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In vitro Cytogenetic Effects of the *Euphorbia grisophylla* Aqueous Extract in Human Peripheral Lymphocytes

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Abstract

The effects of the aqueous extract obtained from the aerial parts of *Euphorbia grisophylla* M.L.S.Khan, a herbaceous member of the Euphorbiaceae family, which is common in other parts of the world outside the Antarctic continent, on genotoxic, cytotoxic, and oxidative parameters were investigated. Experiments were carried out under short-term and in vitro conditions with sublethal concentrations of the test substance extract. According to the first results, the plant extract did not significantly affect the oxidative stress indicators in cell culture. Similarly, it increased the micronucleus ratio in lymphocytes insignificantly, but the concentration-effect relationship is significant in the 24-hour treatment. Again, the test substance did not induce an evident reversion-type mutation in the Ames test. However, the percentage of damaged cells in the Comet test and the genetic damage index increased significantly except for the lowest concentration. The cytotoxic effect observed in the context of the same pattern with these findings is remarkable. In conclusion, the slightly clastogenic and cytotoxic effect of *Euphorbia grisophylla* aqueous extract, not based on the oxidative attack, may provide a new projection for further research, like cancer therapy.

Keywords: *Euphorbia grisophylla*, Cytogenotoxicity, Micronucleus (MN) test, Ames/Salmonella test, Comet assay

1. Introduction

Euphorbiaceae family is a widespread plant group on the earth with more than 5000 taxa described by now. The considerable diversity of this plant is in the tropical region and is known to predominate in some ecosystems [1]. In this study, the test substance was isolated from the aerial parts of *Euphorbia grisophylla* M.L.S.Khan (Herbarium catalog barcode no: K001080042) by an aqueous extraction method. This species is used for ethnomedical purposes in Malatya and elsewhere in Anatolia (Turkey).

The ancient folkloric belief that natural herbal products protect and improve bodily/mental health constitutes the main framework of traditional-based herbal therapy. The traditional use of natural resources other than plants for healing has mediated the birth of modern pharmacognosy. In this context, the World Health Organization (WHO) defines traditional medicine as therapeutic methods that existed before the development and spread of modern medicine and are still used today [2]. Traditional medicine, which has economic importance in a rapidly growing health system, still maintains its significance. In Africa, 80% of the population prefers traditional medicine to help meet their health needs. Conventional medicine still has a prominent place in Asian and Latin American societies with the impact of historical accumulation and cultural heritage. About 40% of all healthcare services in China are traditional medicine focused. Today, the popularity of complementary alternative medicine practices is increasing again in many developed countries. The proportion of the population who use this option at least once in their life is 48% in Australia, 70% in Canada, 42% in the USA, 38% in Belgium, and 75% in France [3].

In the beginning, the whole plant or parts (such as roots, leaves, flowers, fruits, and seeds) were used directly without any pretreatment as a medicine; today, the using extract, tincture, distillation, and derivative products of them have become widespread.

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At the beginning of this century, 11% of the 252 drugs considered vital by the WHO were phytochemicals of flowering plant origin alone. Medicines such as codeine, quinine, and morphine contain plant-derived ingredients. Natural products, used since ancient times, have been the backbone of the traditional healing system on a global scale, as an integral part of the population, history, and culture. The incorporation of identified compounds extracted from plants into modern drug discovery and development processes only began in the 19th century. Bioactive natural products play a central and critical role, especially in the evolution of innovative drugs with antibacterial and antitumor effects. Today, although the popularity of synthetic solutions has increased due to their optimized production cost, easy quality control, and rapid action, the dependence on natural products is still at high levels. To date, around 70,000 plant species have been scanned for medicinal use. According to currently available information, the plants especially suitable for ethnopharmacological use may be a primary source for early drug discovery [4]. Of these, 48.6% of small molecules used in cancer therapies are either natural products or derivatives directly obtained from them [5]. The output of a study supporting this view indicated that 122 identified compounds obtained from 94 plant species are used as drugs worldwide [6]. The first commercial pure natural product to be marketed for therapeutic use was morphine, marketed by Merck in 1826. The first semi-synthetic pure aspirin based on salicin, a natural product also isolated from white willow (*Salix alba*), was introduced to the market by Bayer in 1899. Development in the pharmaceutical industry has led to the introduction of early drugs, some of which are still in use, such as cocaine, codeine, digitoxin, quinine, and pilocarpine. Similarly, agents such as Paclitaxel, Artemisinin, and Silymarin are a few of the new plant-derived compounds marketed as drugs. Paclitaxel, isolated from the Pacific yew tree (*Taxus brevifolia*), is one of the drugs prescribed against lung, ovarian, and breast cancer. Likewise, Artemisinin extracted from the traditional Chinese plant sweet wormwood (*Artemisia annua*) is used to combat multidrug-resistant malaria. Silymarin obtained from the thistle seeds (*Silybum marianum*) is used for liver disease therapy. With the analytical reinterpretation of clinical data in light of the molecular approach, old molecules have found new application areas. For example, the alkaloid Forskolin from *Coleus forskohlii* and some phytochemicals isolated from *Stephania glabra* show promise as activators of adenylate cyclase and nitric-oxide, which may help prevent conditions such as obesity and atherosclerosis. In recent years plant-derived drugs containing functional molecules have been introduced to the market. For example, the Arteether agent, a semi-synthetic natural product obtained from Artemisinin that contains endoperoxide sesquiterpene, is used in malaria treatment. Nitisinone, which is isolated from *Callistemon citrinus*, is used in the treatment of tyrosinemia. Galantamine extracted from *Galanthus nivalis* is a natural alkaloid for Alzheimer's treatment. Semi-synthetic Apomorphine, derived from morphine isolated from *Papaver somniferum*, is used in Parkinson's disease therapy. The active substance called Tiotropium, a derivative of atropine extracted from *Atropa belladonna*, is used as an active ingredient in chronic obstructive pulmonary disease, Dronabinol and Cannabidiol obtained from *Cannabis sativa*, and the active compound of Capsaicin isolated from *Capsicum annuum* are used as pain relievers. The advantages and disadvantages of using plants as a starting point for drug development are still an important matter of discussion. However, the long-standing preference for medicinal plants in drug discovery should come as no surprise because the most striking feature of natural products is that they still have a largely unused or unknown structural diversity [4]. Euphorbiaceae is the largest-fifth family of flowering plants, with about 7,500 species classified in 300 genera [7] Members of this taxon are widely used in ethnopharmacology, especially in traditional Chinese medicine, around the world. These plants are known for their special inflorescences and latex [8]. Ingenol mebutate, one of the secondary metabolites isolated from many euphorbia species, is a highly active antitumoral ingredient approved by the US Food and Drug Administration and the European Medicines Agency for the topical treatment of human non-melanoma skin cancer and actinic keratosis [9].

The herbal extract used as the test substance in this study was obtained from the aerial parts of *Euphorbia grisophylla* (Herbarium catalog no: K001080042 [10]) herbaceous plant from the Euphorbia family by aqueous extraction method. The test substance, containing many functional bioactive components, was evaluated in terms of genotoxic, cytotoxic, and oxidative potential in this study. Despite its widespread folkloric use and a substantial number of active ingredients, the number of genotoxicity and cytotoxicity studies conducted with the euphorbia plant has very little. *Euphorbia grisophylla* inhabited in the rural area of Malatya province of Turkey is used for drinking water purification in the region. When the latex (milk) of the plant drip into turbid water, it precipitates the particles suspended in the water and reduces the number of microorganisms living in the water (Anonymous). Euphorbia juice is used externally for the removal of warts, and also eczema, and rheumatic diseases by applying it to the related area for itching and pain relief [11]. The isolates, obtained from different Euphorbia species by various methods, contain bioactive molecules such as jatrophanediterpene, jatropha polyester [12], tiglianediterpene, cyclobutane-type triterpene [13], and quercetin [14] again, in many parts of Anatolia, euphorbia is used for treating warts and open infections. It also has laxative and pain-relieving effects. Finally, although it is not very common, it is known that milk/juice of the euphorbia using in fishing (Anonymous).

As it is known, if a medicinal/aromatic plant itself or a component is used for food or therapeutic purposes, it is necessary to reveal its effects on human health. It is vitally important to investigate the genetic and epigenetic activity on the cell genome of any agent, even herbal. There are reliable short-term genotoxicity tests for determining the possible genotoxic and cytotoxic risks of an agent. Short-term genotoxicity tests cover all methods used to determine whether a chemical is genotoxic or cytotoxic.

A reactive substance can determine cell fate by affecting genetic and epigenetic (based on gene expression) mechanisms. The ability of direct or indirect effects to potentially damage the cell genome structure is called genotoxicity. A healthy cell activates the DNA repair mechanism or apoptosis pathways in the cell to manage post damage process. However, if the cell does not take sufficient initiative in repairing the damage, risky mutations may cause cell death or loss of cell division control (abnormal proliferation). Generally, changes in the genome are permanent and hereditary. Here genotoxicity tests determine the potential of any agent to cause cytotoxicity or cancer by damaging the genetic material in cells at any time. These tests are widely used to determine the genotoxic and cytotoxic effects of many natural or synthetic agents. Here, this study aims to reveal the cytogenetic effects of the aqueous extract obtained from the aerial parts of the *Euphorbia grisophylla* (Euphorbia) plant.

2. Material Method

2.1. The scientific basis of the study

All of the experiments in this study were performed under in vitro conditions. The study used the aqueous extract of the aerial parts of *Euphorbia grisophylla* (EAE) as the test substance. Peripheral blood taken from 4 healthy volunteer donors (two females and two males) who did not smoke and do not use drugs (26-28 years old) was used as test cells.

Çukurova University Local Ethics Committee confirmed the experimental study plan (2019, 93 Decision number: 12). Sublethal concentrations (0,625, 1,25, or 2,5 mg/ml) of EAE were determined as the test compound. The maximum sub-lethal concentration was determined by preliminary studies carried out according to items 22 and 24 of Test No. 473 of the OECD protocol (Organization for Economic Co-operation and Development)[15]. The treatment period was selected for 24 or 48 hours, corresponding to 1 or 2 cycles of lymphocyte division in the culture. All data were evaluated by One Way Analysis of Variance (ANOVA), a post hoc LSD test (IBM SPSS Statistics 25). In group comparisons, the standard significance level was accepted as $P \leq 0.05$.

2.2. Micronucleus (MN) Test and Microscopic Examination

In vitro MN test was performed based on the method developed by Rothfuss et al. (2000) [15]. Test concentrations were prepared by diluting the euphorbia extract (EAE) in distilled water. Blood samples from donors (1/10 heparinized) were inoculated into karyotyping media (PB-MAX™, Catalog number: 12557013) in the amount of 0.2 ml under sterile conditions and cells were cultured at 37±1°C for a total of 68 hours. EAE was added to the culture medium 24 or 48 hours before the end of the culture, with a final concentration of 0.625, 1.25, or 2.5 mg/ml. Test concentrations of EAE were determined by preliminary studies based on cytotoxicity (OECD Guideline No 487, item 31) [16]. The concentration that creates 55±5% cytotoxicity (2.5 mg/ml) in lymphocytes was chosen as the highest concentration in the study. At the end of the culture, harvesting, staining, and microscopic examination of the MN experiment were carried out according to Ila et al. (2015) [17].

Cell nucleus and micronucleus separation, Titenko-Holland et al. (1997) and Fenech (2000) were made according to the principles determined [18,19].

The most important advantage of the cytokinesis blocking method is that it allows the determination of chromosomal aberration and nucleus division dynamics (cytotoxicity) in the dividing cell population. The procedure for cytotoxicity was performed by counting mononuclear, binuclear, and multinuclear (>2 nuclei) cells formed following the addition of cytochalasin-B (Sigma-Aldrich CAS number 14930-96-2). The Nucleus Division Index (NDI) was calculated according to the following formula proposed by Eastmond and Tucker (1989) [20].

$$NDI = \frac{(1 \times M1 + 2 \times M2 + 3 \times M3 + 4 \times M4)}{N}$$

NDI provides information regarding the cytotoxic potential of an agent. According to this formula, M1 is the number of cells with one nucleus, M2 with binuclear, M3 with trinuclear, M4 with quad nuclear, and "N" with the total number of cells [21].

2.3. Comet Assay

To detect DNA strand breaks single-cell gel electrophoresis technique [23] developed by Singh et al. (1988) was modified and applied in this study. For this experiment, a stock was prepared by isolating lymphocytes from whole human blood via the Ficoll Gradient Centrifugation method [24].

For each variant, 100 cells were visually scored, and images were recorded. The cells are listed in 5 groups from undamaged to very high damaged.

Grade 0 → undamaged nucleus with no tail,

Grade 1 → nucleus with very-short tail length,

Grade 2 → nucleus with less tail length,

Grade 3 → nucleus with a long tail,

Grade 4 → nucleus with too much tail length

The calculation method proposed by Pitarque et al. (1999) was used to determine the total genetic damage index [22]. Damage score levels calculated with the following formula are given as the genetic damage index (GDI).

$$GDI = \frac{0 \times \text{Grade } 0 + 1 \times \text{Grade } 1 + 2 \times \text{Grade } 2 + 3 \times \text{Grade } 3 + 4 \times \text{Grade } 4}{\text{Grade } 0 + \text{Grade } 1 + \text{Grade } 2 + \text{Grade } 3 + \text{Grade } 4}$$

The mean of comet cells (Grade 2, 3, and 4) was evaluated as the damaged cell ratio (DCR) and added to the data table [23,24].

$$DCR = \text{Grade } 2 + \text{Grade } 3 + \text{Grade } 4$$

2.4 Ames/Salmonella Bacterial Reverse Mutation Test

In this study, the test substance (aerial part aqueous extract of Euphorbia grisophylla) was investigated by the Ames/Salmonella test method, whether causing point mutation. In the experiment, the TA98 strain of Salmonella typhimurium was used to detect frameshift, and the TA100 strain was used to detect base substitution mutations. Test agent concentrations of 0, 0.625, 1.25, or 2.5 mg/plate were used. Furthermore, 4-Nitro-o-phenylenediamine (4-NPD) for the TA98 strain and sodium azide (NaN_3) for the TA100 strain were included in the study as positive controls. Experiments were carried out in triplicate for each test concentration. Husunet et al. (2022) procedure was followed for the execution of this experiment [23].

2.5. Determination of Total Oxidant Capacity and Total Antioxidant Response

Total oxidative capacity (TOC) and antioxidant response (TAR), which are oxidative stress parameters, were determined spectrophotometrically using the cell culture supernatant in the micronucleus experiment. The commercial Rel Assay diagnostics kits were used to investigate TOC and TAR values in these measurements. Calibration of the TOC test was performed using hydrogen peroxide (µmol H2O2 equivalent/L). The oxidant capacity in the sample was determined by measuring of color intensity formed in the presence of oxidant spectrophotometrically (wavelength 530 nm). [27]. The determination of TAR induced by the test substance is based on the reduction fact of the dark blue-green ABTS (2,2 azinobis (3-ethyl benzothiazoline-6-sulfonic acid) radical in the test kit by the antioxidant agents in the sample. For determining the TAR level, absorbance values at 660 nm were taken into account [25].

The oxidative stress index (OSI) was calculated according to the formula below through the TOC and TAR data [25,26].

$$OSI = \frac{\text{TOC (µmol H2O2 equivalent / L)}}{\text{TAR (µmol Trolox equivalent / L)}}$$

3. Results and Discussion

3.1. Results

The components detected as a result of GC-MS (Agilent 7890B /7019B) analysis in the aqueous extract obtained from the aerial parts of the *Euphorbia grisophylla* plant are listed in the table below (Table 1). The three most abundant phytochemicals among these components are respectively 2,3-butanediol, [S-(R*,R*)]-, tetramethylpyrazine, and acetic acid.

Table 1. Contents of phytochemical composition determined in *Euphorbia grisophylla* plant extract by GC/MS analysis

Phytochemical components	**Ratio %**
2,3-Butanediol, [S-(R*,R*)]-	48,71
Tetramethylpyrazine (Ligustrazine)	18,04
Acetic acid	11,35
Hexanoicacid	5,36
Benzylalcohol	2,11
Nonanoicacid	1,82
PhenylethylAlcohol	1,57
Pyrazine, trimethyl-	1,55
Hexanoicacid, 2-methyl-	1,21
n-Decanoicacid	1,06
2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	0,96
Acetoin	0,81
Phenyl-.beta.-D-glucoside	0,81
Octanoicacid	0,80
2,3-Dimethyl-5-ethylpyrazine	0,65
2-Furanone, 2,5-dihydro-3,5-dimethyl	0,63
Octan-2-one, 3,6-dimethyl-	0,57
3,6-Dimethyl-3,6-dihydro-pyran-2-one oxime	0,54
1-(3H-Imidazol-4-yl)-ethanone	0,50
Ethanone, 1-(1H-pyrrol-2-yl)-	0,47
Propanoicacid, 2-methyl-	0,47

3.1.1 Micronucleus Test Findings

Variable MN rates were detected in human peripheral lymphocytes treated for 24 or 48 hours with three different concentrations of the test substance (0.625, 1.25, and 2.5 mg/ml) (Figure 1). However, the MN increases caused by EAE were not generally as significant as the positive control mitomycin C (MMC). The rate of MN detected only at the highest concentration (2.50 mg/ml, 24 hours) is close to the value observed in MMC. On the other hand, no significant difference was found between the untreated group (0 mg/ml) and the groups treated with EAE in terms of MN findings (Table 2). However, the concentration-effect relationship in MN data was statistically significant ($P<0.05$) (Fig.2, Fig. 3).

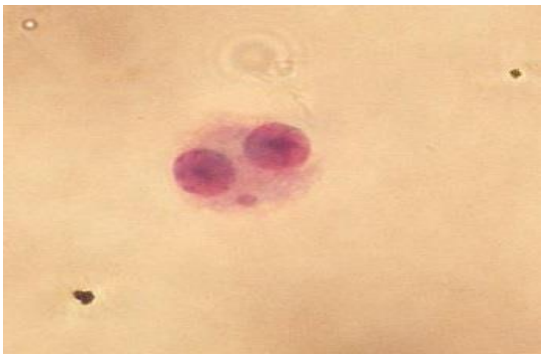

Figure 1. The binuclear cell containing one micronucleus, (1.25 mg/ml EAE, 24-hour treatment) x400.

Table 2. Total MN ‰ and nuclear division index (NDI) detected in human peripheral lymphocytes treated with different concentrations of *Euphorbia grisophylla* extract (EAE) for 24 or 48 hours

Test substance	Treatment		Total ‰ MN± SE	Distribution according to the number of nuclei				NDI ± SE
	Time (h)	Cons. (mg/ml)		1	2	3	4	
MMC*	24	0,25µg/ml	21.00±4.143	2925	1059	13	3	1.3167±0.0586
EAE**	0	0	3.00 ±0.577	1956	1521	145	89	1.5899±0.0825
EAE	24	0.625	5.25 ±0.478 a_2	2553	1392	31	24	1.4265±0.0571
EAE	24	1.25	9.50 ±2.598 a_1	2632	1293	53	22	1.3722±0.0535 b_1
EAE	24	2.5	13.25 ±2.719	2624	1293	55	28	1.3422±0.0655 b_1
MMC	48	0,25µg/ml	28.50±18.500	1847	124	23	6	1.094±0.0170
EAE	0	0	3.00 ±0.577	1956	1521	145	89	1.5899±0.0825
EAE	48	0.625	5.50 ±1.190 a_3	2574	1347	83	44	1.4534±0.0732 a_2
EAE	48	1.25	9.50 ±3.013 a_2	2819	1138	26	17	1.3212±0.0574 b_2
EAE	48	2.5	12.00 ±3.415 a_1	3490	489	17	5	1.2194±0.0910 b_3

*MMC: Mitomycin C (Positive control); **EAE: Euphorbia aqueous extract

a: The difference with the positive control group is significant; b: The difference with the untreated group is significant. a_1b_1: P≤0.05; a_2b_2: P≤0.01; a_3b_3: P≤0.001.

EAE had a decreasing effect on the nuclear division index (NDI). Except for the lowest concentration (0.625 mg/ml), the test substance significantly reduced NDI compared to the untreated group (P<0.05) (Table 2). In addition, the concentration-effect relationship was significant in decreasing of NDI in 48 hours of EAE treatment (Fig. 4).

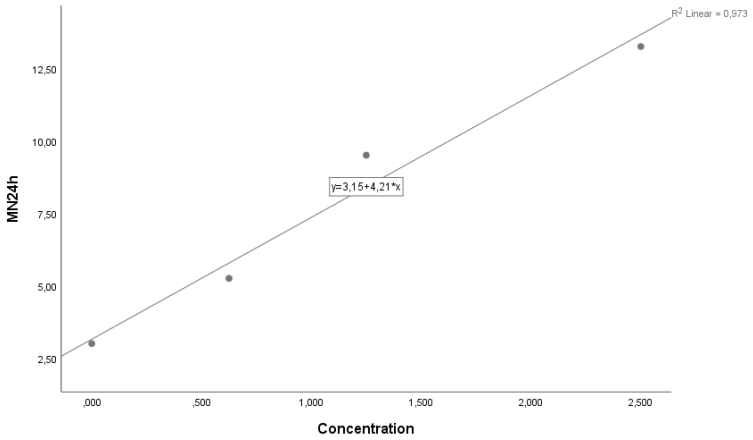

Figure 2. The regression line and correlation coefficient showing the concentration-effect relationship in the MN data detected in lymphocytes treated with different concentrations of EAE for 24 hours

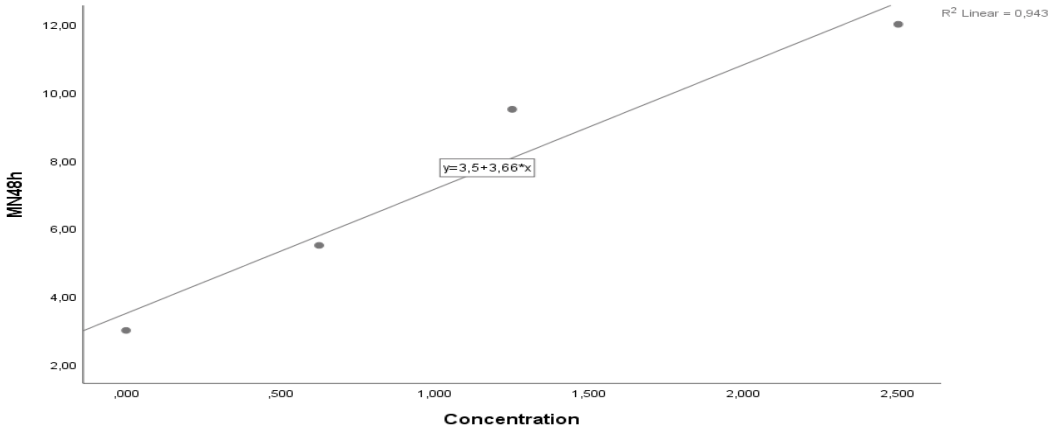

Figure 3. The regression line and correlation coefficient showing the concentration-effect relationship in the MN data detected in lymphocytes treated with different concentrations of EAE for 48 hours

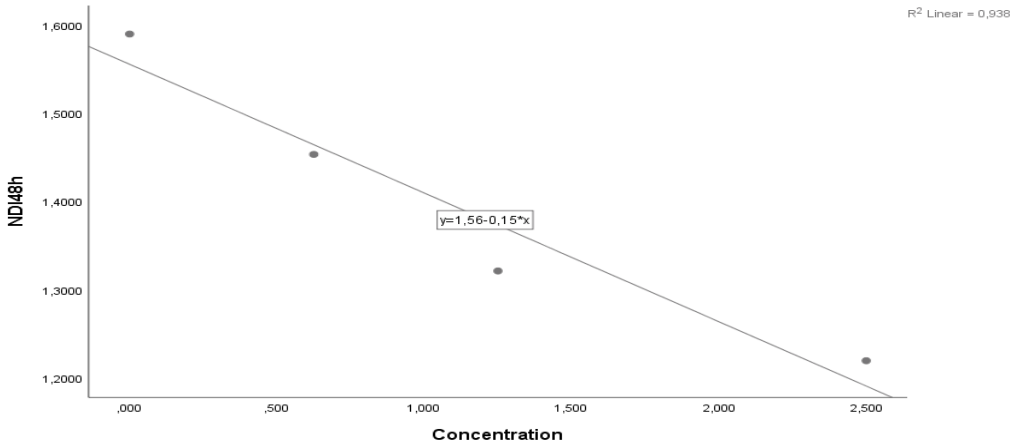

Figure 4. The regression line and correlation coefficient showing the concentration-effect relationship in the NDI data detected in lymphocytes treated with different concentrations of EAE for 48 hours.

3.1.2. Comet Test Findings

DNA strand breaks in human peripheral lymphocyte cells treated with EAE were investigated by the comet assay. DNA damages increased in lymphocytes exposed to 0.625, 1.25, or 2.5 mg/ml concentrations of spurge plant extract (EAE) for 1 hour. This observation in the Comet assay is relatively consistent with the MN findings. In this experiment, the percentage of damaged cells (DCR) and the genetic damage index (GDI) increased significantly (P≤0.01) at the other concentrations, except for the lowest concentration of the test substance (0.625 µg/ml). At the highest dose, the GHI data was significantly higher (P≤0.05) even than the positive control (Table 3). In this experiment, the concentration-effect relationship (P≤0.01) was significant only in the DCR data (Fig. 5).

Table 3. Damaged Cell Ratio (DCR) and Genetic Damage Indices (GDI) detected in human peripheral lymphocytes treated with different concentrations of EAE

Test Substance	Cons. (mg/ml)	Damaged Cell Ratio (%) (DCR)	Genetic Damage Indices (GDI)
H_2O_2*	100 µM	66.00 ± 2.00	2.09 ± 0.11
EAE**	0	16.50 ± 0.50	1.15 ± 0.02
EAE	0.625	18.00 ± 1.00	1.13 ± 0.05
EAE	1.25	29.50 ± 1.50b_2	1.67 ± 0.02b_2
EAE	2.5	64.00 ± 0.50b_3	2.81 ± 0.19 a_1b_3

*H_2O_2: Positive control; **EAE: Euphorbia aqueous extract. **a**: The difference with the positive control group is significant; **b**: The difference with the untreated group is significant. a_1: P≤0.05; b_2: P≤0.01; b3: P≤0.001

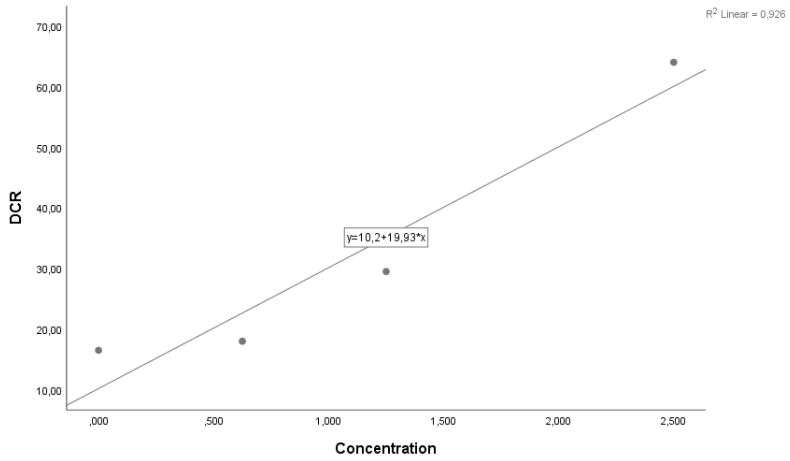

Figure 5. The regression line and correlation coefficient showing the concentration-effect relationship in the damaged cell ratio (DCR) data detected in lymphocytes treated with different concentrations of EAE for 1 hour in Comet assay

3.1.3. Ames Test Findings

The potential to transform into prototrophs by reverse mutation of auxotroph Salmonella typhimurium mutant strains (TA98 and TA100) treated with different concentrations of EAE was observed; however, the difference was not significant between the untreated and test groups (P>0.05) (Table 4).

Tablo 4. The mean number of revertant colonies in Salmonella typhimurium TA98 and TA100 strains treated with EAE at different Concentrations

Test Substance	Concentration (mg/plate)	TA98	TA100
4-NPD(1)	20 µg/plate	47.3333±2.7284	-
NaN_3 (2)	1 µg/plate	-	352.0000±33.7786
EAE*	0	32.6666±4.6666	324.0000±28.4780
EAE	0.625	27.3333±7.2188	300.3333±16.7564
EAE	1.25	43.0000±2.8867	318.3333±18.2239
EAE	2.5	44.3333±8.8191	323.0000±10.0166

(1) 4-NPD: 4-Nitro-o-phenylenediamine; (2) NaN_3: Sodium azide; *EAE: Euphorbia aqueous extract

3.1.4. Total Oxidant and Total Antioxidant Test Findings

Total oxidant capacity (TOC) and total antioxidant response (TAR) values of blood culture medium treated for 24 or 48 hours with 0.625, 1.25, or 2.5 mg/ml concentrations of *Euphorbia grisophylla* aqueous extract were all within the confidence interval. Small fluctuations possibly related to the test substance were not statistically significant (P>0.05). There were non-significant differences in the oxidative stress index (OSI) values calculated from TOC and TAR data (Table 5).

Table 5. Total oxidant capacity (TOC), total antioxidant yanıt (TAR), and oxidative stress index (OSI) value caused by *Euphorbia grisophylla* aqueous extract (EAE) in human peripheral lymphocyte culture

Treatment					
Test Substance	Time (hour)	Cons. (mg/ml)	TOC±SH	TAR±SH	OSI±SH
MMC*	24	0.25 µg/ml	4.9828±0.6356	1.778±0.2644	2.9047±0.4088
EAE**	24	0	3.6938±0.8934	1.6805±0.2541	2.6419±1.1462
EAE	24	0.625	5.3298±0.5175	1.70875±0.1347	3.1391±0.2527
EAE	24	1.25	5.8503±0.9179	1.58625±0.1239	3.7824±0.6931
EAE	24	2.5	6.321±1.2745	1.9425±0.3680	3.2816±0.4260
MMC	48	0.25 µg/ml	2.8507±1.3670	1.9917±0.2880	1.7382±1.080
EAE	48	0	3.6938±0.8934	1.6805±0.2541	2.6419±1.1462
EAE	48	0.625	3.4210±0.8437	1.8985±0.1559	1.9528±0.6783
EAE	48	1.25	4.5860±0.5157	0.9963±0.3485	6.0248±1.4160
EAE	48	2.5	3.8673±0.8822	1.8445±0.1236	2.1274±0.5035

*MMC: Mitomycin C (Positive control); **EAE: Euphorbia aqueous extract

3.2. Discussion

In this study, Euphorbia grisophylla aerial part water extract (EAE) was investigated for genotoxic, cytotoxic, and oxidative potential. Micronucleus (MN) findings, one of the endpoint assays, correlate with the clastogenic and or aneugenic effect. In this test findings, the statistically insignificant increase in the number of MN was due to the increase in the extract concentration. In addition, MN formations induced by EAE are significantly lower than the positive control (mitomycin C, MMC). The similarities between the MN increases detected at high concentrations of the test substance and the DNA damage findings in the comet test draw attention. The fact that the outputs of these two experiments seem to support each other may be related to the possible clastogenic activity of the test substance. However, insignificant increases in the number of revertant colonies observed in the Ames test indicate that EAE did not induce the point mutation. The clastogenic and cytotoxic effect of EAE may be related to disruption of the DNA synthesis/repair function, oxidative stress, or the inadequacy of oxidative energy production.

Although the oxidative stress index (OSI) measured in the cell culture medium showed partial fluctuations, it was not statistically significant. The cause for this result is either the TOC did not significantly increase, or TAR did not respond to the current situation. The fact that Euphorbia extract possibly reduces the amount of dissolved oxygen in the liquid medium may help to explain the present results. According to previous studies supporting this view, Euphorbia extract significantly reduces the amount of dissolved oxygen depending on the dose [27,28]. Decreased dissolved oxygen probably suppresses oxidative stress parameters and oxidative ATP production in cells. This approach also supports the argument that the test substance is possible genotoxic and apparent cytotoxic.

Previous studies have data that supports some of our findings and contradicts others. When it comes to the clastogenic potential, including DNA breaks, with results of a few comprehensive studies conducted recently generally show similarities with the DNA damage findings we have detected. But a small number of them have reported that euphorbia products do not damage DNA and even have DNA protective activity, contrary to our findings. In one study, the whole extract and fractions of *Euphorbia rigida* significantly stimulated DNA strand breaks [29]. Similarly, in the micronucleus test performed with *Euphorbia hyssopifolia* ethanolic extract in the hepatocarcinoma cell line (HepG2), some concentrations induced severe DNA damage that led to death [30]. *Euphorbia triaculeata* extract exhibited a genotoxic effect by increasing the mean DNA damage percentage in a time-dependent manner against MCF-7 and PC 3 cancer lines, but this did not occur in the HEPG2 cancer cell line [31]. In the comet experiment with the MCF-7 cell line, *Euphorbia hirta* extract exhibited a genotoxic effect by increasing the average DNA damage rate in a time-dependent manner [32]. In the *Allium cepa* test performed with the extract of the same euphorbia species, an increase in chromosome aberrations and a decrease in the mitotic index were observed in response to the high extract concentration [33]. Contrary to the above, both the aqueous extract and the latex of the *Euphorbia tirucalli* are not genotoxic in human leukocyte cells in vitro [34]. Some euphorbia species have DNA protective activity; the methanolic extract of *Euphorbia dracunculoides* showed a healing effect against oxidative stress and DNA damage caused by CCI4 in rat liver [35].

2.3-butanediol, one of the most abundant components in the test substance extract, did not show embryotoxic effects in 10 days pregnant albino Wistar rats [36]. In a study with human blood, the 30% concentration of 2.3-butanediol showed relatively low toxicity for erythrocytes. However, the time-dependent hemolytic effect draws attention. The hemolysis rate detected 5 hours after treatment of the agent reached 2%, 6% after 21 hours, and 60% after 46 hours [37].

Hexanoic acid, another component of euphorbia extract, has shown a growth-arresting effect in early childhood malignant tumors [38]. This result is consistent with our cytotoxicity findings.

According to the Ames test results in our study, the test substance did not cause a significant increase in colony numbers of *Salmonella typhimurium* mutant auxotrophic strains (TA 98 and TA 100). Here the extract did not induce frameshift and base shift mutations in the auxotrophic strains. Similarly, in different studies, complete extracts and fractions of some medicinal plants, including euphorbia, did not produce a positive mutagenic effect in *Salmonella typimurium* mutant strains (TA98 and TA100), regardless of whether there was metabolic activation or not [29]. The natural or lyophilized Euphorbia splendens latex did not have an acute toxic effect on bioluminescent *Photobacterium phosphoreum*. In addition, it did not cause mutagenic activity in *Salmonella typhimurium* TA98 and TA100 strains in the presence or absence of S9 [39]. According to these results, Euphorbia extract does not induce nucleotide substitution and frameshift mutations.

In the cell culture used in our study, the aqueous extract of spurge did not induce any oxidative stress parameters (TOC and TAR) and thus oxidative stress index. However, in a different study, the antioxidant effect of *Euphorbia hirta* and the galactoside and glucoside derivatives isolated from *Euphorbia kansui* were discovered to have antioxidative and anti-fatigue properties in mice [40,41]. An extract combination (KIOM-79) obtained from a group of medicinal plants, including *Euphorbia pekinensis*, was studied in rat pancreatic beta (RINm5F) cells. Against oxidative stresses induced by streptozotocin (STZ) in RINm5F (rat insulinoma) cells, KIOM-79 showed a potential cytoprotective effect by inhibiting the ERK pathway [42]. In contrast to the above study, euphorbia factor L1 (EFL1), a lathyran-type diterpenoid obtained from the *Euphorbia lathyris* plant, decreased human gastric mucosal epithelium cells (GES-1) survival. Moreover, this agent increased LDH leakage and induced abnormal ROS, MDA, and SOD production in GES-1 cells. Some parameters such as reduced mitochondria membrane potential (MMP), pyknotic nucleus, fragmented DNA, and increased apoptosis rate are reporters of mitochondria-mediated apoptosis. EFL1 has reduced the survival of GES-1 cells by several mechanisms, including oxidative stress, mitochondria-mediated apoptosis activation, and inhibition of the PI3K/AKT/mTOR pathway [43]. The above result was confirmed by genotoxicity data, which damages DNA and

increases oxidative damage at 1% and 10% concentrations of *Euphorbia tirucalli* (Aveloz) extract. Since aveloz contains phorbol ester, it can act as a genotoxic agent [44]. Contrary to the previous finding, *Euphorbia dracunculoides* methanolic extract showed an ameliorative effect against CCI4-induced oxidative stress and DNA damage (hepatic toxicity) in rat liver [35]. *Euphorbia bicolor* latex extract showed antioxidant activity by scavenging free radicals in vitro analyses. [45]. In a different study, diterpenoids isolated from ethyl acetate extract obtained from the aerial parts of *Euphorbia antiquorum* significantly suppressed the production of the lipopolysaccharide-induced oxidation marker nitric oxide (NO) in murine macrophage J774.A1 cells [46].

The difference between our findings and the results of some studies may be due to differences in plant species, habitat, harvest time, extraction method, and test protocol. Our experiments were carried out directly on a single type of healthy cell culture medium in vitro. Possibly, the lack of homeostatic reflexes of cells to an exogenous effect may be one of the reasons for the discrepancy between study findings.

In our study, the applied *Euphorbia grisophylla* plant extract decreased the nuclear division index (NDI) value non-randomly compared to the control. In another study, extracts from Euphorbia platyphyllos showed cytotoxic effects on human breast metastatic carcinoma (MCF-7) cells in a dose- and time-dependent manner in vitro. It has been evaluated that this cytotoxicity is due to the genotoxic effect [47]. Contrary, no significant cytotoxic effect of *Euphorbia splendens* latex was detected in Chinese hamster ovary (CHO) cells [39]. The ethanolic extract isolated from *Euphorbia hyssopifolia* species did not show significant cytotoxic activity in the MTT test in HepG2 cell culture. However, the 1.0 mg/ml concentration of the extract induced severe cell damage and cytotoxic effect leading to death in the micronucleus test [30]. There is significant toxicity in the MTT test performed with a methanolic extract of a different species of spurge (*Euphorbia triaculeata*) [31]. The increase in methanolic extract concentrations of *Euphorbia hirta* significantly decreased the mitotic index in *Allium cepa* cells. In the current situation, the mitodepressive nature of the methanolic extract is remarkable. [33]. In a study including in silico approaches to examine the apoptogenic potential of *Euphorbia peplus* methanolic extract (EPME) in rats, EPME induced strong renal and cardiac p53 expression and moderate cardiac TNF-a expression, which are markers of apoptosis in rats. Moreover, according to molecular docking simulation data, di-(2-ethylhexyl) phthalate (DEHP), one of the components of the extract, was predicted to have a higher docking affinity to the unique apoptosis dimer interface. Here DEHP docked and inhibited to apoptosis dimer interface that the mouse double minute 2 (MDM2) homologous-p53, compared to the standard reference compound. Long-term exposure to EPME adversely affects heart and kidney tissues, possibly due to stimulating inflammatory and apoptotic activities. According to in silico data, EPME inhibits MDM2-mediated p53 degradation, suggesting possible anticancer potential in kidney and heart tissues [48]. One of the different diterpenoids isolated from *Euphorbia neriifolia* exhibited moderate cytotoxicity on HepG2 and multi-drug resistant HepG2/Adr cells. While another component of the same plant exhibited significant cytotoxicity in the HepG2 cell line, it did not show any cytotoxic activity against the HepG2/Adr cell line [49]. The semi-synthetic ingenol derivative (ingenol-3-dodecanoate, IngC) from *Euphorbia tirucalli* caused dose-dependent cytotoxicity and effective colony reduction. Exposure to IngC regulated a broad spectrum of signaling effectors relevant to survival and cell cycle management by inducing S-phase arrest associated with overexpression of the p21CIP/WAF1 pathway. In addition, an IngC derivative directed glioma cells to autophagy through the accumulation of LC3B-II. When combined with a specific autophagic inhibitor, bafilomycin A1, cells exhibited increased cytotoxic sensitivity [50]. In a study related to the induction of apoptosis, the hydroalcoholic extract of *Euphorbia microsciadia* increased the cytotoxic effect on the metastatic mammary epithelium (MDA-MB-231) cell line in a dose-dependent manner. According to qRT-PCR data, the same extract increased the expression levels of Let-7, miR-15, miR-16, miR-29, and miR-34a molecules, which caused increased apoptosis [51]. Zhou et al. (2021) observed that 80% ethanol extract obtained from the aerial parts of *Euphorbia helioscopia* exhibited cytotoxic activities against six different kidney cancer cell lines [52]. Another study has reported that seven compounds, three of them novel, isolated from *Euphorbia peplus*, did not show cytotoxic activities against five human tumor cell lines at 40 µM concentration [53]. One of the eight compounds, ingol-3.7.12-triacetate-8-benzoate, isolated from *Euphorbia royleana* has shown a modulatory effect on the P-glycoprotein-mediated multidrug-resistant cancer cell (HepG2/DOX) line without apparent cytotoxic effect. This effect increased the effectiveness of the anticancer drug doxorubicin (DOX) and proved to be approximately 105 times stronger than the positive drug verapamil [54]. As a result of the phytochemical analysis of *Euphorbia kopetdaghi* aerial part extract, which grows wild in the northeastern regions of Iran, two new different cyclomyrcinol macrocyclic diterpenes with apoptotic effects were discovered. The apoptotic potential of these compounds has been associated with excessive ROS production and loss of mitochondrial membrane potential ($\Delta\Psi m$). One of the compounds abovementioned, copetdaghinane A, inhibits the growth of MCF-7 breast cancer cells through the mitochondrial apoptotic pathway activation [55]. On the other hand, none of the compounds in *Euphorbia antiquorum* root extracts showed cytotoxic activity [56]. Fraction compounds of Euphorbia umbellata

latex hexane extracts have caused morphological changes compatible with apoptosis induction against leukemic cells, such as cell cycle alteration, depolarized cell population increase, and caspase 3/7 activation. The bio-guided fractionation study suggested that latex samples of *Euphorbia umbellata* synergistically enhance the activity of phytocomplexes used against leukemia cells and that this cytotoxicity may be due to apoptosis [57]. The chloroform fraction (Em-C) of *Euphorbia milii* methanol extract showed significant cytotoxicity against HepG2 cells compared with the standard compound 5-fluorouracil (5-FU). However, Em-C did not show similar significant effects on the human cervical cancer cell (HeLa) line [58]. GRC-2, a prostratin analog isolated from Euphorbia grandicornis, is ten times more potent than prostratin used to inhibit the growth of human non-small cell lung cancer (NSCLC) (A549) cells. According to flow cytometry analysis, GRC-2 and prostratin induce apoptosis by inhibiting cell cycle progression in the G2/M phase. There is evidence that the cytotoxic effect of GRC-2 and prostratin accompany Protein kinase C-δ (PKC-δ) and Protein kinase D (PKD) activation and nuclear translocation as well as ERK hyperactivation. Here, prostratin and its more potent analog, GRC-2, has reduced cell viability in A549 cells, partly through activation of the PKC-δ/PKD/ERK pathway [59]. In a similar study, two new daphnane-type diterpenoids (fischerianin A and fischerianin B) and two known diterpenoids (langduin A and langduin A6) were isolated from the extract of the dry roots of *Euphorbia fischeriana* Steud. These four compounds have shown moderate inhibitory activity against human cancer cell lines (A375, HepG2, HL-60, K562, and HeLa) [60].

4. Conclusion

Some of the research on Euphorbia plant extract and its components are in vivo studies. However, cancer cell lines have been used in most in vitro studies. The originality of our experiments is the application of *Euphorbia grisophylla* plant extract to healthy cells in vitro under short-term treatment. According to our findings, *Euphorbia grisophylla* extract showed a clear and consistent cytotoxic effect. In most previous studies, plant extracts belonging to Euphorbia species have cytotoxic and antioxidant potential in cancer cell lines.

As mentioned above, according to our findings, spurge extract had a partial cytogenotoxic effect on lymphocytes. The cause for this effect has not been clearly explained. This situation may have resulted from the extract's retention of the dissolved oxygen present in the culture medium. The oxygen binding potential has been experimentally confirmed but not published yet. Already, the practice of using euphorbia in fishing with the conventional method among the people forms the basis of this idea. Presumably, the fact that the extract does not also stimulate oxidative stress indicators may be related to the oxygen binding/retention characteristic in the environment.

In summary in this study, moderate genotoxic and prominent cytotoxic effects of *Euphorbia grisophylla* extract at subtoxic concentrations were observed in cultured healthy human peripheral lymphocyte cultures. However, the agent has not caused significant changes in parameters related to oxidative stress. The fact that a very small number of similar studies have been carried out before increases the importance of this project. Original scientific research to be carried out by impartial laboratories with euphorbia extract or components in different cell systems and with innovative methods, alone or in combination, will be groundbreaking in that area. Moreover, it is a strong possibility that the findings obtained from these studies will donate new paradigms and actors to the pharmaceutical industry.

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Conflicts of Interest

There is no statement of conflict of interest for this study.

References

[1] Can, L., (2012). İstanbul Üniversitesi Fen Bilimleri Enstitüsü Yüksek Lisans Tezi.

[2] WHO., (2022). Traditional, Complementary and Integrative Medicine. https://www.who.int/health-topics/traditional-complementary-and-integrative-medicine#tab=tab_1. [accessed June 8, 2022].

[3] WHO., (2002). Traditional Medicine Strategy (2002–2005). WHO/EDM/TRM/2002.1. Geneva, Switzerland: World Health Organization. https://apps.who.int/iris/bitstream/handle/10665/67163/WHO_EDM_TRM_2002.1_eng.pdf?sequence=1&isAllowed=y. [accessed June 9, 2022].

[4] Veeresham, C., (2012). Natural products derived from plants as a source of drugs. Journal of Advanced Pharmaceutical Technology & Research. 3(4): 200. doi: 10.4103/2231-4040.104709.

[5] Newman, D.J., Cragg, G.M., (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. Journal of Natural Products. 75(3): 311–35. doi: 10.1021/NP200906S.

[6] Fabricant, D.S., Farnsworth, N.R., (2001). The value of plants used in traditional medicine for drug discovery. Environmental Health Perspectives. 109 Suppl 1(Suppl 1): 69–75. doi: 10.1289/EHP.01109S169.

[7] Gillespie, L.J., Armbruster, W.S., (1997). A Contribution to the Guianan Flora: Dalechampia, Haemutostemon, Omp ha lea, Peru, Plukene tia, and Tragia (Euphorbiaceae) with Notes on Subfamily Acalyphoideae. Washington: Smithsonian Institution Press.

[8] Salehi, B., Iriti, M., Vitalini, S., Antolak, H., Pawlikowska, E., Kręgiel, D., et al., (2019). Euphorbia-Derived Natural Products with Potential for Use in Health Maintenance. Biomolecules 2019, Vol. 9, Page 337. 9(8): 337. doi: 10.3390/BIOM9080337.

[9] Seca, A.M.L., Pinto, D.C.G.A., (2018). Plant Secondary Metabolites as Anticancer Agents: Successes in Clinical Trials and Therapeutic Application. International Journal of Molecular Sciences. 19(1). doi: 10.3390/IJMS19010263.

[10] IPNI., (1970). Euphorbia grisophylla M.L.S.Khan | Plants of the World Online | Kew Science. https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:346693-1. [accessed June 9, 2022].

[11] Baytop, T., (1999). Türkiye'de bitkiler ile tedavi: Geçmişte ve bugün. 2nd ed., İstanbul: Nobel Tıp Kitabevleri.

[12] Hohmann, J., Forgo, P., Csupor, D., Schlosser, G., (2003). Isolation and Structure Determination of New Jatrophane Diterpenoids from Euphorbia platyphyllos L. Helvetica Chimica Acta. 86(10): 3386–93. doi: 10.1002/HLCA.200390282.

[13] Haba, H., Lavaud, C., Harkat, H., Alabdul Magid, A., Marcourt, L., Benkhaled, M., (2007). Diterpenoids and triterpenoids from Euphorbia guyoniana. Phytochemistry. 68(9): 1255–60. doi: 10.1016/J.PHYTOCHEM.2007.01.017.

[14] Chaabi, M., Freund-Michel, V., Frossard, N., Randriantsoa, A., Andriantsitohaina, R., Lobstein, A., (2007). Anti-proliferative effect of Euphorbia stenoclada in human airway smooth muscle cells in culture. Journal of Ethnopharmacology. 109(1): 134–9. doi: 10.1016/J.JEP.2006.07.016.

[15] Rothfuss, A., S.P., B.S., V.T., E.E., K.R., V.W., & S.G., (2000). Induced micronucleus frequencies in peripheral lymphocytes as a screening test for carriers of a BRCA1 mutation in breast cancer families - PubMed. Cancer Research. https://pubmed.ncbi.nlm.nih.gov/10667592/. [accessed May 27, 2022].

[16] OECD TN 487., (2016). OECD/OCDE 487: In Vitro Mammalian Cell Micronucleus Test | READ online. OECD Guideline for the Testing of Chemicals. https://read.oecd-ilibrary.org/environment/test-no-487-in-vitro-mammalian-cell-micronucleus-test_9789264264861-en#page1. [accessed June 10, 2022].

[17] İla, H.B., Topaktaş, M., Arslan, M., Büyükleyla, M., İstifli, E.S., (2015). Evaluation of the genotoxic or mutagenic effects of thermal stress on cultured human lymphocytes. Turkish Journal of Biology . 39: 98–103. doi: 10.3906/biy-1404-55.

[18] Titenko-Holland, N., Windham, G., Kolachana, P., Reinisch, F., Parvatham, S., Osorio, A.M., et al., (1997). Genotoxicity of malathion in human lymphocytes assessed using the micronucleus assay in vitro and in vivo: a study of malathion-exposed workers. Mutation Research. 388(1): 85–95. doi: 10.1016/S1383-5718(96)00140-4.

[19] Fenech, M., (2000). The in vitro micronucleus technique. Mutation Research. 455(1–2): 81–95. doi: 10.1016/S0027-5107(00)00065-8.

[20] Eastmond, D.A., Tucker, J.D., (1989). Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody. Environmental and Molecular Mutagenesis. 13(1): 34–43. doi: 10.1002/em.2850130104.

[21] Fenech, M., (1997). The advantages and disadvantages of the cytokinesis-block micronucleus method. Mutation Research. 392(1–2): 11–8. doi: 10.1016/S0165-1218(97)00041-4.

[22] Pitarque, M., Vaglenov, A., Nosko, M., Hirvonen, A., Norppa, H., Creus, A., et al., (1999). Evaluation of DNA damage by the Comet assay in shoe workers exposed to toluene and other organic solvents. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 441(1): 115–27. doi: 10.1016/S1383-5718(99)00042-X.

[23] Husunet, M.T., Mısırlı, R.Ç., Istıflı, E.S., Ila, H.B., (2022). Investigation of the genotoxic effects of patent blue V (E131) in human peripheral lymphocytes and in silico molecular docking. Drug and Chemical Toxicology. 45(4): 1780–6. doi: 10.1080/01480545.2021.1878208.

[24] Tarpey, M.M., Wink, D.A., Grisham, M.B., (2004). Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations. American Journal of Physiology. Regulatory, Integrative and Comparative Physiology. 286(3). doi: 10.1152/AJPREGU.00361.2003.

[25] Erel, O., (2005). A new automated colorimetric method for measuring total oxidant status. Clinical Biochemistry. 38(12): 1103–11. doi: 10.1016/J.CLINBIOCHEM.2005.08.008.

[26] Erel, O., (2004). A novel automated method to measure total antioxidant response against potent free radical reactions. Clinical Biochemistry. 37(2): 112–9. doi: 10.1016/J.CLINBIOCHEM.2003.10.014.

[27] Akpa, L.E., Ajima, M.N.O., Nwani, C.D., Onu, C.A., (2009). Toxic effect of Euphorbia tirucalli (Pencil tree) sap on Tilapia zillii fingerlings. Environment Conservation Journal. 9(2): 247–51.

[28] Idowu, A.A., Soetan, M.O., Akinde, A., Popoola, O.C., (2019). Effect of Euphorbia hirta leaf extracts on histopathology of juvenile Clarias gariepinus. Nigerian Journal of Animal Science. 21(1): 96–109. doi: 10.4314/tjas.v21i1.

[29] Basaran, A.A., Yu, T.W., Plewa, M.J., Anderson, D., (1996). An investigation of some Turkish herbal medicines in Salmonella typhimurium and in the COMET assay in human lymphocytes. Teratogenesis, Carcinogenesis, and Mutagenesis. 16(2): 125–38. doi: 10.1002/(SICI)1520-6866(1996)16:2<125::AID-TCM6>3.0.CO;2-K.

[30] de Sousa Araújo, S., Fernandes, T.C.C., Cardona, Y.T., Almeida, P.M. de., Marin-Morales, M.A., dos Santos, A.V., et al., (2015). Cytotoxic and genotoxic effects of ethanolic extract of Euphorbia hyssopifolia L. on HepG2 cells. Journal of Ethnopharmacology. 170: 16–9. doi: 10.1016/J.JEP.2015.04.044.

[31] Al-Faifi, Z.I.A., Masrahi, Y.S., Aly, M.S., Al-Turki, T.A., Dardeer, T., (2017). Evaluation of Cytotoxic and Genotoxic Effects of Euphorbia Triaculeata Forssk. Extract. Asian Pacific Journal of Cancer Prevention : APJCP. 18(3): 771–7. doi: 10.22034/APJCP.2017.18.3.771.

[32] Ping, K.Y., Darah, I., Chen, Y., Sasidharan, S., (2013). Cytotoxicity and genotoxicity assessment of Euphorbia hirta in MCF-7 cell line model using comet assay. Asian Pacific Journal of Tropical Biomedicine. 3(9): 692–6. doi: 10.1016/S2221-1691(13)60140-9.

[33] [Mohamed, H.M., Aly, M.S., (2018). Evaluation of genotoxicity of Euphorbia triaculeata Forssk. extract on mice bone marrow cells in vivo. Toxicology Reports. 5: 625–31. doi: 10.1016/J.TOXREP.2018.05.007.

[34] de Oliveira, L.F.S., Fuentefria, A.M., da Silva Klein, F., Machado, M.M., (2015). Antifungal activity against Cryptococcus neoformans strains and genotoxicity assessment in human leukocyte cells of Euphorbia tirucalli L. Brazilian Journal of Microbiology : [Publication of the Brazilian Society for Microbiology]. 45(4): 1349–55. doi: 10.1590/S1517-83822014000400027.

[35] Batool, R., Khan, M.R., Majid, M., (2017). Euphorbia dracunculoides L. abrogates carbon tetrachloride induced liver and DNA damage in rats. BMC Complementary and Alternative Medicine. 17(1): 223. doi: 10.1186/S12906-017-1744-X.

[36] Priscott, P.K., (1985). The effects of acetaldehyde and 2,3-butanediol on rat embryos developing in vitro. Biochemical Pharmacology. 34(4): 529–32. doi: 10.1016/0006-2952(85)90185-6.

[37] Pubchem., n.d. 2,3-Butanediol | C4H10O2 - PubChem. 2,3-Butanediol. https://pubchem.ncbi.nlm.nih.gov/compound/2_3-Butanediol#section=Toxicity-%20%20%20%20%20%20%20Summary. [accessed May 30, 2022].

[38] Atasever-Arslan, B., Yilancioglu, K., Bekaroglu, M.G., Taskin, E., Altinoz, E., Cetiner, S., (2015). Cytotoxic effect of extract from Dunaliella salina against SH-SY5Y neuroblastoma cells. General Physiology and Biophysics. 34(2): 201–7. doi: 10.4149/GPB_2014034.

[39] Schall, V.T., Vasconcellos, M.C., Valent, G.U., Sato, M.I.,., Furlan, E. v., Sanchez, P.S., (1991). Evaluation of the genotoxic activity and acute toxicity of Euphorbia splendens latex, a molluscicide for the control of schistosomiasis. Brazilian Journal of Medical and Biological Research . 24(6): 573–82.

[40] Karim, K., Jura, M.R., Sabang, S.M., (2015). Uji Aktivitas Antioksidan Ekstrak Daun Patikan Kebo (Euphorbia Hirta L.). Jurnal Akademika Kimia. 4(2): 56–63.

[41] Yu, F., Lu, S., Yu, F., Feng, S., McGuire, P.M., Li, R., et al., (2006). Protective effects of polysaccharide from Euphorbia kansui (Euphorbiaceae) on the swimming exercise-induced oxidative stress in mice. Canadian Journal of Physiology and Pharmacology. 84(10): 1071–9. doi: 10.1139/Y06-052.

[42] Kang, K.A., Lee, K.H., Kim, S.Y., Kim, H.S., Kim, J.S., Hyun, J.W., (2007). Cytoprotective effects of KIOM-79 on streptozotocin induced cell damage by inhibiting ERK and AP-1. Biological & Pharmaceutical Bulletin. 30(5): 852–8. doi: 10.1248/BPB.30.852.

[43] Zhu, A., Sun, Y., Zhong, Q., Yang, J., Zhang, T., Zhao, J., et al., (2019). Effect of euphorbia factor L1 on oxidative stress, apoptosis, and autophagy in human gastric epithelial cells. Phytomedicine : International Journal of Phytotherapy and Phytopharmacology. 64. doi: 10.1016/J.PHYMED.2019.152929.

[44] Machado, M.M., de Oliveira, L.F.S., Zuravski, L., de Souza, R.O., Fischer, P., Duarte, J.A., et al., (2016). Evaluation of genotoxic and cytotoxic effects of hydroalcoholic extract of Euphorbia tirucalli (Euphorbiaceae) in cell cultures of human leukocytes. Anais Da Academia Brasileira de Ciencias. 88(1): 17–28. doi: 10.1590/0001-3765201520140076.

[45] Basu, P., Hornung, R.S., Averitt, D.L., Maier, C., (2019). Euphorbia bicolor (Euphorbiaceae) Latex Extract Reduces Inflammatory Cytokines and Oxidative Stress in a Rat Model of Orofacial Pain. Oxidative Medicine and Cellular Longevity. 2019. doi: 10.1155/2019/8594375.

[46] Choodej, S., Hanthanong, S., Aree, T., Pudhom, K., (2020). Diterpenoids from the aerial parts of Euphorbia antiquorum and their efficacy on nitric oxide inhibition. Phytochemistry. 180. doi: 10.1016/J.PHYTOCHEM.2020.112523.

[47] Aslantürk, Ö.S., Çelik, T.A., (2010). Aydın yöresinde kullanılan bazı tıbbi bitkilerin antioksidant ve sitotoksik etkilerinin araştırılması. (2010).

[48] Abd-Elhakim, Y.M., Abdo Nassan, M., Salem, G.A., Sasi, A., Aldhahrani, A., ben Issa, K., et al., (2019). Investigation of the In-Vivo Cytotoxicity and the In Silico-Prediction of MDM2-p53 Inhibitor Potential of Euphorbia peplus Methanolic Extract in Rats. Toxins. 11(11): 642. doi: 10.3390/toxins11110642.

[49] Li, J.C., Feng, X.Y., Liu, D., Zhang, Z.J., Chen, X.Q., Li, R.T., et al., (2019). Diterpenoids from Euphorbia neriifolia and Their Related Anti-HIV and Cytotoxic Activity. Chemistry & Biodiversity. 16(12). doi: 10.1002/CBDV.201900495.

[50] Silva, V.A.O., Rosa, M.N., Tansini, A., Martinho, O., Tanuri, A., Evangelista, A.F., et al., (2019). Semi-Synthetic Ingenol Derivative from Euphorbia tirucalli Inhibits Protein Kinase C Isotypes and Promotes Autophagy and S-phase Arrest on Glioma Cell Lines. Molecules (Basel, Switzerland). 24(23). doi: 10.3390/MOLECULES24234265.

[51] Mahmoudian-Sani, M.-R., Asadi-Samani, M., (2020). Modulation of MicroRNAs by Euphorbia Microsciadia Boiss in MDA-MB-231 Cell Line: New Possibilities in Breast Cancer Therapy. Recent Patents on Anti-Cancer Drug Discovery. 15(2): 174–84. doi: 10.2174/1574892815666200630102944.

[52] Zhou, M., Ma, Q., He, L., Chen, Y.H., Zhu, B.Y., Wang, J.H., et al., (2021). Cytotoxic jatrophane diterpenoids from the aerial parts of Euphorbia helioscopia. Journal of Asian Natural Products Research. 23(8): 731–7. doi: 10.1080/10286020.2020.1769611.

[53] Chen, Y.N., Lu, Q.Y., Li, D.M., Li, Y.Y., Pu, X.X., Li, B.T., et al., (2021). Three new diterpenoids from Euphorbia peplus. Natural Product Research. 35(21): 3901–7. doi: 10.1080/14786419.2020.1749609.

[54] Shaker, S., Sang, J., Yan, X.L., Fan, R.Z., Tang, G.H., Xu, Y.K., et al., (2020). Diterpenoids from Euphorbia royleana reverse P-glycoprotein-mediated multidrug resistance in cancer cells. Phytochemistry. 176. doi: 10.1016/J.PHYTOCHEM.2020.112395.

[55] Riahi, F., Dashti, N., Ghanadian, M., Aghaei, M., Faez, F., Jafari, S.M., et al., (2020). Kopetdaghinanes, pro-apoptotic hemiacetialic cyclomyrsinanes from Euphorbia kopetdaghi. Fitoterapia. 146. doi: 10.1016/J.FITOTE.2020.104636.

[56] Tran, T.N., Sichaem, J., Nguyen, V.K., Nguyen, H.H., Cao, T.T., Nguyen, T.P., et al., (2022). New diterpenoids from the stems of Euphorbia antiquorum growing in Vietnam. Natural Product Research. 36(2): 1–8. doi: 10.1080/14786419.2020.1789634.

[57] Cruz, L.S., Kanunfre, C.C., de Andrade, E.A., de Oliveira, A.A., Cruz, L.S., de Faria Moss, M., et al., (2020). Enriched Terpenes Fractions of the Latex of Euphorbia umbellata Promote Apoptosis in Leukemic Cells. Chemistry & Biodiversity. 17(9). doi: 10.1002/CBDV.202000369.

[58] Chohan, T.A., Sarfraz, M., Rehman, K., Muhammad, T., Ghori, M.U., Khan, K.M., et al., (2020). Phytochemical profiling, antioxidant and antiproliferation potential of Euphorbia milii var.: Experimental analysis and in-silico validation. Saudi Journal of Biological Sciences. 27(11): 3025–34. doi: 10.1016/J.SJBS.2020.08.003.

[59] Tsai, J.Y., Rédei, D., Hohmann, J., Wu, C.C., (2020). 12-Deoxyphorbol Esters Induce Growth Arrest and Apoptosis in Human Lung Cancer A549 Cells Via Activation of PKC-δ/PKD/ERK Signaling Pathway. International Journal of Molecular Sciences. 21(20): 1–12. doi: 10.3390/IJMS21207579.

[60] Xie, R., Xia, G., Zhu, J., Lin, P., Fan, X., Zi, J., (2021). Daphnane-type diterpenoids from Euphorbia fischeriana Steud and their cytotoxic activities. Fitoterapia. 149: 104810. doi: 10.1016/J.FITOTE.2020.104810.