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Determination of bioactive compounds and biological properties of olive fruit extract *(Olea europaea* L.)

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Olive (*Olea europaea* L.) fruit is an important plant that has been traditionally used for both nutritional and therapeutic purposes. However, the complete chemical profile of bioactive compounds found in olive fruit and their biological effects have not yet been fully elucidated. Therefore, detailed examination of the phenolic content and biological activities of olive fruit extract is of great importance for the discovery and use of naturally derived functional compounds. Polyphenols are known for their ability to mitigate the adverse effects of reactive oxygen species (ROS). This study aimed to determine the phenolic acid content of *Olea europaea* L. extract and investigate its biological properties, including antimicrobial, anticancer, and enzyme inhibition activities. Additionally, target gene prediction analyses for key phenolic compounds were conducted. The phenolic content was analyzed via liquid chromatography-mass spectrometry (LC-MS) which revealed high levels of oleuropein, protocatechuic acid, luteolin, and vanillic acid. Common target gene predictions for these phenolic acids were performed via the sRplot database. Enzyme inhibition and anticancer activity studies were carried out on MCF-7 and HCT-116 cells using the *Olea europaea* extract. The results showed that the *Olea europaea* extract at a concentration of 75 µM/mL effectively inhibited the hCAI enzyme (IC50: 63.12±11.37 µM/mL; Ki: 39.18±4.02 µM/mL) and significantly inhibited the hCAII enzyme (IC50: 39.45±7.02 µM/mL; Ki: 45.33±5.11 µM/mL). Furthermore, the extract significantly inhibited acetylcholinesterase (AChE) (IC50: 52.30±8.21 µM/mL; Ki: 34.50±3.15 µM/mL). Notably, the extract had significant cytotoxic effects on the MCF-7 (78.19±4.85 µg/mL) and HCT-116 (80.19±7.03 µg/mL) cell lines. In conclusion, the *Olea europaea* extract is rich in phenolic acids, indicating strong antioxidant, antiangiogenic, anticarcinogenic, and anti-inflammatory properties. These findings can contribute to further investigations in new drug design.

Keywords: Acetylcholinesterase, Carbonic anhydrase, Anticancer activity, Antimicrobial activity, Phenolics

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Olea europaea (*O. europaea*) is in the Oleaceae family. *O. europaea* is an economically valuable plant widely used in traditional medicine and in ethnobotany worldwide, including Türkiye. The olive tree has important biological properties and is rich in phenolic substances. The main phenolic compound is oleuropein[1,2]. The structure of this compound was first discovered by Bourquelot and Vintilesco in 1908 and could only be identified in 1960[3]. Oleuropein is reported to be the heterosidic ester of elenolic acid and hydroxytyrosol[4]. The olive (*O. europaea L.*) is a small evergreen tree that grows slowly and can live for more than 1,000 years. It has been cultivated in the Mediterranean basin for thousands of years, domesticating its wild form, the spindle. This species is a typical tree of Mediterranean vegetation, well adapted to drought and poor soils and resistant to salinity. Although mainly distributed along the coasts, today its cultivation is found in all Mediterranean climatic zones of the world. This species is one of the most important trees for the agricultural economy of the Mediterranean region, with more than 70% of the world olive oil production[5].

The pharmacological effects of phenolic compounds found in olives include antioxidant, antidiabetic, antimicrobial, anti-inflammatory, antihypertensive, and anticarcinogenic effects[6,7]. Oxidative stress is an imbalance between increased levels of reactive oxygen species (ROS) and reduced activity of antioxidant mechanisms. Increased oxidative stress can damage the cellular structure and potentially destroy tissues. On the other hand, ROS are required for adequate cell function, including energy production by mitochondria. The paradox is that the disruption of oxidative stress and antioxidant balance is the main cause of tissue damage. Increased oxidative stress can lead to various pathological conditions, such as aging and cancer, neurodegenerative diseases, cardiovascular diseases, diabetes, inflammatory diseases, and intoxications[8-10].

Gene network analysis describes biological interactions between genes. These findings provide a systematic understanding of cellular signaling and regulatory processes. It shows how a set of genes interact with each other to form a functional module and how different gene modules are related[11].

O. europaea has been included important part of medicinal history and used in folk medicine to treat

various diseases. The Mediterranean diet rich in olive products is evidence of the positive effects of *O. europaea* on health and is associated with reduced cases of cancer and cardiovascular disease. This study aims to contribute to the literature on the therapeutic potential of *O. europaea* products for cancer and various global diseases by revealing human carbonic anhydrase I and II (hCA I and II) enzyme inhibition and cytotoxic effects on MCF-7 and HCT-116 cancer cells.

Materials and Methods

Preparation of the samples

The olives (halhali-type- *O. europaea* L) used in the experiments were collected from olive trees growing in Kahramanmaras city province. The fruits were pitted, washed with distilled water, laid on blotting paper and dried overnight at room temperature (25 °C). The dried pits were crushed in a mortar and pestle into a slurry with very small particles. During this process, the small amount of gelatinous substance present in the crushed olive pits, together with the crushed woody matter, causes the formation of a slurry-like structure. On the other hand, the fruits were first pounded in a mortar and pestle and then slurried with a blade homogenizer through tip 3, thus preparing all three olive components for Soxhlet extraction.

Soxhlet extraction

To prevent dispersion of the prepared samples, the samples (30 g of olive fruit and 30 g of olive pits were used to obtain the extract) were placed sequentially in the cartridge in the Soxhlet apparatus[12]. Each sample was first extracted with 400 mL of petroleum ether at 55 °C for 3 h, and lipophilic nonpolar components were removed first. The extraction process was then continued with 400 mL of ethanol. The ethanol used as a solvent was evaporated, and the high-density ethanol extract remaining in the flask was weighed. An extract of 1,700 g of olive fruit and 0,900 g of olive pith was obtained. These extracts were then dissolved in distilled water to obtain stock solutions. All the measurements were carried out, and each sample was divided into 10 equal volumes with plastic-capped tubes, labelled, and stored in a deep freezer at -40 °C until the time of measurement.

LC–MS/MS instrument and chromatographic conditions

Qualitative and quantitative determination of 25 phytochemicals was performed via a Nexera Shimadzu HPLC instrument coupled with a dual MS instrument (Shimadzu, Kyoto, Japan). The liquid chromatography unit was equipped with an LC-30AD binary pump, a DGU-20A3R degasser, an SIL-30AC autosampler, and a CTO-10AS column oven. The separation was performed using an Inertsil ODS-4 C18 3 μm reversed-phase analytical column (150 mm × 4.6 mm). Gradient elution was performed with a 0.5 mL/min flow rate at 40 °C, and the injection volume was 4 mL. The mobile phase consisted of solvent A (water, 5 mM ammonium formate, and 0.1% formic acid) and solvent B (methanol, 5 mM ammonium formate, and 0.1% formic acid). The following elution program was used: 40-90% B at 0-20 min, 90-99% B at 20-23 min, 99-40% B at 23-24 min, and 4% B at 24-29 min. MS detection was performed via a Shimadzu LCMS 8040 model triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operating in both positive and negative ionization modes. LC/MS data were collected and processed with LabSolutions software (Shimadzu, Kyoto, Japan). Multiple reaction monitoring (MRM) mode was used to quantify the analytes. The assay of phenolic compounds was performed following two or three transitions per compound, the first for quantitative purposes and the second and/or third for confirmation.

The limits of detection (LOD = mean concentration of the blank +3 SDs) and limits of quantification (LOQ = mean concentration of the blank +10 SDs) of the LC-MS/MS method are reported for phytochemicals[13].

Gene network analysis

For gene network analysis, we utilized the clusterProfiler tool, which is an effective method for biological term classification and enrichment analyses of gene sets. The GroupGO method classifies genes based on their projections within the Gene Ontology (GO) hierarchy, providing in-depth information about biological processes, cellular components, and molecular functions[14]. RichGO and RichKEGG perform enrichment tests to determine which biological processes or pathways are overrepresented in specific gene clusters compared with reference gene sets, via hypergeometric distribution[15]. To control for false discovery rates (FDRs), q-values are calculated, enhancing the reliability of the results[16]. Additionally, the Compare Cluster function automatically computes enriched functional categories across different gene clusters, aiding researchers in understanding similarities and differences among gene groups. Together, these methods play crucial roles in interpreting biological data and testing hypotheses.

Determination of antimicrobial activity

Strains and growth conditions

In this study, standard strains of bacteria known for their resistance to antibiotics were used. These isolates were identified as two gram-positive bacteria, *Staphylococcus aureus* ATCC 29213; and *Enterococcus faecalis* ATCC 29212, and two gram-negative bacteria, *Escherichia coli* ATCC 25922; and *Pseudomonas aeruginosa* ATCC 27853. The strains were grown in blood medium overnight before they were subjected to the liquid microdilution method. They were then passaged and stored in tryptic soy broth (TSB) at -20°C to for use in repeat experiments.

Preparation of inoculums

The bacterial strains were passaged on blood media and incubated in an oven at 35±2°C for 18-24 h. At least 3-5 similar colonies on the culture plate were selected. These colonies were picked with a pipette and transferred to 4-5 mL of liquid medium (such as tryptic soy broth). The liquid medium was incubated at 35°C until it reached McFarland 0.5 turbidity (approximately 2-6 h).

Antimicrobial test

The microdilution method (MIC) was used to determine the MIC values[17]. Stock compounds were dissolved in Dimethyl sulfoxide (DMSO). Penicillin, fluconazole, and gentamicin were prepared as positive controls. It was prepared at concentrations of 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/mL. DMSO (1%) was used as a solvent control. The compounds were set at initial test control concentrations of 250 µg/mL in MHB. MHB was added to all the wells except the first well. One hundred microliters of each compound was added to the first well. Serial dilutions were then made to the minimum concentration (1 µg/mL). Then, 0.5 McFarland was prepared from the bacteria incubated in Mueller Hinton Broth (MHB) for 1 night. Fifty microliters of bacterial suspensions were added to each well. The mixture was incubated at 37 °C for 24 h, and the microplates were read on a spectrophotometer at a wavelength of 620 nm. The percentage inhibition rates of the microorganisms in the study were determined according to the formula below.

$$\%100 - \left(\frac{\text{Experimental well absorbance - Blank well absorbance}}{\text{Negative control absorbance}} \times \%100 \right)$$

The MIC value was considered the lowest concentration of the compound that inhibited 100% of each microorganism.

Human Carbonic anhydrase (hCAI and hCA II) activity assay

The esterase activities of hCA I and hCA II were tested via Verpoorte′s method[18], and the change in absorbance at 348 nm was detected to determine the inhibitory effects of the *O. europaea* extract according to previous studies.

AchE activity assay

A common method for measuring acetylcholinesterase (AChE) activity begins with the homogenization of biological samples to prepare supernatants. A specific volume of this supernatant (e.g. 50 µL) was mixed with 1 mL of phosphate buffer solution. Then, 50 µL of acetylcholine is added, and the mixture is thoroughly mixed and incubated for a specific period (e.g. 15 min) to allow enzyme activity to occur. During this time, AChE hydrolyzes acetylcholine to produce thiocholine. After the reaction was complete, Ditan was added, and the mixture was stirred again and allowed to sit for a specific duration. The absorbance was subsequently measured at 412 nm via a spectrophotometer. AChE activity is calculated on the basis of the change in absorbance and is typically expressed as µmol acetylcholine/min/gram of protein. Experiments conducted with control groups are crucial for evaluating the effects of inhibitors. This method provides a standard approach for determining AChE activity and facilitates the investigation of the pharmacological potential of natural products, such as olive extracts[19,20].

***In vitro* cytotoxicity analysis**

The MTT test was applied for cytotoxicity analysis of the interaction of *O. europaea* with MCF-7 and HCT-116 cells. It was carried out according to ISO 10993-5 standards.

MTT assay

MCF-7 and HCT-116 cells were detached with 1 mL of trypsin, incubated for 3 minutes, after which an 1 mL of trypsin was added. After incubation, the flask was washed with 10 mL of medium. The cell additional suspension was transferred to a Falcon tube with 5 mL of medium; and centrifuged at 1000 rpm for 5 min, after which the supernatant was discarded. The cell pellet was resuspended in 5 mL of medium. A 20 µL aliquot of this suspension was mixed with 20 µL of trypan blue dye, placed on a Thoma slide,

and counted under a microscope, where live cells appeared colourless and dead cells were blue. This count was used to determine the cell concentration for the MTT assay.

Cell transplantation and MTT

Two 24-well sterile plates were used for MCF-7 and HCT-116 cells. *O. europaea* extract sections were sterilized in 80% ethanol for 24 h, then dried in a sterile cabinet and placed in the wells. The cells, at a concentration determined from previous counts, were added to the wells. For the 24 h measurements, the medium was removed, and 200 µL of MTT solution was added to each well and incubated for 2 h. MTT dye, which turns into dark blue-purple formazan in live cells with active mitochondria, was used to assess cell viability. After incubation, the MTT solution was aspirated, and 400 µL of DMSO was added to dissolve the formazan. The absorbance was measured at 570 nm via an ELISA reader. The cell viability was calculated via the following formula:

$$\%Viability = 100 \times (OD570e / OD570b)$$

[Where OD570e is the absorbance of the test group and OD570b is the absorbance of the control group. A viability rate below 50% indicates cell toxicity]

Elution Method

Following ISO 10993-5 standards, a 1 cm^2 membrane of sterilized *O. europaea* extract was incubated in 330 µl of DMEM for 24h at 37°C. The extract was then added to wells containing MCF-7 and HCT-116 cells. After a 24h incubation, the DMEM was removed, and 200 µL of MTT solution was added, followed by a 2h incubation. The MTT solution was discarded, and 400 µL of DMSO was added. The absorbance was again measured at 570 nm to compare the effect of the DMEM on cell viability with that of the original MTT method.

Results and Discussion

***Olea europaea* phenolic content results**

Owing to its excellent selectivity and sensitivity, LC-MS/MS was used to evaluate phenolic compounds in *O. europaea*. The analytes analyzed are listed in Table 1 with LODs, LOQs, linear ranges, and R2 values. However, in this study, the LODs and LOQs of 25 phenolic compounds were determined via LC-MS/MS (Table 2). A methanol extract of *O. europaea* was used to determine its phytochemical content via LC-MS/MS. The main active phenolic acid contents in *O. europaea* are shown in Fig. 1. The following compounds were measured in the *O. europaea* methanol extract: oleuropein (70.31±0.03 mg/kg), protocatechuic acid (25.80±0.01 mg/kg), luteolin (1691.34±33.34 mg/kg), quercetin (949.17±5.22 mg/kg), kaempferol (820.32±40. 43 mg/kg), caffeic acid (731.50±3. 10 mg/kg), syringic acid (407.54±8.93 mg/kg), luteolin (17.35±12.13 mg/kg), and vanillic acid (12.02±2.12 mg/kg) (Table 1). Studies indicate that oleuropein has

Table 1 — Analytical parameters for phenolic compound detection via LC–MS/MS

Compounds	Retention time (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	LOD (µg/L)	LOQ (µg/L)	Linear regression	Linear range (µg/L)	r^2	Repeatability (n = 6)	
									Means	RSD %
Acetohydroxamic acid	0.406	76.15	58	6.90	23.01	y=216.91x+6165.8	20-750	0.9989	489.34	1.79
Catechin hydrate	2.532	291	139.1	2.05	6.84	y=1717.9x–63.99	10-750	0.9988	485.19	1.19
Vanillic acid	2.762	168.95	65	84.78	282.61	y=48.343x+662.5	250-1000	0.9993	496.07	5.94
Syringic acid	3.001	199.1	140.1	2.88	9.61	y=112.03x+1316.1	10-500	0.9994	483.07	2.91
Resveratrol	3.606	229	135	41.83	139.43	y=733.34x–69955	250-1000	0.999	486.42	2.19
Fumaric acid	0.809	115.2	71.1	7.91	26.38	y=100.91x–1701.62	40-750	0.9989	499.19	4.02
Gallic acid	1.278	169.1	124.9	3.92	13.06	y=305.07x–1859.3	10-100	0.9981	471.32	6.98
Caffeic acid	2.836	179	135	2.87	9.58	y=1227.2x–5396.5	10-100	0.9948	474.22	3.69
Phloridzin dihydrate	3.594	435.1	273.1	81.80	272.67	y=120.23x–9479.5	250-1000	0.9989	500.19	4.32
Oleuropein	3.567	539.1	377	7.17	23.90	y=324.26x–5388.8	40-750	0.9997	496.52	5.26
Protocatechuic Acid	3.556	181	108	2.76	9.20	y=1382.2x–4393.1	10-500	0.9967	479.92	4.31
Salicylic acid	3.558	137.2	93	22.88	76.25	y=3838.2x–149277	75-1000	0.9977	520.39	6.81
Ellagic acid	3.681	301.1	228.9	23.74	79.14	y=18.841x+911.46	100-1000	0.9967	502.25	3.45
Myricetin	3.644	317	179.1	4.34	14.45	y=588.4x–4990.6	20-500	0.9987	492.54	3.39
2-Hydroxy-1.4-naphthoquinone	3.664	173.1	145	2.07	6.91	y=461.45x–4553.8	10-500	0.9989	540.11	10.83
hydroxybenzoic acid	3.555	137.2	93.1	8.92	29.74	y=3831.2x–94423	40-500	0.9996	477.66	4.75
Silymarin	3.996	481.1	453.1	8.00	26.70	y=199.91x+950.97	40-750	0.9997	478.05	3.41
Quercetin	3.891	301.1	150.9	7.79	25.98	y=150.09x–422.87	20-500	0.9997	487.60	2.99
Naringenin	3.952	271	150.9	68.40	228.10	y=700.8x–26469	250-1000	0.9997	481.52	2.73
Butein	3.935	271	134.9	38.50	128.20	y=62.943x–2793	100-1000	0.996	492.32	3.25
Luteolin	4.069	285	150.9	6.40	21.40	y=1389x–40923	40-1000	0.9988	491.54	2.73
Kaempferol	4.298	285	117	3.90	13.00	y=62.513x–821.08	20-1000	0.9982	491.68	3.31
Alizarin	4.594	239	211	15.30	51.10	y=26.512x–1721	60-2000	0.9991	512.49	8.30
Curcumin	4.672	367.1	216.9	12.80	42.70	y=1908.9x–8252.1	40-1000	0.9994	509.57	4.95
Thymoquinone	3.337	165	137	7.64	25.47	y=349.23x–2887.4	20-500	0.9971	482.18	2.71

Table 2 — Results of phenolic compound detection in *Olea europaea* L via LC–MS/MS

Compounds	Means±sds mg/kg	Compounds	Means±sds mg/kg
Acetohydroxamic acid	< LOQ	Myricetin	< LOQ
Catechin hydrate	< LOQ	2-Hydroxy-1,4-naphthoquinone	< LOQ
Vanillic acid	12.02±2.12	4-hydroxybenzoic acid	< LOQ
Syringic acid	< LOQ	Silymarin	< LOQ
Resveratrol	< LOQ	Quercetin	< LOQ
Fumaric acid	< LOQ	Naringenin	< LOQ
Gallic acid	3.03±0.78	Butein	< LOQ
Caffeic acid	2.5±0.9	Luteolin	17.35±12.13
Phloridzin dihydrate	< LOQ	Kaempferol	< LOQ
Oleuropein	70.31±0.03	Alizarin	< LOQ
Protocatechuic acid	25.80±0.01	Curcumin	< LOQ
Salicylic acid	< LOQ	Thymoquinone	< LOQ
Ellagic acid	< LOQ		

[Values are below the limits of the quantification and not determined value of 52.30 ± 8.21 µM/mL and a Ki value of 34.50 ± 3.15 µM/mL. As a control, tacrine exhibited an IC50 value of 90.11 ± 4.69 µM/mL and a Ki value of 86.13 ± 2.13 µM/mL]

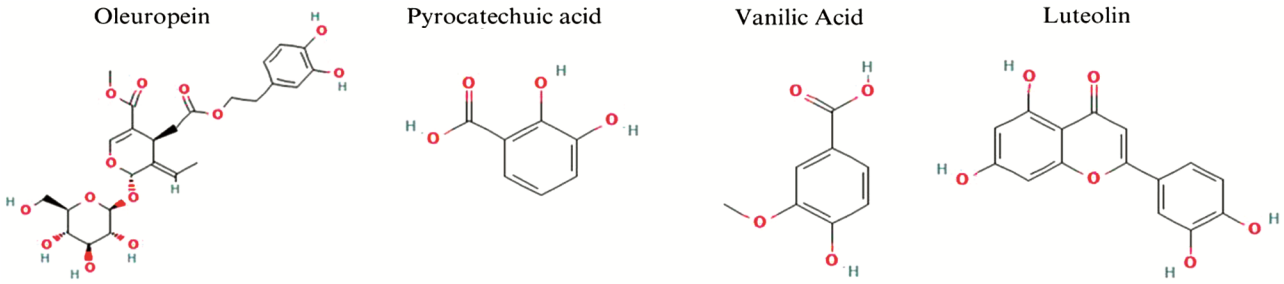

Fig. 1 — Active phenolic acid contents in *Olea europaea*.

antioxidant, anti-inflammatory and cardiovascular health-improving properties[20,21].

Flavonoids such as luteolin (1691.34 mg/kg) and quercetin (949.17 mg/kg) are compounds present in high amounts in olive fruit, increasing the antioxidant capacity of this fruit. The potential health benefits of flavonoids include inhibiting cancer cells, showing anti-inflammatory effects, and reducing cardiovascular disease risks[22,23]. The high levels of compounds such as kaempferol and caffeic acid in our study are among the important factors supporting the health benefits of olive fruit. Kaempferol is a flavonoid known for its antioxidant properties and may play an active role in the prevention of diseases such as cancer, cardiovascular diseases and diabetes[24].

In this study, the phenolic acid content, enzyme inhibition, antimicrobial activity, and anticancer activities of extracts obtained from the fleshy parts and pits of olive fruits were examined. In addition, high phenolic acid contents were evaluated via pharmacological gene enrichment analysis. According to our research findings, the equivalent oleuropein level of 1.00 mg extract was determined to be 70.31 µg in olive fruit extracts containing only hydrophilic biomolecules after the removal of nonpolar components. In a study conducted with some olive cultivars grown in Turkey, the total phenolic matter content per gram of raw olive fleshy parts was determined to be 421, 275, and 251 µg in the Uslu, Gemlik, and Ayvalık cultivars, respectively[25,26]. These values are characteristic of raw olives, and depending on the processing methods and techniques used in table olives, the amount of phenolic substances present decreases at certain rates. Similarly, the same losses occur in olive oil, the most important product of the olive industry, depending on the methods and techniques used[27].

Pharmacological gene network analysis results of phenolic acid-rich in *Olea europaea*

The genes encoding oleuropein, protocatechuic acid, luteolin, and vanillic acid with high phenolic acid contents were retrieved from Swiss target prediction and analyzed via a Venn diagram (Fig. 2). As a result, oleuropein, procatechic acid, luteolin, and vanillic acid compounds were associated with 13 common genes. These genes were *CA3, TYR, CA13, CA14, CA4, CA9, CA5, AKR1C3, CA1, CA6, CA2, CA12,* and *CA7* (Table 3).

We performed gene enrichment analysis on 13 common genes. The molecular function, biological process, cellular component, and KEGG pathway relationships of these common genes were determined. In terms of molecular function, we identified alditol-NADPH-1 oxidoreductase activity, bile acid binding activity, carbonate dehydratase activity, carbon-oxygen lyase activity, hydrolase activity, monooxygenase activity, and NADP-retinol dehydrogenase activity relationships (Fig. 3A). In the cellular component category, actin-based cell projection, an anchored component of the outer side of the plasma membrane, the basolateral plasma membrane, the chitosome, an intrinsic component of the outer side of the plasma membrane, the melanosome membrane, the microvillus, microvillus membrane myelin sheath, and the pigment granule membrane relationship were observed (Fig. 3B). In the biological process category, we identified relationships among bicarbonate transport, intracellular pH production, one-carbon metabolic processes, pH reduction, positive regulation

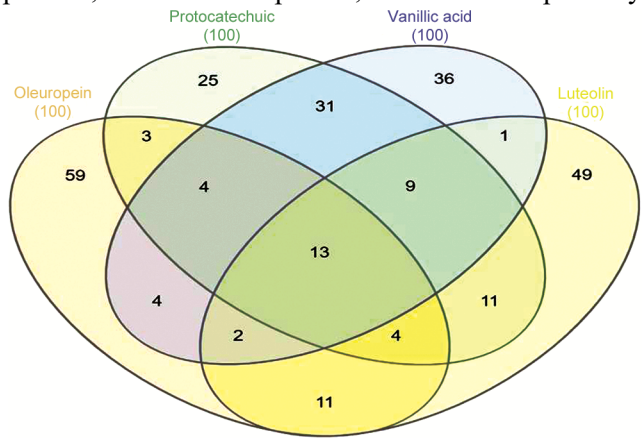

Fig. 2 — Venn diagram of the common gene relationships of *Olea europaea* bioactive compounds.

Table 3 — Common gene relationships of *Olea europaea* bioactive compounds

Names	Total	Elements
Luteolin Oleuropein Protocatechuic acid Vanilic Acid	13	CA3 TYR CA13 CA14 CA4 CA9 CA5A AKR1C3 CA1 CA6 CA2 CA12 CA7
Oleuropein Protocatechuic acid Vanilic Acid	4	CA5B MMP8 TYMS ALB
Luteolin Oleuropein Protocatechuic Acid	4	CYP19A1 PTGS2 CDK2 PARP1
Luteolin Oleuropein Vanilic Acid	2	MMP2 MMP9
Luteolin Protocatechuic Acid Vanilic Acid	9	AURKB TTR MAOA ALK ESR1 IGF1R AKR1B1 ESR2 ACHE
Oleuropein Protocatechuic Acid	3	DRD1 PTGS1 GBA
Oleropein Vanilic Acid	4	MME MMP1 SLC29A1 HSP90AA1
Luetolin Oleuropein	11	PLG MMP13 TNKS2 MMP3 TNKS ADORA1 SYK ADORA2A DRD4 ABCB1 TOP1
Protocatechuic Acid Vanilic Acid	31	POLA1 SERPINE1 RXRG IDO1 CTBP2 LCK FYN CNR2 OGA MAOB LDHA RXRA ELANE LAP3 SQLE PIN1 FUT7 PLA2G4B LDHB ERN1 BCL2L1 CDA KDM2A PTPRB MCL1 TUBB1 POLB SRD5A2 COMT TPMT RXRB
Luetolin Protocatechuic Acid	11	HSD17B1 KDR MET AKR1C1 AXL EGFR PTK2 GPR35 AKR1C2 NEK2 SRC
Luteolin Vanilic Acid	1	KDM4E
Oleuropein	59	HLCS NADK SLC37A4 MMP7 SLC28A3 TNF YARS MAP3K7 ITGB1 ITGA4 ADAM17 FNTA FNTB SLC28A2 TACR2 MAPK1 PGF SLC5A1 IMPDH1 PRKACA HCAR2 LGALS4 SLC5A2 MAP2K1 CDK2 CCNA1 CCNA2 MARS PIK3CA LGALS1 TRPM2 ST6GAL1 ALDH2 IL2 VEGFA PDE5A F9 JUN IRAK4 ADORA3 ADK MGMT HSPA8 PRKCA EPHX2 NRAS SLC2A1 SLC5A4 PRSS1 PNP ADORA2B GBA2
Procatechuic Acid	25	TLR4 ADRA2C RARG STS ADRB1 RARA DYRK1A FASN KDM5C ADRA2B BCL2 ENPEP ADRB2 ADRA2A DAO ABAT ADRA1D HTR3A RARB ALDH5A1 ADRA1A DYRK1B PHF8 CLK1 OPRM1
Vanilic Acid	36	ACE HMGCR KDM4A ECE1 GPR17 CPA1 SLC13A5 GGH NAALAD2 PLAU FABP3 NGFR GRM2 TRPM8 FABP4 TBXAS1 FTO KMO MIF DPP4 KDM6B KDM4C SHBG FABP5 DTYMK DBF4 CDC7 APEX1 KDM3A LIG1 FBP1 FUCA1 SLC16A1 AMPD3 ADA ST14 CASP3
Luteolin	49	NUAK1 XDH HSD17B2 ABCG2 CDK6 GLO1 CAMK2B ALOX12 PTPRS CCNB3 CDK1 CCNB1 CCNB2 AKR1C4 AMY1A MPO ALOX5 CXCR1 BACE1 AKR1B10 AKT1 PIK3R1 GRK6 PYGL CDK5R1 CDK5 F2 PLA2G1B ARG1 PLK1 ABCC1 CD38 NOX4 FLT3 PFKFB3 CDK1 AKR1A ESRRA AVPR2 AR CSNK2A1 GSK3B DAPK1 ALOX15 TERT MMP12 CFTR APP CYP1B1 PKN1 AHR PIM1 NEK6

of synaptic transmission, GABAergic regulation of chloride transport, regulation of the intracellular pH, secondary metabolic processes, and synaptic transmission (Fig. 3C). We found a relationship between arachidonic acid metabolism, bile secretion, collecting duct acid secretion, folate biosynthesis, gastric acid secretion, nitrogen metabolism, ovarian steroidogenesis, proximal tubule bicarbonate reclamation, steroid hormone biosynthesis, and tyrosine metabolism in the KEGG pathway (Fig. 3D).

Target gene predictions of oleuropein, protocatechuic acid, luteolin, and vanillic acid structures were extracted from the bioinformatics-based Swiss target prediction database, and Venn diagram analysis was performed for common gene relationships. Venn diagram analysis showing the associations of oleuropein, procatechic acid, luteolin, and vanillic acid with hCAI and hCAII. Gene enrichment analysis with enzyme inhibition was performed in relation to molecular functions, biological processes, cellular components, and KEGG pathways, resulting in new drug designs for the treatment and prevention of several global diseases such as glaucoma, diabetes, and cancer.

Olea europaea extract AChE, hCA I and hCA II results

O. europaea extract AChE, hCA I and hCA II enzyme inhibition results are shown in Table 4. The *O. europaea* extract concentrations were compared with those of AZA. Compared with AZA the *O. europaea* extract (75 µM/mL) was more effective at inhibiting hCA I and hCA II. *O. europaea* extract at a 75 µM/mL concentration was more effective at inhibiting hCA I (IÅ0: 63.12±11.37 µM/mL; Ki: 39.18±4.02 µM/mL), and hCA II (IC50: 39.45±7.02 µM/mL; Ki: 45.33±5.11

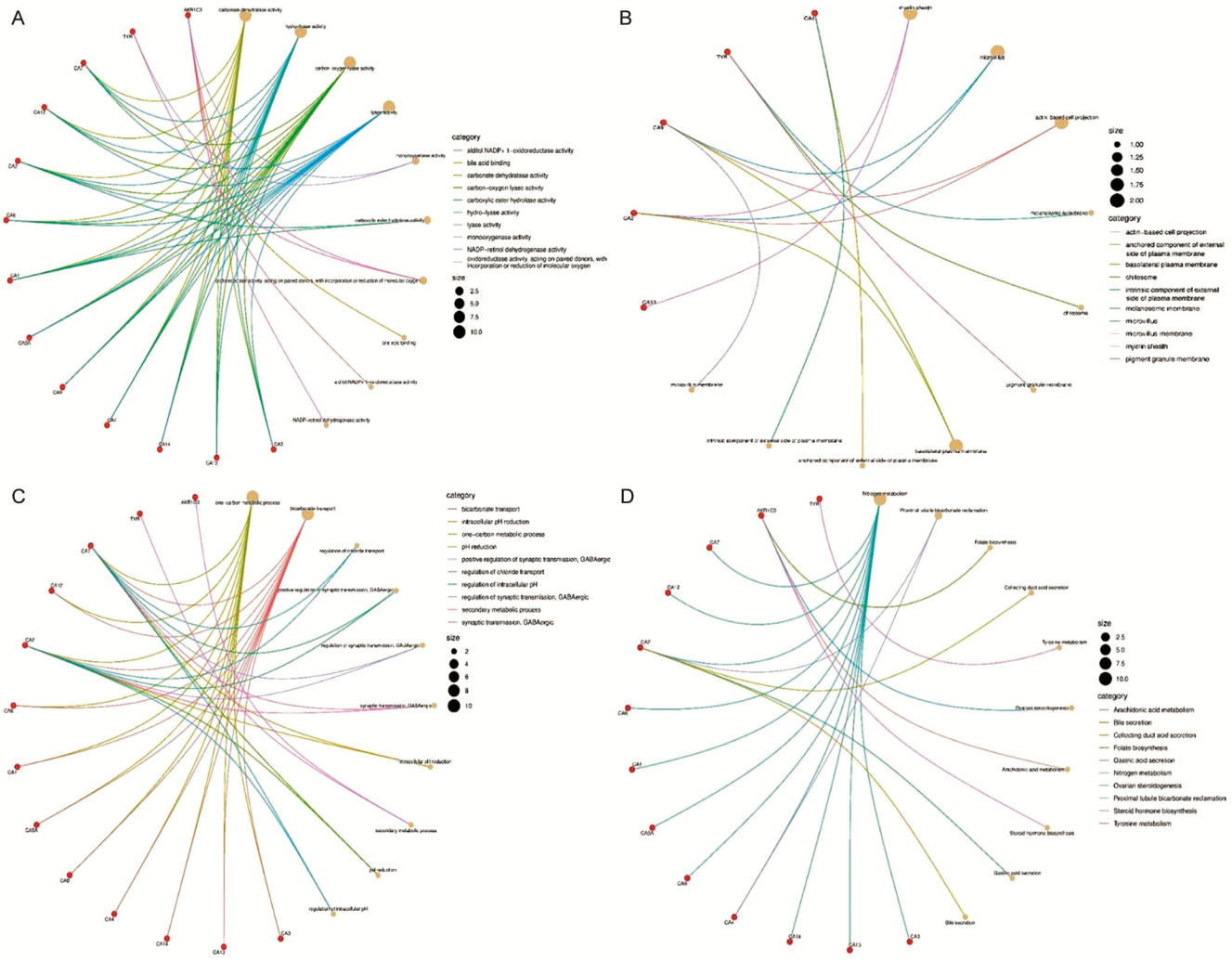

Fig. 3 — **R**elationships of genes common to procatechuic acid, vanillic acid, oleuropein and luteoin, **(A)** Molecular function, (B) Cellular component, (C) Biological process, (D) KEGG pathway.

Table 4 — *Olea europaea* enzyme inhibition results

	hCA I	r^2	hCA II	r^2	AChE	r^2	hCA I	hCA II	AChE
O. europaea 25 µM/mL	113.24±6.4	0.9101	115.20±2.98	0.9014	121.03±5.63		122.05±6.12	155.09±3.78	-
O. europaea 50 µM/mL	103.12±8.15	0.8910	93.45±4.33	0.9235	95.12±3.78		112.18±4.02	105.33±5.11	-
O. europaea 75 µM/mL	63.12±11.37	0.9872	39.45±7.02	0.9715	52.30±8.21		39.18±4.02	45.33±5.11	-
AZA	101.00±1.97	0.9815	87.10±5.55	0.9892	-		118.21±4.05	99.17±6.21	-
TAC					90.11±4.69				86.13±2.13

µM/mL) enzymes than acetazolamide (hCA I IC50: 101.00±18.37; hCA I Ki: 118.21±4.05; hCA II IC50: 87.10±8.15; hCAII Ki: 99.17±6.21). The oleuropein extract significantly inhibited of acetylcholinesterase (AChE), with an IC50 value of 52.30 ± 8.21 µM/mL and a Ki value of 34.50 ± 3.15 µM/mL. As a control, tacrine exhibited an IC50 value of 90.11 ± 4.69 µM/mL and a Ki value of 86.13 ± 2.13 µM/mL when assessed for its inhibitory effect on acetylcholinesterase (AChE). These findings indicate its potential as an effective inhibitor of AChE, suggesting that oleuropein may have pharmacological applications in conditions related to cholinergic dysfunction.

Recently, natural product-derived carbonic anhydrase (CA, EC 4.2.1.1) inhibitors have led to the development of new drug designs for the treatment and prevention of several global diseases, including glaucoma, diabetes, and cancer. For this purpose, the inhibitory effects of the *O. europaea* extract on hCA I and II isoenzymes were evaluated in the present study. *O. europaea* extracts at a concentration of 75 µM/mL inhibited hCA I and hCA II enzymes.

Aggul *et al.* reported that tyrosol, hydroxytyrosol, luteolin, diosmetin, caffeic acid, luteolin 7-O-glycoside, and apigenin 7-O-glycoside obtained from *O. europaea* leaves had inhibitory effects against hCA isozymes I and II. Another study revealed that oleuropein and verbascoside obtained from olive oil had inhibitory effects on hCA I and II isoenzymes[28]. In the present study, we found that the *O. europaea* extract inhibited hCA I and hCA II enzymes at a concentration of 75 µM/mL. We suggest that this is due to the presence of oleuropein, procatechic acid, luteolin, and vanillic acid, which are the phenolic acids we found in our *O. europaea* extract.

The measurement results of acetylcholinesterase (AChE) activity demonstrated the effects of the olive extract on AChE. The AChE activity in the control group was determined to be 12 µmol acetylcholine/min/gram protein, whereas with olive extract, this value was measured at 5 µmol acetylcholine/min/gram protein. These findings indicate that olive extract inhibits AChE activity by approximately 58%. These findings highlight the potential neuroprotective properties of olive extract and emphasize that AChE inhibition is an important target in the treatment of neurological disorders such as Alzheimer′s disease. These results support the need for further research into the health benefits of olive products.

Measuring acetylcholinesterase (AChE) activity is crucial for neurological health, particularly in identifying potential therapeutic targets for neurodegenerative diseases such as Alzheimer′s disease. The results obtained demonstrate the inhibitory effect of the olive extract on AChE, with an inhibition percentage calculated to be 58%. These finding suggest that olive components may possess neuroprotective properties. Notably, oleuropein, an important phenolic compound found in olive leaves and fruits, has an inhibitory effect on AChE activity[29,30]. Research suggests that oleuropein and other phenolic compounds may contribute to preserving cognitive function through AChE inhibition, in addition to their antioxidant, anti-inflammatory, and anticancer properties, which highlight the positive effects of natural sources on neurological health. Furthermore, the inclusion of olive products in the Mediterranean diet supports the potential of this diet to reduce the risk of cognitive decline[32]. The components of the Mediterranean diet may influence AChE activity by combating inflammation and oxidative stress, thereby altering the course of conditions such as Alzheimer′s disease.

However, further research is needed to understand the mechanisms underlying the inhibition of AChE by olive extracts. Specifically, in-depth studies on the effects of different phenolic compounds, olive extracts, and other natural products on AChE activity can help elucidate this potential. Additionally, it is essential to explore how these findings can be evaluated for clinical applications and to comprehensively investigate their impacts on human health. In conclusion, olive extract stands out for its potential to inhibit AChE activity, opening new avenues for research to better understand the health benefits of olive products properties [31].

Table 5 — *Olea europaea* cytotoxicity results

	HCT116 (µg/mL) IC50±STD	MCF-7(µg/mL) IC50±STD
Cisplatin	84.21±4.01	82.01±9.12
Olea europaea L (25 µM/mL)	25.36±24.39	26.10±11.36
Olea europaea L (50 µM/mL)	46.21±6.21	47.61±6.78
Olea europaea L (75 µM/mL)	80.19±7.03	78.19±4.85

***In vitro* cytotoxicity of *Olea europaea* extract**

The *O. europaea* cytotoxicity results are given in Table 5. We studied the cytotoxicity of the *O. europaea* extract at concentrations of 25 µM, 50 µM/mL, and 75 µM in MCF-7 and HCT-116 cells. We observed that the *O. europaea* extract (at a concentration of 75 µM/mL) showed similar cytotoxicity to cisplatin in MCF-7 and HCT-116 cells. The cytotoxic effect was lower than that of cisplatin at other concentrations (Table 4). In terms of the cell viability profiles, approximately 80% of the cell viability profiles, were observed in *MCF-7* and *HCT-116* cells after treatment with cisplatin or *O. europaea* extract (75 µM/mL). The effect of the *O. europaea* extract on cell viability was low at other concentrations (Fig. 4 A&B). The cell proliferation images of the *O. europaea* extracts are shown in (Fig. 5 A&B). Additionally, 75 µM/mL *O. europaea* extract had a good cytotoxic effect on MCF-7 (78.19±4.85 µg/mL) and *HCT-116* (80.19±7.03 µg/mL) cells.

Han *et al.* examined the anticarcinogenic effects of oleuropein and its degradation product, hydroxytyrosol, on a breast cancer cell line (MCF-7). They reported that 200 µg/mL oleuropein or 50 µg/mL hydroxytyrosol significantly reduced cell proliferation and induced cell apoptosis[33]. Similarly, Hassan *et al.* exposed breast cancer (MCF-7) cells to 100 and 200 µM oleuropein and observed the inhibition of cell proliferation and the induction of apoptosis at these concentrations[34]. Elamin *et al.* reported that 200 µM oleuropein significantly inhibited cell proliferation and induced cell apoptosis in human breast cancer cells (MCF-7)[35]. In recent studies, certain concentrations of oleuropein were used to treat breast cancer cells (MCF-7). The IC50 value of oleuropein at the 24th hour was 256.1 µM, the IC50 value at the 48th hour was 247.5 µM, and the IC50 value at the 72nd hour was 222.5 µM. At the 48 hour IC50, cell apoptosis was induced, and at the same concentration, oleuropein had a strong antioxidant effect[36]. Samara *et al.* studied 51 analogs of oleuropein in several human cancer cell lines and reported that analog 24, which is nontoxic to normal cells, has the highest level of inhibitory activity in vitro (human colon

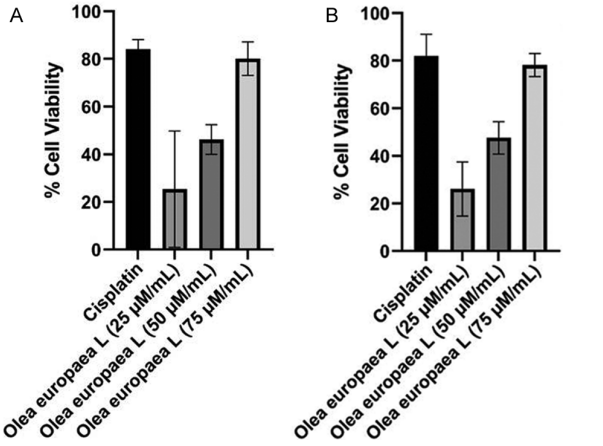

Fig. 4 — *Olea europaea* promotes cell viability and proliferation HCT-116 Cells (A), MCF-7 Cells (B).

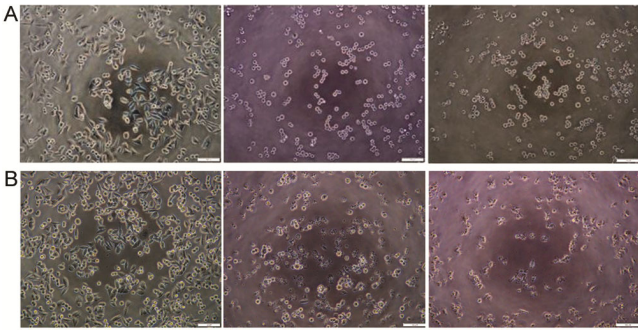

Fig. 5 — *Olea europaea* proliferation image of (A) HCT-116, (B) MCF-7 cells treated with cisplatin for 24 h.

cancer cells HCT-116, human cervical carcinoma cells *HeLa*; and MCF-7 cells) and *in vivo* (B16-F10 mouse melanoma cells)[37]. Importantly was the finding that Oleuropein analog 24 promoted natural immune responses from natural killer cells and limphokine-activated killer cells.

Several authors have shown that oleuropein promotes apoptosis in cancer cells, such as HeLa cells[38], HepG2 human hepatoma cells[39], SH-SY5Y human neuroblastoma cells[40], and HCT-116 cells[41]. Taken together, these findings provide significant insights into the contribution of Oleuropein in treating cancer cells, revealing several targets of its pro-apoptotic activity, such as activation of the JNK pathway, suppression of PI3K/AKT signaling, and activation of *caspase-9* and *3* gene expression.

All these observations from scientific studies suggest that oleuropein can be used as a potential chemotherapeutic agent for cancer treatment. When the relationship between oleuropein and cancer was examined; in the literature, more studies on the effects of oleuropein on breast cancer were reported. There are relatively few studies on other cancer types. *O. europaea* extracts, which contain specific compounds, have been shown to be effective in various *in vitro* and *in vivo* cancer models. The source of *O. europaea* cytotoxicity has not yet been fully characterized; however, compounds such as oleuropein and verbascoside have independent cytotoxic effects on animal cancer models. Initial results from animal models are promising but need to be translated into a clinical setting. Treatments using these compounds are likely to be well tolerated and represent a promising direction for future research[42].

Antimicrobial evaluation results of *Olea europea* extract

The antibiogram zone diameters of the *O. europaea* extracts are given in Table 6. The antimicrobial effects of the *O. europaea* extracts are shown in Table 7. The *O. europaea* extract had the highest antimicrobial activity at 75 µM/mL. The antimicrobial activity decreased as the concentration decreased.

Since the discovery of penicillin, very important progress has been made in the fight against infectious diseases. However, since obtaining penicillin naturally is a very difficult and expensive process, semisynthetic and synthetic penicillins have started to be produced by developing easier and cheaper methods over time. This was followed by the discovery of other antibiotics and their synthetic production in laboratories on a large scale. Increasing antibiotic consumption has led to significant problems, including the development of resistance in microorganisms and the occurrence of adverse side effects. In the following years, as with most other health problems, people turned to natural products and complementary medicine practices in the fight against infection. Many nutrients and medicinal plants have started to be preferred and consumed intensively in infectious diseases because of their antimicrobial, antiviral, antifungal, and anti-inflammatory properties. These include products that we frequently consume in our daily lives, such as linden, rosehip, lemon, olive, sage tea, mint, and thyme.

In terms of the antimicrobial effects of our extracts, compared with ampicilin, *O. europaea* (75 µM/mL) was more effective against *S. aureus* and *E. faecalis*. However, the MICs values of *O. europaea* at 50 µM/mL and 25 µM/mL in the other two extracts were lower than those of ampicillin. Similarly, many studies in the literature have revealed the antimicrobial effects of olive products. Thielmann *et al.* reported the antimicrobial effects of olive fruit extracts[43]; Boo *et al.* reported the antimicrobial and antioxidant effects of olive leaf extracts[44]; Cicerale *et al.* reported the antimicrobial and anti-inflammatory effects of olive and olive oils[45]; and Masoko *et al.* reported the antifungal and anti-inflammatory effects of *O. europaea* leaf extracts.

These findings suggest that olive fruit extract (*O. europaea L.*) is a valuable natural resource for health with its high antioxidant capacity and antimicrobial effects. Especially phenolic compound (e.g. oleuropein) are thought to play an important role in these biological activities. The results obtained indicate that olive fruit extract can be used in the development of functional foods, natural preservatives or pharmaceutical supplements. However, further studies on the bioavailability, *in vivo* effects and

Table 6 — Antibiogram zone diameters

	S. aureus / Zone diameter	*P. aeruginosa* / Zone diameter	*E.facealis* / Zone diameter	*E.coli* / Zone diameter
Olea europaea L (25 µM/mL)	1 mm	1 mm	1 mm	1 mm
Olea europaea L (50 µM/mL)	4 mm	5 mm	3 mm	3 mm
Olea europaea L (75 µM/mL)	8 mm	10 mm	6 mm	7 mm
Ampicilin	10 mm	10 mm	9 mm	9 mm

Table 7 — Antimicrobial effects of *Olea europaea* extracts

	S. aureus /MIC	*P. aeruginosa* / MIC	*E. coli* / MIC	*E. facealis* /MIC
Olea europaea L (25 µM/mL)	100	75	100	100
Olea europaea L (50 µM/mL)	25	50	100	100
Olea europaea L (75 µM/mL)	25	50	50	12,5
Control	Bacterial growth	Bacterial growth	Bacterial growth	Bacterial growth
Ampicillin	25	25	25	25

toxicological safety of the compounds are needed to fully evaluate this potential. In this context, our study contributes to the literature on the biological potential of olive fruit extract and provides.

Conclusion

Phenolic compounds in *O. europaea* (olive) fruit were analysed using LC-MS/MS. According to the data obtained from the methanol extract, the amounts of active compounds such as oleuropein, protocatechuic acid, luteolin, quercetin, kaempferol and vanillic acid were determined. *O. europaea* extract significantly inhibited hCA I, hCA II and AChE enzymes and showed a stronger effect compared to the standard inhibitors acetazolamide and tacrine. *O. europaea* extract showed significant cytotoxic effect in MCF-7 and HCT-116 cancer cells at a concentration of 75 μM/mL and this effect was found to be similar to cisplatin. At lower concentrations, cytotoxicity decreased significantly. These results indicate that *O. europaea* extract has anticancer potential in a dose-dependent manner. In conclusion, interest in *O. europaea* extract products is increasing because of their beneficial effects on human health. The polyphenols present in these extracts are garnering increasing attention for their impact on the production of reactive oxygen species. The phenolic acid content of the olive extract derived from the anklet species, cultivated in Kahramanmaras Province Turkey, includes oleuropein, protocatechuic acid, luteolin, and vanillic acid. The efficacy of *O. europaea* in inhibiting human carbonic anhydrase I (hCA I) and human carbonic anhydrase II (hCA II) enzymes suggests its potential in the development of novel drug designs for the treatment and prevention of various global diseases, including glaucoma, diabetes, and cancer. Additionally, the extract demonstrated significant cytotoxicity against MCF-7 and HCT-116 cancer cells, indicating its promising therapeutic potential. Moreover, the inhibition of acetylcholinesterase (AChE) by the olive extract further underscores its role in neuroprotection, suggesting potential applications in the management of neurological disorders such as Alzheimer′s disease.

Conflict of interest

The authors declare no conflict of interest.

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