Cumhuriyet Science Journal
CSJ

e-ISSN: 2587-246X
ISSN: 2587-2680

Cumhuriyet Sci. J., Vol.39-1(2018) 127-143

Cytotoxic and Genotoxic Effects of Aqueous Extracts of *Rosmarinus officinalis* L., *Lavandula stoechas* L. and *Tilia cordata* Mill. on *in vitro* Human Peripheral Blood Lymphocytes

Tulay ASKIN CELIK*, Ozlem Sultan ASLANTURK

Department of Biology, Faculty of Arts and Sciences, Adnan Menderes University, Aydın, TURKEY

Received: 07.07.2017; Accepted: 30.11.2017 *http://dx.doi.org/10.17776/csj.405629*

Abstract: The aim of this study was to investigation of *in vitro* cytotoxic and genotoxic effects of infusion and decoction aqueous extracts obtained from *Rosmarinus officinalis, Lavandula stoechas* L. and *Tilia cordata* Mill. on human peripheral blood lymphocytes.

For this purpose, lymphocytes taken from 3 females, were treated with aqueous extracts of *R. officinalis* (1.5 and 3 mg/ml), *L. stoechas* (0,4 and 1,2 mg/ml) and *T. cordata* (1 mg/ml and 3 mg/ml) for 48 hours. PBS was used as negative control and Mitomycin C (MMC) (0,25 µg/ml) was used as positive control in experiments. Cytotoxic effect of extracts on lymphocytes was determined by calculating mitotic index. Also, chromosome aberrations micronucleus formations were determined.

All extracts considerably decreased the cell division in lymphocytes depending on the increased concentration in comparison with control groups ($p<0,01$). Also cells in metaphase decreased. Thus, no chromosomal aberration was observed in lymphocytes. However, extract treatments induced the formation of micronucleus in lymphocytes when compared to control groups ($p<0,01$). 3 mg/ml decoction extracts of *L. stoechas* and *T. cordata* showed the highest cytotoxic effect on lymphocytes, and the highest genotoxic effect appeared after 48 h treatment with 1.2 mg/ml decoction extract of *L. stoechas*.

***Keywords*:** Cytotoxic effect, genotoxic effect, human peripheral lymphocytes, *Lavandula stoechas, Rosmarinus officinalis, Tilia cordata*

Rosmarinus officinalis L., *Lavandula stoechas* L. and *Tilia cordata* Mill. Sulu Ekstrelerinin *in vitro* İnsan Periferal Kan Lenfositleri Üzerindeki Sitotoksik ve Genotoksik Etkileri

Özet: Bu çalışmanın amacı *Rosmarinus officinalis*, *Lavandula stoechas* L. ve *Tilia cordata* Mill'den elde edilen sulu ekstrelerin (infüzyon ve dekoksiyon), insan periferal kan lenfositleri üzerindeki in vitro sitotoksik ve genotoksik etkisinin araştırılmasıdır.

Bu amaçla, 3 kadından alınan lenfositler, *R. officinalis* (1.5 ve 3 mg / ml), *L. stoechas* (0,4 ve 1,2 mg/ ml) ve *T. cordata* (1 mg / ml ve 3 mg / ml) sulu ekstreleri ile 48 saat süreyle muamele edilmiştir. Denemelerde PBS negatif kontrol olarak, Mitomisin C (MMC) (0,25 µg / ml) ise pozitif kontrol olarak kullanılmıştır. Ekstrelerin lenfositlerdeki sitotoksik etkisi, mitotik indeks hesaplanarak belirlenmiştir. Ayrıca, lenfositlerideki kromozom aberasyonları ve mikronukleus oluşumları da belirlenmiştir.

Deneyde kullanılan sulu ekstreler, kontrol gruplarına kıyasla konsantrasyon artışına bağlı olarak, periferal lenfosit hücrelerinde bölünmeyi önemli ölçüde azaltmıştır ($p <0,01$). Yine, metafazdaki hücrelerin sayısı da azalmıştır. Bu nedenle, periferal lenfositlerde kromozom incelemesi yapılamamıştır. Bununla birlikte, sulu ekstre muameleleri periferal lenfositlerde kontrol gruplarına kıyasla mikronukleus oluşumuna neden olmıuştur ($p <0,01$).

* Corresponding author. *Email address: tcelik@adu.edu.tr*
http://dergipark.gov.tr/csj ©2016 Faculty of Science, Cumhuriyet University

L. stoechas ve *T. cordata'nın* 3 mg / ml dekoksiyon ekstreleri, 48 saatlik muameleden sonra lenfositler üzerinde en yüksek sitotoksik etkiyi göstermiştir. En yüksek genotoksik etki ise 1.2 mg / ml *L. stoechas* dekoksiyon ekstresi ile muameleden sonra ortaya çıkmıştır.

***Anahtar Kelimeler*:** Sitotoksik etki, genotoksik etki, insan periferal kan lenfositleri, *Lavandula stoechas*, *Rosmarinus officinalis*, *Tilia cordata*

1. INTRODUCTION

Medicinal plants have always played an important role in the treatment of human diseases all over the world. Bioactive components of medicinal plants are important for discovering new drugs such as chemotherapeutics [1, 2]. According to ethnomedicinal uses, some plant compounds are used in therapies for different widespread diseases, including cancer. However, unconsciously and excessive usage of medicinal plants can cause poisoning and death in human and animals.

Rosmarinus officinalis L. belongs to Lamiaceae (Labiatea) family and commonly referred to as rosemary. Rosemary has antibacterial [3,4], effective chemo preventive and antimutagenic properties [5]. It has also been reported that extracts of this plant have anti-carcinogenic, cognition-improving and certain glucose level lowering properties, hepatoprotective, antidiabetic, antioxidant, antiproliferative, antiviral, antimicrobial, antinociceptive and antidepressant, among others [6,7]. It has been previously reported that rosemary extracts and their components show inhibitory effects on the growth of breast, liver, prostate, lung and leukemia cancer cells and represses the initiation and promotion of tumorigenesis of melanoma and glioma in animal models [8]. Rosemary have also andioxidant capacity due to the presence of phenolic subtances such as carnosol, rosmanol, carnosic acid, methyl carnosate, rosmarinic and caffeic acids [9].

Tilia cordata Mill. (Lime tree, Linden) is a tall deciduous tree native throughout Europe as far north as 65 in latitude, which can grow to heights approaching to 30 metres. It is found in the wild and purposely planted in gardens. It is also cultivated in Europe and North America while the material of commerce originates mainly from Balkan countries such as Bulgaria, Romania, former Yugoslavia, Turkey and in part from China [10].

The lime flowers have been used as a diaphoretic to promote perspiration, tranquiliser and to treat headaches, indigestion and diarrhoea, chills, bronchitis, fever, inflammations and influenza infections. Traditionally lime flowers were added to baths to quell hysteria and steeped as a tea to relieve anxiety-related indigestion, heart palpitation and vomiting [11, 12,13].

Lavandula is an important member of Labiatae (Lamiaceae) family. Lavandula species are widely distributed in the Mediterranean region and cultivated in France, Spain and Italy. In Turkey mainly two species, *L. angustifolia* and *L. stoechas* and their subspecies and hybrid forms grow wildly or they are cultivated [14,15]. *L. stoechas* is known as French lavender [16] and it is used for various diseases of central nervous system (epilepsy and migraine), treatment of wounds, reduce to blood sugar, as antispasmodic, antiseptic, antimicrobial, sedative, diuretic and analgesic [17-19]. Several essential oils from *Lavandula* have been reported to possess antinociceptive, gastroprotective, anti-inflammatory, analgesic, antiplatelet, antithrombotic, and antifeedant effects [20], neuroprotective, antioxidant, anti-cholinesterase, anticonvulsant, sedative, antidepressant and antispasmodic properties [21]. *Lavandula* species contain different secondary metabolites, such as monoterpenes, diterpenes, sesquiterpenes [20-22] and phenolic compounds such as flavonoids, phenolic acids, coumarins, tannins [23].

Determination of the potential genotoxic effect of a chemical substance is possible with short-term genotoxicity tests such as Chromosome Aberration (CA) [24, 25], mitotic index (MI), sister chromatid exchange (SCD) [26], and micronucleus (MN) tests [27,28, 29]. The mitotic index (MI) is used as a biomarker of cell proliferation and measures the proportion of cells in the M-phase of the cell cycle. The decrease or inhibition of MI is assessed as a delay in cell death or cell proliferation kinetics [30,31]. High CA frequency may be an indication of high cancer risk, regardless of the reason for initiating CA increase because it has been reported that CA formation may also result from incorrect repair of chain fractures in DNA [32-34,35].

R. officinalis, L. stoechas, and *T. cordata* have been used to alternative medicine for many years in Turkey [18]. Their increasing use in recent years is clear evidence of public interest in having alternatives to conventional medicine. Although numerous studies are present about various effects of these plants, there are very few studies reporting the cytotoxic and genotoxic effects of them on human lymphocyte cells. The aim of this study is to investigate the cytotoxic and genotoxic effects of aqueous extracts from rosemary *Rosmarinus officinalis* L., *Lavandula stoechas* L. *Tilia cordata* Mill. on human peripheral lymphocytes

2. MATERIAL and METHODS

2.1 Plant Material

In this study, human peripheral blood was used as a test system and *Lavandula stoechas* L (French lavander), *Rosmarinus officinalis* L (Rosemary), and *Tilia cordata* Mill (Lime) were used as a test substance. Aerial parts of *Lavandula stoechas* L (French lavander) were collected from Aydin-Çine Dam Area (Aydın, Turkey), at the flowering stage, in April 2006, *Rosmarinus officinalis* L (Rosemary) were collected from Adnan Menderes University, Central Campus Road (Aydın, Turkey), in March 2007, and *Tilia cordata* Mill (Lime) was purchased in August 2007 from a local market. Plant identification was carried out by Dr. Özkan Eren and Dr. Mesut Kırmacı who are botanist at Adnan Menderes University, Faculty of Art and Science, Department of Biology. Voucher specimens of the plants have been deposited in Adnan Menderes Herbarium (AYDN 1209 for *Lavandula stoechas* L, AYDN 2470 for *Rosmarinus officinalis* L., AYDN 2471 for *Tilia cordata* Mill). The plant aerial parts were cleaned and air-dried at room temperature in the shade, and then powdered.

2.2. Preparation of extracts

Aqueous extracts of plants were prepared by infusion and decoction methods. The concentrations of the extracts used in the experiments were determined according to the traditional usage of the plants by Turkish people; 0.4 and 1.2 mg / ml for *L. stoechas*; 1.5 mg / ml and 3 mg / ml for *R. officinalis;* 1 mg / ml and 3 mg / ml for *T. cordata.*

For infusion extraction, 150 ml boiled distilled water was added to 15 g plant material for 10 min and then extracts were filtered. For decoction extraction, 150 ml distilled water was added to 15 g plant material and boiled for 10 min. Then extracts were filtered. Filtered extracts were freezed and lyophilized. After lyophlization, they were sealed in glass bottles and stored at -20 °C until they were used.

2.4. Lymphocyte culture and extract treatments

The peripheral bloods of three healthy, 20-25 years old, non-smoking female volunteers, that they are not exposed to radiation or drug, were taken after free informed consent. A 5 ml blood sample was aseptically collected in heparinized sterilized glass tubes from each volunteer. The volunteers did not present disease symptoms and reported not having used prescription drugs for at least 30 days prior to collection.

Peripheral blood cell cultures and preparation of preparations were made according to Evans (1984) [36]. In addition, this study was prepared according to IPCS guidelines. Peripheral blood samples were transferred to sterile culture tubes

containing 2.5 ml Chromosome Medium B in 6 drops (0.2 ml) under sterile conditions and blood samples were left for 24 hours incubation at 37ºC [37]. Experiments were repeated three times.

For two different concentrations of different plants prepared by dissolving in PBS (1.5 mg / ml and 3 mg/ml *R. officinalis*, 0.4 mg/ml and 1.2 mg/ml for *L. stoechas* and 1 mg/ml and 3 mg/ml for *T. cordata* prepared aqueous extracts were added to the culture tubes at 24 hours of lymphocyte cultures and incubation continued for 48 hours. In addition, lymphocyte cultures that were not treated with plant extracts as negative control (PBS) were used in each experiment, Mitomycin-C (MMC) added to the culture tube as a positive control with a final concentration of 0.25 µg / ml.

The hypotonic solution was added dropwise to the cells to prevent clumping and the cell suspension was homogenized by pipetting. After each tube was added 5 mL of hypotonic solution (0.075 M KCl), the sealed tube was incubated at 37 ° C for 20 min. At the end of the supernatant the cell suspension was centrifuged at 800 g for 10 min and the supernatant discarded. Then cold fixative (3: 1, methanol: glacial acetic acid) was added, as with the addition of the hypotonic solution, so that each tube was 5 ml, with gradual and stirring.

Cells treated with 10 min fixative at room temperature were centrifuged at 800 g for 10 min. Centrifuged and the supernatant discarded and the fixative added. This process has been repeated 3 times. The liquid remaining at the end of the fixative treatment was completely clear. After the last centrifugation, the supernatant was discarded in the form of 0.5-0.7 ml of liquid at the bottom and the preparation process was started.

Cells remaining in the bottom of the tube are mixed with pasteur pipet, and the cell suspension and the spreading of the cells and chromosomes on the slide are performed by dropping the cell suspension (3 to 4 drops per each slide) on the per each slides which have been previously cleaned and kept in the refrigerator in distilled water. During the dropping of the cell suspension to the slides, care was taken that the droplets do not fall over. Slides were dried for 24 hours at room temperature.

2.4.3. Staining of slides

Prepared slides were stained with 4% Giemsa prepared in Sörensen buffer [38].

2.4.4. Microscopic Analysis

The slides were examined with an immersion lens (10x100 = 1000 MB) on an Olympus BX-50 binocular light microscope.

2.4.5. Calculation of Chromosomal Damages (Aberration, CA), Mitotic Index (MI) and Micronucleus (MN)

A total of 100 cells per slides (300 cells from 3 slides) with well-dispersed chromosomes found in the metaphase from each individual preparation were examined to determine structural and numerical chromosomal aberrations (CA) that would occur in peripheral lymphocytes after extract treatments. The percentage of chromosomal aberrations were calculated [39, 40]. The mitotic index (MI) [41] and the number of micronucleated cells (MN) [42, 43] were calculated by calculating the number of metaphases found in 1000 cells per culture as well as chromosomal aberrations.

2.5. Statistical Analysis

CA, MI and MN data obtained from control and treated groups were analyzed by One-Way ANOVA in SPSS 11.5 package program and the results were compared with negative control and positive control group. The significance of the statistical differences between the control groups and the treatment groups were determined by Duncan's Multiple Range Test.

3. RESULTS

The *in vitro* cytotoxic and genotoxic effects of aqueous extracts obtained from three different plants (*R. officinalis, L. stoechas,* and *T. cordata*) on peripheral lymphocytes were assessed separately for each plant.

3.1 *Rosmarinus officinalis* L.

The *in vitro* cytotoxic and genotoxic effects of *R. officinalis* infusion and decoction extracts (1.5 mg/ml and 3 mg/ml) on lymphocytes after 48 hours treatment are summarized in Table 1 and Figures 1. *R. officinalis* infusion and decoction extract treatments decreased MI (1 mg/ml = 3.54±4.45%; 3 mg/ml = 0.56 ± 0.96% for infusion extract treatment; 1 mg/ml = 4.33 ± 2.51%; 3 mg/ml= 0.67± 0.58% for decoction extract treatment). Decrease of MI were statistically significant (p <0.01) when compared with the negative control (PBS; 8.90±0.17%).

Table 1. Mitotic Index, micronucleus, and chromosomal aberrations in human lymphocyte cultures treated with infusion and decoction extracts of *R. officinalis.*

Groups	Treatment time (h)	Concentrations (mg/ml)	Chromosome Aberrations			Total Chromosome aberrations (%) ± SD	Micronuclei (MN) (‰) ± SD	Mitotic index(MI) (%) ± SD
			Chromosome breakage (%) ± SD	Acentric Fragment (%) ± SD	Dicentric Chromosome (%) ± SD			
Control (PBS)	48 h		-----	-----	-----	-----	-----	8,90±0,17
Positive control (MMC)	48 h	0,25µg/ml	1,10±0,17	1,83±0,29	-----	2,93±0,93*	7,89±2,50*	1,67±1,66*
Infusion extract	48 h	1,5	0,22±0,38	0,22±0,38	0,22±0,38	1,22±0,43	2,11±2,83	3,54±4,45*
Infusion extract	48 h	3	-----	-----	-----	-----	8,00±0,67*	0,56±0,96*
Decoction extract	48 h	1,5	-----	-----	-----	-----	6,11±0,84*	4,33±2,51*
Decoction extract	48 h	3	-----	-----	-----	-----	7,78±0,51*	0,67±0,58*

*p<0,01

The results of MN test are given in Table 1. MN results showed that 3 mg/mL concentration of *R. officinalis* infusion extract (8.00±0.67 ‰) increased MN compared with the negative control (PBS= (0.00 ‰) and positive control (MMC = 7.89± 2.50 ‰). Micronuclei formation is increased in relation with the increase of extract concentration. These results showed that the *R. officinalis* infusion extracts triggered the formation of micronucleus more than the decoction extract. This difference between infusion and decoction extracts were statistically significant (p <0.01) (Table 1). Consequently, the data obtained from *R. officinalis* suggested that infusion and decoction extracts significantly reduced the cell division in human peripheral blood lymphocytes with increasing concentration, so lead to a cytotoxic effect.

After treatment of the infusion and decoction extracts obtained for 48 hours, chromosomal aberrations, (CAs) (chromosomal breakage, ascentric fragment, dycentric chromosome) in lymphocytes was observed after treatment of only 1.5 mg/ml infusion extract (1.22 %), 3 mg/ml infusion and decoction extract did not lead to CAs at both treatment concentrations. chromosomal aberrations were found 2.93% in the positive control, but in negative control CAs were not found. CAs value in positive control was statistically significant in comparison with negative control (p <0.01) (Table 1, Figure 1).

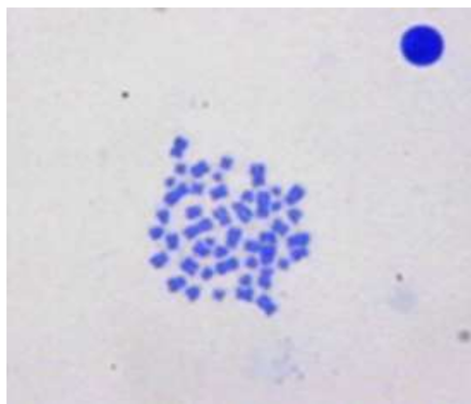

Figure 1a. Control-metaphase**.**

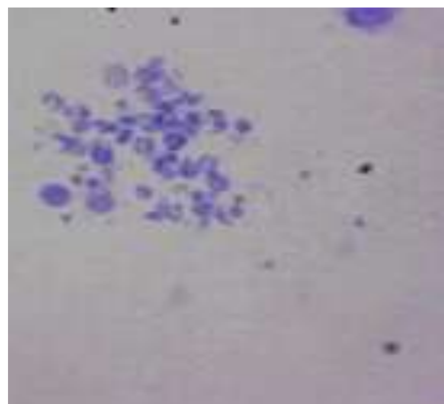

Figure 1b. Positive control breakage in metaphase.

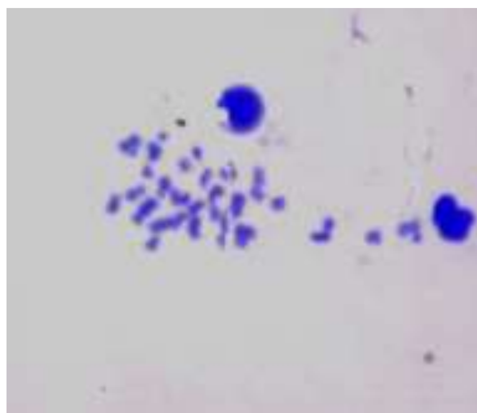

Figure 1c. Dicentric chromosome.

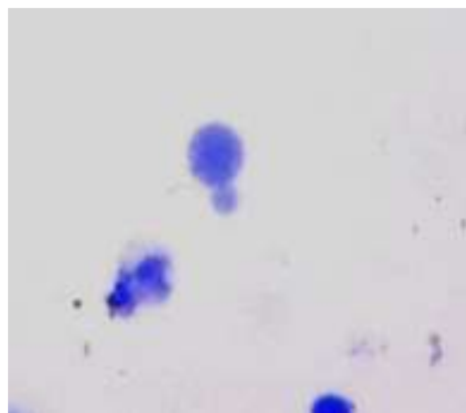

Figure 1d. Micronucleus.

Figure 1. Aberrant cells in human peripheral lymphocytes by infusion and decoction extracts of *Rosmarinus officinalis* L. (M.B. 100x).

3.2 Lavandula stoechas L.

The effects of *L. stoechas* infusion and decoction extracts (0.4 mg/ml and 1.2 mg/ml) on lymphocytes after 48 h are summarized in Table 2 and Figures 2. Results showed that after 48 h of peripheral lymphocytes culture in the presence of 0.4 and 1.2 mg/ml concentrations of *L. stoechas* infusion and decoction extracts, MI decreased (0.4 mg/ml=1.44±0.19 %; 1.2 mg/ml=1.11±0.16 % for infusion extract treatment; 0.4 mg/ml=0.0%; 1.2 mg/ml= 0.33±0.58% for decoction extract) compared with the negative control (PBS) (8.90±0.17 %) and positive control (MMC = 1.67 ± 1.66%). This decrease was statistically significant ($p < 0.01$) (Table 2, Figure 2). Significant reduction in cell division observed in peripheral lymphocytes suggests that compounds found in *L. stoechas* extracts may caused cytotoxic effect on human peripheral lymphocytes.

Table 2. Mitotic Index, micronucleus, and chromosomal aberrations in human lymphocyte cultures treated with infusion and decoction extracts of *L.stoechas*.

Groups	Treatment time (h)	Concentrations (mg/ml)	Chromosome Aberrations			Total Chromosome aberrations (%) ± SD	Micronuclei (MN) (‰) ± SD	Mitotic index (MI) (%) ± SD
			Chromosome breakage (%) ± SD	Acentric Fragment (%) ± SD	Dicentric Chromosome (%) ± SD			
Control (PBS)	48 h		-----	-----	-----	-----	-----	8,90±0,17
Positive control (MMC)	48 h	0,25µg/ml	1,10±0,17	1,83±0,29	-----	2,93±0,93*	7,89±2,50*	1,67±1,66*
Infusion extract	48 h	0,4	-----	-----	-----	-----	9,89±0,69*	1,44±0,19*
Infusion extract	48 h	1,2	-----	-----	-----	-----	12,00±0,33*	1,11±0,16*
Decoction extract	48 h	0,4	-----	-----	-----	-----	11,44±0,51*	0,00±0,00*
Decoction extract	48 h	1,2	-----	-----	-----	-----	13,33±0,34*	0,33±0,58*

*p<0,01

The results of MN test are given in Table 2. Despite the absence of MN in the negative control, the MN ratio in the positive control was 7,89 ‰ (p <0.01). MN results showed that *L. stoechas* infusion extract (0.4 mg/mL= 9.89.00 ± 0.69 ‰ ; 1.2 mg/ml= 12.00 ± 0.33 ‰) increased MN. Increase of MN formation in lymphocytes after infusion extract treatments was found statistically significant in comparison with the negative and positive control groups (p <0.01). Treatment with decoction extract also resulted in the formation of MN at high levels in lymphocytes (respectively; 11.44 ‰ and 13,33 ‰). This increase in decoction extract treatment groups is statistically significant (p <0,01) when compared with the negative and positive control group. These results showed that the *L stoechas* decoction extracts triggered the formation of micronucleus more than the infusion extract (Table 2, Figure 5). Apoptotic cells were also found in lymphocytes after treatments 1.2 mg/ml *L. stoechas* infusion and decoction extracts of (Fig. 2).

Chromosomal damages (chromosomal breakage, ascentric fragment, dysenteric chromosome) in peripheral lymphocytes were observed only in the positive control group and total chromosomal aberration was 2.93% (p <0.01). In the negative control group and extract treatment groups, CAs were not found (Table 2, Fig. 2).

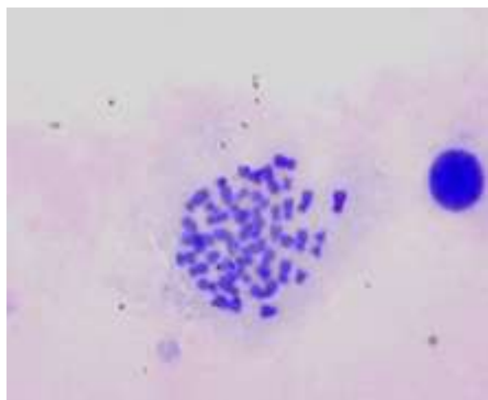

Figure 2a. Control – metaphase.

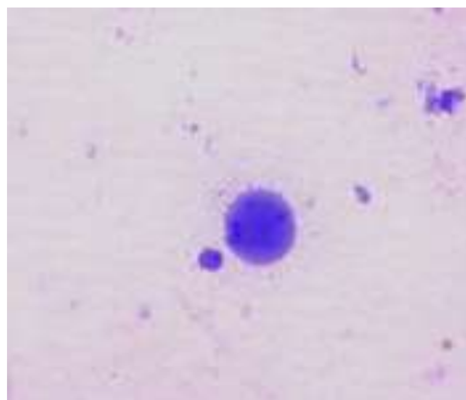

Figure 2b. Positive control – micronucleus.

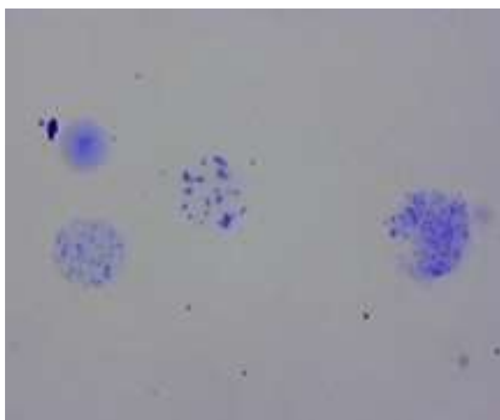

Figure 2c. Apoptotic cells

Figure 2. Aberrant cells in human peripheral lymphocytes by infusion and decoction extracts of *L. stoechas* L. (M.B. 100x).

3.4 Tilia cordata Mill.

The effects of *T. cordata* infusion and decoction extracts (1 mg/ml and 3 mg/ml) for 48 h are summarized in Table 3 and Figures 3. These results showed that after 48 h of peripheral blood culture in the presence of different concentrations of *T. cordata* the infusion and decoction extracts, significantly reduced MI and the mitotic index value of 8.90±0.17 % in the negative control group was lower than the positive control (1.67±1.66 %) in the 1 mg / ml infusion extract (1.33±2.31%). In groups treated with 3 mg / ml infusion extract and 1 to 3 mg / ml decoction extract, the extract treatment completely inhibited the cleavage of lymphocytes and reduced the MI value. Microscopic examinations of the preparations of these groups did not reveal any metaphase cells (Table 3 and Fig. 3). This decrease in MI in peripheral lymphocytes as a result of extract treatment is also statistically significant ($p <0.01$). The significant reduction in cell division in peripheral lymphocytes treated with *T. cordata* infusion and decoction extracts of depending on the concentration suggests that the compounds contained in the *T.cordata* extracts cause cytotoxic effect in peripheral lymphocytes.

The effects of *T. cordata* infusion and decoction extracts (1 mg/ml and 3 mg/ml) on lymphocytes after 48 h are summarized in Table 3 and Fig.3. Results showed that after 48 h of peripheral lymphocytes culture in the presence of 1 and 3 mg/ml concentrations of T.cordata infusion and decoction extracts, MI decreased (1 mg/ml=1.33±2.31 %; 3 mg/ml=0.00 % for infusion extract treatment; 1 and 3 mg/ml=0.0 % for decoction extract treatment) compared with the negative control (PBS) (8.90±0.17 %) and positive control (MMC=1.67±1.66%). This

decrease was statistically significant (p <0.01) (Table 3, Fig. 3). Significant reduction in cell division observed in peripheral lymphocytes suggests that compounds found in *T. cordata* extracts may caused cytotoxic effect on human peripheral lymphocytes.

Table 3. Mitotic Index, micronucleus, and chromosomal aberrations in human lymphocyte cultures treated with infusion and decoction extracts of *T.cordata.*

Groups	Treatment time (h)	Concentrations (mg/ml)	Chromosome Aberrations			Total Chromosome aberrations (%) ± SD	Micronuclei (MN) (‰) ± SD	Mitotic index (MI) (%) ± SD
			Chromosome breakage (%) ± SD	Acentric Fragment (%) ± SD	Dicentric Chromosome (%) ± SD			
Control (PBS)	48 h		-----	-----	-----	-----	-----	8,90±0,17
Positive control (MMC)	48 h	0,25µg/ml	1,10±0,17	1,83±0,29	-----	2,93±0,93	7,89±2,50*	1,67±1,66*
Infusion extract	48 h	1	-----	-----	-----	-----	10,11±1,07*	1,33±2,31*
Infusion extract	48 h	3	-----	-----	-----	-----	11,34±0,58*	0,00±0,00*
Decoction extract	48 h	1	-----	-----	-----	-----	9,67±1,20*	0,00±0,00*
Decoction extract	48 h	3	-----	-----	-----	-----	12,00±0,33*	0,00±0,00*

*p<0,01

MN formation increased after extract treatments depending on increasing extract concentrations and this increase was found statistically significant when compared with controls (p<0.01). Treatment with decoction extract also resulted in the formation of MN at high levels in lymphocytes (respectively; 9.67±1.20 ‰ and 12.00±0.33 ‰). This increase in decoction extract treatment groups are statistically significant (p <0,01) when compared with the negative and positive control group. These results showed that the *T. cordata* decoction extracts triggered the formation of micronucleus more than the infusion extract. This increase was statistically significant (p <0.01) (Table 3, Figure 3). As a result of infusion and decoction extracts treatment, no chromosomal damage was observed in peripheral lymphocyte cells (Table 3). However, cells with single MN, as well as two or more micronucleus and cells with more than one nucleus were found (Figure 3).

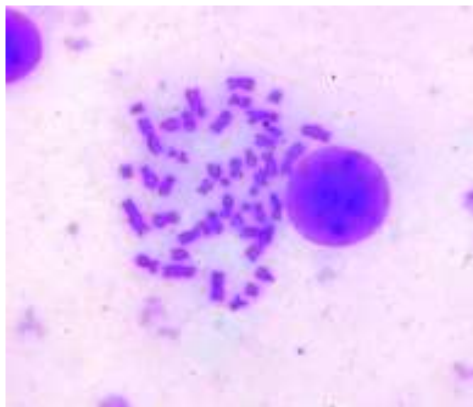
Figure 3a. Control – metaphase.

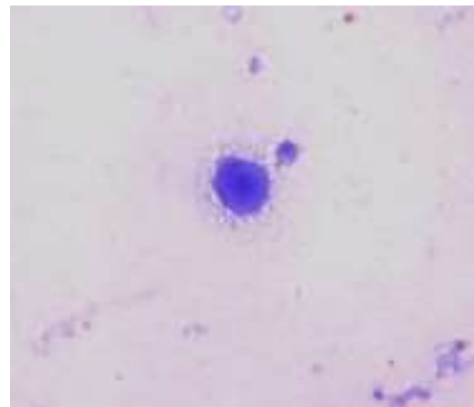
Figure 3b. Positive control-micronucleus

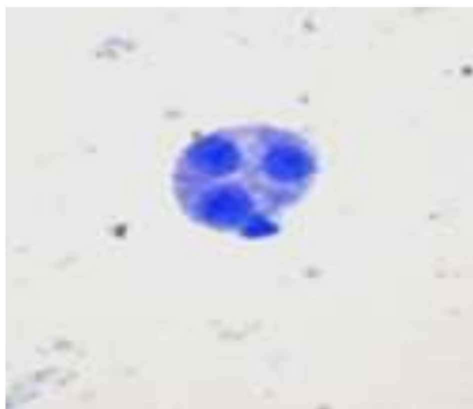
Figure 3c. Three nucleated cell.

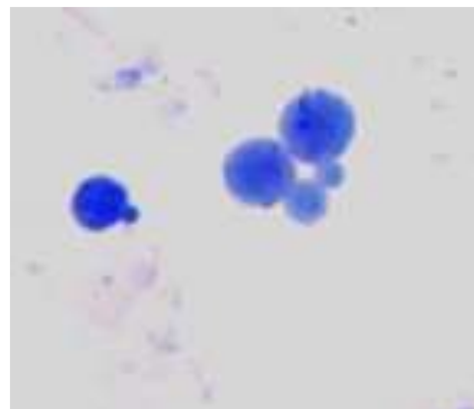
Figure 3d. Cell with two micronuclei.

Figure 3. Aberrant cells in human peripheral lymphocytes by infusion and decoction extracts of *T. cordata* Mill. (M.B. 100x).

Comparing infusion and decoction extracts obtained from three different plants, *T. cordata* has been the most effective plant on inhibition of mitotic index. The *R. officinalis* infusion and decoction extracts were least effective on MI. All three plant infusion and decoction extracts decreased the MI in a dose-dependent manner. Most micronucleus on peripheral lymphocyte cells was formed by *L. stoechas, T. cordata*, and *R. officinalis* infusion and decoction extracts, respectively.

CAs in peripheral lymphocyte cells was observed only after *R. officinalis* infusion extract treatment, other plant extracts not caused CAs in peripheral lymphocyte cells.

All three plants used in the experiments were found to have different effects on cell division and chromosomes in peripheral lymphocyte cells. It can be concluded that all three plants have both cytotoxic and genotoxic effects on peripheral lymphocytes.

DISCUSSION

In this study, *in vitro* cytotoxic and genotoxic effects of infusion and decoction extracts of *L. stoechas*, *R. officinalis,* and *T. cordata* on human peripheral blood lymphocytes were investigated. The aqueous extract (infusion and decoction) were prepared according to traditional usage. Peripheral lymphocytes have been widely used for detecting genotoxic effects of various agents in a great number of studies, since they are considered to be adequate for detecting general exposure. In addition, these cells are in a non-proliferative stage (G0) and have a long half-life (about 3 years) [44-46].

The mitotic index (MI), and micronucleus (MN) analysis methods are cytogenetic tests that are used both *in vivo* and *in vitro*. The MI analysis used to characterize proliferating cells and identify compounds that inhibit or induce mitotic progression [47]. MI is used as indicators of adequate cell proliferation biomarkers. MI measures the proportion of cells in the M-phase of

the cell cycle and its inhibition could be considered as cellular death or delay in the cell proliferation kinetics [30]. Jain and Andsorbhoy (1988) [48] reported that various chemical compounds suppress the functions of cell energy production centers, slow down cell division as a result of the reduction of ATP synthesis, and produce cytotoxic effect.

In this study, MI in peripheral lymphocytes treated with *L. stoechas*, *R. officinalis* infusion and decoction extracts for 48 h were significantly lower than in the negative control. The decrease in MI was found to be dose dependent (p <0.01). This may be attributed to several factors, one of which could be the fact that many cells, during the time remained in the circulation (at G0). Another factor to be considered could be that mitotic delay, by permitting the repair of genotoxic lesions, might also modify the frequency of cells that undergo mitosis at a given time in culture, thus changing the frequency of cells with two nuclei or more at harvest [49]. The decreases of the MI in the peripheral blood lymphocytes indicate that *L. stoeach*, *R. officinalis,* and, *T.cordata* infusion and decoction extracts may suggest as cytotoxic agents. Conclusions regarding the MI values obtained in our study suggest that the compounds contained in the extracts used may have cytotoxic effect in human peripheral blood lymphocytes. The results are also in accordance with the literature. Studies using various plant extracts and test systems have shown that a large proportion of plant extracts have cytotoxic effects on different cell lines [31, 40-51]. The cytotoxic effect observed with plant extract applications is evidenced by the decrease in MI values [31]. In another study, the effects of three different *T. cordata* flower extracts on normal and tumor lymphocyte proliferation were investigated [52]. According to the results of this study, aqueous, dichloromethane and ethanol extracts all showed antiproliferative effect on tumor cells. The dichloromethane extract was proved to be most active with an EC50; this was 11 and 1000 times lower than the EC50. Moreover, aqueous and dichloromethane extracts showed the highest inhibitory effects expressed as maximum efficacy. Another study has shown that *Lavandula antineae* hydromethanolic extract has a toxic effect against salty shrimp nauplii. In addition, it was found that the lethal level was directly proportional to the concentrate of the extract. The observed lethality of this plant extract to *Brine shrimps* showed the presence of potent cytotoxic and probably antitumor components [53]. According to Meyer et al., (1982) [54] a crude plant extract is considered as toxic (active) if it has an LC50 value of less than 1000 µg/ml. Cheung and Tai., (2007) [55] showed that the crude ethanolic rosemary extract had anti-proliferative effect on human leukemia and breast carcinoma cells. Addition of rosemary extract delayed the oxidation of lipid fraction of minced meatballs during storage in the freezer. The antioxidative effect was related to the concentration of the active compounds present in the extract [56, 57]. Studies showed that there are biologically active compounds in rosemary essential oil exhibiting cytotoxic, antioxidant, anti-carcinogenic and cognition-enhancing properties [6, 58]. Out findings are in accordance with these studies mentioned above.

In *R.officinalis* and *T. cordata*, compounds such as caffeic acid, rosmarinic acid, chlorogenic acid are compounds of phenol group as chemical structure. Phenolic compounds and their derivatives cause both cytotoxic and genotoxic damage to humans and animals [59-61]. Phenolic compounds are thought to exhibit this effect by inhibiting DNA polymerase, which causes an increase in sister chromatid exchange. Capasso and Cristinzio (1992) [62] conducted studies on plants grown in agricultural fields irrigated with polyphenol-containing wastewater, and found that polyphenol compounds in waste water can be separated into prophase granulation, heterogeneous chromatin distribution, metaphase accumulation, anaphase bridge formation, telophase early or late cytokinesis, and heterozygous solubility in chromosomes.

Micronuclei (MN) are small, extranuclear bodies apart from the main nucleus of a cell. They are formed when whole chromatids, chromosomes and acentric fragments are left behind and eventually excluded from the daughter nuclei at mitosis [64-67]. In general, micronucleus analysis is utilized in both genotoxicity testing and biomonitoring of genotoxic exposure and effect in humans. MN are particularly considered a useful biomarker of genotoxic effects in populations exposed to genotoxicants in occupational settings. In principle, the micronucleus assay (MN assay) allows the detection of both aneugenic agents (inducing numerical chromosome alterations) and clastogenic agents (inducing chromosome breakage) and can be applied, in addition to

peripheral lymphocytes, to cells exfoliated from buccal, nasal and urothelial mucosa [47, 67-69].

Our study has demonstrated an association between levels of MN formation and the genotoxic risk of plant extracts. Increasing MN rates showed that they could have genotoxic and carcinogenic effects at high concentrations. *L. stoeach*, *R. officinalis* infusion and decoction extracts increased rates of MN on peripheral lymphocytes cells. In the current study, we also detected that two different extracts of three plants generally induced MN formation; however, that increment was statistically significant at the highest concentration for 48 h treatment. In addition, a dose-dependent increase in MN formation was recorded for 48 h treatment ($p < 0.01$). Extracts from the plants used in the study led to a statistically significant increase in micronucleus (MN) in peripheral lymphocytes (Tables 1, 2 and 3). This data is consistent with literature data showing that chemical compounds contained in extracts have significant genotoxic effects on chromosomes and/or mitotic index of human peripheral blood lymphocytes [70].

In addition to the MN, apoptotic cells found in lymphocytes after treatments 1.2 mg/ml *L. stoechas* infusion and decoction extracts. Also, lymphocytes with three or more nuclei were also found after treatments of *T. cordata* infusion and decoction extracts (Fig. 3). These results are similar to those obtained in our previous study in which we investigated cytotoxic and genotoxic effects of *L. stoechas* aqueous extracts using *Allium Test* [71].

Cytogenetic biomarkers such as chromosomal aberrations (CAs), sister chromatid exchanges (SCEs), and MN have for many years been used for surveillance of human genotoxic exposure and cytogenetically visible early effects [67]. The use of these biomarkers is based on the fact that most established human carcinogens are genotoxic in short-term tests and capable of inducing chromosome damage [72]. The analysis of structural CAs in peripheral blood lymphocytes has been used for more than 30 years as a biomarker of early effects of genotoxic carcinogens [73]. The frequency of CAs, determined by ordinary metaphase analysis, has in several studies been shown to be associated with a higher cancer risk [27, 73-79]. Because of these findings, the traditional CA assay is still much used in studies of human exposure to chemical genotoxins and ionizing radiation.

CAs were observed only in lymphocytes treated with 1.5 mg/ml *R. officinalis* infusion extract. When compared to control and solvent control for 48 h treatment, plant extracts did not caused CAs. *R. officinalis* infusion extract (1.5 mg/ml) caused structural CAs which means that *R. officinalis* infusion extract as a clastogen can trigger the formation of CAs by breaking the phosphodiester backbone of DNA**.** However, L. *stoechas* and *T. cordata* did not cause structural chromosomal aberrations.

It is possible that compounds at high concentrations in plant extracts used as test materials lead to irreversible DNA damage in lymphocytes (genotoxic effect) and cell death (cytogenetic effect) by inhibiting mitotic events. Previous studies have shown that extracts from Tilia sp. act on cell proliferation at low concentrations [80] and antiproliferative [81] at high concentrations, while extracts from *Rosmarinus officinalis* affect *Aspergillus parasiticus* [82] have been shown to be effective.

In this study, in terms of *in vitro* cytotoxic effect resulting from the treatments of the extracts prepared at two different concentrations and cytotoxic and genotoxic effects to human peripheral lymphocytes for 48 hours, the highest *in vitro* cytotoxic effect was observed by *T. cordata, T. cordata* was followed by *L. stoechas* and *R. officinalis.* In terms of genotoxic effect *in vitro*, the plants were found to have the order L. stoechas> T. cordata> *R. officinalis* in comparison among themselves.

Especially in recent years, the cytotoxic and genotoxic effect of herbal extracts, which are widely used together with modern methods of medicine, raises the problem of reliability that will arise if these extracts are used for a long time. Plants are an important source of biologically active compounds. Harmfull effects of many of biologically active compounds, especially on hereditary material, are still unknown. For this reason, unconscious usage or misusage of plants for whatever purpose may lead to serious problems that can lead to death.

ACKNOWLEDGMENTS

The authors are thankful to the financial support of Adnan Menderes University Scientific Research Foundation (Project No: FEF-07014). We would like to thank Dr. Özkan Eren and Dr. Mesut Kırmacı for botanical identification of *T. cordat, L. stoechas* and *R. officinalis.*

Disclosure statement

No potential conflict of interest was reported by the authors.

REFERENCES

[1]. Harvey A. L., Natural products in drug discovery, Drug Discov Today., 13 (19/20) (2008) 894-901.

[2]. Limem-Ben Amor I., Boubaker J., Ben Sgaier M., et al., Phytochemistry and biological activities of Phlomis species, J Ethnopharmacol., 125 (2009) 183-202.

[3]. Karamanoli K., Vokou D., Menkissoglu U., Constantinidou I. H., Bacterial colonization of phyllosphere of Mediterranean aromatic plants, J. Chem. Ecol., 26 (2000) 2035.

[4]. Ozcan K., Antioxidant activities of rosemary, sage, and sumac extracts and their combinations on stability of natural peanut oil.J. Med. Food., 6 (2003) 267-270.

[5]. Minnunni M., Wolleb U., Mueller O., Pfeifer A., Aeschbacher H.U., Natural antioxidants as inhibitors of oxygen species induced mutagenicity, Mutat. Res., 269 (1992) 193-200.

[6]. Faixova Z., Faix S., Biological effects of Rosemary (*Rosmarinus officinalis L.*) essential oil, Folia Veterinaria., 52 (3-4) (2008) 135-139.

[7]. Fernandez L. J., Zhi N., Aleson C. L., Perez A. J. A., Kur V., Antioxidant and antibacterial activities of natural extracts, application in beef meat balls, Meat Science., 69-3 (2005) 371-380.

[8]. Huang H.C., Huang C. Y., Lin-Shiau S. Y., Lin J. K., Ursolic acid inhibits IL-1beta or TNF-alpha-induced C6 glioma invasion through suppressing the association ZIP/p62 with PKC-zeta and downregulating the MMP-9 expression, Mol. Carcinog., 48 (2009) 517-531.

[9]. Gonzalo V., Molina S., González-Vallinas M., García-Risco M.R., Fornaril T., Reglero G., Ramírez de Molina A., Production of Supercritical Rosemary Extracts and their Effect on Tumor Progression, J.Supflu, 79 (2012) 101-108.

[10]. Assessment report on Tilia cordata Miller, Tilia platyphyllos Scop., Tilia x vulgaris Heyne or their mixtures, flos EMA/HMPC/337067/2011.

[11]. Blumenthal M., Busse W. R., Goldberg A., Gruenwald J., Hall, T.,Chance, W.R., Robert, S.R., Klein, S., editors. The Complete German Commission E Monographs. American Botanical Council, Austin Texas 1998, 163.

[12]. Blumenthal M., Goldberg A., Foster S., Herbal Medicine-Expanded Commission E Monographs. American Botanical Council, Integrative Medicine Communication, Boston, MA, 2000, pp. 240-243.

[13]. Thomson A., Healing plants (a modern herbal); Mc Graw Hill Book Company; London, England, 1987.

[14]. Davis P.H. (ed.), Flora of Turkey and the East Aegean Islands. Vol. 7. Edinburgh University Press, Edinburgh, 1992, pp. 947.

[15]. Gören A. C., Topçu G., Bilsel G., Bilsel M., Aydoğmuş Z., Pezzuto J. M., The chemical constituents and biological activity of essential oil of *Lavandula stoechas* ssp. Stoechas, Z. Naturforsch. 57c (2002) 797-800.

[16]. Hsu C.K., Chang C.T., Lu H.Y., Chung Y. C., Inhibitory effects of the water extracts of *Lavendula* sp. on mushroom tyrosinase activity, Food Chem. DOI: 10.1016/j.foodchem.2007.02.008.

[17]. Gamez M.J., Jimenez J., Risco S., Zarzuelo A., Hypoglycemic activity in various species of genus Lavandula. Part I: *Lavandula stoechas* L. and *Lavandula multifida* L. Pharmazie. 42 (1987) 706-707.

[18].Baytop T., Therapy with Medicinal Plants in Turkey (Past and Present). 2nd Edition. Nobel Medical Publishers, 1999, Istanbul.

[19].Gilani A.H., Aziz N., Khan M.N., Shaheen F., Jabeen Q., Siddiqui B. S., Herzig J. W., Ethnopharmacological evaluation of the anticonvulsant, sedative and antispazmodic activities of *Lavandula stoechas* L., J. Ethnopharmacol., 71 (2000) 161-167.

[20].González-Coloma A., Delgado F., Rodilla J. M., Silva L., Sanz J., Burillo J., Chemical and biological profiles of *Lavandula luisieri* essential oils from western Iberia Peninsula populations, Biochem Syst Ecol., 39 (2011) 1-8.

[21].Sosa S., Altinier G., Politi M., Braca A., Morelli I., Loggia D., Extracts and constituents of *Lavandula multifida* with topical anti-inflammatory activity, Phytomedicine., 12-4 (2005) 271-277.

[22].Dob T., Dahmane D., Agli M., Chelghoum C., Essential oil composition of *Lavandula stoechas* from Algeria, Pharm Biol., 44 (2006) 60-64.

[23].Upson T. M., Grayer R. J., Greenham J. R., Williams C. A., Al-Ghamdi F., Chen F.H., Leaf flavonoids as systematic characters in the genera *Lavandula* and *Sabaudia,* Biochem Syst Ecol., 28 (2000) 991-1007.

[24].Carrano A.V., Natarajan A.T., International Commission for protection against environmental mutagens and carcinogens, Mutat Res., 204-3 (1988) 379-406.

[25].Anderson D., Human biomonitoring, Mutat. Res., 204 (1988) 353-541.

[26].Tucher J. D., Preston R. J., Chromosome Aberrations, Micronuclei, Aneuploidy, Sister Chromatid Exchanges, and Cancer Risk Assessment, Mutat Res., 365 (1-3) (1996) 147-159.

[27].Hagmar L., Brøgger A., Hansteen I. L., Heim S., Högstedt B., Knudaen L., Lambert B., Linnainmaa K., Mitelman F., Nordenson I., Reuterwall C., Salomaa S., Skerfving S., Sorsa M., Cancer risk in human predicted by ıncreased levels of 69 chromosomal aberrations in lymphocytes: Nordic study group on the health risk of chromosome damage , Cancer Res., 54 (1994) 2919-2922.

[28].Heddle J.A., Cimino M.C., Hayashi M., Romagna F., Shelby M.D., Tucker J.D., Vanparys P., Mac Gregor J. T., Micronuclei as an index of cytogenetic damage: past, present, and future, Environ. and Mol. Mutagen., 18 (1991) 277-291.

[29].Fenech M., Biomarkers of genetic damage for cancer epidemiology, Toxicology, 181 (2002) 411-416.

[30].Rojas E., Herrera L.A., Sordo M., Gonsebatt M.E., Montero R., Rodriguez R., Ostrosky-Wegman P., Mitotic index and cell proliferation kinetics for identification of antineoplastic activity, Anticancer Drugs, 4 - 6 (1993) 637-640.

[31].Gadano A. B., Gurni A.A., Carballo M.A., Argentine folk medicine: Genotoxic effects of *Chenopodiaceae* family, Journal of Ethnophamacology, 103 (2006) 246-251.

[32].Bonassi S., Hagmar L., Strömberg U., Montagud A.H., Tinnerberg H., Forni A., Heikkilä P., Wanders S., Wilhardt P., Hansteen I.L., Knudsen L.E., Norppa H., Chromosomal aberrations in lymphocytes predict human cancer independently of exposure to carcinogens, Cancer Res., 60 (2000) 1619-1625.

[33].Bonassi S., Znaor A., Norppa H., Hagmar L., Chromosomal aberrations and risk of cancer in humans: an epidemiologic perspective, Cytogenet. Genome Res., 104 (2004) 376-382.

[34].Bonassi S., Ugolni D., Kirsch-Volders M., Strömberg U., Vermeulen R., Tucker J.D., Human population with cytogenetic biomarkers: review of the literature and future prospectives, Environ. and Mol. Mutagen., 45 (2005) 258-270.

[35].Savage J. R. K., Update on target theory as applied to chromosomal aberrations, Env. Mol. Mutagen., 22 (1993) 198-207.

[36].Evans H. J., Handbook of mutagenicity test procedures. In: Kilbey, B.J., Legator, M., Nichols, W. and Ramel, C. (Eds.), Human peripheral blood 63 lymphocytes for the analysis of chromosome aberrations in

mutagen tests. Second edition, Elsevier Science Publishers, BV. 1984, pp. 405-427.

[37].Rencüzoğulları E., Topaktaş M., The relationship between quantities of bromodeoxyuridine and human peripheral blood with determination of the best differential staining of sister chromatids using Chromosome Medium-B, Fen ve Mühendislik Bilimleri Dergisi, 5-3 (1991) 19-24.

[38].Speit G., Haupter S., On the mechanism of differential giemsa staining of bromodeoxyuridine substituted chromosomes. II. differences between the demostration of sister chromatid differentiation and replication patterns, Hum. Gen., 70 (1985) 126-129.

[39]. Savage J.R., Classification and relationships of induced chromosomal structual changes, J Med Genet., 13-2 (1976) 103-122.

[40].Tice R.R., Boucher R., Luke C.A., Shelby M.D., Comparative cytogenetic analysis of bone marrow damage induced in male B6C3F1 mice by multiple exposures to gaseous 1,3 butadiene, Environmental Mutagenesis, 9 (1997) 235-250.

[41].Roncada T., Vicentini V.E.P., Mantovani M.S., Possible modulating actions of plant extracts on the chromosome breaking activity of MMC and Ara-C in human lymphocytes in vitro, Toxicology in vitro, 18 (2004) 617-622.

[42].Fenech M., The in vitro micronucleus technique , Mutat Res., 455(1-2) (2000) 81-95.

[43].Kirsch-Volders M., Sofuni T., Aardemac M., Albertini S., Eastmond D., Fenech M., Ishidate Jr. M., Kirchner S., Lorge E., Morita T., Norppa H., Surralles J., Vanhauwaert A., Wakata A., Report from the in vitro micronucleus assay working group, Mutat. Res., 540 (2003) 153-163.

[44].Murray E.B., Edwards J.W., Micronuclei in peripheral lymphocytes and exfoliated urothelial cells of workers exposed to 4,4_-methylenebis-(2- chloroaniline) MOCA, Mutat. Res., 446 (1999) 175-180.

[45].Amorin M.I., Mergler D., Bahia M.O., Dubeau H., Miranda D., Lebel J., Burdano R.R., Lucotte M., Cytogenetic damage related to low levels of methyl mercury contamination in the Brazilian Amazon, An. Acad. Bras. Cienc., 72 (2000) 497-507.

[46].Thierens H., Vral A., Morthier R., Aousalahm B., De Ridderm L., Cytogenetic monitoring of hospital workers occupationally exposed to ionizing radiation using the micronucleus centromere assay, Mutagenesis., 15 (2000) 245-249.

[47].Holland N., Bolognesi C., Kirsch-Volders, M, Bonassi S, Zeiger E, Knasmueller S, et al., The micronucleus assay in human buccal cells as a tool for biomonitoring DNA damage: the HUMN project perspective on current status and knowledge gaps, Mutat Res., 659(1-2) (2008) 93-108.

[48].Jain A.K., Andsorbhoy R.K., Cytogenetical studies on the effects of some chlorinated pesticides. III. Concluding remarks, Cytologia (Tokyo), 53 (1988) 427-436

[49].Eroğlu H.E., Aksoy A., Hamzaoğlu E., Budak U., Albayrak S., Cytogenetic effects of nine Helichrysum taxa in human lymphocytes culture, Cytotechnology., 59-1 (2009) 65-72.

[50].Lazutka J.R., Mierauskien J., Slap G., Dedonyt V., Genotoxicity of dill (*Anethum graveolens* L.), peppermint (*Mentha piperita* L.) andpine (*Pinus sylvestris* L.) essential oils in human lymphocytes and *Drosophila melanogaster,* Food Chem. Toxicol., 39 (2001) 485-492.

[51].Rencüzoğulları E., İla H.B., Kayraldız A., Diler S. B., Yavuz A., Arslan M., Kaya F. F., Topaktaş M., The mutagenic and anti-mutagenic effects of *Ecballium elaterium* fruit juice in human peripheral lymphocytes, Russian Journal of Genetics, 42-6 (2006), 623-627.

[52].Barreiro Arcos M.L., Cremaschi G., Werner S., Coussio J., Ferraro G., Anesini C., *Tilia cordata* Mill. extracts and scopoletin (isolated compound): Differential cell growth effects on lymphocytes, Phytother. Res., 20 (2006) 34-40.

[53].Krimat S., Dob T., Toumi M., Metiji H., Kesouri K., Chelghoum C., Evaluation of phytochemicals, antioxidant and cytotoxic activities of *Lavandula antineae* Maire

endemic medicinal plant from Algeria, Informatics Journals, 6-3 (2014) https://doi.org/10.18311/ajprhc/2014/517.

[54].Meyer B. N., Ferrigni N. R., Putnam J. E., Jacobsen L. B., Nichols D. E., McLaughlin J. L., Brine shrimp: a convenient general bioassay for active plant constituents, Planta Medica, 45 (1982) 31-34.

[55].Cheung S., Tai J., Anti-proliferative and antioxidant properties of rosemary *Rosmarinus officinalis*, Oncol. Res., 1-7 (2007) 525-531.

[56].Lopez-Bote C.J., Gray J. L., Gomaa E. A., Flegal C.J., Effect of dietary administration of oil extracts from rosemary and sage on lipid oxidation in broiler meat, Br. Poult. Scrl., 39 (1998) 235-240.

[57].Karpinska M., Borowski J., Danowska-Oziewicz M., Antioxidative activity of rosemary extract in lipid fraction of minced meat balls during storage in a freezer, Nahrung, 44 (2000) 38-41.

[58].Santos P.A.S.R., Avanço G.B., .Marcelino R.I.A, Janeiro V.M.C., Valaderes M.C., Machinski M., Assessment of Cytotoxic Activity of Rosemary (*Rosmarinus officinalis* L.), Turmeric (*Curcuma longa* L.), and Ginger (*Zingiber officinale* R.) Essential Oils in Cervical Cancer Cells (HeLa), The Scientific World Journal. 2016, ID 9273078, 8 pages

[59].De Marco A., Romanelli M., Stazzı M.A., Vitagliano E., Induction of micronucleated cells in *Vicia faba* and *Allium cepa* root tips treated with NTA, Mutat. Res., 171 (1986) 145-148.

[60].Williams G.M., Methods for evaluating chemical genotoxicity, Ann. Rev. Pharmocol. Toxicol., 29 (1989) 189-211.

[61].Migliore L., Baraler B.E., Giorgelli F., Minnunni M., Scarpato R., Loprieno N., Genotoxicity of emthyglyoxal: cytogenetic damage in human lymhocytes in vitro and intestinal cells of mice, Carcin., 11- (1990), 1503-1507.

[62].Capasso R., Cristinzio G., Evidente A., Scognamiglio F., Isolation spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste water, Phytoch., 31-12 (1992): 4125-4128.

[63].Heddle J.A., Hite M., Kirkhart B., Mavournin K., MacGregor J.T., Newell G.W., Salamone M.F., The induction of micronuclei as a measure of genotoxicity. A report of the US environmental protection agency gene tox program, Mutat. Res., 123 (1983) 61-118.

[64].Ford J.H., Schultz C.J., Correll, A.T., Chromosome elimination in micronuclei: a common cause of hypoploidy, Am J Hum Genet., 43-5 (1988) 733-40.

[65].Lindholm C., Norppa H., Hayashi M., Sorsa M., Induction of micronuclei and anaphase aberrations by cytochalasin B in human lymphocyte cultures, Mutat Res., 260-4 (1991) 369-75.

[66].Ford J.H., Correll A.T., Chromosome errors at mitotic anaphase, Genome., 35-4 (1992) 702-5.

[67].Albertini R.J., Anderson D., Douglas G.R., Hagmar L., Hemminki K., Merlo F., Natarajan A.T., Norppa H., Shuker D.E., Tıce R., Waters M.D., Aitio A., IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. International Programme on Chemical Safety, 463- 2 (2000) 111-72.

[68].Pastor S., Gutierrez S., Creus A., Xamena N., Piperakis S., Marcos R., Cytogenetic analysis of Greek farmers using the micronucleus assay in peripheral lymphocytes and buccal cells, Mutagenesis, 16-6 (2001) 539-45.

[69].Norppa H., Falck G.C.M., What do human micronuclei contain?, Mutagenesis, 18-3 (2003) 221-233.

[70].Ballardini M., Scarpato R., Kotwal G.J., Barale R., In vitro mutagenicity studies of the antiretrovirals AZT, didanosine, and 3TC and a plant antiviral extract secomet-V derived from the *Trifolium* species, Ann. N.Y.Acad. Sci., 1056 (2005) 303-310.

[71].Aşkın Çelik T., Aslantürk Ö.S., Cytotoxic and genotoxic effects of *Lavandula stoechas* aqueous extracts, Biologia, 62-3 (2007) 292-296.

[72].Waters M. D., Stack H.F., Jackson M.A., Genetic toxicology data in the evaluation of

potential human environmental carcinogens, Mutat Res., 437-1 (1999) 21-49.

[73].Hagmar L., Stromberg U., Bonassi S., Hansteen I.L., Knudsen L.E., Lindholm C et al., Impact of types of lymphocyte chromosomal aberrations on human cancer risk: results from Nordic and Italian cohorts, Cancer Res., 64-6 (2001) 2258-63.

[74].Bonassi S., Abbondandolo A., Camurri L., Dal Prá L., De Ferrari M., Degrassi F., Forni A., Lamberti L., Lando C., Padovani P. et al., Are chromosome aberrations in circulating lymphocytes predictive of future cancer onset in humans? Preliminary results of an Italian cohort study , Cancer Genet Cytogenet., 79-2 (1995) 133-5.

[75].Hagmar L., Bonassi S., Strömberg U., Brøgger A., Knudsen L.E., Norppa H., Reuterwall C., Chromosomal aberrations in lymphocytes predict human cancer: a report from the European Study Group on Cytogenetic Biomarkers and Health (ESCH), Cancer Res., 58-18 (1998) 4117-21.

[76].Liou S.H., Lung J.C. Chen Y.H.,Yang T., Hsieh L.L., Chen C.J., Wu T.N., Increased chromosome-type chromosome aberration frequencies as biomarkers of cancer risk in a blackfoot endemic area, Cancer Res., 59-7 (1999) 1481-4.

[77].Bonassi S., Hagmar L., Stromberg U, Montagud AH, Tinnerberg H. et al., Chromosomal aberrations in lymphocytes predict human cancer independently of exposure to carcinogens. European Study Group on Cytogenetic Biomarkers and Health, Cancer Res., 60-6 (2000) 1619-25.

[78].Smerhovsky Z., Landa K., Rössner P., Brabec M., Zudova Z. et al., Risk of cancer in an occupationally exposed cohort with increased level of chromosomal aberrations, Environ Health Perspect., 109-1 (2001) 41-5.

[79].Bonassi S., Norppa H., Ceppi M., Stromberg U., Vermeulen R., Znaor A. et al., Chromosomal aberration frequency in lymphocytes predicts the risk of cancer: results from a pooled cohort study of 22 358 subjects in 11 countries, Carcinogenesis, 29-6 (2008) 1178-83.

[80].Anesini C., Werner S., Borda E., Effect of *Tilia cordata* flower on lymphocyte proliferation: participation of peripheral type benzodiazepine binding sites, Fitoterapia, 70 (1999) 361-367.

[81].Manuele M.G., Ferraro G., Anesini C., Effect of *Tilia x viridis* flower extract on the proliferation of a lymphoma cell line and on normal murine lymphocytes: contribution of monoterpenes, especially limonene, Phytotherapy Research, 22 (2008)1520- 1526

[82].Tantaoui-Elaraki A., Beraoud L., Inhibition of growth and aflatoxin production in *Aspergillus parasiticus* by essential oils of selected plant materials, J. Environ. Pathol. Toxicol. Oncol., 13-1 (1994) 67-72.