**In-vitro and in-vivo anti-breast cancer activity of OEO (Oliveria decumbens vent essential oil) through promoting the apoptosis and immunomodulatory effects**

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**Abbreviations:**  
OEO  
Oliveria decumbens essential oil  
GC/MS  
Gas chromatography/Mass spectrometry  
MTT  
(3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide)  
DCFH2-DA  
2', 7' dichlorodihydrofluorescein diacetate  
MMP  
Mitochondrial membrane potential  
ROS  
Reactive oxygen species  
AO  
Acridine orange

**ABSTRACT:**

*Ethnopharmacological relevance: Oliveria decumbens vent is a valuable plant in Iran, used as a vegetable. Traditionally, the aerial parts of this plant are used to treat the cancer-related symptoms, inflammation, pain, and feverish conditions. However, the scientific evidence related to its traditional effects especially the possible cellular and molecular mechanisms needs to be illuminated.  

**Aim of the study:** The main objectives of our study were to explore in-vitro anti-cancer properties of OEO in 2D and 3D conditions, to understand the mechanism of OEO in the induction of death in cancer cells, and to identify in-vivo anti-tumor effect of OEO and induced immunomodulatory effects.  

**Material and methods:** OEO was extracted by hydrodistillation and analyzed by GC-MS method. To evaluate the cytotoxic effect of OEO on 4T1 cancer monolayer cells (2D culture) and spheroids (3D cultures), MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was used. Fluorescence staining, various flow cytometry techniques, colorimetric assay, electrophoresis, and comet assay were performed to understand the anti-cancer mechanisms of OEO and determine the death mode in treated 4T1 cells. In animal studies, mouse mammary tumor model was established, the anti-tumor effect of OEO was investigated and ultimately by using the ELISA cytokine assay, immunostimulatory of OEO was studied.  

**Results:** According to GC/MS analysis, thymol, carvacrol, γ-cymene, and γ-terpinene were identified as main components of OEO. Based on MTT assay, OEO inhibited viability in 4T1 cancer cell without any significant effect on L929 normal cells in 2D, also the anti-proliferative effects of OEO on 4T1 spheroids (3D) was significant but less extent. Our results revealed that OEO induces apoptosis through ROS generation, mitochondrial membrane potential (ΔΨm) disruption, caspase3 activation, and DNA damage. Evaluating the effectiveness of OEO on 4T1 tumor-challenging mice and cytokine assay confirmed anti-tumor effects of OEO and development of an immune response related to Th1 expansion.  

**Conclusion:** These data shed light on the apoptotic mechanisms related to OEO cytotoxicity and introduced this compound as a candidate in cancer therapy.

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**1. Introduction**

In spite of the development of conventional therapies to combat cancer, including chemotherapy, these therapies cause damage to many tissues and increase the resistance leading to treatment failure. Hence, to minimize these problems, the finding of alternatives of cancer therapy is important, among which herbs have been known as a significant source of novel bioactive compounds for chemotherapeutic development (Roy et al., 2018). Numerous researches in drug discovery from medicinal plants show various pharmacological properties of these plants including anticancer activity. Phytochemical agents obtained from plants work by various mechanisms of action targeting cancer progression and inducing the apoptotic death (Roleira et al., 2015; Roy et al., 2017). Essential oils are natural complexes composed mainly of terpenes in addition to some other non-terpene components. These components exhibit multiple anti-inflammatory, anti-microbial, and anti-oxidant properties and commonly use in pharmaceutical and food industries. An antioxidant is an agent that, when presents at low concentrations, significantly prevents oxidation of a substrate (Carocho et al., 2018). Some antioxidant agents may lose their antioxidant...
activity at high concentrations and therefore act like a pro-oxidant agent (Vertuani et al., 2004). Some essential oils and their components display cancer suppressive activity on a number of human cancer cell lines including breast cancer (Blowman et al., 2018). Apoptosis is the common mode of cell death caused by many anticancer drugs. In apoptotic death, caspase activation plays a central role leading to cleavage of many substrates and ultimately apoptotic events such as DNA fragmentation. Immunotherapy is a relatively modern approach in cancer therapy, by which the host immune system is stimulated to destroy cancer cells. The antitumor efficacy of some chemotherapeutic agents can be achieved through eliciting the immune cells such as Th1 and cytotoxic T lymphocytes, in addition to direct inhibitory effects on cancer cells (Ye et al., 2017). *Oliveria decumbens vent.* is a traditional medicinal herb belongs to the Apiaceae family and grows in west-southern mountains of Iran. The hydrophytopic property of *Oliveria* essential oil (OEO) allows passing through the membrane and reaching inside the cell. This plant has been used in the traditional treatment of a spectrum of diseases. There are some Persian medical sources that show this plant (also called “Shavasara or Moshk choopan”) has been used as a healing agent for inflammation, cancer, and infections traditionally (Gahreman and Okhovat, 2010; Khorasani, 2004; Tonekaboni, 2008). Anti-oxidant, anti-inflammatory and anti-microbial activity of OEO has been investigated in previous studies, however, the anticance activity of OEO is less clear (Jamali et al., 2018). 4T1 is a mammary carcinoma cell line with highly tumorigenic and invasive properties. Based on these properties, the 4T1 mouse tumor is a suitable animal model for human breast cancer (Pulaski and Ostrand-Rosenberg, 2000). In the current study, our aim was to consider the in-vitro effects of OEO on cell survival and death mode in 4T1 cells in 2D and 3D culture and to study the anti-cancer mechanism induced by OEO in 4T1 monolayers. After that, the anti-tumor activity of OEO was investigated in-vivo in a mouse model followed by the analysis of immunomodulatory effects associated with OEO.

2. Material and methods

2.1. Preparation and analysis of OEO

*Oliveria decumbens* (the local names are “den”, “denak” and “moshkhorak”) is the only species of the genus *oliveria* (the plant name has been checked with [https://www.gbif.org and http://www.ipni.org/](https://www.gbif.org and http://www.ipni.org/)) from Apiaceae family (herbarium number: 55078). The aerial parts of *Oliveria* were collected from the mountainous areas of Fars in Iran (two kilometers around the position of 29°34'18.4"N 51°32'46.5"E). For extraction of essential oil, dried plants were subjected to hydroydistillation for 3 h in an all-glass Clevenger-type apparatus (Herbal Exir Co., Mashhad, Iran). In this method, plant materials were soaked in water. The water was boiled to produce steam bearing the most volatile chemicals. The steam was then chilled, and the resulting distillate was collected. The essential oil normally float on top of the aromatic water and was separated from aromatic water via a decanting funnel. The essential oil was dehydrated over anhydrous sodium sulfate and stored at 4 °C.

OEO was analyzed by an Agilent 7890A series gas chromatograph (Agilent, Palo Alto, CA, USA) which is equipped with a flame ionization detector (FID) on a fused silica capillary HP-5 column (30 m × 0.32 mm i.d. and film thickness 0.25 μm). The injector volume was 0.1 μl and split ratio of carrier gas (helium) was 1/40. The temperature was set 60°C–240°C. The detector and injector temperatures were set at 250 and 240°C respectively. GC/MS was carried out by use of Agilent gas chromatograph coupled with Agilent 5975C mass spectrometer equipped with a column HP-5MS. Ionization source temperature was set at 280°C and the temperature and carrier gas were the same as above. The constituents of OEO were recognized by comparison of Retention indices (calculation for volatile constituents using a homologous series of n-alkanes C8-C25 indices) with those described in the texts (wiley7n.l and NIST05a.L libraries).

2.2. Preparation of OEO emulsion

At first, OEO-water mixture was formed by adding of OEO (1.0 mL) to water (100 mL). Then, polysorbate-20 (100 μg/mL) was added to this mixture. The mixture was incubated (for 24 h and at 35 °C) and finally, a milky emulsion was formed.

2.3. 4T1 and L929 cell culture

The spontaneously metastasizing murine mammary adenocarcinoma cell line (4T1) and mouse normal fibroblast cell line (L929) were purchased from National Cell Bank of Iran (Pasteur Institute, Iran). L929 and 4T1 cell lines were cultured in RPMI-1640 (Gibco) and DMEM (Gibco) medium respectively. These media were supplemented with 10% FBS (Gibco) and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). Both cell lines were incubated in a humidified incubator (containing 5% CO₂ at 37 °C) and passaged using trypsin/EDTA (Gibco) and PBS (phosphate-buffered saline) solution.

2.4. Formation of spheroids

For spheroid formation, a combination of two methods, hanging drop and liquid overlay, was used. The confluent cultures of 4T1 were trypsinized, washed in PBS and resuspended in the medium. Drops (50 μl) containing 3500 cells/drop were placed on the inner surface of a 60 mm dish lid, which was inverted over dishes containing 10 ml sterilized PBS to humidify the culture chamber. Hanging drop cultures were incubated for 3 days. The formed small spheroids at the bottom of drops were harvested using the wide-mouth tips. Then spheroids were seeded into wells of poly-HEMA precoated 96-well plates. For the preparation of these plates, 50 μl of poly-HEMA solution (0.5 μl poly-HEMA (Sigma) in 95% ethanol) was seeded in each well and then air dried was used for 3 days at 37 °C prior to the procedure. In the following, spheroids with homogenous size were seeded into each well of the round bottom of poly-HEMA pre-coated 96-well plate filled by 200 μl complete medium. The plates were incubated in a humidified incubator (at 37 °C with 5% CO₂). After 3 days, it was taken some photos using invert microscope (Ax overt 25, Zeiss, Germany at 5 × and 10 × magnitudes) and employed Image J software to estimate the size of spheroids. Formed spheroids were with diameters ranging from 500 to 600 μm.

2.5. Cytotoxicity assay in 2D and 3D cell cultures

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay was used to investigate cell viability. Briefly, the 4T1 and L929 cells (1 × 10⁶ cells/well) were grown in 96-well cell culture plates and after 24 h were exposed to various concentrations of OEO (0–200 μg/ml) for 24 h. To study the cytotoxic activity in 4T1 spheroids, after exposure of spheroids to OEO (0–200 μg/ml) for the same duration, spheroids were transferred to 96-well flat bottom plates, supernatant was removed and 200 μl of MTT reagent (5 mg/ml in PBS; Sigma-Aldrich) was added to each well and incubated for 4 h at 37 °C. Subsequently, the MTT solution was removed, and 100 μl of DMSO was replaced in each well and incubated for 30 min. Finally, the intensity of the purple formazan product was measured through the optical density (OD) at 492 nm by using an ELISA reader (Model wave xx2, BioTek, USA). Consequently, IC₅₀ (the concentration at which 50% cell proliferation is inhibited) was estimated from the concentration-response curves.

2.6. Fluorescent staining and apoptosis analysis

Apoptosis and cell viability were determined by fluorescent staining with ethidium bromide (EB)/acridine orange (AO). The untreated and
treated cells were harvested and centrifuged. The cell pellets were washed with cold PBS and then a mixture of EB/AO solution (1:1, v/v) was added to the cell suspension at a final concentration of 100 μg/mL. Finally, the stained cells were observed by a fluorescence microscope (Axioskop 2 plus, Zeiss, Germany).

2.7. Annexin V/propidium iodide staining and apoptosis analysis

Apoptotic 4T1 cells treated with OEO were identified using a FITC (fluorescein isothiocyanate) labeled Annexin V/propidium iodide (PI) apoptosis detection kit (BioVision). Initially, the cells were treated for 4 h with the IC50 of OEO, then were harvested, washed in cold PBS and stained with PI and FITC-conjugated Annexin V. Finally, stained cells were analyzed using flow cytometry (Partec PAS, Germany).

2.8. DNA damage analysis using DNA laddering assay

4T1 cells were cultured in 60 mm dishes, and harvested after treatment with IC50 doses of OEO and doxorubicin as a positive control. Then based on standard phenol/chloroform extraction procedure, DNA extraction was performed. Extracted DNA was dissolved in TE buffer and loaded on a 2% agarose gel. After staining the gel with EB, DNA was visualized using a gel doc system.

2.9. DNA damage analysis using comet assay

DNA damage induction of OEO in 4T1 cells was determined using the alkaline comet assay. 4T1 cells cultured in 6 well plates (3 × 10^4 cells/well) were treated with IC50 concentration of OEO for 4 h. The cells were then harvested and centrifuged. Pellets were re-suspended in ice-cold PBS buffer. Cell suspensions (1 × 10^6) were made in 100 μl of 1% low-melting-point agarose (in PBS) and pipetted into a frosted glass slide coated with a thin layer of hot 1% normal-melting-point agarose in PBS, covered with a coverslip, and incubated for 30 min in 4 °C by applying an electric current of 0.6 V/cm. Then to remove alkali, the slides were washed slowly in neutralization buffer (denaturing buffer) (300 mM NaOH, 1 mM EDTA, pH 13) for 30 min to allow unwinding of DNA and finally subjected to the electrophoresis for 1 h at 4 °C. Then the comet slides were placed in a chilled electrophoresis buffer (denaturing buffer) (300 mM NaOH, 1 mM EDTA, pH 13) for 30 min to allow unwinding of DNA and finally subjected to the electrophoresis for 30 min in 4 °C by applying an electric current of 0.6 V/cm. Then to remove alkali, the slides were washed slowly in neutralization buffer (0.4 M Tris, pH 7.5) for 5 min and then stained with 50 μl 1X SYBR green and incubated for 15 min in the dark and finally photographed using a fluorescent microscope (Axioskope 2 plus, Zeiss, Germany). At least 100 comets were scored and analyzed using open comet software. Tail length, tail DNA percent, tail moment and olive moment were considered in the comet analysis (George et al., 2018).

2.10. Reactive oxygen species (ROS) quantitation

Intracellular ROS generation was measured using 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH2-DA, Sigma-Aldrich). ROS in the cells causes the oxidation of DCFH and forms the highly fluorescent product 2′, 7′-dichlorofluorescein (DCF). The cells treated with OEO for 12 h were harvested, washed with PBS and incubated with DCFH-DA (20 μM) for 15 min in the dark. Finally, the intracellular fluorescence was evaluated using flow cytometry (excitation: 485–495 nm; emission: 525–530 nm, FACSCalibur, BD Biosciences).

2.11. Mitochondrial membrane potential (∆Ψm) assessment

Changes in the inner mitochondrial transmembrane potential (∆Ψm) were estimated using Rhodamine123 (Rh123) in 4T1 cells exposed to IC50 of OEO for 12 h. Treated and untreated cells were harvested, washed with PBS and incubated in 1 mL of Rh123 (50 μM) at 37 °C for 20 min in the dark. Then the cells were washed twice with PBS to remove extracellular Rh123. Finally, the fluorescent intensity of the Rh123 in the cells was scored immediately by flow cytometry (excitation: 488 nm; emission: 525–530 nm, FACSCalibur, BD Biosciences).

2.12. Caspase-3 activity assay

The caspase activity was measured using caspase-3 colorimetric activity assay kit (BIOMOL International, USA) based on the fluorometric detection of the chromophore p-nitroaniline (p-NA) after the cleavage of the synthetic peptide Ac-DEVD-pNA. Briefly, apoptosis was induced in 4T1 cells by different concentrations of OEO. Cells were trypsinized and lysed with the lysis buffer on ice. After centrifugation (12000g for 5 min), the supernatants were collected and their protein value was estimated by the Bradford method. Finally, equal amounts of protein (100 μg), 5 μL of caspase-3 substrate (AcDEVD-pNA, 2 mmol/L) and assay buffer were added to each reaction mixture and were incubated for 3 h at 37 °C. Finally, caspase-3 activity was calculated at 405 nm with a microplate reader.

2.13. Animals

Female BALB/c mice, 5–6 weeks old and with weights about 19 ± 1.0 g, were purchased from Pasteur Institute, Karaj, Iran and were allowed to adapt to the laboratory conditions for at least 1 week before the experiment. All animals were kept under standard housing conditions in polycarbonate cages at 85% relative humidity, 22 ± 3 °C temperature with a 12 h regular light-dark cycle with standard diet pellets and tap water (Azizi et al., 2017). The experiments were performed with approval from the animal ethics committee of Animal Care and Ethics Committee of the University of Tehran.

2.14. Animal model and treatment

The 4T1 tumor models were generated by injection of 1 × 10^6 cells in 50 μL PBS into the back of female BALB/c mice subcutaneously. For the experiment, 35 mice were divided into 7 groups. The first group was a control group without any injection of 4T1 cells, vehicle (sesame oil) and OEO. Group 2 was the control group without any injection of 4T1 cells but with OEO. The third group, negative control, was injected with vehicle alone. Group 4 was kept as tumor control, injected by 4T1 cells and without any treatment. Group 5 was the tumor group treated only by vehicle. Groups 6 and 7 were the treatment groups. Group 6 (major group) was injected by 4T1 cells and treated by OEO (intraperitoneal injection, every 2 days for 2 weeks) when the tumor volume reached to 4–5 mm (14 days after tumor inoculation). Group 7 was exposed to the 4T1 cell lines and OEO simultaneously to study the prevention role of OEO from tumor incidence. In this study, a high LD50 dose (the dose necessary to produce lethality in 50% of the mice) of 900 mg/kg for toxicity was determined and finally, the injected doses were normalized to be 450 mg/kg. All mice finally were killed (5 weeks after tumor inoculation), and the spleens were isolated for ex-vivo cytokine production.

2.15. Measurement of tumor growth and spleen

During the autopsy, tumors were isolated, measured with a caliper. Tumor volumes (mm3) were calculated by the following formula: as the volume = (tumor length) x (tumor width)^2/2. In addition, spleen and tumors weighed on digital scales (Ren et al., 2018).

2.16. Cytokine ELISA assay

At the end of the study, spleens were removed aseptically and dissected. Single spleen cell suspensions were prepared by gentle
homogenization, and red blood cells (RBCs) and red blood cells (RBCs) were disrupted with RBC lysis buffer (20 mM Tris, 160 mM NH4Cl, pH 7.4). The splenocytes were adjusted to 2.5 × 10^6 cells/ml in RPMI1640 (Gibco Life Technologies, Germany) supplemented with 10% fetal bovine serum (Invitrogen, Paisley Germany), 100 μg/ml streptomycin, and 100 IU/ml penicillin (Sigma, Germany) 2 mM l-glutamine. Cells were then seeded in 24-well plates and stimulated with 10 μg/ml E. coli LPS (Sigma, L2630) or 10 μg/ml Concanavalin A (Con A, Sigma C7275), at 37 °C in a humidified atmosphere of 5% CO2 for 72 h. Cytokine levels (IL-2, IL-4, IL-10, IL-6, IL-1β, TGF-β, TNF-α and IFN-γ) were determined by using ELISA kits (R&D System, Minneapolis, MN). The assays were carried out according to the manufacturers’ instructions. Values were normalized to total protein content as pg of cytokine per mg protein (mean ± SD, n = 5) (Kianmehr et al., 2015).

### 2.17. Liver biochemical tests

To the assessment of OEO toxicity, peripheral blood was collected into appropriate microtubes and their sera were separated using centrifugation at 3000 × g for 25 min. Then the levels of two liver enzymes, serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) of each sample were measured.

### 2.18. Statistical analysis

The results were presented as the mean ± standard deviation of three or more independent experiments. The significant differences between means were determined by the t-test and one-way ANOVA followed by Turkey’s post-test when statistical significance was P-value ≤ 0.05.

### 3. Results

#### 3.1. The chemical composition of essential oil

OEO was analyzed qualitatively by GC/MS. OEO was found to be rich in phenolic compounds including thymol (25.54%), carvacrol (23.12%), p-cymene (22.07%) and γ-terpinene (17.80%). In general, the monoterpenoid phenols (carvacrol and thymol) form the main components of OEO, but OEO is also composed of monoterpenes (p-cymene and γ-terpinene) (Fig. 1A, Table 1). Since amount of these contents change with some conditions such as collection time, geographical variation, etc., some previous studies reported a different amounts of OEO components, however, in all reports thymol is the main agent (Amin et al., 2005; Hajimehdipoor et al., 2010).

#### 3.2. Spheroids formation

3D cell culture models are better models than 2D cultivation to gain a deep understanding of the biology of cancer. In fact, Multicellular spheroids are mainstay in-vitro models to resemble tumor-like micro-environments and thus use in drug screening. The same anticancer drugs show different effects in 3D and 2D systems so that the cells in 3D cell condition, display a lower sensitivity to some chemotherapeutic drugs compare to 2D culture. To study the effect of OEO on 4T1 cellular spheroids, we used a combination of hanging drop and liquid overlay methods that resulted in spheroid formation. In fact, spheroids were formed and gradually increased in diameter in 5 days. Analysis of phase-contrast images of spheroids indicated that the average diameter of spheroids was calculated 500 ± 5 μm (Fig. 1B).

#### 3.3. Cytotoxicity induction by OEO in 2D and 3D culture

OEO was applied at various concentrations on L929, 4T1 cells, and 4T1 spheroids. MTT assay was performed to detect the anti-proliferative activity of the OEO in these cell lines. In this assay, yellow tetrazolium MTT is reduced in mitochondria to purple formazan by succinate dehydrogenase. Our results showed that OEO does not have any inhibitory effect on L929 (IC50>200) while induces cytotoxicity in 4T1 cells with IC50 of 47.3 μg/ml (Fig. 1C). On spheroids, OEO treatment induced cytotoxicity with IC50 of 130.4 μg/ml (Fig. 1C). Thus, however, the OEO induces its cytotoxic effect in the spheroids, higher IC50 exhibits more resistant of 4T1 spheroids to inhibition by OEO compared to 4T1 monolayer cells. Therefore, these studies demonstrated that OEO is powerful anti-proliferative compounds in 4T1 cells and spheroids, producing concentration-dependent effects.

#### 3.4. Apoptosis induction by OEO in 4T1 cells

**Fluorescence microscopic analysis:** Using fluorescence microscopy and two dyes of AO and EB, cell death was qualitatively investigated. Ultimately, OEO was found to induce apoptosis in treated cell lines. Indeed, cells stained with AO, are green viable cells, while cells stained with EB are orange late apoptotic cells with condensed or fragmented chromatin and, stained cells with both AO and EB, represent green and orange early apoptotic cells with condensed or fragmented chromatin. Therefore, according to the staining, the chromatin condensation and loss of membrane integrity, it was shown that the mode of cell death was apoptosis in 4T1 cells treated with OEO (for 24 h) (Fig. 2A).

**Annexin V/PI staining:** Flipping of phosphatidylserine (PS) to the outer surface of the cell is one of the properties of apoptosis. The calcium-binding protein of Annexin V binds to PS and thus FITC fluorescence labeled to Annexin V (Annexin V-FITC) can spot PS in apoptotic cells. Since Annexin V is able to contact the ruptured plasma membrane, thus necrotic cell's membrane is detectable too. Therefore co-staining with PI can be useful to distinguish the apoptotic and necrotic cells. Cells in late apoptosis are positively stained with both Annexin V and PI, in early apoptosis are positively stained with Annexin V, whereas negative staining for both FITC-Annexin-V and PI are recognized as alive cells.

Flow cytometry-based detection with Annexin V-FITC/PI can quantify apoptosis in treated 4T1 cells. Our findings showed that OEO significantly induces early apoptosis (FITC-Annexin-V+/PI-) in 4T1 cells (after 4 h treatment) (Fig. 2B). The percentage of apoptosis in treated cells compare to that of control cells measured by flow cytometry showed that OEO can be a strong inducer of apoptosis in cancer cells.

#### 3.5. DNA fragmentation induced by OEO

**DNA ladder:** Nucleases like CAD (Caspases-activated DNase) degrades DNA during apoptosis. DNA fragmentation is an important hallmark of apoptosis(Zhang and Ming, 2000). Our results showed that treatment of 4T1 with OEO and doxorubicin (an inducer of DNA fragmentation) after 24 h led to DNA cleavage and observation of ladder pattern on an agarose gel (Fig. 2C).

**Comet assay:** To confirm the ability of OEO in the induction of DNA fragmentation, alkaline comet assay (the single cell gel electrophoresis) was performed(Ganapathy et al., 2016). “Hedgehog tails” which are traces of damaged DNA were observed in the fluorescent microscopy images of 4T1 cells treated with OEO while the control cells possess the intact “heads” without any tails. Therefore, the amount of DNA migration increased after 24 h treatment with IC50 of OEO. Collectively, the results indicated that the apoptosis induced by OEO is associated with DNA damage. Tail DNA percentage, tail moment, tail length and olive tail moment from the comet assay analysis were showed in (Fig. 3A).

#### 3.6. Caspase-3 dependent death induced by OEO

Caspase-3 plays a central role in the apoptotic signaling pathway.
Caspase-3 cleaves a wide range of cellular substrates at the C-terminal side of the aspartate residue of the sequence DEVD (Asp-Glu-Val-Asp). The caspase-3 assay is based on the cleavage of Ac-DEVD-pNA as a colorimetric substrate of caspase-3 resulting in the release of pNA. The absorbance of pNA at 405 nm allowed determination of the several-fold increase in caspase-3 activity in treated cells compared to untreated cells (Fig. 3B). Our findings indicated the apoptotic effect of OEO via a caspase-3 dependent pathway in treated cells.

3.7. MMP reduction by OEO

Since Mitochondrial dysfunction participates in the triggering of apoptosis and is central in the apoptotic pathway, to extra evaluate whether OEO is involved in this mechanism, mitochondrial membrane potential (MMP) in the 4T1 cells was estimated by Rh123 staining using as a colorimetric substrate of caspase-3 resulting in the release of the pNA. The absorbance of pNA at 405 nm allowed determination of the several-fold increase in caspase-3 activity in treated cells compared to untreated cells (Fig. 3B). Our findings indicated the apoptotic effect of OEO via a caspase-3 dependent pathway in treated cells.

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Table 1

Chemical compositions of Oliveria decumbens essential oil (OEO) identified by retention index and gas chromatography and gas chromatography-mass spectrometry.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Retention index</th>
<th>Relative percent in OEO</th>
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<tbody>
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<td>a-Thujene</td>
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<td>a-Pinene</td>
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</tr>
<tr>
<td>Myristicin</td>
<td>1520</td>
<td>3.43</td>
</tr>
</tbody>
</table>
| Retention indices were determined using retention times of n-alkanes as standard on fused silica capillary HP-5 column that were injected after essential oil under the same chromatographic conditions.

Caspase-3 cleaves a wide range of the cellular substrates at the C-terminal side of the aspartate residue of the sequence DEVD (Asp-Glu-Val-Asp). The caspase-3 assay is based on the cleavage of Ac-DEVD-pNA.
a flow cytometer. Mitochondrial transmembrane potential (ΔΨm) alteration is an indication of the mitochondrial permeability in the cell death systems (Zorova et al., 2018). Indeed, Fluorochrome of Rh123 incorporates into cells dependent on their mitochondrial transmembrane potential. Therefore, reduction of ΔΨm and quenching of Rh123 is along with the mitochondrial membrane permeability. The results showed that in treated cells with OEO, MMP decreased and the percentage of cells with depolarized ΔΨm (increase in Rh123 fluorescence means depolarization) increased compared to untreated cells (Fig. 4B). These results confirmed that treatment of 4T1 cells with OEO can induce apoptosis through the mitochondrial pathway. Additionally, since MMP alteration is related to the ROS level, intracellular ROS was considered in the following.

3.8. Intracellular ROS generation by OEO

Intracellular ROS plays an important role in the toxicity induced by some antitumor compounds and in the regulation of cellular apoptosis. To deepen into the OEO effects, we determined whether the OEO could modulate the ROS level. ROS generation was evaluated in the treated 4T1 cells using DCFH-DA oxidation and forming of fluorescent DCF subjected to flow cytometry. Our results showed that ROS levels in treated 4T1 cells were higher than that in control group (about 7 fold) (Fig. 4A). These results were in compliance with the obtained outcomes in ΔΨm studies.

3.9. Tumor growth inhibition by OEO

On the day the mice were killed, the mice weight, tumors volume and tumor weight were measured. The results indicated that group 6, which underwent OEO injection, in spite of the higher body weight, displayed lower tumor volume and tumor weight compared to other tumor-bearing control groups (Fig. 5A, B, C). Therefore our results exhibited a significant decreasing effect of OEO on the growth rate of the tumor. Additionally, obtained data from group 7, exposed to 4T1 cell line and OEO simultaneously to study the prevention role of OEO from tumor incidence, showed no effect of OEO on preventing tumorigenesis and also decreasing tumor growth.

3.10. Spleen weight

Spleen weight is associated with tumorigenesis and is an indicator of stress hematopoiesis (Liu et al., 2015). Our results showed a significant effect of 4T1 tumorigenesis on spleen weight. Our analysis indicated that spleen weight was lower in the tumor-bearing group which received OEO (group 6) compared to other tumor-bearing groups (Fig. 5D). This could indicate a decrease in erythropoiesis adopted from the tumor.

3.11. Enzymatic tests

The serum level of SGOT and SGPT was measured. We explored that OEO immunization causes no change in the serum level of these enzymes and therefore has no remarkable effect on liver function (Fig. 5E).
3.12. Cytokine determination in spleen cell culture

In our study, the cytokine level of splenocytes was measured using a sandwich ELISA assay (R&D System). Elevated levels of proinflammatory cytokine IL-1β, inflammatory mediator IL-6, immunosuppressive cytokine IL-10, and multifunctional cytokine TGF-β and low levels of pro-inflammatory cytokines IL-2, IFN-γ, and TNF-α were detected in untreated and vehicle-treated turmeric mice comparing to control groups. Our results showed that the levels of IL-10, IL-6, TGF-β, and IL-1β reduced in OEO-treated 4T1 inoculated mice (group 6), however, IL-2, IFN-γ, and TNF-α increased in this group compared to other tumor-bearing groups. In addition, our results displayed that IL-4 in splenocytes were not changed significantly in all groups (Fig. 6).

Based on these results and the calculation of IFN-γ/IL10 (Fig. 6), the Th1 type of immune response is more likely involved in tumor-bearing mice treated with OEO than other mice.

4. Discussion

Chemotherapy is considered the most common modality of cancer treatment. A chemotherapeutic should induce cytotoxic effects and reduce tumor growth, with the least damage to normal cells. In spite of extensive efforts for minimization of side effects and enhancement of drug efficacy, due to the inability to achieve the success, chemotherapy application is still very restricted in most of the cancers. Hence, to improve the chemotherapy options, there is a need for alternative agents with low side effects. Todays, plant products are interesting agents in cancer therapy, because of their actions and limited toxicity. These natural components can trigger several signaling pathways and ultimately regulate cell proliferation, apoptosis, etc (Cragg and Newman, 2005). For example, phenolic-rich plants exhibit the high growth inhibition of various cancer cells such as colon, prostate, and cervical cancer cells (Mazewski et al., 2018; Singh et al., 2018).

Due to lipophilic properties, the essential oils such as OEO have a unique ability to penetrate the cell membrane and reach inside the cell (Amorati et al., 2013; Gautam et al., 2014). In this study, by analyzing GC/MS and observing the abundance of thymol and carvacrol, OEO was identified as a phenol-rich substance and then its anticancer effects on 4T1 cell line in-vitro and 4T1 tumor in-vivo were evaluated. The growth inhibitory effect of OEO on 4T1 cells without any toxicity on L929, obtained from the MTT assay, showed selectivity effect of OEO on cancer cells and strengthened the suitability of OEO as an effective agent.

Although the success of monolayer cultivation in the investigation of drugs is significant, 2D is not able to imitate the complexity of clinical tumors. In fact, compacted spheroids from 3D cell culture are a bridge between simple in-vitro 2D culture and the in-vivo tumors.

Therefore, in this work, formed 4T1 spheroids were exposed by
OEO. Our data obviously confirmed the cytotoxicity of OEO. However, obtaining higher IC50 in spheroid studies showed that OEO has a lower cytotoxicity effect on spheroids compared to 2D monolayer cells. Since cell death induced by most drugs is mediated by triggering the apoptotic pathways, in the following to confirm the apoptotic mode death recognized under a fluorescent microscope, exposure of phosphatidylserine at the cell surface was explored through AnnexinV-(FITC) flow cytometry assay. In addition, DNA fragmentation which is the result of triggering of DNase enzymes by caspase activity was revealed in the treated 4T1 cells with OEO. Detection of caspase3 activity in untreated, treated cells showed the enhancement of caspase3 activity in OEO-treated cells which is essential in apoptosis. Generally, the level of oxidative stress in cancer cells is high. This property can lead to cancer development, however, this level of ROS can cause the susceptibility of cancer cells to additional ROS (Bazhin et al., 2016; Maroof et al., 2012). Apoptosis induced by several anticancer drugs is associated with enhancement of ROS. However, related mechanisms are not clear. In this research, investigation of the effect of OEO on ROS generation showed that treatment of cells with OEO increases ROS level.

Although increasing the mitochondria membrane depolarization increases the ROS level, extra ROS also cause to loss of mitochondrial membrane potential (ΔΨm). Indeed, during the early apoptosis, an important property is mitochondrial dysfunction which is a key event leading to caspase activity and finally the intrinsic pathway of apoptosis. Our findings demonstrated that OEO is able to decrease MMP and thus it suggested that OEO induces the apoptotic mitochondrial pathway in OEO-treated 4T1 cell lines.

It is worth to mention that in this study, very low concentrations of OEO were used and thus OEO is not able to behave like a pro-oxidant and change the culture medium in the used concentrations. According to previous studies, this essential oil has high antioxidant power and can scavenge the active oxygen and nitrogen radicals. Therefore, OEO not only does not produce active oxygen and nitrogen radicals such as hydrogen peroxide, but it is also capable of eliminating excess radicals.

Fig. 5. A) The effect of OEO on tumor growth and spleen in-vivo. B, C) Final tumor weight and volume in 4T1 model groups (p ≤ 0.05). D) Spleen weight index in control and 4T1-bearing groups (p ≤ 0.05). Tumors and Spleens were isolated and weighted, and spleen weight index were calculated as percentage of organ weight (gram, g) per total mouse body weight gram (g). E) The serum level of SGOT and SGPT (No significant difference was found in this measure).
in the culture medium (Esmaeili et al., 2018; Karami et al., 2019). Therefore, the effect of OEO on cancer cells is a direct effect resulting from permeability in the cell.

To further prove the relevance of our results, the efficacy of OEO on the inhibition of tumor growth in a mouse model of 4T1 breast cancer (in-vivo) was investigated. Our findings based on the reduction of tumor size and weight signified that OEO is effective in decreasing tumor growth. Additionally, in our tumor models, splenomegaly was significantly observed, however in the tumor groups with OEO injection, the spleen weight reduced. This reduction can indicate a decrease in hematopoiesis adopted from the tumor (DuPre’ and Hunter Jr, 2007; Liu et al., 2015). Furthermore, since the exposure to OEO induced no noticeable toxicity regarding animal weight and liver function, OEO could be a promising drug for breast cancer in the future.

The immune system plays an important role in tumorigenesis and also cancer therapy. An evaluation of the expression of antitumor cytokines is interesting to confirm the relation between OEO treatment and immune system towards tumor rejection. Therefore, in the present work, we studied the effect of OEO on immune responses of mice-bearing 4T1 breast cancer.

Th1 pathways through activating of cytotoxic T lymphocytes, Natural Killer (NK) cells and macrophages can defend against tumors. Indeed, the change of Th1/Th2 balance can influence the direction of the immune response, so that, enhancing the shift from Th2 immune response to Th1 pattern results in anti-tumor immunity (Kohn et al., 2011). TNF-α is a multifunctional cytokine with the important roles in apoptosis, cell survival, inflammation, and immunity. This cytokine is involved in tumor regression while some reports have also explained the role of TNF-α in tumor promotion (Yazdi et al., 2012). IL-2 plays a central regulatory role in the immune system and is necessary for the growth and activity of T cells, NK cells, and macrophages. IFN-γ plays a key role in cancer therapy due to a decrease in tumor growth and also a reduction in the metastasis (Fooladi et al., 2016). In the present study, the TNF-α, IL-2 and IFN-γ measurements as the Th1 cytokines showed a significant increase in the production of these cytokines in OEO-treated tumor-bearing mice compared to other tumor groups.

The previous studies also have shown the anti-tumor effects of some other agents attributed to increased IFN-γ and IL-2 and therefore NK activity (Lin and Zhang, 2004; Tsavaris et al., 2002; Yeap et al., 2015). Indeed, NK cells can influence the magnitude of T cell responses, specifically Th1 function through the production of cytokines such as IFN-γ and IL-2 that control the downstream immune responses and finally lead to death and therefore play a major role in the rejection of tumors (DeNardo and Coussens, 2007; Rezvani and Rouce, 2015). Therefore based on high levels of IFN-γ, IL-2 and TNF-α in OEO-treated tumor-bearing mice, we suggested that OEO has anti-tumor effects probably

Fig. 6. Measurement of cytokine levels secreted by splenocytes. Spleen cells were isolated and the cytokine levels produced from splenocytes stimulated by LPS or ConA were calculated in different groups (*p < 0.05).
through activating these innate anti-cancer cells. Generally, in cancer patients, the level of Th2 cytokines, IL-4, and IL-10 are high or unchanged (Razali et al., 2016). Our data from the evaluation of IL-4 showed that OEO affects slightly on the production of this cytokine in tumor mice. Numerous studies considered IL-10 as an immune suppressive cytokine which often acts indirectly and obstructs the Th-1 cytokine production (de LeBlanc et al., 2005). High levels of IL-10 decreases cytotoxic T cell responses during tumor growth resulted in inhibition of anti-tumor immunity. However, Dennis et al. showed that IL-10 is also essential for Th1 cell function and thus anti-tumor activity (Dennis et al., 2013). In this study, the reduction of IL-10 level, as a Th2 indicator, was obvious in OEO-treated tumor-bearing mice.

TGF-β with an anti-inflammatory activity drives the shift toward Th2 responses via IL-10-mediated pathways and directly through inhibition of the Th1-type responses in tumor-challenging mice (Maeda and Shiraishi, 1996). This cytokine is in high levels in tumors environment and contributes to the inhibition of T cells proliferation, causing immune-suppressive and angiogenesis properties. Additionally, Arteaga et al. showed that anti-TGF-β antibodies inhibit the progression of mammary carcinomas and increase mouse spleen NK cell activity (Arteaga et al., 1993). In addition, several cytokines such as TGF-β and IL-10 plays an important role in tumor escape from NK immune surveillance through inhibition of NK cell function (Maroof et al., 2012).

Our studies from the investigation of TGF-β production from splenocyte culture showed that OEO significantly decreases TGF-β level in mice-bearing 4T1 cancer and therefore it probably does not block the NK cells activity and T cell proliferation.

IL-1 family molecules, especially IL-1β acts with VEGF in maintaining angiogenesis and tumor invasiveness. High IL-1β levels in the tumor microenvironment have been observed in many studies in cancer patients. Our study showed a reduction of IL-1β in OEO-treated tumor mice. Since 4T1 is a cancer with high invasive capacity, this result can lead to a reduction of the chance of tumor invasion (Voronov et al., 2014).

Interleukin-6 (IL-6) is a cytokine with a dual role in Th1/Th2 differentiation and therefore tumor-promoting and tumor-inhibitory effects (Diehl and Rincón, 2002) (Knüpfel and Preiß, 2007). Sullivan et al. exhibited the effect of IL-6 in the induction of an epithelial-mesenchymal transition phenotype and thus metastasis in breast cancer cells (Sullivan et al., 2009).

In our cytokine assay, IL-6 reduced in splenocyte culture of OEO-treated tumor mice compared to other tumor groups. In this study, increased levels of IL-2, INF-γ, and TNF-α but no change in IL-4 level along with low levels of IL-6, IL-10, IL-1β, and TGF-β suggested the expansion of Th1 and development of an anti-tumor immune response by OEO. Therefore, taken together, our findings suggest that OEO may play a role in attenuating tumor growth by altering the cytokine milieu and anti-tumoral cell activation during 4T1 cell carcinogenesis.

5. Conclusion

In this study, we revealed that OEO shows the in-vitro capability to control the proliferation of 4T1 breast cancer cell lines in 2D and 3D cultures. Considering the 2D results, this inhibitory effect of OEO is via ROS generation, MMP reduction and finally inducing apoptosis. This outcome was even observed in OEO-treated tumor mice, confirming that OEO in spite of inducing the death in tumor cells, displays the anti-tumor effects due to the development of an anti-tumor immune response. Thus, OEO can be a potential compound with many benefits in breast cancer therapy. However, molecular events and biochemical pathways involved in anticancer activity of OEO are needed to be investigated further in the future.

Author contributions

Tahereh Jamali performed the experiments, analyzed data and wrote the main manuscript text. Email address: taheremjamali@gmail.com.

Gholamreza Kavoosi extracted, analyzed, supplied the essential oil and gave some advice in this project. Email address: ghkavoosi@shirazu.ac.ir.

Susan K. Ardestani supervised the project. Email address: Ardestany@ut.ac.ir.

Declaration of competing interest

The authors declare no competing interests.

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References


Tonekaboni, M.M.m., 2008. Tohfatul-Mo’menin Institute, Tehran, Iran.


