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The synergistic effect of eucalyptus oil and retinoic acid on human esophagus cancer cell line SK-GT-4

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Abstract

Background: In order to improve cancer patients' chances of survival, scientists have prioritized finding alternatives to chemotherapy, focusing their efforts on natural sources. The current study investigates the anti-cancer action of retinoic acid and Eucalyptus oil in esophageal cancer and studies their combined effect as well as the cellular pathways that each trigger as part of ongoing research in this field. As a model of esophageal cancer, the SK-GT-4 cancer cell line was treated with a series of concentrations of both materials.

Results: The concentrations of Eucalyptus oil (10, 100, 1000, and 1500 g/mL) and Retinoic acid (5, 100, 150, and 200 M/mL) were used for treatment of cells. The MTT test was used to assess the anti-cancer activity of Eucalyptus oil and Retinoic acid, and qPCR was used to determine cellular pathways. Our findings show that both Eucalyptus oil and Retinoic acid inhibit cancer cell growth significantly. Our findings revealed that the IC50 values for eucalyptus oil were 63 g/mL and 111.3 M l/mL for retinoic acid. Furthermore, the impact was at the level that causes apoptosis. The findings suggested that any herbal substance could act as an inducer of the caspase-9-dependent pathway. The caspase-8-dependent pathway, on the other hand, was restricted to retinoic acid.

Conclusion: Our research discovered that the two chemicals worked together to create a synergistic effect. This synergistic effect could be attributed to a close connection between external and internal apoptotic pathways, which inhibits SK-GT-4 cell growth.

Keywords: Synergistic effect, Eucalyptus oil, Retinoic acid, Apoptotic pathway, Cell line SK-GT-4

Graphical Abstract

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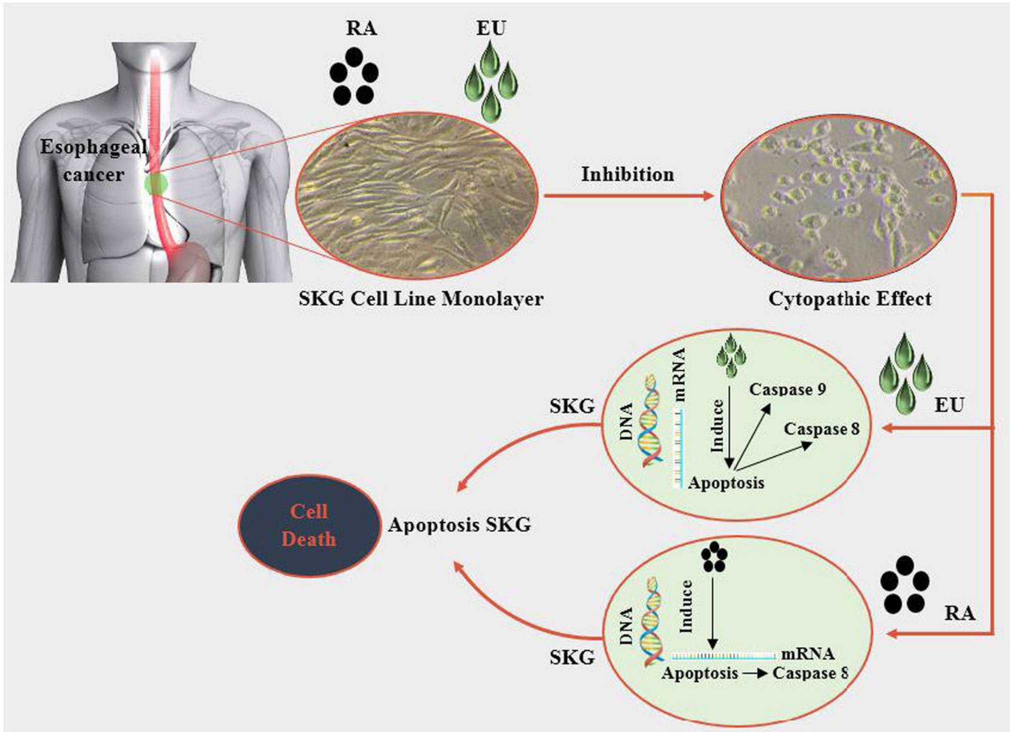

Background

Despite the fact that cancer research has been ongoing for a long time, it remains one of the world's most serious diseases. Chemotherapy has long been the treatment of choice for cancer [1, 2]. Chemotherapy drugs are currently available in fifty different formulations to treat more than 200 different cancers. Chemotherapy has a number of drawbacks, the most serious of which is its impact on healthy cells [3, 4]. Natural plant compounds have been widely used in pharmaceuticals for many years, and they have aided in the development of modern treatments [5]. These natural compounds have served as models for drug design, synthesis, and semi-syntheses [6].

There are over 200,000 known plant natural product structures. The majority of plant-based drug research has resulted in the development of anti-cancer drugs. Natural plant products are classified chemically into four groups based on their metabolic origin: alkaloids, phenylpropanoids, polyketides, and terpenoids [5]. Essential oils (EOs) and other phytoproducts are natural products that have gained popularity due to their chemical and biological properties. Eucalyptus, a species of tree belonging to the Eucalyptus genus, which contains over 800 species worldwide [7], is one of the plants with the most diverse natural products. One of the most commonly used essential oils in aromatherapy is eucalyptus. These oils have been shown to effectively treat a wide range of diseases. Anti-inflammatory, antiulcer, antidiabetic, antinociceptive, antipyretic, anti-diarrheal, antibacterial, and antifungal properties are evaluated [8, 9].

Furthermore, previous research has focused on the anti-cancer properties of Eucalyptus spp. essential oils, such as Eucalyptus torelliana, Eucalyptus camaldulensis, Eucalyptus Bentham, Eucalyptus globulus, Eucalyptus torquata, Eucalyptus sideroxylon, and Eucalyptus benthamii [10–12]. Retinoic acid (RA) is an active metabolite of vitamin A [13], a fat-soluble vitamin found in leafy greens, spinach, carrots, and yellow and orange fruits [14, 15]. It is found in plant-derived foods such as leafy greens, spinach, carrots, and yellow and orange fruits. Retinoic acid, which is required for cell growth and differentiation [14], has a variety of effects, including regulating embryonic development and generating differentiation, proliferation, Apoptosis, and resistance in cancer cells, among other things. Retinoids have been shown to have significant anti-cancer activity through non-genomic pathways (via extranuclear and non-transcriptional effects) in addition to their typical genomic action (binding to nuclear receptors and regulating the expression of downstream target genes) [13]. Previous research has shown that RA can help prevent various types of cancer, including breast, ovary, prostate, bladder, skin, and oral cavity cancers.

However, RA is not a particularly effective cancer treatment [16]. With the recent shift toward synergistic cancer treatment, combination therapy has become more widely used. This is due in part to the obvious benefit of attacking the disease from multiple angles, and research has recently focused on synergistic cancer treatment. Despite the fact that numerous effective combination-therapy therapies have been developed over the last few decades, these studies have yielded positive results [17–20]. Given the continued high number of cancer-related fatalities each year, there is an ongoing need to develop effective anti-cancer therapeutic regimens. Because currently available anti-cancer chemo-drugs do not target specific cancers and cause a variety of side effects and issues in the clinical management of many different types of cancer [21], there is an urgent need for innovative, effective, and nontoxic natural chemicals that are nontoxic and do not cause side effects [22, 23]. Although previous research indicates that Eucalyptus spp. and RA have anti-cancer properties, the underlying combination effect, particularly between Eucalyptus polybractea and RA, is unknown. The purpose of this research was to see if E. polybractea essential oil has anti-cancer properties.

Furthermore, no previous research has evaluated the combined effect of RA and E. polybractea essential oil as an anti-cancer agent, to our knowledge. In the current study, SK-GT-4, a human esophageal cancer cell line, was used, and a mixture of two natural chemicals of botanical origin was used to learn about their role in inhibiting malignant cells. Furthermore, their molecular mechanisms in cancer cells were studied.

Materials and methods

Maintenance and proliferation of cell cultures

The SK-GT-4 cancer cell line was provided by the IRAQ Biotech Cell Bank Unit in Basrah, Iraq. After reaching confluence, the cells were maintained in RPMI-1640 supplemented with 10% Fetal bovine, 100 units/mL penicillin, and 100 g/mL streptomycin. In brief, after wishing cell culture once with two milliliters of Trypsin-versene solution, cells were detached from flask with two milliliters of Trypsin-versene solution, and suspended cells with RMPI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 g/mL streptomycin were seeded in a 25-cm flask at 37. The suspension cells were divided into two halves for proliferation cells, which were then reseeded in a fresh flask in 5 mL RPMI-1640 supplemented with 10% Fetal bovine serum and incubated at 37 °C and 5% Co_2 for 24 h. When the cell culture reached 50% confluence, this procedure was repeated twice a week [24].

Cytotoxicity assays

The cytotoxicity of eucalyptus oil and retinoic acid on the SK-GT-4 cell was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. After trypsinization, cells were suspended in complete RMPI 1640 medium in a 25-cm flask, then seeded at 1*10 cell/100 µL per well in a 96 well plate and incubated at 37 °C in a Co_2 atmosphere for 24 h. Cells were treated with four different concentrations of pure Eucalyptus oil derived from Eucalyptus polybractea (purchased from Felton Grimwarde and Bosisto's (FGB), Pty Ltd 61 Clarinda Rd, Oakleigh Sth, Vic 3167) (10, 100, 1000, and 1500 g/mL) and four different concentrations of Retinoic acid (purchased from Sigma Aldrich) (5, 100, 150, and 200 µM/mL). Untreated cells that received only 0.1% dimethyl sulfoxide (DMSO) medium and serum-free medium served as vehicle and negative control groups, respectively. The MTT assay was used to determine cell viability after 72 h of exploration. As a result, an MTT solution (10 l MTT (5 mg/mL) and 90 l serum-free media) was added to each well. The precipitates were dissolved in 100 L of DMSO after a 2-h incubation period. A microplate reader was used to measure absorbance at 620 nm [25]. The values displayed are the means and standard deviations of three independent experiments performed in triplicate. The viability rate of cells was estimated using the following equation:

The proliferation rate (PR) is calculated as $(\text{PR}) = B/A * 100$, where A is the mean optical density of untreated wells, B is the optical density of treated wells, and the inhibition rate (IR) is calculated as $(\text{IR}) = 100 - \text{PR}$ [26].

Inhibition concentration that kills 50% (IC50) of cells

The IC50 values of Eucalyptus oil and Retinoic acid were calculated using the GraphPad Prism 8 program software. The SK-GT-4 cell line's IC50 was calculated using three replicates of inhibition for each concentration.

The combined effect of eucalyptus oil and retinoic acid

The results were also examined using Compusyn software version 1 [27]. Cells were given three concentrations of eucalyptus oil (10, 100, and 1000 g/mL) and retinoic acid (5, 100, and 200 M/mL). Cells that had not been treated served as a control group. In a humidification incubator, treated and untreated cells were incubated for 72 h at 37 °C with 5% Co_2. The MTT assay was used to calculate the rate of inhibition after exposure.

Cell morphology study

After the cell culture formed a confluent monolayer, cells seeded on coverslips (7 × 105 cells/cover) inside plate 6 were exposed to the IC50 concentrations of Eu and RA. The other coverslips, on the other hand, were left untreated as a control. The plate was then covered with adhesive paper and incubated for 24 and 72 h at 37 °C in a humidified atmosphere of 5% Co_2. Each treated and untreated coverslip has two replicates in the assay. After 24 h of treatment, a duplicate of each treated coverslip was stained in acridine orange/ethidium bromide AO/EB and immediately examined by fluorescence microscopy [28]. Simultaneously, the other treated and untreated caps were stained with hematoxylin and eosin dye after 72 h and examined under a light microscope to assess morphological changes.

Gene expression of Caspase 8 and 9 genes of SK-GT-4 cells lines treated with eucalyptus essential oil and retinoic acid

The suspension cells, after trypsinization, were seeded in 24 well plates 7 × 10^5, then incubated at 37c with atmosphere 5% Co_2. After gaining a monolayer, every three wells of plate treated with a concentration of IC50 of essential oil and Retinoic acid, respectively, and three other left as untreated wells which served as the control group. Then, the plate is incubated at 73c and under a humidified atmosphere of 5% Co_2 for 24 h. After the trypsinization process, the RNA was extracted with kits. The RNA extracted from cells according to the GEBEzol™ TriRNA Pure Kit steps transformed to cDNA according to the steps of BIONEER AccuPower® RocKetScript™ RT PreMix. Then, the genes required to determine the amount of their gene expression were amplified. And use the Hmn rRNA 18 s as a reference gene. The following table (Table 1) represents the sequences of the primers used.

Table 1 Sequences of primers of the studied genes (caspase-8, caspase-9, and Hmn rRNA 18S)

Genes	Primers	Base pairs
Caspase-8	5′-CATCCAGTCACTTTGCCAGA-3′ (FWD) 5′-GCATCTGTTTCCCCATGTTT-3′ (REV)	128
Caspase-9	5′-GTTTGAGGACCTTCGACCAGCT-3′ (FWD) 5′-CAACGTACCAGGAGCCACTCTT-3′ (REV)	129
Hmn Rrna 18S	5′-ATCTGTCAATCCTGTCCGTGT-3′ (FWD) 5′-GGAGTATGGTTGCAAAGCTGA-3′ (REV)	

The statistical analyses

Gene expression data were analyzed using SPSS software, and variance was analyzed according to the ANOVA test.

Results

Cytotoxicity assays of eucalyptus and retinoic acid in SK-GT-4 cell line

MTT colorimetry was used to assess the effect of Eucalyptus oil and Retinoic acid on the SK-GT-4 cell line in the study. The cytotoxic effect of Eucalyptus oil and Retinoic acid on the viability of the SK-GT-4 cell line was demonstrated by this assay. After 72 h, data from the MTT assay revealed that Eucalyptus oil and Retinoic acid inhibit the proliferation of SK-GT-4 cells. The effect of eucalyptus oil was dose-dependent, so the impact increased as concentrations increased (Fig. 1A). The value of viability for a low concentration of Eucalyptus oil (10 g/mL) is 78%, while the values for other concentrations (100, 1000, and 1500 g/mL) are (32, 26, and 24) %, respectively. The current study confirms that the inhibition concentration (IC50) of Eucalyptus oil is 63 g/mL. Furthermore, the effect of Retinoic acid increased

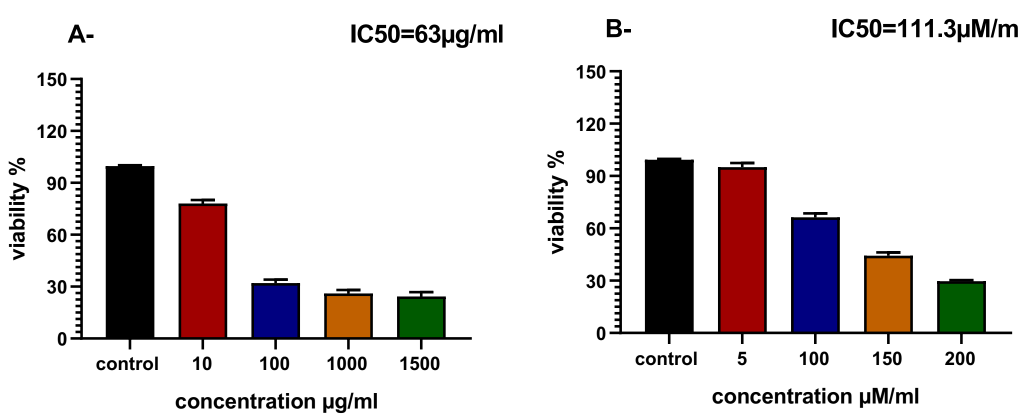

Fig. 1 Effect of both substances on the viability of Esophagus cancer cell line SK-GT-4 after 72 h of treatment ($n = 3$) **A** treatment with eucalyptus oil and **B** treatment with retinoic acid

Table 2 Value of CI for non-constant combination (EU + RA) of esophagus carcinoma SK-GT-4 treated for 72 h (*n* = 3)

Points	Concentration eucalyptus (μg/mL)	Concentration retinoic acid (μM/mL)	Effect	CI
1	10	5	0.59	0.20414
2	100	100	0.58	2.82187
3	1000	200	0.60	13.4156

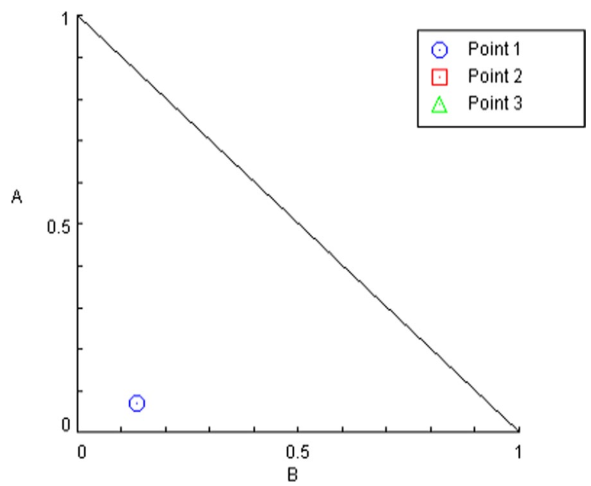

Fig. 2 Isobologram analysis showing the synergistic effect between EU and RA in the lowest concentration on SK-GT-4 cell line, after 72 h of treatment (Regarding points 1, 2, 3 note Table 2)

in a direct proportion to its concentration (Fig. 1B). As a result, the viability of a low concentration of Retinoic acid (5 M/mL) is 93%. In comparison, the viability value of concentrations (100 and 150) M/mL is (66.3 and 44.3) %, respectively, while the high value of viability was 29.7% with a concentration of 200 M/mL Retinoic acid. The current study confirms that the inhibition concentration that kills 50% of the bacteria (IC50) is 111.3 M/mL.

The combined effect of eucalyptus oil and retinoic acid

The result of the compusyn Isobologram software showing combination index CI data for three concentrations of both EU and RA after a 72-h exposure period, CI data indicate a synergistic effect between EU and RA in the lowest concentration due to CI 1, while the two other concentrations have antagonism effect due to CI > 1 (Table 2 and Fig. 2).

Cell morphology study

The morphological changes analysis

The shape of untreated SK-GT-4 appears as a fibroblast-like cell, but when treated with IC50 dose of EU and RA, it undergoes different changes and loses its distinctive shape as a fibroblast-like cell; additionally, the cytopathic effects of SK-GT-4 are increased with an increase in the treatment period. Furthermore, when SK-GT-4 was treated with the IC50 of EU and RA, shrinking cells were observed after 24 h, indicating the early dead stage, and round and aggregation cells were observed after 72 h. This refers to the final shape as the cytopathic effect of SK-GT-4 on dead cells is a swelling cell. Furthermore, due to dead cells, cells-spaces clear in SK-GT-4 tissue culture and then undergo lysis (Fig. 3).

Apoptosis analyzes

According to the AO/EB staining, the untreated SK-GT-4 cells had a fusiform shape with green cytoplasm and nuclei after 6 h of incubation (Fig. 3A, B). However, after 6 h of treatment with the IC50 doses of EU and RA, a number of treated SK-GT-4 cells exhibited signs of phenotypic apoptotic changes, including lysis of cytoplasm with yellow-green shrinkage nucleus as an early apoptotic cell and some cells with colored red nuclei and cytoplasm as late apoptotic cells (Fig. 4C–F).

Effects of eucalyptus oil and retinoic acid on the expression of caspase-8 and 9 genes in SK-GT-4 cells

Figures 5 and 6 show the effects of Eucalyptus oil and retinoic acid on the expression of caspase 9 and 8 genes in SK-GT-4 cells. Caspase 9 gene expression was significantly increased in cells treated with EU and RA. However, caspase 9 expression was significantly higher in EU treatment than in RA treatment. The level of significance between the values of Caspase 9 gene expression in untreated cells and cells treated with EU was (0.000), and the level of significance between cells treated with RA and untreated cells was (0.004) at a level of significance (0.05). Treatment with Eucalyptus oil increased Caspase 9 expression approximately 227-fold, whereas treatment with Retinoic acid increased Caspase 9 expression approximately 81-fold. Caspase 9 had a greater fold increase in the presence of eucalyptus oil than Retinoic acid. This indicated that Eucalyptus oil had a greater effect on caspase 9 than Retinoic acid (Fig. 5). The caspase eight gene expression was significantly increased in cells treated with retinoic acid. The level of significance between Caspase 8 gene expression values in untreated cells and cells treated with RA was (0.000) at a significance level of (0.05). On the other hand, the EU had little effect on caspase 8 expression. The level of significance between Caspase 8 gene expression values in untreated cells and cells treated with EU was (0.091) at a significance level of (0.05) (Fig. 6). Treatment with Eucalyptus oil increased Caspase 8 expression approximately 2.2-fold, whereas treatment with Retinoic acid increased Caspase 8 expression approximately 30.4-fold. Caspase 8

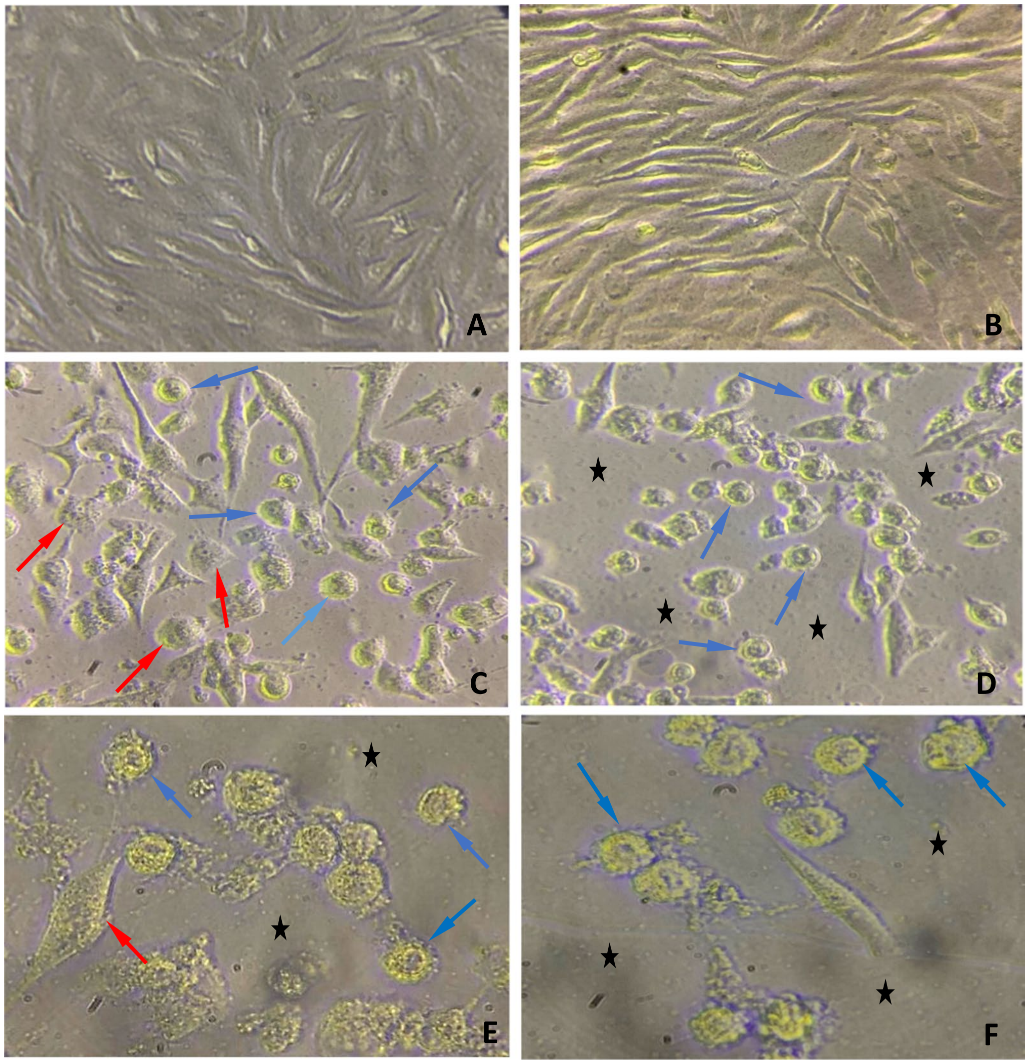

Fig. 3 Morphological analysis of SK-GT-4cell line following unstained. **A** Untreated cells after 24 h incubation, the SK-GT-4 show as fibroblast-like cell, ×20. **B** Untreated cells 72 h incubation, the SK-GT-4 show as fibroblast-like cell, ×20. **C** Treated cells with IC50 of EU after 24 h, blue arrows refer to the rounded cells as dead cells, red arrows refer to shrinking cells, ×20. **D** Treated cells with IC50 of EU after 72 h, in the filed most cells, shows as a rounded cell as dead cells (blue arrows), and appear more space clear from cells (star), ×20. **E** Treated cells with IC50 of RA after 24 h blue arrows refer to the rounded cells as dead cells, red arrows refer to swelling cells, and appear space between cells (star), ×40. **F** Treated cells with IC50 of RA after 72 h, blue arrows refer to the rounded cells as dead cells, and appear space between cells (star), ×40

increased by a much smaller factor in the presence of eucalyptus oil than Retinoic acid. This meant that caspase 8 was less sensitive to Eucalyptus oil than it was to Retinoic acid. Retinoic acid induced the caspase 8 and 9-dependent pathways in SK-GT-4 cells, whereas Eucalyptus oil induced only the caspase 9-dependent pathway.

Discussion

Chemotherapy has numerous side effects, prompting researchers to seek alternative methods that are both more effective against tumors and less harmful to the host. Natural plant products are one of the alternative methods proposed [5, 29, 30]. As a result, research has focused on describing active compounds in plants such as Eucalyptus [7, 31–33] and those that are a source of vitamin A (retinol) [34–37]. This is to determine its efficacy in inhibiting various cancers [36–40]. Previous research has shown that both Eucalyptus essential oil and retinoic acid have cytotoxic effects on cells [41–44]. Essential oils isolated from Eucalyptus genes are natural oils that are widely used in medicine, including cancer prevention and treatment [45]. The essential oil and

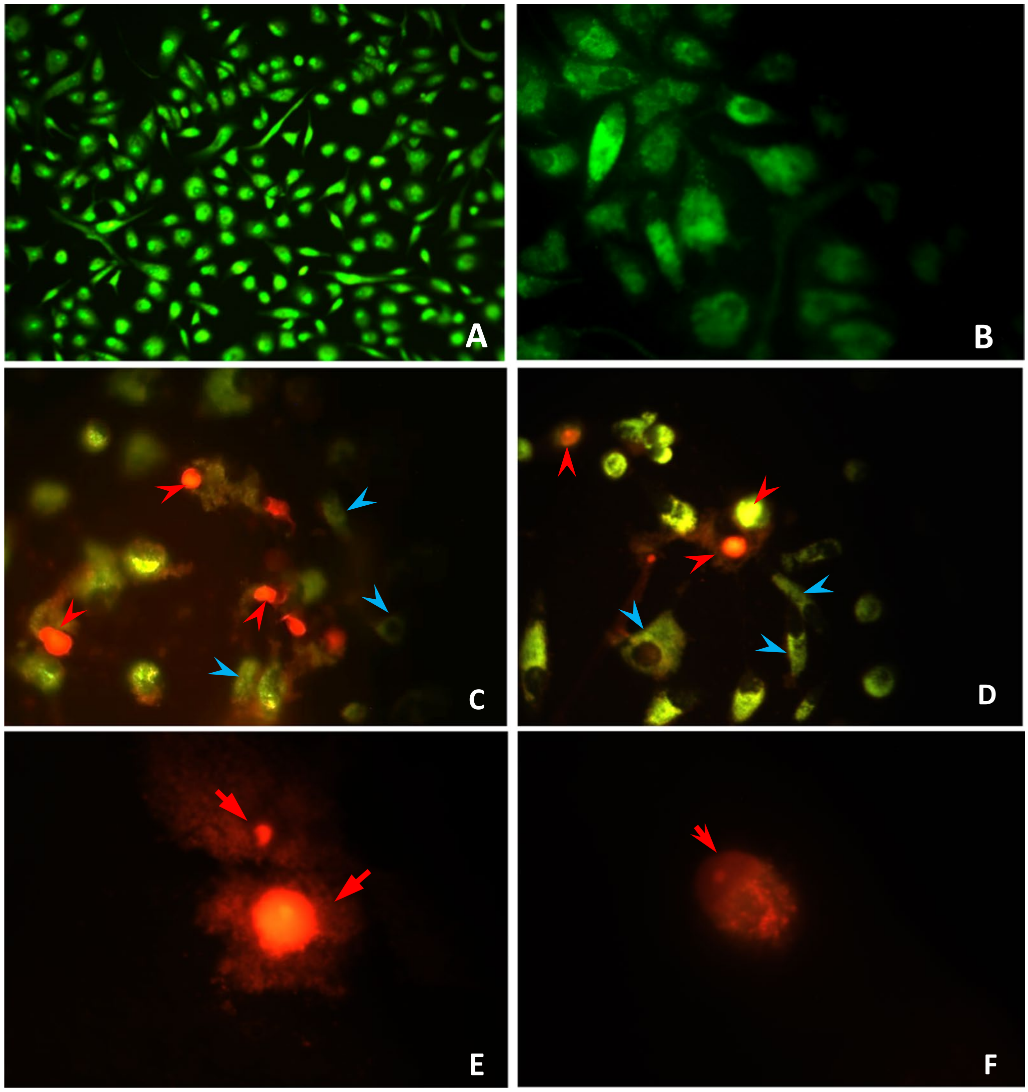

Fig. 4 Morphological analysis of SK-GT-4 cells line following acridine orange/ethidium bromide staining. **A** Untreated cells ×10. **B** Untreated cells ×40. **C** Treated cells with IC50 of EU, the viable cells (blue head arrows), the apoptotic cells (red head arrows) ×40. **D** Treated cells with IC50 of EU, the proapoptotic and apoptotic cells (red head arrows), the viable cells (blue head arrows) ×40. **E** Treated cells with IC50 of RA, the apoptotic cells (red head arrows) ×100. **F** Treated cells with IC50 of RA, the apoptotic cells (red head arrows) ×100

monoterpenoids of Eucalyptus sp. are responsible for inhibiting the growth of numerous human cancer cell lines. PC-3, Hep G2, Hs578T, and MDA-MB-231 [46], EAC [43], WEHI-3, HT-29, and HL-60 [40], HeLa and Jurkat [32], and mcf7 and Hep G-2 [47]. Furthermore, the essential oil of E. polybractea has a cytotoxic effect on the human esophageal cancer cell line SK-GT-4, and our MTT assay data revealed that the essential oil evoked a concentration-dependent cytotoxic effect on SK-GT-4. Because the essential oil contains many bioactive components such as cineol (82%), limonene and terpineol (3.67%), sabinene (1.98%), and others [48]. As a result, the essential oil has a wide range of activities, including cytotoxicity against cancer cell lines [40, 49]. Despite this, 1,8 cineol has the highest concentration in the essential oil of Eucalyptus polybractea [48]. Previous research has shown that 1,8 cineol alone has less or no toxic effects on cancer cell lines than oil [50, 51]. This clearly shows that proportion does not always account for the greatest share of total bioactivity and cytotoxicity of eucalyptus oil due to its constituents combined. Retinoic acid is a plant compound that belongs to the terpenoids family [52]. It is known that it promotes cell reproduction and differentiation in normal tissues and that it plays a role in the

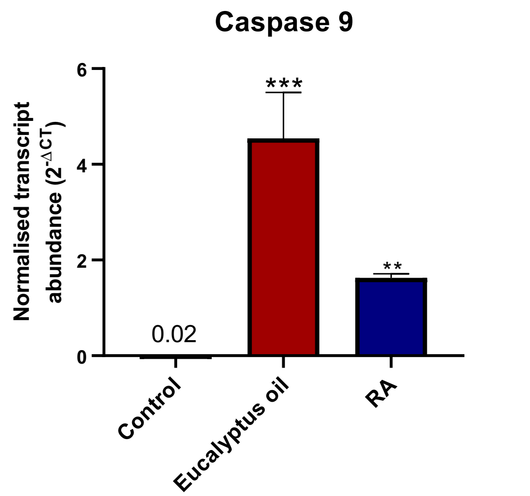

Fig. 5 Effect of EU and RA on gene expression levels for Caspase-9

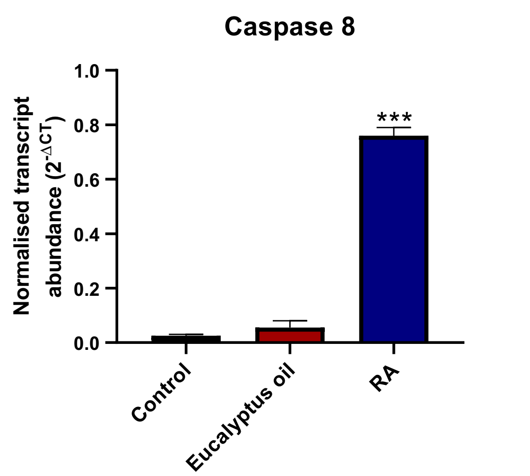

Fig. 6 Effect of EU and RA on gene expression levels for Caspase-8

embryonic development of some tissues, including nerve tissue [14, 37]. Whereas acts as a growth inhibitor for tumor masses [13]. Retinoic acid and its derivatives have frequently been used as anti-cancer agents against a variety of cancers, including breast cancer [34–36], lung cancer [35, 53], ovarian cancer [54], and cervix cancer [55]. The current study found that it is toxic to the human esophageal cancer cell line SK-GT-4. Its toxicity increased as concentration increased. In human cells, RA has two important receptors: Retinoic A Receptor (RARa, RARB, RARY) and Retinoic X Receptor (RXR), both of which are nuclear receptors [16]. The effectiveness of those nuclear receptors is responsible for the inhibitory activity against various tumors [56]. The current study was able to achieve the half-cells inhibitor concentration IC50 of both EU and RA using the mtt technique. The IC50 concentration values varied depending on the species of Eucalyptus tree from which the oil was extracted and the cell line type. On the WEHI-3, HT-29, and HL-60 cancer lines, the IC50 concentration of the oil extracted from the E. camaldulensis tree was (16.1 g/mL, 50.5 g/mL, and 42.1 g/mL, respectively [40]. On the MCF7 cancer cell line, the IC50 concentration of the extracted oil was 6.76 g/mL for E. sideroxylon and 5.22 g/mL for E. torquata [8]. On the human esophageal cancer cell line, the IC50 concentration for the oil extracted from E. polybractea is 63 g/mL. (SK-GT-4). These morphological changes are the result of biochemical and molecular events in treated SK-GT-4 cells [57]. A cell that has undergone Apoptosis has a distinct morphology in terms of shape, size, cytoplasm, and nucleus. We interpret the changes in SK-GT-4 cells as a progression of different stages of apoptosis. Since we discovered a link between the EU and RA, we can conclude that Apoptosis occurs. We looked at genes that are involved in both intrinsic and extrinsic pathways. Previous research has found that Eucalyptus oil and retinoic acid are involved in apoptosis and/or cell cycle arrest [25, 42, 43, 58–61]. The current study confirms this by examining the image of Acridine Orange—Ethidium bromide and analyzing gene expression. Apoptosis can occur in mammalian cells via two pathways: the caspase-dependent apoptosis pathway is the classic programmed cell death pathway, with two signaling mechanisms: the extrinsic pathway promoted caspase-8, 10, and 7. Caspase-9, 12, and 6 promote the intrinsic pathway [62]. The essential oil isolated from the Eucalyptus genus contains numerous bioactive compounds capable of inducing cell death pathways [42, 43, 63]. Our findings show that E. polybractea essential oil can induce apoptosis in the SK-GT-4 cell line by increasing caspase-9 mRNA levels. The essential oil isolated from the Eucalyptus genus contains numerous bioactive compounds capable of inducing program cell death pathways [42, 43, 63]. Monoterpenes are compounds found in the essential oil of Eucalyptus spp. that have been shown to prevent cancer at various stages [64]. The monoterpene Eucalyptus essential oil 1,8 cineole promoted the P38 gene, which splits PARP and activates caspase-3 in two types of human colorectal cancer cell lines [33]. In addition, trepinen-4-ol, a monoterpene found in Eucalyptus essential oil, activated the intrinsic apoptosis pathway by upregulating caspase-9 and 3 in A549 and CL1-0 cells [49]. Terpinen-4-OL also caused G1 phase arrest in AE17 and B16 murine cells [65]. P-menth-1-ene-4-7 (EC-1) [43], another compound isolated from Eucalyptus, increased gene expression of P53 and Bax genes related to apoptosis in EAC cells. Apoptosis was induced and the

GolG1 phase was arrested in mcf7 at low concentrations of RA [66–68]. The role of retinoic acid in tumor prevention is dependent on its receptors (RAR and RXR) [69–71]. RA receptors bind to conventional gene sites (RARE) [37]. This promotes the extrinsic Apoptosis pathway, which is regarded as a critical regulator of a caspase cascade. Independent of RARE, retinoic acid increases caspase-8 expression gene expression. However, it has been established that the various members of the death receptor-mediated apoptosis pathway are attributed to the recruitment of procaspase-8 upregulation. Finally, the procaspase-8 is cleaved and activated; when this happens, the cell enters Apoptosis [72]. Furthermore, RA and its variants promote apoptosis by binding to regulator proteins such as NF-KB [73], IFN-Y [74], VEGF [75], and TGF-B [76]. In the Jurkat cell line, retinoid-related molecules cause the released cytochrome C to activate the gene expression of caspase-9 and 3 [77]. The current study has confirmed that RA induces the extrinsic and intrinsic apoptosis pathways by upregulating caspase-8 and 9 gene expression. These findingswere confirmed by Hong and Lee-Kim [34] on mcf7 treated with RA isomers. The receptors RAR and RXR of RA have inhibited the wnt/b-catenin pathway, leading to apoptosis in the unorthodox pathway of RA. These receptors bind in the CREB region of the gene to promote caspase-8 gene expression [72]. Recent research has concentrated on the antagonistic or synergistic relationship between essential oils and chemotherapy [51]. Retinoic acid, on the other hand, is used in combination therapy with other drugs or materials [25]. In the current study, the essential oil is combined with RA to investigate their role as a combination therapy concurrently. The molecular reasoning related to their targets was used to create a combined EU and RA. Although essential oil and retinoic acid alone have cytotoxic effects and can induce apoptosis in human esophageal cancer cell SK-GT-4, encouraging results in preventing SK-GT-4 have been observed when combined with the EU and RA. This combination's inhibitor rates are 59%, 58%, and 60% at three concentrations. These findings revealed an antagonism effect on SK-GT-4 at the second and third concentrations, whereas have a synergistic effect on SK-GT-4 at the first concentration. The combined results show a synergistic strategy to improve Apoptosis only at the lowest concentration. The intrinsic apoptosis pathway (caspase-9) induces essential oil, whereas the extrinsic and intrinsic apoptosis pathways induce retinoic acid (caspase-8 and 9). As a result, the increased level of apoptosis may be regulated by a close interaction between these two apoptosis pathways at the same time. These events resulted in an increase in the inhibition rate of SK-GT-4, particularly at the lowest combination concentration. The combined results suggest a promising strategy for increasing Apoptosis by simultaneously stimulating extrinsic and intrinsic pathways at the lowest concentration.

Conclusion

It is well-understood that the goal of combination studies in tumor treatments is to increase the possibility and degree of therapeutic responses while decreasing the cytotoxicity of chemotherapeutic agents. The current study found that combining low concentrations of essential oil of Eucalyptus polybractea and retinoic acid stimulated apoptosis in SK-GT-4 more than high concentrations. This bioactive combination concentration may have a lower cytotoxic effect on normal cells. As a result, we recommend conducting additional research to better understand the roles and effects of Eucalyptus polybractea essential oil and retinoic acid.

Abbreviations

RA: Retinoic acid; EU: Eucalyptus oil; PR: Proliferation rate; IR: Inhibition Rate; AO/EB: Acridine Orange/Ethidium Bromide; IC50: The Half Maximal Inhibitory Concentration 50%; h: Hours; MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay; RPMI-1640: Roswell Park Memorial Institute (media.

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Authors' contributions

SMJF and AAA contributed funding and put forward the idea of the research, and the STA-S and AAAAAA-A contributed to conducting laboratory experiments and writing. All authors read and approved the final manuscript.

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Availability of data and materials

Preparation of Eucalyptus oil concentrations:
1. 94 µL oil + 6 µL methanol = 940 µg from oil per 100 µL.
2. N1 × V1 = N2 × V2
940 µg × = con. ? × 500 µL sfm (serum free media)
Preparation of Retinoic Acid concentrations:
1. A stocks are prepared from retinoic acid (1 mg/1 mL) using sfm.
2. N1 × V1 = N2 × V2
1000 µg × = con. ? × 500 µL (sfm)
There are no additional data because all the data related to the research was mentioned in the build of the research within the methods and results.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

There are no financial and non-financial competing for all research participants.

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