

Efficacy and toxicity of anise oil as a potential topical wound healer: a cell culture study

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Abstract. – OBJECTIVE: We studied the cytotoxic effects of topical anise oil on NIH/3T3 fibroblast cells using a cell culture assay.

MATERIALS AND METHODS: NIH/3T3 fibroblast cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (10%) and penicillin/streptomycin under standard cell culture conditions in a humidified incubator containing 5% carbon dioxide. For the MTT cytotoxicity experiment, NIH/3T3 cells were plated in triplicate at a concentration of 3×10^3 per well in 96-well plates and incubated for 24 hours. The cells were treated with anise oil concentrations ranging from 3.13 to 100 μM , and the plates were cultured for 24, 48, and 72 hours under standard cell culture conditions. For assessment by confocal microscopy, NIH/3T3 cells were seeded on sterilized coverslips in 6-well plates at a concentration of 10^5 cells per well in triplicate. For 24 hours, cells were treated with 100 μM of anise oil. Three wells that were not treated with anise oil served as the control group.

RESULTS: The MTT findings demonstrated that anise oil is not cytotoxic to NIH/3T3 fibroblast cells. Anise oil stimulated cell growth and triggered cell division at all three incubation intervals of 24, 48, and 72 hours. The maximum growth was obtained in the applied highest concentration of 100 μM anise oil. At doses of 25, 50, and 100 μM , there was also a statistically significant improvement in cell viability. At 72 hours of incubation, dosages of 6.25 and 12.5 micro of anise oil were shown to be viability-inducing for NIH/3T3 cells. In the confocal microscopy pictures, it was found that anise oil was not cytotoxic on NIH/3T3 cells at the applied maximal dose. The experimental group of NIH/3T3 cells exhibited the same cell morphology as the untreated control group. In both sets of NIH/3T3 cells, the nucleus was round and undamaged, and the cytoskeleton was determined to be compact.

CONCLUSIONS: Anise oil is not cytotoxic on NIH/3T3 fibroblast cells and initiates cell growth. Anise oil could be used topically to enhance wound healing after surgical procedures if clinical trials will confirm experimental data.

Key Words:

Anise oil, NIH/3T3 Cells, Cell culture, Cytotoxicity, Wound healing.

Introduction

Anise (*Pimpinella anisum*), a member of the Umbelliferae family, has been used as a carminative, fragrant, antiseptic, and galactagogue in traditional Iranian medicine (mainly its fruits) for centuries. *Pimpinella anisum* has a rich history of therapeutic use. Aniseeds have been found to have a wide variety of effects on the digestive system and other systems, including antibacterial, antifungal, antiviral, antioxidant, muscle-relaxing, analgesic, and anticonvulsant action. Besides helping with dysmenorrhea and menopausal flashes, it can help lessen morphine dependency. Aniseeds were shown to have a hypoglycemic impact, a hypolipidemic effect, and a lipid peroxidation, lowering effect in diabetic individuals. "Trans-anetole, estragole, γ -hymachalen, para-anisaldehyde, and methylcavicol" are the most crucial aniseed essential oil¹.

Aniseed contains 1.5-6.0% volatile oil, primarily trans-anethole, 8-11% lipids rich in fatty acids, including palmitic and oleic acid, around 4% carbohydrates, and 18% protein². In the family Umbelliferae, the medicinal herb *Pimpinella anisum* L. has been used for thousands of years. Other research has indicated that its essential oil includes the "chemicals eugenol trans-anethole,

methylchavicol, anisaldehyde, estragole, coumarins, scopoletin, umbelliferone, estrols, terpene hydrocarbons, polyenes, polyacetylenes”³. This annual grassy plant grows 30-50 cm tall, with white flowers and tiny green to yellow seeds in “the Eastern Mediterranean, West Asia, the Middle East, Mexico, Egypt, and Spain”⁴. In August and September, *P. anisum* is cultivated mainly for the seeds (fruits) that it produces. Aniseeds are a popular spice because of their essential oil, which has culinary, medicinal, and carminative uses. Aniseed use by nursing mothers boosts milk production and soothes gassy newborns⁵. Anise serves as a flavoring and aromatic component in a variety of foods, including fish items, ice cream, chocolates, and gums^{4,6}.

In the present study, we investigated the topical anise oil’s cytotoxic effects on NIH/3T3 fibroblast cells. This is cell culture study, MTT colorimetric assay and confocal microscopic evaluation were performed to evaluate the cytotoxic effects.

Materials and Methods

This study was undertaken at the ENT Department of Eskisehir Osmangazi University, working alongside the Department of Biology within the Faculty of Science at Eskisehir Technical University.

Cell Culture

NIH/3T3 fibroblast cells (Commercially available at <https://www.atcc.org/products/crl-1658>) (February 12, 2023) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with fetal bovine serum (10%) and penicillin/streptomycin in standard cell culture conditions in a humidified incubator with CO₂ (5%). Cells were passaged each third day and were used for all experimentations at a confluency of 85%.

MTT Cytotoxicity Assay %

NIH/3T3 cells were seeded in triplicates in 96-well plates with a concentration of 3x10³ per well and were incubated for 24 hours. Concentrations of anise oil ranging from 3.13 to 100 µM were applied to the cells and plates were incubated for 24, 48, and 72 hours under standard cell culture conditions. After the incubation period 20 µL of MTT dye (5 mg/mL in PBS) was added per well and further incubated for 2 hours. At the end of this period liquids of all wells were aspirated and 200 µL of dimethyl sulfoxide (DMSO) was

added in each well to dissolve the formazan crystals. Absorbances were read with an ELISA reader (BioTek HTX Synergy – Winoosky, VT, USA) at a wavelength of 560 nm. Viability percentages were calculated as mean SD values on an excel.

Confocal Microscopy

NIH/3T3 cells were seeded on sterilized coverslips in 6-well plates in triplicates at a concentration of 10⁵ cells in each well. Cells were treated with 100 µM dose of anise oil for 24 hours. Three wells were not treated with anise oil and were used as control group. After the incubation with anise oil cells were fixed in room temperature in glutaraldehyde and stained with phalloidin for 1 hour. The stained cells were washed with primary blocking solution and stained with acridin orange for 10 minutes in room temperature. Subsequently, cells were observed and visualized under a confocal microscope (Leica SP5-II – Wetzlar, Germany).

Statistical Analysis

Statistical evaluations were realized on Graph-Padprism 6 programme (San Diego, CA, USA) by using One-way ANOVA and post-Test Tukey. *p*-values were taken in consideration as significant at a level of <0.05.

Results

MTT results indicated that anise oil does not exert cytotoxicity on NIH/3T3 fibroblast cells. In all three incubation times of 24-, 48-, and 72-hours anise oil triggered cell proliferation and caused cell growth. This effect can be seen in the graphs in Figures 1, 2, and 3. The highest growth was recorded in the applied highest concentration of 100 µM anise oil. Also, statistically significant augmentation in cell viability was recorded at concentrations of 25, 50, and 100 µM. At an incubation period of 72 hours also doses of anise oil of 6.25, and 12.5 were detected to be viability triggering doses for NIH/3T3 cells.

In the confocal microscopy images, it was detected that anise oil was not cytotoxic on NIH/3T3 cells at the applied highest dose. The cell morphology was not changed in the experimental group of NIH/3T3 cells compared to the untreated control cells. In both group of NIH/3T3 cells the nuclei were circular and undamaged as well as the cytoskeleton was found to be compact (Figure 4 and Figure 5).

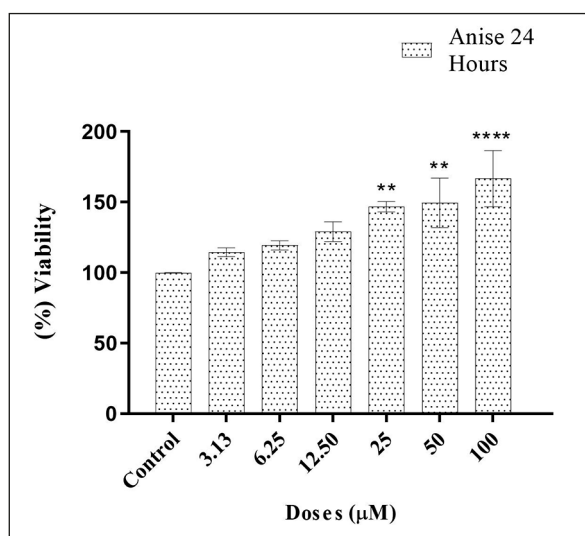


Figure 1. Viability percentages of NIH/3T3 cells treated with Anise Oil for 24 h (** $p \leq 0.0014$, **** $p \leq 0.001$).

Discussion

Aniseed is well known for its positive effect on delivery and boosted milk production in antiquity^{7,8}, and it is still prescribed for various ailments in traditional medicine. Primarily due to the presence of trans-anethole, the fruit, and essential oil are utilized in traditional therapies, such as for the relief of “coughs, respiratory congestion, migraines, gastrointestinal (GI) distress, and colic; treatment of skin infections, as a tranquilizer and aphrodisiac, and to enhance lactation⁹⁻¹⁵”. Recent

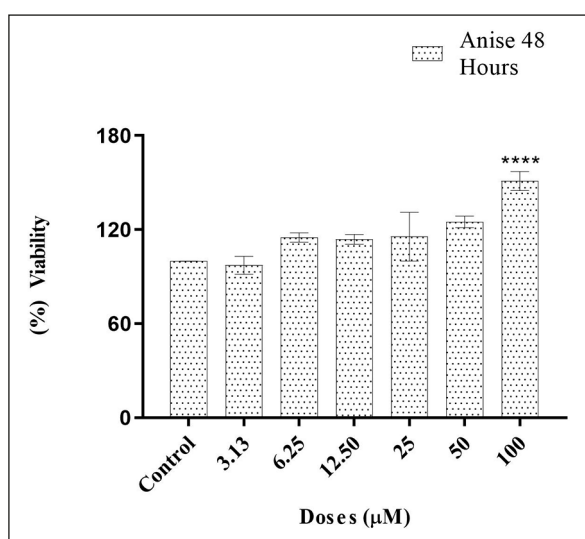


Figure 2. Viability percentages of NIH/3T3 cells treated with Anise Oil for 48 h (**** $p \leq 0.001$).

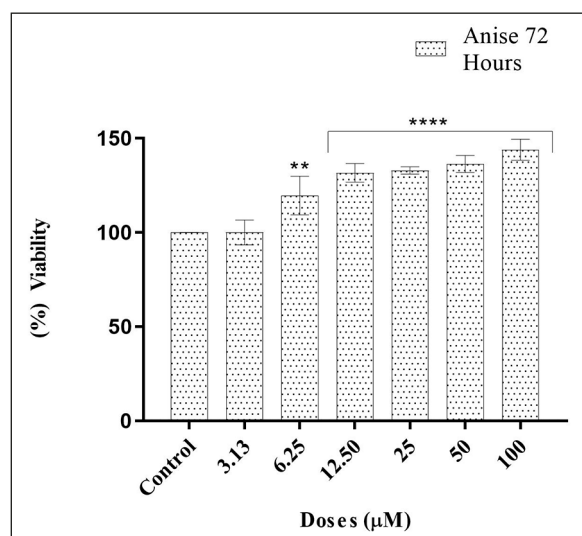


Figure 3. Viability percentages of NIH/3T3 cells treated with Anise Oil for 72 h (** $p \leq 0.0014$, **** $p \leq 0.001$).

attempts have been made to determine aniseed’s effectiveness against “diabetes, dysmenorrhea, and menopausal hot flashes”^{10,16}, as well as its antioxidant, anti-inflammatory, and antibacterial characteristics.

Using the disc diffusion method, the antibacterial effects of anise fruit extracts in water, 50% (v/v) methanol, acetone, and petroleum ether were tested against four pathogenic microorganisms. Only aqueous and methanol extracts were

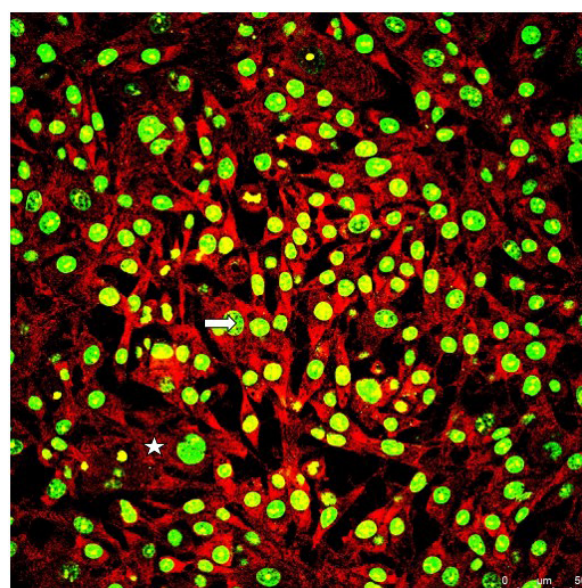


Figure 4. Confocal microscopy image of NIH/3T3 cells treated with Anise Oil or 24 h. Arrow-nucleus (Green). Asterisk-cytoskeleton (Red). Scale bar: 0-50 µm.

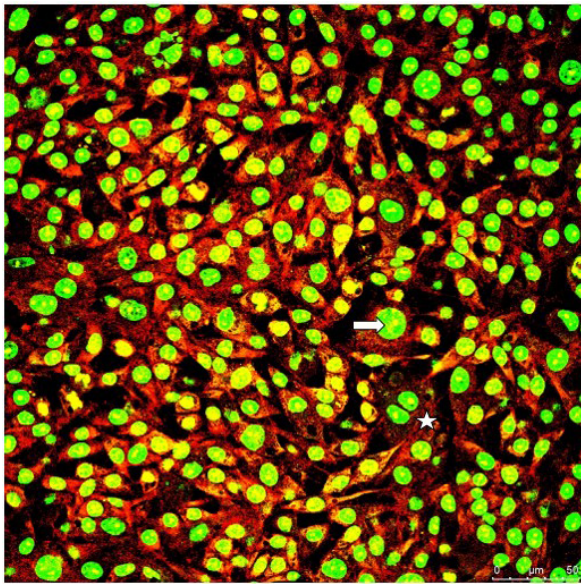


Figure 5. Confocal microscopy image of untreated NIH/3T3 cells. Arrow-nucleus (Green). Asterisk-cytoskeleton (Red). Scale bar: 0-50 μm .

bactericidal, and the aqueous extract was more efficient than the methanolic one against all test microorganisms. In contrast, extracts of acetone and petroleum ether failed to halt the growth of the dangerous test bacteria¹⁷.

In the present study, we investigated the topical anise oil's cytotoxic effects on NIH/3T3 fibroblast cells. MTT results indicated that anise oil does not exert cytotoxicity on NIH/3T3 fibroblast cells. In all 3 incubation times of 24-, 48-, and 72-hours anise oil triggered cell proliferation and caused cell growth. The highest growth was recorded in the applied highest concentration of 100 μM anise oil. Also, statistically significant augmentation in cell viability was recorded at concentrations of 25, 50 and 100 μM . At an incubation period of 72 hours also doses of anise oil of 6.25, and 12.5 were detected to be viability triggering doses for NIH/3T3 cells.

In the confocal microscopy images, it was detected that anise oil was not cytotoxic on NIH/3T3 cells at the applied highest dose. The cell morphology was not changed in the experimental group of NIH/3T3 cells compared to the untreated control cells. In both group of NIH/3T3 cells the nuclei were circular and undamaged as well as the cytoskeleton was found to be compact.

Using the disc diffusion technique, the antimicrobial properties of aniseed water and ethanolic extracts were examined against ten bacterial species and *Candida albicans*. In this investigation,

the ethanolic extract inhibited the growth of all examined microorganisms except *Candida albicans*. However, water extract was ineffective against Gram-negative bacteria, *Pseudomonas aeruginosa*, and *Escherichia coli*, but effective against *Candida albicans*³. The antibacterial activity of the alcoholic extracts of *Pimpinella anisum* seeds was also demonstrated against "*Micrococcus luteus* and *Mycobacterium smegmatis*"¹⁸.

Migraine sufferers ($n = 22$) were given an aniseed oil cream to rub onto their temples and foreheads for six weeks¹⁹. Therapy with a cream containing aniseed oil markedly decreased the number and duration of episodes, but not the intensity, in contrast to users of a placebo cream. Chronic rhinosinusitis without polyps²⁰ patients ($n = 26$) were instructed to use nasal drops containing 200 g of an aniseed water extract in almond oil in each nostril every 12 hours for four weeks. Fluticasone was sprayed up the noses of a different group of people. When the effects of aniseed extract therapy and fluticasone dosage were compared, CT scans showed that both treatments significantly reduced inflamed paranasal sinus mucosa and improved sinonasal symptoms. Comparing these two groups revealed that drops containing aniseed were substantially more efficient than fluticasone at reducing rhinological symptoms.

In the investigation by Hashemnia et al²¹, full-thickness excisional wounds were produced on the backs of diabetic male Sprague-Dawley rats. The rats were randomly assigned to one of four treatment groups: 1 mL of basal cream, 3% doxycycline, 10% *Pimpinella anisum* for 14 days, or a control group. Five animals from each group were euthanized at 7-, 14-, and 21-days post-injury, and macroscopic, histological, and oxidant/antioxidant analyses were performed on wounds. Sixty rats were utilized in the investigation. During the trial, a substantial reduction in wound size was seen in rats treated with *Pimpinella anisum* compared to other groups. In addition, treatment with *Pimpinella anisum* reduced the number of lymphocytes, enhanced the number of fibroblasts, and raised the number of fibrocytes during the later phases of wound healing.

Boskabady and Ramazani-Assari evaluated the relaxing action of *Pimpinella anisum* on isolated guinea pig tracheal chains and its probable mechanism. In this study, the bronchodilatory effects of anise aqueous, ethanol extracts, and essential oil were examined on isolated guinea pig tracheal chains precontracted by 10 mM methacholine under two conditions: nonincubated (group 1)

and incubated with 1 mM propranolol and 1 mM chlorpheniramine (group 2). Compared to controls, aqueous and ethanol extracts, essential oil, and theophylline (1 mM) exhibited substantial relaxing effects²².

It was determined whether or not three different “hydroalcoholic extracts of the aerial parts” of *Pimpinella anisum* (ethanol: water; 40:60, 60:40, and 80:20) had antispasmodic and relaxing effects on rat anococcygeus smooth muscle. All three hydroalcoholic extracts counteracted the contraction induced by acetylcholine. Two different hydroalcoholic extracts did not produce relaxation in this investigation, but the extract containing 60% ethanol (HA60) relaxed “acetylcholine-precontracted tissues” in a concentration-dependent manner. The stimulation of the NO-cGMP pathway is primarily responsible for the relaxant action, as revealed by research into the underlying processes²³.

Anise’s anti-inflammatory and analgesic effects on mice were examined. The results demonstrated that anise oil had anti-inflammatory properties equivalent to indomethacin and analgesic properties comparable to 100 mg/kg aspirin and 10 mg/kg morphine at 30 minutes²⁴.

On the hypersensitive host *Chenopodium amaranticolor*, the effects of the essential oils of *Foeniculum vulgare* and *Pimpinella anisum* were evaluated against PVX (potato virus), TMV (tobacco mosaic virus), and TRSV (tobacco ring spot virus). At 3000 ppm, the essential oil inhibits PVX, TMV, and TRSV²⁵.

It was explored if aniseed treatment may reduce tissue damage caused by various toxic insults²⁶⁻²⁸. Inconsistent results may have been attributable, in part, to the aniseed fraction tested, the many animal models and dose regimens employed, and the various target organs and tissues studied. In contrast, in animal models, trans-anethole protects against liver, lung, and gastrointestinal tract²⁹ damage caused by various agents³⁰⁻³³.

Limitations

This is a study conducted with NIH/3T3 fibroblast cells. The results will be used if clinical trials will confirm experimental data that is the limitation of our study.

Conclusions

Anise oil is not cytotoxic on NIH/3T3 fibroblast cells and initiates cell growth. Anise oil

could be used topically to enhance wound healing after surgical procedures³⁴ if clinical trials will confirm experimental data.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

This is a cell-culture study conducted by commercially available NIH/3T3 cells; therefore, ethics committee approval was not needed.

Informed Consent

This is a cell-culture study conducted by commercially available NIH/3T3 cells; therefore, there was no need to take informed consent.

Funding

There are no funds for this study.

Authors’ Contribution

Ibrahim Sungur: Planning, designing, literature survey, active intellectual support. Nuray Bayar Muluk: Planning, designing, literature survey, interpretation of the results, active intellectual support, writing, submission. Canan Vejselova Sezer: Planning, designing, data collection, literature survey, statistical analysis, interpretation of the results, active intellectual support. Hatice Mehtap Kutlu: Planning, designing, data collection, literature survey, interpretation of the results, active intellectual support. Cemal Cingi: Planning, designing, literature survey, data collection, interpretation of the results, active intellectual support, English editing.

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