Cumhuriyet Tıp Derg 2013; 35: 14-24
Cumhuriyet Med J 2013; 35: 14-24

Original research-Orijinal araştırma *http://dx.doi.org/10.7197/1305-0028.1757*

Thyme essential oil inhibits proliferation of DLD-1 colorectal cancer cells through antioxidant effect

Kekik uçucu yağı antioksidan etki aracılığı ile DLD-1 kolorektal kanser hücrelerinin proliferasyonunu inhibe ediyor

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Abstract

Aim. It is a very hot topic to investigate the effects and the mechanisms of action of biological active extracts such as thyme essential oil on various cancer cells. This paper examines the ability of thyme essential oil to inhibit the growth of DLD-1 colorectal cancer cells and if it is the case, determine the contribution of antioxidant effect to this ability. **Methods.** To do this, we treated human DLD-1 colorectal cancer cells with thyme essential oil and assessed its effects on cell proliferation by using real time cell analyzing system (Xcelligence System). Furthermore, we applied the same concentrations to the L929 fibroblast cells in order to determine if thyme essential oil has cytotoxicity on normal cells in the same concentrations it showed anticancer effects. Then, antioxidant ability of thyme essential oil was evaluated by using DPPH assay. **Results.** Thyme essential oil treated DLD-1 cells exhibited decreasing cell index values in a concentration-dependent manner. An inhibitor concentration 50 (IC_{50}) value of 0.347 mg/mL was achieved. On the other hand, when thyme essential oil was tested on L929 fibroblast cells, it was observed that thyme essential oil did not show cytotoxic effect on L929 fibroblast cells. IC_{50} value was 22 mg/mL for this experiment. Besides, thyme essential oil showed a strong antioxidant effect on DPPH assay. **Conclusion.** When the antiproliferative and the antioxidant effects of thyme essential oil are considered, it can be concluded that thyme essential oil may be an alternative option in the treatment of colorectal cancer.

Keywords: Thyme, cytotoxicity, DLD-1, colorectal cancer, oxidative stress, Xcelligence system

Özet

Amaç. Kekik uçucu yağı gibi biyolojik aktif ekstraktların çeşitli kanser hücreleri üzerindeki etkilerini ve etki mekanizmalarını araştırma konusu oldukça önemli bir konudur. Bu çalışmada kekik uçucu yağının insan DLD-1 kolorektal kanser hücrelerinin büyümesini inhibe edip etmediği, eğer ediyorsa bu inhibisyona antioksidan etkisinin katkısını belirlemek amaçlanmıştır. **Yöntem.** İnsan DLD-1 kolorektal kanser hücrelerine kekik uçucu yağı uygulayarak, uçucu yağın hücre proliferasyonu üzerindeki etkilerini gerçek zamanlı hücre analiz sistemi (Xcelligence Sistemi) ile değerlendirdik. Ayrıca kekik uçucu yağının antikanser etkinlik gösterdiği konsantrasyonların normal hücrelerde sitotoksik olup olmadığını belirlemek amacıyla L929 fibroblast hücrelerine de aynı konsantrasyonları uyguladık. Daha sonra kekik uçucu yağının antioksidan etkinliğini DPPH yöntemini kullanarak değerlendirdik. **Bulgular.** Kekik uçucu yağı uygulanan DLD-1 hücreleri, konsantrasyona bağımlı olarak hücre indeksinde azalan değerler gösterdi. IC_{50} değeri 0,347 mg/mL olarak bulundu. Diğer taraftan kekik uçucu yağı L929 fibroblast hücreleri üzerine uygulandığında sitotoksik etkisi gözlenmedi. Bu deney için IC_{50} değeri 22 mg/mL olarak bulundu. Bunun yanında kekik uçucu yağı DPPH yönteminde güçlü antioksidan etkinlik gösterdi. **Sonuç.** Kekik uçucu yağının antiproliferatif ve antioksidan etkileri dikkate alındığında, kolorektal kanser tedavisinde alternatif bir seçenek olabileceği sonucuna varılmaktadır.

Anahtar sözcükler: Kekik, sitotoksisite, kolorektal kanser, oksidatif stress, Xcelligence system

Geliş tarihi/Received: October 05, 2012; **Kabul tarihi/Accepted:** January 31, 2013

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Introduction

Cancer continues to be a worldwide killer, despite the enormous amount of research and rapid developments seen during the past decade. According to the recent statistics, cancer accounts for about 23% of the total deaths in the USA and is the second most common cause of death after heart disease [1]. Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and ‘‘westernized’’ diets [2].

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cancer cases and 608,700 deaths estimated to have occurred in 2008. While CRC death rates have been decreasing in several Western countries, largely resulting from improved treatment and increased awareness and early detection, rates continue to increase in many countries with more limited resources and health infrastructure, particularly in Central and South America and Eastern Europe [2, 3]. CRC can develop on the grounds of inflammatory bowel disease (IBD), being the most common cancer among such patients. The three most important high risk conditions for CRC are IBD and the hereditary syndromes of familial adenomatous polyposis and hereditary nonpolyposis CRC syndrome [3, 5]. Modifiable risk factors for CRC include smoking, physical inactivity, overweight and obesity, red and processed meat consumption, and excessive alcohol consumption [2, 3].

Oxidative stress results from the metabolic reactions that use oxygen and represents a disturbance in the equilibrium status of prooxidant/antioxidant reactions in living organisms. The excess reactive oxygen species can damage cellular lipids, proteins, or DNA inhibiting their normal function. Oxidative stress induces a cellular redox imbalance which has been found to be present in various cancer cells compared with normal cells; the redox imbalance thus may be related to oncogenic stimulation. Permanent modification of genetic material resulting from “oxidative damage” incidents represents the first step involved in mutagenesis, carcinogenesis, and ageing. DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions have been noted in various tumours, strongly implicating such damage in the etiology of cancer [4].

Concerning the mechanisms of carcinogenesis, it has been hypothesized that inflammation results in neoplastic transformation by enhancing epithelial cell turnover in the colonic mucosa. Mucosal biopsies from patients with ulcerative colitis (UC) demonstrate higher rates of mitosis and apoptosis, especially in areas of active inflammation. Oxidative stress and oxidative cellular damage are important features of UC. The activities of phagocytic leukocytes are increased in UC patients, resulting in enhanced generation of pro-oxidant molecules. Oxidative stress in inflamed tissue can pave the way for malignant tumors and nitric oxide may contribute to the pathogenesis of CRC [5].

Systemic therapy with cytotoxic drugs is the basis of the most effective treatments of disseminated cancers. However, the responses of tumors to chemotherapeutic regimens vary, and failures are frequent owing to the emergence of drug resistance. The use of medicinal plants for the treatment of diseases is as old as mankind. Essential oils and their

components are becoming increasingly popular as naturally occurring bioactive agents [6]. There have been great efforts to find safe and potent natural antioxidants from various plant sources. As harmless sources of antioxidants, wild herbs, spices, fruits, nuts, and leafy vegetables have been investigated for their antioxidant properties, for example thyme [7].

Thyme [*Thymus fallax*. Fisch. Mey. (Labiatte)] is an aromatic herb that is used extensively to add a distinctive aroma and flavour to food. The leaves can be used fresh or dried for use as a spice. Essential oils extracted from fresh leaves and flowers can be used as aroma additives in food, pharmaceuticals, and cosmetics. Thyme also possesses various beneficial effects, e.g., antiseptic, carminative, antimicrobial, and antioxidative properties [8]. Additionally, dietary supplementation with thyme oil maintained significantly higher superoxide dismutase and glutathione peroxidase activities and total antioxidant status. At non-toxic concentrations, thyme extract was also identified as a natural antimutagen with the possibility of enhancement of error-free DNA repair [6]. Even though some reports on the use of Labiatae species for the treatment of cancer are available, no extensive research has been carried out against different types of human cancer cell lines under in vitro conditions which otherwise provide important experimental evidences to select the plant extracts with potential anticancer activities for further investigations [7].

Based on provided information, the aim of this study is to evaluate whether thyme has anticancer activity and whether this activity is arising from its antioxidant activity. We decided to examine the in vitro cytotoxic effect of its essential oil against human colorectal adenocarcinoma DLD-1 cell lines. The findings may support the further utilization of thyme as a source of natural antioxidants.

Material and methods

T. fallax plants were collected from Imranli-Yoncabayiri, Taslikyamac (1950 m), Sivas, Turkey. The taxonomic identification was made during flowering season late February 2006. The voucher specimen was identified and deposited at the Herbarium of the Department of Biology, Cumhuriyet University in Sivas in Turkey (CUFH-Voucher No: ED 11011). The air-dried and finely ground aerial parts of *T. fallax* were subjected for 3 h to water distillation using a clevenger- type apparatus (Yield 2.9% v/w). The oil was dried over anhydrous sodium sulphate and after filtration stored at +4°C.

DPPH assay

The method is based on the reduction of alcoholic DPPH solutions in the presence of a hydrogen donating antioxidant. DPPH solutions show a strong absorption band at 517 nm appearing as a deep violet colour. The absorption vanishes and the resulting decolorization is stoichiometric with respect to the degree of reduction. The remaining DPPH, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant [9]. Fifty microliter of various concentrations of the extracts in methanol was added to 5 mL of a 0.004% methanol solution of DPPH. After 30 min of incubation at room temperature, the absorbance was read against a blank at 517nm. Inhibition free radical DPPH in percent (I %) was calculated in the following manner: where A blank is the absorbance of the control reaction (containing all reagents except the test compound), and A sample is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against extract concentration. Synthetic antioxidant reagent butylated hyroxytoluene (BHT) was used as the positive control and all tests were carried out in triplicate.

Cell Culture

Human colon adenocancer cells DLD-1 were purchased from the Sap Institute. L929 fibroblast cell line from mouse also was used. Cells were multiplied in three passages,

frozen in aliquots and stored in liquid nitrogen. The cells were maintained in DMEM with phenol red and $NaHCO_3$. The culture medium was supplemented with 10% heat inactivated FBS, %1 penicilin and streptomycin. Cells were grown in T-25 cm^2 culture flasks in a humidified atmosphere containing 5% CO_2 at 37^oC.

xCELLigence system

The xCELLigence system was used according to the instructions of the supplier (Roche Applied Science and ACEA Biosciences). The xCELLigence system consists of four main components: the RTCA analyzer, the RTCA DP station, the RTCA computer with integrated software, and disposable E-plate 16. The RTCA DP station fits inside a standard tissue-culture incubator, while an analyzer and laptop computer with software will be on the outside. The core of the xCELLigence system is the E-plate 16: this is a single use, disposable device used for performing cell-based assays on the RTCA DP instrument, which has similar application like commonly used 96-well micro titer plate. However the E-plate 16 differs from standard 96-well micro titer plates vastly with its incorporated gold cell sensor arrays in the bottom, which contributes cells in-side each well to be monitored and assayed. The E-plate 16 has a low evaporation lid design: the bottom diameter of each well is 5.0mm±0.05mm; with a total volume of 210±5 µL, approximately 80% of the bottom areas of each well is covered by the circle-on-line electrodes, which is designed to be used in an environment of +15 to +40°C, relative humidity 98% maximum without condensation [10]. The electronic impedance of sensor electrodes is measured to allow monitoring and detection of physiological changes of the cells on the electrodes. The voltage applied to the electrodes during RTCA measurement is about 20mV (RMS) [10]. The impedance measured between electrodes in an individual well depends on electrode geometry, ion concentration in the well and whether or not cells are attached to the electrodes [10]. In the absence of cells, electrode impedance is mainly determined by the ion environment both at the electrode/solution interface and in the bulk solution. In the presence of cells, cells attached to the electrode sensor surfaces will act as insulators and thereby alter the local ion environment at the electrode/solution interface, leading to an increase in impedance [10]. Thus, the more cells that are growing on the electrodes, the larger the value of electrode impedance. The RTCA associated software allows users to obtain parameters such as: average value, maximum and minimum values, standard deviation (SD), half maximum effect of concentration (EC50), half maximum inhibition of concentration (IC_{50}), cell index (CI), and in addition graphics. The data expressed in CI unit scan is exported to Excel for any type of mathematical analysis [10].

Cell growth and proliferation assay using xCELLigence system

DLD-1 cells were grown and expanded in tissue-culture flasks. After reaching ~75% confluence, the DLD-1s (passage 6) were washed with PBS, afterwards detached from the flasks by a brief treatment with trypsin/EDTA. Subsequently, 100 µL of cell culture media at room temperature was added into each well of E-plate16. After this the E-plate16 was connected to the system and checked in the cell culture incubator for proper electrical contacts and the background impedance was measured. Meanwhile, the cells were resuspended in cell culture medium and adjusted to 20.000 cells/mL. 100 µL of cell suspension was added to the 100 µL medium containing wells on E-plate16. After 30 min incubation at room temperature, E-plate16 was placed into the cell culture incubator. Finally, proliferation of the cells was monitored every hour for a period of up to 72h via the incorporated sensor electrode arrays of the E-Plate16. The electrical impedance was measured by the RTCA-integrated software of the xCELLigence system as a dimensionless parameter termed CI.

Cytotoxicity assay using xCELLigence system

First, the optimal seeding concentration for proliferation experiments of the DLD-1 was determined. After seeding the respective number of cells in 100 µL medium to each well

of the E-plate16, the proliferation of the cells was monitored every 30 min by the xCELLigence system. Approximately 24 h after seeding, when the cells were in the log growth phase, the cells were exposed to varied concentrations of thyme oil. The concentration of stock solution was also used to evaluate the effects of high concentrations. Controls received either medium only or medium+methanol or medium+thyme oil.

Statistical analysis

All data are expressed as mean ± SEM. Statistical comparisons between groups were performed using general linear models of analysis of variance (ANOVA) followed by the Tukey test and a t test when appropriate and P-values of less than 0.05 were considered to be statistically significant.

Results

Antioxidant effect of thyme essential oil was evaluated with the DPPH method. In the DPPH test the ability of a compound to act as donor for hydrogen atoms or electrons was measured spectrophotometrically. Both the oil and the pure compounds were able to reduce the stable radical DPPH to the yellow colored diphenylpicrylhydrazine. The strongest effect was measured for the thyme essential oil with an IC_{50} of 0.382 mg/mL and for the butylhydroxytoluene with an IC_{50} of 0.010 mg/mL. The results are shown in Table 1, Figure 1 and Figure 2. Thyme essential oil was found to have significant antioxidant effect.

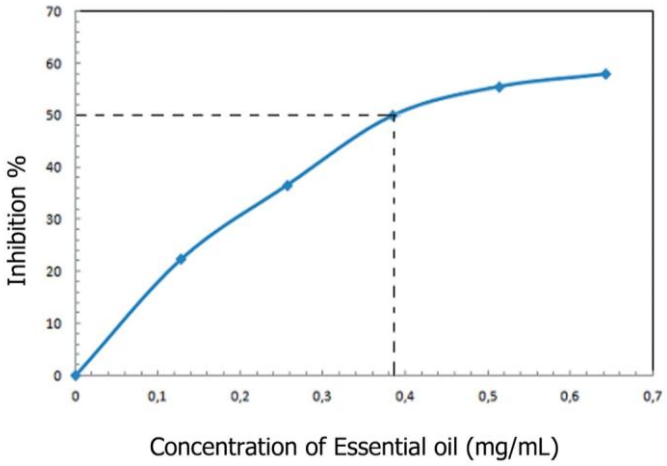


Figure 1. % Inhibition of thyme essential oil.

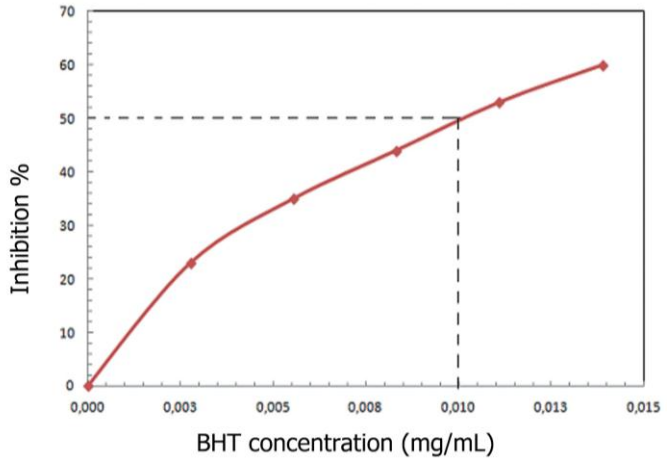


Figure 2. %Inhibition of BHT concentration.

Table 1. Concentration of thyme essential oil and Butylhydroxytoluene (mg/mL) for a 50% inhibition in the DPPH assay.

	IC_{50} (mg/mL)
Thyme	0,382
BHT	0,010

Monitoring of cytotoxicity in real-time using xCELLigence system

We used the 20.000 cells/well concentration in the xCELLigence assay to examine the anti-proliferative effects elicited by thyme essential oil, as the 20,000 cells/well concentration which has an optimal treatment window between 16-24 hours. Thyme essential oil treated DLD-1 cells exhibited decreasing cell index values in a concentration-dependent manner. While 43, 0.80, 0.68, 0.48 and 0.34 mg/mL concentrations showed complete cytotoxic effect and decreased CI to 0.1. While 0.16 mg/mL thyme decreased CI to 0.5, 0.08 mg/mL thyme decreased CI to 1.6. Both showed statistically significant cytotoxic effect when compared to the control ($p<0.05$) (Figure 3). Twenty-four hours after treatment with thyme oil in DLD-1 cells, an IC_{50} value of (IC_{50}) 0.347 mg/mL was achieved (Figure 5). In addition to the DLD-1 CRC cells, the cytotoxic effects of thyme essential oil were tested on L929 fibroblast cells. Thyme essential oil did not show cytotoxic effect on L929 fibroblast cells. It was observed that while 43 mg/ml concentration complete and 0.8 mg/mL concentration showed partial cytotoxic effect, the rest of the concentrations did not show any cytotoxicity on L929 fibroblast cells. Twenty-four hours after treatment with thyme oil in L929 cells, an IC_{50} value of (IC_{50}) 22 mg/mL was achieved (Figure 4).

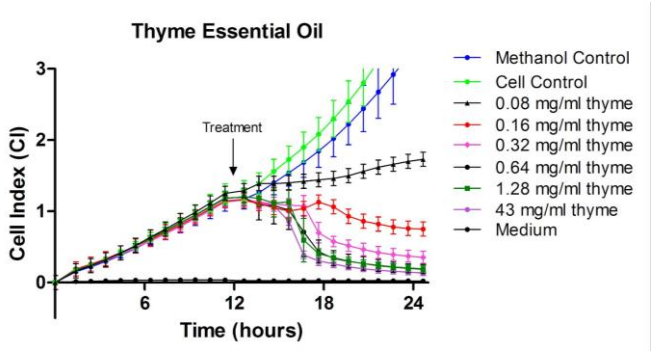


Figure 3. Real-time monitoring of cytotoxic effect of thyme essential oil on DLD-1 human colon adenocancer cell line using RTCA.

- **Methanol control:** Wells that containing only methanol-(thyme solvent).
- **Cell control:** Wells that containing only DLD-1 cells without thyme.
- **Medium:** Wells that containing only medium without thyme and cell.

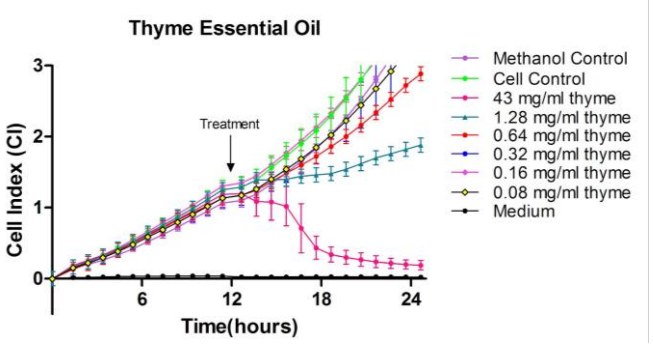


Figure 4. Real-time monitoring of cytotoxic effect of thyme essential oil on L929 fibroblast cell line using RTCA.

- **Methanol control:** Wells that containing only methanol-(thyme solvent).
- **Cell control:** Wells that containing only L929 cells without thyme.
- **Medium:** Wells that containing only medium without thyme and cell.

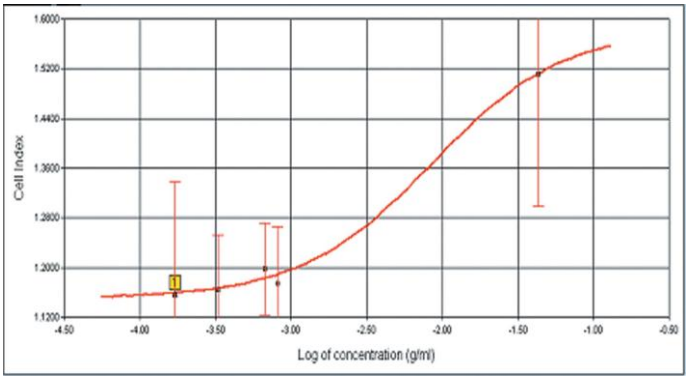


Figure 5. Calculation of IC_{50} of thyme essential oil (IC_{50}=0.347 mg/mL).

Discussion

CRC continues to be a major healthcare concern, accounting for the second leading cause of cancer deaths for both men and women, and estimated to cause 49.920 deaths in the USA in 2009 alone [11].

The main treatments for advanced stage CRC include chemotherapy, radiotherapy, targeted therapy and surgery etc. Surgery can be performed wherever applicable in cases with liver or lung metastasis. Adjuvant chemotherapy or adjuvant chemotherapy combined with targeted therapy before surgery may be considered in cases where chemotherapy may render the lesion resectable. For the unresectable colon cancer associated with diffuse metastatic lesions, the chemotherapy or/and targeted with single or multiple medicines can be adopted such as 5-fluorouracil/calcium folinate, Capecitabine, Irinotecan, Oxaliplatin, Bevacizumab, Cetuximab and Jesper antibody (NCCN 2010: 51). At present, one study showed that the 5-year survival rate of colon cancer of stage IV was only 8.1% after treatment with Western medicine [12]. The median survival of CRC patients of stage IV were prolonged to 17.9 months by adding Bevacizumab on the basis of the program 5-fluorouracil / calcium folinate [13]. The treatment of combined FOLFIRI regimen and Bevacizumab could prolong the patients' overall survival time to 28 months [14]. Although good results have been achieved with advances in cancer treatment, conventional protocols are not satisfactory. So scientists working on cancer research are trying to find out new therapeutic options including biologically active derivatives.

In recent years major research has been focused on the biologically active derivatives of medicinal plants for the development of novel potential drugs for several pathologies with significant social impact [15, 16]. The use of natural products from the extracts of medicinal plants in the treatment of skin, respiratory, neuromuscular and mental health disorders and also in obstetrics and gynecology is already known [17-19]. The potential anti-tumor activity of the medicinal plants has been recently described in many studies [20-22]. Extensive research on biologically active compounds from essential oils has proved them to be potent anti-bacterial, anti-fungal and anti-oxidant agents [23-26].

In our study, we showed that thyme essential oil has concentration-dependent cytotoxic effect on DLD-1 CRC cells. Twenty-four hours after treatment with thyme oil, an IC_{50} value of (IC_{50}) 0.347 mg/mL was achieved. By the time similar concentrations of thyme essential oil were applied on L929 fibroblasts cells in order to see if thyme essential oil has a toxic effect on normal body cells. It was observed that thyme essential oil did not

show any cytotoxic effect on L929 cells with an IC50 value of (IC_{50}) 22 mg/mL. This may show that thyme essential oil could kill cancer cells selectively. In accordance with the present study, Danielle Berrington and Namrita Lall [27] have shown that Thymus vulgaris with many other plant extracts have antitumor effect on Cervical Epithelial Carcinoma (HeLa) cells. The IC50 value of *Thymus vulgaris* for HeLa cells was 36.13 µg/mL. Furthermore the essential oil of Thymus vulgaris which is basically the same compound we used was tested on head and neck squamous cell carcinoma by Sertel et al. [28] and was found to have toxicity with an IC_{50} value of 369µg/mL. This study has very high correlation with the present study in terms of IC50 values. We achieved almost same IC_{50} value with a different kind of cancer type. Yi et al. [29] has tested five culinary and medicinal herbs grown under greenhouse conditions on SW-480 human colon adenocarcinoma cell line. Their results showed that thyme extracts which was prepared differently when compared to our study significantly inhibited SW-480 colon cancer cell growth and IC_{50} value was 138.5µg/mL which is partially fitting to the present study.

Although it is well known that chronic inflammation is strictly related to the human cancers, the exact mechanism by which chronic inflammation results in carcinogenesis is still unclear. Persistent inflammation is believed to result in increased cell proliferation as well as oxidative stress, leading to the development of dysplasia [30]. Oxidative stress develops particularly in inflammatory reactions because the inflammatory cells, activated neutrophils, and macrophages produce large amounts of reactive oxygen and nitrogen species. DNA damage caused by oxidative stress in the characteristic damage-regeneration cycle is a major contributor to CRC development in ulcerative colitis patients. Thus, oxidative stress-induced cellular damage may provide a mechanistic basis for many of the events thought to drive UC-associated colon carcinogenesis in humans and animal models, including specific gene alterations, genetic instability and aberrant methylation [31].

Studies in animal models of UC have provided support as to the involvement of oxidative stress in inflammation-driven CRC. Seril et al. [32] have recently examined the role of nitric oxide (NO) in UC-associated colorectal carcinogenesis using the DSS-induced and iron-enhanced model of chronic UC in inducible nitric oxide synthase (iNOS) knockout mice. These results showed that there is no difference in UC-associated cancer development in iNOS+/+ and iNOS-/- mice, suggesting that in the absence of iNOS, other factors, such as eNOS, may play a role in nitrosative stress and UC-associated carcinogenesis in this model system [32].

In the present study, antioxidant effect of thyme essential oil was evaluated with the DPPH method. The strongest effect was measured for the thyme essential oil with an IC_{50} of 0.382 mg/mL and for the butylhydroxytoluene with an IC_{50} of 0.010 mg/mL. Thyme essential oil was found to have significant antioxidant effect. Our results are consistent with the study of Aziza et al. [33]. They have concluded that the essential oil of T. vulgaris has a potential antioxidant activity and a dose-dependent protective effect against aflatoxin toxicity. Also in a study Undeger et al. [34] which examined the genotoxicity of thymol and carvacrol using comet assay in V79 Chinese hamster lung fibroblast cells, it has been found that thymol and carvacrol displayed a concentration-dependent antioxidant capacity.

In conclusion, in the present study, we found that thyme essential oil has strong and selective cytotoxic effect on CRC cells and its antioxidant capacity may be at least partly related to this cytotoxic effect. Further studies are needed to fraction and isolate subfractions of thyme essential oil and define which subfraction is responsible for this cytotoxic and antioxidant effect.

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