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Original Article

The Cytotoxic and Genotoxic Potential of *Euphorbia macroclada* Boiss. Extract on Colon Cancer Cells

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Abstract

Introduction: Cancer continues to be the second most common cause of death globally. There is currently any proven treatment medication for cancer. Chemotherapy is one of the modern therapeutic approaches that not only kills cancer cells but also has major negative effects on healthy cells. 80% of people worldwide still get their basic medical care from traditional medications, according to data from the World Health Organization. Scientists have created new approaches to treating cancer by combining radiation, chemotherapy, and surgery with a variety of phytochemicals derived from different plant species. This is because plants and active pharmaceutical ingredients derived from plants have minimal to no side effects and high activity. The *Euphorbia macroclada*, found in different locations of Turkey, has long been utilized by the public for its traditional medicinal properties in treating a range of illnesses. Research conducted in current scientific databases has established that *Euphorbia macroclada* exhibits significant antioxidant activity.

Methods: This study aims to evaluate the potential antiproliferative effect of the dichloromethane extract of the *Euphorbia macroclada* on HT-29 and HCT-116 cancer cell lines using the *in vitro* MTT technique. Additionally, the possible genotoxic activity using the Comet assay on human colon cancer cells were also done.

Results: The IC_{50} concentration of *Euphorbia macroclada* was determined to be 133,835 µg/mL in HCT-116 cells and 111,215 µg/mL in HT-29 cells after 24 h. Concurrently with the MTT results, it was established that there was an increase in DNA damage in a dose dependent manner than those of control.

Conclusion: Following the collection of these data with the *Euphorbia macroclada*, it has been determined that dichloromethane extract of the *Euphorbia macroclada* has the potential to serve as a therapeutic agent for the treatment of colon cancer. On the other hand, in vivo and in vitro studies on this subject are needed.

Keywords: *Euphorbia macroclada*, colon cancer, cytotoxicity, genotoxicity

1. Introduction

Cancer is a highly detrimental illness that has a significant impact on the human population. The disease is defined by the persistent and uncontrolled growth of cells in the human body. There is a persistent need for novel medicines to address and mitigate this potentially fatal illness. Presently, the available treatments encompass chemotherapy, radiation, and pharmacologically synthesized medications. Existing techniques, such as chemotherapy, are restricted in their application because they have adverse effects on host tissues that are not the intended target. Consequently, experts have directed their attention on the utilization of alternative medicines and therapies in the battle against cancer. Currently, there is a trend towards using natural products that are believed to have fewer adverse effects than standard treatments like chemotherapy. As a result, there is a growing need for alternative medicines that contain naturally occurring substances with anticancer properties (1). Plants are essential elements of both traditional medicine used globally and current medication development research. This is because they are inexpensive, readily available, and have little side effects (2).

Euphorbia species, referred to as "euphorbia" in Turkish, have been utilized in traditional medicine in Turkey and various regions globally for the treatment of dermatological diseases, wounds, warts, gonorrhea, migraines, cancer, and intestinal parasites (3). There has been a growing interest in Euphorbia species due to their varied structures and therapeutic significance. These species possess a range of biological activities including cytotoxic, antitumor, antibacterial, anti-inflammatory (4), antiproliferative, antiviral, antidiarrheal, antimicrobial, and antipyretic-analgesic effects (5). Research indicates that numerous varieties of Euphorbia are used for medicinal purposes, specifically in the management of ailments such as respiratory infections, skin irritations, digestive disorders, inflammatory infections, body discomfort, microbiological infections, and snake or scorpion envenomation. (6-10).

Euphorbia species preparations are used in traditional therapy as skin medicine to alleviate various skin conditions such as itching, warts, eczema, hair loss, acne, dermatitis, boils, sunburn, calluses, rashes, irritation, and pustules. These preparations possess antiseptic, disinfecting, and emollient features (8).

Euphorbia macroclada (*E. macroclada*) is an important plant species commonly found in various regions of Central, Eastern and Southern Anatolia. Among these regions, the most common cities are Ağrı, Ankara, Antalya, Bitlis, Burdur, Çorum, Elazığ, Erzincan, Erzurum, Eskisehir, Gümüshane, Hakkari, Kars, Kayseri, Malatya, Maras, Mardin, Niğde, Osmaniye, Sanliurfa, Tunceli, Van and Yozgat (11). The fact that the plant spreads over such a wide area shows its ability to adapt to different environmental conditions. The use of the plant's latex in traditional medicine reflects its long-standing cultural and medicinal importance in the region. This latex is traditionally used to treat various digestive disorders such as constipation, ulcers, hemorrhoids, and various skin problems such as wounds, stretch marks, warts, eczema, and fungal infections, arthritis, scorpion stings, bee stings, malaria, and body parasites (12).

The current scientific literature study reveals that comprehensive investigation has been done on numerous species of Euphorbia, however only a few investigations have been carried out on *E. macroclada.* Kirbag et al. evaluated the potential antimicrobial activities of *E. macroclada, E. aleppica, E. szovitsii var*. and found that they had antibacterial and antifungal activity (13).

Mahmoudi et al. considering the paucity of studies on the antifungal effects of *E. macroclada latex*, aimed to evaluate the antifungal activity of *E. macroclada* latex in hospitalized patients. As a result of this study, it was determined that *E. macroclada* latex showed antifungal activity against some pathogenic Candida species. (14).

The literature also includes studies on the antioxidant properties of *E. macroclada*. A study was conducted to examine the antioxidant activity in several extracts of *E. macroclada*. The results

of the research revealed that all tested extracts of *E. macroclada* exhibited more antioxidant activity compared to BHT and α-tocopherol (15).

In another study the chemical composition and antioxidant properties of *E. macroclada* were examined.The results indicated that both the ethanolic and aqueous extracts of this plant exhibited significant antioxidant activity, suggesting that it could potentially be utilized in the prevention of certain diseases associated with oxidative stress (16).

A further study was conducted to analyze the chemical composition and antioxidant capabilities of *E. gaillardotii* and *E. macroclada*. The levels of rutin, hesperedin, and hyperoside in the extract of *E. macroclada* were significantly higher compared to the extract of *E. gaillardotii*. In contrast to the absence of rosmarinic acid in the *E. gaillardotii* extract, a significant amount of rosmarinic acid was detected in the *E. macroclada* extract. Therefore, the antioxidant activity of the extract from *E. macroclada* stems and leaves can be attributed to the presence of a significant quantity of e chlorogenic and rosmarinic acid, rutin and quercetin compounds known for its antioxidant properties (17).

Recent investigation has indicated that Euphorbia species possess various pharmacological activities, including *in vitro* anti-cancer effects, owing to their abundant synthesis of bioactive chemicals (18). The plant is believed to have cytotoxic properties through various mechanisms, including its impact on cell proliferation and differentiation, inhibition of apoptosis and metastasis, excessive formation of reactive oxygen species, and promotion of angiogenesis (19).

This study aimed to evaluate the antiproliferative properties of the dichloromethane extract of *E. macroclada* on colon cancer cell lines (HT-29 and HCT-116) using the MTT assay. Furthermore, our objective was to examine its genotoxic potential by employing the Comet assay on human colorectal cancer cells, marking the first instance of such investigation.

2. Methods

2.1 Data collection and identification of plant sample

The aerial parts of the *E. macroclada* were collected from its natural environment and crowded populations in the Tunceli province of Türkiye's eastern Anatolia region. The taxonomic description of the species was made using appropriate scientific sources. The plant dried at room temperature and in the shade. Additionally, a sample of it has been kept as voucher specimen (MARE 20618) at Herbarium of Marmara University Faculty of Pharmacy.

2.2 Preparation of crude extract

The aerial parts of the *E. macroclada* plant were dried at room temperature and ground into powder. Dichloromethane extract will be obtained by the maceration method of the aerial parts. After the maceration process, the liquid part were filtered through filter paper. The solvents were then filtered via filter paper and evaporated under low pressure in a rotary evaporator, with the obtained dried extracts stored in the refrigerator at +4 ºC.

2.3 Preparation of the extracts

The dried residues (10 mg) were dissolved in 100 µL of dimethyl sulfoxide (DMSO) and diluted to a final volume of 2 mL with distilled phosphate buffer saline (PBS) then filtered through 0.22 µm microbiological filters. The concentrations of 10, 20, 50, 100, and 200 µg/mL were achieved by further dilution.

2.4 Determination of antiproliferative activity

Human colorectal cancer cell lines, HT-29 (ATCC, HTB-38) and HCT-116 (ATCC, CCL-247), were used in this study to investigate the effects of dichloromethane extract of *E. macroclada*. HT-29 and HCT-116 cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C and 5% CO_2 atmosphere. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphentyltetrazolium bromide) method was used to determine the cytotoxic effects on HT-29 and HCT-116 cancer cells (20).

The cells (1 × 10^4 cells per well) were seeded in 96-well plates and cultured for 24 h. After that, the cells were treated with different concentrations of dichloromethane extract of *E. macroclada*. The plates were incubated for 24 h. Then the medium was discharged from the 96-well plate and 10 µL of 3-(4,5 – dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) was added per well. Additionally the plate kept for 4 h in 5% CO_2 humidified incubator at 37°C to allow reaction of yellow colored MTT reduced by mitochondrial dehydrogenases in viable cells to form pink to purple because of formazan. Excess MTT was taken off, and the resulting formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO). The optical density (OD) was read at 570 nm using 630 nm as reference wavelength on a multiwell plate reader (Biotech Instruments, Winooksi, VT, USA). All the experiments were repeated twice, and each treatment was run in triplicate.

The percentage of cell viability was calculated the following formulae:

% Cell viability= Mean (OD) of treated cells / Mean (OD) of the untreated cells × 100

2.5 Determination of genotoxicity

The genotoxic effects of *E. macroclada* on HT-29 and HCT-116 cells were evaluated by using alkaline single cell gel electrophoresis assay (Comet Assay) according to Singh et al. (21) with slight modification. Comet Assay was conducted *in vitro* in control, positive control (hydrogen peroxide) and plant extract groups. To determine the genotoxic potential of *E. macroclada* cells were seeded into 6-well cell culture plates (approximately 5x10^5 cells per well) with cell culture medium and incubated at 37 ºC in 5% CO_2 for 24 h. for cell proliferation. After 24 h, three concentrations of *E. macroclada* (100, 200 and 400 µg/mL in 1% DMSO) were added to the cells and incubated for another 24 h at 37ºC, here DMSO (0.1 %) was used as control. A concentration of 50 µM H_2O_2, which is known to cause DNA damage, has been used as a positive control. After incubation, the cells were washed with PBS, harvested using trypsin/EDTA and collected for centrifugation at 400 x g for 5 min. at 4 ºC. The supernatant was discharged, and the cell density was adjusted to 1x10^6 cells/ml by using cold PBS. 10 µL cell suspension was mixed with 90 µL of 0.6% low melting agarose (LMA) and added to the slides precoated with 1% high melting agarose. After solidification of the agarose, the slides were immersed in lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 10) for 1 h at 4°C. Upon removing the slides from the lysing solution, they were washed with cold PBS and placed in a horizontal electrophoresis tank side by side. DNA was allowed to unwind for 20 min. in freshly prepared alkaline electrophoresis buffer containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0). Then, electrophoresis was run at 25 V, 300 mA for 20 min. at 4 °C under minimal illumination to prevent further DNA damage. The slides were washed three times with a neutralization buffer (0.4 M Tris, pH 7.5) for 5 min. at 4 ºC and then treated with ethanol for another 5 min. before staining. Dried microscope slides were stained with ethidium bromide (20 µg/mL in distilled H_2O; 50 µL/slide) covered with a coverslip and analyzed using a fluorescence microscope (Olympus BX51, Japan) at a 400 × magnification. Percentage of DNA in the tail (% DNA_T) were scored using Comet Image Analysis-BABSOFT, and A total of 100 cells in triplicate per group were used to calculate the DNA damage. The DNA percentage in tail was used as the primary measure of DNA damage according to Hartmann et al. (22).

2.6 Statistical analysis

All data were expressed as the mean and standard error of the mean, derived from the repetition of each test at least three times. Statistical analyses were conducted using the SPSS 20.0 software. Student t-test was used to assess IC_{50} values derived from MTT test results.

The Comet assay findings were assessed using one-way analysis of variance (ANOVA) with the One-Way ANOVA test. Post-hoc analyses, specifically comparing the differences between groups, were conducted using Fischer's least significant difference (LSD) test. Comet data were expressed as the mean ± standard deviation (SD) of the data. *p*-values of less than 0.05 were considered statistically significant.

3. Results

The antiproliferative effect of *E. macroclada* dichloromethane extract on human colon cancer cell lines HT-29 and HCT-116 was assessed using the MTT assay. Various concentrations of *E. macroclada* extract (10-20-50-100 and 200 µg/mL) were administered to both cell lines, and the viability percentages were determined after 24 hours. The results indicate a dose-dependent inhibition of cell proliferation in HT-29 cells treated with *E. macroclada* extract (Fig 1A). The Half Maximal Inhibitory Concentration (IC_{50}) value for HT-29 cells was calculated as 111.215 µg/mL. *E. macroclada* inhibited 79.5% of HT-29 cells at a concentration of 200 µg/mL.

The genotoxic potential of *E. macroclada* was assessed by exposing cancer cells to various dosages of *E. macroclada* (ranging from 100 to 400 µg/mL for HCT-116 cells and from 50 to 400 µg/mL for HT-29 cells) for 24 hours. The extent of DNA damage was measured using the Comet Assay. Nuclei exhibiting DNA damage displayed a comet-like structure characterized by a luminous head and a trailing tail, while nuclei with intact DNA exhibited a spherical shape devoid of a tail. Each figure depicted a characteristic comet tail formed by the observed cells. The data was collected from at least 100 cells on two slides in each experiment. Significant alterations in the percentage of tail DNA were observed between the control cells and cells

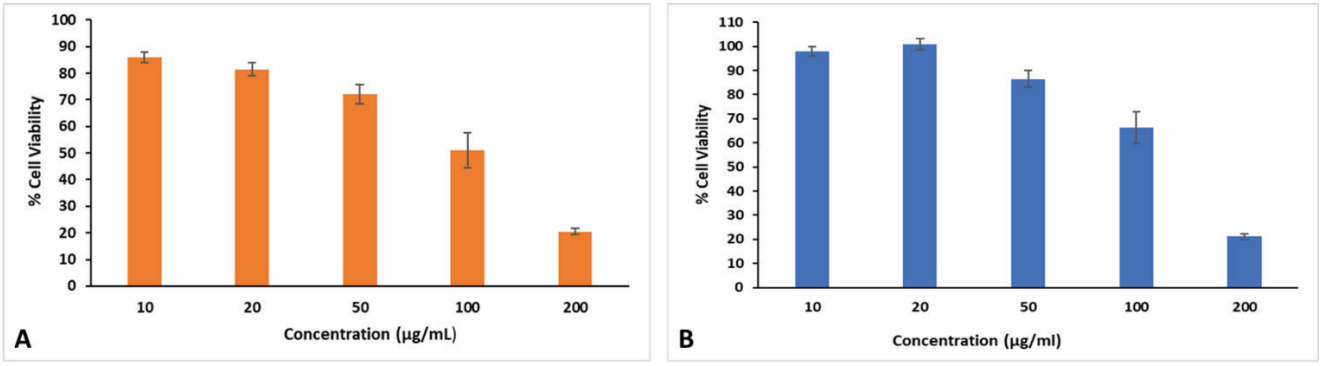

Figure 1. The antiproliferative effect of *E. macroclada* on HT-29 (A) and HCT-116 (B) cells.

In HCT-116 cells, viability showed a dose-dependent decline following exposure to *E. macroclada* extract, particularly at concentrations of 50 µg/mL and above (Fig 1B). At a concentration of 200 µg/mL, *E. macroclada* demonstrates an inhibition of 78.9% on HCT-116 cells, while the IC_{50} value for these cells was calculated as 133.835 µg/mL.

that were exposed to *E. macroclada,* as indicated in Table 1 and Table 2. As it is seen tables significant increase in the percentage of DNA damage and length of comet tail in a concentration-dependent manner in cancer cells exposed to *E. macroclada*. After 24 h. *E. macroclada* treatment, cancer cells showed different sizes, fragmentation and comet structures with increased

Table 1. The genotoxic effect of *E. macroclada* extracts on HCT-116 cells

	%DNA_T				
	Control	**Positive Control**	**100 µg/mL**	**200 µg/mL**	**400 µg/mL**
HCT-116	23.82 ± 0.56	52.64 ± 2.75***	32.16 ± 0.39$^{*,+++}$	45.44 ± 2.02$^{***,+}$	All Cells were Death

Data are represented as mean±standard deviation. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.$^{*}p$ <0.05, $^{**}p$<0.01 and $^{***}p$ <0.001 compared with the control group,^{+}p<0.05, ^{++}p<0.01 and ^{+++}p<0.001 compared with the positive control group.

% DNAT : Percentage of DNA in the tail

Table 2. The genotoxic effect of *E. macroclada* extracts on HT-29 cells

	%DNA_T					
	Control	**Positive Control**	**50 µg/mL**	**100 µg/mL**	**200 µg/mL**	**400 µg/mL**
HT-29	25.49 ± 0.87	64.72 ±1.79***	35.75 ± 4.29$^{***,+++}$	48.27 ± 0.11$^{***,+++}$	70.94 ± 1.10$^{***,+}$	All Cells were Death

Data are represented as mean±standard deviation. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. $^{*}p$ <0.05, $^{**}p$<0.01 and $^{***}p$ <0.001 compared with the control group, ^{+}p<0.05, ^{+}p<0.01 and ^{+++}p<0.001 compared with the positive control group.
% DNA_T : Percentage of DNA in the tail

% tail intensity when compared to the normal cells. The results indicated that *E. macroclada* induced DNA damage in a concentration-dependent manner and there were significant changes in the tail % of DNA between the normal cells and cancer cells at all doses.

4. Discussion

Despite significant improvement in chemotherapeutic strategies for cancer treatment, current methods still fall short in effectively combating the disease. This statement is mainly due to the non-specific targeting of tumor cells by drugs, their adverse side effects, and the emergence of drug resistance through various mechanisms. Consequently, there is a desperate need to explore alternative therapeutic agents that can enhance the efficacy of existing chemotherapeutics. In this context, the potential application of compounds isolated from natural sources as chemotherapeutic or chemopreventive agents is increasingly recognized.

The genus Euphorbia, which includes over 5000 species, is valued as a therapeutic resource in traditional medicine. Numerous scientific articles have shown the utilization of Euphorbia species in the treatment of various ailments, including microbiological infections, malaria, cancer, ringworm, tuberculosis, as well as sexually transmitted diseases like syphilis and gonorrhea (8, 23, 24). In addition, the latex from Euphorbia plants has been utilized for the treatment of lice infestations and mange, which is seen in animals. Furthermore, it has been documented that this substance has been used in the therapeutic management of parasitic illnesses, such as measles. Moreover, the investigation discussed the utilization of Euphorbia species for the management of respiratory ailments such as asthma, cough, and pneumonia (8, 23, 25).

The Euphorbiaceae family is known for its wide variety of diterpenoids that possess therapeutic activities such as antiproliferative, anti-inflammatory, and immunomodulatory properties (8). Notably, some diterpenoids from *Euphorbia esula L.* have shown antitumor activity against both multidrug-resistant (MDR) and non-resistant human gastric cancer cells (26, 27). Recently, an extract derived from *Euphorbia esula L.* was reported to exhibit antiproliferative activity against various types of cancer such as lung, cervix, stomach, breast, and liver. Additionally, diterpenoids isolated from the ethanol extract of *Euphorbia helioscopia* have demonstrated cytotoxic effects in renal cancer cell lines (28, 29).

However, investigations on the effects of plant extracts from the *Euphorbia* genus on human colon cancer cell lines *in vitro* is still limited. In a study, the latex of *E. trigona Mill.* was tested on the HT-29 colon cancer cell line and found to be inactive (30). In Aliomrani and colleagues' study, it was reported that the dichloromethane extract of *E. turcomanica* exhibited cytotoxic effects on HT-29 cells, with an IC_{50} value of 115 µg/mL as determined through their investigation (31).

The literature review reveals that numerous studies have demonstrated that *E. macroclada* have high antioxidant activies. However, the antiproliferative effect of the plant has only been the subject of two studies. One of the investigations aimed to examine the harmful effects of dichloromethane, ethylacetate, and methanol extracts from *E. macroclada* Boiss and the plant's latex on the MDA-MB-468 breast cancer cell line using the MTT method. The findings of this research study showed that the dichloromethane and

ethylacetate extracts exhibited cytotoxic properties against the MDA-MB-468 cell line, however the methanol extract and latex did not display cytotoxicity at the evaluated quantities. According to the findings of the study, *E. macroclada* Boiss non-polar extracts had shown greater cytotoxic action (32).

In the second study, using the MTT method, Tas et al. (2018) investigated the effects of acetone extracts of *E. macroclada* Boiss flower stems and leaves on the human breast cancer cell line (MCF-7) and mouse fibroblast healthy cell line (L-929). The study has found that the cytotoxicity of the extracts changed based on the concentration and duration of exposure, and the viability of cells decrease as the concentration increased. The acetone extract derived from the leaves of the plant exhibited superior efficacy compared to other extracts at all measured time intervals. Furthermore, it was found that after 72 hours of incubation, the leaf extract of *E. macroclada Boiss* exhibited greater cytotoxic activity in MCF-7 cell lines when compared to the control (L-929) (33).

The findings from these two investigations provide evidence about the potential of *E. macroclada* to be utilized as an anticancer agent. There is currently no research in scientific literature indicating the cytotoxic effects of *E. macroclada* on colon cancer cell lines.

In our study, the IC_{50} concentration of *E. macroclada* was determined to be 111.215 µg/mL in HT-29 cells and 133.835 µg/mL in HCT-116 cells. These findings suggest that the dichloromethane extract of *E. macroclada* is more effective in a dose-dependent manner on HT-29 cells when compared to HCT-116 cells.

Additionally, the genotoxic effects of the dichloromethane extract of *E. macroclada* on colon cancer cell lines (HT-29 and HCT-116) significantly increased in a dose-dependent manner in correlation with the MTT results.

5. Conclusion

Based on our research, it can be concluded that the components of *E. macroclada* may have cytotoxic effects on colon cancer cells. Furthermore, the genotoxic effect of the plant extract has been demonstrated for the first time. However, further research is needed to purify and identify the active components in order to develop a new cytotoxic agent.

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