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Technical Note

Antiproliferative activity and cytotoxic effect of essential oil and water extract from *origanum Vulgare* L.

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ABSTRACT

Origanum vulgare L., an aromatic and medicinal plant has been used extensively for the food and pharmaceutical industry. In this work, the essential oil was generated by steam distillation and the compounds were identified by GC-MS analysis. Carvacrol was found as a major product. *O. vulgare* was extracted with water (60 °C) to yield the water extract as well. The antiproliferative activity and cytotoxic effect of essential oil and extract were investigated using MTT [3- (4,5-dimethyl-thiazol-2-yl) -2,5-diphenyl tetrazolium bromide] and LDH (Lactate dehydrogenase) techniques, respectively. A549 (human lung carcinoma), Hep3B (hepatocellular carcinoma), HT29 (human colon carcinoma), MCF7 (human breast adenocarcinoma) cancerous cell lines, FL (human amnion cells) normal cell lines were used for essential oil and extract, while cisplatin and 5-FU were exploited as standards. The essential oil revealed the significant activity against A549 (IC_{50}, 27.2 µg/mL), Hep3B (IC_{50}, 7.4 µg/mL), and MCF-7 (IC_{50}, 7.1 µg/mL) cancerous cell lines as compared to standards. Moreover, extract displayed outstanding activity against Hep3B (IC_{50}, 27.2 µg/mL) and MCF-7 (IC_{50}, 10.8 µg/mL) cell lines. The activity of essential oil may be due to carvacrol since carvacrol is the main constituent of essential oil with a high percentage (90.4%) or due to the synergistic effect of the compounds in the essential oil.

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INTRODUCTION

Plants have been consumed widely for food and medicine since ancient times due to their bioactive contents. After the discovery of spectroscopy in the 19th century, the structures of bioactive compounds in the plant began to be elucidated. Hence, the plants became the focus of science, and a lot of bioactive compounds were isolated and identified from plants that were used for drug discovery and development. Also, the corresponding bioactive

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compounds inspired synthetic chemists to synthesize these compounds [1-4].

Origanum genus (Lamiaceae family), an aromatic and medicinal plant is represented by twenty-three species and six hybrids in Turkey flora, 14 of which are endemic [5]. *Origanum* species have been used widely for culinary and traditional medicine against fever, allergy, hypertension, diabetes, menstrual pain, stomach pain, cough, rheumatism, headache, insomnia, pyretic, inflammatory, respiratory infection. Moreover, these species were reported to exhibit considerable biological effects including antimicrobial, antiparasitic, antioxidant, antidiabetic, anticancer activities [6]. *Origanum* is native to Europa, North Africa, Asia, however, most species of this genus are distributed along to the Mediterranean region, mainly in Turkey. It was also presented in North America, where it grew in many states along the east and west coasts. Phytochemical study on *Origanum* species revealed the isolation of bioactive compounds displaying considerable biological activities [7-10].

O. vulgare known as oregano is one of the most famous aromatic plants, with a strong traditional history as a spice and medicinal herb, but also as a well-established origin of valuable plant-based remedies in modern phytotherapy. *O. vulgare* is both wild-collected and cultivated for commercial, Turkey is the most important supplier of Mediterranean oregano. The common name of "oregano" has been used for other species such as *O. onites, O. majorana, O. minutiflorum, O. syriacum. O. vulgare* contains essential oils with changeable composition, as well as secondary metabolites such as terpenoids, flavonoids, tannins, phenolic compounds [11]. The essential oils of oregano are well documented. Due to the variation of *O. vulgare,* its chemical compositions are also variable. The essential oils consist of monoterpene, sesquiterpene as well as phenolic compounds. Carvacrol, thymol, p-cymene, terpinene, and linalool are the major constituents [12]. The essential oil compounds vary according to the geographical area, harvesting time, plant ripening stage, and environmental conditions [13-15].

The *Origanum* species, *O. vulgare* subsp. *hirtum, O. vulgare* subsp. *glandulosum* and *O. vulgare* subsp. *gracile* possessing high essential oil content contains carvacrol, thymol, *p*-cymene, and γ-terpinene as major products [16]. *O. vulgare* essential oils (Ov-OEO) display a considerable effect against a broad spectrum of pathogenic bacteria. Hence, they represent an efficient alternative as an antimicrobial agent against bacterial strain [17]. Since essential oils are hydrophobic molecules, they have greater permeability across the cell membrane causing the expansion of cellular contents. Moreover, OvEO showed considerable biological effects such as antioxidant, anti-inflammatory, antidiabetic, antifungal, antiparasitic, antitumoral, wound healing, hypoglycemic, and anti-Alzheimer properties [18-23]. Carvacrol and thymol represent the major compounds, constituting virtually 78-85% of the OvEO and they are responsible for the biological properties [24]. The ethanol extract of *O. syriacum* and *O. vulgare* was reported to have an antiproliferative effect against adenocarcinoma of breast cell line (MCF7) [25]. In addition, the essential oil of *O. vulgare* contained the 4-terpineol as a major product and displayed a high cytotoxic effect on HT-29 cell lines [26] as well as an antiproliferative effect on stomach cancer [27].

Since the *O. vulgare* includes a high percentage of essential oil, this plant is cultivated in our university, the essential oil of *O. vulgare* has been used extensively in food, and culinary, we aimed to find out the essential oil content of *O. vulgare* and to investigate the antiproliferative and cytotoxic effects of essential oil and water extract on A549, Hep3B, HT29, MCF7 cancerous cell lines, and FL normal cell lines.

MATERIAL AND METHODS

Chemicals

MTT, 5-fluorouracil and cisplatin were supplied from Roche Diagnostics GmbH, Germany. All solvents and chemicals were bought from Sigma-Aldrich.

Plant Material

O. vulgare was cultivated in the Aromatic and Medicinal Plant Field of Tokat Gaziosmanpasa University under the control of Prof.Dr. Isa Telci.

Essential Oil Generation and Extract Preparation

O. vulgare dried powder (190 g) and distilled water (1.0 L) were added to a quartz glass flask (2.0 L) and Clevenger apparatus was connected to the flask. After the steam distillation for 2 h, water was removed by decantation, and essential oil (1. 66 g, 0.87%) was stored at +4 °C to analyse [28, 29]. *O.vulgare* powder (20.0 g) was heated with distilled water (200 mL) at 60°C for 3 hours. After the filtration, the water was removed by a rotary evaporator to yield the water extract (1.7 g) that was kept in the fridge for analysis (+4°C).

GC-MS Analysis

The essential oil (20 mg) was dissolved in acetone (1.2 mL). Perkin Elmer Clarus 500 GC-MS with BPX5 (30 m × 0.25 mm ID, 0.25 μm film) column was used for analysis. The injection volume and injection temperature were fixed as 2.0 μL and 250 °C respectively. The helium was the carrier gas with a flow rate of 1 mL/min and the splitting rate was 50:1. The oven temperature was started at 50 °C, increased per minute at 5 °C up to 100 °C. The oven program was started at 50 °C, increased by 5 °C per minute to 100 °C, and kept at this temperature for 2 minutes, then the temperature was increased by 3 °C per minute to 220 °C and kept this temperature for 2 minutes. The total time was adjusted to 30 min. The ionization energy was 70 eV and

the ion source temperature was 250 °C. The identification of compounds was executed by retention index and electron impact (EI) mass spectrum with the comparison with the library (NIST, Wiley, and Pfleger) [30].

The Effects of Extract and Essential Oil on Cancer Cells *In Vitro*

MCF-7 (human mammary gland adenocarcinoma, ATCC® HTB22 ™), Hep3B (human liver hepatocellular carcinoma, ATCC® HB8064 ™), A549 (human lung carcinoma, ATCC® CCL185 ™), and HT29 (human colorectal adenocarcinoma, ATCC® HTB38 ™) cancer cell lines and FL (human epithelial amnion cell, ATCC® CCL62 ™) normal cell lines were used. A sterile laminar cabinet was used under aseptic conditions during cell growth and activity measurements. MTT proliferation test, LDH cytotoxicity test, and morphological analyses were carried out after the cells formed sufficient density at 37 ° C and 5% CO_2 conditions in suitable media containing 10% FBS (Fetal Bovine Serum) and 2% PenStrep (Penicillin-Streptomycin). To ensure that the tests were healthy, studies were carried out after the cells had reached the logarithmic growth phase [31]. The experiments were repeated three times.

GI_{50}, TGI, LC_{50}, and IC_{50} Parameters of the Extract and Essential Oil

In this study, MTT test was used to measure the effects of extract and essential oil on cell proliferation [32]. This test protocol was applied after the extract and essential oil and cancer cell lines were incubated for 24 hours. Briefly, The second stock is prepared by diluting 1/9 of the 5 mg / mL MTT stock solution with RPMI 1640 medium that does not contain phenol red, and the old broth of the incubated cells is removed and replaced with this MTT solution and incubated for another 4 hours. After incubation, the results are calculated as % cell inhibition according to the following formula; % Inhibition [1- (A test substance / A control)] × 100.

To determine the IC_{50} concentrations of the extracts (the concentration that inhibits the proliferation of 50% of the cells in the environment), logarithmically increasing extract concentrations are prepared at a certain interval. Then, these concentrations are tested on the cells by MTT methods and their absorbance values are found. The IC_{50} value is calculated with the help of an Excel program over the logarithmic curve prepared from these absorbance values. The GI50, TGI, and LC50 parameters of the extracts were calculated using the formulas below. [(Ti-Tz) / (C-Tz)] × 100 = 50 with the condition that Ti ≥ Tz for the concentration value (GI50) that reduces the growth by 50%; Concentration value (TGI) reducing growth by 100% Ti = Tz; [(Ti-Tz) / Tz] × 100 = -50 provided that Ti <Tz for the concentration value (LC50) that kills the cells in the environment at 50%. Here, Tz; zero points, C; control growth, Ti; is the inhibition caused by the test substance.

Cytotoxic Effects of Essential Oil and Extract on Cells

Whether the extract and essential oil are cells cytotoxic or cell cytostatic was determined by the LDH method [33]. Depending on the substance tested, an increase in the number of cells dying during the incubation time will result in an increase in LDH in the culture supernatant. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme that is found in most cells and is stable. For this purpose, the LDH cell cytotoxicity kit was used according to the manufacturer's procedure. Briefly, the change in the amount of formazan formed as a result of LDH enzyme activity was measured and the evaluation was made according to the following formula; % Cytotoxicity = [(Substance Absorbance - Low Control) / (High Control - Low Control) × 100].

Statistical Analysis

Statistical analysis was executed by GraphtPad Prism software (version 8.00) with one-way ANOVA, multiple comparisons tests. The results were reported as mean values ± SDs of three independent assays ($P < 0.05$).

RESULTS AND DISCUSSION

Antiproliferative Effect of Extract and Essential Oil

The cell proliferation inhibitory effects of extract and essential oil were conducted against four cancer cell lines and one normal cell line, as summarized in Table 1. Here, cisplatin and 5-fluorouracil (5-FU) were used as reference antitumor drugs (Table 2). The essential oil was more effective on A549 (TGI 20.04 µg/mL, IC_{50} 27.16 µg/mL), Hep3B (TGI 7.44 µg/mL, IC_{50} 7.36 µg/mL) and MCF7 (TGI 7.24 µg/mL, IC_{50} 7.07 µg/mL) cell lines (Table 1) than water extract of O. vulgare.

The essential oil showed less antiproliferative effect against HT29 (TGI >500 µg/mL and IC_{50} >500 µg/mL) cell line than positive controls (cisplatin and 5-FU) (Table 2). Among them, the LC50 values of the essential oil were significant in MCF7 (LC50 100.16 **µg/mL**) cells and A549 cells (LC50 341.08 **µg/mL**) (Table 1). The extract and essential oil had within acceptable GI50 values (1.53-4.67 µg/mL and 1.92-9.51 µg/mL, respectively) when tested on the all cell line. Antiproliferative activity of essential oil was a bit high on normal FL cells compared to cancer cells (Table 1). However, FL cells may have lower mitochondrial activity than cancer cells. Because MTT assay measures only the mitochondrial activity of living cells, the anticancer effect of the compounds on the FL cells is probably incorrectly measured. To solve this problem, the LDH and MTT test results were evaluated together in the decision-making process. When the toxicity test was examined, it was found that essential oil (% 17.47) had the least toxic effect on normal cells than cancer cells (Table 3). In comparison of essential oil and extract, it was observed that essential oil had higher antiproliferative effect than extract on A549, Hep3B, MCF7 cell lines.

Table 1. GI_{50}, TGI, LC_{50}, and IC_{50} values for Water extract and Essential oil*

μg/mL	*Essential oil*				*Water extract*			
	GI_{50}	TGI	LC_{50}	IC_{50}	GI_{50}	TGI	LC_{50}	IC_{50}
A549	4.67±0.8^{c}	20.04±1.7^{c}	341.08±17.3^{d}	27.16±1.7^{c}	9.51±0.9^{c}	>500	>500	>500
HT29	1.61±0.1^{a}	>500	>500	>500	1.92±0.1^{a}	>500	>500	>500
Hep3B	2.60±0.4^{b}	7.44±0.8^{b}	66.81±2.8^{b}	7.36±0.8^{bA}	3.72±0.2^{b}	27.88±1.8^{c}	>500	27.20±1.0^{cB}
MCF7	2.34±0.4^{b}	7.24±0.9^{b}	100.16±5.4^{c}	7.07±0.5^{bA}	2.15±0.2^{a}	11.24±0.9^{a}	>500	10.76±0.8^{aB}
FL	1.53±0.1^{a}	3.14±0.4^{a}	21.36±1.6^{a}	3.11±0.2^{aA}	3.12±0.2^{b}	15.60±1·0b	>500	15.35± 0.9^{bB}

*Values are given as the mean ± SD of three experiments. Values followed by the same letter in the column are not significantly different ($P < 0.05$). The capital letters indicate the statistical analysis of row, values followed the same letter in the row are not significantly different ($P < 0.05$). The comparison was executed for the same cell lines for IC_{50}.

Table 2. GI_{50}, TGI, LC_{50}, and IC_{50} values for 5FU and Cisplatin*

μg/mL	*5FU*				*Cisplatin*			
	GI_{50}	TGI	LC_{50}	IC_{50}	GI_{50}	TGI	LC_{50}	IC50
A549	9.34±0.8^{c}	63.25±2.9^{b}	>500	69.79±3.4^{d}	7.83±0.5^{d}	54.23±2.1^{c}	241.37±15.6^{a}	60.49±3.3^{d}
HT29	9.27±0.7^{c}	94.23±3.3^{d}	>500	65.19±2.8^{c}	4.16±0.3^{a}	51.67±2.8^{b}	236,37±19.4^{a}	40.39±2.1^{a}
Hep3B	8.36±0.8^{b}	91.28±3.9^{d}	>500	62.89±2.7^{b}	5.41±0.4^{b}	39.15±1.7^{a}	290.79±20.1^{b}	48.69±1.9^{b}
MCF7	7.83±0.5^{a}	47.47±2.8^{a}	>500	74.19±4.1^{e}	6.14±0.5^{c}	50.93±2.9^{b}	>500	63.79±2.2^{e}
FL	8.21±0.6^{b}	82.36±3.7^{c}	>500	59.09±3.6^{a}	6.79±0.5^{c}	55.07±3.0^{c}	349.11±17.7^{c}	52.79±2.7^{c}

*Values are given as the mean ± SD of three experiments. Values followed by the same letter are not significantly different ($P < 0.05$).

Table 3. % Cytotoxic values of water extract and essential oil (EO)

% Cytotoxicity	A549	Hep3B	MCF7	HT29	FL
EO	22.86^{c}	18.48^{c}	19.63^{d}	20.32^{d}	17.47^{b}
Water extract	25.26^{d}	19.41^{d}	16.83^{c}	18.04^{c}	16.65^{b}
Cisplatin	8.63^{a}	8.46^{a}	10.71^{b}	11.23^{b}	8.33^{a}
5FU	9.19^{b}	9.67^{b}	7.69^{a}	7.91^{a}	8.44^{a}

Values are given as the mean ± SD of three experiments. Values followed by the same letter are not significantly different ($P < 0.05$).

Overall, the high antitumor activity for essential oil may be related to its unique three-dimensional structure. This study vigorously expressed that three parameters (GI50, TGI, and LC50) of essential oil met substantially with NCI-60 criteria enough to pass into further pharmacological investigations.

Cytotoxic Effect of Essential Oil and Extract

In the present study, cytotoxic activities of extract and essential oil on cell lines were measured with the aid of an LDH cytotoxicity kit based on measuring the amount of cytoplasmic LDH, an indicator of membrane integrity. Table 3 shows that the extract and essential oil for FL cells (17%) were not cytotoxic but were toxic for cancer cells. However, the essential oil has therapeutic efficacy since it has only safe in FL cells at IC_{50} and below concentrations. Here, our findings expressed that essential oil has a cytostatic effect on the cells. The cytotoxic effect of the extract was determined to be more than essential oil.

Chemical Analysis of Essential Oil

The quantitative analysis of the chemical profile of essential oil from *O. vulgare* is shown in Table 4. The table includes retention times, retention indices, retention indices in literatures [34, 35] and the percentage of the compounds identified. Essential oils include significant secondary metabolites that have a broad spectrum of biological effects. Many essential oils have been used in the cosmetic and pharmaceutical industries [36]. In this work, *O. vulgare* essential oil includes carvacrol (90.4%) as a major product. In reported work carvacrol was detected as the percent of 58.1%[37] in *O. vulgare*. Another research indicated that the main essential oil compound of *O. vulgare* was 4-terpineol (41.2%) [26].

Hence, this medicinal plant could be a source of carvacrol. The isolation of pure compounds from plants requires extensive chromatographic techniques. Moreover, the isolation of compounds from essential oils is much more difficult. Hence, the essential oil from *O. vulgare* containing carvacrol with high percentage could be a great

Table 4. Chemical composition of essential oil from *O.vulgare*

Compounds	RT	[a]RI	[b]RI	%
Tyranton	3.47	846	844	0.58
Myrcene	5.49	981	991	0.35
α-Terpinene	6.55	1021	1018	0.17
p-Cymene	6.97	1027	1026	4.21
γ-Terpinene	7.95	1063	1062	1.19
Trans- sabinenehydrate	8.55	1085	1074	0.40
Cis-sabinenehydrate	9.91	1091	1097	0.28
4-Terpineol	13.07	1179	1178	0.74
α-Terpineol	13.81	1208	1207	0.14
Thymol	17.27	1290	1290	0.22
Carvacrol	18.11	1301	1299	90.42
β-Caryophyllene	22.94	1417	1418	0.83
β-Bisabolene	26.01	1510	1509	0.21
Caryophylene oxide	29.68	1581	1581	0.26
Total %				99.98

[a]Retention index (RI) calculated with respect to *n*-alkana, [b]Retention index (RI) in literatures, % yield was calculated with FID

advantage for use in food and pharmaceutical industries. Besides, it is possible to present the mechanism of pure compounds.

Carvacrol is the monoterpenoid found mostly in essential oils of *Origanum* species. It is extensively used as a food flavouring, additive, and preservative. Carvacrol is also used in cosmetic products as a fragrance. The *in vivo* and *in vitro* studies revealed the carvacrol had pharmaceutical properties such as anticancer, antifungal, antibacterial, anti-inflammatory, antioxidant, hepatoprotective, and spasmolytic. Besides, it was presented that the carvacrol could be a possible candidate for Covid-19 [38].

CONCLUSION

Antiproliferative activity and cytotoxic effect of essential oil and extract from *O. vulgare* were carried out. The essential oil and extract displayed considerable antiproliferative and cytotoxic effects on some cancer cells. Since the major compound of *O. vulgare* essential oil was carvacrol, the antiproliferative effect may have been caused by carvacrol. Essential oil was determined to have excellent antiproliferative effect on A549, Hep3B, MCF7 cancer cell lines. Moreover, the extract activity was displayed to be effective on Hep3B, MCF7 cell lines. In comparison essential oil and extract, it was found that essential oil has more antiproliferative effect than that of the extract. However, the cytotoxicity of extract was more than the essential oil. *O. vulgare* could be an excellent source of carvacrol. This work contributes to the knowledge on the use of this medicinal plant. These results also suggested the potential feasibility of *O. vulgare* as well as its possible applications in the pharmaceutical industry.

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AUTHORSHIP CONTRIBUTIONS

Authors equally contributed to this work.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

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