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Article

Dill seed oil as a possible contraceptive agent: antiangiogenic effects on endothelial cells

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Dill (*Anethum graveolens* L.) essential oil is wide spread in the food, beverage and pharmaceutical sectors. Dill is a member of the Apiaceae (Umbelliferae) family. It has the following biological activities: antioxidant, antifungal, antibacterial, antimicrobial, antihyperlipidemic, antihypercholesterolemic, antispasmodic, antiproliferative and anti-inflammatory. Aqueous extract of dill seed has reported effects on sex hormones and infertility potential. Moreover, boiled dill seed has an impact on reducing labor duration in giving birth. Implantation and placentation are necessary for a healthy pregnancy in the early stages. Angiogenesis is responsible for these essential processes. This study aimed to investigate dill seed oil's cytotoxic and antiangiogenic effects on rat adipose tissue endothelial cells (RATECs). Dill seed oil showed dose-dependent cytotoxicity on RATECs. It disrupted endothelial tube formation and depolymerized F-actin stress fibers. According to this study, depolymerization of F-actin stress fiber by dill seed oil could inhibit angiogenesis by suppressing endothelial cell proliferation, tube formation and motility. In other words, dill seed oil can be a new anti-angiogenic agent and a novel contraceptive.

Keywords: *Anethum graveolens*. Angiogenesis. Dill seed oil. Endothelial cells. Contraceptive.

INTRODUCTION

Apium plants belong to the Apiaceae family. They are among the herbs used in traditional medicine (Salehi *et al.,* 2019). There are about 20 types of flowering plants in this genus. (Sowbhagya, Srinivas, Krishnamurthy, 2010; Salehi *et al.,* 2019). They are medium to tall, biennial and perennial plants. They grow to one meter in swampy areas in subtropical and temperate regions. The leaves are pinnate or bipinnate with small white flowers. These plants are grown worldwide for their green leaves, bulbous roots, seeds (fruits), and petioles (Malhotra, 2012; Salehi *et al.,* 2019). It contains the phytochemical components bergapten, flavonoids, glycosides, furanocoumarins, furocoumarin, limonene, psoralen, xanthotoxin, and selinene (Salehi *et al.,* 2019). *Anethum graveolens* is commercially cultivated for its leaves (celery, smallage), roots (celeriac), seeds, and essential oil because of its characteristic smell and health benefits (Mencherini *et al.,* 2007; Salehi *et al.,* 2019). *Anethum graveolens* has various applications in food production as a flavoring ingredient or spice owing to its unique aroma and essential oil. Essential oils have been common constituents of plant foods and spice mixtures for centuries due to their therapeutic effects (Sowbhagya, Sampathu, Krishnamurthy, 2007; Sowbhagya, Srinivas, Krishnamurthy, 2010; Salehi *et al.,* 2019). One of the members of the plant family Apiaceae, dill, is an annual or biennial herb (Sintim *et al*., 2015) Dill grows up to 90-120 cm tall (Shyu *et al*., 2009). It has slender branched stem, finely divided leaves, small umbels (2-9

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cm diameter) of yellow flowers, and long spindle-shaped roots. The dill seed is aromatic, carminative, stomachic, mildly diuretic, and stimulant. Most of the time, it is used while flavoring in meats, stews, pastries, and vinegar as a whole or ground (Sintim *et al*., 2015; Weisany, Raei, Ghassemi-Golezani, 2016). Carvone and phellandrene are the most significant EO compounds in this plant, and in the fully grown seeds, d-carvone and d-limonene are the most critical compounds (Weisany, Raei, Ghassemi-Golezani, 2016). The quantity of carvone and α-phellandrene largely determines the properties of dill oil, with the characteristics of an herb oil predominating if the carvone content is less than 35% (Callan *et al*., 2007). The role of medicinal plants in human health services worldwide is undeniable. Moreover, some essential oil (EO) components of the medicinal plants are used in the industry (Weisany, Raei, Pertot, 2015).

Dill has the following properties: antioxidant (Sintim *et al*., 2015; Satyanarayana *et al*., 2004; Kazemi, 2015; Oshaghi *et al.,* 2016), antifungal (Fatope *et al*., 2006; Kaur, Arora, 2009; Ma *et al*., 2015), antimicrobial (Delaquis *et al*., 2002; Wahba, Ahmed, Ebraheim, 2010), antihyperlipidemic and antihypercholesterolemic (Yazdanparast, Alavi, 2001; Monsefi *et al*., 2014; Danesi *et al*., 2016; Zhenjing *et al*., 2018), antibacterial (Ma *et al*., 2015; Delaquis *et al*., 2002), antiinflammatory (Kazemi, 2015) and antiproliferative (Monsefi *et al*., 2014; Nakano *et al.,* 1998; Tsyganov *et al*., 2016). Dill is useful in treating several gastrointestinal diseases such as flatulence, gas, indigestion, stomachache, and colic as a folk remedy (Norman, 1990; Duke, 2001; Zhenjing *et al*., 2018). There is an antispasmodic effect of dill seed oil on the gastrointestinal tract (Fleming, 2000). Moreover, in nursing mothers, dill fruit enhances milk production (Norman, 1990; Zhenjing *et al*., 2018). The aromatic water of dill fruit (concentrated dill water) has a soothing effect on the digestive system. It is benefical in relieving hiccups and colic in babies, (Ishikawa, Kudo, Kitajima, 2002), bad breath, cough, cold, flu, and menstrual cramp pains (Weisany, Raei, Ghassemi-Golezani, 2016). It has been reported that dill seed extracts in mice have antisecretory and mucosal protective effects (Hosseinzadeh *et al*., 2002). Its antibacterial activity against a panel of rapidly growing mycobacteria with minimum inhibitory concentration (MIC) was in the range of 2-128 μg/ml (Stavri, Gibbons; 2005). Dill is beneficial while lowering blood cholesterol levels in hypercholesterolemic animals and humans (Danesi *et al*., 2016). Dill seed has an appetite-enhancing effect (Kazemi, 2015).

According to Monsefi *et al*. (2014) dill seed stimulated progesterone release and affected the menstrual cycle. Flavonoids such as kaempferol, myristicin, and vicenin are present in dill seeds. Among them kaempferol and vicenin have phytoestrogen features (Monsefi *et al*., 2014; Jana, Shekhawat, 2010; Gómez-Coronado *et al*., 2004). Phytoestrogens are nonsteroidal components similar to natural estrogens, an example of which is 17β-estradiol. They attach to alpha and beta estrogen receptors, and result in biological effects such as cell growth, differentiation, and maintaining general homeostasis of reproductive and other systems (Hall, Couse, Korach, 2001; Ososki and Kennely, 2003)

Successful implantation between a mother and an embryo is a complicated operation. Uterine, immunologic, thrombophilic, and embryonic factors affect this complex process. Successful implantation permits the provision of oxygen and nutrients and leads to adequate placental development. Implantation and placentation are necessary for the early stages of a healthy pregnancy (Jung *et al*., 2016). The process in which new capillaries grow from pre-existing capillaries and postcapillary venules is called angiogenesis. This is a tightly controlled process. Apart from wound healing, the development of the corpus luteum, and embryonic development, this process seldom occurs under normal conditions. However, persistent unregulated angiogenesis causes many problems (Wang *et al*., 2004).

The present study investigated the cytotoxic and antiangiogenic effects of dill seed oil in RATECs. The purpose of this study is to evaluate the potential use of dill seed oil as an angiogenic agent and as a contraceptive.

MATERIAL AND METHODS

Plant material

Ripe seeds of dill (*Anethum graveolens* L.) were purchased. Kemal Hüsnü Can Başer established the authenticity of the dill seeds and a sample was deposited

at the Department of Pharmacognosy Depository with number 2004:34. Crushed seeds were hydrodistilled using a Clevenger type apparatus with 3.6% oil yield on a dry weight basis.

Analysis of the essential oil

The oil was analyzed by capillary GC (Gas Chromatography) and GC/MS using an Agilent GC-MSD (Gas Chromatography - Mass Spectrometry) system.

GC and GC/MS analysis

GC-MS conditions:

The oils were analyzed by capillary GC/MS using an Agilent GC-MSD system (Agilent Technologies Inc., Santa Clara, CA). The column was an HP-Innowax FSC column (Hewlett-Packard-HP, U.S.A.) (60 m × 0.25 mm i.d., with 0.25 μm film thickness). Helium was the carrier gas (0.8 mL/min). The GC oven temperature was kept at 60°C for 10 min and raised 240°C at a rate of 1°C/min. The split flow was at 40 mL min^{-1} with a 40:1 split ratio. The injector temperature was 250 °C. Mass spectra were taken at 70 eV with a mass range of *m/z* 35-450.

GC conditions:

The GC instrument was an Agilent 6890N GC system fitted with a FID detector at 300°C. To obtain the same elution order with GC-MS, there was a simultaneous autoinjection on a duplicate of the same column with the same operational conditions.

Identification of compounds

Essential oil components were identified by comparison of their mass spectra with those in the Baser Library of Essential Oil Constituents, Wiley GC/MS Library, Adams Library, and MassFinder Library and confirmed by comparison of their retention indices. A homologous series of *n*-alkanes were the reference points in the calculation of relative retention indices (RRIs). The relative percentages of the separated compounds were calculated from FID chromatograms.

The analysis results are expressed as the mean percentage as listed in Table 1.

TABLE I - Main components of the dill seed oil. [3]

	Main components	%
1203	Limonene	38.2
1645	*cis*-isodihydrocarvone	1.5
1751	Carvone	56.4
2384	Dillapio	0.8

Cell culture

Growth of Rat Adipose Tissue Endothelial Cells (RATECs (Koparal et al., 2004) occurred in Dulbecco's Modified Eagle's Media DMEM (Sigma, St.Louis, MO, USA) which contains 10% heat-inactivated fetal calf serum (Sigma), 9.2% NaHCO3 and 1% penicillin/streptomycin (Sigma). RATECs were cultured in a humified atmosphere, containing 5% CO_2 at 37°C.

In vitro cytotoxicity assay

The colorimetric MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Mossmann, 1983) determined the cytotoxic effects of dill seed oil. First, stock solutions of dill seed oil were prepared in dimethyl sulfoxide (DMSO), stored at 4°C, and diluted in a fresh, complete medium. Cells ($3x10^3$/well) were seeded in 96-well microtiter culture plates in a final volume of 100 μl. After attachment for 24 h, the culture medium was removed, and the cells were treated with different concentrations of dill seed oil. There were eight replicate wells per concentration. Untreated controls (medium) and solvent controls (DMSO at a final concentration of 0.1% v/v) were conducted in parallel. After removal of the supernatant fluid, 200 μl/well DMSO was added and the mixture was shaken for 5 min. The absorbance was measured at 570 nm using a using a microplate reader (Bio-Tek, ELX808IU, USA). There were three or more replications in all

experiments. Statistical analysis of the MTT assay was performed with SPSS (Statistical Package for Social Sciences) software. One way ANOVA (Analysis of Variance), and Tukey test followed this evaluation. A value of $p<0.05$ was the threshold for statistical significance.

In vitro angiogenesis assay

Koparal *et al*. (2004) indicated that RATEC colonies showed the potential to form capillary-like structures when cultured on Matrigel. The matrigel tube formation process was as previously described (Quchi *et al*., 2004). RATECs were serum starved by culturing in endothelial cell basal medium-2 (EBM-2; Cambrex Bio Sciences, CC3156) with 1% FBS for 4 h. Serum starved cells were plated at a density of $1,5_X10^4$ cells/well on Matrigel. It coated the wells of 96-well plates after equilibration with EBM-2 medium (containing dill seed oil when indicated). Endothelial cells formed a network structure at 12 h, when they were cultured on Matrigel. Photographs were taken as representatives by using an Olympus CKX41 microscope.

Cytoskeletal immunofluorescence

Fluorescent phalloidin stained the F-actin cytoskeleton by the method described by Rubin *et al*., (1991) with slight modifications. Untreated control, solvent control, and dill seed oil-treated RATEC monolayers grown on glass coverslips (Marienfeld, Germany) were fixed with 3.7% (w/v) paraformaldehyde-phosphate buffer saline (PBS) for 15 min at 37 °C. The cells were then permeabilized with 0.5% Triton X-100 (v/v) in PBS for 5 min at 37 °C. Preparations were washed three times with PBS. 5 µg/ml tetramethylrhodamine B isothiocyanate (TRITC)-labelled phalloidin (Sigma) stained the cells for 1 h at 37 °C. RATECs were rinsed with PBS. Fluorescent images were viewed using an Olympus fluorescence microscope.

RESULTS AND DISCUSSION

Chemical analysis of dill seed oil

The GC-GC/MS analysis results are available in Table I. Limonene and carvone were the main components of dill seed oil, with 38% and 56% ratios, respectively.

Cytotoxicity

The effect of dill seed oil on the proliferation of RATECs was examined. The mitochondrial dehydrogenase (MTT) assay, evaluated the effect of dill seed oil on cell proliferation. The percentage of growth inhibition by dill seed oil at various concentrations on RATECs was determined as the percentage of viable-treated cells compared to viable cells of untreated controls. Dill seed oil treatment showed a remarkable dose-dependent decrease in cell viability.

The cells were incubated with increasing doses of dill seed oil (12,5µM, 25µM, 50µM and 100 µM). At the end of four days of treatment, the cell quantity was counted using a tetrazolium salt-based assay. According to the results, the inhibitory effect on cell proliferation was dose and time –dependent (Figure 1).

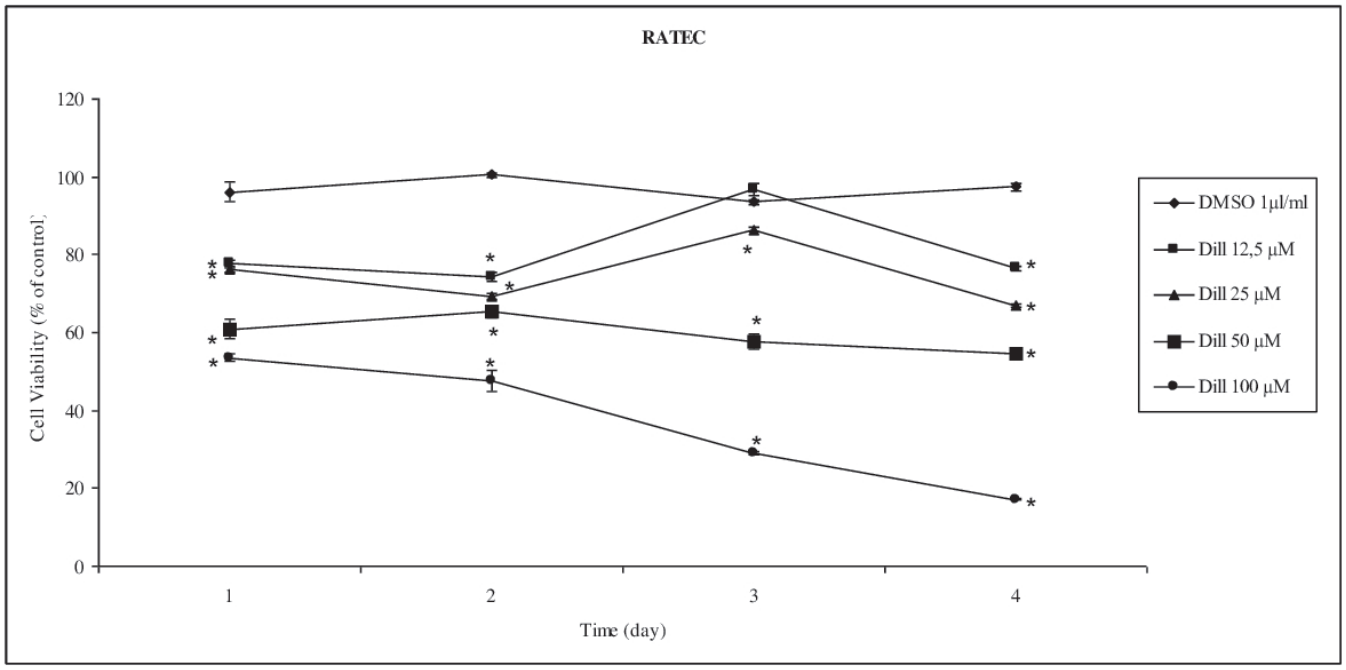

FIGURE 1 - Dill seed oil inhibits cell proliferation. Cell proliferation was measured using the MTT colorimetric assay after one, two, three and four days of exposure. The results are expressed as the mean ± SD. * Indicates a statistically significant difference from the control group by the Tukey test ($p< 0.05$).

In vitro antiangiogenic activity

Endothelial cells must rearrange themselves into a tube in the later stages of angiogenesis to create a new small blood vessel. The endothelial cell tube formation assay was used here as an *in vitro* model of this process. In the control and solvent control groups, RATECs on a Matrigel substratum displayed high motility and differentiated into well-defined network-like structures within 12 h. Dose-dependent inhibition of tube formation was observed when dill seed oil (12,5-100 µM) was added at the time of Matrigel planting. Photographs of cellular morphology were taken in four arbitrary areas per well to quantify the inhibitory effect of dill seed oil (Figure 2).

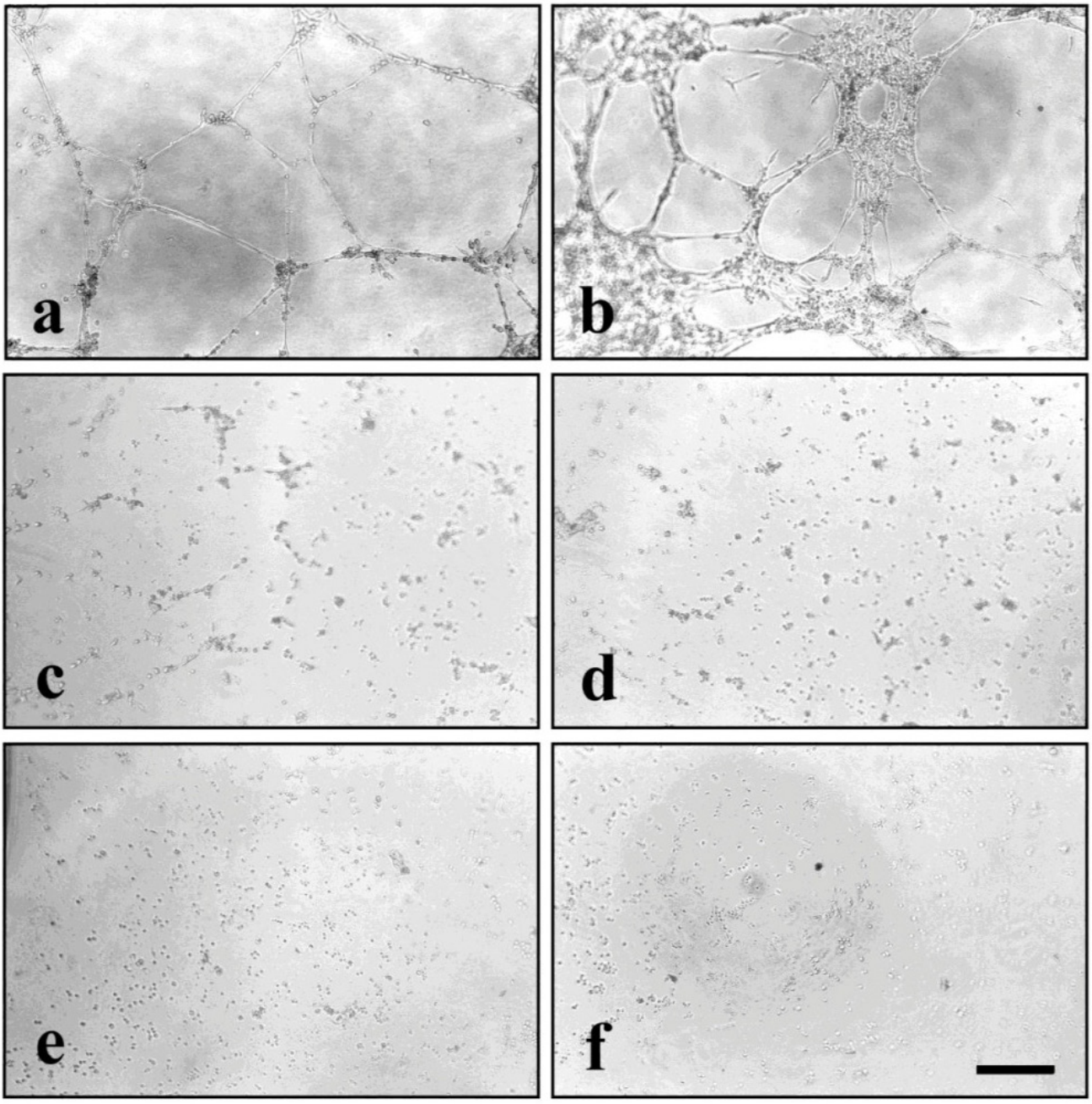

FIGURE 2 - Effect of dill seed oil on rat adipose tissue endothelial cell (RATEC) tube formation. (a) Control cells and (b) solvent control cells. RATECs were treated with 12,5 µM dill (c), 25 µM dill (d), 50 µM dill (e), 100 µM dill (f). The images are representative of independent triplicate assays. Scale bar:125 µm.

Disruption of the endothelial cytoskeleton

The organization of the cytoskeletal structure of F-actin filaments is necessary to maintain and modulate cellular morphology, structural integrity, and permeability of the endothelium (Carbajal, Schaeffer, 1999). Therefore, the impacts of dill seed oil on the cytoskeletal architecture were investigated with specific staining of F-actin filaments after 24 h of treatment. F-actin filaments in the center position were the stress fibers, and the periphery of the cells was the dense peripheral bands before applying dill seed oil.

RATECs were grown on coverlips. The endothelial cytoskeleton of the RATECs was in a normal distribution. Dill seed oil treatment caused dramatic disruption of the organization of F-actin filaments within the cells. There were reductions in the density of F actin filaments both in the center and peripheral regions of the cells along with the increased concentration of dill seed oil. According to the F-actin staining, apart from unaligned F-actin bundles along the periphery of the cell, dill seed oil treatment led to the disorganization of straight, parallel stress fibers (Figure 3).

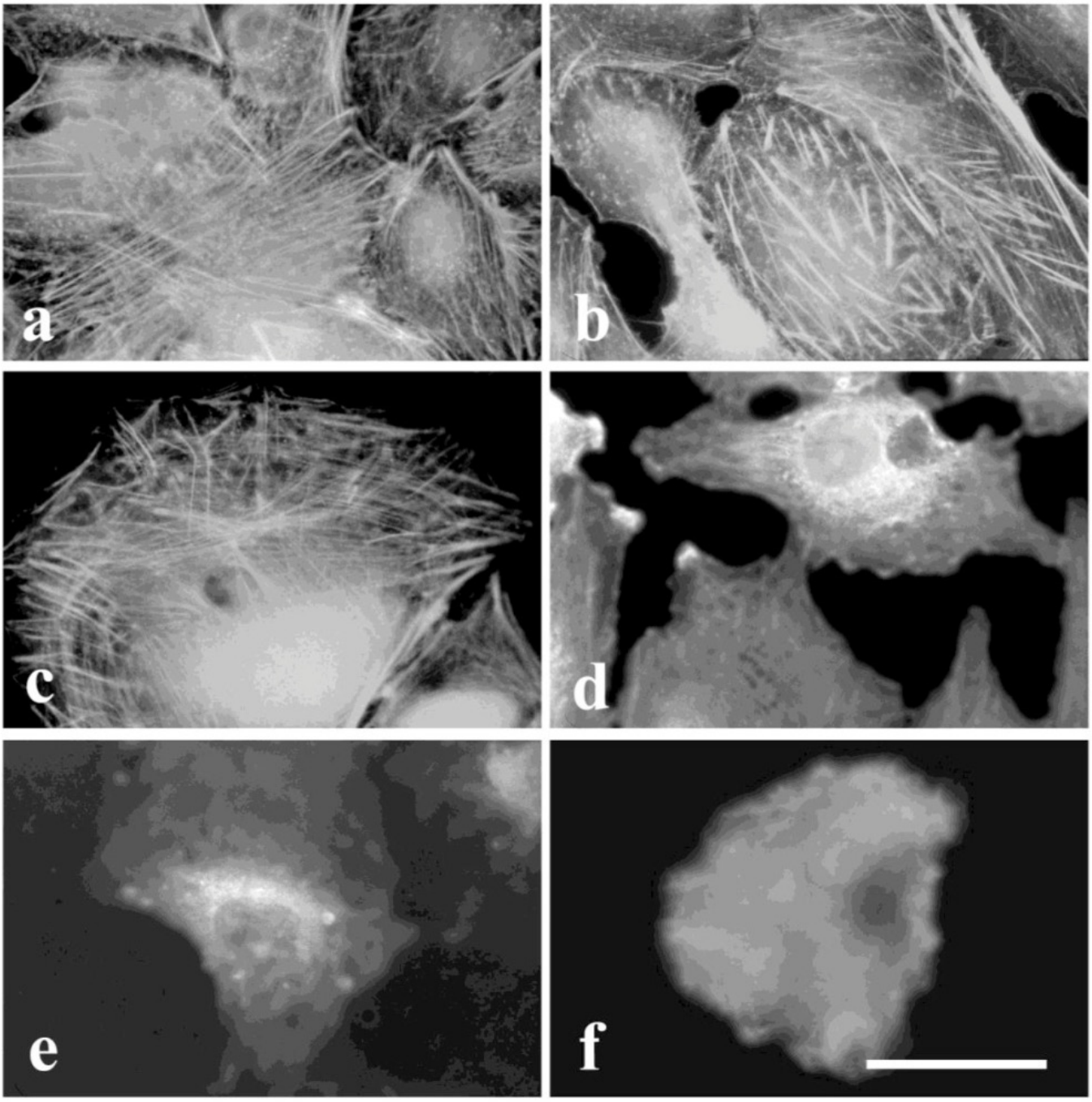

FIGURE 3 - Fluorescence microscopic images of F-actin. F-actin filaments were stained with (TRITC)-labeled phalloidin. (a) Control cells and (b) solvent control cells demonstrate stress fibers and normal actin distribution in bundles along the periphery of the cell. Increasing doses of dill seed oil c: 12,5 µM, d: 25 µM, e: 50 µM and f: 100 µM disrupts F-actin filaments. The images are representative of independent triplicate assays. Scale bar: 125 µm.

DISCUSSION

A search for endothelial cell growth inhibitors was the first experimental strategy to examine new antiangiogenic compounds. Activation of endothelial cells, which rest in the parent vessel, occurs by an angiogenic signal. Then, these cells are stimulated to synthesize and release degradative enzymes that allow endothelial cells to migrate, proliferate and finally differentiate to make way for capillary tubules. There is a possibility for any of these steps to be a potential target for pharmacological intervention (Quesada, Muñoz-Chápuli, Medina, 2006)

Many plants are natural sources of antiangiogenic compounds (Liu *et al*., 2006). Dill seed oil is a natural plant extract traditionally used as a food additive. Dill seeds flavor cakes and pastries, soups, salads, potatoes, meats, sauerkraut, and pickles (Preedy, 2015).

According to Monsefi *et al*. (2014), dill seed oil extract led to the survival of corpus luteum, and enlarged its granulosa lutein cells smooth endoplasmic reticulum (SER), which reacts to the high secretion of progesterone. Dill seed induced prolongation of the diestrus phase of the estrous cycle, and it inhibited ovulation in the following cycle (Monsefi *et al*., 2006 (a); Monsefi *et al*., 2006 (b)). These results and steroidal contraceptive pill performance are similar, so it is likely that the aqueous extract of dill seed directly acts on the ovary and leads to an increase in progesterone levels by affecting the progesterone-producing cells of the corpus luteum (Monsefi *et al*. 2014).

The contractive effects of Dill seeds, which have been in the laboratory, on myometers have been observed in an earlier study (Lis-Balchin, Hart, 1997; Hekmatzadeh *et al*., 2014). Additionally, oxytocin, which plays an important role in uterine contraction, is released with dill seeds (Hekmatzadeh *et al*., 2014). The report of Zagami *et al*. (2012) has shown that dill seed infusion (1 tablespoon whole dill seed seeped in a half or whole cup boiling water for 3-4 min) leads to an increase in the number of contractions and a reduction in the duration of the first stage of labor. The benefits of this plant during the postpartum period were reported by Mahdavian *et al*. (2001).

According to the report of Hekmatzadeh *et al*. (2014), boiled dill seeds are effective in reducing labor duration. Because they have some components such as limonene and tannin, dill seeds may increase the contractions of the uterus and may positively affect the delivery process (Hekmatzadeh *et al*., 2014). Their study had compatible results with their previous study. there is no connection between dill and pain labor intensity, and Hekmatzedh *et al*. (2011). According to them there is no connection between dill and pain and intensity in labor.

The fact that dill caused a decreased fertility index and diestrus phase prolongation, but dill leaf aqueous did not induce these changes was reported by Monsefi *et al*. (2014). They reported that dill seed is useful as a medicinal herb with its infertility potential. In this study, we investigated the *in vitro* cytotoxic and antiangiogenic effects of dill seed oil on rat adipose tissue endothelial cells.

In this study, there was 38.2% limonene, 1.5% cis-isodihydrocarvone, 56.4% carvone and 0.8% dillapiol in the content of dill seed oil (Table I). Stammati *et al*. (1999) reported that with different concentrations of (S) (+) carvone, inhibition of cell viability and colony-forming ability of Hep-2 cells were found to be dose-dependent. In addition, carvone led to the fragmentation of nuclei which is typical for a condensed apoptotic phenotype in Hep-2 cells. In the angiogenic process, the proliferation and survival of endothelial cells are significant (Loutrari *et al*., 2004). Lazutka *et al*. (2001) reported that essential oil from dill seed has cytotoxic and genotoxic effects on human lymphocytes. According to the results of our studies, the inhibitory effect of dill seed oil on RATEC proliferation depended on dose and time. This result resembles Stammati's and Lazutka's works (Stammati *et al*., 1999; Lazutka *et al*., 2001).

According to the report of Monsefi *et al*. (2006a) dill can be used by women who have irregular menstrual cycles as a regulatory agent or it can be an antifertility agent. Degradation of the basement membrane of existing blood vessels, migration and proliferation of endothelial cells, and organization of endothelial cells into capillary tubes are the processes that can be considered the basic steps of the angiogenesis process (Klagsbrun, Moses 1999). Meanwhile, the ability of endothelial cells to form capillary tubes is a specialized function of this cell type. The result of vascular tube formation is a finely

tuned balance between proliferation, migration and differentiation (Soeda *et al*., 2000).

In this study, there was a model in which the culture of RATECs on Matrigel causes the formation of tube-like structures, similar to capillary blood vessels characteristic of angiogenesis. Our data suggest that dill seed oil can be a strong angiogenic inhibitor able to decrease tube formation of rat adipose tissue endothelial cells. Furthermore, the results of the cytoskeletal study were similar: F-actin in RATECs was obviously and rapidly disorganized after exposure to dill seed oil.

In summary, the conclusions from the experimental data are as follows: Dill seed oil displayed potent anti-angiogenic activity *in vitro*, inhibited the proliferation of rat adipose tissue endothelial cells (RATECs), depolymerized F-actin stress fibers, and disrupted endothelial tube formation. These findings indicate that dill seed oil is a new anti-angiogenic agent, and its development as a new contraceptive is possible.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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