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Methi Lutein prevents Oxidative Stress induced DNA Damage and Cytotoxicity

Leela Srinivas and Thammanna Gowda S.S.
Adichunchanagiri Bitotechnology and Cancer Research Institute, B.G.Nagara-571448, Mandya, Karnataka, INDIA

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Abstract

The detrimental effect of oxidative stress has been known since a long, which may cause the deleterious effect on DNA and finally leads to death. The present investigation revealed the DNA protectant effect of Lutein anti-oxidant purified from fresh Methi (Trigonellafoenumgraecum) leaves. Ferrous sulphate and ascorbic acid (10:100µM) induced the lipid peroxidation and fragmentation in DNA in human lymphocytes model systems. Methi lutein inhibits the formation of lipid peroxidation and DNA fragmentation in fresh human lymphocytes to the magnitude of 80% and 76% respectively. On treatment of Cow dung smoke condensate with human lymphocytes could lead to the adduct formation in human DNA which was controlled by the Methi Lutein (20µM) effectively. U.V radiation induced DNA double strand break and cross linking was strongly controlled by the Methi lutein in Calf thymus DNA when compared to the standard antioxidants BHA and α-tocopherol. Methi lutein might also avoid the cytotoxicity induced by the H_2O_2 and Fe:AA in fresh lymphocytes by maintaining the 80% viability even after 2 hours of treatment.

Keywords : Lipid peroxidation, DNA, Antioxidants.

Introduction

The damage to DNA is very specific and significant which lead to the alteration of base pairs and passed on to the succeeding generations. The severe damage of DNA leads to the mutation and this mutational state enforce the DNA to codes for pathologically significant proteins hence the organism susceptible for various types of diseases including cancer[1]. U.V radiations, inter-chelating agents, polycyclic aromatic hydrocarbons and oxygen radicals are the DNA damaging agents. 65% of melanoma and 90% of non-melanoma cancer occurred due to the U.V radiation[2]. The damage caused by the radiations is cross linking of base pairs, single and double strand breaks[3]. Single strand breaks does not pose a serious problems but the double strand breaks lead to the chromosomal aberrations[4]. Whereas the PAH molecules like benzopyrene and benzanthracene are well known carcinogens[5].

Hydrogen peroxide, hydroxyl radicals, Superoxide radicals are important ROS causes significant threat to the DNA and also proteins, lipids[6,7]. High concentration Reactive oxygen species (ROS) as well as reactive nitrogen species (RNS) formed from the cellular redox process that leads to the oxidative stress which is responsible for chronic and degenerative diseases such as cancer, rheumatoid arthritis, cataract, aging, cardiovascular and neurodegenerative diseases respectively[8,9,10,11]. Oxidative DNA damage could lead to cancer development[12, 13]. Initiation and promotion of cancer are associated with chromosomal defects and oncogene activation due to free radicals. A common form of damage is the formation of hydroxylated bases of DNA, which are the main event in chemical carcinogenesis. Oxidative DNA damage also produces a multiplicity of modifications in the DNA structure including base and sugar lesions, single and double strand breaks, DNA-protein cross-links and base-free sites. Oxidative stress is also responsible for brain damage after stroke[14].

Antioxidants play an important role in the prevention of diseases caused by the oxidative stress[15]. Epidemiologic studies have shown that a high fruit and vegetable intake is associated with a lower risk of chronic diseases[16-8]. The carotenoids found in fruit and vegetables can act as a major group of antioxidants, thereby preventing damage from harmful reactive oxygen species, which are continuously produced in the body from normal cellular functions and from exogenous sources[19]. Earlier studies in our laboratory proved that, the dietary antioxidants such as water soluble Turmerin peptide could prevent the lymphocytes and DNA damages caused by the cowdung smoke condensate and fuel smoke condensate effectively[20,21]. In this study we have shown that, the Methi lutein prufied from Methi leaves (Trigonellafoenumgraecum) is proved to be non-toxic and natural protectant for DNA damage caused by oxidative stress in cellular and calf thymus DNA.

Material and Methods

Materials: Ethidium bromide, BHA, Calf thymus DNA (CT-DNA), SDS, Agarose, t-BOOH, Thiobarbutaric acid, Diphenylamine (DPA) were purchased from sigma chemicals Co., USA. H_2O_2, EDTA, Ascorbic acid, BHA,

Dimethylsulfoxide (DMSO), ferrous sulphate, sodium acetate, Deoxyribose, Trisodium citrate, Dextrose were from Himedia Pvt. Ltd., Bombay, India. All the other chemicals and reagents were of analytical grade.

Methods: Isolation of lymphocytes: Lymphocytes were isolated from 20ml blood drawn from the healthy, non-smoking young volunteer[22]. Blood was collected in ACD (85mM citric acid 71mM trisodium citrate, 165mM D glucose) in the ratio 5:1. Four volumes of haemolysing buffer (150mM NH_4Cl in 10mM Tris buffer, pH 7.4) were added, mixed well, incubated at 4^0C for 30 min. Centrifuged at 1200rpm for 12 min, the supernatant was discarded, pellet was washed again with 5ml of haemolysing buffer and the pellet containing cells were washed thrice with 10 ml of solution B (250mM m-inositol in 10mM phosphate buffer pH 7.4) and suspended in same solution. The cell viability was determined by tryphan dye blue exclusion method[23]. To 10µl of lymphocyte sample added the cell number was counted. The dead cells, being permeable to tryphan blue, appear blue against white colour of the viable cells.

The survival rate of lymphocytes was determined[23] at time intervals 20th, 40th and 60th minutes of incubation. Viability was tested by trypan blue exclusion and it was exceeded 96% in each isolation. Percentage viability was calculated using the formula.

$$\% \text{ Viability} = \frac{\text{Number of viable}}{\text{Total number}} \text{ X } 100$$

Lymphocytes with viability over 90% were used to evaluate the preventive effects of Methi lutein againstoxidative DNA damage due to MDA, LOP and cytotoxicity.

Pro oxidants: The prooxidants such as lipid oxidation product (LOP), t-BOOH, H_2O_2, Ferrous sulphate:Ascorbate (Fenton reactant), cow-dung smoke condensate were studied for their ability to induce oxidative lymphocyte cell damage and DNA damage.

Isolation of DNA from human lymphocytes: Lymphocytes were isolated (Ref Phenol –chloroform method) from the fresh blood as described in the method from the healthy donor and were washed thoroughly with HBSS pH 7.4. Cells were homogenized with Buffer X (0.3 M Tris pH-8 containing 0.2M sucrose, 0.1M NaCl and 0.01M EDTA). To this 125µl of 10% SDS was added while vortexing and incubated at 65^oc for 30min. The contents were cooled and 350µl of 8M potassium acetate was added and kept in ice for 60min. The contents were centrifuged at 5000 rpm for 20 min and the supernatant was transferred to fresh centrifuge tube. To this equal volume of cold Phenol-Chloroform mixture was added and shaken slowly to isolate DNA. Aqueous layer was collected and washed twice with phenol-chloroform mixture as mentioned above. Adding equal volume of ice-cold propanol precipitated DNA. DNA precipitate was washed with 70% propanol dissolved in 50µl of 10mM PBS. The final concentration of DNA was calculated by its absorbance at 260nm.

$$\text{DNA } (\mu g/ml) = \frac{\text{Final dilution x OD at 260nm}}{\text{20 x Vol. of the sample}}$$

Agarose gel electrophoresis: DNA gel electrophoresis was carried out using 0.6% agarose prepared in TAE buffer containing 0.5µg\ml of Ethidium Bromide. Electrophoresis was carried out using Tris-TAE buffer (40mM Tris, 20mM Sodium acetate, 18mM Nacl, 2mM EDTA, pH 8.0). Bands visualized under U.V transilluminator

Preparation of DPA solution: 10-ml glacial acetic acid added to 150mg DPA in a 50ul polypropylene tube and mixed thoroughly by repeated inversion until complete distillation. To this 150ml concentrated H_2SO_4 was added mixed thoroughly. Later 50µl of acetaldehyde solution added and mixed thoroughly.

Preparation of cow dung smoke condensate: Smoke condensate from cow dung cake was prepared according to Nakayama et al[24].2 grams of the cowdung cake was smouldered for 15 mins and the ensuing smoke was condensed into 4 ml of 10mM ice-cold PBS pH 7.4 (10mM potassium phosphate buffer with 0.8% NaCl). 50ul of DMSO was added to solubilize the water insoluble lipopilic components and to stabilize ROS. The condensate obtained was filtered through glass wool and the clear filterate was collected and used within 30 minutes after collection. Care was taken, not to include the black charred material. In all the experiments involving smoke condensate, the condensate was added at a concentration corresponding to an Optical density of 1 at 271 m, the signature wavelength of PAH put together[21,25]. This corresponded to 1: 100 dilution of the smoke condensate.

Ferrous sulphate: Ascorbic acid induced peroxidation–MDA levels in Lymphocytes by Methi lutein: Lymphocytes (1 x 10^6 cells) suspension was treated with ferrous sulphate: ascorbate which is the known inducer of lipid peroxidation at the concentrations ranging from 0.5:5 to 3:30µmole in 1ml of HBSS, pH 7.4 and incubated at 37^0C for 1 hour. Lymphocytes (1x10^6 cells) suspension was pre-treated with or without Methi lutein, BHA and α-tocopherol at concentration ranging from 25-100 µg in HBSS, pH 7.4 at 37^0C for 20 minutes, ferrous sulfate: ascorbate (2.20umole) was added, final volume was made to 1ml with HBSS, pH 7.4 and incubated at 37^0C for 1 hour, centrifuged at 1500rpm, for 10 min at 4^0C. An aliquot of supernatant was incubated with 100ul of 8.1% SDS, 2.5ul of 2% BHA and 0.5ml of thiobarbituric acid-HCl (15% W/V), Trichloroacetic acid in 0.375%TBA and 0.25N HCl) at 70^0C for 20 minutes. After cooling 1ml of n-butanol was added, vortexed, centrifuged at 5000 rpm and the absorbance of supernatant was measured at 532nm and quantified the level of

malondialdehyde (MDA) using the extinction coefficient of MDA ($1.56 \times 10^5\ M^{-1}cm^{-1}$)[26].

Quantitative analysis of DNA fragmentation by diphenylamine method: The inhibitory effect of antioxidant on prooxidants induced DNA fragmentation in lymphocytes was studied by diphenylamine reaction method[27]. This method separated from chromosomic DNA upon centrifugal sedimentation. This includes lysis of cells to release the nuclear DNA, centrifugation of two fractions (corresponding to intact and fragmented DNA). The precipitation of DNA, hydrolysis and colorimetric quantitation upon staining with DPA that binds to deoxyribose.

100ul of cell suspension, corresponding to 1 x 10^6 cells were pretreated with or without Methi lutein or BHA and α-tocopherol at the concentrations ranging from 0-100ug in 0.5ml HBSS then added ferrous sulphate : ascorbate (2:20umole) or t-BOOH (125µM) in total volume of 1ml of HBSS and incubated at 37^0C for 60 minutes. Viability was tested and centrifuged at 1200-1500rpm, 20 min, 4^0C and processed for the estimation of DNA fragmentation by diphenylamine reaction method as follows.

Supernatants were transferred carefully into fresh tube A. The pellets were transferred to the tube B were lysed in 1ml of TTE solution (TE buffer -10mM TrisHCl and 1mM EDTA) pH 7.4 with 0.2% Triton X 100) and vortexed vigorously that allows the release of fragmented chromatin from nuclei, after lysis due to triton X 100 and disruption of nuclear structure following Mg++ chelation by EDTA in TTE solution. The DNA was separated from chromatin by centrifugation at 1500 rpm in centrifuge tube at 4^0C, 10 min, the supernatant was carefully transferred to the tubes T and the pellets from the tube B suspended in 1ml of lysis buffer -TTE solution. To the pellets (b) and supernatants (T and S), 1ml of 25% TCA was added and vortexed vigorously and incubated for 24 hour for overnight precipitation at 4^0C. The samples were centrifuged for 20 min at 1500rpm at 4^0C. Supernatants were discarded by aspiration with the help of pipette and the DNA was then hydrolyzed by adding 160ul of 5% TCA to each pellet and heated for 15 min at 90^0C. A blank was also prepared with 160µl of 5% TCA alone. Then to each sample tubes, 100ul of freshly prepared DPA solution (150mg DPA in 10ml glacial acetic acid was mixed thoroughly by repeated inversion of polypropylene tube until complete dissolution. Added 150µl of concentrated H_2SO_4 and 50µl acetaldehyde solution (16mg/ml) and mixed thoroughly by vortexing. Incubated at ambient temperature for 24 hour to allow for the development of colour (Burton, 1956) and the absorbance read at 600nm. The control was without test compound and the proportion of fragmented DNA and the % inhibition was calculated as follows.

$$\% \text{ DNA fragmentation} = \frac{\text{Absorbance of Test (T)}}{\text{Absorbance of Test (T)} + \text{Absorbance of Blank}}$$

$$\% \text{ inhibition of DNA fragmentation} = \frac{\text{DNA fragmentation (control)} - \% \text{ DNA fragmentation (test)}}{\% \text{ DNA fragmentation}} \times 100$$

Prevention of cow dung smoke condensate induced DNA damage in human lymphocytes by Agarose gel electrophoresis: Lymphocytes were isolated according to David et al (David et al 1986) with some minor modifications. These lymphocytes suspension (1 x 10^6cells) were pre-treated with or without Methi lutein (20µM) or α-tocopherol (400µM) in 0.5 ml HBSS, pH 7.4 at 37^0C for 20 minutes, then 100µl of the smoke condensate (O.D corresponding to 1 at 271nm)/100ul of the H_2O_2 (144µM) was added and final volume was made to 1ml with HBSS, pH 7.4 and incubated at 37^0C for 60 minuts, then centrifuged at 1000 - 1200rpm, 15-20 minutes at 4^0C.

Cells were washed thoroughly with HBSS pH 7.4 and cells were homogenized with buffer H (0.3M tris pH 8 containing 0.2M sucrose, 0.1m NaCl and 0.01M EDTA). To this, 125ul of 10% SDS was added while vortexing the mixture and incubated at 65^0C for 30 min. The contents were cooled and 350ml of 8M potassium aceate was added and kept in ice for 60min. The contents were spun down at 5000 rpm for 20 minutes and the supernatant was transferred to a fresh eppendorff tube. Then DNA was extracted by saturated phenol: chloroform: amylalcohol mixture (25:24:1). Mixed well and centrifuged at 6000rpm for 10 minutes. The same step was repeated thrice with organic phase. DNA was precipitated from the organic phase by the addition of equal volume of chilled isopropanol and dissolved in 50ul of 1X TAE (1.5mM Tris base, 0.57 ml glacial acetic acid, 0.05m EDTA) buffer. DNA was estimated by UV absorbance and the final concentration of the DNA was calculated by using the formula.

$$\text{DNA (µg/ml)} = \frac{\text{Final dilution x OD 260nm}}{\text{20 x volume of the sample}}$$

The human lymphocyte DNA was taken in 0.5ml of HBSS pH 7.4 and the known volume of smoke condensate and H_2O_2 was added with and without Antioxidants and Methi lutein final volume was made to 1ml with HBSS incubated at 37^0C for 1 hour. Reaction was stopped in ice bath. 5µg of the DNA was loaded to the agarose gel (0.7%) and the bands were visualized under UV-transilluminator.

U.V radiation induced DNA damage protection by Methi lutein: Calf thymus DNa was sheared 90 times using 21 guaze needle and CTDNA (1mg/ml) was subjected to UV radiation (345nm) in the presence or absence of Antioxidants and Methi lutein in the presence of germicidal UV lamp (Hanovia Lamp) for 60 min at 370C in 20 mM phosphate buffer saline containing pH 7.4. At regular intervals of 30 minutes, the reaction mixture corresponding to the 5 ug of calf thymus DNA was drawn and loaded to the 0.6% of the agarose gel. Bands were visualized under U.V transilluminator to determine the protection offered by antioxidants.

Time course study of the effect of H_2O_2, Ferrous sulphateAscorbate and effect of antioxidants on the viability of lymphocytes: Lymphocyte cells (1 x 10^6) were treated with H2O2 and ferrous sulphate:ascorbate (2:20umole) in the presence or absence of Methi lutein, antioxidants in 1ml HBSS pH 7.4 at 37^0C. After the desired incubation time up to 6 hours, the viability of the cells was determined by tryphan blue exclusion analysis[22] and the percentage of viable cells was calculated as described in the section.

Statistical analysis: All the experiments involving the quantitative parameters were repeated at least three times and the values were represented as Mean+ SD/SEM. Significance of the observation was tested by students T test and the P value less than 0.05 was considered as significance. Significance is indicated by the symbol.

Results and Discussion

Various types of Reactive oxygen species such as hydrogen peroxide, hydroxyl radicals and superoxide radicals cause significant threat to the DNA proteins and lipids. ROS induces the oxidative stress which intern causes the DNA damage and modifications in the DNA structure including base and sugar lesions, single and double strand breaks.

Due to the effect of ferrous sulphate and ascorbic acid, the MDA formation was takes place and was inhibited by Methi lutein to the tune of 80%, whereas α-tocopheral and BHA offered the inhibition to the range of 55 and 45% at the maximum concentration of 50 µg as shown in figure-1. The Ferrous sulphate and ascorbic acid acted upon the lipid membrane of the lymphocytes and could lead to the formation of MDA. Being a hydroxyl carotenoid, lutein inhibited the formation of MDA by preventing the oxidation of membrane in lymphocytes.

In addition to this, ferrous sulphate and ascorbic acid (1:10) also induced the DNA fragmentation in human lymphocytes. As oxidizing agents, they induced the oxidation and thereby lead to the formation of ROS which causes the oxidative trauma in the cell due to high reactivity. Ferrous sulphate being a transition metal with variable valencies involved in the generation of oxygen from radicals. Ferrous involved in the formation of superoxide by reduction and hydroxyl radicals through Fenton reactant which severely caused the DNA damage.

When ferrous sulphate and ascorbic acid added in the ratio of 1:10 umole concentration to the human lymphocytes DNA, they induced the double strand break due which could be due to the formation and effect of hydroxyl radicals. When they treated with Methi lutein, it could inhibit the DNA fragmentation to the tune of 76% at the concentration of 20µM which acts as an effective inhibitor than standard antioxidant BHA and α-tocopherol. Both of them prevented the DNA fragmentation to the extent of 61 and 59% respectively at the concentration of 400µM (figure-2)**.**

DNA damage activated by the cow dung smoke condensate was protected by Methi lutein (figure-3). Smoke condensate contains polycyclic aromatic hydrocarbons (PAH) which induces the metabolically activated microsomal enzymes of the body and finally targeted the DNA. Due to the attack of active forms of enzymes on DNA, there is a formation of adduct. In addition to this, various metal ions present in the PAH, led to the formation of Fenton reactants which are very reactive. They attack the nucleophiliccentres of the DNA and alter the covalent structures of the DNA. The Methilutein donate the extra electrons and prevents the breakdown of covalent bonds and hence, avoid the DNA damage in human lymphocytes DNA. It protected the DNA damage up to 95% and which is more than the protection offered by BHA (86%) and α-tocopherol (93%).

Treatment of calf thymus DNA with U.V rays causes the DNA damage due to the formation and effect of OH^- radicals. The OH radicals are produced by splitting of water molecule. The effect of DNA damage due to the radiation could be cross linking of base, single and double strand break. The double strand break led to the chromosomal aberrations due to the erroneous re-joining of strands. Methi lutein at the concentration of 20nM avoids the strand breaks even after 60 minutes of exposure.

In the presence of Methi lutein, the % of damage was less than 10% as shown in figure- 4.

Being a known toxic agent, H_2O_2 caused the toxicity in human lymphocytes and the % viability was decreased to 18% after 2 hours incubation with H_2O_2 (figure-5). Hydrogen peroxide could be diffused through the membrane of lymphocytes and reach the nucleus where it converted into OH^- radicals with the help of metal ions. However, the % viability was significantly retained to 85% in the presence of Methi lutein (12.5µg) even after 2 hours. α-tocopherol (150µg) could also retained 80 % viability. The present work specified that the efficiency of Methi lutein in giving protection against the cytotoxicity induced by the H_2O_2 and this proved to that Methi lutein by itself is non-toxic.

Conclusion

The above results proved that, Methi lutein can prevent the damages in DNA in both human and calfthymus model systems competently at the lower concentration than the standard and known Antioxidants. It also proved to be the effective anti-cytotoxic agent. Hence Methi leaves can be used as the effective inhibitor of DNA damage thereby we can prevent the oxidative stress induced diseases such as the Arthritis, CVD, Alzheimer's, Cancer. Further it is proved to be the nontoxic, easily available dietary source.

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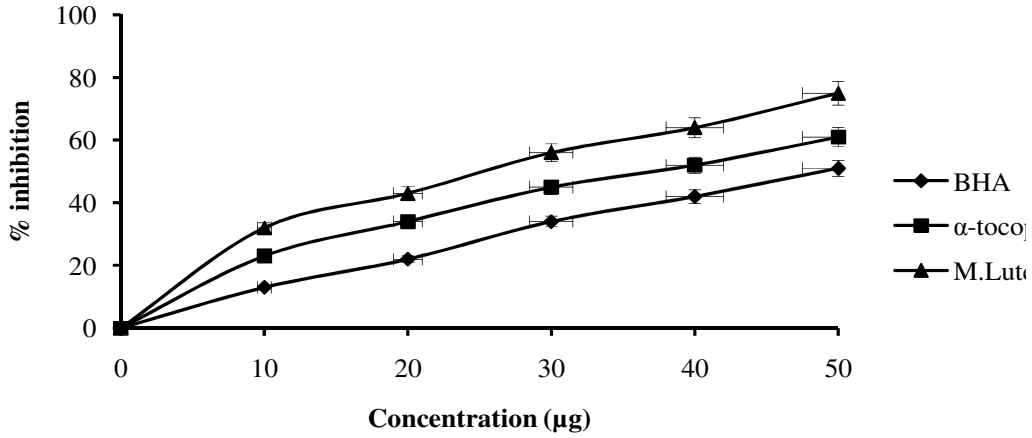

Figure-1
Inhibitory effect of Methi lutein on Ferrous sulphate:Ascorbate induced lipid peroxidation (MDA equivalent) formation in lymphocytes

Lymphocytes (1 x 106 cells) in 0.5ml of HBSS, pH 7.4 ± Methi lutein or BHA or α-tocopherol at indicated concentration and incubated at 37^0C for 20 mins. Then ferrous suphate: ascorbate (2:20µmole) was added ± antioxidants and incubated at 37^0C for 1hour. Reaction stopped by adding 1ml of ice-cold HBSS pH 7.4 centrifuged at 5000 rpm. Aliquot of lysate from each sample were taken for the estimation of TBARs as described in methods. Total lipid peroxides (MDA equivalents) were calculated using the molecular extinction coefficient of MDA (1.56 x 10+5 M-1cm-1). Values are mean of six experiments.

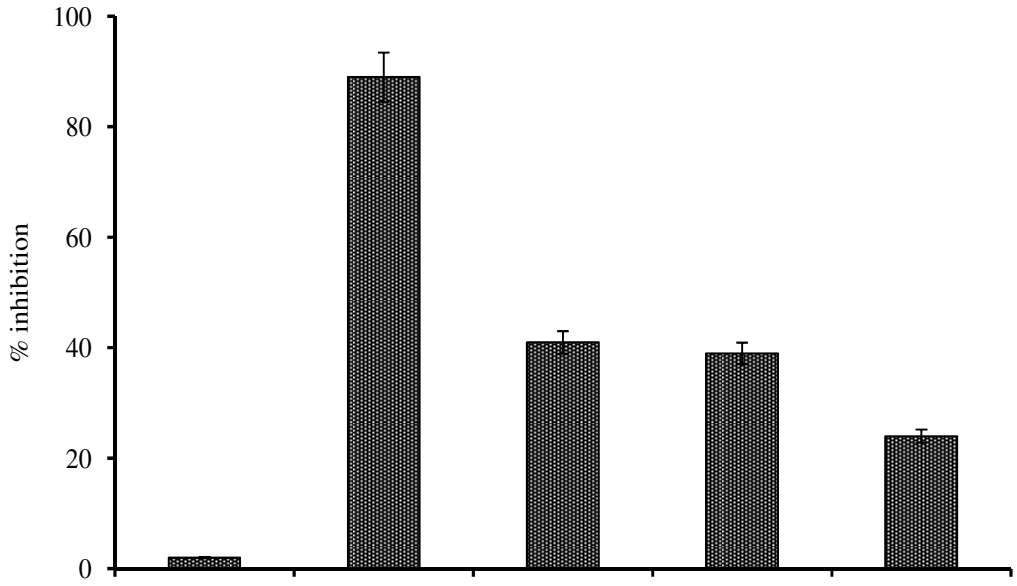

Figure-2
Inhibition of Fe:AA induced DNA fragmentation by Methi lutein-diphenylamine method

Lymphocyte suspension (1x 10^6 cells) pretreated with or without Methi lutein (20 µg) or BHA (400 µM), α-tocopherol (400 µM). Then Fe:AA (2:20 µmole) was added and final volume was made to 1ml with HBSS, pH 7.4 and and incubated at 37^0C, 1hr. Fragmented double stranded DNA was quantitated colorimetrically at 600nm with diphenylamine reaction as described in methods. The control was without test compound and the % inhibition was calculated accordingly. The value are mean ± SD (n=6)

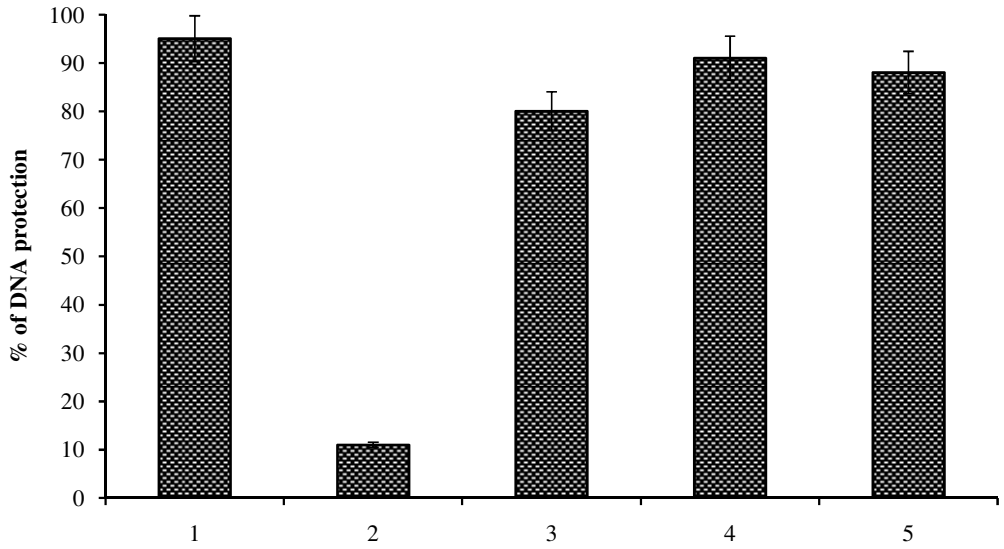

Figure-3
Analysis of Cow dung smoke condensate induced DNA damage in human lymphocytes

Lane 1–DNA, Lane 2 – As (1) + Smoke condensate (20µl), Lane 3 – As (2) + BHA (400µM), Lane 4 – As (2) + Methi lutein (20µM), Lane 5 – As (2) + _- Tocopherol (400µM). Human lymphocyte DNA in 0.5ml of HBSS pH 7.4 ± 20µl smoke condensate ± Antioxidants final volume was made to 1ml with HBSS incubated at 370C for 1 hour. Reaction was stopped in ice bath. 5µg of the DNA was loaded to the agarose gel (0.7%) and the bands were visualized under UV-transilluminator.

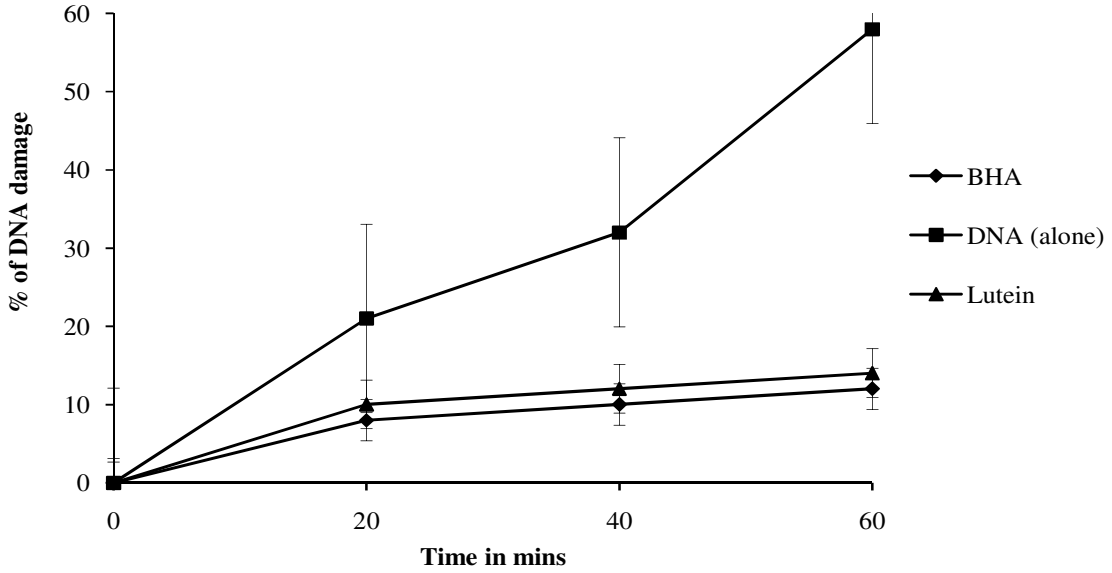

Figure-4
Prevention of U.V radiation induced DNA damage in Calfthymus DNA

Sheared calfthymus DNA (1mg/ml PBS, pH 7.4) ± M. Lutein (20nM) + BHA (400µM), subjected to U.V radiation (345nm), 60 min, 37^0C, 200µl aliquot + 3µl of Ethidium bromide solution mixed well, fluorescence measured (ex:520nm and em: 590nm).

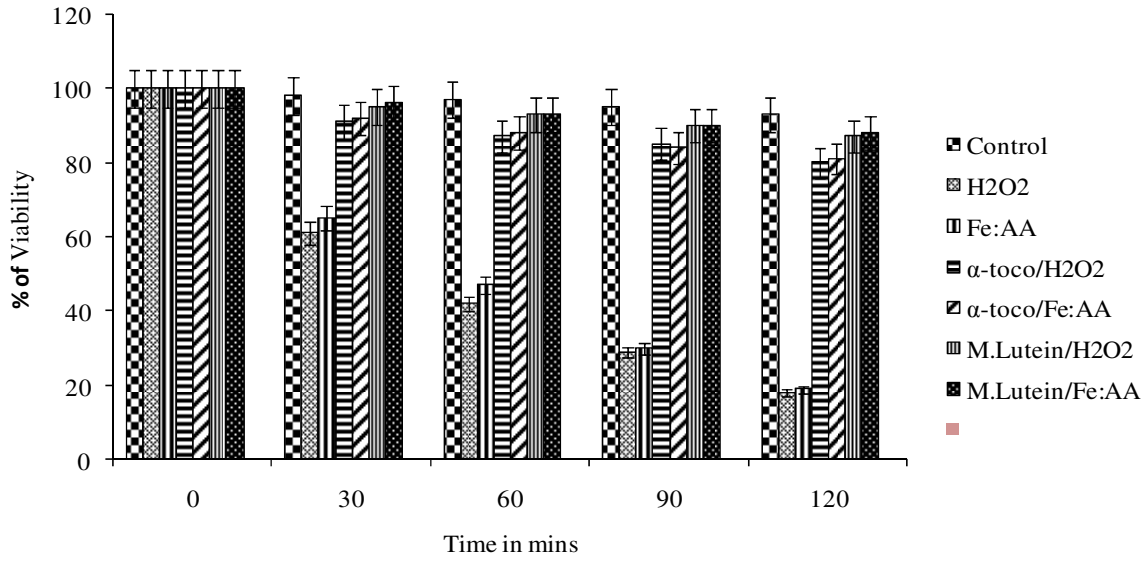

Figure-5
Effect of Methi lutein on the viability of lymphocytes on treatment with H_2O_2- time course study

Lymphocytes (10^6 cells) were pretreated with or without methi lutein and antioxidants at indicated concentrations in 0.5ml HBSS pH 7.4, then H_2O_2 (144µM)/Fe:AA (10:100uM) was added, incubated at 37^0C for indicated time periods in final volume of 1ml HBSS, pH 7.4. After the desired incubation time (up to 120 mins), viability of the cells was determined for every interval of 30 mins up to 2 hours by tryphan blue exclusion and the percentage of viable cells were calculated as mentioned in methods.

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