In Vitro and In Vivo Anticancer Activity of Ferula Gummosa Essential Oil Nanoemulsions (FGEO-NE) for the Colon Cancer Treatment

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Abstract
This survey was performed to aim of synthesize Nano emulsion from Ferula gummosa essential oil (FEGO-NE) and to evaluate its anti-tumor effect. First, Ferula gummosa essential oil was analyzed by GC–MS method, and then the Nano emulsion was synthesized as O/W and after characterization by DLS, Zeta potential, AFM, FESEM and TEM methods, its toxicity was evaluated by MTT method. Then its pro-apoptotic effects were evaluated by qPCR (Caspase3, 9, Bax and Bcl-2) method and AO/PI staining. The cancer induction model was used to evaluate the antitumor effects in Balb/C mice. The anti-angiogenic and antioxidant effects were evaluated by qPCR (VEGF, CAT and SOD) method. The results of physico-chemical studies showed the formation of droplet with dimensions of 24.6 nm, dispersion index of 0.41 and zeta potential of − 28.5 mV with a spherical morphology. The Nano emulsion synthesized at a concentration of 2.9 μg/mL inhibited about 50% of ht-29 cells, while up to a concentration of 4 μg/mL showed no inhibitory effect on normal cells. Increase of caspase 3, 9 and Bax and decrease of BCL-2 gene expression along with increase of apoptotic cells in AP/PI staining confirmed induction of apoptosis by FEGO-NE. The FEGO-NE showed an inhibitory effect on angiogenesis and an additive effect on the expression of antioxidant genes. In addition, the reduction of tumor volume (69.72% in 14 days) in samples treated with FEGO-NE was confirmed. The results of this study showed that FEGO-NE by various mechanisms are able to inhibit cancer cells and have a reducing effect on induced tumors in the in vivo model. These results suggest FEGO-NEs as a suitable candidate for cancer therapy studies.

Keywords Ferula gummosa essential oil · Nano emulsion · Apoptosis · Cytotoxic · Antiangiogenic · Anti-tumor

Introduction
Cancer is a deadly multifactorial disease that begins with the uncontrolled growth of cells and invades surrounding cells and tissues, leading to tumor formation and spread [1]. Lack of diagnostic techniques and standard treatment methods lead to the mortality increase due to this disease. The most common current treatment is the use of chemical drugs; however, these drugs are designed to target cells with high proliferation rates and are unable to differentiate cancer cells from normal cells with high proliferation rates. This can lead to some irreversible side effects [2]. Gradual resistance of cancer cells to treatment is one of the most important problems in common cancer treatments (chemotherapy and radiotherapy). Therefore, the development of new therapies is one of the goals of immuno pharmacological studies in order to increase the effectiveness of treatment [3].

Effective strategies for targeting cancer cells include induction of apoptosis, cell cycle inhibition, and angiogenesis, so natural products with such capabilities are valuable resources in suppressing cancer [2]. Bioactive compounds act through various mechanisms such as disruption of cellular signal transduction pathways, cell cycle change, interference with microtubules, and topoisomerase inhibitors [4]. Also, some studies have reported the anti-angiogenesis effects and antioxidant power of some phytochemical compounds such as flavonoids, polyphenolic acids, phenolic diterpenes, catechols and tannins, etc. [5].

Among plant-derived compounds, essential oils (EO) have anti-cancer properties. Although treatment with these compounds cannot replace chemotherapy and radiotherapy, it can be used in combination with cancer therapy to reduce the side effects of medications. Hence, such compounds can
be used to improve the health of patients and as a source of new anticancer compounds [6]. Essential oils contain 20–60 low molecular weight compounds (alkaloids, phenols and terpenes) that introduced by the FDA (FDA) and the EPA (Environmental Protection Agency) as GRAS (generally safe) substances [7]. Despite the high therapeutic effects, the susceptibility of these compounds to various chemical reactions, such as oxidation, causes instability and reduces or eliminates their effectiveness [8]. Other functional limitations of essential oils included their poor physical and chemical properties, including lack of solubility in water (lipophilic compounds of essential oils), high fluctuations and their rapid half-life noted [9].

Nano capsulation technology is one of the promising approaches for transporting and loading essential oils. The nanometer dimensions of these carriers cause the penetration of bioactive compounds into deeper tissues and increase their cellular uptake. In addition to increasing biological activity, they provide the possibility of controlling and modulating the release of active substances in the desired location. Finally, such an approach can increase solubility and reduce fluctuations and protect of bioactive compound in interaction with the environment [10].

In this study, Ferula gummosa essential oil (FGEO) was used due to its phytochemical components. This essential oil contains terpenoids such as α-pinene, which are known to be anti-cancer compounds [12]. The presence of hydrophobic and non-polar active compounds in this essential oil definitely reduces its solubility and thus its bioavailability. Therefore, to improve the bioavailability, this essential oil was encapsulated by amphiphilic molecules and formulated as a nanoemulsion [13]. The overall purpose of this study was to synthesize nanoemulsions from Ferula gummosa (Barieh) essential oil to increase bioavailability and to evaluate its anti-cancer effects.

Materials and Methods

Material

FGEO and Tween 80 (T-80) were purchased from Merck Co. The RPMI and DMEM cell culture medium, Streptomycin/ Penicillin, FBS (Fetal Bovine serum), tetrazolium dye MTT 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, and dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich. The human colon cancer (HT-29) and normal (HFF) cell lines were provided from Cell Bank of Ferdowsi University of Mashhad, Iran.

GC–MS Analysis

Gas chromatography-mass spectrometry (GC–MS; Agilent Technologies, Santa Clara, CA, USA) were used to analyze the FGEO components. The transfer line temperature and ion-source temperature were 250 °C and 200 °C, respectively. The temperature program was set at 60 °C (1 min) and 220° C (15 min) and the heating rate was set at 5 °C/min. The scan-range was 40–450 amu with a cycle time of 0.25 s.

FGEO-NE Emulsification

In order to achieve the optimal formulation, in this study, different volumes of Tween 80 (6, 12, and 18 mL) were used as surfactants. First, essential oil (3 ml) was added to the desired volume of surfactant and then deionized distilled water was added to the solution (until the final volume of 100 mL). The ultrasonic waves (hielscher-UP400-24 kHz, Germany) at 150-W and 20 kHz frequency were utilized for 2 min to optimize the emulsification [14].

FGEO-NE Physical Properties

The Dynamic light scattering (DLS) method was utilized to estimate the droplets’ Z-average as the FGEO-NE valid size index. Additional size approval tests were performed applying high-resolution atomic-force microscope (AFM), Field emission electron microscopy (FESEM), and transmission electron microscopy (TEM). The FESEM was carried out according to Hongwu Sun et al. protocol. Briefly, a thin layer of the FGEO-NE solution was dried and placed on the microscope glass gold-coated slide to be analyzed [17].

Cell Culture

The colon cancer cells (HT-29) and normal human foreskin fibroblasts (HFF) were cultured in RPMI and DMEM cell culture media (respectively) containing FBS (10%).
penicillin/streptomycin (1%) in 5% CO₂ incubator at 37 °C and 95% humidity.

**MTT Assay**

The FGEO-NE cytotoxicity on both HT-29 and HFF cells was studied by seeding 5 × 10^3 cells/well in a 96-well culture and incubating for 24 h. The cells were treated with different FGEO-NE concentrations (0.5, 1, 2, 4, and 8 µg/mL) for three incubation times (24, 48, and 72 h). The wells’ culture medium was then refreshed by a fresh MTT (5 mg/mL)-supplemented media and incubated for 4 h at 37 °C. The second refreshment phase was carried out by substituting the DMSO-supplemented fresh media and mixing for 10 min. Finally, the plate reader spectrophotometer (Stat Fax 2100) was utilized to record the samples’ absorbance at 570 nm. The cells’ viability was measured according to the following equation [18, 19]:

\[
\text{Cell viability}\% = \left( \frac{\text{Sample absorbance}}{\text{Control absorbance}} \right) \times 100
\]

**Gene Expression Measurements**

Following the HT-29 treatment at different concentrations of FGEO-NE (1.5, 3, and 4.5 µg/mL) and 48 h incubation, the cells were harvested to extract RNA utilizing the RNeasy Mini kit (Qiagen, Hilden, Germany). The samples’ total RNA content was then triggered for synthesizing the cDNA libraries by the Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany). The primer sets’ sequence of target genes including Cas-3, Cas-9, BAX, Bcl-2, SOD, CAT, and VEGF were designed for cDNA amplification. The GAPDH gene was applied as the housekeeping gene (Table 1). Finally, the SYBR Green PCR Master Mix (Qiagen, Hilden, Germany) was applied for measuring a comparative Real time-PCR analysis [20]. In order to perform the reaction, a mixture with a volume of 20 µl including of Syber Green (10 µL), specific primer (2 µL), of CDNA (1 µL) and DW (7 µL) was prepared and analyzed by CFX-96 Biorad.

**Acridine Orange/Propodium Iodide (AO/PI) Staining**

First, 1 × 10^6 cells/well in a 6-well culture plate was cultured and after 24 h, the cells were treated with 1.5, 3, and 4 µg/ml of FGEO-NE. After 48 h of incubation, the cells were harvested and put separately on glass slides. The cells’ staining was carried out by adding 1 µL fluorescent staining solution containing AO (100 µg/mL) and PI (100 µg/mL) to each cell suspension droplets (25 µL) and then covered with a coverslip. The apoptotic cell morphology was studied utilizing a fluorescent microscope (OLYMPUS, Japan) [21].

**Murine Colon Cancer Model**

This experiment was performed in full compliance with ethical principles and obtaining a code of ethics (IR.IAU. MSHD.REC.1399.070). Initially, 32 male Balb/C mice were purchased from Pasteur Institute of Iran and after transfer to the laboratory and adaptation to the new environment; cancer induction was done in each sample with injection of 100 µL of CT-26 cell suspension (3 × 10^5) subcutaneously. After observing the tumors, the samples were treated (intraperitoneal injection) with different concentrations of the FGEO-NE once in every 2 days for 14 days, no treatment was performed in the control samples. Tumor size was measured on days of treatment using a digital caliper and after the fourteenth day, the samples were killed with chloroform and the tumors were removed and transferred to 10% formalin. Tumors after incision using microtome were stain and the obtained slides were examined by the pathologist.

**Statistics**

Utilizing SPSS 21 software the one-way ANOVA tests were applied for all statistical calculations. The p-values (p-value < 0.001) were considered as statistically significant levels.

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**Table 1** The sequences of target primer sets (Cas-3, Cas-9, BAX, Bcl-2, VEGF, CAT, and SOD) and control (GAPDH) gene

| Gene     | Forward             | Reverse             |
|----------|---------------------|---------------------|
| GAPDH    | 5’TGGGTGGCAGTGATGGCATGG 3’ | 5’GGAGAGGAGGACCGAGCC 3’ |
| Cas-3    | 5’CTGGACTGTGGCATTGAGAC 3’ | 5’ACAAAGCGACTGTGAATGACC 3’ |
| Cas-9    | 5’CCAGATTCGCAAAACAGG 3’ | 5’GAGCACCACATCAACAAATCC 3’ |
| BAX      | 5’TCTGGCTGACGGTTGGTCATCCA 3’ | 5’CTCCATGTACCTGCTCCAGTCGT 3’ |
| Bcl-2    | 5’GTATGTTGTTGGAGAGCGTCAC 3’ | 5’CATGAGGCAACCAGGTGTATG 3’ |
| VEGF     | 5’CTGGCTGCTTTGGCTGATTTG 3’ | 5’TTCACATTGTGTTGCTGTAG 3’ |
| CAT      | 5’GGAGAGGAGGACCGAGCC 3’ | 5’AGTCAGGCTGGACCTGATG 3’ |
| SOD      | 5’GGAGAGGAGGACCGAGCC 3’ | 5’CACATTGTGCTGCTGTAG 3’ |
Results

GC–MS Analysis

Data from the standard mass spectrum reported 51 components, accounting for 99.5% of the total frankincense essential oil (Table 2). Chromatogram results showed that alpha and beta-pinene accounted for about 17.9% and 58.5% of the total essential oil composition (respectively).

FGEO-NE Characterization

The results of different formulations based on the amount of surfactant showed that the formulation containing 9% surfactant (9 ml T-80) has the smallest nanoemulsion size (16 nm) with the acceptable dispersion index (0.530), which was considered as the selected formulation (Table 3). Regarding the Steinfeld et al. findings, Nano emulsions with less than 0.7 PDI values show a mono-distribution formulation, which is required for the reliable Z-average values [22].

Examination of the samples stability during 16 days after storage showed an increase in particle size to 24.6 nm and a decrease in the dispersion index to 0.414 (Fig. 1A/left), which indicates the stability of the formed nanoemulsion. In addition, the results of sample ζ potential showed that the FGEO-NE have a surface charge of −28.5 mV, which according to the findings of previous studies; this value is within an acceptable range (−16 to −30 mV) to confirm the stability of nanoparticles [23]. Also, the TEM, FESEM, and AFM analysis verify the droplets’ size which had been measured by the DLS method (Fig. 1B, C). Examination of FGEO-NE morphology in TEM images shows particles with spherical, cylindrical and polyhedral morphology that according to the results of some studies, this morphology (Polymorphic) causes high efficiency of nanoemulsion.

FGEO-NE Cytotoxicity

The cytotoxicity effect of FGEO-NE against HT-29 as a cancer cells in comparison with HFF as normal cells was investigated by MTT method. As shown in the diagram (right), the toxicity of FGEO-NE against HT-29 cells depends on concentration and time. The toxicity effect of FGEO-NE on HT-29 cells 24 h after treatment shows that the lowest inhibition is observed at a concentration of 2 μL/mL (17%) and with increasing concentration to 4 and 8 μL/mL, the inhibition increases to 45% and 97% (Fig. 1 right). The median concentration (IC_{50}) of FGEO-NE against cancer cells 24, 48 and 72 h after treatment was reported to be about 4.3, 2.9 and 1.08 (Table 4) that indicating the time-dependent cytotoxic effects of FGEO-NE. Evaluation of inhibition of HFF cells in treatment with different concentrations of FGEO-NE (0.5, 1, 2 and 4 μL/mL) did not show a significant difference with untreated cells. However, with increasing the treatment concentration to 8 μg/mL, the inhibition rate increased above 90% in all three time efficiencies, indicating the concentration-dependent effects of FGEO-NE against normal cells (Fig. 1 left). Therefore, in this study, a concentration of 4 and 8 μL/mL of FGEO-NE were reported as a safe and toxic dose (respectively). Comparison of the FGEO-NE toxicity against normal and cancer cells confirmed the effect of its selective toxicity on cancer cells. This may be due to the complex diversity in cancer cell biochemical responses comparing with normal types. Moreover, high levels of metabolic activities in cancer cells make them vulnerable to cytotoxic compounds due to their involved antioxidant defense network. (See Fig. 2)

FGEO-NE and the HT-29 Cell Death Type

HT-29 Gene Expression Profile Following to HT-29 treatment with different concentrations of FGEO-NE, a significant up-regulation was detected for both anti-oxidant (CAT and SOD) and apoptotic (Cas-3, BAX, and Cas-9) gene expression. This is while the anti-apoptotic Bcl-2 and angiogenesis VEGF mRNA levels were faced with a meaningful down-regulation. The FGEO-NE treatment doses significantly lead to the CAT and SOD genes overexpression (p-value = <0.001) (Fig. 3). On the other hand, the HT-29 gene expression profile reflects the induction of apoptosis pathways and suppression of angiogenesis response in cancer cells following FGEO-NE treatments. Therefore, FGEO-NE can simultaneously neutralize both cancer cell strategies, anti-apoptosis and angiogenesis induction, despite of its cyto-protective impact (CAT and SOD up-regulating), which makes it appropriate to be used as an efficient anti-cancer therapeutic.

Fluorescent AO/PI Staining Following to HT-29 treatment plane, the AO/PI stained HT-29 cells revealed a significant positive relationship between the number of apoptotic cells and FGEO-NE concentrations, which approves the successful apoptotic pathways in response to FGEO-NE treatment. Figure 4 shows the occurrence of apoptosis in cells treated with FGEO-NE compared to untreated cells. In the control group, due to the infiltration of AO as a vital dye into healthy cells, most cells are green, while in FGEO-NE-treated samples, due to damage to the cell membrane, PI dye penetrated the damaged cells and caused to emitted red color in cells.

FGEO-NE Anti-Cancer Potential in the Murine Colon Cancer Model

Figure 5 shows images taken of tumor sections in two magnifications of 100 and 400. As shown in Fig. 5A and B, in the
Table 2 Essential oil compounds obtained by GC–MS analysis

| No. | Compound | % |
|-----|----------|---|
| 1   | α-Pinene, (1S)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene | 17.9 |
| 2   | Camphene, Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1S)- | 0.1 |
| 3   | Cyclohexane, 1,1′-dodecylidenebis[4-methyl | 0.7 |
| 4   | β-Pinene, Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1R)- | 58.5 |
| 5   | β-Mycrene, β-Pinene, Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)- | 2.8 |
| 6   | Decane | 0.2 |
| 7   | 3-Carene | 7.2 |
| 8   | Benzene, 1-methyl-3-(1-methylethyl), Benzene, 1-ethyl-2,4-dimethyl- | 0.3 |
| 9   | D-Limonene, Cyclohexene, 1-methyl-5-(1-methylethenyl)-, (R)- | 1.5 |
| 10  | α-Pinene, β-Ocimene | 0.2 |
| 11  | β-Ocimene, 3-Carene | 0.1 |
| 12  | Cyclohexene, 1-methyl-4-(1-methylethylidene)-, Cyclohexene, 3-methyl-6-(1-methylethylidene)- | 0.2 |
| 13  | Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene-, [1S-(1α,3α,5α)]-Bicyclo[3.1.1]heptan-3-ol, 6,6-dimethyl-2-methylene- | 0.7 |
| 14  | Pinocarvone, Sabinone | 0.3 |
| 15  | (3E,5Z)-1,3,5-Undecatriene, (E,E)-1,3,5-Undecatriene, (3Z,5E)-1,3,5-Undecatriene, 5-Undecen-3-ylene, (E)- | 0.4 |
| 16  | (3Z,5E)-1,3,5-Undecatriene, 5-Undecen-3-ylene, (E), (3E,5Z)-1,3,5-Undecatriene | 0.1 |
| 17  | Benzenemethanol, α,α,4-trimethyl-, m-Cymen-8-ol | 0.2 |
| 18  | (-)-Myrtenol, Bicyclo[3.1.1]hept-2-ene-2-methanol, 6,6-dimethyl- | 0.7 |
| 19  | trans-3-Caren-2-ol | 0.1 |
| 20  | Fenchyl acetate, α-FENCHYL ACETATE, Bicyclo[2.2.1]heptan-2-ol, 1,3,3-trimethyl-, acetate, (1S-exo)- | 2.0 |
| 21  | Benzene, 2-methoxy-1-methyl-4-(1-methylethyl), Benzene, 1-ethyl-2,4-dimethyl-, Benzene, 2-methoxy-4-methyl-1-(1-methyl) | 0.1 |
| 22  | 3-Methylbut-2-enoic acid, 4-cyanophenyl ester, 3-Methylbut-2-enoic acid, 4-nitrophenyl ester, 1,5-Heptadien-4-one, 3,3,6-trimethyl-, 3-Methyl-2-butenolic acid, cyclobutyl ester | 0.1 |
| 23  | Bornyl acetate, Bicyclo[2.2.1]heptan-2-ol, 1,3,3-trimethyl-, acetate, (1S-endo)-Acetic acid, 1,7,7-trimethyl-bicyclo[2.2.1]heptan-2-yl ester, Bicyclo[2.2.1]heptan-2-ol, 1,7,7-trimethyl- | 0.1 |
| 24  | 3(10)-Caren-3-ol, 4-acetoacetic acid ester, trans-Pinocarvyl acetate | 0.1 |
| 25  | 4,7,7-Trimethylbicyclo[4.1.0]hept-3-en-2-one, 2-Cyclohexen-1-one, 3-methyl-6-(1-methylethylidene)- | 0.1 |
| 26  | α-Terpiny acetate | 0.5 |
| 27  | 1,2,4-Metheno-1H-indene, octahydro-1,7a-dimethyl-5-(1-methylethyl)-, [1S-(1α,2α,3α,4α,5α,6α,7β,8α*] | 0.2 |
| 28  | Copaene, Ylangene, α-Cubebene | 0.1 |
| 29  | Cyclohexane, 1-ethyl-1-methyl-2,4-bis (1-methylethylidene)-, [1S-(1α,2α,3α,4α,5α,6α,7β,8α*]Cyclohexane, 1-ethyl-1-methyl-2,4-bis (1-methylethylidene)-, (1α,2β,4β)- | 0.1 |
| 30  | 1H-3a,7-Methanoazulene, octahydro-3,8,8-trimethyl-6-methylene-, [3R- (3α,3aβ,7β,8α)]-Cedrene, (3R,3aR,7R,8aS)-3,8,8-Trime- | 0.1 |
| 31  | 2-(4α,8-Dimethyl-2,3,4,4a,5,5a-hexahydronaphthalen-2yl)propan-1-ol, Tetracyclo[6.1.0.0(2,4).0(5,7)]nonane,3,3,6,6,9,9 hexamethylene-(1α,2α,3α,4a,5aβ,6aβ) | 0.1 |
| 32  | Isoledene, (1R,3aS,8aS)-7-Isopropyl-1,4-dimethyl-1,2,3,3a,6,8a-hexahydroazulene | 0.1 |
| 33  | (3aR,4R,8R,8aS)-3a,4,8a-Trimethyl-7-methyleneoctahydro-4,8-methanoazulene -rel | 0.2 |
| 34  | 1,4,7-Cyclodecatriene, 1,5,9,9-tetramethyl-, Z.Z.Z-Humulene | 0.1 |
| 35  | γ-Muurolene | 0.1 |
| 36  | Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethyl)-, [4aR-(4aα,7α,8α)]- | 0.2 |
| 37  | 2H-3a,9a-Methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-, [3R- (3α,5α,9α,9αα)]-2,2,5a,9- | 0.5 |
| 38  | Oxamide, N-(4-methobenzyl)-N′-(1-methylpropyl)- | 0.1 |
| 39  | Naphthalene, 1,2,3,5,6,8a-hexahydro-4,4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-, 1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydroazulene | 0.2 |
| 40  | (-)-α-Panasinsen | 0.1 |
| 41  | Cyclohexanemethanol, 4-ethenyl-α,α,4-trimethyl-3-(1-methylethyl)-, [1R- (1α,3α,4β)]-3,7-Cyclodecadiene-1-methanol, α,α,4,8-tetramethyl-, [s-(Z,Z)] | 0.1 |
| 42  | 1,5-Cyclodecadiene, 1,5-dimethyl-8-(1-methylethylidene)-, (E,E)- | 0.2 |
control sample, the tumor tissue is uniform and no change is observed. In samples treated with different concentrations of FGEO-NE, some areas, which are seen more densely colored, show areas where the cells have undergone apoptosis and the tumor tissue is out of uniformity. As can be seen, the amount of apoptotic areas increases with increasing concentration of FGEO-NE and in samples treated with 100% FGEO-NE, apoptotic areas increased significantly and its cells are observed in dark color. Examination of tumor size in treated samples compared to control samples (Fig. 5C) shows that the volume of tumors in control samples and samples treated with 50% FGEO-NE concentration increased over time. In samples treated with 75% of FGEO-NE, an increase in tumor size was reported until the fifth day, while in the following days, tumor volume showed a decreasing trend, and this result shows the inhibitory effect of FGEO-NE on tumor volume. The inhibitory effect of 100% FGEO-NE concentration on tumor size was observed from the first day of treatment. In fact, treatment with 100% FGEO-NE showed an inhibitory effect of about 69.7% on tumor growth.

Discussion

The increase in the incidence of colorectal cancer in humans and the lack of safe and effective treatments make the treatment of this disease face serious challenges [24]. In this regard, more targeted therapeutic strategies including nanocarrier systems to deliver bioactive compounds and drugs for more effective treatment of this disease have been used recently.

In this study, the chemical composition of Ferula gummosa essential oil (FGEO) was determined by chromatographic method and followed the FGEO was formulated to increase bioavailability and improve therapeutic properties in the nanoemulsion system. The results of chromatography showed that the highest percentage of essential oil ingredients is including β-Pinene, α-Pinene, Camphene, β-Myrcene, Decane, D-Limonene which is comparable to the compounds specified in Ferula gummosa and frankincense essential oil [25, 26].

In this study, formulation optimization was investigated using different concentrations of surfactant and the smallest nanoemulsions were obtained from a combination of 3% essential oil, 9% surfactant and 88% water by using ultrasonic method. Investigation the stability of FGEO-NEs after 16 days of storage confirmed a slight change in particle size (24.6 nm) and a decrease in the dispersion index (0.414). Investigation of zeta potential of FGEO-NEs reported the presence of repulsive force to prevent their agglomeration. Various methods are used to synthesize of nanoemulsions [27, 28], Among these, ultrasonic method is known as a low-cost, clean, fast and easy method [29], so in this investigation, similar to the some previous studies [29–32], this method was used to synthesize FGEO-NE.

The synthesis of nanoemulsions requires the presence of compounds called surfactants, which are active surface molecules that, due to their amphiphilic nature, reduce interfacial stress and cause dispersion of two immiscible phases [33]. Twin 80 surfactant is a non-ionic surfactant which makes it possible to form nanoemulsions with smaller dimensions in the presence of this surfactant [34]. Similar to the present study, in the synthesis of turmeric, basil,
The anti-tumor effects of FGEO-NE on inhibition of tumor growth in murine colon cancer model showed that FGEO-NE treatment, in addition to reducing the size of tumors, causes apoptotic and necrotic areas in tumor tissue, indicating its anticancer effect of FGEO-NE. Similar to the present study, in a study conducted in 2018, the pro-apoptotic effects of carvacrol nanoemulsion were confirmed via mitochondrial mediated apoptosis (Increase and decrease the expression of BAX and BCL-2 respectively), and very potent antitumor effects of this compound were reported in tumor-bearing mice [49]. Similarly in 2016, the antitumor effects of Nano emulsion loaded with Pipirline (10 mg/kg) were reported in mice with melanoma tumor [50].

Angiogenesis is a process that occurs in tumors and helps them survive and multiply. Inhibition of this process stops the supply of nutrients needed by cancer cells and is an effective way to control cancer. Some anticancer drugs target cancer cells in this way [2]. Inhibition of vascular endothelial growth factor (VEGF), which plays an important role in angiogenesis, is a major indicator of the anti-angiogenic behavior displayed by nanoemulsions. In this study, as expected, treatment of cells with FGEO-NE reduced the expression of VEGF gene as the main gene involved in the angiogenesis process. These results were also confirmed in the study of the anti-angiogenic effect of betulin nanoemulsion [51]. Also, in previous studies, the anti-angiogenic effect of nanoemulsion of lemon essential oil and memecylon was investigated and confirmed [52, 53]. Also, our findings reflect the overexpression of the antioxidant gene (CAT and SOD) in HT-29 cancer cells, which is expected to be up-regulated in response to toxic compounds [54]. In other words, the increasing FGEO-NE treatment doses induce the CAT and SOD genes up-regulation in HT-29 cells. This clearly reflects the improvement of HT-29 survival pathways following FGEO increasing concentrations. However, it is not dominant enough to prevent apoptosis in HT-29 cells.

Conclusion

In conclusion, *Ferula gummosa* essential oil nanoemulsion causes a significant cell-specific apoptotic death in HT-29 human colon cancer cells. It has also targeted two main cancer survival strategies including apoptotic suppression and angiogenesis induction in HT-29 colon cancer cells. Therefore, the FGEO-NE is suggested to be used as a cell-selective natural anticancer tool in human colon cancer therapy. However, there are still several apoptotic and angiogenesis genes that have to be studied to detect its exact anticancer mechanism.
Fig. 1 The FGEO-NE characterization data: A/left: The FGEO-NE size measurement during 16 days storage. A/right: The droplets surface charge (zeta potential) is at -28.5 mV. B/left: FESEM image shows the single and agglomerated nano-droplets (scale bar: 1 µm). B/right: TEM image of the FGEO-NE (scale bar: 30 nm). C: The white line has 189 nm lengths and its small internal red line shows the FGEO-NE droplet diameter (~24.34 nm)

Fig. 2 The FGEO-NE cytotoxic impacts on both colon cancer (HT-29) and normal (HFF) cell lines. The charts refer the cells viability percent under different FGEO-NE treatment doses (8, 4, 2, and 1 µg/mL). The “*: and “***” indicate the level of statistical significance (p-value < 0.05 and p-value < 0.001)

Fig. 3 The HT-29 colon cancer cell line gene expression profile. The fold changes values refer to the significant overexpression of Cas-3, cas-9, BAX, CAT, and SOD. This is while the Bcl-2 and VEGF genes have been down-regulated following to increasing FGEO-NE treatment doses (1.5, 3, and 4.5 µg/ml). The “***”, “**”, and “*” indicate the p-values <0.001, <0.01, and 0.05, respectively)

Table 4 The IC50 values of FGEO-NE in colon cancer (HT-29) and normal (HFF) cell lines; each measurement has been measured in triplicate manner (N = 3)

| Incubation times | IC50 doses for HT-29 (µg/mL) | IC50 doses for HFF (µg/mL) |
|------------------|-------------------------------|----------------------------|
| 24 h             | 4.380                         | 6.220                      |
| 48 h             | 2.923                         | 6.080                      |
| 72 h             | 1.087                         | 6.091                      |
Fig. 4 AO/PI fluorescent staining of the HT-29 colon cancer cells. The apoptotic dead and normal alive cells have been shown with orange and green colors, respectively. Following to increasing treatment doses of FGEO-NE (1.5, 3, and 4.5 µg/ml) the numbers of apoptotic dead HT-29 cells have significantly increased (Color figure online).

Fig. 5 Inhibitory effects of FGEO-NE on tumor growth in a mouse model of colon cancer. Histopathological results of tumors isolated from control and treated specimens at 100 (A) and 400 (B) magnifications. Tumor volume changes in treated samples compared with controls (C), the sample of tumor-bearing mouse (D).
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Declarations

Conflict of interest The authors declare that there are no conflict of interest.

Ethical Approval This material has not been published in whole or in part elsewhere. The manuscript is not currently being considered for publication in another journal. All authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content. Ethical Approval code: IR.IAU.MSHD.REC.1399.070.

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