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# ANTIMICROBIAL ACTIVITY OF CLEMENTINE PEEL ESSENTIAL OIL WITH ITS CYTOTOXIC AND IN VITRO WOUND HEALING POTENTIAL ON NIH-3T3 FIBROBLAST CELLS

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**Abstract**

*Citrus essential oils has many area of use such as skin and hair care, insomnia, improving digestion, and boosting immune system. The present study is aimed to determine the biological activities of clementine (Citrus clementina Hort. ex Tan), one of the mandarin species, peel essential oil (CPEO) that grow in Turkey. Antimicrobial activity of CPEO was evaluated using disc diffusion method against Escherichia coli, Staphylococcus aureus, and a fungi Candida albicans. The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide)-colorimetric monocyte mediated cytotoxicity assay was applied using NIH-3T3 cells and IC50 value was calculated. CPEO was also evaluated for its in vitro wound healing effect on the fibroblast cell migration and proliferation using scratch wound assay technique. Results of the present study indicated that CPEO has remarkable antimicrobial activity. The highest inhibition zone was observed against C. albicans as 20.67±0.58 mm. IC50 value obtained for CPEO was 52.50±1.19 µg/mL. Fibroblast cells showed higher migration than the control group to heal the artificial scar after treatment of CPEO concentrations below the IC50 dose. The results of the present study indicated that the CPEO may be useful in effective management of skin care applications and wound healing products with its potent activity against pathogen microorganisms.*

**Keywords: Clementine, antimicrobial, cytotoxicity, wound healing, fibroblast**

# KLEMENTİN ESANSİYEL YAĞININ ANTİMİKROBİYAL AKTİVİTESİ İLE NIH-3T3 FİBROBLAST HÜCRELERİ ÜZERİNE SİTOTOKSİK VE İN VİTRO YARA İYİLEŞME POTANSİYELİNİN BELİRLENMESİ

**Öz**

*Narenciye esansiyel yağları cilt ve saç bakımı, uykusuzluk, sindirimin düzenlenmesi ve bağışıklık sisteminin geliştirilmesi gibi pek çok alanda kullanıma sahiptir. Bu çalışma, Türkiye'de yetiştiriciliği yapılmakta olan bir mandalina türü olan klementin'in (Citrus clementina Hort. ex Tan) kabuğundan elde edilen esansiyel yağın biyolojik aktivitelerinin belirlenmesi amacıyla planlanmıştır. Klementin kabuk esansiyel yağının (CPEO) antimikrobiyal aktivitesi E. coli, S. aureus ve bir maya türü olan C. albicans'a karşı disk difüzyon yöntemi ile belirlenmiştir. Sitotoksisitesi MTT kolorimetrik analizi ile NIH-3T3 fibroblast hücreleri kullanılarak tespit edilmiştir. Ayrıca stratch-yara iyileşme tekniği kullanılarak CPEO'nın fibroblast hücrelerinin göçü ve proliferasyonu üzerine etkinliği in vitro yara iyileşme aktivitesi olarak değerlendirilmiştir. Sonuçlara göre CPEO'nın kayda değer antimikrobiyal aktiviteye sahip olduğu gözlenmiştir. En yüksek inhibisyon zonu C. albicans'a karşı 20.67±0.58 mm olarak ölçülmüştür. Sitotoksik aktivite analizine göre IC50 değeri 52.50±1.19 µg/mL olarak belirlenmiştir. CPEO için IC50 değerinin altında uygulanan dozlarda fibroblast hücreleri kontrol grubuna kıyasla daha hızlı sürede proliferasyon sağlayarak yapay yaranın iyileşmesine yönelik yüksek hareket göstermiştir. Mevcut çalışma sonuçlarına göre CPEO, patojenik mikroorganizmalara karşı sahip olduğu antimikrobiyal karakterin yanı sıra cilt uygulamaları ve yara iyileşmeye yönelik ürünlerin geliştirilmesinde kullanılma potansiyeline sahiptir.*

**Anahtar Kelimeler: Klementin, atimikrobiyal, sitotoksisite, yara iyileşme, fibroblast**

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## 1. Introduction

The relation between natural products and health has gaining much interest as people pay much attention to their diet. There are new technologies to improve the functionality of foods. To enhance the potential properties of food, natural additives are used, e.g. to provide antimicrobial characteristics.

The antimicrobial activities of extracts and essential oils from several plants have been recognized and the spices obtained from these materials are used traditionally as flavoring agents in foods for ages. Natural antimicrobial agents that present in weeds, herbs, spices and plants have been intensively studied recently. Essential oils and phenolic compounds are known to be the active antimicrobial components [1].

Essential oils obtained from plants have been studied for their antimicrobial activities [2-5]. Essential oils and the main components of them possess a wide spectrum of biological activity, so they are of great importance in several scientific fields; from biochemistry and food chemistry to pharmacology, biotechnology and pharmaceutics [6]. They are considered to be generally recognized as safe (GRAS) so can be used for human consumption [7]. Besides being antimicrobial, plant-based bioactive components are recently known to play preventive role against the incidence of common diseases like cardiovascular-neurodegenerative disorders and cancer [8].

Fruits are generally used as an ingredient for desserts, but they have significant economic value for their essential oil (EO) due to their aromatic compounds [9]. The genus Citrus belong the Rutaceae family includes about 17 species distributed throughout the tropical and temperate regions [10], [11]. Citrus flavours are used in different food sectors as beverage, confectionary, cookies and desserts [12], [13]. Mandarin which is the second largest Citrus fruit crop after orange is cultivated in countries with temperate summers and warm winters as Southern Aegean and Mediterranean costs of Turkey, Clementine (Citrus clementina Hort. ex Tan.) is one of several Citrus species of the mandarin group. Turkish clementine production has been increasing year by year. The production period for clementine fruit in Turkey extends from mid-October to the end of December [14].

The objective of the present work was to define the potential inhibitory effects of the clementine peel essential oil on the growth of pathogenic microorganisms including Candida albicans, Escherichia coli and Staphylococcus aureus. Cytotoxic effect and wound healing capacity of MO against NIH-3T3 fibroblast cells were also determined.

## 2. Material-Methods

### 2.1 Plant

The essential oil was obtained from the clementine peel (*Citrus clementina* Hort. ex Tan.), a hybrid mandarin species growing in Mugla (Turkey) after drying and milling the peels. Essential oil (EO) was obtained by hydro distillation, using a Clevenger apparatus (Edutek Instrumentation, Haryana, India) with 100 g of dry plant material and 1000 mL of water.

### 2.2 Microbial strains

Antimicrobial activities were determined against *Candida albicans* ATCC 10239, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923 that were provided from Culture Collection of Mugla Sitki Kocman University (MUKK). Bacterial strains were cultured in Mueller Hinton Broth (MHB) and incubated at 37°C for 18–24 h. The yeast was cultured in Sabouraud Dextrose Broth (SDB) at 30°C for 24–48 h. Microbial inocula was adjusted to match the 0.5 McFarland turbidity standard dilution. After maintaining the cultures of microorganisms, they were maintained in their appropriate agar slants at 4°C and used as stock cultures throughout the assays.

### 2.3 Antimicrobial activity of CPEO

Disc diffusion method was used to determine the antimicrobial activity of the CPEO [15]. Briefly, 20 mL of Sabouraud Dextrose Agar (SDA) (for *C. albicans*) and Mueller Hinton Agar (MHA) (for *E. coli* and *S. aureus*) sterilized and cooled to 45–50°C. 1000 µl of each microorganism culture was injected to sterile plates, media was poured under aseptic conditions and distributed homogeneously. 20 µl of CPEO was impregnated into the sterile blank discs of 6 mm in diameter. The discs were put into the inoculated agar plates for each microorganism. Plates were incubated at appropriate temperatures for required incubation time of each microorganism. Antimicrobial activity was evaluated by measuring the zone of inhibition (mm) around the CPEO-loaded discs. Discs of amoxicillin (10 µg/disc), gentamicin (10 µg/ disc) and nystatin (30 µg/disc) were used as positive controls. Studies were performed in triplicate.

### 2.4. Cell Culture

NIH 3T3 (mouse embryonic fibroblast cell line) provided from American Type Culture Collection (ATCC, Manassas, VA, USA), was grown in Dulbecco's Modified Eagle's Medium (DMEM)-high glucose, supplemented with 10% heat-inactivated Fetal Calf Serum (FCS), L-glutamine (2 mM), antibiotic-antimycotic solution (10.000-unit penicillin, 10 mg streptomycin and 25 µg amphotericin B per mL). Cells were maintained at 37°C, in a humidified atmosphere of 5% $CO_2$. After being detached from culture flasks with 0.05% trypsin solution, cells were passaged (sub-cultured). Exponentially growing cells were seeded onto a cell culture dish (60x10 mm) at a density of 75×104/cm2 and were maintained in those culture dishes for 24 h prior to CPEO treatment.

### 2.5. Cytotoxicity

Cytotoxicity evaluation of the CPEO on fibroblast cells were screened using MTT colorimetric assay [16]. Prior to the addition of essential oil, fibroblast cells were cultured in 96-well plates in the medium described above, at plating density of 10.000 cells per well (200 µL), and incubated at 37°C, 5% $CO_2$, and 100% relative humidity for 24 h. CPEO was solubilised in dimethylsulphoxide (DMSO) (10%) and diluted in respective medium. The medium was replaced after 24 h incubation with the respective medium containing the oil at various concentrations (250, 125, 62.5, 31.25, 15.625 and 7.8125 µg/mL). The plates were incubated at the same conditions for 24 h. Sextuplicate (6 replicates) was

maintained and the medium with DMSO (without the CPEO) served as control. 20 µL mL of MTT (5 mg/mL, prepared in phosphate-buffered saline) was added to each well after 24 h and incubated at 37°C for additional 3 h. The medium containing MTT was then poured off and 100 µL of DMSO was used to solubilize the formed formazan crystals in each well. Plates were put in an orbital shaker for 15 minutes at orbital shaker and the absorbance was measured at 570 nm using microplate reader (Thermo Scientific Multiskan FC, Thermo Fischer, Vantaa, Finland). The % cell inhibition was determined using the following formula (1) and the graph between % cell inhibition and concentration were plotted, from which $IC_{50}$ was calculated:

$$(\%) = [100 \times (Sample_{abs})/ (Control_{abs})] \quad (1)$$

### 2.6. *In vitro* scratch- wound healing assay

Scratch wound assay which measures the expansion of a cell population on surfaces was used to assess the spreading and migration capabilities of 3T3 fibroblasts [17]. Briefly, a linear wound was generated in the cell monolayer, using a sterile 100 µl plastic pipette tip. Cellular debris was removed by washing the culture dishes with Dulbecco's phosphate buffered saline solutions. Thereafter, cells were treated with 50, 25, 12.50 and 6.25 µg/mL CPEO and maintained in culture for a period of 24 h. Control group was prepared as the cells cultured in basal medium with DMSO. Representative images from each cell culture dish of the scratched areas were photographed using a Leica DM IL microscope (Leica Microsystems, Wetzlar, Germany) to estimate the relative migration of the cells. Experiments were performed in triplicate.

## 3. Results and Discussion

Antimicrobial potential of the CPEO was evaluated against pathogenic strains including yeast, Gram-positive and Gram-negative bacteria by disc diffusion method. The zone of inhibition measurements as millimeters (mm) are given in Table 1.

CPEO was observed to show promising activity against the tested microorganisms. Taking into consideration of the inhibition zone measurements, the highest antimicrobial activity was observed for *C. albicans* as 20.67±0.58 mm. The activity was relatively moderate for *S. aureus* (14.67±1.15 mm).

Table 1. Antimicrobial activity of CPEO.

| | Inhibition zone (mm) | |
|---|---|---|
| | CPEO | Antibiotics |
| *C. albicans* | 20.67±0.58 | 25.33±0.58 (Nystatin) |
| *E. coli* | 12.67±0.58 | 19.33±1.15 (Gentamicin) |
| *S. aureus* | 14.67±1.15 | 26.00±1.00 (Amoxicillin) |

Citrus essential oils that have high volatile content are reported to reduce/inhibit the fungal growth in a dose-response matter [18]. Such an antifungal activity is supposed to be generated by a major compound itself or synergistic and/or antagonistic effects of different compounds [19, 20]. Kirbaşlar et al. [14] studied the chemical compositions of the volatile extract samples of clementine (*C. clementina* Hort. ex Tan.) peel grown in Turkey by GC (gas chromatography) and GC/MS (gas chromatography/mass spectrometry). They reported that the main component as limonene (88.12–89.28%) followed by myrcene (4.64–3.77%) and the oxygenated compounds as linalool (1.02–1.24%) and decanal (0.71–0.72%).

Linalool, citral, caryophyllene oxide, α-pinene, α-terpineol have been reported to have antifungal and antibacterial activity [21, 22] and are highly found in C. reticulata oil Jayaprakasha et al. [23] reported that the antimicrobial activity of different fractions from Citrus peel suppressed the growth of gram positive bacteria at concentrations lower than that required for gram negative bacteria. Veldhuizen et al. [24] suggested that the antimicrobial activity of the amphipathicity of the phenolic compounds explain their interactions with biomembrane and thus the antimicrobial activity.

The cytotoxic activity of the essential oil extracted from C. clementina peel was evaluated in normal cell line based on cell viability, using MTT colorimetric assay. The viability of the NIH-3T3 cells exposed to the CPEO were measured and expressed in terms of relative absorbance of EO-treated cells, in comparison to control cells. CPEO was observed to show cytotoxic effect on the NIH 3T3 cell lines in a dose-dependent pattern and the $IC_{50}$ value was determined as 52.50±1.19 µg/mL (Figure 1). Baik et al. (2008) studied 14 kinds of citrus oils and reported that the majority of the essential oils showed no cytotoxicity human dermal fibroblast.

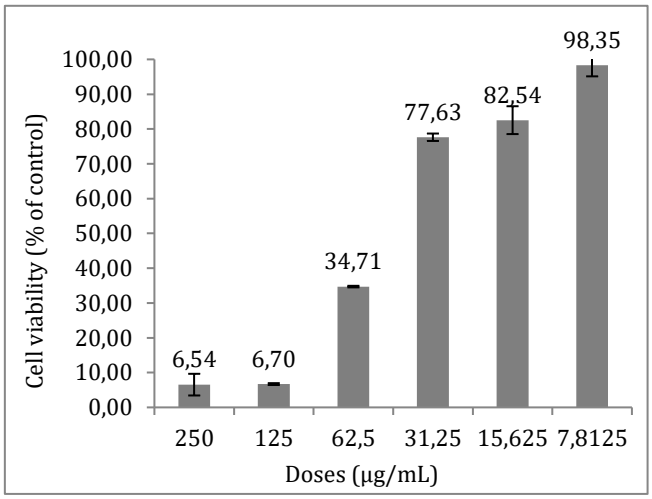


Figure 1. Cytotoxic effects of clementine essential oil on NIH-3T3 fibroblasts evaluated by MTT colorimetric assay. Results are presented as viability ratio compared to the control group. Values were expressed as the mean of six replicates

To investigate the effect of CPEO on fibroblasts' migration and proliferation, stratch-wound healing assay was applied to NIH-3T3 fibroblasts and images were taken at regular intervals. Concentrations of CPEO were determined according to the MTT assay, so the doses

below the $IC_{50}$ value were applied (50, 25, 12.50 and 6.25 µg/mL). After clementine oil treatment to cell culture plates, fibroblasts were stimulated of the cell migration. When compared to the control group, scar completely healed on CPEO treated-group plates after 24 hours (Figure 2).

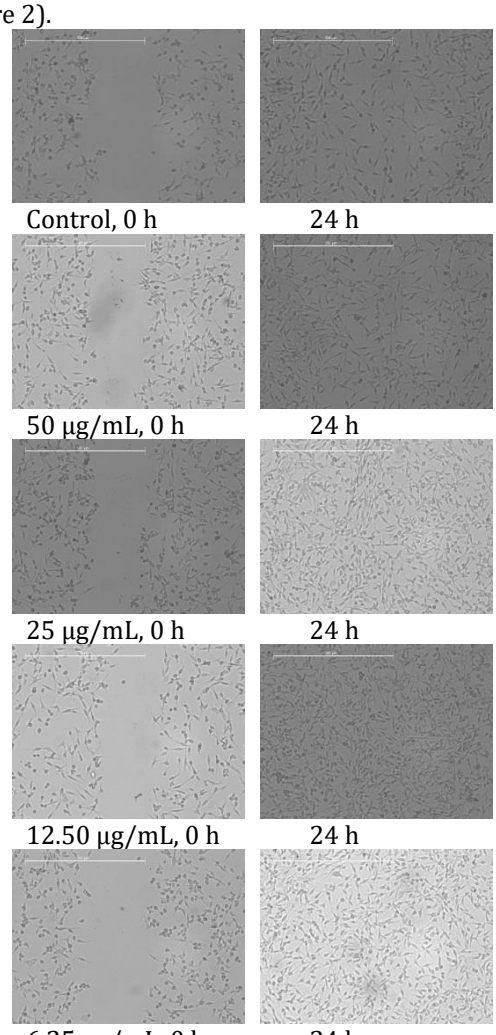


Figure 2. Images of *in vitro* stratch assay for 0. and 24. hours.

## 4. Conclusion

The present study revealed out the certain biological activities of CPEO that are directly related to pharmaceutical and natural therapies. Besides being non-toxic at tested doses, clementine oil was found to have a potential stimulating effect on fibroblast cells which may be a useful property for wound healing and skin care treatments. With its promising antimicrobial potential, the results indicated that CPEO may be considered as an appropriate alternative to chemical and synthetic additives that are used in the food and cosmetic industry, satisfying the requirements for safety and the demands of consumers on natural products. Further toxicological and clinical in vivo studies are required to prove the safety of the CPEO.

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