

***In Vitro* Antimutagenicity of *Allium Tuncelianum* Ethanol Extract Against Induction of Chromosome Aberration by Mutagenic Agent Mitomycine C**

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Summary

Allium Tuncelianum is a garlic species which is locally produced in Tunceli, Turkey. Garlic contains several biologically active compounds known as organosulfur compounds which are reported to have anticarcinogenic and antimutagenic effects. Present study examined the *in vitro* antigenotoxic activity of ethanolic extracts of *A. Tuncelianum* in human lymphocyte cells by chromosome aberration test. Samples of *A. Tuncelianum* were extracted by ethanol using soxhlet extractor. The extract was tested on human lymphocyte cell culture *in vitro* chromosome aberration test. The effect of ethanol extract of *A. Tuncelianum* at concentrations of 0.10, 0.15, 0.20, 0.25 µl/mL were screened for chromatid and chromosome breaks, chromosome exchange as well as chromatid union and polyploid cells against negative acetone and positive control Mitomycine C. A significant decrease in the frequency of chromosomal aberration was observed for all treatments with *A. Tuncelianum* ethanol extract at 24 h. In conclusion, ethanol extract of *A. tuncelianum* significantly reduced the chromosomal aberration rate as compared with the culture treated with MMC. The results could also indicate that *A. Tuncelianum* could be protective against mutagenic and carcinogenic compounds when consumed through the diet.

Keywords: *Allium Tuncelianum*, Antimutagenic, Chromosomal aberration, Toxicity

Mutajenik Ajan Mitomisin C ile Uyarılmış Kromozom Aberrasyona Karşı *Allium Tuncelianum* Etanolik Ekstraktının *In Vitro* Antimutajenik Etkinliği

Özet

Allium Tuncelianum, Tunceli/Türkiye'de lokalize olmuş bir sarımsak türüdür. Sarımsak bitkisi organosülfür bileşikler adı verilen, antikarsinojenik ve antimutajenik etkileri olduğu daha önceden bildirilmiş olan biyolojik yönden aktif komponentler içermektedir. Bu çalışmada *A. Tuncelianum* etanolik ekstraktının *in vitro* antigenotoksik aktivitesi, kromozom aberrasyon testi ile insan lenfosit hücre kültüründe çalışılmıştır. *A. Tuncelianum* ekstraktı etanol ile soxhlet kullanılarak elde edilmiştir. Bu araştırmada *A. Tuncelianum*'un etanolik ekstraktı insan lenfosit hücrelerinde kromozom aberrasyon testi ile antigenotoksik aktivitesi çalışılmıştır. *A. Tuncelianum*'un etanolik ekstraktı, 0.10, 0.15, 0.20 ve 0.25 µl/mL konsantrasyonlarda aseton (negative kontrol) ve Mitomisin C (pozitif kontrol)'ye karşı kromatid ve kromozom kırıkları, kromozom değişimi, kromatid birleşimi ve poliploidi bakımından taranmıştır. Uygulamadan 24 saat sonra, kromozomal aberrasyon frekansı bakımından *A. Tuncelianum*'un etanolik ekstraktı tüm konsantrasyonlarda önemli bir düşüş ortaya koymuştur. Sonuç olarak *A. Tuncelianum*'un etanolik ekstraktı MMC uygulanan kültür hücreleri ile karşılaştırıldığında kromozomal aberrasyonda önemli bir düşüşe neden olmuştur. Bu sonuçlara göre *A. Tuncelianum*'un mutajenite ve karsinojeniteye karşı koruyucu gıda olarak diyetle tüketilebileceği düşünülmektedir.

Anahtar sözcükler: *Allium Tuncelianum*, Antimutajenik, Kromozomal aberrasyon, Toksikite



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INTRODUCTION

It is known that diet rich in vegetables and fruits has the potential to lower the risk of cancer ¹. Recently, there has been a great deal of interest about the anti-mutagenic and anti-carcinogenic potential of compounds derived from plants and natural food stuff ². Garlic which is produced and consumed worldwide has drawn attention for its protective effects against a number of disorders ^{1,3}. Epidemiological studies showed that garlic contains many biologically and pharmacologically active compounds ³. It has been used for medical purposes since the ancient times, and its use for cancer treatment dates back to 3500 years ago ³. Garlic has antimutagenic, anticarcinogenic, anti-thrombotic, antineoplastic and antioxidant properties ^{2,4}. Experimental and epidemiological studies have shown that there is a correlation between garlic consumption and lower rate of cancer incidences ¹⁻³. It is reported that organosulfur compounds found in garlic confer its antitumor action ⁴. Furthermore, anti-mutagenic, anti-carcinogenic and antitoxic effects of garlic are also attributed to its active components including sulphydryl groups and organosulfur compounds as diallyl sulphide, diallyl disulphide, ajoene, allixin, allyl mercaptans and allyl methylsulphides found in the plant ⁵. Components of garlic selectively inhibit tumor proliferation by a number of mechanisms including control of DNA repair, chromosomal stability and regulation of cell cycle ³. Recent evidences suggest that inhibitory effect of garlic extract on adenosine deaminase and cyclic adenosine monophosphate cAMP phosphodiesterase enzymes play an important role in its biological and pharmacological actions ³. *Allium tuncelianum* is grown in the eastern part of Turkey, and it is an endemic genus which is peculiar to this region ⁶. It was reported that the biological activity of species varies with respect to the several factors including geographical location in which the plant grows, extraction method and section of the plant ⁷.

Genotoxic effects of chemical agents on human could be investigated through the screening tests which are utilized in genetic toxicology. These tests include chromosomal aberration test (CA), micronucleus test and sister chromatide exchange test (SCE) at the chromosomal level, Ames test at the gene level and single cell gel electrophoresis COMET assay at the molecular level ⁸. Treatment of cells with a DNA-damaging agent could result in irreparable lesions in both strands of DNA chain. Chromosomal breaks due to this type of insults can be determined in cells during metaphase stage. Although various cell lines could be used for this test, use of human lymphocytes is mostly preferred ⁹. The purpose of *in vitro* chromosomal aberration test in human lymphocytes is to determine structural chromosomal aberrations ¹⁰.

Although there are some studies about the various effect of garlic plant, *A. tuncelianum* is an endemic genus

which is specific to Tunceli district. There is no previously performed study on the effect of this genus for the anti-mutagenic effect.

In this study, the aim was to evaluate the antigenotoxic activity of crude ethanolic extracts of *A. tuncelianum* on human lymphocyte cell culture *in vitro* by using chromosome aberration test.

MATERIAL and METHODS

Collection of Plant Material

Samples of *A. tuncelianum* used in the study were collected from rural areas in Ovacik district of Tunceli province, Turkey in July 2011. The plants were dried in a room without receiving direct sun light. The plants were identified by biologist Mehmet N. Yilmaz Kafkas University, Faculty of Art and Sciences, Department of Biology.

Extraction of Garlic

The extraction procedure was performed by the method of Jabar and Mossawi ¹¹. Briefly, a sample of 100 g minced garlic was allowed to stand in 500 ml of ethanol at room temperature for 20 h, and then the mixture was filtered. The liquid portion was taken and evaporated at 30°C in a rotary evaporator ¹¹.

Chromosome Aberration Test with Human Lymphocytes

Human peripheral blood cells were used as a test system. Peripheral venous blood was collected from healthy, non-smoking adults aged 18-22 years old. Donors provided written, informed consent at the time of donation that the blood samples collected will be used in this study. Whole blood 0.4 mL was added to 5 mL chromosome medium Biochrome. Because longer exposure times caused considerable inhibition of cell division, the essential oil of *A. tuncelianum* was added after 48 h of culture initiation. Human lymphocytes were exposed to different concentrations 0.10, 0.15, 0.20, 0.25 µl/mL of ethanol extract. Negative acetone and positive control [mitomycin-C MMC, 0.3 µg/mL] was included in each experiment. Colchicine 0.06 µg/mL was added to each culture at 70 h. Cells were collected by centrifugation 377 X g, 10 min, and resuspended in a hypotonic KCl solution 0.4% for 30 min at 37°C. Following this process, cells were centrifuged again and fixed in a cold methanol: acetic acid 3:1 mixture for 35 min at +4°C. At the end of this procedure, cells were treated with fixative twice. Then, slides were made by dropping concentrated cell suspensions. Air-dried slides were stained for 15-20 min with 5% Giemsa stain pH 6.8 prepared in Sorensen buffer. One hundred metaphases per culture were analyzed for the presence of chromosomal aberrations (CA). The number of CAs was obtained by calculating the percentage of metaphases at each concentration and

treatment period that showed structural and numerical chromosome aberrations. Chromatid and chromosome breaks, chromosome exchange and chromatid union, and polyploid cells were screened at all treatment concentrations ¹².

Statistical Analysis

All statistical analyses were performed using Graph Pad In Stat version 3.05 for Windows 95 Graph Pad Software, San Diego California USA. The frequency of chromosomal aberrations in the cell cultures was analyzed using Fisher's exact test. Differences between the negative control and a series of treated groups were determined with Dunnett's t-test.

This study was approved by Kafkas University, Faculty of Medicine Ethics Committee with no. B.30.2.KAU.0.20.71.100/15.

RESULTS

A significant decrease in the frequency of chromosomal aberration was observed for all treatments with *A. Tuncelianum* ethanol extract at 0.10, 0.15, 0.20, 0.25 µl/mL at 24 h compared with the negative control Acetone and mitomycin C (MMC: 0.3 µg/mL), which was used as a positive control (Table 1). Examination of lymphocyte chromosomes revealed that chromosome exchange and chromatid union as well as chromatid and chromosome breaks in both the positive control and in treatment groups at all concentrations were present. Polyploidy was observed at concentrations of 0.20 and 0.25 µl/mL for the extract, and upon treatment with the positive control. Statistical analysis demonstrated significant increases in CA frequency in a dose dependent manner 24 h after addition of ethanol extract as compared with the negative control

($r=0.95$). Mitomycin treatment also caused a significant increase $P<0.001$ in the frequency of aberrant cells. Ethanol extract of *A. Tuncelianum* at all concentrations clearly inhibited the mitotic index in human lymphocytes Table 1.

Treatment of ethanol extract of *A. Tuncelianum* significantly reduced the chromosomal aberration rate as compared with the culture treated with MMC.

DISCUSSION

A significant difference in the frequency of chromosomal aberration was observed between ethanol extract of *A. Tuncelianum* at all concentrations; 0.10, 0.15, 0.20, 0.25 µl/mL and MMC group as well as negative control $P<0.05$. Bhattacharya *et al.* reported that garlic causes micronuclei induction in the mouse bone marrow cells ⁴. In this study, the high aberration frequency observed as a result of applying *A. Tuncelianum* ethanol extract at 0.20 and 0.25 µl/ml could be linked to the toxic effect of the plant type as reported. Antigenotoxic effect of different garlic species was reported previously. The protective effect of water extract of garlic against asbestos Rhodesian Chrysotile-induced mutagenicity on human peripheral lymphocytes was reported *in vitro*. Simultaneous treatment of Rhodesian Chrysotile and garlic extract caused a significant decrease in micronucleus formation, sister chromatid exchange and chromosome aberration frequency ⁴. Furthermore, Bhuvaneswari *et al.*² studied the protective effect of water extract of garlic and tomatoes against 7,12-dimethylbenz[a]anthracene DMBA-induced buccal pouch carcinogenesis in hamsters. It was observed that mean tumor load and incidence were effectively suppressed in group treated with garlic and tomatoes. In another study, the antigenotoxic effect of garlic organo-sulfur compounds OSCs including allicin DADSO, diallyl

Table 1. Frequencies of Chromosome Aberrations and Mitotic Index in Cultured Human Lymphocytes Treated with *A.tuncelianum* ethanol extract and *A.tuncelianum* plus MMC

Table 1. *A. tuncelianum* etanol ekstraktı ve *A.tuncelianum* etanol ekstraktı ile MMC uygulanmış insan lenfosit hücrelerinde kromozom aberrasyon ve mitotik indeks

Test Substance	Treatment		Structural Aberrations				Numerical Aberrations	Frequency of Aberrant Cell ± SEM(%)	Mitotic Index ± SEM(%)
	Period (h)	Doses (µL/ml)	ctb	csb	cse	cu	p		
Negative Control	24	1	1	-	-	-	-	0.2±0.2	4.3±0.3
Mitomycine C	24	0.3	17	2	3	8	1	6.2±2.9*	1.7±1.2*
<i>Allium tuncelianum</i> ethanol extract	24	0.10	1	-	1	2	-	0.8±0.3	3.2±0.5*
		0.15	2	-	1	1	-	0.8±0.3	2.7±0.4*
		0.20	3	-	2	-	-	1.0±0.6	2.4±0.6*
		0.25	2	1	3	4	1	2.2±0.5	2.1±0.4*
<i>Allium tuncelianum</i> ethanol extract + MMC	24	0.10	14	3	3	5	-	5.0±2.3*	3.3±0.5*
		0.15	13	1	3	5	-	4.4±2.3	3.6±0.2*
		0.20	9	-	4	6	-	3.8±1.7	3.9±0.3*
		0.25	5	-	2	4	-	2.2±1.0	4.1±0.4*

ctb: chromatid break, csb: chromosome break, cu: chromatid union, cse: chromosome exchange, negative control (1% distilled water), MMC: (0.3 µg/ml mitomycin-C (24 h), p: polyploidy, * $P<0.05$ as compared to negative control (Dunnett's test). Mitotic index

sulfide DAS, diallyl disulfide DADS, S-allyl cysteine SAC and allyl mercaptan AM was tested in the human hepatoma cell line HepG2 using comet assay. The organosulfur compounds at test concentrations ranging from 5 to 100 μM did not affect cell viability and caused no DNA damage. SAC and AM significantly reduced DNA break in dimethylnitrosamine treated Hep G2 cells. In addition, garlic organosulfur compounds decreased the genotoxicity of hydrogen peroxide and methyl methanesulfonate. These studies indicate that garlic organosulfur compounds have antigenotoxic effects¹. Choudhury *et al.*⁸ studied the protective effect of garlic and mustard oil against clastogenic activity of sodium arsenite by evaluating clastogenic activity in the bone marrow cells of rats with respect to the chromosomal aberration. The aqueous extract of dietary garlic and mustard oil applied for 30 days was reported to decrease the clastogenic effect of sodium arsenite.

Environmental pollution and accumulation of toxic chemicals due to widespread use of chemical agents as in pesticides result in increased cancer rates in humans¹³. It was reported that anti-genotoxic compounds consumed within the diet could decrease the increased number of these cases¹. Plant extracts rich in polyphenols have antimutagenic potentials and DNA-protecting effects¹⁴. The mechanism of protection against cancer by *Allium* species and OSCs were reported to involve inhibition of mutagenesis, inhibition of adduct formation, free-radical scavenging and effects on cell proliferation and tumor growth³. Mutagenic and carcinogenic compounds such as polycyclic aromatic hydrocarbons exert their effects primarily through the production of electrophilic intermediates leading to mutations in microsomal enzymatic reactions. Antigenotoxic agents found in natural substances affect various cellular pathways including endogenous sequestration of mutagens via various enzymes⁹. Anticancer effects of garlic are reported to be due to the presence of OSCs which are released during processing¹⁵. In addition, previous studies showed that the constituents of garlic especially sulfhydryl compounds have anticarcinogenic and antimutagenic effects. The protective effect of garlic is also attributed to scavenging of potentially toxic and mutagenic electrophiles and free radicals as well as modification of phase I and II enzymes involving in detoxification pathways. Furthermore, the ally group of garlic constituents increases GST level which helps the detoxification of mutagens and carcinogens⁹. The apparent effect of *A. Tuncelianum* on anticarcinogenic effect observed *in vitro* chromosomal aberration test could involve all of these mechanisms.

In conclusion, increasing number of different environmental pollutants, toxins and chemicals pose a great risk for human health in terms of increasing cancer ratios

in today worlds. It is important to reduce or alleviate the adverse effects of environmental carcinogens and mutagens through daily diet. The results of this study showed that *A. tuncelianum* could be protective against mutagenic and carcinogenic compounds by preventing mutagenic effects.

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