



Research Article

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## **Ameliorative Effect of Eugenol and Anethole on Arsenic Induced Oxidative DNA Damage in Cultured Human Peripheral Blood Lymphocytes**

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### **ABSTRACT**

Arsenic contamination is one of the major health concerns all over the world and associated with various types of cancer and pathological effects. The production of reactive oxygen species (ROS) plays a crucial role in arsenic mediated toxicity. Several studies have shown that population constantly exposed to arsenic have substantial oxidative stress that, in turn, induces DNA damage. In the present work eugenol and anethole were investigated for their protective effect against arsenic mediated oxidative DNA damage in peripheral blood lymphocytes. Comet assay and lipid peroxidation was used as biomarker of genotoxicity and oxidative stress respectively. A dose dependent increase in tail moment and lipid peroxidation was observed when lymphocytes were treated with sodium arsenite. Treatment of arsenic (50 $\mu$ M) along with eugenol (20 $\mu$ M) and anethole (50 $\mu$ M) showed a significant decrease in the tail moment and lipid peroxidation in cultured peripheral blood lymphocytes. The decrease in the tail moment and lipid peroxidation was significantly higher in combined supplementation of eugenol and anethole as compared to individual administration. The results of the present study suggests ameliorative role of eugenol and anethole against arsenic induced genotoxic and oxidative damage in cultured human peripheral blood lymphocytes.

**Keywords:** Arsenic, Anethole, Eugenol, Oxidative stress, genotoxicity.

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### **INTRODUCTION**

Arsenic (As) induced genotoxicity is one of the most prevalent health concern throughout the world.

Epidemiologic studies have indicated that environmental exposure of arsenic can lead to carcinogenesis. [1] Arsenic is a metalloid element and

found in nature as organic and inorganic arsenic (iAs). Later is considered as a class I human carcinogen and associated with manifestation of various tumors and several other pathologies. [2] Numerous studies have elucidated that populations persistently exposed to As had a significant induction of a strong oxidative stress with generation of free radicals in cells that, in turn, mediates DNA damage as well as lipid peroxidation and decreased glutathione levels. [3] In a study Pei *et al* [4] has observed the induction of DNA damage in peripheral blood lymphocytes with increased apoptosis at a very low levels of arsenic.

Phytochemicals found in commonly used dietary elements such as fruits, vegetables, cereals, spices and beverages have been studied with significant health benefits. [5] The consumption of these phytochemicals considered as an effective strategy for reducing oxidative stress, DNA damage, cardiovascular diseases and cancer. [6] In a recent study, white pepper seed extract (*Piper nigrum*) was found to possess immunomodulatory potential in macrophage cells. [7] Likewise naturally occurring alkanylbenzenes trans anethole (1-methoxy-4 propenylbenzene) and eugenol (4-allyl-1-hydroxy-2-methoxy benzene) are two common food flavouring agents acknowledged for preventative effects. [8]

Eugenol is the chief component of clove oil. It is also present in other spices such as cinnamon, basil and nutmeg. It used mainly in baked products, beverages. Eugenol has been reported for anticancerous properties in melanoma cancer cells by arresting the cells in S-phase and inhibiting the E2F1 transcriptional activity. [9-10] It also exhibited antiseptic, antigenotoxic, anti-inflammatory, antioxidant, analgesic and anti-bacterial properties. [11]

Anethole is volatile constituent of more than 20 spices. [8] It is a major component of sweet and bitter fennel and anise. Trans anethole is used in chewing gums, baked goods, candy and ice-creams. Antigenotoxicity of trans anethole was studied in mice [12] and found to increase antigenotoxicity with increased dosage.

Comet Assay [13] is considered as one of the significant biomarker for the estimation of the DNA damage in the form of single strand breaks (SSB) in any accessible cells. In this assay, a damaged cell appears as a comet, with head and tail regions, where later signifies extent of DNA damage. [14] For the analysis of oxidative stress lipid peroxidation amongst the reliable biomarker. In the presence of oxidative stress free radicals form poly unsaturated fatty acids (PUFAs). These complexes were measured by spectrophotometer and found to play a significant role in the study of oxidative DNA damage and cytotoxicity. [15] In the present work we explored the individual and combined modulatory effects of eugenol and anethole against arsenic induced oxidative DNA damage in cultured human peripheral blood lymphocytes.

## MATERIALS AND METHODS

### Preparation of chemicals

Sodium arsenite, trans anethole and eugenol (Sigma Aldrich) were prepared in double distilled water and Dimethyl sulphoxide (DMSO) respectively. The stock solution (1mg/ml) was sterile filtered (0.22µm, Himedia) and stored at -20°C until use.

### Subjects and Sampling

In sodium heparin coated vacutainer tubes 5 ml venous blood was collected from healthy volunteers for lymphocyte culture set up. A consent form and questionnaire was also got filled by the donors for their consent and health status history. Before commencing present work, protocols were duly approved by the Human Ethical Committee of Kurukshetra University.

### Human lymphocyte culture

Short term Peripheral blood lymphocyte (PBL) cultures were set up using earlier studied protocol of Moorhead *et al* [16] with minor amendments. Cultures were set up in duplicate with the addition of 0.4 ml whole heparinized blood into culture vials comprising 5 ml of RPMI 1640 medium (Himedia) with L-glutamine (1%), fetal calf serum (20%) (Himedia), penicillin 100 UI/ml, streptomycin 100µg/ml solution (Himedia), and phytohaemagglutinin (2%) (Bangalore Genei). The cultures were incubated in 5% CO<sub>2</sub> at 37°C for different time periods.

### Single cell gel electrophoresis (SCGE) or comet assay

For comet assay or single cell gel electrophoresis assay the technique of Singh NP [13] was used with minor modifications. Short peripheral blood cultures were set up. Sodium arsenite was added to culture tubes in different concentrations (10 to 100µM) to analyse its DNA damaging effect. Simultaneously antimutagenic potential of eugenol and anethole was also studied at dosage of 10 and 50µM respectively. The cultures were incubated for 24 hours at 37°C and ± 5% CO<sub>2</sub>. The cells were harvested by centrifugation, fixed. The harvested cells were suspended in 0.5% (w/v) low melting agarose and layered over a frosted microscopic slide coated with first layer of 1% normal melting agarose for firm gripping. Then slides were kept at 4°C for solidification for ten minutes. A third layer was made with 0.5% (w/v) low melting agarose, slides were kept for another ten minutes. Afterwards slides were immersed in lysis buffer (NaCl 2.5 M), (Na<sub>2</sub>EDTA 0.1M), Tris (10 mM), NaOH (0.3M), Triton X-100(1%) and DMSO (10%) in a solution of pH 10. The slides were kept for two hours in lysis at 4°C. Then slides were transferred to electrophoresis chamber containing electrophoresis buffer (300mM NaOH, 1mM Na<sub>2</sub>EDTA, pH 13.0) and pre-immersed for 20 minutes for unwinding the DNA and followed by electrophoresis for 20 minutes (300mA, 25V) at 4°C.

Slides were then kept in neutralizing buffer (Tris 0.4M, pH 7.5) for ten minutes, stained with ethidium bromide (20µg/ml) and later screened under a fluorescence microscope. The cells were subjected to image analysis using Comet IV software. The magnitude of DNA damage was analysed by measurement of tail moment

which was calculated as the percentage of DNA in the tail multiplied by the length between the center of the head and tail.

#### Lipid peroxidation assay

Lipid peroxidation is analysed by the formation of thiobarbituric acid reactive substances (TBARS). Levels of TBARS in cell culture lysate were measured by spectrophotometer at 535nm by the technique defined by Ohkawa *et al* [15] with minor modifications. Short term PBL were set up. Arsenic (50µM) treated cultures were supplemented with eugenol and anethole concentrations of 10 and 20µM respectively for the study of antioxidative potential. Control was also run simultaneously along with DMSO as a negative control and EMS as a positive control. The cultures were incubated in 5% CO<sub>2</sub> at 37°C for 24 hours. Treated cell cultures were harvested and washed with PBS (1%). The pellet was lysed using lysis buffer (Tris buffer, MgCl<sub>2</sub>, EDTA, mercaptoethanol and glycerol) following centrifugation at 6000 rpm for 20 minutes at 4°C. Supernatant was used for protein estimation by Lowry's method.

The reaction mixture tubes containing cell lysate, 0.1ml of 10% SDS, 0.6ml of 20% of glacial acetic acid, 0.6 ml of 0.8% TBA, and water (final volume 3.0ml) were placed in boiling water bath for 1 hour and instantly placed on ice bath for 10 minutes. Centrifugation was performed at 5000 rpm for 10 min and supernatant was observed spectrophotometrically at 535nm against a blank deprived of cell lysate.

#### Statistical analysis

All the experiments were performed in duplicates and results were articulated as mean ± S.E /mean ± S.D. Student t test was used for comparing paired sample tests. Significance difference was measured at  $P < 0.05$ . All tests were performed using statistical software package system SPSS 16.0 (IBM).

## RESULTS

Various biomarkers are available for the assessment of genotoxicity and cytotoxicity. Comet and Lipid peroxidation assays are considered as reliable, sensitive and simple methods for the analysis of DNA damage and oxidative stress respectively. In the present study, comet assay and lipid peroxidation were studied as biomarkers of oxidative DNA damage caused by sodium arsenite in human peripheral blood lymphocytes. There was dose dependent increase in tail moment observed in cultures treated with sodium arsenite at concentration ranging from 10 to 100µM. Arsenic at concentration of 50µM showed significantly higher genotoxicity.

Eugenol and anethole were also analyzed for having any genotoxic effects in a separate culture set up. They were found to be non genotoxic as there was no significant increase in tail moment as compared to control untreated sample. Individually both eugenol and anethole showed a protective effect against arsenic mediated genotoxicity in a dosimetry manner. There

was significant dose dependent decrease ( $P < 0.05$ ) in tail moment in comparison to the PBL treated with arsenic (Table 1 and Fig. 1). Eugenol showed a significant decrease at the 20µM dosage, while anethole found to be effective at concentration of 50µM. However the combined effect of eugenol and anethole showed a significantly higher decrease in the tail moment in comparison of individual administration.

In lipid peroxidation assay oxidative stress was measured in term of TBARS formation, similar trend as of tail moment was observed in TBARS levels. There was significantly higher TBARS levels in arsenic treated culture as compared to control cultures (Table 2 and Fig. 2). Eugenol and anethole exhibited an ameliorative potential against arsenic mediated oxidative stress. There was significant decrease in lipid peroxidation (TBARS levels) observed when arsenic treated cultures were supplemented with eugenol (20µM) and anethole (50µM). The decrease was significantly higher when administered in combination instead of individual treatment.

**Table 1: Antigenotoxic effect of Eugenol and Anethole against Arsenic induced DNA damage cultured human lymphocytes**

Treatments	Concentrations used (µM)	Tail moment ± S.D
Control	Untreated	2.11 ± 0.27
DMSO (negative control)	20µl	1.37 ± 0.12 <sup>a</sup>
EMS (positive control)	2µl	67.52 ± 6.13 <sup>b</sup>
Arsenic (As)	50	35.78 ± 1.69 <sup>c</sup>
As +Eugenol	50+20	11.98 ± 1.51 <sup>d</sup>
As + Anethole	50+50	17.15 ± 2.87 <sup>d</sup>
As+ Anethole+ Eugenol	50+20+50	9.37 ± 0.91 <sup>d</sup>

a $P > 0.05$  (non-significant as compared to untreated), b $P < 0.05$  (significant as compared to untreated), c $P < 0.05$  (significant as compared to untreated), d $P < 0.05$  (significant as compared to arsenic treatment), S.D-standard deviation

**Table 2: Antioxidative effect of Eugenol and Anethole against Arsenic induced DNA damage cultured human lymphocytes**

Treatments	Concentrations used (µM)	TBARS ± S.E
Control	Untreated	4.07 ± 0.19
DMSO (negative control)	20µl	2.43 ± 0.07 <sup>a</sup>
EMS (positive control)	2µl	27.28 ± 0.63 <sup>b</sup>
Arsenic (As)	50	20.5 ± 0.38 <sup>c</sup>
As +Eugenol	50+20	12.24 ± 0.41 <sup>d</sup>
As + Anethole	50+50	13.11 ± 0.16 <sup>d</sup>
As+ Anethole+ Eugenol	50+20+50	10.44 ± 0.21 <sup>d</sup>

a $P > 0.05$  (non-significant as compared to untreated), b $P < 0.05$  (significant as compared to untreated), c $P < 0.05$  (significant as compared to untreated), d $P < 0.05$  (significant as compared to arsenic treatment), S.E-standard error

## DISCUSSION

Arsenic mediated genotoxicity is a matter of serious concern all over the world. Arsenic treatment showed dose-dependent augmentation in the incidences of sister chromatid exchanges (SCE), increased the ROS generation, comet frequency, cell cycle proliferative index in peripheral blood lymphocytes. [17-18] A recent review report on arsenic toxicity in west Bengal called arsenic a grave health concern with 20 million lives at stake. [19]

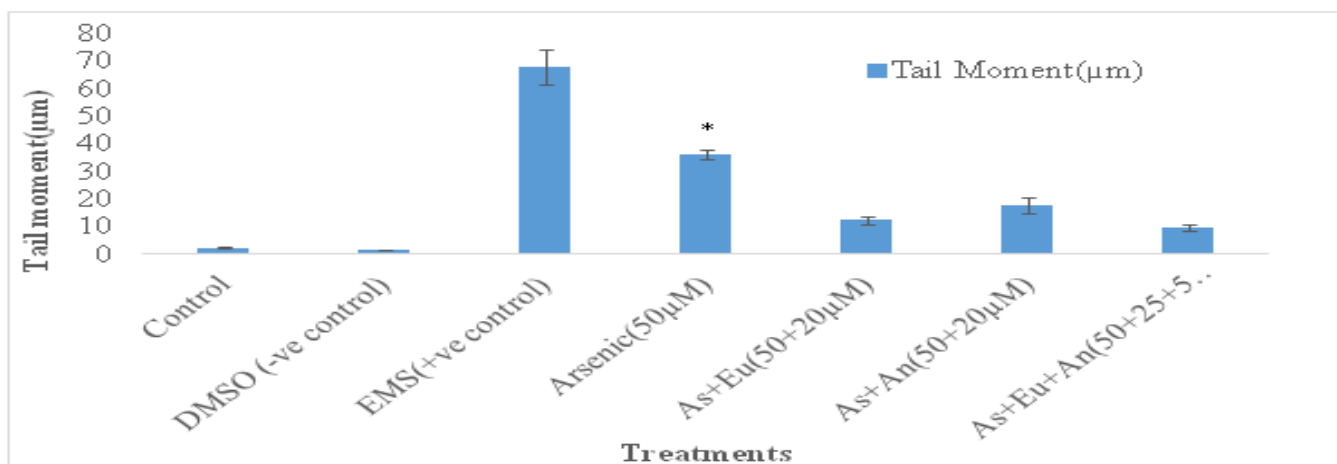


Fig. 1: Dose dependent decrease in tail moment with supplementation of eugenol and anethole in cultured lymphocytes against arsenic mediated genotoxicity. \*P<0.05 [significant as compared to untreated], As: Arsenic, An: Anethole and EMS: Ethyl methane sulphonate.

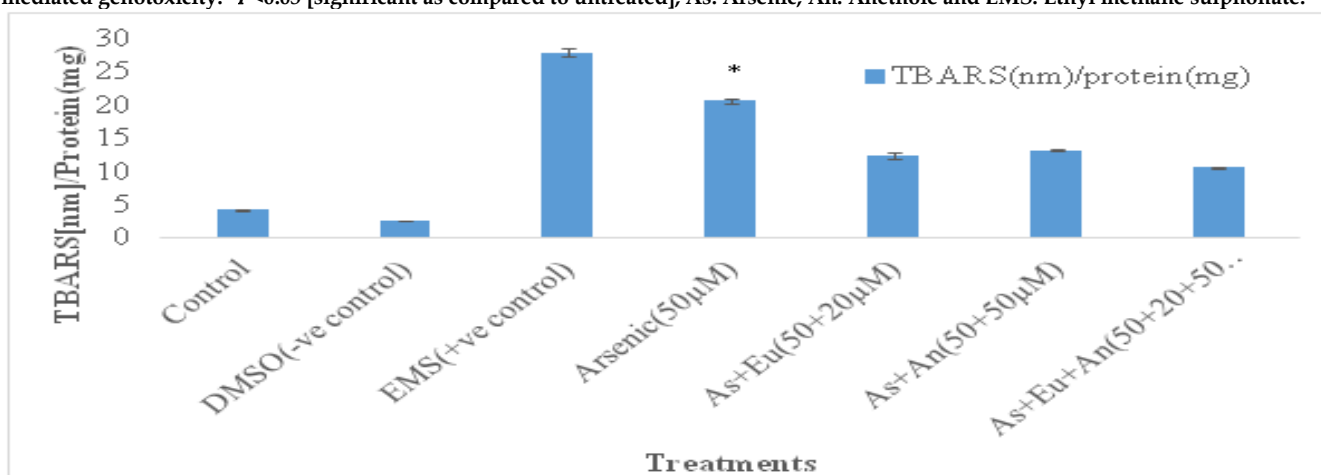


Fig. 2: Dose dependent decrease in TBARS levels with supplementation of eugenol and Anethole in cultured lymphocytes against arsenic mediated oxidative stress. \*P< 0.05 [significant as compared to untreated]; As: Arsenic, Eu: eugenol, DMSO: dimethyl sulphoxide and EMS: ethyl methane sulphonate.

Dietary phytochemicals present in commonly used vegetables, fruits, spices have been explored and found to play an important role as antitumor, antioxidant and antimutagenic agents. [6] In a study conducted by Nagababu *et al* [20], eugenol proved to be an active antioxidant agent both *in vivo* and *in vitro* analysis. Eugenol found to protective against iron and Fenton reagent mediated lipid peroxidation in *in vitro*. In another study eugenol and 6 bromoeugenol has shown antioxidant activity. [21] The antioxidant activity was analysed using 2, 2-diphenyl-1 picrylhydrazyl (DPPH) radical scavenging method. Anethole has not explored much as eugenol for antigenotoxic and antioxidant potential although in one study Shahat *et al* [22] have found antioxidant potential of fennel against oxidative stress. Later was also studied for genotoxicity and antigenotoxicity in *Drosophila melanogaster* using somatic mutation and recombination test (SMART) and showed a significant protective potential in eyes spot against Methyl methane sulphonate. [23]

In the present work we have analysed both individual and combined effect of eugenol and anethole against arsenic induced oxidative DNA damage in PBL. The results of the present study have shown a protective effect of eugenol and anethole against arsenic mediated

genotoxicity and oxidative stress. There was a significant decrease in lipid peroxidation levels and comet tail moment at arsenic toxic dosage (50µM) in cultures, when supplemented with eugenol (20µM) and anethole (50µM) separately. While in combination, there was a several fold increase in ameliorative potential of eugenol and anethole against arsenic mediated genotoxicity. Our results agree with the study conducted by Abraham *et al* [12] in mice where trans-anethole and eugenol were assessed for antigenotoxic effects. The doses of trans-anethole (40-400 mg/kg body weight) and eugenol (50-500 mg/kg weight showed anti-genotoxic effects against cyclophosphamide (CPH), procarbazine (PCB), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and urethane (URE) in mouse bone marrow micronucleus test.

Previous studies were limited to individual effects of phytochemicals; however the upcoming focus is on combinatorial effects of phytochemicals. This can be seen in the recent findings illustrating ameliorative additive or synergistic interactions of phytochemicals against oxidative stress, DNA damage induction and carcinogenesis. [24] To the best of our knowledge, no study has been conducted on healthy population



regarding the combined effect of eugenol and anethole against arsenic mediated genotoxicity and oxidative stress. The present work suggests that the use of indigenous food (eugenol and anethole) ingredients can act as a safer preventative medicine.

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