International Journal ofNature and Life Sciences

IJNLS

https://dergipark.org.tr/tr/pub/ijnls

e-ISSN: 2602-2397https://doi.org/10.47947/ijnls.1397191

Article

Potential Anti-Cancer Effects of Extra Virgin Olive Oil and Its Phenolic Extracts on Hepatocellular Carcinoma Cells

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Abstract: In this study, three types of Extra Virgin olive oils (EVOO) grown in different regions of Türkiye (Kilis Yaglik, Ayvalik, Izmir Sofralik) and their phenolic extracts (EVOOP) were evaluated for their anti-cancer activity in hepatocellular carcinoma (HCC) cells (Hep40) and a complete profiling of the fatty acid, sterol and polyphenol content of these olive oils was performed by HPLC and GC method. It was shown that genetic diversity and differences in growing conditions of the olive oils studied significantly modified the phenolic composition. The biophenol content was found as 655.4 mg/kg, 508.75 mg/kg and 197.86 mg/kg in Kilis Yaglik, Izmir Sofralik and Ayvalık respectively. The highest content of oleocanthal was found in İzmir Sofralik EVOO (142.00 mg/kg) and its anti-proliferative effect was found to be high. The highest amount of hydroxytyrosol was found in Kilis Yaglik (42.14 mg/kg) and the highest amount of tyrosol was found in Izmir Sofralik (43.86 mg/kg). It was shown that there was a significant difference in the responses of polyphenols in Hep40 cells. The direct use of olive oil in Hep40 cells and the comparison with EVOOPs were evaluated for the first time in this study. The evaluation of the anti-cancer effect of EVOOs and EVOOPs was tested by MTT and the IC50 value of Ayvalik EVOO was found to be the lowest at %12.84. In EVOOPs, Izmir Sofralik was the most effective in Hep40 cells with an IC50 value of 35.40 µg/mL.

Key words: Hep40, Hepatocellular carcinoma, Mediterranean diet, Olive oil Polyphenols, Olive oil.

Citation: Kahraman, G., Özkaya, M. T., & Yıldırım Ö. (2023). Potential anti-cancer effects of extra virgin olive oil and its phenolic extracts on hepatocellular carcinoma cells. International Journal of Nature and Life Sciences, 7 (2), 112-122.

Received: November 28, 2023
Accepted: December 08, 2023
Online Published: December 30, 2023

1. Introduction

Extra virgin olive oil (*Olea europaea* L. *var. Türkiye*) (EVOO) is one of the main components of the Mediterranean diet and is obtained directly from the olive fruit by mechanical extraction without any further processing. Its chemical composition consists of major and minor components (Martín-Peláez et al., 2013; Serreli & Deiana, 2020). The major components, including glycerols, represent more than 98% of the total fat weight, while about 2% consists of minor components (aliphatic and triterpene alcohols, antioxidants, etc.). EVOO contains over 230 chemical compounds. Its main antioxidants are carotenes and phenolic compounds (Abid et al., 2023).The polyphenolic composition of olive oil depends on several factors such as geographical region, agro-climatic conditions, fruit ripeness, harvest time, cultivars, storage conditions and oil extraction technology. Genetic variations between cultivars appear to influence the amount of polyphenols present in olive oil. Therefore, polyphenolic compounds show both qualitative and quantitative differences among olive oils (Kouka et al., 2019; Özkaya, 2021). Recent data from various studies (in vivo and in vitro) show that olive oil components act on receptors, signaling kinases and transcription factors associated with cellular stress and inflammation, pathways responsible for cell cycle regulation and play a protective role in cancer development (Markellos et al., 2022).

One of the most important carcinogenic factors is diet, and it is estimated that more than 30% of all cancers are preventable, often simply by modifying dietary intake. Therefore, the observation that populations living in the Mediterranean region have a lower incidence of cancer compared to other regions is an important observation, especially for the identification and characterization of foods and food components that can prevent cancer (Finicelli et al., 2022; Mentella et al., 2019).

Epidemiological evidence and many case-control studies support that the Mediterranean diet reduces the risk of cancer and that, in particular, olive oil consumption is inversely related to cancer prevalence, and this effect is due to the antioxidant, anti-inflammatory and antitumor properties exerted by the phenolic compounds of olive oil (Coccia et al., 2016; Spagnuolo et al., 2022).

Hepatocellular carcinoma (HCC) is the sixth most commonly diagnosed malignancy (5th in men and 7th in women) and the third most lethal malignancy. HCC is the most common histological subtype, accounting for approximately 85-90% of primary liver cancers worldwide, and its incidence has more than tripled in the last 30 years (1.5-0.7/100,000) (Koulouris et al., 2021). The high mortality of HCC is attributed to the lack of effective treatment and chemotherapy

resistance, which often occurs with systemic chemotherapy (Donne & Lujambio, 2022; Soto-Alarcon et al., 2018). Thus, the frequent emergence of chemoresistance, which is one of the main problems in the treatment of HCC patients, creates an active area of research for agents that can be coupled to standard chemotherapy treatment to delay resistance, allowing drug dose and toxicity reduction (Daher et al., 2018; Seydi et al., 2023; Zhao et al., 2022).

These tumor-active compounds that can be coupled to the anti-cancer drugs may provide key compounds that can be used in combination with growth factor inhibitors as a new option to overcome drug resistance and reduce side effects (Hosseini-Zare et al., 2021). Recent studies have clinically evaluated tumor-active compounds such as polyphenols, alkaloids and inorganic compounds in HCC. Preclinical and clinical studies are investigating the effects of these compounds on antiviral, anti-inflammatory, antioxidant, anti-angiogenic and anti-metastatic activities (Newman, 2018). There are many studies investigating the effects of phenolic compounds in EVOO on cancer. In the liver, EVOO has been shown to inhibit inflammation by reducing the production of tumor necrosis factor-α, a pro-inflammatory cytokine, thereby preventing liver damage that leads to HCC (Hatimy & Kadmiri, 2021). In addition, polyphenols suppress HCC cell proliferation and induce HCC cell apoptosis by inhibiting NF-κB activation (George et al., 2021; Vachliotis et al., 2023).

Since anti-tumor compounds found in food target more than one pathway, their addition to treatment in combination with anti-cancer drugs allows different mechanisms to reduce the development of anti-cancer drug resistance. In recent years, the addition of compounds to anti-cancer drugs has been studied in many areas to overcome the altered regulatory cell pathways that may be responsible for the drug resistance mechanism and to further minimize the associated side effects (Rahaiee et al., 2020). Thus, there has been growing interest in the collective effects of chemotherapeutics in combination with EVOOPs. Numerous studies indicate that EVOOPs can reduce toxic effects or anti-cancer effects in various cancer cells when used in combination with EVOOPs (Torić et al., 2019). The biological activity of EVOOPs, especially hydroxytyrosol, tyrosol and their derivatives, oleuropein, oleacein and oleocanthal, has been extensively studied on various cancer cell types. Their anti-cancer properties have been attributed to the phenolic compounds found in olive oil (Moral & Escrich, 2022; Serra et al., 2022).

There are some studies investigating the effects of EVOOPs on HCC. However, there is no comprehensive study in the literature describing the full effects of the use of EVOO in the liver on in vitro models. To fill this gap, the current study examines EVOO applied in pure form without any cell treatment compared with the effects of EVOOPs. To investigate how these two conditions (EVOO and EVOOPs) inhibit cell proliferation in liver cells, EVOO obtained from three olive varieties grown in different regions of Türkiye were used for these experiments against EVOOPs obtained by methanolic extraction method. We have shown that regional and genetic differences of EVOO change both qualitative and quantitative properties and this in turn has different effects on liver cancer cells.

2. Materials and Methods

2.1. Chemicals and reagents

All chemicals used were from commercial sources and were purchased as follows: Trypsin - EDTA 0.25% trypsin (Serana, Germany) and dimethyl sulfoxide (DMSO) from OriGen (CryoPurtm, Malta), -(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) from VWR Ambresco (CAS No: 298-93-1), Sorafenib from Sigma-Aldrich (Cas No: 2984461-73-1, China), penicillin/streptomycin from Multicell (Cat No: 450-201-EL), L-glutamine from Sigma Aldrich (Lot: RNBF4742, UK), DMEM low glucose from EuroColone (Cat No: ECM0066L, Pero, Italy), MEM non-essential from Biowest (ID No: X0557-100), methanol and all standards from Sigma Aldrich. Extra virgin olive oil (EVOO) samples obtained from olives of different varieties in Türkiye (*Olea europaea* L. *var. Türkiye*). These three samples were taken from different lots of an endemic mono culture; Ayvalik (*Olea europaea* L. *Cvs. Ayvalik*) (Çanakkale, Türkiye), *Kilis yaglik* (*Olea europaea* L. *Cvs. Kilis yaglik*) (Gaziantep, Türkiye) and *Izmir sofralik* (*Olea europaea* L. *Cvs Izmir sofralik*) (Izmir, Türkiye) were obtained from Buta Assos, Masmana and Hedef Ziraat companies respectively. EVOO samples were stored in amber glass bottles at +18 °C in the dark until analysis.

2.2. Phenolic compound extraction from EVOO

The selected EVOOs were subjected to extraction in order to obtain the polyphenolic extracts. Specifically, liquid-liquid extraction was carried out according to the method proposed by Emanuele Boselli, 2009 (Boselli et al., 2009), with some modifications and automation of the procedure. Exactly 40 g of EVVO were weighed and extracted from each oil with 80 mL of MeOH:H2O (CH3CN/H2O 80:20 v/v). The methanolic samples were vortexed for three minutes and then centrifuged at 5000g for 25 minutes at +4°C. After removing the methanolic phase from the sample, the experiment was repeated with the remaining oil. After collecting the methanolic phase, the experiment was repeated with 80 mL MeOH:H2O 90:10 (v/v) methanol. The methanolic phases were combined and filtered with a 0.45 nm polyvinylidene fluoride (PVDF) filter. The methanolic phase was dried to dryness under reduced pressure (approximately 0.25 atm) using a Rotavapor (Buchi labortechnik, Switzerland). The extract was dissolved in DMSO to a stock concentration of 50 mg/ml. Stock concentrations were divided into 500 µL and stored at -20 °C until used in experiments.

2.3. Determination of total phenolic compounds

Total phenolic compounds were determined by the Folin-Ciocalten method (Rizvi et al., 2023). Briefly, into a 3 mL plastic cuvette were added successively: 1350 µL distilled water, 25 µL FC reagent diluted 1:2 with water, 25 µL oil extract and, after 3 min, 50 µL saturated sodium carbonate solution. After 90 min incubation in the dark, the absorbance was measured at 765 nm. Total phenolics were quantified using a calibration curve (R2 > 0.99) established with gallic acid (≥99% purity). The results were expressed as mg gallic acid equivalent (GAE) per kg oil (mg GAE kg -1 oil).

2.4. Sterol analysis

The sterol composition was determined according to the method established by the International Olive Council (COI/T.20/Doc. No.26/Rev. 5/2020). Identification and quantification of the sterols were carried out by gas chromatography

(GC) using a capillary column (30 m x 0.25 mm id x 0.25 mm film) and a flame ionization detector (FID). Injector, column and detector temperatures were set at 280, 260 and 290 °C respectively. Quantification was performed by addition of an internal pattern (α-cholestanol) and apparent β-sitosterol was calculated as the sum of β-sitosterol, Δ5,23-stigmastadienol, clerosterol, sitostanol and Δ5,24-stigmastadienol.

2.5. Fatty acid composition

The fatty acid composition was determined according to the International Olive Oil Council (COI/T.20/DOC. 28/Rev. 2 - 2017) (IOC, 2017) Methyl esters were prepared by vigorous shaking of an oil solution in n-heptane (0.1 g in 2 mL) with 0.2 mL of 2-methanolic potassium hydroxide and analyzed on an Agilent GC system (Agilent 6850, USA) equipped with a FID and a capillary column DB-23 of 60 m length × 0.25 mm inner diameter and 0.25 µm layer thickness. The carrier gas was helium at a rate of 1.0 mL/min. Injector, oven and detector temperatures were 250, 230 and 280 °C respectively. Results were expressed as relative area percentage of the total. The injection volume was 1 µL. Fatty acids were identified by comparing their retention times with those of reference compounds.

2.6. Cell culture

The human hepatocellular carcinoma cell line (Hep40) was maintained in Dulbecco's modified Eagle's medium (DMEM low glucose) (Gibco, Paisley, UK) containing 10% (v/v) fetal bovine serum (FBS) (Gibco, Paisley, UK), 1% (v/v) L-glutamine (Pero, Italy), 1% (v/v) MEM non-essential amino acids (Biowest, UK), 100 IU/mL penicillin and 100 µg/mL streptomycin (Multicell), at 37 °C a humidified atmosphere and 5% CO2.

2.7. Cell treatment with EVOO

Cells were seeded in 96-well plates at 1.5×104 cells in 100 µL per well in DMEM low glucose culture medium with FBS and cultured at 37°C in a humidified atmosphere and 5% CO2 for 24 hours. Each olive oil sample was filtered at 0.22 nm prior to cell culture. The medium was then replaced with DMEM containing prepared olive oil dissolved in fresh culture medium at 0%, 1%, 2.5%, 5%, 10%, 15% and 20% (v/v). They were incubated for 24 and 48 hours at 37 °C in a humid atmosphere and 5% CO2. Control cells were left untreated. All experiments were performed under the same conditions and each concentration was replicated in triplicate wells and all experiments were performed in triplicate.

2.8. Cell treatment with EVOOP extract

Cells were seeded in 96-well plates at $1.5x10^4$ cells per well in 100 µl DMEM low glucose medium with FBS and cultured at 37°C in a humidified atmosphere and 5% CO2 for 24 hours. The medium was then replaced with fresh medium. Extract was added at concentrations of 0 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL and 50 µg/mL. They were incubated for 24 and 48 hours at 37 °C in a humid atmosphere and 5% CO2. Control cells were treated with 0.1% DMSO. All experiments were performed under the same conditions and each concentration was replicated in triplicate wells and all experiments were performed in triplicate.

2.9. MTT assay

Cell viability was assessed using the MTT assay kit (Cas No: 298-93-1, Ambresco). Briefly, $1.5x10^4$ Hep40 cells per well were cultured in DMEM in a 96-well plate. After 24 hours of incubation, a series of EVOO and EVOOP concentrations diluted in serum-free DMEM were administered for 24 and 48 hours. Then 10 µL of MTT reagent (5 mg/mL) was added to each well. After 4 hours of incubation, the absorbance was monitored at 570 nm using a BioTek ELx800 microplate reader. Data were calculated as the percentage of viability using the following equation: viability % = $A_{control}$-$A_{sample}/A_{control}$x100 (A: absorbance) Experiments were performed in triplicate and also at 3 different independent times.

2.10. Statistical analysis

Each experiment was repeated at least three times and statistical significance was determined using GraphPad Prism8.4.3 and data are expressed as mean ± standart deviation (SD). Two-way ANOVA test was used to compare groups, and one-way ANOVA of variance was used to compare fold changes between EVOOs or EVOOPs. Microsoft Excel was used for IC50 calculations. In the tests, the means in columns with * (p<0.05), ** (p<0.01), *** (p<0.001) and **** (p<0.0001) were considered statistically significant. Values above these are reported as not statistically significant.

3. Results

In this study, olive oils from three EVOOs grown in Türkiyewere characterized and their anti-proliferative activities were measured in Hep40 to evaluate their anti-cancer activity. These EVOOs were first treated directly with cells and then phenolic compounds extracted by methanolic extraction method were subjected to MTT evaluation for 24 and 48 h anti-proliferative activity in Hep40 cell line.

3.1. Efficiency of the extraction of three different types of EVOO

Each olive oil was weighed exactly 40 g and extracted with 80:20 (v/v) methanol/water under the same conditions, and the amounts of dry extract weighed at the end of the extraction are given in Table 1. During the extraction of extra virgin olive oil, the dry extract yield was found to be lowest for Ayvalik EVOO and highest for Kilis Yaglik EVOO.

Table 1. Efficiency of three different types of EVOO.

Varieties of Olive Oil	Oil (g)	MeOH:H2O (80:20 v/v) (mL)	Dry Extract (mg)
Ayvalik EVOO	40	80	95,7
İzmir Soralik EVOO	40	80	126,1
Kilis Yaglik EVOO	40	80	137,5

3.2. Characterization of the compounds present in three different types of EVOO

The compositions of biophenolic compounds, fatty acid composition and sterol content of three EVOO samples obtained from three different olive cultivars (Ayvalik, Kilis Yaglik and Izmir Sofralik) are shown in Table 2. Regarding EVOOs, total biophenol was the highest in Kilis Yaglik olive oil, reaching 655.4 mg/kg, 3.21 and 1.28 times higher than in Ayvalik EVOO and Izmir Sofralik EVOO, respectively. Furthermore, cholesterol (0.28%) and arachidic acid (3.01%) were the highest in Kilis Yaglik EVOO. Among the varieties, the amount of each total sterol varied greatly. Of all the EVOOs, Kilis Yaglik EVOO had the highest phenolic content (71.66 mg GAE/100g).

Table 2. Olive oil composition of three different EVOO in Türkiye.

		Ayvalik EVOO	Kilis Yaglik EVOO	Izmir Sofralik EVOO	
Analysis	**Unite**	**Result**	**Result**	**Result**	**Limit Value**
Sterol Composition					
Brassicasterol	%	Not detected	Not detected	Not detected	≤0,1
Delta-7-Stigmastenol	%	0,46	0,46	0,48	≤0,5
Erythrodiol and Uvaol (In Total Sterols)	%	2,22	0,55	1,36	≤4,5
Campesterol	%	2,75	2,95	2,8	≤4
Cholesterol	%	Not detected	0,28	Not Detected	≤0,5
Stigmasterol	%	0,93	0,6	0,81	<Campestero
Total Beta-Sitosterol	%	93,11	93,65	94,41	> 93
Total Sterol	mg/kg	1689,8	156135,8	31891	> 1000
Faty Acids Composition					
Arachidic Acid (C20:0)	%	0,44	0,51	0,41	≤0,6
Behenic Acid (C22:0)	%	0,14	0,14	0,12	≤0,2
Gadoleic Acid (C20:1)	%	0,33	0,29	0,33	≤0,5
Heptadesenoic Acid (C17:1)	%	0,27	0,16	0,12	≤0,6
Lignoceric Acid (C24:0)	%	0,07	0,1	0,08	≤0,2
Linoleic Acid (C18:2)	%	11,22	5,94	7,92	2,5-21
Linolenic Acid (C18:3n3)	%	0,73	0,72	0,82	≤1
Margaric Acid (C17:0)	%	0,18	0,1	0,07	≤0,4
Myristic Acid (C14:0)	%	Not Detected	Not Detected	Not Detected	≤0,03
Oleic Acid (C18:1)	%	68,88	72,03	72,3	55-83
Palmitic Acid (C16:0)	%	14,33	16,1	14,45	7,5≤-≤20
Palmitoleic Acid (C16:1)	%	0,82	1,06	1,1	0,3≤-≤3,5
Stearic Acid (C18:0)	%	2,6	2,83	2,29	0,5-5

Biophenolic Compounds	mg/kg	197,86	655,4	508,75
Phenol	mg GAE/100g	46,93	71,66	62,89

3.3. The direct application of different EVOOs to the Hep40 cell line leads to inhibition of cell proliferation

Cell viability was evaluated through deployment of the MTT assay. There was a primary focus on evaluating the impacts of three distinct extra virgin olive oils (Ayvalik, Kilis Yaglik and Izmir Sofralik). Cells were subjected to treatments with EVOOs at varying concentrations (0% to 20%) over a 24- and 48-hour period, as illustrated in Figure 1, following 22 nm filtration. It is noteworthy that in cells administered varying doses of EVOOs, inhibitions of cell viability were more significantly pronounced (****p<0.0001) at 20% and beyond, in comparison to each other over the entire concentration range. Our findings demonstrate that the impact of Ayvalik EVOO on cell proliferation inhibition was notably greater (Figure 2).

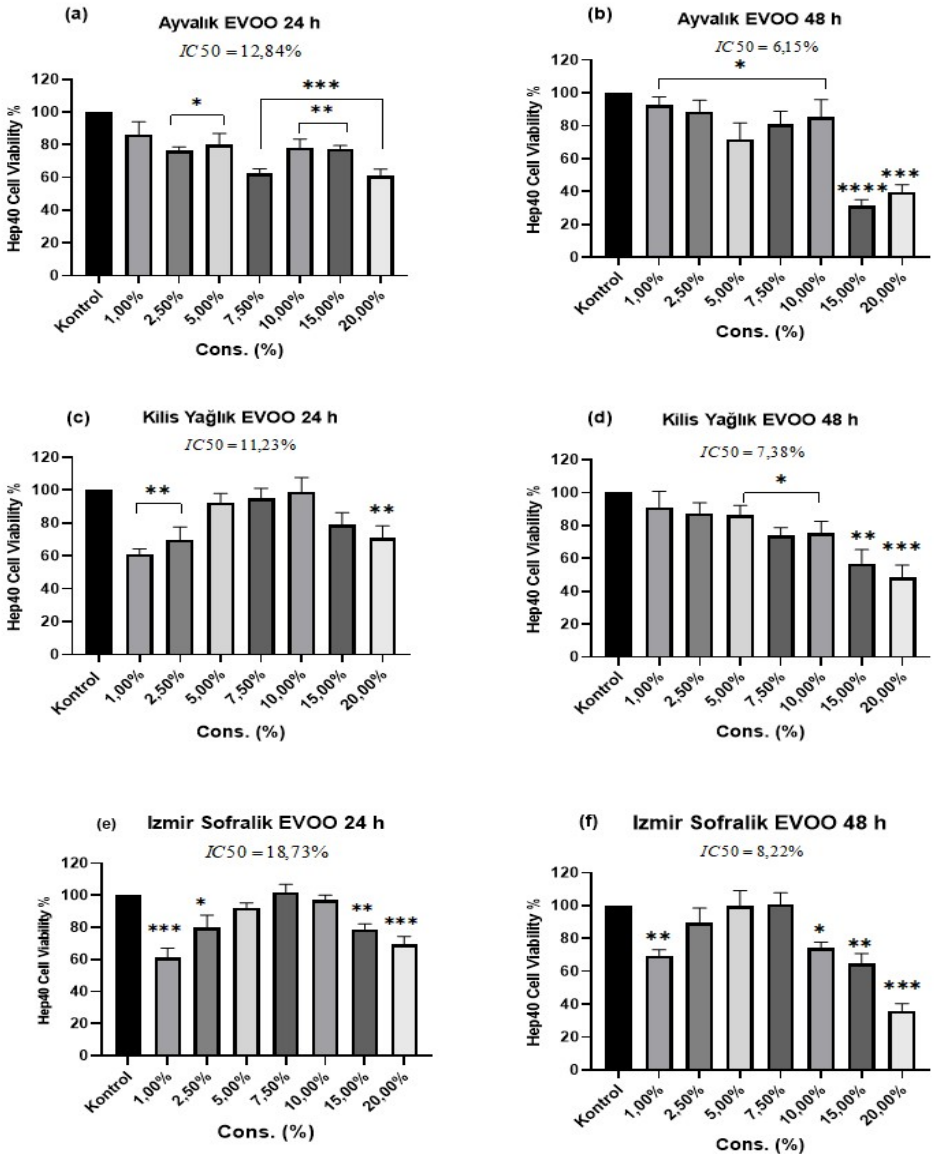


Figure 1. Effect of the direct application of extra virgin olive oils (EVOOs) on the viability of Hep40 cells.

The viability of Hep40 cells: a growth curve and time analysis in the Hep40 cell line. Hep40 cells were incubated with EVOO at different dose concentrations for 24 and 48 hours: using Ayvalik 24 and 48 hours (a and b), Kilis Yaglik 24 and 48 hours (c and d), and Izmir Sofralik 24 and 48 hours (e and f). During the initial 24 and 48-hour period of testing, we measured the IC50 value of various brands of EVOO from three different regions, namely Ayvalik (a and b), Kilis Yaglik

(c and d), and Izmir Sofralik (e and f). The results from three experiments demonstrate that a:12.84±0.92%, b: 6.15±0.78%, c: 11.23±1.0%, d: 7.38±0.65%, e: 18.75±0.95%, and f: 8.22±0.71%. Additionally, the values for a ($p<0.001$), b ($p<0.005$), c ($p<0.001$), d ($p<0.001$), e ($p<0.005$), and f ($p<0.005$) showed considerable variation from the control. All means were accompanied by ±SD values.

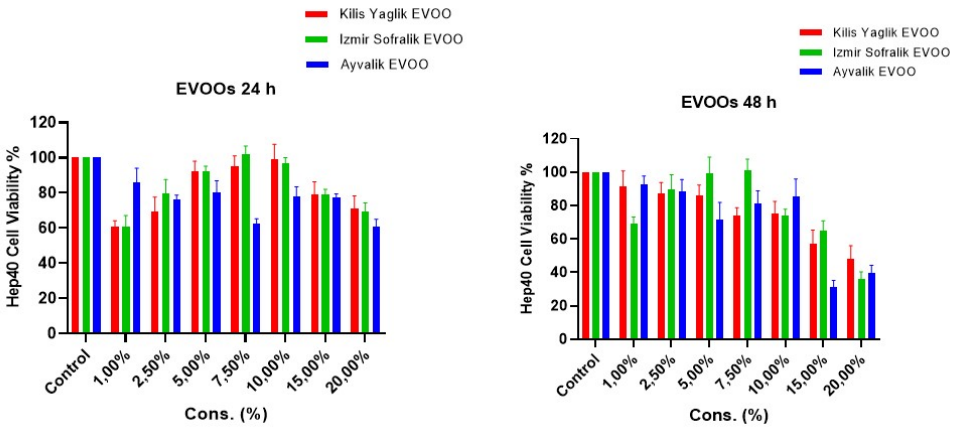


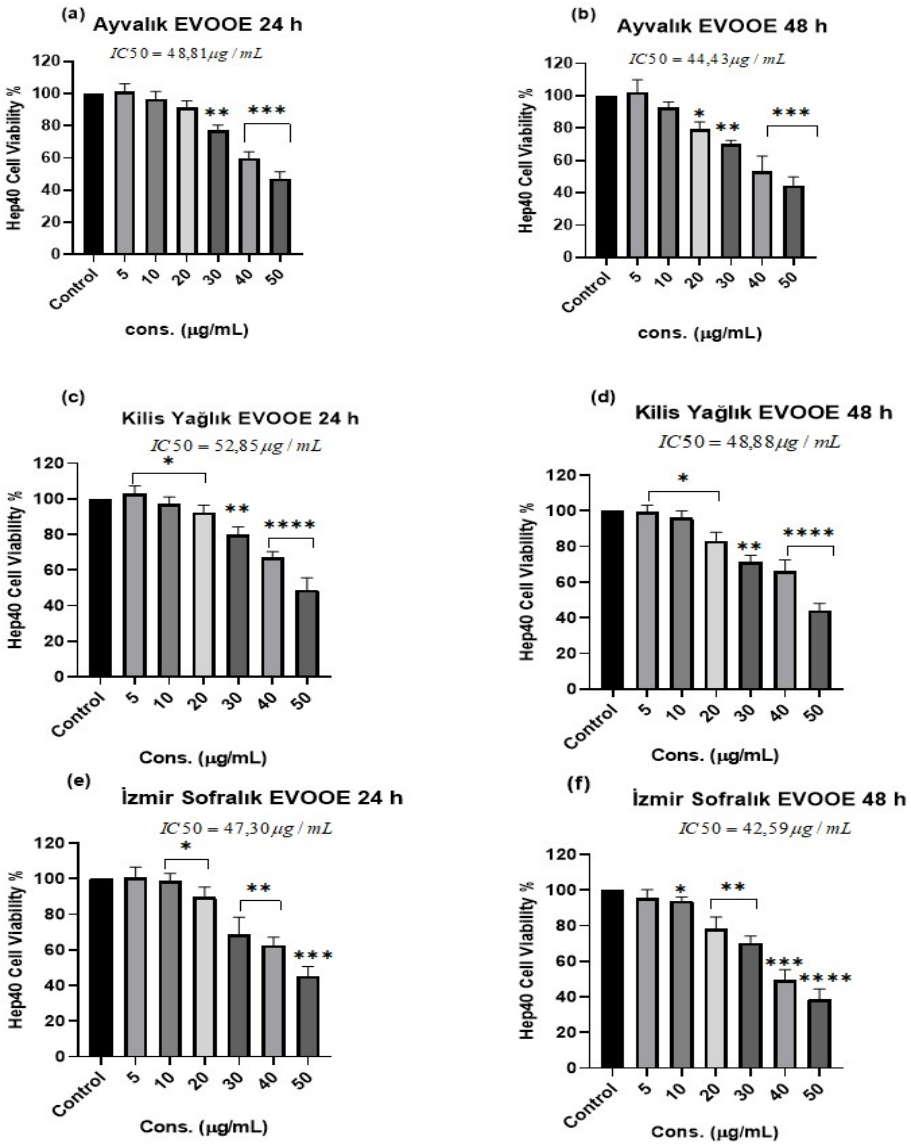
Figure 2. Comparison of Hep40 cell viability among different EVOOs.

Although the dose had a reduced effect on Hep40 cell viability at 24 and 48 hours, it was observed that Ayvalik EVOO was more effective than the other two EVOOs. The difference between the EVOOs was determined to be significant with a p-value of (a): $p>0.05$ ($p:0.9978$) considered insignificant and (b): $p<0.05$ ($p:0.0370$) being significant. These results are representative of three experiments and the data are presented with a ± SD.

Several epidemiological studies have indicated that certain components of olive oil may have a significant impact on cancer prevention (Yang et al., 2020). This study analysed tests performed on EVOO samples with an enriched cell medium at different concentrations. The minor water-soluble components of olive oil include many compounds that have been the focus of cancer research and have been positively identified. Our current investigation uncovers the fact that unextracted portions of olive oil, for instance sterols and fatty acids, possess an inclination to diminish cell viability in cancer cells. It is known that extra virgin olive oils contain a variety of phenolic compounds, mainly derived from oleuropein and ligstroside. The absence of glucose prompts the elenolic ring moiety to open, thereby creating fresh phenolic structures in various tautomeric forms, comprising mono- and dialdehyde variants. These phenolic compounds give rise to oleacin and oleocanthal, as stated by Xie et al (2021). In vitro studies have revealed the purified form of oleocanthal to be lethal. However, Goren et al. (2019) found that olive oils containing an average oleocanthal content lead to a decrease in cell viability, whereas olive oils without oleocanthal have no effect on cell viability. The tested EVOO samples exhibited varying degrees of inhibition on the proliferation of Hep40 cells due to differences in their oil composition to compound ratios. Over time, the viability of Hep40 cells decreased at higher concentrations of olive oil (20% and 15% v/v). Technical term abbreviations such as "v/v" were explained when first used. This may have been caused by the release of water-soluble phenolic compounds into the environment, without the need for another solvent. The use of olive oil at a lower concentration (1-10% v/v) may have initiated the inhibition of cell proliferation through some water-soluble phenolic compounds. However, this effect was less pronounced than the cell division stage, leading to enhanced cell viability within 48 hours. To enhance clarity, we assessed the anti-proliferative potential of each type of olive oil under identical conditions in the Hep40 cell line.

3.4. Effect of phenolic extract on HCC cell viability

Hep40 cells were exposed to varying doses of EVOOPs, ranging from 0 to 50 µg/mL, over a period of 24 and 48 hours. The findings indicate that the phenolic extract reduced cell count in a dose- and time-dependent fashion (Figure 3). Notably, among the EVOOPs, the Izmir Sofralik EVOO phenolic extract resulted in a marked decrease in cell number. While some phenolic extracts from olive oil have shown substantial reduction in cell growth as compared to Izmir Sofralik, it was observed that the Izmir Sofralik EVOO phenolic extract had significant effects within just 24 hours of application. Furthermore, after 48 hours, it led to extensive cell death (Figure 4).



EVOOE: Extra Virgin Olive Oil Extract

Figure 3. Inhibition of Hep40 cell proliferation by phenolic extracts of EVOO.

Hep40 cells were treated with three different extracts of phenolic compounds from EVOO at varying concentrations (0-50 µg/mL) for 24 and 48 hours. The IC50 values of these extracts were measured at 24 and 48 hours. Ayvalik extract had IC50 values of 48.81±1.93 µg/mL (a) and 44.43±2.35 µg/mL (b); Kilis Yaglik extract had IC50 values of 55.85±2.68 µg/mL (c) and 48.88±1.98 µg/mL (d); Izmir Sofralik extract had IC50 values of 47.30±3.01 µg/mL (e) and 42.59±2.78 µg/mL (f). The findings present the outcomes of three experiments with Mean ± SD values provided for the data. Notably, the results of a: ($p<0.005$), b: ($p<0.005$), c: ($p<0.0001$), d: ($p<0.005$), e: ($p<0.0001$), and f: ($p<0.0001$) were all significantly different from the control.

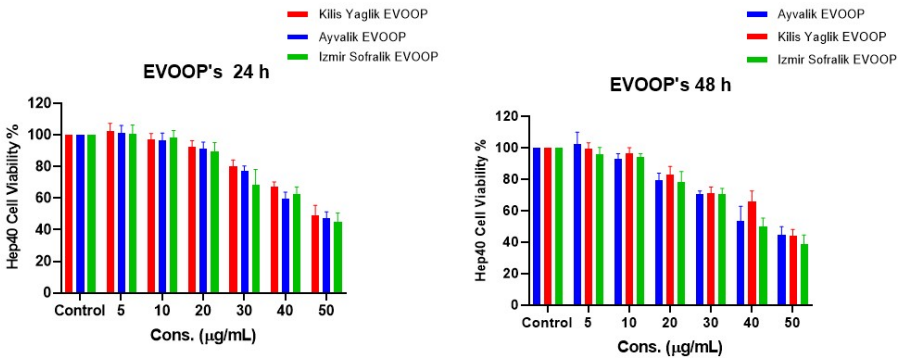


Figure 4. Comparison of the effects of EVOOPs on Hep40 cell viability.

The Hep40 cell line was exposed to different doses of EVOOPs (0-50 µg/ml), followed by measurement of cell viability at 24 and 48 hours post-treatment (a and b, respectively). The results are presented as mean ± standard deviation. Statistical analysis revealed significant variations between the treatment groups and the control ($p<0.005$).

All extracts showed a dose-dependent cytotoxic response on Hep40 cells. The various chemical compositions of each extract can exert a significant impact on the sample's toxicity. Izmir Sofralik EVOOP had the most considerable impact on Hep40 cell viability, being the second richest regarding phenol content. Izmir Sofralik EVOOP significantly decreased cell viability in Hep40 cells, whereas Ayvalik EVOOP and Kilis Yaglik EVOOP exhibited a tendency to reduce cell viability after 48 hours of treatment.

4. Discussion

The study presents a comprehensive profile of different olive oils, fatty acids, sterols, and phenolics from three selected Türkiye cultivars, based on their regional distribution. Additionally, the study evaluates the response of extra-virgin olive oils (EVOOs) and extra-virgin olive oil phenols (EVOOPs) to cytotoxicity in Hep40 cells to determine their effect on liver cancer. Various studies link the Mediterranean diet to lower incidences of different cancer types (Toledo et al., 2015; Turati et al., 2014). Chemotherapy is widely considered as the primary approach for cancer treatment despite its high cytotoxicity rates and severe side effects. However, combining anti-cancer drugs with EVOOPs may present a more effective and safer therapeutic strategy due to the synergistic effects of EVOOPs found in olive oil. These findings suggest that a combination of EVOOPs and anti-cancer drugs may lead to improved chemotherapeutic efficacy and reduced toxicity (Bakrim et al., 2022; Bouyahya et al., 2022). The heightened attention towards the biological properties of polyphenols stems from the escalating occurrences of numerous ailments, particularly cancer, and the urgency to identify secure and efficient treatment approaches (Motamedi et al., 2022; Panigrahy et al., 2022; Yadav & Ahmaruzzaman, 2023). Multiple in vitro and in vivo investigations divulged that EVOOPs have great potential as chemopreventive and anti-cancer agents (Hashim et al., 2014; Kapoor et al.) and endorse the remedial impacts of anti-cancer medications (Antoniou & Hull, 2021). The findings of this study indicate that EVOOs and EVOOPs can impede cell proliferation on liver cancer cells. Specifically, the Ayvalik variety of EVOOs and the Izmir Sofralik type of EVOOPs demonstrated anti-proliferative effects on the Hep40 cell line. Furthermore, the investigation of cytotoxicity on the same cell line established that both EVOO and its extracts exhibited a relationship that is dependent on the dosage. The qualities of olive oils are remarkably affected by factors such as geography, climate, and the characteristics and variety of the fruit (Özkaya, 2021; Rodríguez-López et al., 2020). Numerous studies indicate that olive oil extracts exhibit potency in altering cancer cells (Monti et al., 2001; Negro et al., 2019). Nonetheless, this research aims to examine the conduct of olive oil and its extracts upon exposure to liver cancer cells. Our investigation has found no data regarding the direct effects of olive oil on liver cancer cell lines.

Liver cancer ranks fifth as the most common neoplasm and is the third most frequent cause of death globally (Choi et al., 2023; Huang et al., 2022; Rumgay et al., 2022; WHO, 2022). Chemotherapy resistance is a common development in HCC due to the tumor's heterogeneous nature (Fang et al., 2022; Tang et al., 2020). Many studies exhibit the promise of tumor-active compounds having distinctive cell-killing mechanisms to combat drug resistance and decrease side effects (Emma et al., 2021; Jain et al., 2021). In the study, it was demonstrated that the olive oil compounds possess the capability to restrain the growth of Hep40 cells. An MTT assay was conducted to examine the impact of EVOO on cancer cells, while HPLC and GC were employed for the characterization of EVOO compounds.

The phenolic fraction of EVOO appears to be varied and the phenolic compounds display diverse structural differences. The heterogeneity of extra virgin olive oil (EVOO) is influenced by several factors, such as the type of olive fruit, the location of cultivation, agricultural practices, the level of maturity during harvest, and the techniques used in olive oil extraction, processing, and storage. The phenolic concentrations in EVOOs may also be affected by the duration elapsed since the harvest (Özkaya, 2021). From the three distinct regions of Turkey, olive oil samples were collected, and among them, Ayvalik variety had the lowest phenol content, which was 42.59 mg GAE/100g, whereas the other varieties had more phenol contents, for Kilis Yaglik (71.66 mg GAE/100g) and Izmir Sofralik (62.89 mg GAE/100g). Nonetheless, Ayvalik variety demonstrated the greatest effectiveness while directly implementing EVOO on Hep40 cells.

A systematic review and meta-analysis investigated the link between olive oil consumption, cancer risk, and prognosis, examining 45 studies (17,369 cases and 28,294 controls) and 8 cohorts (with 12,461 event cases out of a total

of 929,771 cohorts) conducted from 1990 to 2022, where the highest olive oil consumption was 31%. (RR = 0.69, 95%CI: 0.62-0.77), breast (RR = 0.67, 95%CI: 0.52-0.86), gastrointestinal (RR = 0.77, 95%CI: 0.66-0.6), upper respiratory tract (RR = 0.74, 95% CI: 0.60-0.91) and urinary tract cancers (RR = 0.46, 95%CI: 0.29-0.72). A meta-analysis of 35 observational studies, including Mediterranean and non-Mediterranean participants, utilizing both multivariate and univariate analyses and comprising approximately 100,000 people from all subgroups, revealed a negative correlation between olive oil consumption and cancer prevalence (Markellos et al., 2022). Another randomized study showed that women who followed a Mediterranean diet with enriched olive oil had a 62% lower incidence of invasive breast cancer when compared to a control group who were advised to limit their dietary fat intake(Toledo et al., 2015). The research was carried out in Spain for a period of 6 years, with a total of over 4000 participants. The study design was a 1:1:1 randomized, single-blind, controlled field trial. Numerous in vitro cancer model studies have demonstrated that the phenolic compounds present in olive oil possess anti-cancer properties.

This study suspended all oil components with the cell medium to investigate the relationship between olive oil and cell growth. The results show that olive oil-enriched culture medium specifically inhibited cancer cell growth. IC50 values for Ayvalik were 12.84% and 6.15% at 24 and 48 hours respectively, for Kilis Yaglik were 11.23% and 7.38%, and for Izmir Sofralik were 28.73% and 8.22%. Interestingly, the inhibitory effect of olive oil on cancer cell proliferation was significantly higher at concentrations of 20% and 15%. Moreover, all olive oils demonstrated an inhibitory effect on cell proliferation following 48-hour treatments compared to 24-hour treatments. As no research has investigated the direct use of oil at high concentrations in the in vitro cancer model, it is believed that this outcome is caused by the presence of oil droplets covering the surface of the wells, which prevents the cells from obtaining the necessary oxygen for growth. Elevated suspended doses (ranging from 1% to 10%) impeded cell proliferation in the initial 24 hours of treatment, but after 48 hours, the cells started to regrow and proliferate. Olive oil potentially induced this effect by transferring some of its water-soluble phenolic compounds to the medium sans the requirement of additional solvents. It may have exhibited a hindering effect on cell growth within 24 hours, but ultimately increased cell viability within 48 hours. Notably, the water-soluble minor components took longer to take effect compared to the cancer cell's growth curve. No direct application of oils to cells has been studied, but these essential oils have been researched for their efficacy against cancer, specifically in breast and melanoma cancers, with positive results (Jovtchev et al., 2018; Kulig et al., 2022). However, there is currently no research available on the anti-proliferative effect of olive oil with regards to liver cancer cells. The differing abilities of the olive oils investigated in our study to suppress the proliferation of Hep40 cells may be attributed to the heterogeneity of the oil. The differing abilities of the olive oils investigated in our study to suppress the proliferation of Hep40 cells may be attributed to the heterogeneity of the oil. Furthermore, this disparity could potentially stem from the combined interaction between the individual components of the oil. To comprehensively ascertain the cultivar distinctions, qualitative and quantitative analyses such as Q-TOF and library screening are necessary.

The findings of our MTT experiments, investigating the cytotoxicity of EVOOPs against HCC, are detailed in the results section. We applied extracts from three different olive oils, obtained by the methanolic extraction method, within a concentration range of 0 to 50 µg/mL. Our aim was to assess the impact of these extracts on Hep40 cells, following 24 and 48 hour applications. After incubating for 24 and 48 hours, the IC50 values for Ayvalik, Kilis Yaglik, and Izmir Sofralik were found to be 48.81 µg/mL, 52.85 µg/mL and 47.30 µg/mL respectively. Notably, the EVOOPs derived from Izmir Sofralik, the phenolic compound-ranked second, proved to be more effective on Hep40 cells.

5. Conclusions

The study findings indicate that the impact on cell viability is not related to the total phenolic compounds' quantity. The Hep40 cells were subjected to various olive oils and their extracts. Amongst all the oils extracted through methanol (Table 1), it was observed that the Izmir Sofralik EVOOP variety performed the best in inhibiting Hep40 cell growth while obtaining the highest amount of dry weight. Technical terms were explained when used for the first time. The anti-proliferative effects were independent of the total phenolics amount in both instances. The rationale for researching functional foods in the context of complementary therapies by cancer patients is that cancer causes substantial emotional, psychological, and physical distress. However, data on the therapeutic benefits of some natural foods are scant, and they may have consequential side effects and interactions with anti-cancer drugs. The addition of complementary therapies to standard chemotherapy may have adverse effects. To summarize, examining the use of tumor-active compounds in cancer treatment enables the identification of bioactive compounds that can enhance the effectiveness of anti-cancer medication while also regulating the cancer patients diet.

Conflicts of Interests

Authors declare that there is no conflict of interests

Financial Disclosure

This research received financial support from the Coordination of Scientific Research Projects at Ankara University (Project number: 21L0430007).

Statement contribution of the authors

This study's experimentation, analysis and writing, etc. all steps were made by the authors.

Acknowledgements: We express our gratitude to Ankara University Scientific Research Projects Coordination Office for their support. Additionally, we acknowledge with gratitude the contributions of Hedef Ziraat (Izmir Sofralik), Buta Assos (Ayvalik), and Masmana (Kilis Yaglik), the companies from Türkiye that supplied olive oil for this study.

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