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# Investigation of the cytotoxic, anti-proliferative and anti-angiogenic effects of Toluhydroquinone on Caco-2 cell line

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**ABSTRACT**

Colorectal cancer is one of the most common types of cancer in the world. The treatment options for colorectal carcinoma generally consist of surgery, radiotherapy and chemotherapy. The drug resistance to chemotherapy agents used in the current cancer treatment has brought about the finding of new drug molecules from some plant and aquatic species in the treatment approaches. Some species of aquatic biota create novel biomolecules as potential drugs for cancer and other diseases. Toluhydroquinone belongs to thesegroups of biomolecules and it shows anti-oxidative, anti-inflammatory and anti-angiogenic properties. In this study, we tested the cytotoxic and anti-angiogenic effects of Toluhydroquinone on Caco-2(Human colorectal carcinoma cell line) cells. It was observed that the amount of closure of the wound space, colony forming ability (*in vitro* cell survivability) and formation of tubule-like structures in matrigel decreased in comparison to the control group. As a result of this study, Toluhydroquinone has cytotoxic, anti-proliferative and anti-angiogenic properties on the Caco-2 cell line.

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## Introduction

In recent times, many studies have been conducted to investigate the potential of using biomolecules isolated from some marine species as therapeutic agents in various diseases, especially cancer. Although the mechanism of action of some natural marine products in a wide range of diseases has been understood, most have not yet been clarified (1). Numerous natural products from marine species and their derivatives as drug candidates are currently being tested at different phases for the treatment of various diseases (2). Natural products, especially those containing groups such as quinones and saponins that can be isolated from plants and cordyceps, are examples of anticancer drug candidates (3). Natural products, especially those containing groups (quinones, alkaloids, saponins, polyphenols etc.) that can be isolated from plants and marine species, are examples of anticancer drug candidates (4). Toluhydroquinone is a secondary metabolite known to have anti-inflammatory and anti-angiogenic effects, which can be isolated from some species of aquatic biota as any other novel biomolecules used as a potential drug for cancer and other diseases (5). The only study about the anti-cancer effect of Toluhydroquinone is in esophageal cancer: Toluhydroquinone exerts its anti-cancer effect through apoptosis induction (6). The anti-inflammatory property of Toluhydroquinone was demonstrated in lipopolysaccharide-stimulated RAW264.7 cells (7).

Cancer is one of the most common and painful human diseases in the world, and its economic impact is considerable. Colorectal cancer ranks 3rd in cancer-related deaths (8). Colorectal cancer is a type of cancer that occurs due to uncontrolled cell divisions in the large intestine for various reasons. As in all cancer types, although the rate of environmental influence is higher in the etiology of colorectal cancer, genetic influence is also observed. It is noted that the incidence of colorectal cancer increases in developed countries, especially in relation to diet (5, 9) surgery, radiotherapy and chemotherapy are some options for colorectal carcinoma treatment. However, drug resistance emerges due to the chemotherapy agents used in current cancer treatment. This brings up the necessity for the discovery of new drug molecules. The biggestadvantage of using these biomolecules in cancer treatment is that they have less toxic effects on healthy cells (10).

Many studies show that secondary metabolites are effective in cancer treatment. These secondary metabolites can be obtained from plants, as in the example of saffron, or they can be isolated from marine sources (11). Evaluation of the cytotoxicity of metabolites derived from Aspergillus species was performed against both liver and colon cancer cell lines. It has been determined that Caco-2 is more sensitive to this metabolite and shows its cytotoxic and apoptotic effect more in colon cancer cells (12). Okadaic acid, pectenotoxin-2, 13-desmethylspirolide C and yessotoxin, which are marine secondary metabolites, are some examples in which the cytotoxic effect on the Caco-2 cell line was revealed. Different treatment combinations were tried with okadaic acid as the main component and different degrees of cytotoxic effect were determined (13). When Brown marine algae *Gongolaria baccata* extract is applied to Caco-2 cells, it protects human intestinal Caco-2 cells against oxidative damage by modulating GSH concentration, MDA production, ROS production and antioxidant

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enzyme activities in colorectal carcinoma cells (14).

In our study, we tested cytotoxic and anti-angiogenic activities of Toluhydroquinone on caco- 2 cell line. The colony formation ability of Caco-2 cells treated with 2 different doses of Toluhydroquinone decreased remarkably. This study is the first to examine the cytotoxic and anti-angiogenic effects of Toluhydroquinone in the Caco-2 cell line.

## Materials and Methods

### Experimental reagents

Toluhydroquinone (Sigma-Aldrich 112968) stock solution (10 mM) was made by dissolving in sterile distilled water and stored at -20 °C.

### Cell culture

The cell culture was carried out with standard cell culture methods. Human colorectal adenocarcinoma cells (Caco-2) were accessed from Yeditepe University Department of Medical Genetics Research Group and cultured in a Dulbecco's Modified Eagle Medium (Sigma Aldrich) with 10 % (v/v) Fetal Bovine Serum (BioSera) and 1 % (v/v) penicillin-streptomycin (GenMarkBio) at 37 °C and 5 % CO2. Human vascular endothelial cells (HUVECs) were obtained from Bahc¸es¸ehir University Department of Medical Genetics Research Group and cultured in Dulbecco's Modified Eagle Medium (Sigma Aldrich) with 10 % (v/v) Fetal Bovine Serum and 1% (v/v) penicillin-streptomycin (GenMarkBio) at 37 °C and 5 % CO2. CCD-1072Sk (Human skin fibroblast) cells were procured from Bezmialem Vakif University Department of Medical Genetics Research Group as a control group and cultured in Eagle Minimum Essential Medium (Sigma Aldrich) with 10 % (v/v) Fetal Bovine Serum and 1 % (v/v) penicillin-streptomycin (GeneMarkBio) at 37 °C and 5 % CO2. All cells used in the study were under 25 passages for all experiments.

### Cell proliferation assay

MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), one of the cytotoxicity tests with tetrazolium salts, is a positively charged compound. It can be reduced intracellularlyafter by easily crossing the membrane of eukaryotic cells. The formazan crystal formed as a result of the reduction is insoluble in water and gives a purple color with DMSO (Dimethyl sulfoxide) (15). MTT(Gold Biotechnology®, St Louis, MO, USA) was used for the proliferation assay. It was carried out in accordance with the protocol of the product. Caco-2 cells and CCD-1072Sk cells ($10^4$ cells/well) were seeded at 96-plate and incubated overnight. The same dose scale (0-40 µM) was applied to these two cells. After 24h incubation, each well was aspirated and MTT solution was added. The absorbance was measured at 570 nm with a microplate reader (Multiscan Go, Thermo Scientific).

### Wound healing assay

The wound-healing test was performed to analyze cell migration. Caco-2 cells ($10^4$ cells/well) were plated to confluency in the 6-well dishes with a complete medium for 24 h. After incubation, the medium was aspirated and the surface of the plate was gently scratched with a pipette tip (100 µl volume) from top to bottom. Cells were treated with different doses of Toluhydroquinone. The cell migration was observed by phase contrast microscopy 24 h after treatment with Toluhydroquinone.

### Colony Formation Assay

The clonogenic activity sensitively shows the ability of cancer cell's survive by themselves (16). It is performed to test the single-cell colony formation potential of Toluhydroquinone-treated Caco-2 cells (17). Cells were seeded on a 6-well plate in a low density (250/well) after 24h incubation with Toluhydroquinone. 10 days later, colonies that were stained with crystal violet were counted with ImageJ Software.

### Tube Formation Assay

The method valued as vasculogenic mimicry is to determine the vascularization ability of endothelial cells on matrigel (18). The conditioned media(CM) was prepared with Caco-2 cells. The Toluhydroquinone-treated cells were incubated with serum-free media for 72h. Then the media was collected and centrifuged at 1000 rpm for 20 minutes. The supernatant was filtered through a 0.22 µm filter and stored at -20 °C. HUVECs were seeded (3 x $10^4$ cells/well) on a matrigel coated 24-well plate (Corning Biocoat, Australia) with CM and a complete medium mixture(1:1). Tube-like structures were monitored using the phase contrast inverted microscope (Zeiss Axiovert A.01, Germany). Images were analyzed with Wimasis imaging analysis software (Wimasis GmbH, Munich, Germany).

### Statistical analysis

Image analyses were performed with Wimasis Image Analysis Software. Data obtained from Wimasis software and IC50 values were calculated using GraphPad Prism software V8 (Graph-Pad Software, La Jolla, CA). Statistical differences between the control and treated groups for cytotoxicity and cellular anti-angiogenic activity measurements were analyzed by one way-Anova test. p values less than 0.05 was considered as statistically important.

## Results

### Toluhydroquinone shows cytotoxic effects

Caco-2 cells were treated with prepared Toluhydroquinone at various concentrations from 0-40 µM for 24h. There was no significant change at the 0-20 µM concentration scale whereas cell viability was significantly decreased at the concentration 20 to 30 µM range. Also, CCD-1072Sk cells were not affected by this scale of Toluhydroquinone doses. The IC50 value of Toluhydroquinone for Caco-2 cells is approximately 22 µM, as shown in Figure 1. The IC50 value inhealthy cell line CCD-1072Sk cells was determined as 112.5 µM as a negative control.

### Toluhydroquinone reduces colony formation of Caco-2 cells

The colony forming capability was tested with the effect of 20 µM and 25 µM Toluhydroquinone on Caco-2 cell. It was found that the colony-forming ability of Caco-2 cells treated with Toluhydroquinone was significantly reduced compared to the control group. As a result of the 10-day colony formation process, we determined that the number of colonies decreased significantly as the applied drug dose increased, as shown in Figure 2.

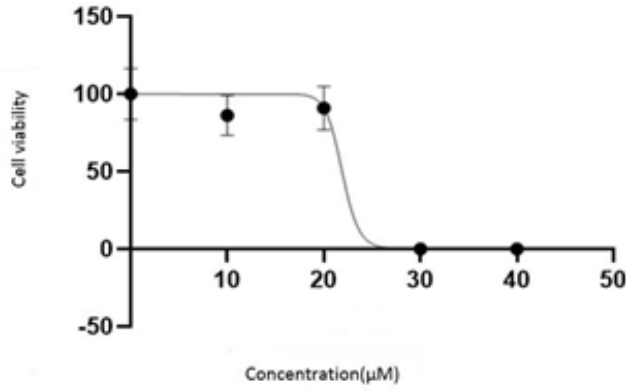



**Figure 1.** Effects of Toluhydroquinone on Caco-2 cell proliferation. To examine whether Toluhydroquinone presents anti-proliferative effects, Caco-2 cells were treated with a variety of concentrations of Toluhydroquinone for 24 h. The IC50 value was calculated with GraphPad Prism software V8 (Graph-Pad Software, La Jolla, CA). One-way ANOVA test was used for the statistical evaluation of the results, Data were expressed as percentages of the control (100%) as mean ± S.D (*p < 0.1).

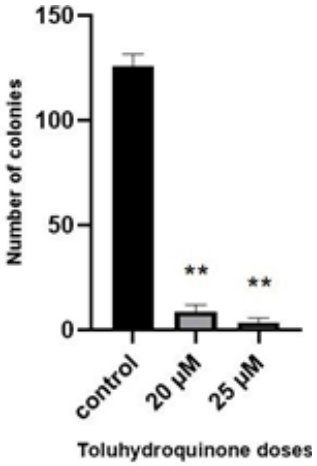
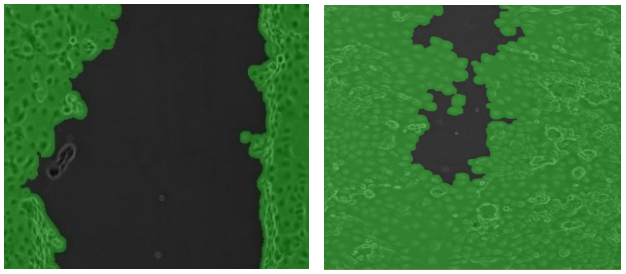
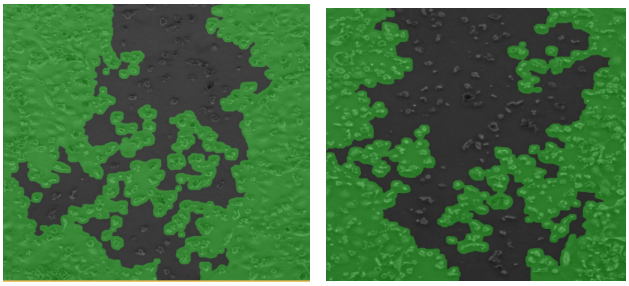



**Figure 2.** Toluhydroquinone decreases the colony formation ability of Caco-2 cells. 2D colony formation ability of Caco-2 cells were analyzed and quantified after 20 and 25 µM Toluhydroquinone treatment. Data were expressed as percentages of the control (100%) as mean ± S.D (**P < 0.05).



(A) After Injury (B) non-treated cells



(C) 20 µM treated cells (D) 25 µM treated cells

**Figure 3.** Wound healing capability decrease with Toluhydroquinone-treated cells. The scratch area in the plate was photographed and analyzed by Wimasis Image Analysis Software. A. The first image of Caco-2 cells after injury. B. Non-treated group cells after 24 h injury was able to close the wound area almost all. C. 20 µM treated Caco-2 cells after 24h injury. D. 25 µM treated Caco-2 cells after 24h injury. All presented data were generated by three independent experiments. (100x Zeiss Axiovert A.01, Germany).

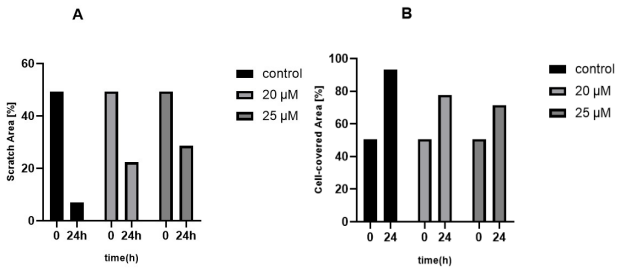



**Figure 4.** Toluhydroquinone treatment reduces the migration ability of Caco-2 cells. Scratch assay was used to determine the effect of Toluhydroquinone on the migration ability of Caco-2 cells. A. The scratch area in the plate was photographed and analyzed by Wimasis Image Analysis Software. B. The area covered by cells was photographed and analyzed by Wimasis Image Analysis Software. All data were presented as mean and three independent experiments. Data were expressed as percentages of the control (100%) as mean ± S.D (*P < 0.1).

**Toluhydroquinone inhibits the migration of Caco-2 cells.**

The scratch assay was used to assess the effect of Toluhydroquinone on the migration ability of Caco-2 cells. It was measured to what extent the Toluhydroquinone-treated Caco-2 cells could close the wound cavity in 24 hours. As seen in Figure 3 and Figure 4, it was determined that while the wound space areaincreased depending on the increasing doses, on the contrary, the cell-covered areas decreased.

**Toluhydroquinone minimizes the formation of tubular structures**

The anti-angiogenic effect of Toluhydroquinone was tested with a tube formation assay. After treatment of Caco-2 cells with Toluhydroquinone, we calculated the number of the total loop, total branching points and total tube length using Wimasis Image Analysis Software. All of these counted values decreased significantly at increasing Toluhydroquinone concentrations in comparison with control groups, as shown in Figure 5 and Figure 6. It points out that Toluhydroquinone reduces tubular structures with 20 and 25 µM concentration values. Referring to these results, it can be decided that Toluhydroquinone has an anti-angiogenic effect on Caco-2 cells with determined doses.

## Discussion

Understanding angiogenesis and developing related approaches are very important in the treatment of colorectal cancer due to the limitations of current therapies. There are few research studies on the relationship of Toluhydroquinone with cancer and other diseases. However, hydroquinone (HQ) is a very similar benzene derivative. The only difference between Toluhydroquinone is the addition of methyl group to the benzene ring. In the literature, various

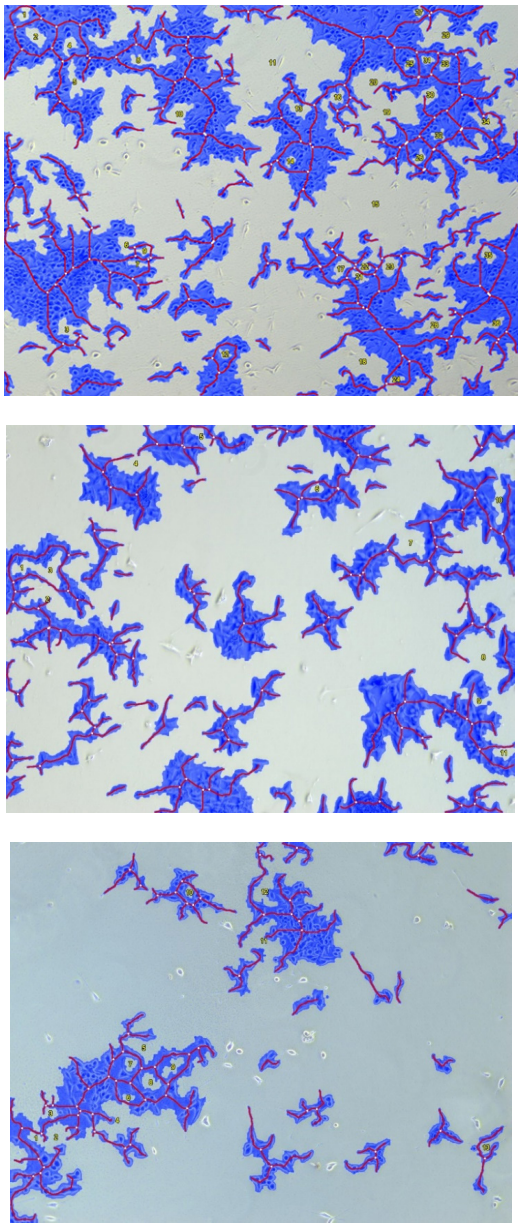


**Figure 5.** Images of Caco-2 cells treated with tube formation test at different doses of Toluhydroquinone, obtained with Wimasis Image Analysis Software. Depending on the increasing drug dose, the ability to form tubular structures decreases from top to bottom. HUVEC cells seeded with Condition Media from untreated Caco-2 cells (top), HUVEC cells seeded with Condition Media from Caco-2 cells treated with 20 µM Toluhydroquinone(middle), HUVEC cells seeded with Condition Media from Caco-2 cells treated with 25 µM Toluhydroquinone(bottom). All presented data were generated by three independent experiments. (100x Zeiss Axiovert A.01, Germany).

studies showed the anti-cancer effect of hydroquinone and its derivatives in different types of cancer cells and animal models. In the study of Byeon, the effect of hydroquinone in an in vivo colon cancer model was studied. They found that hydroquinone decreased the number of tumors up to 50% in a dose-dependent manner in the mouse. In this study, the anti-angiogenic potential of HQ was also demonstrated with CAM assay. Similar to our results with Toluhydroquinone, HQ inhibited new blood vessel formation up to 70% (3). Another derivative of HQ, XG-d, also showed an anti-cancer effect in colon cancer both in *in-vitro* and *in-vivo*. It was demonstrated that its effect is

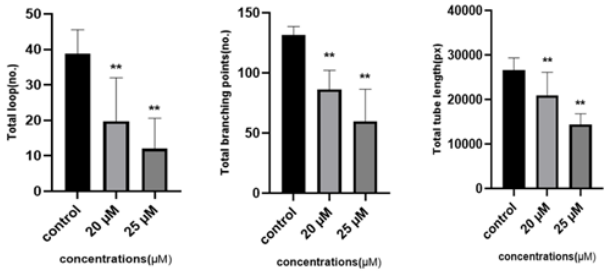



**Figure 6.** Toluhydroquinone inhibits total loop(left), total branching points(middle), and total tube length(right) with increased drug doses. Quantification of these parameters were examined with Wimasis Image Analysis Software. All data were presented as mean and three independent experiments. Data were expressed as percentages of the control (100%) as mean ± S.D (**P < 0.05).

mainly through the induction of apoptosis. Hydroquinone analog 4-[(Tetrahydro-2H-pyran-2-yl) oxy] phenol induces C26 colon cancer cell apoptosis and inhibits tumor growth in vivo (19). It was stated in the study of Whibley et. al. that 6 quinone-related matters isolated from *L. millecra* shows the cytotoxic effect on some esophageal and cervical cancer cell lines as 95% mortality after 48 h at 50 µM (6). In addition, these compounds have differential IC50 values from 9,5 to 83,3. The IC50 value obtained from our study was below the IC50 value of 3 of these components. In this sight, it is interpreted that they are similar due to the close cytotoxic values. It has been reported by Garc´ıa-Caballeroac et al., in 2013, that tubule-like structures are reduced by the application of Toluquinol to BAECs (Bovine Aortic Endothelial Cells) and HT29(Human Colon Adenocarci noma) cells, and the cytotoxic value in HT29 cells is between 8.3 and 8.9 (20). In our study, which we conducted with a similar molecule, Toluhydroquinone, it was observed that the tubular structures were reduced, while the IC50 value was found to be higher. This difference detected in the IC50 value suggests that the activity of different molecules is also different and is due to the use of different cell lines. It is also very important to investigate the effects of secondary metabolites on tumoral pathways. In this context, a study was conducted by Hwang P. et al. to determine the effects of Toluhydroquinone on tumor necrosis-related pathways; The IC50 value of Toluhydroquinone on LPS-stimulated RAW 264.7 cells isolated from Aspergillus sp. was determined as 4 µM, and it was determined that it showed anti-inflammatory (7). The result of this study is similar to our study presented in this paper in terms of the cytotoxic effect of Toluhydroquinone on the tumor. HUVEC cells are frequently used in angiogenesis studies. Although the application dose varies due to the different cytotoxic effects of the substance in different cells, our findings show parallelism with this study that Toluhydroquinone inhibits angiogenesis at high doses (5). In the scope of the aforementioned study, a tube formation test was used directly on the medium in which HUVEC cells were grown, while in our study, conditioned media of treated Caco-2 cells was used.

Although there are similar studies on Toluhydroquinone in the literature, there is no study investigating its effect on angiogenesis in the Caco-2 cell line. In this study, we tested cytotoxic and anti-angiogenic effects of Toluhydroquinone in Caco-2 cells and important results have been obtained. Toluhydroquinone reduced the colony-forming

ability in Caco-2 cells noticeably. While the control group closed the wound almost completely, it was observed that the amount of closure of the wound space decreased as the dose increased in the other groups. It was observed that treated cells with different doses of Toluhydroquinone tend to decrease the formation of tubule-like structures in comparison with the control group. As a result of this study, we determined that Toluhydroquinone has cytotoxic, antiproliferative and anti-angiogenic properties on the Caco-2 cell line. The study presented in this article contributed to the development of data on the utility of anti-angiogenic novel biomolecules in colorectal cancer.

There are related studies with Toluhydroquinones in the literature. The results of our study also support these studies. The main contribution of this study to the literature is to demonstrate the cytotoxic and anti-angiogenic effects of Toluhydroquinone on Caco-2 cells and to guide future studies. Liposomal or nanoparticle drug-loaded systems for colorectal cancer treatment are quite remarkable (21). In light of the data obtained from this study, Toluhydroquinone will be tested in different cell lines and its effects will be determined by transferring Toluhydroquinone to the nanoparticle and liposomal system.

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**Author contribution**
Conceptualization, Mine Kuçak; methodology, Mine Kuçak and Ummuhan Demir; validation, Mine Kuçak and Ummuhan Demir; Formal Analysis, Mine Kuçak, M. Hamza Muslumanoglu and Ummuhan Demir; investigation, Mine Kuçak; writing-original draft preparation, Mine Kuçak; writing-review & editing, Mine Kuçak and Ummuhan Demir; visualization, Mine Kuçak; supervision, M. Hamza Muslumanoglu; project administration, Mine Kuçak and M. Hamza Muslumanoglu. All authors have read and agreed to the published version of the manuscript.

**Conflict of interest**
The authors declare no competing interests.

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