, Thespesia populneus* and *Azima tetracantha* extracts obtained by method 1. Tomato seedlings pre-treated with plant leaf extract prepared by method 1 showed lower tail moment value, compared to control. Tomato seedlings pre-treated with distilled water and subjected to EMS treatment (control) recorded the highest tail moment value of 148.61μm (Table 5).

**4.3.2 Pre-treatment of tomato seedlings with plant leaf extract prepared by method 2, before EMS treatment, on DNA damage protection**

Tomato seedlings treated with plant leaf extracts obtained by method 2 prior to EMS treatment influenced tail moment value. The value ranged from 0.00μm to 148.61μm. Tomato seedlings treated with *Azima tetracantha* extracts obtained by method 2, prior to EMS treatments, recorded lowest tail moment of 0.00μm which was followed by treatment with *Thespesia populneus, Myxopyrum serratulum, Pongamia pinnata* extracts. The highest tail moment value of 148.61μm was recorded in tomato seedlings pre-treated with distilled water and subjected to EMS treatment (control). Tomato seedlings pre-treated with plant leaf extract obtained by method 2 and subjected to EMS treatment showed lower tail moment value, compared to the control (Table 6).

**Table 5. Effect of pre-treatment of tomato seedlings with plant leaf extracts prepared by method 1 on EMS induced DNA damage**

| Plant leaf extracts | Tail moment (μm) mean | Tail intensity (%) mean | Tail length (μm) mean |
|---|---|---|---|
| *Azima tetracantha* | 0.02 | 3.76 | 59.55 |
| *Cissampelos pareira* | 0.30 | 2.40 | 66.60 |
| *Myxopyrum serratulum* | 0.46 | 6.60 | 50.37 |
| *Pongamia pinnata* | 0.00 | 0.00 | 103.46 |
| *Thespesia populneus* | 0.14 | 8.86 | 64.75 |
| Distilled water (Control) | 148.61 | 74.20 | 330.53 |

**Table 6. Effect of pre-treatment of tomato seedlings with plant leaf extracts prepared by method 2 on EMS induced DNA damage**

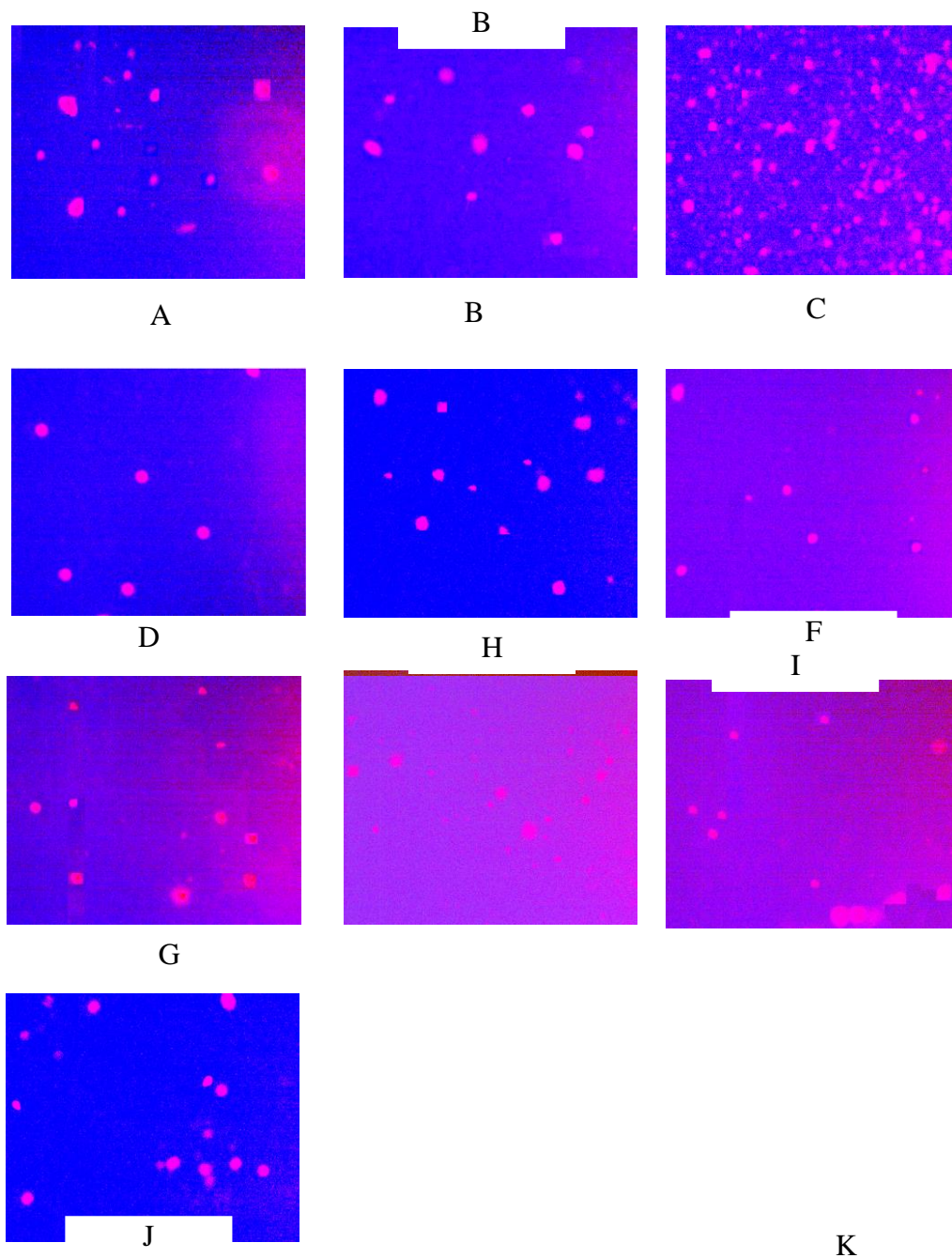| Plant leaf extracts | Tail moment (μm) mean | Tail intensity (%) mean | Tail length (μm) mean |
|---|---|---|---|
| *Azima tetracantha* | 0.00 | 0.00 | 57.98 |
| *Cissampelos pareira* | 62.08 | 21.50 | 194.57 |
| *Myxopyrum serratulum* | 18.40 | 14.92 | 49.17 |
| *Pongamia pinnata* | 21.82 | 30.70 | 70.90 |
| *Thespesia populneus* | 1.42 | 15.11 | 54.87 |
| Distilled water (Control) | 148.61 | 74.20 | 330.53 |

**Plate 4:** Comet images of nuclei taken from tomato leaves treated with plant leaf extract (method 1 and method 2), before EMS. A) *Azima tetracantha* (method 1), B) *Azima tetracantha* (method 2), C) *Cissampelos pareira* (method 1), D) *Cissampelos pareira* (method 2), E) *Pongamia pinnata* (method 1), F) *Pongamia pinnata* (method 2), G) *Myxopyrum serratulum* (method 1), H)*Myxopyrum serratulum* (method 2), I) *Thespesia populneus* (method 1), J) *Thespesia populneus* (method 2), K) Control respectively, before EMS.

### 4.3.3 Pre-treatment of tomato seedlings with plant leaf extract obtained by method1, before MMS treatment, on DNA damage protection

The study on pre-treatment of plant leaf extract obtained by method 1, before MMS treatment, in tomato seedlings influenced tail moment. The values ranged from 0.00 μm to 258.89 μm (Table 7). The lower tail moment (0.00μm) was recorded in tomato seedlings treated with *Pongamia pinnata* extract prepared by method 1, before MMS treatments, followed by treatments with *Thespesia populneus*, *Cissampelos pareira* and *Myxopyrum serratulum* extracts prepared by method 1. Tomato seedlings pre-treated with plant leaf extract obtained by method 1 and subjected to MMS treatment showed lower tail moment value compared to control. The tomato seedlings pre-treated with distilled water and subjected to MMS (control) recorded the highest tail moment of 258.89 μm. This was followed by the values in tomato seedlings pre-treated with *Azima tetracantha* extracts prepared by method 2 before MMS treatments (67.50 μm).

### 4.3.4 Pre-treatment of tomato seedlings with plant leaf extract obtained by method 2, before MMS treatment on DNA damage protection

Pre-treatment of tomato seedlings with plant leaf extract obtained by method 2, before MMS treatment influenced the tail moment. Tail moment values recorded were ranged from 0.00μm to 258.39μm (Table 8). The lower tail moment values (0.00μm) were recorded in *Thespesia populneus* and *Cissampelos pareira* extracts prepared by method 2 treated tomato seedlings, prior to MMS treatment, followed by the treatment with *Myxopyrum serratulum*, *Azima tetracantha* and *Pongamia pinnata* extracts prepared by method 2 treated tomato seedlings prior to MMS treatment. The highest tail moment (258.89 μm) was recorded in tomato seedlings pre-treated with distilled water and subjected to MMS (control). Tomato seedlings treated with plant leaf extracts obtained by method 2 and subjected to MMS treatment showed lower tail moment value compared to control.

**Table 7. Effect of pre-treatment of tomato seedlings with plant leaf extracts prepared by method 1 on MMS induced DNA damage**

| Plant leaf extracts | Tail moment (μm)mean | Tail intensity (%) mean | Tail length (μm) mean |
|---|---|---|---|
| *Azima tetracantha* | 67.49 | 25.22 | 203.03 |
| *Cissampelos pareira* | 6.73 | 4.09 | 80.36 |
| *Myxopyrum serratulum* | 10.94 | 20.73 | 55.72 |
| *Pongamia pinnata* | 0.00 | 0.00 | 129.10 |
| *Thespesia populneus* | 0.04 | 0.74 | 65.62 |
| Distilled water (Control) | 258.89 | 97.02 | 581.66 |

**Table 8. Effect of pre-treatment of tomato seedlings with plant leaf extracts prepared by method 2 on MMS induced DNA damage**

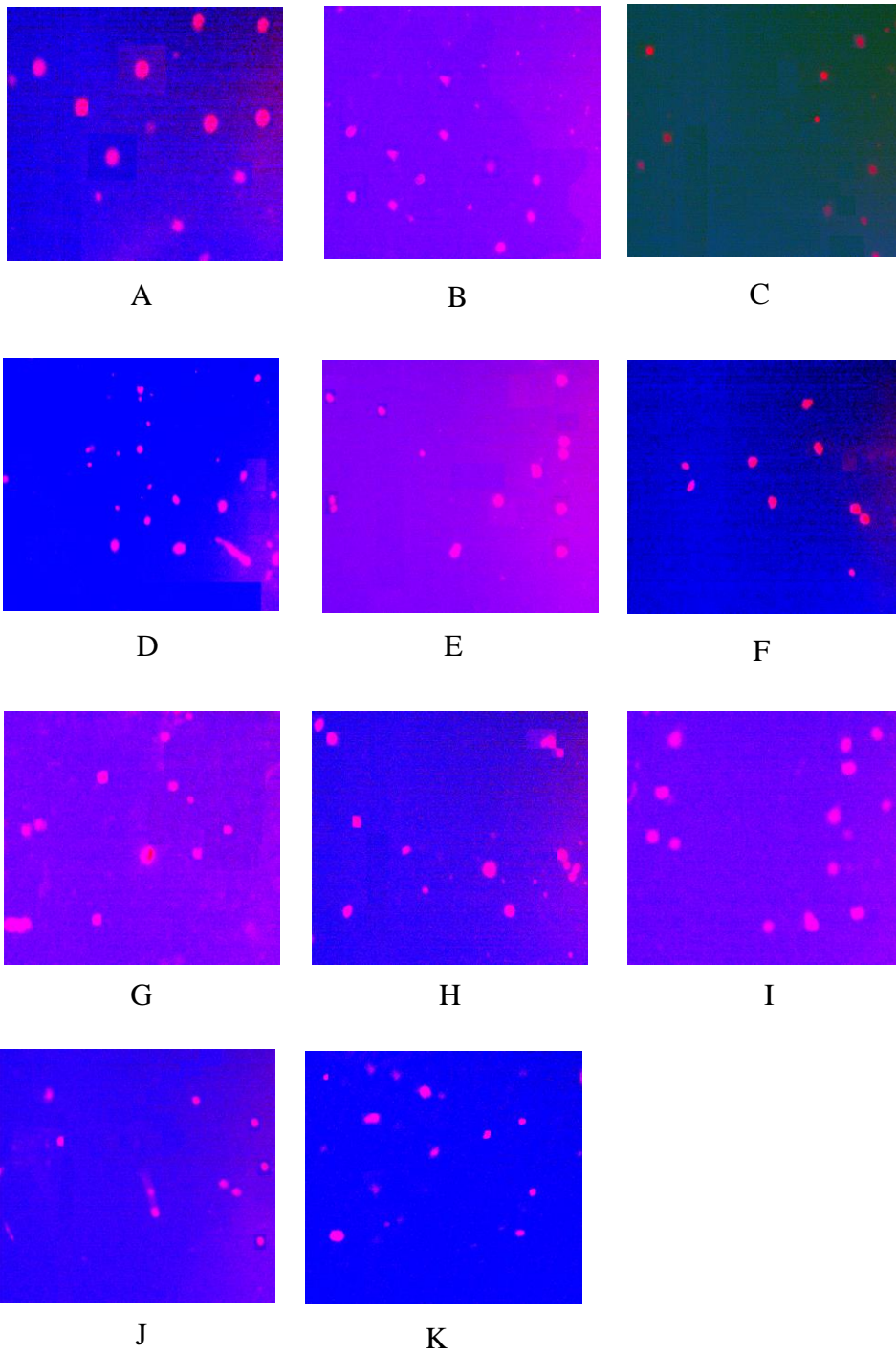| Plant leaf extracts | Tail moment (μm) mean | Tail intensity (%) mean | Tail length (μm) mean |
|---|---|---|---|
| *Azima tetracantha* | 3.34 | 9.48 | 49.79 |
| *Cissampelos pareira* | 0.00 | 0.01 | 52.57 |
| *Myxopyrum serratulum* | 0.02 | 3.70 | 73.74 |
| *Pongamia pinnata* | 5.41 | 29.15 | 40.78 |
| *Thespesia populneus* | 0.00 | 0.00 | 56.80 |
| Distilled water (Control) | 258.89 | 97.02 | 581.66 |

**Plate 5:** Comet images of nuclei taken from tomato seedlings leaves treated with plant leaf extract, A) *Azima tetracantha,* B) *Cissampelos pareira*, C) *Pongamia pinnata*, D) *Myxopyrum serratulum*, E) *Thespesia populneus* (of method 1), F) *Azima tetracantha*, G) *Cissampelos pareira*, H) *Pongamia pinnata,* I) *Myxopyrum serratulum*, J) *Thespesia populneus* (of method 2), K) Control respectively, before MMS treatment.

4.4 POST-SUPPLEMENTATION TREATMENT

**4.4.1 Post treatment of tomato seedlings with plant leaf extract prepared by method 1, after EMS treatment, on DNA damage repair**

Post treatment of tomato seedlings with plant leaf extract obtained by method1, after EMS treatment in tomato seedlings influenced tail moment value. Tail moments recorded were ranged from 0.51 μm to 229.67 μm. The lower tail moment (0.51μm) was recorded in tomato seedlings post treated with *Azima tetracantha* extract prepared by method1, after EMS treatment, followed by treatment with *Cissampelos pareira, Myxopyrum serratulum, Thespesia populneus, Pongamia pinnata* extracts prepared by method 1, after EMS treatment. The highest tail moment (229.67 μm) was recorded in case of tomato seedlings post treated with distilled water, after EMS treatment (control). In this study tomato seedlings post treated with plant leaf extract obtained by method 1, after EMS treatment recorded lowest tail moment value compared to control (Table 9).

**4.4.2 Post treatment of tomato seedlings with plant leaf extracts obtained by method 2, after EMS treatment on DNA damage repair**

Post treatment of tomato seedlings with plant leaf extracts obtained by method 2, after EMS treatment in tomato seedlings influenced tail moment. Tail moments values recorded (Table 10) were ranged from 0.00 μm to 229.67 μm. The lowest tail moment (0.00 μm) was recorded in case of tomato seedlings post treated with *Myxopyrum serratulum* extract obtained by method 2, after EMS treatment and followed by treatment with *Pongamia pinnata, Azima tetracantha, Cissampelos pareira* and *Thespesia populneus* extracts prepared by method 2, after EMS treatment. The highest tail moment (229.67 μm) was recorded in case of tomato seedlings post treated with distilled water after EMS treatment

**Table 9. Effect of post-treatment of tomato seedling with plant leaf extracts prepared by method 1 on EMS induced DNA damage**

| Plant leaf extracts | Tail moment (µm) mean | Tail intensity (%) mean | Tail length (µm) mean |
|---|---|---|---|
| *Azima tetracantha* | 0.51 | 3.71 | 73.07 |
| *Cissampelos pareira* | 11.68 | 17.22 | 72.87 |
| *Myxopyrum serratulum* | 47.85 | 17.99 | 149.76 |
| *Pongamia pinnata* | 73.89 | 28.79 | 222.25 |
| *Thespesia populneus* | 66.80 | 30.98 | 179.71 |
| Distilled water (Control) | 229.67 | 82.24 | 525.15 |

**Table 10. Effect of post-treatment of tomato seedlings with plant leaf extracts prepared by method 2 on EMS induced DNA damage**

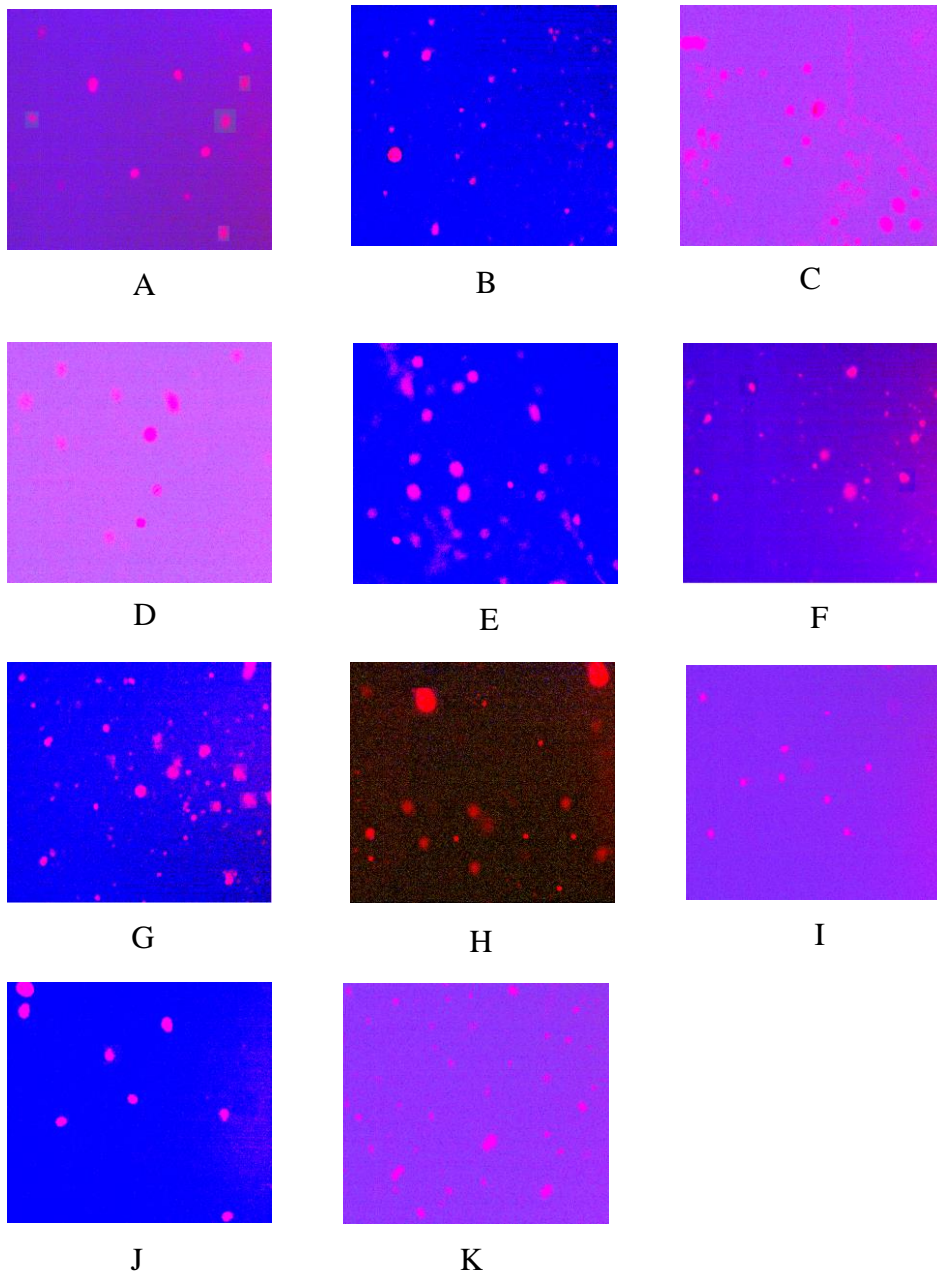| Plant leaf extracts | Tail moment (µm) mean | Tail intensity (%) mean | Tail length (µm) mean |
|---|---|---|---|
| *Azima tetracantha* | 8.95 | 13.44 | 64.12 |
| *Cissampelos pareira* | 11.56 | 7.93 | 60.60 |
| *Myxopyrum serratulum* | 0.00 | 0.00 | 91.27 |
| *Pongamia pinnata* | 0.10 | 1.29 | 58.92 |
| *Thespesia populneus* | 63.67 | 38.53 | 167.92 |
| Distilled water (Control) | 229.67 | 82.24 | 525.15 |

**Plate 6:** Comet images of nuclei taken from tomato leaves treated with plant leaf extract, A) *Azima tetracantha,* B) *Cissampelos pareira,* C) *Pongamia pinnata,* D) *Myxopyrum serratulum,* E) *Thespesia populneus* (of method 1), F) *Azima tetracantha,* G) *Cissampelos pareira, H) Pongamia pinnata,* I) *Myxopyrum serratulum,* J) *Thespesia populneus* (of method 2), K) Control respectively, after EMS.

(control). Tomato seedlings post treated with plant leaf extract prepared by method 2, after EMS treatment recorded lower tail moment value compared to control.

**4.4.3 Post treatment of tomato seedlings with plant leaf extract prepared by method 1, after MMS treatment on DNA damage repair**

Post treatment of tomato seedlings with plant leaf extracts obtained by method 1, after MMS treatment in tomato seedlings influenced tail moment values ranged from 0.00 μm to 214.49 μm. Tomato seedlings treated with *Cissampelos pareira* extract prepared by method 2, after MMS treatment showed lower tail moment of 0.00 μm, followed by treatment with *Thespesia populneus, Azima tetracantha, Pongamia pinnata and Myxopyrum serratulum* extracts obtained by method 1, after MMS treatment. The highest tail moment of 214.49 μm recorded in tomato seedlings treated with distilled water, after MMS treatment (control). Tomato seedlings post treated with plant leaf extract prepared by method 1, after MMS treatment recorded lowest tail moment value compared to control (Table 11).

**4.4.4 Post treatment of tomato seedlings with plant leaf extracts obtained by method 2, after MMS treatment on DNA damage repair**

The study on post treatment of plant leaf extracts obtained by method 2, after MMS treatment, in tomato seedlings influenced tail moment values. The values ranged from 0.00 μm to 214.49 μm (Table 12). The treatment of *Azima tetracantha* extract prepared by method 2, in tomato seedlings, after MMS treatment showed lower tail moment (0.00 μm), followed by the values of treatment with *Thespesia populneus*, *Pongamia pinnata*, *Myxopyrum serratulum* and *Cissampelos pareira* extracts prepared by method 2, after MMS treatment in tomato seedlings. The treatment of tomato seedlings with distilled water, after MMS treatment (control) was recorded the highest tail moment of 214.49 μm.

**Table 11. Effect of post-treatment of tomato seedlings with plant leaf extracts prepared by method 1 after MMS treatment on DNA damage repair**

| Plant leaf extracts | Tail moment (µm) mean | Tail intensity (%) mean | Tail length (µm) mean |
|---|---|---|---|
| *Azima tetracantha* | 98.01 | 50.93 | 249.15 |
| *Cissampelos pareira* | 0.00 | 0.00 | 50.27 |
| *Myxopyrum serratulum* | 150.64 | 53.98 | 359.11 |
| *Pongamia pinnata* | 98.10 | 46.58 | 242.51 |
| *Thespesia populneus* | 15.08 | 17.49 | 62.19 |
| Distilled water (Control) | 214.49 | 78.84 | 490.88 |

**Table 12. Effect of post-treatment of tomato seedlings with plant leaf extracts prepared by method 2 on MMS induced DNA damage**

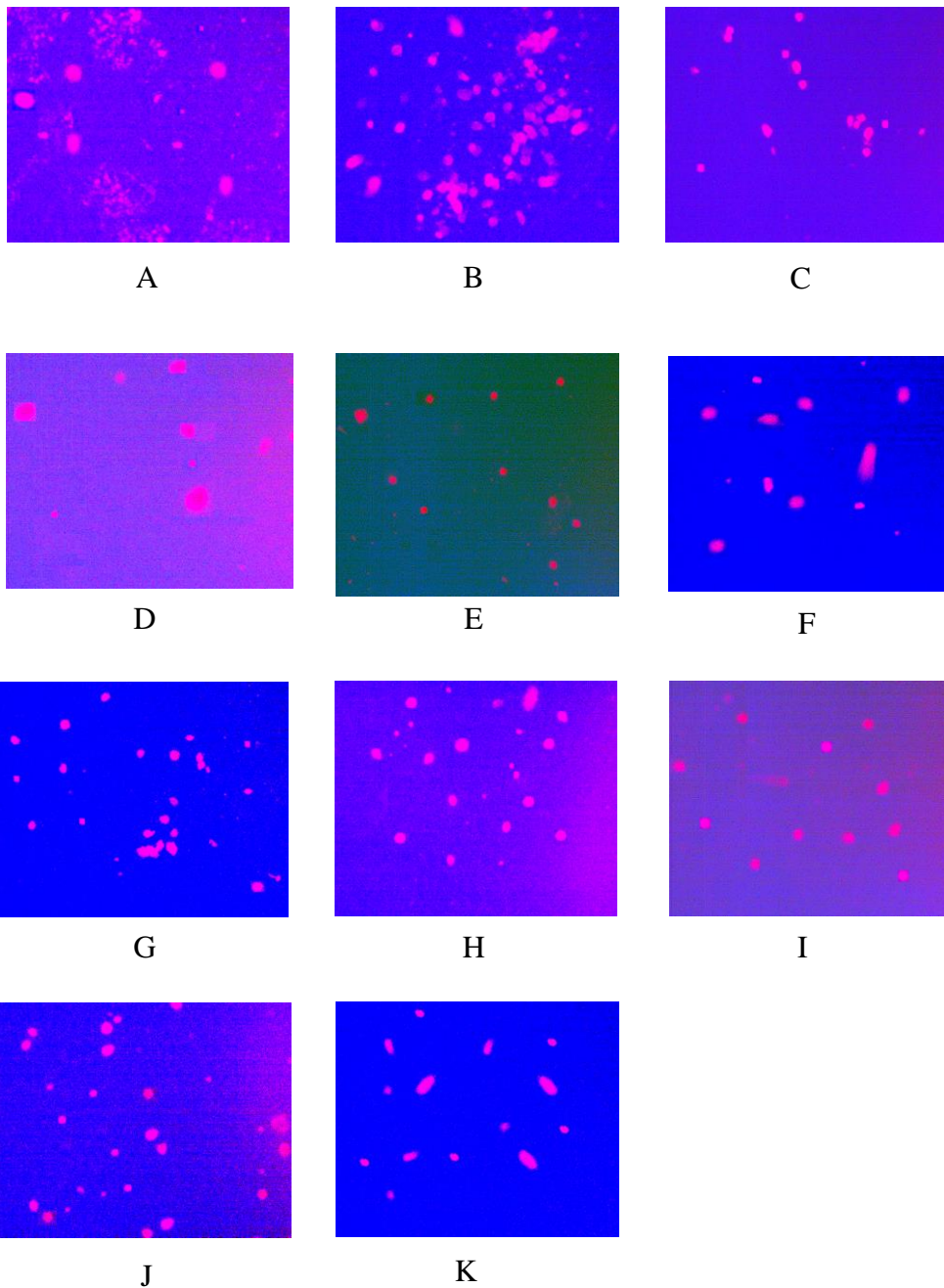| Plant leaf extracts | Tail moment (µm) mean | Tail intensity (%) mean | Tail length (µm) mean |
|---|---|---|---|
| *Azima tetracantha* | 0.00 | 0.00 | 81.87 |
| *Cissampelos pareira* | 0.04 | 0.74 | 53.65 |
| *Myxopyrum serratulum* | 0.00 | 0.31 | 74.46 |
| *Pongamia pinnata* | 0.03 | 5.79 | 46.64 |
| *Thespesia populneus* | 0.03 | 6.11 | 48.03 |
| Distilled water (Control) | 214.49 | 78.84 | 490.88 |

**Plate 7:** Comet images of nuclei taken from tomato leaves treated with plant leaf extract, A) *Azima tetracantha*, B) *Cissampelos pareira*, C) *Pongamia pinnata,* D) *Myxopyrum serratulum,* E) *Thespesia populneus* (of method 1), F) *Azima tetracantha,* G) *Cissampelos pareira, H) Pongamia pinnata*, I) *Myxopyrum serratulum,* J) *Thespesia populneus* (of method 2), K) Control.

Tomato seedlings treated with plant leaf extract prepared by method 2, after MMS treatment recorded lowest tail moment value compared to control.

## 4.5 CO-SUPPLEMENTATION TREATMENT

### 4.5.1 Simultaneous treatment of plant leaf extract prepared by method 1 and EMS in tomato seedlings on DNA damage protection

The study on simultaneous treatment of plant leaf extracts obtained by method 1 and EMS in tomato seedlings influenced tail moment values. The values ranged from 0.00 μm to 196.42 μm. Treatment with *Myxopyrum serratulum* extract prepared by method 1 absolutely inhibited the EMS from inducing DNA damage in tomato seedlings. Inhibition was also shown by *Pongamia pinnata*, *Azima tetracantha*, *Cissampelos pareira* and *Thespesia populneus* extracts prepared by method 1. The highest tail moment value of 196.42 μm was recorded in tomato seedlings treated in distilled water (control). Tomato seedlings simultaneously treated with plant leaf extracts obtained by method 1 and EMS recorded significantly lowest tail moment value compared to control (Table 13).

### 4.5.2 Simultaneous treatment of plant leaf extract prepared by method 2 and EMS on DNA damage protection

The study on simultaneous treatment of plant leaf extracts prepared by method 2 and EMS in tomato seedlings influenced tail moment values. The values ranged from 0.00 μm to 196.42 μm. Treatment with *Myxopyrum serratulum* extract prepared by method 2 absolutely inhibited the EMS from inducing DNA damage in tomato seedlings. Significant inhibition was also shown by *Pongamia pinnata, Cissampelos pareira*, *Thespesia populneus* and *Azima tetracantha* extracts prepared by method 1. The highest tail moment value (196.42 μm) was recorded in tomato seedlings treated in distilled water (control). Tomato seedlings simultaneously treated with plant leaf extracts obtained by method 2 and EMS recorded lower tail moment values compared to control (Table 14).

**Table 13. Effect of simultaneous treatment of tomato seedlings with plant leaf extracts prepared by method 1 and EMS on DNA damage protection**

| Plant leaf extracts | Tail moment (µm) mean | Tail intensity (%) mean | Tail length (µm) mean |
|---|---|---|---|
| *Azima tetracantha* | 3.30 | 23.70 | 27.70 |
| *Cissampelos pareira* | 3.43 | 30.43 | 17.16 |
| *Myxopyrum serratulum* | 0.00 | 0.00 | 74.06 |
| *Pongamia pinnata* | 1.42 | 10.85 | 31.17 |
| *Thespesia populneus* | 58.40 | 21.61 | 184.19 |
| Distilled water (Control) | 196.42 | 78.09 | 464.36 |

**Table 14. Effect of simultaneous treatment of tomato seedlings with plant leaf extracts prepared by method 2 and EMS on DNA damage protection**

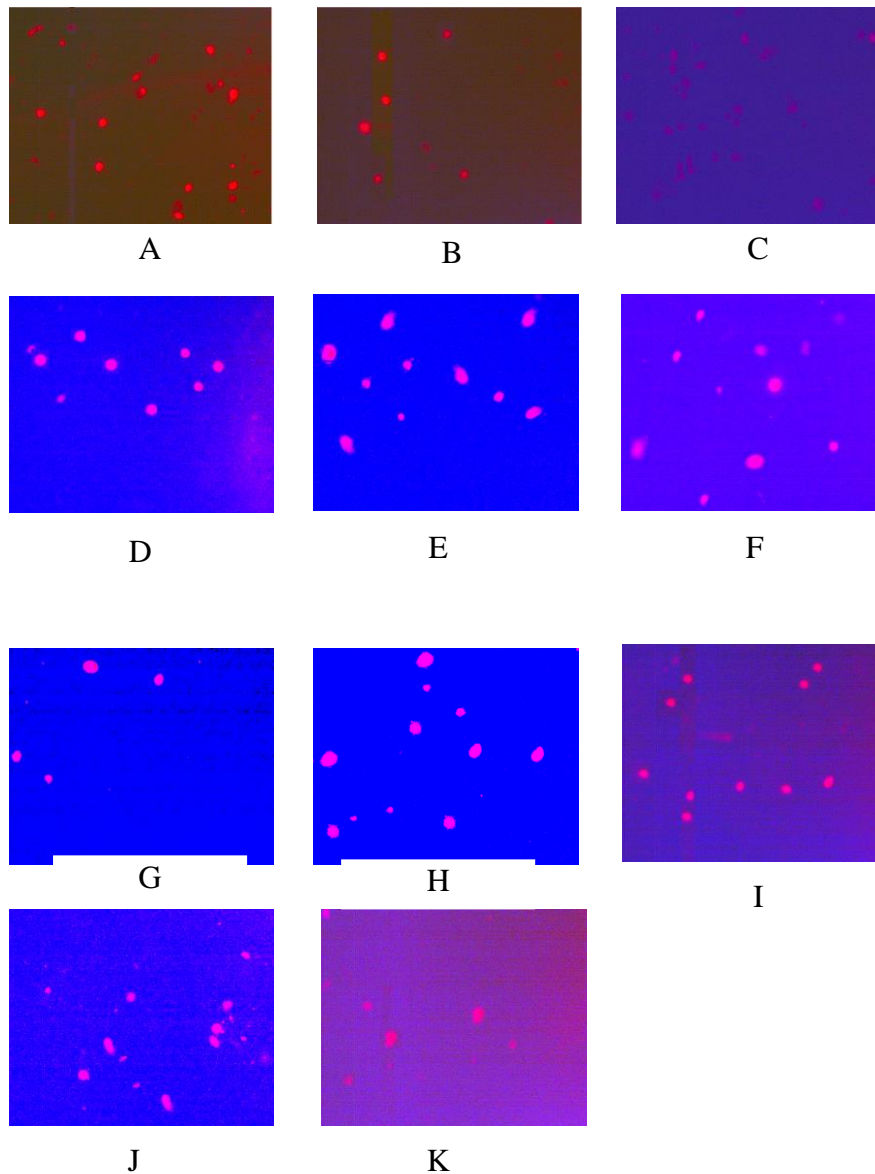| Plant leaf extracts | Tail moment (µm) mean | Tail intensity (%) mean | Tail length (µm) mean |
|---|---|---|---|
| *Azima tetracantha* | 114.98 | 46.58 | 267.82 |
| *Cissampelos pareira* | 69.76 | 35.05 | 185.62 |
| *Myxopyrum serratulum* | 0.00 | 0.23 | 78.13 |
| *Pongamia pinnata* | 51.64 | 34.36 | 145.69 |
| *Thespesia populneus* | 81.90 | 38.06 | 236.74 |
| Distilled water (Control) | 196.42 | 78.09 | 464.36 |

**Plate 8:** Comet images of nuclei taken from tomato leaves treated with plant leaf extract (method 1 and method 2) and ethyl methanesulphonate (EMS) simultaneously. A) *Azima tetracantha* B) *Cissampelos pareira* C) *Pongamia pinnata* D) *Myxopyrum serratulum* E) *Thespesia populneus* (of method 1) F) *Azima tetracantha* G) *Cissampelos pareira H) Pongamia pinnata* I) *Myxopyrum serratulum* J) *Thespesia populneus* (of method 2), K) Control.

**Table 15. Effect of simultaneous treatment of tomato seedlings with plant leaf extracts prepared by method 1 and MMS on DNA damage protection**

| Plant leaf extracts | Tail moment (μm) mean | Tail intensity (%) mean | Tail length (μm) mean |
|---|---|---|---|
| *Azima tetracantha* | 20.12 | 9.62 | 78.55 |
| *Cissampelos pareira* | 0.00 | 0.00 | 51.12 |
| *Myxopyrum serratulum* | 0.11 | 1.27 | 49.98 |
| *Pongamia pinnata* | 0.00 | 0.00 | 56.53 |
| *Thespesia populneus* | 49.42 | 19.40 | 145.58 |
| Distilled water (Control) | 269.01 | 96.54 | 575.16 |

**Table 16. Effect of simultaneous treatment of tomato seedlings with plant leaf extracts prepared by method 2 and MMS on DNA damage protection**

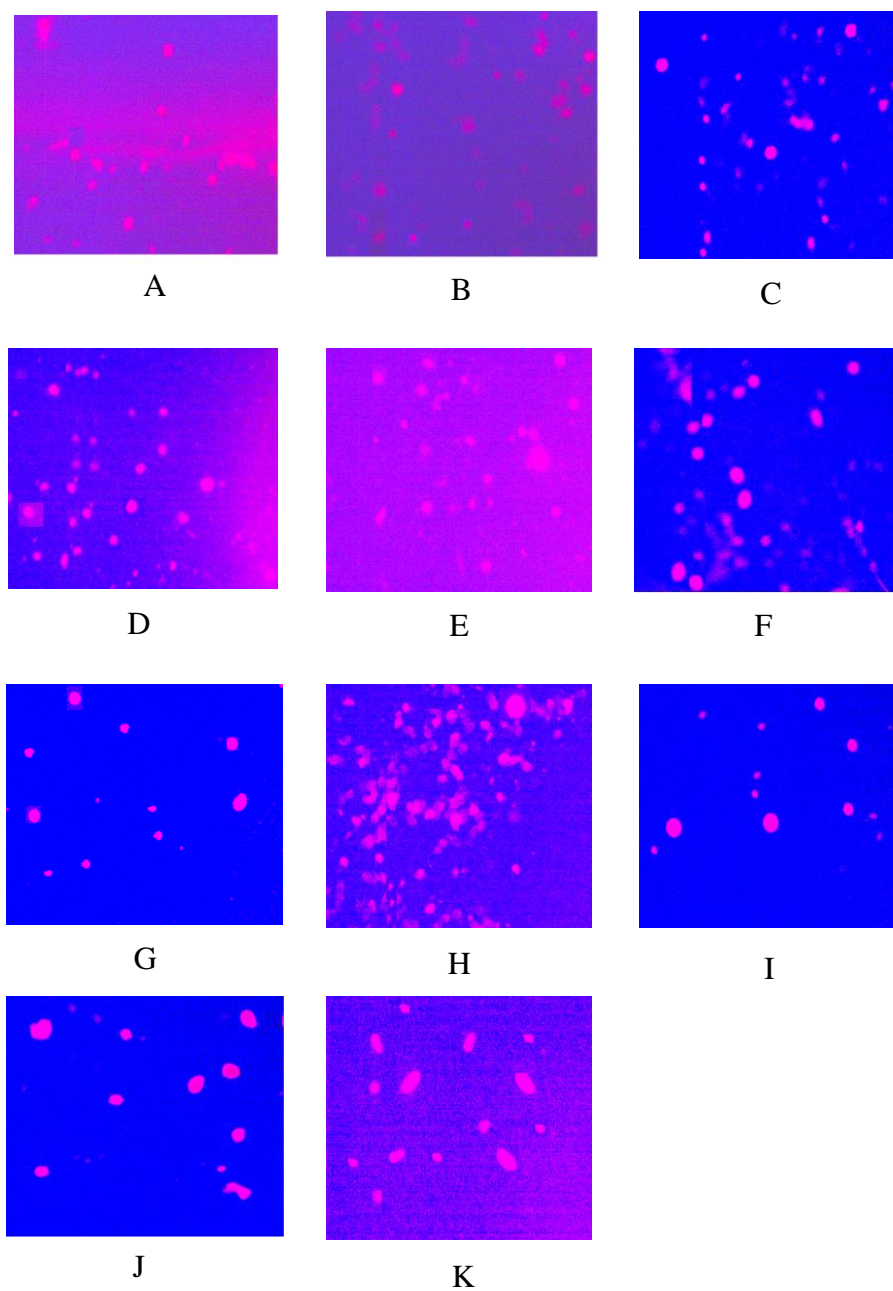| Plant leaf extracts | Tail moment (μm) mean | Tail intensity (%) mean | Tail length (μm) mean |
|---|---|---|---|
| *Azima tetracantha* | 81.90 | 38.06 | 233.53 |
| *Cissampelos pareira* | 60.21 | 30.18 | 176.02 |
| *Myxopyrum serratulum* | 0.38 | 4.97 | 74.97 |
| *Pongamia pinnata* | 135.91 | 50.55 | 355.97 |
| *Thespesia populneus* | 87.96 | 41.69 | 235.28 |
| Distilled water (Control) | 269.01 | 96.54 | 575.16 |

**Plate 9:** Comet images of nuclei taken from tomato leaves treated with plant leaf extract (method 1 and method 2) and MMS simultaneously. A) *Azima tetracantha,* B) *Cissampelos pareira,* C) *Pongamia pinnata,* D) *Myxopyrum serratulum,* E) *Thespesia populneus* (of method 1), F) *Azima tetracantha*, G) *Cissampelos pareira,* H) *Pongamia pinnata,* I) *Myxopyrum serratulum,,* J) *Thespesia populneus* (of method 2) K) Control

### 4.5.3 Simultaneous treatment of plant leaf extract prepared by method 1 and MMS on DNA damage protection

The study on simultaneous treatment of plant leaf extracts obtained by method 1 and MMS in tomato seedling influenced tail moment values. The values ranged from 0.00 μm to 269.01 μm. Treatments with *Pongamia pinnata* and *Cissampelos pareira* extracts prepared by method 1 absolutely inhibited the EMS from inducing DNA damage in tomato seedlings. Significant inhibition was also shown by *Myxopyrum serratulum*, *Azima tetracantha* and *Thespesia populneus* extracts prepared by method 1. The significantly highest tail moment value (269.01 μm) was recorded in tomato seedlings treated with distilled water (control). Simultaneous treatment of plant leaf extracts prepared by method 1 and MMS in tomato seedlings recorded lowest tail moment values compared to control (Table 15).

### 4.5.4 Simultaneous treatment of plant leaf extract obtained by method 2 and MMS on DNA damage protection

The study on simultaneous treatment of plant leaf extracts obtained by method 2 and MMS in tomato seedling influenced tail moment values. The values ranged from 0.38 μm to 269.01 μm (Table 16). Treatments with *Myxopyrum serratulum* extracts prepared by method 1 absolutely inhibited the EMS from inducing DNA damage in tomato seedlings. Significant inhibition was also shown by *Cissampelos pareira, Azima tetracantha* and *Thespesia populneus* extract prepared by method 1 and MMS in tomato seedlings. The highest tail moment value (269.01 μm) was recorded in tomato seedlings treated with distilled water (control). Simultaneous treatment of plant leaf extract obtained by method 2 and MMS in tomato seedlings recorded lowest tail moment values compared to control.

# Discussion

## 5. DISCUSSION

Genomic integrity is under constant threat in all species. These threats come in many forms. There are agents that damage DNA, spontaneous chemical changes and errors in DNA metabolism, leading to a variety of alterations in the normal DNA structure, resulting in single and double strand breaks, chemically modified bases, abasic sites, bulky adducts, inter and intra strand cross links and base pairing mismatches. Failure to repair such damages may lead to many direct and indirect effects on cells and organism viz., mutations, genetic recombination, the inhibition or alteration of cellular processes, chromosomal aberration, tumorigenesis and cell death.

There are reports showing that the extracts / tissues of several plants are effective in protecting the DNA from damages of mutagens (Pool-Zobel et al., 1997). The diet supplemented with tomato, carrot or spinach products resulted in a significant decrease in endogenous DNA damage. The consumption of polyphenol rich fruit juices enhanced antioxidant status, reduced oxidative DNA damage and stimulated immune cell functions (Bub et al., 2003). The diets high in fruits and vegetables are associated with reduced cancer risk and other degenerative disorders related to DNA damage (Ganguly, 2001). Phytochemicals contained in vegetables and fruits including flavonoids and other types of polyphenolic compounds, have been demonstrated to support the DNA repair process (He et al., 2006 and Pomerleau et al., 2006).

An attempt was made to validate the usefulness and involvement of a few plant species, the leaves of which have been included in traditional food supplements, in Kerala, in protecting DNA and assisting in the repair process. The plants selected for the study were *Azima tetracantha* (Ashenku), *Cissampelos pareira linn* (Malatangi), *Myxopyrum serratulum* (Chaturamulla), *Pongamia pinnata* (Ungu) and *Thespesia populneus* (Poovarasu). These plants species are traditionally being used as supplements in food preparations like Karkidaka Kanji

(marunnu kootu) in Kerala, assuming that their consumption increases immunity and general health. The main objective of the study was to test the effect of the green leaf extracts of these plants on the protection and repair of DNA, employing comet assay (single cell gel electrophoresis technique).

The comet assay - SCGE (Single Cell Gel Electrophoresis) has become very popular for measuring the level of DNA damage and the effectiveness of repair processes (Collins, 2004). Its sensitivity and simplicity make it an invaluable tool with widespread application in studies to assess DNA damage and repair in genotoxicity testing, ecological monitoring, as well as human studies. Increased DNA migration results from the induction of DNA single-strand breaks, alkali labile sites, and incomplete excision repair sites at the time of lysis. Tail length, percentage of total DNA in the tail, and the tail moment all reflect DNA damage. The tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed/broken pieces (represented by the intensity of DNA in the tail).

Ethyl methanesulfonate (EMS) was used in the present study, to induce DNA damage in tomato seedlings at different concentrations (0 mM to 4 mM, overnight treatment). It induced a dose dependent increase in DNA damage in leaf nuclei (Fig. 1). Gichner (2003) reported the DNA damaging effect of EMS applied for two hours on roots of tobacco. The damage increased with increasing concentration of EMS. Ethyl methanesulfonate is a mutagenic, teratogenic, and possibly carcinogenic organic compound. It produces random mutations in genetic material by nucleotide substitution; specifically by guanine alkylation.

Methyl methanesulfonate (MMS) was used in the present study to induce DNA damage in tomato seedlings at different concentrations (0 mM to 4 mM, with 12 hour treatment). It was observed that there was significantly higher tail moment value than control and there was no dose dependent response (Fig. 2). Mouchet et al. (2005) observed a significant increase in DNA damage in larvae
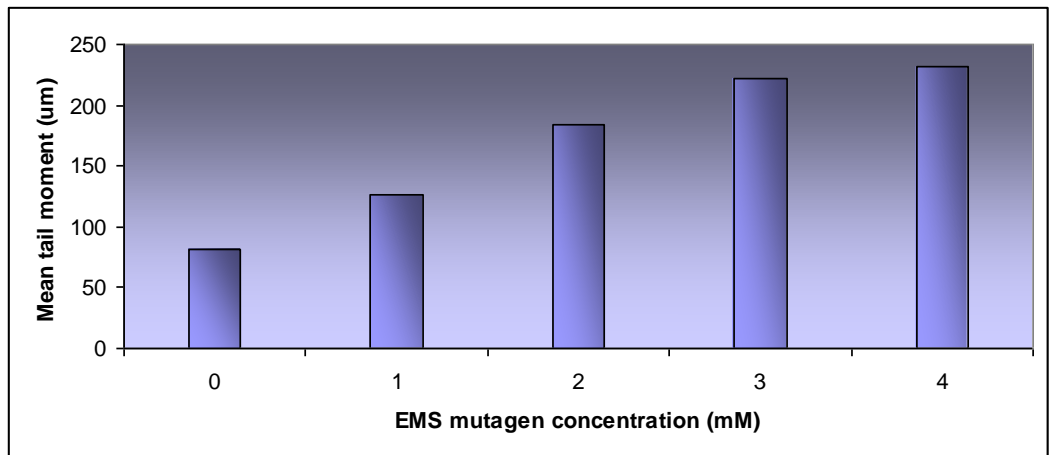
**Fig. 1. Comet assay of nuclei taken from tomato leaf exposed to EMS at varying concentrations.**
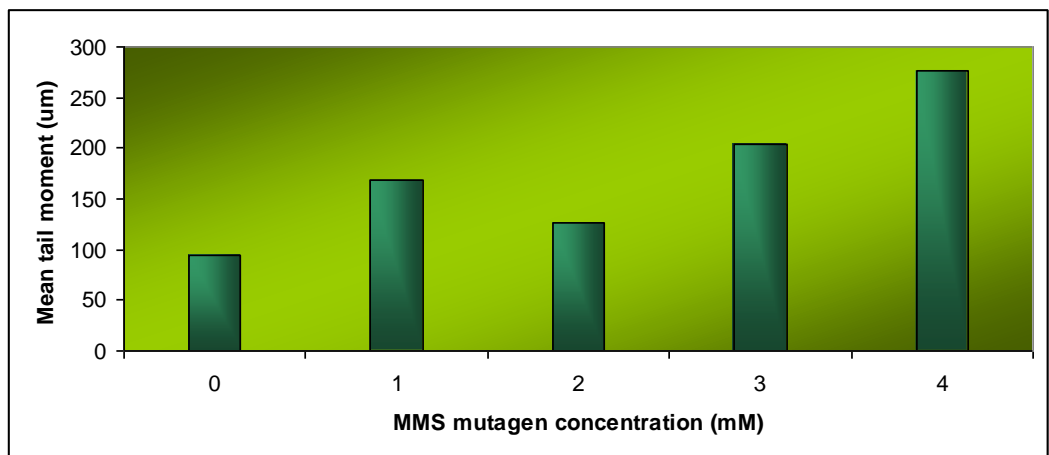


**Fig. 2. Comet assay of nuclei taken from tomato leaf exposed to MMS at varying concentrations.**

exposed to MMS at the different doses compared to the control and there was no dose response relationship. Methyl methanesulfonate is an alkylating agent and a carcinogen. It methylates DNA on N7-deoxyguanine and N3-deoxyadenine. Originally, this action was believed to directly cause double-stranded DNA breaks, because homologous recombination-deficient cells are particularly vulnerable to the effects of MMS (Lundin, 2005). However, it is now believed that MMS stalls replication forks, and cells that are homologous recombination-deficient have difficulty repairing the damaged replication forks.

Two methods of extraction were tried for all the five test plants. In method 1, 1g plant sample was put in 50 ml distilled water, kept overnight in shaker at 400rpm and filtered using Whatman filter paper. Filtrates were evaporated by keeping on water bath till the volume was reduced to 10 ml. It was used for the DNA repair assay. In method 2, 1g plant sample was put in 50ml distilled water, boiled for 30 minutes and filtered using blotting paper. Filtrates were boiled by keeping in water bath till the volume was reduced to 10 ml, and it was used for the DNA repair assay. Genotoxicity of the extracts of each test plants was studied in tomato seedlings, prior to use in DNA repair assay. Both the extracts showed significantly lower tail moment values than control. Hence it was concluded that they did not cause any DNA damage in tomato seedlings, and were effective to be used in DNA repair assay.

In pre-supplementation experiment, the tomato seedlings were incubated in test plant leaf extract for 12h, before treating with mutagens overnight. Immediately after treatment, the leaf nuclei were isolated from tomato seedlings. Three comet slides per treatment were prepared, images were taken through fluorescence microscope and the tail moment values were recorded, using the imaging software of Perceptive instruments comet VI. All the test plant leaf extracts, prepared by two methods of extraction procedure, showed protection against both the EMS (4 mM) and MMS (4 mM) mutagens. Belloir et al. (2006) studied the antigenotoxic activity of several garlic organosulfur compounds
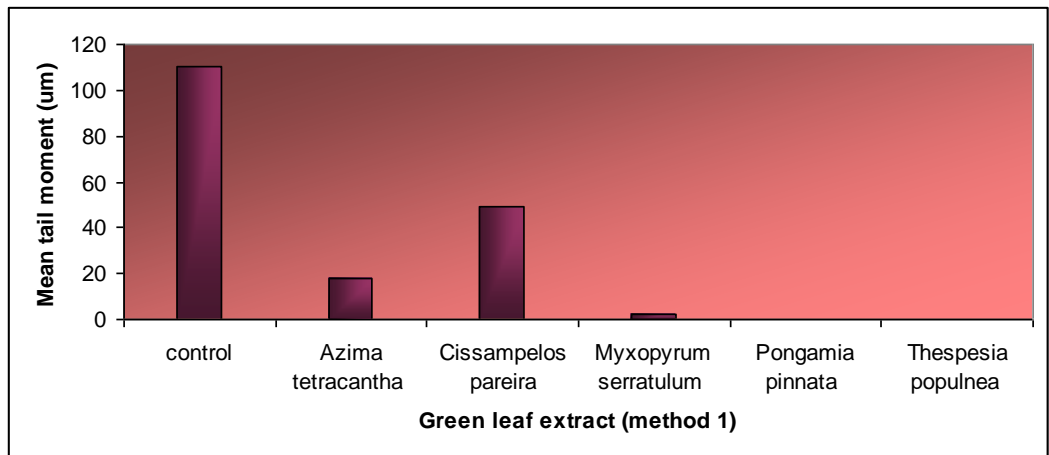
**Fig. 3. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extract prepared by method 1.**
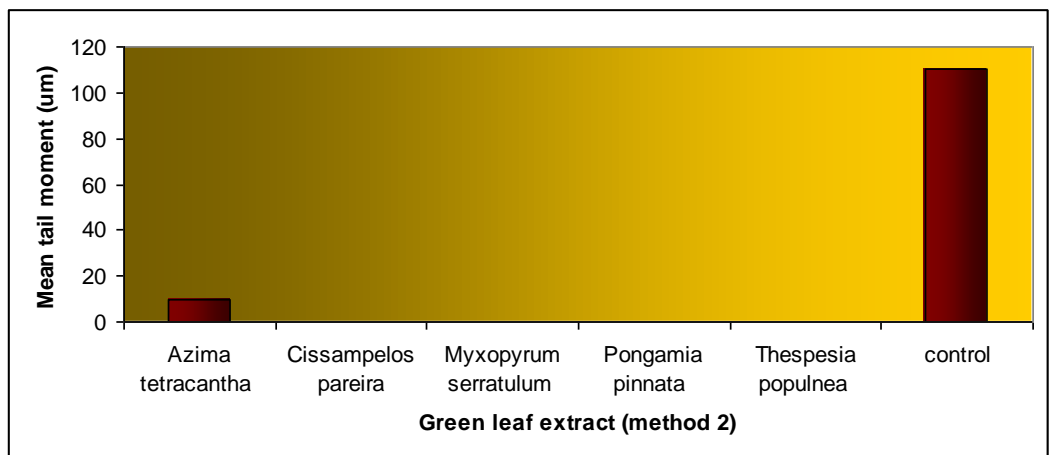


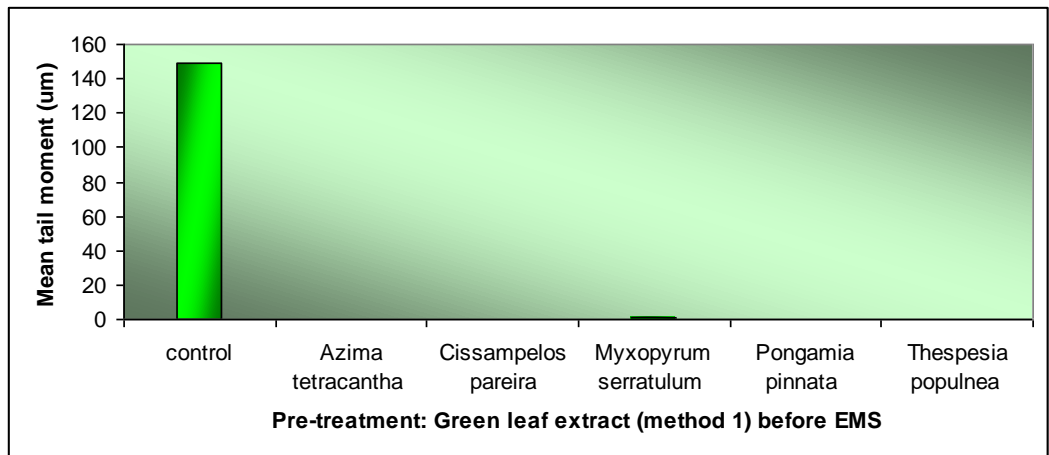**Fig. 4. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extract prepared by method 2.**

**Fig. 5. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extracts (method 1), prior to EMS treatment.**
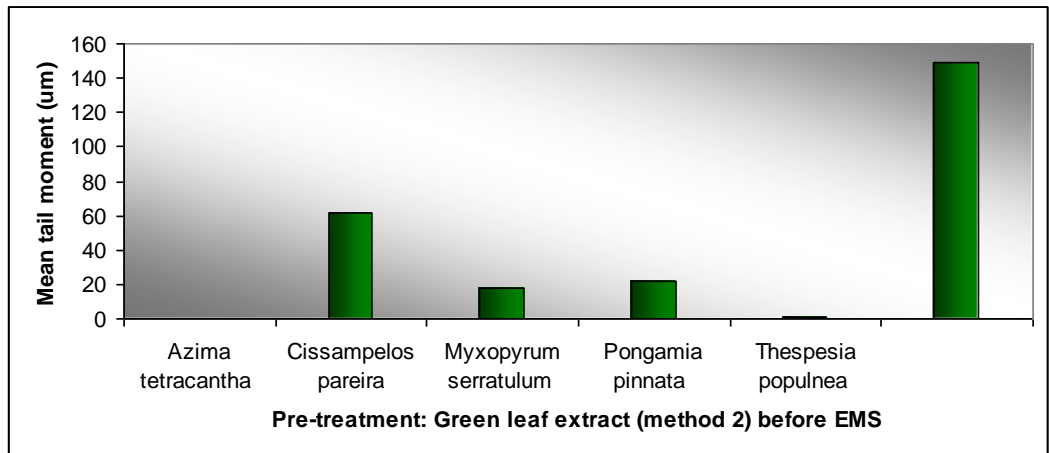


**Fig. 6. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extracts (method 2), prior to EMS treatment.**
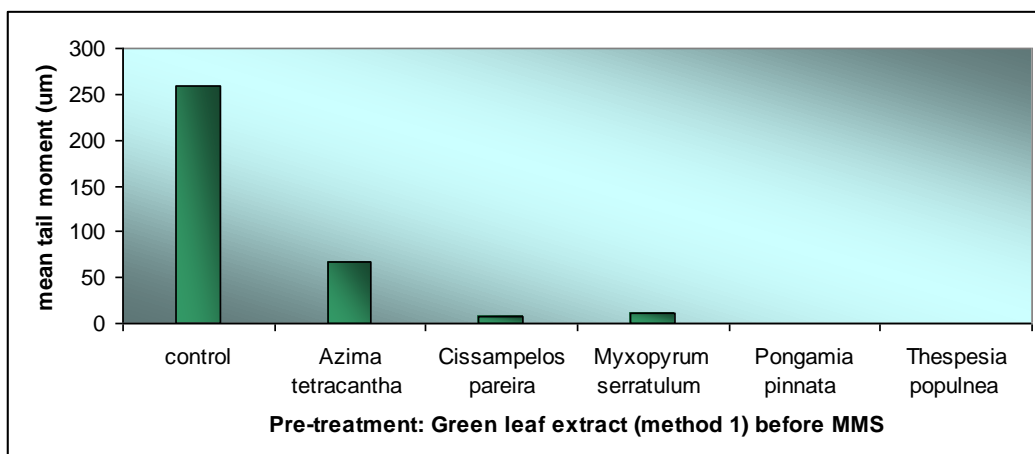
**Fig. 7. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extracts (method 1), prior to MMS treatment.**
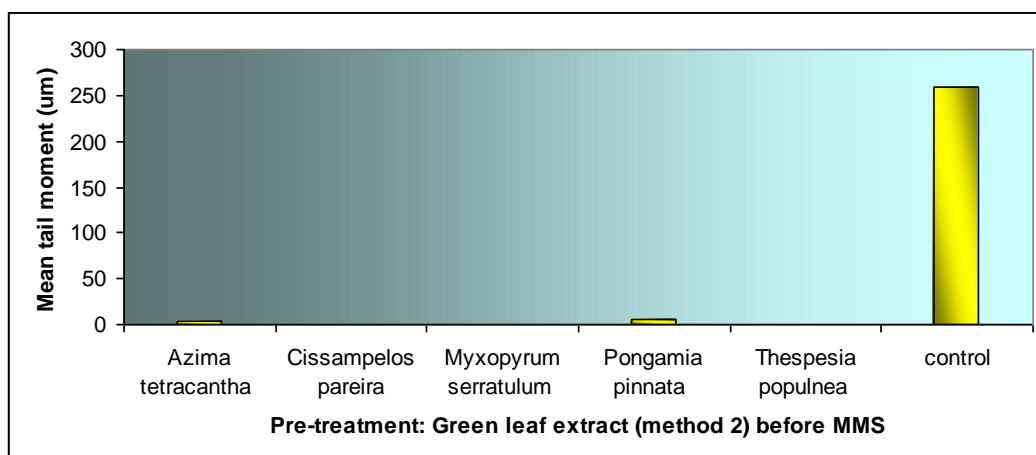


**Fig. 8. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extracts (method 2), prior to MMS treatment.**

(OSC) in human cell line, using comet assay. The aflatoxin B1 genotoxicity was significantly reduced by all the OSC tested. S-allyl cysteine and allyl mercaptan significantly decreased DNA breaks in cells treated with dimethylnitrosamine.

In co-supplementation studies tomato seedlings were simultaneously treated with test plant leaf extract and the mutagens for 12h. Immediately after treatment, the leaf nuclei were isolated from tomato seedlings, three comet slides per treatment were prepared, its images were taken through fluorescence microscope and the tail moment values were recorded using the imaging software of Perceptive instruments comet VI. The studies showed that all the test plant leaf extracts prepared by methods 1 and method 2 extraction procedures showed lower the tail moment value induced by the mutagens, compared to the control. These findings suggest that test plant leaf extract can prevent genotoxicity of EMS (4 mM) and MMS (4 mM) mutagens. This effect could be due to the antioxidative activity of phytochemicals of the test plant extract to scavenge reactive compounds produced by monofunctional alkylating mutagens. Additionally, the phytochemicals may enhance antioxidant enzyme activity. Bub et al. (2003) studied the effects of polyphenol-rich juices on bioavailability of polyphenols and reduction of DNA damage. Juice intervention had no effect on single DNA strand breaks, but significantly reduced oxidative DNA damage. They concluded that consumption of juice reduced oxidative DNA damage and stimulated immune cell functions. Belloir et al (2006) demonstrated that garlic organosulfur compounds displayed antigenotoxic activity in human metabolically competent cells.

In post-supplementation studies tomato seedlings were incubated in mutagens viz., EMS and MMS at 4mM concentration each for 12h prior to incubation in test plant leaf extracts, prepared by following method 1 and method 2 extraction protocols tried over night. Immediately after the treatment, leaf nuclei were isolated from tomato seedlings, three comet slides per treatment were prepared and images were taken through fluorescence microscope. Tail moment values were recorded using the imaging software of Perceptive instruments comet
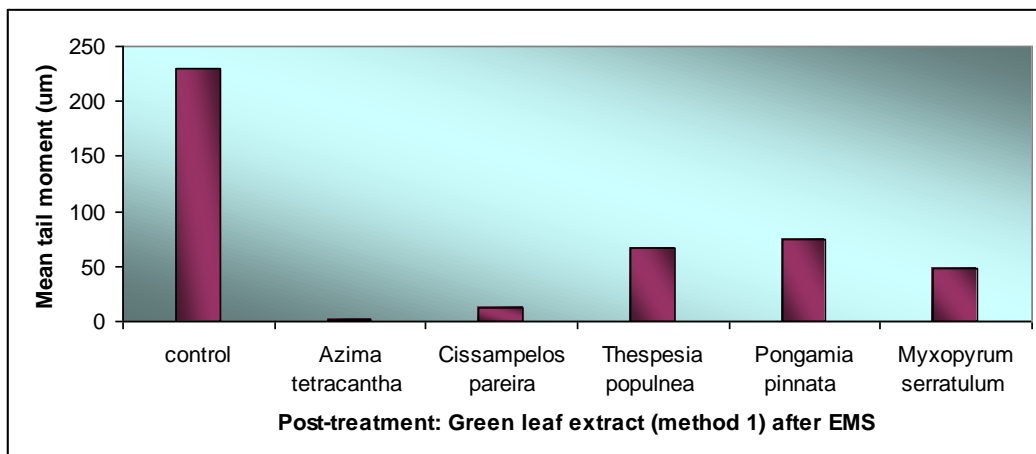
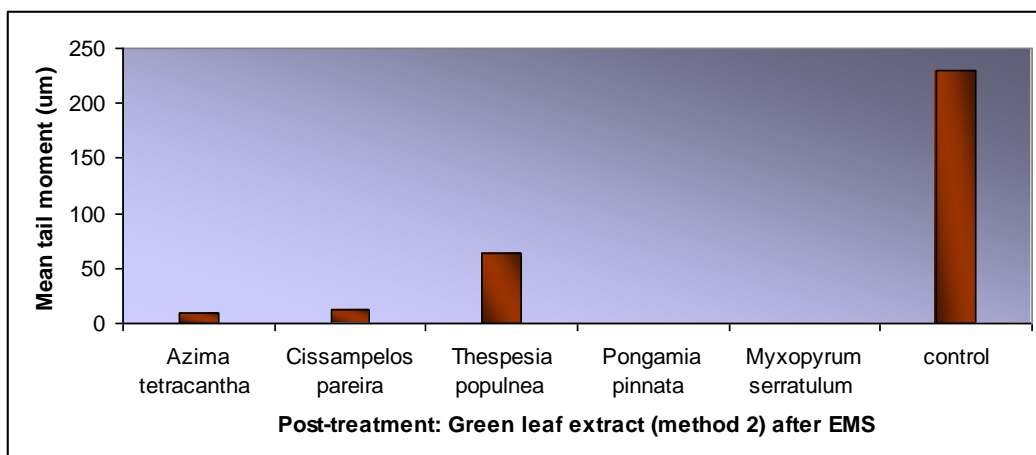**Fig. 9. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extracts (method 1), after EMS treatment.**



**Fig. 10. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extracts (method 2), after EMS treatment.**
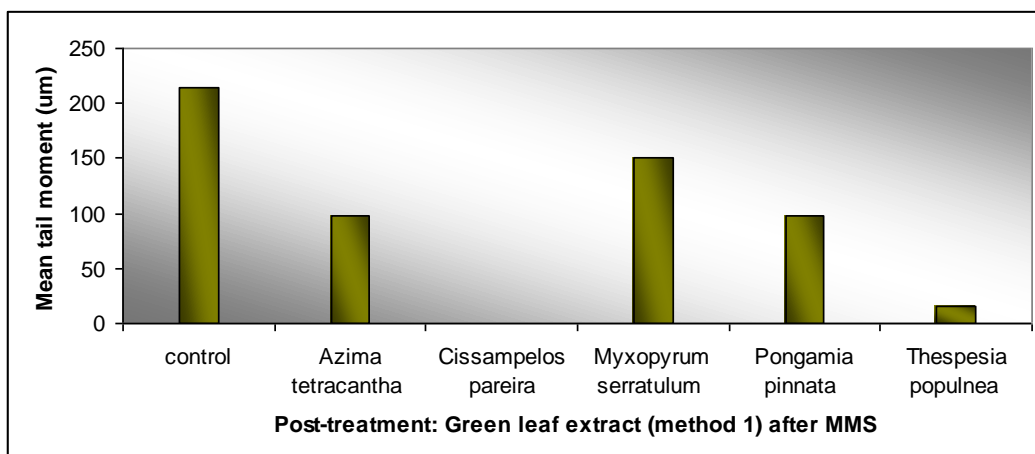
**Fig. 11. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extracts (method 1), after MMS treatment.**
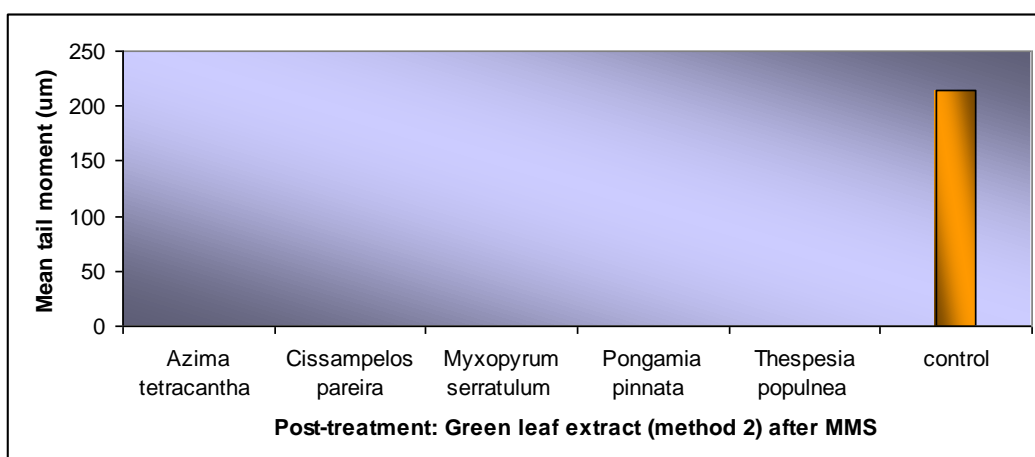


**Fig. 12. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extracts (method 2), after MMS treatment.**

**Fig. 13. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extracts (method 1) and EMS simultaneously.**
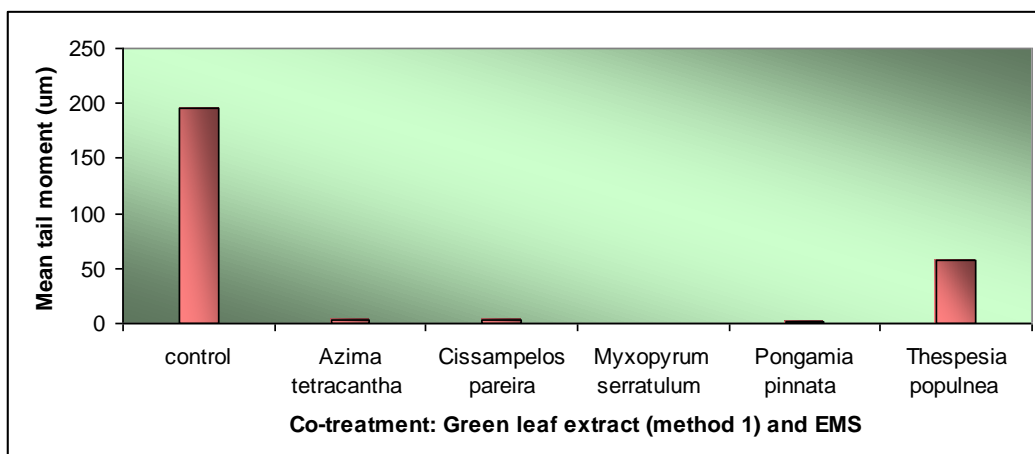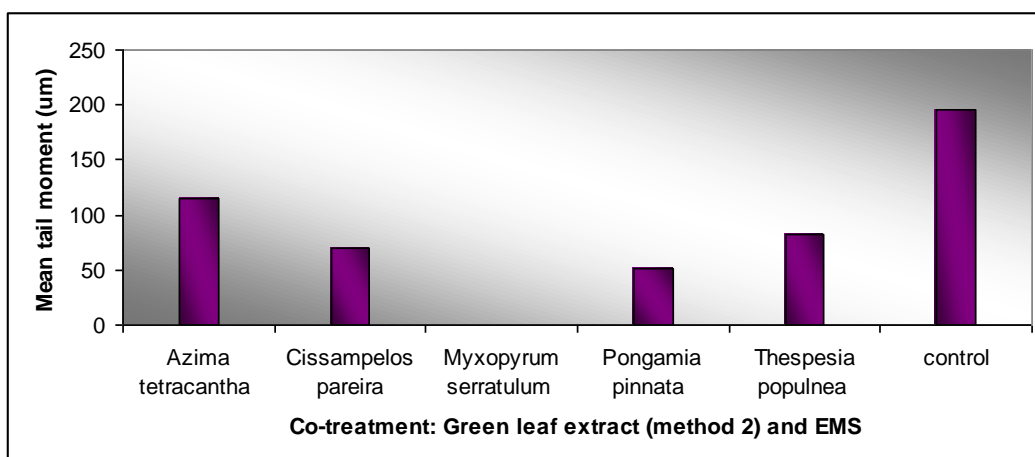


**Fig. 14. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extracts (method 2) and EMS simultaneously.**
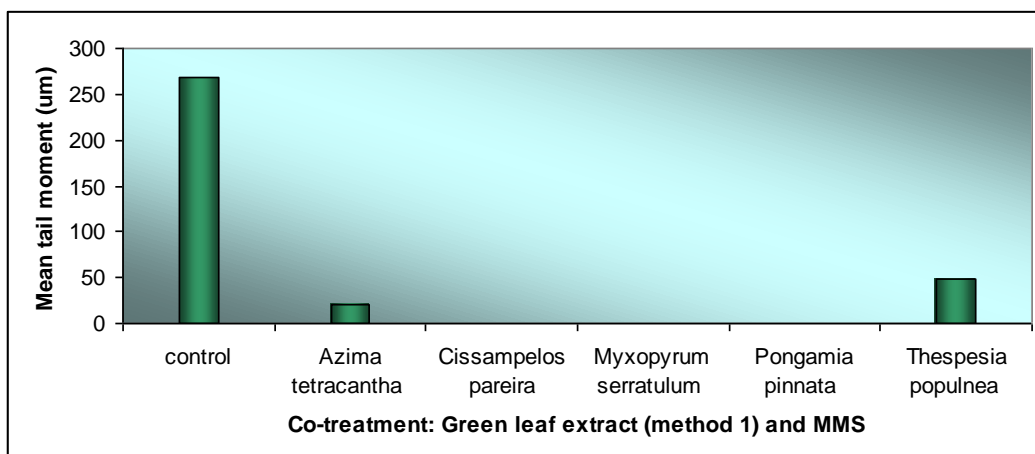
**Fig. 15. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extracts (method 1) and MMS simultaneously.**
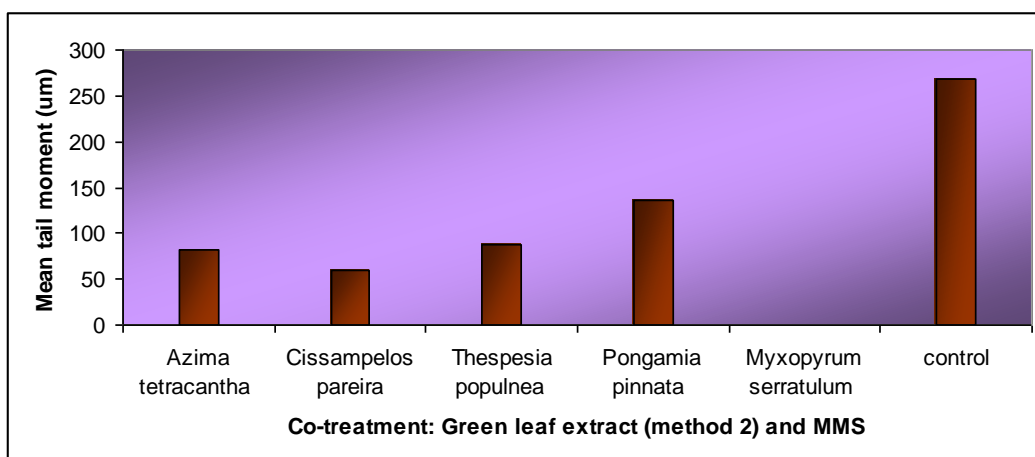


**Fig. 16. Comet assay of nuclei taken from tomato leaf exposed to plant leaf extracts (method 2) and MMS simultaneously.**

VI. The study showed that the leaf extract treatments significantly repaired the DNA damage caused by mutagens. This was true in all the test plant leaf extract treatments, compared to control (EMSdH$_2$O and MMSdH$_2$O). The possible mechanism for reduction of DNA damage by test plant leaf extract may be by stimulation of DNA repair i.e. by up-regulating the expression of genes involved in the repair of DNA damage. Knowles and Milner (2003) observed that garlic organosulfur compounds up-regulated the expression of genes involved in DNA damage repair in human colon tumor cells. Astley et al. (2003) reported that carotenoids and carotenoid rich foods could influence DNA damage: repair by modulation of discrete stages in the DNA repair mechanisms.

Till date no report has been published on the effect of green leaves of ashenku, malatangi, poovarasu, ungu and chaturamulla which are used in traditional food preparations, on DNA repair. This study demonstrated that the test plants are effective in protecting the DNA, in plant cells. Further studies are necessary to test the efficacy of these extracts on the DNA repair process in animal cells. Positive effects of the extracts of these plants on DNA repair in animal cells would be useful in recommending their regular use as food supplements, as a prophylactic measure against the incidence of cancer.

*Summary*

# 6. SUMMARY

A study on "Effect of green leaves used in traditional food preparation on DNA repair" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thirvananthapuram during 2007-2009. The objective of the study was to test the effect of plant leaf extracts of *Azima tetracantha*, *Cissampelos pareira, Myxopyrum serratulum*, *Pongamia pinnata* and *Thespesia populnea,* used in traditional food preparation on DNA repair, employing the Comet Assay. The salient findings of the study are summarized below.

Tomato seedlings treated with two mutagens (Ethyl methanesulfonate and Methyl methanesulfonate) at different concentrations (0 mM-4 mM) overnight under room temperature and dim light, recorded higher tail moment value compared to control. Ethyl ethanesulfonate (EMS) treatment induced a dose dependent increase in DNA damage in leaf nuclei. Methyl methanesulfonate showed higher tail moment value than control but there was no dose dependent response.

All the plant leaf extracts prepared by following method 1 and method 2 extraction protocols showed no genotoxicity effect on treated tomato seedlings. These treatments recorded lower tail moment value, compared to treated in distilled water (control).

Tomato seedlings pre-treated with plant leaf extract prepared by method 1 and method 2 and subjected to mutagens at 4 mM concentration recorded lower tail moment value than the tomato seedlings pre-treated with distilled water and later subjected to mutagens (control).

In the co-supplementation treatment, the tomato seedlings simultaneously treated with plant leaf extract prepared by method 1 and method 2 extraction

procedures and mutagens at 4 mM concentration, recorded lower tail moment value than co-treated with distilled water and mutagens (control).

In post-supplementation treatment, tomato seedlings treated with plant leaf extract obtained by following method 1 and method 2 extractions protocols, after mutagens treatment at 4 mM concentration, recorded lower DNA damage than the tomato seedlings treated with distilled water after mutagen treatment (control).

This study demonstrates that the leaf extracts of test plants are effective in protecting the DNA from damages of mutagens. This may be by phytochemicals and biologically active components (non nutrient) of the test plant leaf extracts. These results can be of use in testing the leaf extracts of test plants in animal system, which in turn can be of use as a prophylactic measure against cancer and other disorders.

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# Appendices

**APPENDIX I**

**Preparation of 0.4 M Tris buffer**

Dissolve 9.7 g Tris (SRL, Mumbai) in 180 ml $dH_2O$, set pH to 7.5 with concentrated hydrochloric acid, add $dH_2O$ to 200ml, store at room temperature

## APPENDIX II

**Preparation of Phosphate buffered saline (PBS)**

Dissolve 50 mg KCl, 50 mg $KH_2PO_4$, 2 g NaCl, 720 mg $Na_2HPO_4.12 H_2O$ in 250 ml $dH_2O$, set to pH 7.4, filter sterilize, store in a refrigerator.

## APPENDIX III

**Preparation of Normal point melting agarose (NPMA)**

Dissolve 1 g NPMA (Himedia Laboratories, India) in 100 ml $dH_2O$, microwave or heat until near boiling.

**APPENDIX IV**

**Preparation of Low point melting agarose (LPMA)**

➢ 1 % LPMA: dissolve 250 mg LPMA (Himedia Laboratories, India) in 25 ml PBS, microwave or heat until near boiling. Prepare 2 to 5 ml aliquots in small vials and store in the refrigerator.

➢ 0.5 % LPMA: dissolve 125 mg LPMA (Himedia Laboratories, India) in 25 ml PBS, microwave or heat until near boiling. Prepare 2 to 5 ml aliquots in small vials and store in the refrigerator

**APPENDIX V**

**Preparation of Electrophoretic buffer**

Make fresh before each electrophoresis run.
Add 30 ml 10 N NaOH and 5 ml 1 mM EDTA to 960 ml dH$_2$O.

Stock solutions:

➢ 10 N NaOH: dissolve carefully 200g NaOH in 500 ml dH$_2$O. Store at room temperature.

➢ 200 mM EDTA (Ethylenediamine-tetraacetic acid, disodium salt 2.H$_2$O): dissolve 14.89 g in 175 ml dH$_2$O, slightly warm, stir, set to pH 10 with 10 N NaOH, add dH$_2$O to 200ml. Store at room temperature.

**APPENDIX VI**

**Preparation of ethidium bromide**

For staining: mix 1 ml stock solution with 9 ml distilled water.
Stock solution: dissolve 10 mg ethidium bromide (SRL, Mumbai) in 50 ml distilled water. Store all solutions at room temperature at dark.

# EEFECT OF GREEN LEAVES USED IN TRADITIONAL FOOD PREPARATION ON DNA REPAIR

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**Abstract of the thesis submitted in partial fulfilment of the requirement for the degree of**

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# ABSTRACT

The thesis entitled "Effect of green leaves used in traditional food preparation on DNA repair" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thirvananthapuram during 2007-2009 with an objective to test the effect of green leaf extracts of plants (*Azima tetracantha*, *Cissampelos pareira, Myxopyrum serratulum*, *Pongamia pinnata* and *Thespesia populneus*) used in traditional food preparations on DNA repair by employing the Comet Assay.

In the first set of treatments in the genotoxicity study, tomato seedlings, treated with two mutagens (Ethyl methanesulfonate and Methyl methanesulfonate) at different concentrations (0-4mM) over night under room temperature and dim light, showed significantly higher DNA damage, compared to control. In the second set of treatments in the genotoxicity study, tomato seedlings, treated with plant leaf extract, obtained by following two methods of extraction protocols, showed no genotoxicity effect.

In all the treatments (pre-supplementation, post-supplementation and co-supplementation treatments) of plant leaf extracts obtained by following two methods of extraction protocol, before, after and simultaneous treatments with mutagens (EMS and MMS at 4mM), in antigenotoxicity studies, tomato seedlings recorded significantly lower tail moment value, compared to control, indicating effective protection of DNA from damages by mutagens. This may be due to phytochemicals and biologically active components (non nutrient) of the test plant leaf extracts. These results can be of use in testing the leaf extracts of test plants in animal system, which in turn can be of use as a prophylactic measure against cancer and other disorders.