Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Antioxidant and Antitumor Activities of *Olea europaea* L. and *Zingiber officinale* in Ehrlich Ascites Carcinoma Bearing Swiss Albino Mice

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Abstract

Cancer kills 10 million people worldwide. In traditional medicine, antioxidant and anticancer plants are used for many ailments. Examining the effectiveness of olive leaf extract and ginger extract in eradicating mice. The main objective of this study was to establish an Ehrlich ascites carcinoma (EAC) tumor model. Eighty mice were separated into eight groups (n = 10) normal control, and (EAC control, standard, OLET, OLAQ, GE, OLAQ+GE, and OLET+GE) that are given 150 mg/kg of the extracts daily for 14 days after tumor induction. The mice were sacrificed, and serum and tissues were collected. All groups were tested for biochemical parameters, cell cycle progression, P53 gene expression, and interleukin-10 levels. Oral olive leaf extract (OLE) and ginger extract (GE) significantly reduced IL-10 levels. The treated groups had increased p53 gene expression and apoptosis. Compared to EAC mice, olive leaf and ginger extracts show in vivo anticancer efficacy and normalize biochemical marker levels. The GC/MS results for the chemical extracts of aqueous and ethanolic olive and ethanolic ginger plants confirmed and endorsed the results of the biological effect assay, with olive leaves and ginger having a greater phenolic content suppressing EAC growth more effectively.

Keywords: *Olea europaea* L.; *Zingiber officinale*; Ehrlich ascites carcinoma; Anticancer; Flavonoids

1. Introduction

Cancer is a major global health issue, that caused 10 million deaths in 2020 [1]. Ehrlich ascites carcinoma (EAC) is a widely used experimental tumor model known for its rapid growth, high transplantability, 100% malignancy, and short lifespan. This model is widely used in cancer research due to its usefulness in testing anticancer drugs [2]. Because of their unique characteristics, EAC cells are suitable for investigating various animal hosts. Plants have been utilized as remedies in many plant cultures and are a source of many effective pharmaceuticals for the pharmaceutical industry owing to the presence of specific bioactive chemicals [3].

Medicinal plants with antioxidant and anticancer properties are commonly used in traditional medicine for numerous illnesses [4]. Medicinal plants have been shown in studies to be a rich source of antioxidants such as flavonoids, phenolics, carotenoids, and vitamins, and the administration of these plants, whether in the form of fresh extracts or chemical components, is often associated with a minor risk of degenerative diseases such as cancer [5]. Previous research has shown that plant-derived substances have antineoplastic effects on mice with EAC through cell growth inhibition and apoptosis [6].

Olea europaea L., an evergreen tree in the Oleaceae family, is found across various Mediterranean countries [7]. Olive leaves a byproduct of the olive oil industry during pruning, are a significant source of bioactive components, such as hydroxycinnamic acid derivatives, hydroxytyrosol, triterpenes, secoiridoids, and flavonoids [8]. Olive leaves contain a greater diversity and amount of polyphenols than olive oil, particularly oleuropein [9]. Oleuropein is effective against colorectal, thyroid, and lung cancer cell lines [10]. Its effects on cell death are based on its pro-apoptotic properties [11]. Studies in animal models have shown that OLE administration prevents breast cancer [12], skin cancer [13], and various soft tissue

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Receive Date: 10 June 2024, Revise Date: 13 August 2024, Accept Date: 05 September 2024
DOI: 10.21608/ejchem.2024.284919.9822

cancers [14]. Earlier studies showed the anti-inflammatory activity of Olea europaea L. extract in animal models [15-17]. Previous investigations have shown antiproliferative properties of olive-derived compounds in several cell lines [18-20].

Flavonoids, which have health-promoting properties such as anti-inflammatory, antimicrobial, antihypertensive, cardioprotective, and antioxidant effects, are among the essential components in olive leaves [21]. The efficacy of olive leaf extract as an anticancer agent was studied by Milanizadeh [22], who reported that the extract reduced the size and weight of cancer cells in mice. It has been demonstrated that OLE polyphenols prevent cancer metastasis in an animal model of breast cancer [22]. Olive leaves are widely used in the food, pharmaceutical, and cosmetic industries due to their various biological properties. In vitro studies show OLE has scavenging characteristics like ascorbic acid and α-tocopherol. It effectively removes hypochlorous acid, a highly reactive oxidant produced by neutrophil myeloperoxidase during inflammation [23].

Ginger (Zingiber officinale), a member of the Zingiberaceae family, is commonly used as a spice or in traditional medicine, particularly in Southeast Asia. Ginger extract has been shown to decrease cancer growth, induce apoptosis, and contain antioxidant properties that eliminate harmful radicals and prevent peroxidation [24]. Ginger has been observed to enhance gastrointestinal tract motility and had analgesic, sedative, antipyretic, and antibacterial activities in laboratory animals. Earlier reports showed the antioxidative activity of ginger extract in animal models [25,26]. Active compounds present in ginger possess a variety of advantageous properties. The effects encompassed are anti-carcinogenic, anti-oxidative, antimicrobial, anti-inflammatory, anti-diabetic, anti-obesity, anti-gastric ulcer, antimicrobial, and antiallergenic [27,28].

Ginger rhizome extract contains zingiberene and its derivatives, which have been linked to anticancer efficacy against colorectal carcinoma [29]. In addition, 6-shogaol, another phenolic ingredient of ginger, lowered the metastatic potential of lung and breast cancer in mice. Studies demonstrate that 6-shogaol has strong anticancer activity both in vivo and in vitro, and it is now regarded as an effective immunotherapeutic agent for cancer therapy [30]. Previous research has demonstrated that ginger has a role in the inhibition of transformation, hyperproliferation, and inflammatory processes involved in various stages of carcinogenesis, angiogenesis, and metastasis [31,32]. Numerous studies have shown that 6-gingerol, a component of ginger, has a role in inducing apoptosis in the prostate cancer cell line LnCaP by raising p53 expression [33], as well as skin carcinogenesis in mice [34].

Multiple studies have demonstrated the potential efficacy of ginger in the treatment of liver cancer, breast cancer, prostate cancer, and colorectal carcinomas, owing to its multifaceted pharmacological mechanisms [35,36]. Ginger extract is a potent antioxidant, that not only reduces toxicity but is also said to have antitumor properties and enhance the effects of many anticancer medications in rats [37].

The therapeutic effects of olive pomace extract and ginger extract on Cryptosporidium parivum in mice have been determined by [38]. However, the potential in vivo benefits of combining olive leaves and ginger extracts on EAC have not been fully explored. Also, the effect of the combined mixture of olive leaves and ginger aqueous and ethanol extracts has not been previously studied for its anticancer effect-induced mice model. This study aimed to explore the antitumor effects of olive leaf extract, and ginger extract and their combination in female Swiss albino mice with EAC. Furthermore, this study aimed to determine the underlying molecular mechanisms involved in tumorigenesis by examining critical biological parameters, such as MDA levels, antioxidants, and markers of apoptosis at both the RNA (p53) and protein (cell cycle) levels.

Experimental

Chemicals and kits

All chemicals were purchased from Sigma-Aldrich, and all chemicals and reagents were of analytical grade.

Extraction of plant materials

Preparation of olive leaf extracts

Olive leaves (*Olea europaea L.*), an emblematic species, were purchased from an herbalist in Mansoura, Egypt. The extraction of the chemical constituents from powdered dried olive leaves was performed using the maceration technique with organic solvent systems, which were chosen based on their polarity: ethanol (100%) and water (100%). The extraction method used was maceration the dry powder samples with ethanol and water as solvent. The extraction begins by grinding the 200 g leaves into fine particles. After that, the samples were heated on a hot plate (IKA C-MAG HS 7, Germany) with continual stirring at 70°C for 5 min. The samples were then set at room temperature for a period of 24 hours in dark prior to being filtered. The subsequent solution was filtered using Whatman No 1 filter paper and concentrated under low temperature using freeze dry treatment. Finally, the extracted samples were kept at −20°C prior to sample analysis [39].

Preparation of ginger extract

Ginger extract was prepared: fresh ginger rhizomes were purchased from an herbalist in Mansoura, Egypt. A mixture of 100 g of ground dry ginger rhizomes and 1000 mL of 70% ethanol and water in a ratio of (80:20) was left for 72 h. The solution was filtered using a muslin cloth and Whatman filter paper No. 1 and then concentrated using a rotary evaporator at 40 °C to produce pure ginger extract (GE). The crude extract solutions were prepared by thoroughly mixing the desired amount of dried ginger extract with sterile distilled water to a final concentration of 100 mg/mL. The extract solution was stored at 4°C until needed [40].

GC–MS analysis of olive leaf extract and ginger extract:

Crude extracts analysis using a Shimadzu GCMS-QP2010 Plus, Japan, the mobile phase is Helium high purity (99.9999% pure) used at a flow rate of 1ml/min. The instrument is equipped with a capillary column DB-5MS (30 m length, 0.25 mm thickness, 0.25m diameter) and technology (NIST) library data. 1 µl sample was injected into the split/splitless inlet in split mode at 250 °C. The column initial temperature was at 70°C (held for 6 min) and was increased to 240°C at 7°C/min and then to 270°C (held for 15 min) at 10°C/min; the injector was at a temperature of 250°C; and the source temperature at 280°C. The split ratio was 100: 1, helium was the carrier gas, the flow rate was 1 mL/min, and the range of scan mode (50–550 amu) was used for data acquisition. Identification of components was achieved based on their retention indices and interpretation of the mass spectrum was conducted using the database of National Institute of Standards and Technology (NSIT). The spectra of the unknown components of crude extracts obtained were compared with the standard mass spectra of known components

stored in NIST library (NISTII). The relative amount of each constituent will be calculated by measuring the corresponding peak area and represented as a percentage of the sum of areas of all peaks. The method was conducted as [41,42] .

In vivo studies

Animals and experimental protocol

Eighty adult female Swiss albino mice weighing 22 to 26 g were obtained from the Animal Farm of Vacsera in Helwan, Egypt. The animals were housed in clean polypropylene cages under a 12-hour light/dark cycle, with controlled temperature and humidity, and were given standard rodent chow and ad libitum access to water. The mice were acclimated for ten days before the beginning of the experiment. The experiments were performed in compliance with relevant guidelines and regulations. All analyses were conducted following the regulations of the Institutional Animal Ethics Committee of Mansoura University in Mansoura, Egypt.

Experimental design

A total of 80 adult Swiss albino mice were randomly divided into eight equal groups of ten mice each. A total of 70 Swiss female mice were inoculated with EAC cells through intraperitoneal injection of 200 microliters of a $2.5x10^6$ cells/mouse solution [43]. The treatments began on the 10^{th} day after EAC cell transplantation and were administered daily for 14 consecutive days. The mice were divided into eight groups as follows: Group I: served as a normal control for healthy mice; Group II: consisted of EAC-bearing mice without treatment; Group III: EAC-bearing mice received Cytoplatin-10 orally at a dose of 150 mg/kg body weight as a standard drug; Group IV: EAC-bearing mice received an ethanol extract (OLET) of olive leaves orally at a dose of 150 mg/kg body weight; Group V: EAC-bearing mice received an aqueous extract (OLAQ) of olive leaves orally at a dose of 150 mg/kg body weight; Group VI: EAC-bearing mice received ginger extracts (GE) orally at a dose of 150 mg/kg body weight; Group VII: EAC-bearing mice received a mixture of OLAQ and GE (1:1) orally at 150 mg/kg body weight; and Group VIII: EAC-bearing mice received a combination of OLET and GE (1:1) orally at a dose of 150 mg/kg body weight. All the mice were administered their respective doses until the end of the experiment. The doses of ginger administered to the animals were chosen based on the studies of Rong et al. [44] and Tajaddini Mahani et al. [45]. The selected dose of olive leaf extract was based on previous studies [46].

Sample collections

At the end of the experiment, the animals were anesthetized by inhaling Isoflurane in an anesthetized jar. Blood samples were withdrawn from the jugular vein using a 25G needle, before being transferred into dry tubes, and centrifuged at 2000 rpm for 20 min. The serum was used to perform IL-10 estimation by ELISA.

Mice were euthanized with CO_2 followed by cervical dislocation and then were dissected to obtain ascites tumor. Ehrlich Ascites fluid was withdrawn with a 1 cc syringe (without a needle) from the muscle wall of the abdominal cavity. EAC cells were centrifuged at 2000 rpm for 15 min, washed with PBS, and stored at -80 °C for RT-PCR determination to determine the relative gene expression of p53 and for flow cytometric analysis of cell cycle progression.

Preparation of the liver homogenates

The liver tissues were exactly weighed and homogenized in phosphate buffer saline to get 10% homogenates. The samples were sonicated for 60 sec at an ultrasonic cycle mode of 15-sec sonication and 5-sec resting time. The homogenates were centrifuged at 6000 r.p.m at 4°C for 15 minutes subsequently the gained supernatants were used for the determination of antioxidants and MDA.

Biochemical parameters and antioxidant assays

To evaluate the antioxidant enzyme activity, various assays were performed. Superoxide dismutase (SOD) activity was determined using the method described in [47]. The catalase activity (CAT) was assessed based on the methods outlined in [48]. The activity of glutathione peroxidase (GSH) was determined following the protocol described in [49]. The lipid peroxide level, or malondialdehyde, was measured using the method outlined in [50].

Antitumor determination

Cell cycle analysis

Flow cytometric analysis was conducted at the Global Center for Scientific Research using a FACSCalibur flow cytometer (Becton Dickinson, Sunnyvale, CA, USA). The cytometer was outfitted with a small, air-cooled, low-power argon ion laser beam emitting at 488 nm.

Analysis of p53 gene expression using RT‑ PCR

The expression of the P53 gene was assessed by real-time PCR using a Rotor-Gene Q (Qiagen, USA), which was used as a housekeeping gene. Complementary DNA (cDNA) amplicons were amplified using Maxima SYBR Green/Fluorescein qPCR Master Mix and specific primers, according to the manufacturer's instructions [51]. The sequences of primers used for the p53 RT–PCR were as follows: 5'GCG TCT TAG AGA CAG TTG ACT 3' and 5'GGA TAG GTC GGC GGT TCA TGC 3', as reported previously [52].

Determination of interleukin 10 (IL-10) levels by enzyme-linked immunosorbent assay (ELISA)

The level of mouse IL-10 in the samples was determined using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit obtained from BioVision, Egypt.

Statistical analysis

The data analysis was carried out using a one-way analysis of variance (ANOVA) with groups as the sole factor, as reported in [53]. The results are presented as the mean ± standard deviation of each group. Pairwise comparisons of means were performed using the Tukey–Kramer multiple comparison test, as described in Steel and Torrie [54].

Results

GC–MS analysis of olive leaf extract and ginger extract:

GC–MS analysis of *Olea europaea L*. aqueous extract yielded a total of 36 components. GC–MS analysis of an ethanolic extract of *Olea europaea L*. yielded a total of 33 components. The chemical constituent's retention (RTs), molecular formulas,

molecular weights (MWs), and concentrations (%) are presented in **Table.1** and **Table.2**. The following primary bioactive compounds were identified in a GC–MS investigation of the aqueous fraction of olive leaves (**Table.1**). The primary bioactive compounds were identified via a GC–MS analysis of the ethanol fraction of olive leaves (**Table.2**). Additionally, minor compounds were present. A total of 58 compounds were identified from the GC/MS analysis of the ethanol extract of *Zingiber officinale.* The major bioactive compounds present in the GC–MS analysis was extracted from ginger (**Table.3**). Additionally, minor compounds were present.

Table.1: Chemical Constituents of the aqueous extract of olive leaves analyzed by GC-MS.

	Chemical Constituents	**Retention Time (RT)**	**concentration (Area %)**	**Molecular Weight (MW)**	**Chemical Formula**
1	Thymine	7.591	1.46	126.11	$C_5H_6N_2O_2$
2	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	9.187	3.75	144.12	$C_6H_8O_4$
3	Coumaran	10.628	3.1	120.15	C_8H_8O
4	5-Hydrxoymethylfurfural	10.934	1.27	126.11	$C_6H_6O_3$
5	Pyrocatechol, 3-methyl-6-pentadecyl-	11.471	0.31	110.11	$C_{22}H_{38}O_2$
6	4-Hydroxy-3-methyl acetophenone	12.272	4.08	150.17	$HOC_6H_3(CH_3)COCH_3$
7	Trans-Caffeic acid	12.549	0.2	180.16	$C_9H_8O_4$
8	p-Carbomethoxy benzaldehyde	13.198	7.76	164.16	$HCOC_6H_4CO_2CH_3$
9	4-(2-Hydroxyethyl)phenol (**Tyrosol)**	14.326	5.26	138.164	$C_8H_{10}O_2$
10	alpha.-Bromo-m-nitrotoluene	14.408	1.41	216.03	$C_7H_6NO_2Br$
11	7,7-Dimethyl-2-oxobicyclo[2.2.1]heptan-1-yl) methanesulfonic acid	14.976	0.41	232.30	$C_{10}H_{16}O_4S$
12	Cyclohexanol, 5-methyl-2-(1-methyl ethyl)-, (1.alpha.,2.beta.,5.beta.)- (carvomenthol)	15.201	0.55	156.26	$C_{10}H_{20}O$
13	1-Phenyl-1,2-ethanediol	15.331	0.34	138.16	$C_8H_{10}O_2$
14	5-tert-Butyl-2,4(1H,3H)-pyrimidinedione	15.442	1.74	168.19	$C_8H_{12}N_2O_2$
15	beta.-(3,4-Dihydroxyphenyl)-L-alanine	15.691	17.71	197.19	$C_9H_{11}NO_4$
16	1,2-Diazaspiro(2.5)octane	15.773	0.91	112.17	$C_6H_{12}N_2$
17	3-tert-Butyl-4-hydroxyanisole	15.944	1.48	180.24	$C_{11}H_{16}O_2$
18	(2E)-3-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-2-propenal	16.249	0.34	178.27	$C_{12}H_{18}O$
19	Eriodictyol-7-O-glucoside	16.472	1	450.4	$C_{21}H_{22}O_{11}$
20	Quercetin-3-O-rutinoside	16.658	2.69	610.5	$C_{27}H_{30}O_{16}$
21	Cyclododecanone	16.881	0.9	182.30	$C_{12}H_{22}O$
22	2-Butanone, 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	17.206	0.76	194.32	$C_{13}H_{22}O$
23	Levodopa	17.536	32.23	197.18	$C_9H_{11}NO_4$
24	Hydroxytyrosol	17.939	0.14	154.16	$C_8H_{10}O_3$
25	1,2,4-Cyclopentanetrione, 3-(2-pentenyl)-	18.18	0.48	180.20	$C_{10}H_{12}O_3$
26	2(3H)-Naphthalenone,4,4a,5,6,7,8-hexahydro-4a-methyl-	18.573	1.26	234.33	$C_{15}H_{22}O_2$
27	4a-Methyl-4,4a,5,6,7,8-hexahydro-2(3H)-naphthalenone	18.644	2.23	164.24	$C_{11}H_{16}O$
28	Allopurinol ribosides	18.692	1.27	268.23	$C_{10}H_{12}N_4O_5$
29	Sphaerobioside	18.808	1.59	578.5	$C_{27}H_{30}O_{14}$
30	5-[(1E)-1-(5,5-Dimethyl-2(5H)-furanylidene)ethyl]-2-methyl-2-vinyltetrahydrofuran	19.493	0.69	234.33	$C_{15}H_{22}O_2$
31	Hexadecanoic acid, methyl ester	20.174	0.28	270.45	$C_{17}H_{34}O_2$
32	Palmitic acid	20.542	1.18	256.4	$C_{16}H_{32}O_2$
33	Oleic acid, methyl ester	21.94	0.45	296.5	$C_{19}H_{36}O_2$
34	9-Octadecenoic acid, (E)-	22.297	0.81	310.5	$C_{20}H_{38}O_2$
35	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha.,8a.beta.)]-	24.389	0.18	204.35	$C_{15}H_{24}$
36	beta.-Sitosterol acetate	24.902	0.15	456.7	$C_{31}H_{52}O_2$

Table.2: Chemical Constituents of the ethanolic extract of olive leaves analyzed by GC-MS

	Chemical Constituents	Retention Time (RT)	concentration (Area %)	Molecular Weight (MW)	Chemical Formula
1	Estragole (Anisole, p-allyl)	11.843	0.18	148.2	$C_{10}H_{12}O$
2	Carvacrol	12.1	0.2	150.21	$C_{10}H_{14}O$
3	4-Hydroxy-3-methylacetophenone	12.296	0.47	150.17	$C_9H_{10}O_2$
4	alpha.-Terpinene	12.883	0.28	136.23	$C_{10}H_{16}$
5	p-Carbomethoxy benzaldehyde	13.215	0.23	164.16	$C_9H_8O_3$
6	1-Pentadecene	13.607	0.08	210.40	$C_{15}H_{30}$
7	γ-Cadinen	14.915	0.23	204.35	$C_{15}H_{24}$
8	beta.-(3,4-Dihydroxyphenyl)-L-alanine (DL-DOPA)	15.57	0.14	197.19	$C_9H_{11}NO_4$
9	3,5-Dimethoxyacetophenone	15.959	0.19	180.20	$C_{10}H_{12}O_3$
10	1-Heptadecene	16.381	0.79	238.5	$C_{17}H_{34}$
11	Cedrol	16.649	0.16	222.37	$C_{15}H_{26}O$
12	Octopamine	17.203	5.08	153.18	$C_8H_{11}NO_2$
13	2(3H)-Naphthalenone,4,4a,5,6,7,8-hexahydro-4a-methyl	18.055	0.95	234.33	$C_{15}H_{22}O_2$
14	Myristic acid	18.394	0.57	228.37	$C_{14}H_{28}O_2$
15	Oleuropein	18.652	5.86	540.51	$C_{25}H_{32}O_{13}$
16	1-Nonadecene	18.773	0.81	266.5	$C_{19}H_{38}$
17	2-Pentadecanone, 6,10,14-trimethyl-	19.307	0.85	268.47	$C_{18}H_{36}O$
18	Palmitic acid	20.659	25.96	256.43	$C_{16}H_{32}O_2$
19	9-Z-Tricosene	20.919	1.42	322.62	$C_{23}H_{46}$
20	1-Octadecanol	21.828	1.9	270.5	$C_{18}H_{38}O$
21	Phytol	22.068	2.71	296.53	$C_{20}H_{40}O$
22	trans-.delta.9-Octadecenoic acid	22.409	31.19	282.5	$C_{18}H_{34}O_2$
23	Stearic acid	22.592	2.95	284.48	$C_{18}H_{36}O_2$
24	Eicosane	23.01	2.03	282.5	$C_{20}H_{42}$
25	Dotriacontane	24.02	4.96	450.9	$C_{32}H_{66}$
26	Beta-Selinene	24.659	1.29	204.35	$C_{15}H_{24}$
27	Tetracontane	25.203	1.07	563.1	$C_{40}H_{82}$
28	Ethyl-p-coumarate	30.769	0.3	192.21	$C_{11}H_{12}O_3$
29	Tetrapentacontane	33.892	0.08	759.4	$C_{54}H_{110}$
30	Trans-Squalene	34.166	4.81	410.7	$C_{30}H_{50}$
31	Pomolic acid	37.988	1.68	472.7	$C_{30}H_{48}O_4$
32	2,6,10,14-Hexadecatetraen-1-ol, 3,7,11,15-tetramethyl-, acetate, (E,E,E)-	38.44	0.03	332.52	$C_{22}H_{36}O_2$
33	Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl-, (E,E,E)-	39.611	0.13	290.5	$C_{20}H_{34}O$

Table.3: Chemical Constituents of the ginger extract analyzed by GC-MS

	Chemical Constituents	**Retention Time (RT)**	**concentration (Area %)**	**Molecular Weight (MW)**	**Chemical Formula**
1	Beta.-Linalool	7.725	0.13	154.25	$C_{10}H_{18}O$
2	Camphor	9.534	1.11	152.23	$C_{10}H_{16}O$
3	Alpha-Terpineol	10.068	0.4	154.25	$C_{10}H_{18}O$
4	Decanal	10.402	0.1	156.26	$C_{10}H_{20}O$
5	Beta-Citronellol	10.757	0.02	156.26	$C_{10}H_{20}O$
6	Geraniol	11.217	0.05	154.25	$C_{10}H_{18}O$
7	2-Undecanone	11.978	0.07	170.29	$C_{11}H_{22}O$
8	(-)-alpha-Cubebene	13.347	0.45	204.35	$C_{15}H_{24}$
9	Caryophyllene	13.567	0.85	204.35	$C_{15}H_{24}$
10	Naphthalene,1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2-(1-methylethenyl)-,[2R-(2.alpha.,4a.alpha.,8a.beta.)]-	14.017	0.08	204.35	$C_{15}H_{24}$
11	gamma.-Elemene	14.175	0.96	204.35	$C_{15}H_{24}$
12	4-Methyl-2(5H)-furanone	14.296	0.09	98.10	$C_5H_6O_2$
13	Beta-Farnesene, (6Z)-	14.513	0.9	204.35	$C_{15}H_{24}$
14	Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, (1S,4S,7R)-	14.627	0.43	204.35	$C_{15}H_{24}$
15	alpha.-Curcumene	14.964	4.99	202.33	$C_{15}H_{22}$
16	(-)-Zingiberene	15.213	9.56	204.35	$C_{15}H_{24}$
17	Alpha-Farnesene	15.307	5.15	204.35	$C_{15}H_{24}$
18	Cyclohexene, 1-methyl-4-(5-methyl-1-methylenehexyl)-	15.359	3.53	204.35	$C_{15}H_{24}$
19	Cadina-1(10),4-diene	15.422	0.87	204.35	$C_{15}H_{24}$
20	Cadina-3,9-diene	15.475	0.81	204.35	$C_{15}H_{24}$
21	Beta.-Sesquiphellandrene	15.589	7.89	204.35	$C_{15}H_{24}$
22	Cyclohexanemethanol, 4-ethenyl-α,α,4-trimethyl-3-(1-methylethenyl)-,[1R-(1α,3α,4β)]-	15.858	1.17	222.36	$C_{15}H_{26}O$
23	Alpha-Bisabolol	15.918	0.53	222.37	$C_{15}H_{26}O$
24	Nerolidol(1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-)	15.994	1.29	222.37	$C_{15}H_{26}O$
25	m-Cymene (Benzene, 1-methyl-3-(1-methylethyl)-)	16.208	0.43	134.22	$C_{10}H_{14}$
26	Citral	16.393	1.24	152.23	$C_{10}H_{16}$
27	Germacrene D	16.703	1.56	204.35	$C_{15}H_{24}$
28	Alpha-Eudesmol(2-Naphthalenemethanol, 1,2,3,4,4a,5,6,8a-octahydro-alpha,alpha,4a,8-tetramethyl-, [2R-(2alpha,4aalpha,8abeta)]-)	16.82	0.37	222.37	$C_{15}H_{26}O$
29	Beta selinene	16.917	2.14	204.35	$C_{15}H_{24}$
30	Zingerone	17.096	4.38	194.23	$C_{11}H_{14}O_3$
31	Paradol	17.25	2.28	278.4	$C_{17}H_{26}O_3$
32	Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl-	17.292	0.6	290.5	$C_{20}H_{34}O$
33	Beta-Caryophyllene oxide	17.376	0.9	220.35	$C_{15}H_{24}O$

Table 3 : continued

	Chemical Constituents	Retention Time (RT)	concentration (Area %)	Molecular Weight (MW)	Chemical Formula
34	Carotol (3a(1H)-Azulenol, 2,3,4,5,8,8a-hexahydro-6,8a-dimethyl-3-(1-methylethyl)-,[3R(3.alpha,3a.alpha,8a.alpha)]-)	17.599	2.59	222.37	$C_{15}H_{26}O$
35	Farnesol (2,6,10-Trimethyl-2,6,10-dodecatrien-12-ol)	17.713	3.05	222.36	$C_{15}H_{26}O$
36	Germacrone ((E,E)-Germacra-3,7(11),9-trien-6-one)	18.155	1.26	218.33	$C_{15}H_{22}O$
37	Mesityl alcohol (2,4,6-Trimethylphenol)	18.276	0.36	136.19	$C_9H_{12}O$
38	Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl-	19.021	2.53	290.5	$C_{20}H_{34}O$
39	Eucalyptol (1,8-Cineole)	19.157	0.83	154.25	$C_{10}H_{18}O$
40	Ledol	19.224	0.45	222.37	$C_{15}H_{26}O$
41	1-Naphthalenepropanol, alpha-ethenyldecahydro-alpha,5,5,8a-tetramethyl-2-methylene-	19.63	2.1	290.5	$C_{20}H_{34}O$
42	Palmitic acid	20.647	1.94	256.42	$C_{16}H_{32}O_2$
43	Naphthalene,1-[1-(3-cyclohexylpropyl)undecyl]decahydro-	21.824	0.37	416.76	$C_{30}H_{56}$
44	Ethyl homovanillate	22.06	0.98	210.23	$C_{11}H_{14}O_4$
45	Ethyl Linoleate (9,12-Octadecadienoic acid (Z,Z)-, ethyl ester)	22.325	1.43	308.5	$C_{20}H_{36}O_2$
46	9-Octadecenoic acid, (E)-	22.387	1.4	310.5	$C_{20}H_{38}O_2$
47	4-Ethyl-2-methoxyphenol (p-Ethylguaiacol)	23.136	1.91	152.19	$C_9H_{12}O_2$
48	Zingerone (2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)-)	23.237	1.23	194.22	$C_{11}H_{14}O_3$
49	10-epi-gamma-eudesmol	23.949	6.27	222.37	$C_{15}H_{26}O$
50	Nonivamide	24.264	0.63	293.4	$C_{17}H_{27}NO_3$
51	Elemol	25.091	6.98	222.37	$C_{15}H_{26}O$
52	Delta-Tocopherol	26.658	0.54	402.7	$C_{27}H_{46}O_2$
53	Gamma-Tocopherol	27.018	0.23	416.7	$C_{28}H_{48}O_2$
54	3,6-Dimethyl-2,3,3a,4,5,7a-hexahydro-1-benzofuran	31.951	0.12	152.23	$C_{10}H_{16}O$
55	4,8,13-Cyclotetradecatriene-1,3-diol, 1,5,9-trimethyl-12-(1-methylethyl)-	33.465	0.05	306.5	$C_{20}H_{34}O_2$
56	Citronellyl acetate	36.902	0.07	198.3	$C_{12}H_{22}O_2$
57	(-)-Menthyl chloride	37.108	0.02	174.71	$C_{10}H_{19}Cl$
58	Butanoic acid, 3,7-dimethyl-2,6-octadienyl ester, (E)-	38.433	0.03	208.33	$C_{14}H_{24}O$

Effect of olive leaf extracts and ginger extract on liver tissue oxidative markers in all studied groups

Table 4 shows the effect of OLE and ginger extracts on liver oxidative indicators in both normal and EAC-induced mice. The treatment of OLE and ginger extracts to EAC-induced animals produced substantial alterations in the oxidative marker SOD. The results showed a significant increase in SOD levels in the groups (standard, OLET, OLAQ+GE, and OLET+GE) compared to the EAC group. The SOD level decreased significantly in the EAC control group as compared to the treatment groups. The normal group had significantly higher SOD activity than the other groups ($P<0.05$).

The EAC control group had substantially higher MDA levels than the treated and normal groups ($p < 0.05$). Treatment with (standard medication, OLAQ, or OLET+GE) resulted in a considerable reduction in MDA levels when compared to EAC, and levels reverted to normal.

However, there was no significant change in CAT levels between the normal and EAC groups. The normal and EAC control groups showed considerably higher CAT activity than the treatment groups ($P<0.001$). The levels of CAT were considerably lower in the GE and OLET+GE groups than the other groups. The EAC control group showed substantially lower GPx levels than the treated and normal groups ($p < 0.05$). Treatment with (standard medication, OLAQ, GE, or OLET+GE) significantly increases GPx levels as compared to the EAC and normal groups. The levels of GPx were considerably lower in the OLET and OLAQ+GE groups than in the normal group.

Table.4: Effect of OLE and ginger extracts on the liver oxidative markers in all studied groups.

Oxidative markers	Normal control	EAC control	Standard	OLET	OLAQ	GE	OLAQ + GE	OLET + GE
MDA(nm/g tissue)	5.95± 0.50^{a}	290± 9.40^{b}	9.68± 5.22^{a*}	21.03± 3.30^{c}	13.81± 1.95^{d*}	21.24± 2.23^{c}	20.36± 3.07^{c}	16.06± 3.63^{c}
SOD (U/g tissue)	740.0± 45.8 ^{a*}	514.2± 42.7^{b**}	728.7± 7.11^{a**}	659.4± 16.1^{a}	606.3± 1.82 ^{c*}	556.6± 16.0^{c}	677.9± 19.9^{a}	708.3± 13.8^{a}
CAT(U/g tissue)	1.97± 0.035^{b}	1.93± 0.068^{bx*}	1.82± 0.030^{a}	1.84± 0.067^{ay}	1.85± 0.063^{ay}	1.73± 0.045^{c}	1.89± 0.009^{a}	1.62± 0.053^{c*}
GPX(mg/g tissue)	1262.3± 12.9^{a}	1156.0± 18.1^{b}	1289.0± 14.6^{a}	1254.9± 15.2^{a}	1383.9± 75.5^{d}	1352.6± 112.9^{c}	1249.6± 17.8^{a}	1274.1± 10.8^{a}

Data represent the means ± SD in each group.

oxidative markers	**P Value**
MDA(nm/g tissue)	a-c different letters between groups are very high significant (P<0.0001) a-d different letters between groups are significant (P<0.05) * non-significant (P>0.05)
SOD (U/g tissue)	b-c different letters between groups are very high significant (P<0.0001) *&**differences are very high significant (P<0.0001) a-b & c different letters between groups are significant (P<0.05)
CAT(U/g tissue)	b-c different letters between groups are very high significant (P<0.0001) a-b different letters between groups are highly significant (P<0.001) x-y different letters between groups are significant (P<0.05) * non-significant (P>0.05)
GPX(mg/g tissue)	b-a*&d different letters between groups are very high significant (P<0.0001) a&d different letters between groups are highly significant (P<0.001) a-b&c different letters between groups are significant (P<0.05)

Cell cycle progression and apoptosis in Ehrlich tissue from the control group and several groups treated with olive leaf extract and ginger extract

Figure.1 shows the results from the cell cycle analysis of the EAC tumor tissues, as identified by flow cytometry of PI-stained cells. The level of apoptosis was significantly greater in the EAC tumor tissues of the treated groups than in those of the EAC-induced group. The proportion of hypodiploid cells in the sub-G1 phase, an indicator of apoptosis, increased by 26.7% in the carcinogen group treated with OLET + GE ($p < 0.0001$). Furthermore, treatment with OLAQ + GE significantly increased the percentage of apoptotic cells by 28.6% compared with that in the EAC control group and other treated carcinogen groups ($p < 0.0001$), as shown in Fig.1.

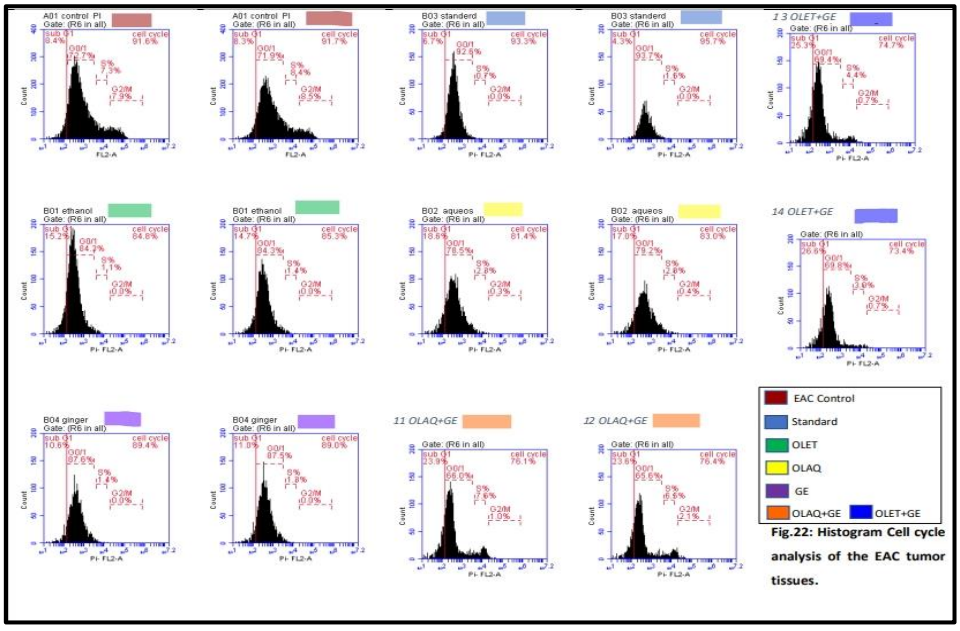

Fig.1

Quantitative PCR (qPCR) data analysis for p53 gene expression

The relative expression of p53 in EAC tissues is shown in (**Figure 2**). The p53 gene expression in the treated groups significantly increased concerning that in the EAC control group ($p < 0.0001$). Administration of OLE extract and ginger extract to the EAC-induced groups caused increased p53 gene expression in the olive extract (OLAQ & OLET) groups and the ginger extract group compared to that in the EAC control group. The combination of (OLAQ and OLET) extracts and ginger extract given to the EAC-induced group in OLAQ+GE and OLET+GE, as well as the administration of the standard drug, resulted in a significant increase in p53 gene expression compared to that in the EAC control group ($p < 0.0001$).

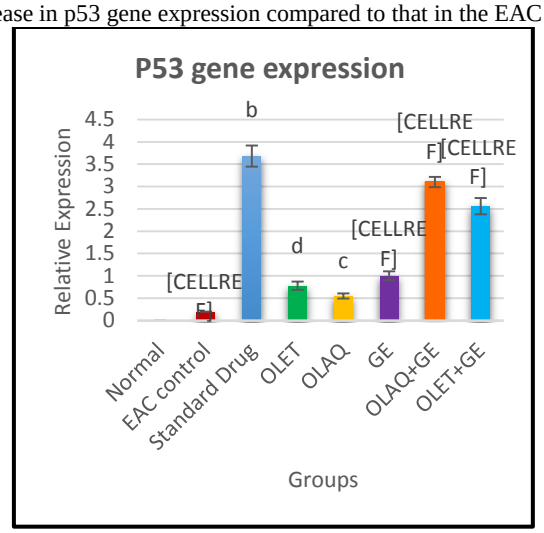

Fig.2: Effect of OLE and Ginger extracts on relative gene expression of P53 in EAC tissues of mice.
The data are displayed as the mean ± SD in each group and were analyzed using one-way ANOVA.
a-g different letters between groups are significant (P<0.0001)
d- c &e different letters between groups are significant (P<0.05)

Effect of olive leaf extracts and ginger extract on IL-10 levels in all studied groups using Enzyme-linked immunosorbent assay (ELISA)

Figure 3 displays the impact of OLE and ginger on IL-10 levels in EAC-induced mice. Compared to those in the EAC control group, the IL-10 levels in the standard and treatment groups were considerably lower ($p< 0.05$). In contrast, the IL-10 levels were greater in the standard and treated groups than in the normal group ($p< 0.05$).

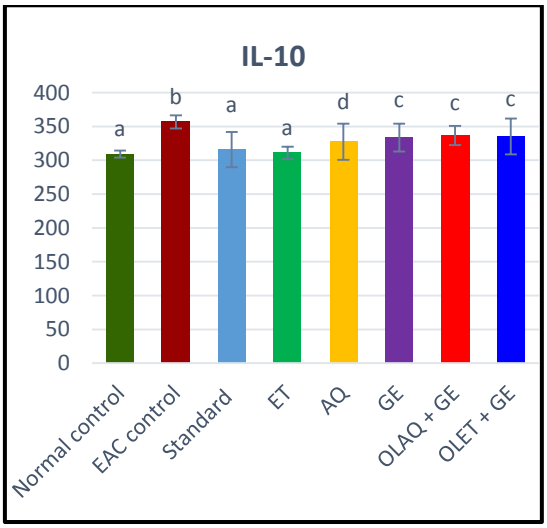

Fig.3: The effect of OLE and ginger extracts on IL-10 level in all studied groups
a-b different letters between groups are highly significant (P<0.001)
b-d different letters between groups are significant (P<0.05)

Discussion

The purpose of this study was to assess the anticancer and antioxidant effects of olive leaf extract, and ginger extract and their combination in treating EAC-induced tumors in female Swiss albino mice. However, cancer cells often exhibit improved resistance to chemical drugs, reducing their effectiveness and causing adverse effects on normal cells. Hence, there has been growing concern about using natural products as an alternative or complementary therapy to conventional chemotherapy [55]. The primary goal of anticancer agents is to inhibit tumor cell proliferation or prevent tumor damage without harming normal cells. Compared with chemical and radioactive products, natural products are considered effective cancer treatments because

they have lower toxicity [24]. Several natural products have been considered for their anticancer effects on numerous experimental models.

Olive (*Olea europaea L.*) and ginger (*Zingiber officinale*) plants contain a variety of bioactive components with antioxidant and anticancer properties, and levodopa has been shown to dramatically suppress the proliferation of ESCC cells by modulating oxidative phosphorylation, NAFLD, and Parkinson disease pathways [56]. Allopurinol is known to operate as a free radical scavenger, antioxidant, and "scavenger" of hypochlorous acid, depending on the dose; hence, the action of larger doses of allopurinol is attributable to its free radical scavenging and antioxidative activity [57]. Coumarans (2,3-dihydrobenzofurans) are antitubercular compounds [58]. The dihydrobenzofuran skeleton has several medicinal applications. For example, Megapodiol is an antileukemic agent [59]. Trans-9-octadecenoic acid is known to be cytotoxic and to have antioxidant and antimicrobial effects [60, 61]. Because of its antioxidant qualities, octopamine decreases ROS levels caused by the ingestion of deep-heated oils, and its nausea-related effects result in enhanced expression of the PPAR gene [62].

Oleuropein combined with doxorubicin has synergistic anticancer effects [63, 64]. Palmitic acid inhibits the growth and spread of prostate cancer cell proliferation and metastasis [65], and palmitic acid reduces stomach cancer development and metastasis by inhibiting the STAT3 signaling pathway [66]. Zingiberene inhibits human colon cancer cell proliferation in vitro and in vivo and may have a role in the prevention of gastrointestinal cancer [67, 68]. Elemol was defined as an active factor in the growth inhibitory effect of Cymbopogon schoenanthus on triple-negative breast cancer (MDA-MB-231) and cervical carcinoma (HEp-2) cells [69], and β-sesquiphellandrene was shown to exhibit anticancer potential [70].

Olive leaf extract (OLE) has been established to have antibacterial and antioxidant effects [71]. Similar chromatographic analyses have been performed on olive plants from various countries, and various biomolecules have been evaluated for biopharmaceutical applications [72, 73]. Phytochemical examination of the *Zingiber officinale* (ginger) extract revealed that the plant contains phenolic chemicals, flavonoids, alkaloids, glycosides, steroids, saponins, and tannins; it has a low sugar and carbohydrate content; and lacks amino acids and proteins. Several investigations have shown that ginger extract contains several of these compounds [74]. These components have been shown to reduce inflammation and have high proliferative and malignant transformation rates. Several studies have shown that several types of polyphenol compounds have anticancer effects, and some of these chemicals arrest the cell cycle in the G1 or G2/M phase due to structural differences [75].

The current investigation discovered that the treated groups had much higher levels of antioxidant enzymes SOD and GPx than the EAC-induced control group, as well as significantly lower levels of MDA and CAT. These results are consistent with earlier research [76]. The current study's EAC-bearing mice showed a significant drop in GPx levels. This observation is congruent with the findings published by [77], who found that a decrease in GPx activity is related with tumor cell growth.

The observed drop-in CAT activity across the treated groups might possibly be used to eliminate H2O2, which is converted into water and oxygen [78]. Furthermore, the depletion was linked to the process of carcinogenesis, which results in an impaired antioxidant level. All flavonoids suppress catalase activity by an uncompetitive manner [79]. As the number of passes grows, the antioxidant catalase's protective mechanism against oxidase's toxic chemical decreases, and the cell develops senescence [80]. In mice, aging induces oxidative damage, reducing antioxidant enzymes like catalase [81]. Our work supports previous studies indicating that Olea europaea L. extract enhances antioxidant enzyme activity [82, 83]. The injection of OLE extracts decreased MDA levels, showing that Olea europaea L. leaf extract has the capacity to reduce oxidative stress in EAC-mice. This observation is consistent with those published by [84].

The results of cell cycle progression and apoptosis in liver tissue from the control and treated groups revealed that the EAC-induced control group had considerably lower levels of apoptosis than the treatment group. The combination of ginger and OLE was significantly more effective at inducing apoptosis in EAC cells than the individual extracts of these two agents. OLE ethanol and aqueous extract alone stimulated apoptosis in more than 15% and 17.8%, respectively, of the Ehrlich cancer cells, while ginger alone increased the percentage of apoptotic cells to 10.8%. When they were combined, a sharp increase in cell death occurred, reaching approximately 24% and 26%, respectively.

An early indication of cellular stress is often an alteration in the cell cycle that results in apoptosis. The present study revealed that treatment with oleuropein caused cell cycle arrest at the G0 phase. This finding is consistent with previous findings that oleuropein upregulates the p53 gene and causes cell cycle arrest in neuroblastoma cells [85]. Moreover, olive polyphenols can stimulate cancer cell apoptosis. Further research revealed that the loss of CDK2 function increased the fraction of cells in the G0/G1 phase and caused G0/G1 phase arrest [86].

The tumor suppressor p53 increased in EAC tumor tissue after treatment with ginger extract, olive leaf extract, or a combination of both. The tumor suppressor p53 is critical for limiting cancer progression by blocking tumor cell proliferation and promoting apoptosis. It is regarded as a critical proapoptotic regulator, yet it is inactivated in more than half of all human malignancies [87]. Preneoplastic and malignant cells have a p53-inducing protective mechanism [88]. The results of quantitative RT–PCR analysis of p53 mRNA levels demonstrated a substantial decrease in p53 gene expression in the EAC control group compared to all treated groups. In contrast, p53 gene expression was significantly greater in all treated groups especially the mixed groups (OLAQ+GE, and OLET+GE) than in the EAC control group. The relative mRNA expression in untreated control EAC cells was 0.2 ± 0.0, whereas it was 3.1 ± 0.115 and 2.56 ± 0.182 in the mixed groups (OLAQ+GE, and OLET+GE) treated EAC cells, respectively.

One of our most notable discoveries was the increase in p53 gene expression levels induced by the addition of olive leaf and ginger extracts. These findings agree with those of [89], who reported that p53 gene mutations are the most common genetic event in various cancers. Despite the role of the tumor suppressor gene p53 in controlling cell proliferation, particularly in stressed cells, our results were consistent with these findings [90]. High-grade serous cancers frequently exhibit p53 status, which is associated with a greater survival probability.

It has been discovered that IL-10, an efficient immunosuppressive cytokine, contributes to several human neoplasms, including prostate, stomach, and colorectal malignancies. IL-10 has been shown to decrease tumor development in animal models [91]. The increased IL-10 levels observed in the EAC control group could be due to regulatory T cells that may have

contributed to dampening inflammation, which agrees with previous findings [92]. Tumor cells in EAC-bearing mice secrete immunosuppressive cytokines, including IL-10, which has been identified as a critical immunomodulatory cytokine for T-cell activity [93]. The production of IL-10 within the tumor microenvironment can be sustained. The decrease in IL-10 should be attributable to the suppression of regulatory cells. Our findings indicate that OLE and GE have strong anticancer action, which may be attributed to a reduction in tumor-induced IL-10 output which agreed with [94, 95]. According to Mocellin et al. [96], tumors release IL-10, an immunosuppressive molecule, which allows malignant cells to avoid immune monitoring. The significant decrease in IL-10, immunosuppressive, may be due to immune surveillance of the host against tumor. Tumor lymphocytes secrete pro-inflammatory cytokines (IL-6 and TNF-α), which boost the production of vascular endothelial growth factor (VEGF), a critical mediator of angiogenesis in tumor cells, leading to a reduction in IL-10 levels in all treated tumor-bearing mice groups [97].

Conclusions

In conclusion, treating mice with olive leaves and ginger extracts has therapeutic effects on EAC cells. Likely, the combination of crude extracts and synergistic antioxidant activities of the bioactive compounds in an aqueous fraction of olive leaf extract such as Levodopa, beta.-(3,4-Dihydroxyphenyl)-L-alanine, p-Carbomethoxybenzaldehyde, 4-(2-Hydroxyethyl)phenol, and the bioactive compounds in ethanol fraction of olive leaf extract such as trans-.delta.9-Octadecenoic acid, Palmitic acid, Oleuropein, Octopamine, All-trans-Squalene, and active phenolic compounds such as Zingiberene, Beta.-Sesquiphellandrene, Alpha.-Farnesene, Alpha.-Curcumene, Gingerdiol3,5 diacetate, Shagaol present in ginger extract are responsible for the potent antitumor activity as reflected in the improvement of immune markers and antioxidant status. The preceding compounds were detected in the extracts of olive leaves and ginger plants by GC–MS. These findings could serve as a stepping stone toward discovering new, safe, and effective antitumor agents.

Abbreviations

"OLE: Olive leaf extract; EAC: Ehrlich ascites carcinoma; GE: Ginger extract
OLAQ: aqueous extract of olive leaves; OLET: ethanol extract of olive leaves group".

Acknowledgment

"We wish to express our scientific appreciation to all the staff members for their help in performing fieldwork. Thanks to all of the study participants."

Data availability

"The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request."

Declaration of competing interest

"The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper."

Authors' contributions

"Amany Yehia: Data curation; Formal analysis; Investigation; Methodology; Writing-original draft. Manar Refaat: Data curation; Methodology; Supervision; Visualization; Writing-original draft; Writing-review & editing. Magdy M. Youssef: Data curation; Methodology; Supervision; Formal analysis; Software; Visualization; Writing-original draft; Writing-review & editing."

Ethics approval and consent to participate

"The Protocol used in this study for the use of female mice as an animal model for cancer research was approved by the Mansoura University Animal Care and Use Committee (MU-ACUC), (Sci-ch-M-2020-23), Faculty of Science, Mansoura University, Mansoura, Egypt. The study was reported in accordance with the ARRIVE guidelines."

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