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Cytotoxic Activity of the Root of *Euphorbia Tehranica* Ethanolic Extract Against Caco-2 Colorectal Cancer Cell Line

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Abstract

Introduction: Efforts are made to identify the new potential components such as anticancer drugs. Medicinal plants belonging to *Euphorbia* genus are widely studied with promising effects. The current study aimed at investigating the effect of ethanolic extract of the root of *E. tehranica*, grown in Iran, on Caco-2 human colorectal cancer cell-line.

Methods: The Caco-2 cells were treated with different concentrations of the root of *E. tehranica* ethanolic extract (25 to 1200 µg/mL) at 24 and 48 hours. Cell growth was evaluated with MTT assay. Anticancer activity of the ethanolic extract was assessed by evaluating the cell viability. Cell viability was determined at the wavelength of 570 nm by MTT method. The IC_{50} (half maximal inhibitory concentration) was determined graphically. One-way ANOVA followed by Tukey's post-test; and P values <0.05 were considered statistically significant.

Results: The findings showed the significant cytotoxic effect of the root of *E. tehranica* ethanolic extract against Caco-2 cell-lines ($P \leq 0.05$). Therefore, the viability of Caco-2 cells reduced with the dose and time-dependent manner, when compared with that of the control group. Also, the differences between the IC_{50} in 24 and 48 hours were 850 and 855 µg/mL, respectively.

Conclusions: It was the first study on the effect of the root of *E. tehranica* ethanolic extract on human colon cancer cell-lines. The results showed that this extract had the capacity to inhibit proliferation of tumor cells. Therefore, it seems to be a good candidate to defend against cancer cells.

INTRODUCTION

Constituents of several medicinal plants are used since ancient times to treat a variety of diseases. Approximately 70,000 plant species are used for medicinal purposes [1]. The genus *Euphorbia* is the largest in the plant family Euphorbiaceae, containing about 2000 known species (from annuals to trees). They have unique flower structures, and the all contain latex [2]. Some of the species are used in the cure of migraines, skin diseases, warts, wounds, intestinal parasites, and gonorrhea, in the world. *Euphorbia* genus has a wide variety of terpenoids (mono, sesqui, and diterpenes to triterpenoids and steroids). Many of these compounds are investigated for their therapeutic effects or their toxicity activity , and some of them are used as medicines [3]. Also, the biological activities of them are antiproliferation, antiviral, antimicrobial, modulability of multidrug resistance, cytotoxic, antifeedant, antidiarrheal, molluscicidal activities [4, 5].

Over 80 species of *Euphorbia* are so far reported from Iran, out of which a few of them are naturalized and the rest are native [6-12]. One of them is *Euphorbia teheranica. E. teheranica* Boiss. (Euphorbiaceae) is a plant widespread in the Tehran region and semi-desert areas of central Iran [13].

Colorectal cancer (CRC) is one of the major cause of death in worldwide [14] and accounts for over 9% of all cancer incidence [15, 16] so, CRC is the third most common cancer worldwide [16]. Also, it is the third most common cancer in Iran. The incidence of CRC varies in different regions of the world and it varies at least 10 times in different regions and has a significant time trend [17, 18]. Recent studa ies showed a rapid rise in the incidence of colorectal cancer in Iran [19, 20]. There are similar incidence rates for cancer of the colon in both genders, and a slight male predominance for rectal cancer [16, 21, 22]. Local treatments such as surgical or radio-there apy make it difficult to cure or prevent recurrence and metastasis of tumor. Hence, the attempt for safe and effective anticancer drugs from natural plants is an important aspect of anticancer investigation [23]. The current study aimed at evaluating the antitumor properties of the root of *E. tehranica* ethanolic extract against Caco-2 human colon cancer cell-line.

METHODS

Plant Material

Euphorbia tehranica was collected from Tehran (Koohsar Park), Iran, in July 2015. The plant was identified by Dr. Shahin Zare in University of Tehran. The specimen (herbarium No. 39917) was deposited in the herbarium of the mentioned center. The roots of plants were dried in a shaded place and then, powdered for later use.

Extraction and Isolation

Plant material was extracted with absolute ethanol and shaken for 24 hours, then was filtered through number 11 Whatman filter paper. The entire extract was roto-evaporated at 40°C and was stored in the dark at -20°C until use [24].The dried mass was dissolved in absolute ethanol for assays. Treatment doses of extracts were 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, and 1200 µg/mL.

Cell Culture

The human colorectal cell-line Caco-2 (adenocarcinoma) was purchased from National Cell Bank of Iran affiliated to Pasteur Institute of Iran, Tehran, Iran and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco-USA) supplemented with 100 U/mL penicillin (Sigma-USA), 100 µg/mL streptomycin (Sigma-USA), and 10% fetal bovine serum (Gibco-USA). The cells incubated at 37 °C in 5% CO_2 atmosphere. The cells were sub-cultured and exponential-phase cells were used throughout the experiments.

Ethanolic Extract Treatment

A total of 80×10^3 dissociated Caco2 cells were plated in 96-well microplates for 24 hours, and 48 hours incubation for toxicity assays. Before testing, the stock solution was prepared in 100% ethanol (Sigma-USA) and diluted into medium to the concentrations in demand. Control cultures received only ethanol. Also, untreated cells were used as the negative control.

Cell Viability Assessment Assay

The test of trypan blue dye exclusion is used to count the number of viable cells. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes such as trypan blue, whereas dead cells do not. The cell suspension is mixed with the dye and then visually examined to determine whether cells take up or exclude the dye. Briefly, 50 µL of cell suspension was mixed with equal parts of 0.4% trypan blue (Biosera-USA) added to the cell suspension to obtain a 1: 2 dilution and mix by pipetting up and down, then incubated for less than three minutes at room temperature. Then, 20 mL of the mix was loaded into each chamber of the hemocytometer. Counts were performed in triplicate by one analyst

under 40X magnification, according to the standard methodology [25].

Cytotoxicity Assessment by MTT Test

Cytotoxicity of the root of *E. tehranica* ethanolic extract at different concentrations assessed against Caco-2 cancer cell-line by 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, Caco-2 cells were dispensed in a 96-well microplate at 80×10^3 cells per well. They treated with serial concentrations of ethanolic extract (25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, and 1200 µg/mL). After 24 and 48 hours of incubation, cytotoxicity was measured. Each concentration was repeated three times; 100 µL of MTT solution (5 mg/mL stock solution in PBS, diluted with culture medium to the final concentration of 0.5 mg/mL) (Sigma-USA) added to each well to produce a total reaction volume of 250 µL. After four hours of incubation at 37°C with 5% CO_2, the supernatants were aspirated, and the formazan crystals in each well were dissolved in 50 µL dimethyl sulfoxide (DMSO) (Sigma-USA). The amount of purple formazan was determined by measuring the absorbance at 570 and 630 nm dual wavelengths with ELISA (the enzyme-linked immunosorbent assay) reader (Model ELx800, Bio Tek, USA). *Euphorbia tehranica* ethanolic extract was not added to the control samples (untreated cells).

Cell viabilities were calculated using the following formula:

Cell viability rate (%) = ($OD_{570/630}$ of treated cells/ OD of control cells) × 100 %.

The IC_{50} (half maximal inhibitory concentration) values from cytotoxicity assays were calculated from dose response curves using linear regression analysis by the JavaScript version of PolySolve (07.20.2013) software.

Statistical Analysis

Data obtained from MTT assay for different concentrations of the root of *E. tehranica* ethanolic extract of triplicate independent measurements within 24 and 48 hours calculated as viability rate was enacted with SPSS version 16 using one-way ANOVA followed by Tukey's HSD post-hoc test. P <0.05 was considered as statistically significant.

RESULTS

Cytotoxicity Assessment of the Root of *E. tehranica* Ethanolic Extract on Caco-2 Cell Line

The current study assessed the cytotoxic effect of the root of *E. tehranica* ethanolic challenges culture. The effects of *E. tehranica* extract on the viability of human colorectal cancer cell line Caco-2 were examined in vitro by incubating the cells in 25, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, and 1200 µg/mL of *E. tehranica* extract for 24 and 48 hours; the cell viability was then measured by MTT assay (Figure 1). The MTT assay results showed that the growth of the treated cells decreased significantly in a concentration-dependent manner.The dose and time-dependent effect of the root of *E. tehranica* ethanolic extract on the growth of Caco-2 cell line was analyzed by one-way ANOVA. Comparison of the mean cell viability of treated cells in two periods of 24 and 48 hours showed a significant difference between all treated groups compared with untreated cells (P <0.001). Also, by increasing the concentration of the root of *E. tehranica* ethanolic extract, the average viability of the cells decreased.

ANOVA followed by Tukey's post-test was used for group comparisons. P values <0.05 were considered statistically significant. Tukey's HSD post-hoc test indicated a significant difference between 400 to 1200 µg/mL treated concentrations and control group (untreated cells) in 24 hours; also there was a significant difference between 200 to 1200 µg/mL treated concentrations and the control group (untreated cells) in 48 hours (P <0.001).

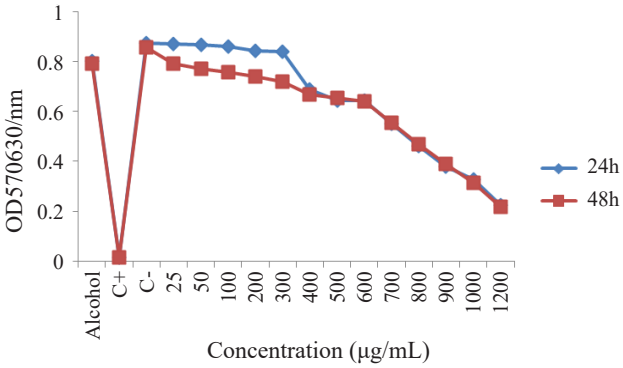


Figure 1: The Effect of The Root of *E.tehranica* Ethanolic Extract at to 1200 µg/mL Concentrations on Caco-2 Human Colorectal Cancer Cell Line 24 Hours and 48 Hours of Incubation. Cell viability was determined by MTT Assay. Alcohol as a solvent control, C^+: treated cells with DMSO 20% as a positive control, C^-: untreated cells as a negative control.

Anticancer Activity Against Caco-2 Colorectal Cancer Cell Line

Twenty-four hours after treatment with the root of *E. tehranica* ethanolic extract, Caco-2 cells viability decreased in a concentration-dependent manner. The viability of Caco-2 cells significantly decreased in 400 to 1200 µg/mL concentrations, compared with untreated cells of the control group (P <0.001) (Figure 2). The highest inhibition of the cell growth was observed after treatment with the root of *E. tehranica* ethanolic extract at 1000 and 1200 µg/mL concentration approximately 62.6% and 74.4%, respectively. Fourth-eight hours after treatment with the root of *E. tehranica* ethanolic extract, Caco-2 cells viability decreased in a concentration-dependent manner. The viability of Caco-2 cells significantly decreased in 200 to 1200 µg/mL concentrations compared with untreated cells as the control group (P <0.001) (Figure 3). The highest inhibition of growth was observed after treatment with the root of *E. tehranica* ethanolic extract at 1000 and 1200 µg/mL concentrations approximately 63.2% and 74.4%, respectively. The root of *E. tehranica* ethanolic extract inhibited Caco-2 cell line proliferation in a time and concentration-dependent manner.

The IC_{50} Was Calculated for the Root of *E. tehranica* Ethanolic Extract

The effective concentration of the root of *E. tehranica* ethanolic extract to determine the IC_{50} value was obtained by regression analyses of concentration-inhibition curves (Figure 4). The IC_{50} value was 850 µg/mL for 24 hours, and 855 µg/mL for 48 hours after treatment.

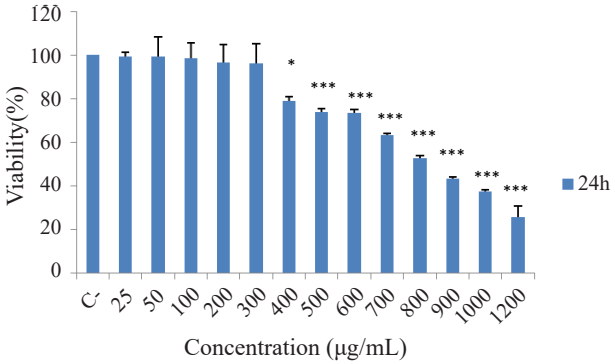


Figure 2: Anticancer Activity of the Root of *E. tehranica* Ethanolic Extract on Caco-2 Cell Line. Cells were incubated with ethanolic extract (25 to 1200 µg/mL) at 37°C for 24 hours. Cells viabilities were evaluated using MTT assays and calculated as a ratio of the control. Data represent the means (SD) of three independent experiments. * Significant reduction in cell viability at P < 0.05, and *** Significant reduction in cell viability at P <0.001, compared with that of the control group.

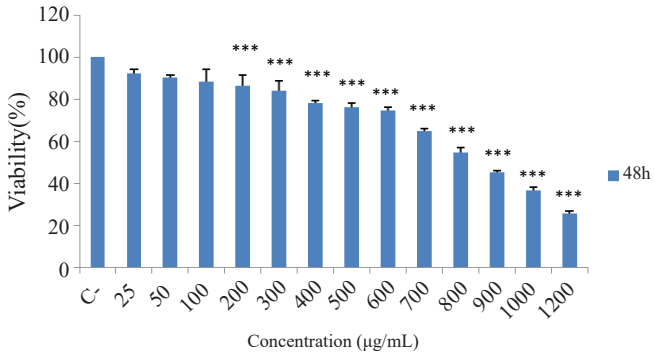
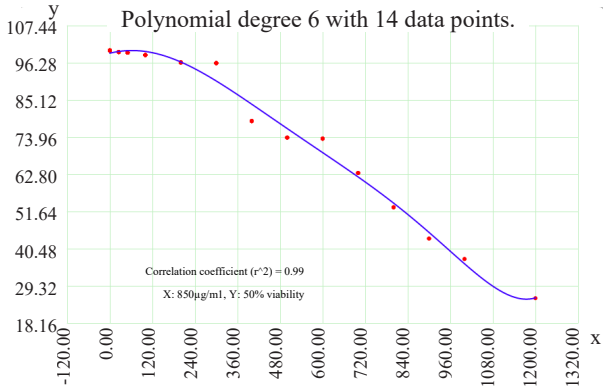


Figure 3: Anticancer Activity of the Root of *E. tehranica* Ethanolic Extract on Caco-2 Cell Line. Cells were incubated with ethanolic extract (25 to 1200µg/mL) at 37 °C for 48 hours. Cells viabilities were evaluated using MTT assays and calculated as a ratio of the control. Data represent the means (SD) of three independent experiments. *** Significant reduction in cell viability at P < 0.001, compared with that of the control.



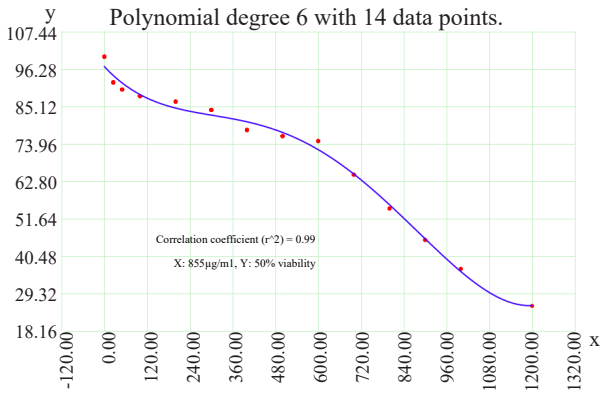


Figure 4: Regression Analyses to Calculate the IC_{50} Values of The Root of *E. tehranica* Ethanolic Extract. The horizontal axis (x) represents the concentration (µg/mL) and the vertical axis (y) represents the percentage of the viability cells. The IC_{50} value was 850 µg/mL for the 24 hours after treatment and 855 µg/mL for 48 hours after treatment.

DISCUSSION

The genus *Euphorbia* is wide-spreading all over the world. The research on *Euphorbia* species show that the some plants can use in traditional medicines or revealed the new activities on modern pharmacological filed. The main biological activities of medical plants include anti-inflammatory, anticancer, antioxidant, antipyretic-analgesic, antimicrobial activity, antifeedant, and molluscicidal activities. The knowledge about the relationships between structure and activity study on diterpenoids gives more detailed information about the active core framework and substituents [5]. The research on medicinal plants and their effective compounds have been attentedin traditional medicine. They might be possibly used in therapy, after pharmacological and toxicological studies

Over the years, natural products are used as promising sources to discover new pharmaceutical agents [26, 27]. Medicinal plants contain powerful compounds in any of the parts, including the bark, stem, resin, leaf, root, flower, fruit, and seed. Such healing properties of plants cure various health problems [28]. *Euphorbia teheranica* Boiss. (*Euphorbiaceae*) is a plant widespread in Tehran region and semi-desert areas of central Iran [13]. Nevertheless, there are no studies on the effect of ethanolic extract of this plant in cancer. The current study aimed at analyzing the anticancer activity of the root of *E. tehranica* ethanolic extract.

Cancer is a class of diseases characterized by unregulated cell growth. The World Health Organization (WHO) reported that approximately 13% of all deaths in the world are caused by cancer each year [29]. Death from colon cancer is the fourth highest among all cancer-related deaths. In the current study, the human colon cancer cell-line Caco-2 was used as the target. The current study observed that the root of *E. tehranica* ethanolic extract induced strong cytotoxic effects, which decreases the viability and growth of Caco-2 cell-line. Response of cells to the drug occurred in a time and dose-dependent manner. IC_{50}, according to the MTT assay was 850 µg/mL for 24 hours, and 855 µg/mL for 48 hours after treatment. Li et al., reported that whole plant of *E. hirta L.* showed significant anticancer activity on HT-29 cell-line for 24 hours [28]. Also, Patil and Magdum [30], and Sidambaram et al., [31] showed that *E. hirta L.* had anti-cancer activity on EL-4 mouse lymphoma cell-line and Hep-2 human larynx epithelioma cell-line, respectively. Prakash and Gupta showed the anticancer effects of ethanolic extract of three plant species namely *Ricinus communis* Linn, *Euphorbia helioscopia, Jatropha curcas* of the family *Euphorbiaceae* against seven human cancer cell lines [32]. Haq et al., reported that the crude methanolic root extract of *Euphorbia wallichii* and its fractions had antioxidant and cytotoxic activities on human cancer cell lines [33]. Sreenika et al., reported the antioxidant and antitumor activity of Ethyl acetate extract of *Euphorbia milii* against breast cancer in vivo and colon cancer in mice [34]. Therefore, findings of the current study, in agreement with the above information, demonstrated a wide range of anti-cancer properties of *Euphorbia* extract.

The current study indicated that the root of *E. tehranica* ethanolic extract had cytotoxicity and anticancer activities. The current study findings demonstrated that the root of *E. tehranica* ethanolic extract was capable of inhibiting growth in Caco-2 cell lines. Future studies are needed to evaluate its molecular mechanisms of action according to anticancer activities. The current results suggested that the root of *E. tehranica* ethanol extract had potent anticancer effects. Taken together, these results suggested that ethanolic extract of the *E. tehranica* may impart interesting biological effects on the cancer cells, which may represent a potential source of chemopreventive agents.

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CONFLICT OF INTEREST

The authors declared no conflict of interests regarding the publication of the paper.

ETHICS APPROVAL

The Ethics Committee of Department approved the protocol of the study.

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