The Potential of Orange Peel Oil as a Suppressor of Cell Proliferation in Animal Feed and Human Nutrition: An Experimental Study

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Abstract

This study aimed to investigate the in vitro cytotoxic activities of orange peel oil on HaCaT cell lines by using an MTT cytotoxicity assay after administering orange peel oil at different doses and time-points. Our objective was to assess the in vitro cytotoxic activities of orange peel oil on HaCaT cell lines. Cell viability was determined with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assays. The HaCaT cells (100 µL) were cultured in plates and treated with different concentrations of orange peel oil (25 µM, 50 µM, 100 µM, 150 µM and 200 µM) for durations of 24 and 48 hours. Cell death was determined by collecting and staining with 0.4% Trypan blue for 5 minutes at room temperature, followed by microscopic examination. There was a significant difference between the doses concerning both time zones (p < 0.05). There was a significant (p<0.05) difference between the control group and all other doses, including 200 µl/mL and 25 µl/mL, 50 µl/mL, 100 µl/mL, and 20% DMSO. Orange peel oil showed toxic effects at all dose levels and time points when compared with the control group. According to the results of our research, and in light of previous investigations, it can be said that orange shell oil may have protective effects such as anti-cancer, anti-microbial, and antioxidant properties, and thus, may be used in human and animal nutrition.

Keywords:
Cytotoxic activities
Essential oil
Cell viability

Article History:
Received: 10.01.2020
Accepted: 03.03.2020
Available Online: 21.05.2020

Introduction

One of the features distinguishing humans from other creatures is the ability to learn and transfer knowledge from generation to generation. This ability has given us a significant advantage in the struggle for survival. People had to deal with plants for many years to meet their basic needs and had detailed information about plants. As a result of these efforts, a lot of information was gathered within thousands of years about the use of plants as food or medicinal products.

Orange is an important tree species belonging to the Rutaceae family. Orange peel oil obtained from the pulp of oranges obtained from these trees has traditionally been widely used for different purposes, including treatment and nutrition.

With 115,650,545 tons, citrus is the most cultivated fruit group in the world. On the other hand, oranges constitute 55.26% of the citrus production in the world. Worldwide the highest orange producing countries are the United States, Brazil, Mexico, Spain, Italy, India, Israel, Egypt, Argentina, and Turkey. Extracts and essential oils derived from medicinal and aromatic plants are widely used in the food industry, cosmetic production, and medicine today.

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Citrus fruits are marketed mainly as fresh fruits or processed juice. Large amounts of shells generated during the processing of citrus fruits are discarded because they do not add value to the product as a by-product.

Citrus essential oils can contain many components (more than 200), including terpenes, sesquiterpenes, aldehydes, alcohols, esters, and are described as a mixture of terpene hydrocarbons, oxygenated compounds, and non-volatile residues.

Citrus avonoids have antioxidant, anticancer (Lai et al., 2013, Im et al., 2014), anti-mutagenic (Hosseinimehr and Karami, 2005; Demir et al., 2009), anti-allergic, anti-inflammatory, and antimicrobial (Hamdan et al., 2013) properties. Additionally, citrus shells were used as a source of animal feed, fiber production, and fuel production (Bampidis and Robinson, 2006; Lashkari and Taghizadeh, 2013).

While cancer cells are destroyed in chemotherapy, which is a conventional treatment method, controlling the disease may fail because it can cause adverse and toxic side effects on normal cells. The alternative solution for the detrimental effects of synthetic agents is the use of natural plants, which have the potential of making an outstanding contribution to modern therapeutics (Sultana et al., 2014).

Orange essential oil (Citrus sinensis L. Rutaceae) produced by the cells in the orange peel is extracted from the shell of the fruit. The main constituent of orange peel oil is D-limonene (more than 90%) (Bauer et al., 2001). In addition to its antioxidant properties, citrus essential oils provide physical and mental energy and help remove toxins and harmful substances from the cells. Adding citrus oils as a food and beverage aroma is a great idea (Fisher and Phillips 2008). In the pharmaceutical industry, citrus oils are used as spice ingredients to hide the unpleasant flavors of medicines. They are also used in the perfume and cosmetics industry (Steuer et al., 2001).

In studies carried out to determine the effects of aromatic plants on performance and other yields in animals, significant improvement in parameters such as feed consumption, feed utilization, live weight gain, and carcass yield were observed by using aromatic plants and essential oils obtained from these plants as growth factors (Güler et al., 2005).

Due to its antimicrobial and antioxidant properties, the use of citrus shell oils as feed additives has recently gained importance. Essential oils and flavonoids are intensely present in the shell of citrus fruits such as orange (Citrus sinensis), lemon (Citrus lemon) and bergamot (Citrus bergamia). These parts contain very high amounts of essential oils such as limonene and linalool (Min-Hsiung, 2009). The antioxidant activity of limonene, which is the most essential component of the orange peel oil, has been reported to be quite high (Roberto, 2010).

The aim of this study was to investigate the in vitro cytotoxic effects of orange peel oil on a non-tumor keratinocyte cell line (HaCaT) using an MTT cytotoxicity assay, after applying the orange peel oil at different doses and at different time intervals.

### Materials and Methods

#### Herbal Extract

The orange peel oil used in this experiment was obtained commercially from Ege Lokman Plant Industry and Trade Co. The orange peel oil I bought commercially was obtained by the cold press method. The active ingredient levels of orange peel oil were investigated by GS-MS (Gas Chromatography and Mass Spectrometry) in Çukurova University Faculty of Fisheries Laboratory (Table 1).

| Table 1. Orange essential oil chemical composition and active ingredient ratios (%) |
|----------------------------------|-----------------|
| Orange Peel Oil                  | (%)             |
| Beta-Myrecene                    | 1.987581        |
| Limonene                         | 97.46377        |
| Camphene                         | 0.018475        |
| Linalool                         | 0.222917        |
| Trans-Limonene Oxide             | 0.005973        |
| Citronella                       | 0.017979        |
| Decanal                          | 0.188161        |
| Alpha-Terpine                    | 0.012065        |
| Alpha-Copaene                    | 0.010914        |
| Germacrene-D                     | 0.005194        |
| Germacrene-B                     | 0.006875        |
| Trans-Caryophyllene              | 0.010227        |
| Beta-Cubebene                    | 0.014546        |
| Valencene                        | 0.028449        |
| Delta-Cadinene                   | 0.006875        |

#### Cell Culture

The immortalized human non-tumorigenic keratinocyte cell line (HaCaT) was acquired from Cell Culture and Biological Resources Unit at Yeditepe University. These cells were seeded at a concentration of 5,000 cell/well on a 96-well plate (BIOFIL, TPC, Switzerland) and conserved in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO), which includes HEPES (Sigma) buffer along with 10% heat-inactivated fetal bovine serum (FCS) (Hyclone Lab., Logan, UT), 100 μg/mL streptomycin (Sigma), and 100 U/mL penicillin (Sigma). The materials were incubated in disposable plastic tissue culture flasks in a 5% CO2/95% air incubator at 37 °C. After incubation, the culture media were removed, the cells were washed with PBS, and MTT cell proliferation assay was performed.

#### Cell Viability (MTT) Assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were used to determine cell viability. The HaCaT cells (100 µL) were cultured in 96-well plates at 2 x 104 cells per well and treated with 25 μM, 50 μM, 100 μM, 150 μM, and 200 μM concentrations of citrus peel oil for durations of 24 and 48 hours. Following treatment, each well was filled with 10 μL of MTT reagent (5 mg/mL) and incubated for 4 hours at 37 °C. Then, the medium was eliminated, and 150 µL of DMSO was added to make the MTT formazan soluble. The absorbance of solubilized MTT formazan products was measured by an ELISA plate reader (Biotek, USA) at 590 nm at 24, 48, and 72 hours.
Cell death was determined by collecting and staining with 0.4% of Trypan blue for 5 minutes at room temperature before microscopic examination. Viable cells were counted via Trypan blue exclusion. Dead cells that stained blue were deemed positive and summed against the total.

Statistical Analysis

The Statistical Package for Social Sciences (SPSS, version 22, IBM, Armonk, New York 10504, NY, USA) was used for data analysis. As descriptive statistics, numerical variables were summarized as mean (± standard deviation). Comparisons between the groups were made with the one-way ANOVA or Kruskal Wallis test. A p-value below 0.05 was considered statistically significant.

Results and Discussion

In this study, we investigated the effect of orange peel oil on the cell viability of human HaCaT keratinocytes. When Table 1 and Figure 1 were examined, it was found that there were significant differences between the two time zones and doses concerning cell viability between the groups (p<0.05).

The Duncan post hoc multiple comparison test was applied to determine the doses between which the differences occurred during the 24-hours. As a result, a significant (p<0.05) difference was found between the control group and all other doses and between 100 µl/mL and 20% DMSO. As a result of multiple comparisons to determine the differences at 48 hours, there were significant differences between the control group and all other doses, as well as between the 200 µl/mL dose and 25 µl/mL, 50 µl/mL, 100 µl/mL, and DMSO 20% (p<0.05). Data in Table 1 reveals that each concentration of orange peel oil shows toxic effects in the HaCaT cell line. Orange peel oil appears to have cancer therapeutic effects.

Citrus essential oils and their components also receive attention from the point of chemoprevention agents in cancer treatment. For example, the Palestine sweet lime essential oil was shown to inhibit inflammation and activate apoptosis of human SW480 colon cancer cells by suppressing the expression of both COX-2 and IL-6 (Jayaprakasha et al., 2012). d-Limonene, a major constituent of citrus essential oil, is recognized as a potential chemotherapeutic agent because it can induce human colon cancer cell apoptosis via the mitochondrial death pathway and suppress the PI3K/Akt pathway (Jia et al., 2013) Perillyl alcohol, an oxygenated monoterpene constituent of citrus essential oil, is effective in the clinical treatment of patients with malignant brain tumors (Chen et al., 2015). The essential oil of blood orange can inhibit angiogenesis, metastasis, and cell death in human colon cancer cells (Murthy et al., 2012).

Many studies have reported that citrus shell oils have antioxidant effects (Turhan et al., 2006; Wilkins et al., 2007; Wang et al., 2008; Al-Saadli et al., 2009; Yapo, 2009; Janati et al., 2012; Oboh and Ademosun, 2012; Fidrianny et al., 2014; Canan et al., 2016). Different kinds of oxidants are present in human food. The oxidants in our diet can induce diseases, such as hypertension, diabetes, arteriosclerosis, cancer, and senescence. Although synthetic antioxidants can be of help, they bear some risks and side effects. Hence, antioxidants originating from plants are attracting attention (Suttirak and Manurakchinakorn, 2014; Stone et al., 2014).

Some studies have demonstrated that orange peel oil can be used as a medical agent that protects against cancer and prevents the growth and proliferation of cancer cells by d-Limonene. The orange essential oil contains large quantities of d-Limonene is present, which has anti-proliferative and apoptosis-inducing effects (Mauro et al., 2013; Crowell and Gould, 1994). Thus, it is used as a chemopreventive and chemotherapeutic agent against multiple types of tumors (Vigushin et al., 1998; Chaudhary et al., 2012).

Table 2. Comparison of cell viability between the different groups and time points

| Time   | Group   | n | Mean ± SD | SE | 95% CI | Min. | Max. |
|--------|---------|---|-----------|----|--------|------|------|
| Hour 24| DMSO%20 | 4 | 0.00825±0.0005 | 0.0025 | 0.00745 | 0.00905 | 0.008 | 0.009 |
|        | Kontrol | 4 | 0.172±0.01951 | 0.00755 | 0.14095 | 0.20305 | 0.153 | 0.193 |
|        | 25µl/mL | 4 | 0.01075±0.0005 | 0.00025 | 0.00995 | 0.01155 | 0.01  | 0.011 |
|        | 50µl/mL | 4 | 0.0135±0.001291 | 0.00645 | 0.01145 | 0.01555 | 0.012 | 0.015 |
|        | 100µl/mL| 4 | 0.02075±0.0025 | 0.00125 | 0.01677 | 0.02473 | 0.018 | 0.024 |
|        | 150µl/mL| 4 | 0.022±0.000816 | 0.000408 | 0.00207 | 0.0233  | 0.021 | 0.023 |
|        | 200µl/mL| 4 | 0.018±0.004234 | 0.002121 | 0.01125 | 0.02475 | 0.013 | 0.022 |
|        | P       | 0 | 0.000       | 0.00025 | 0.00695 | 0.00855 | 0.007 | 0.008 |
| Hour 48| DMSO%20 | 4 | 0.00775±0.0005 | 0.0025 | 0.00695 | 0.00855 | 0.007 | 0.008 |
|        | Kontrol | 4 | 0.266±0.029098| 0.014549 | 0.2197  | 0.3123  | 0.229 | 0.293 |
|        | 25µl/mL | 4 | 0.015±0.004243 | 0.002121 | 0.00825 | 0.02175 | 0.012 | 0.021 |
|        | 50µl/mL | 4 | 0.02075±0.003096 | 0.001548 | 0.01582 | 0.02568 | 0.018 | 0.025 |
|        | 100µl/mL| 4 | 0.02825±0.007719 | 0.00386 | 0.01597 | 0.04053 | 0.021 | 0.039 |
|        | 150µl/mL| 4 | 0.03825±0.00789 | 0.003945 | 0.0257 | 0.0508  | 0.029 | 0.047 |
|        | 200µl/mL| 4 | 0.058±0.018493 | 0.009247 | 0.0207 | 0.08743 | 0.034 | 0.074 |

SD: Standard deviation. SE: Standard error. CI: Confidence interval. *cells with the same letters denote non-significant differences.
However, the beneficial role of orange peel oil and its constituents in cancer treatment needs further elucidation (Lesgards et al., 2014).

Many studies are claiming that essential oils might inhibit pathogenic bacteria in the small intestine. Herbal extracts have an antimicrobial effect against E. coli in poultry and pigs (Bölükbaşı et al., 2007, 2009; Bruggeman, et al., 2002; Kamel, 2001; Mitsch, et al., 2004).

In a study conducted by Erhan and Bölükbaşı in 2017, they looked at the effect of citrus shell oils added to broiler feeds on the numeric density of blood and lymphatic capillaries, and the length and density of jejunal villi. The density score of the blood and lymphatic capillaries of the broilers fed with a diet containing 3 mL/kg orange peel oil was very dense. The blood capillary and lymphatic capillary density scores of the groups consuming 1 mL/kg citrus peel oil (bergamot, lemon, and orange) were low. The increase of the nutrient absorbing surface and the amount of nutrient absorption are related to the rise in the density of blood capillaries. However, they reported that increasing the concentration of lymphatic capillaries would result in an increase in both the absorption surface and the amount of fat absorption.

In animal feed, plant extracts are used as yield enhancers. Yield enhancers serve two purposes. The first objective is to prevent the growth of some pathogenic microorganisms in the digestive system of animals such as Salmonella and the Coliform group, which are the source of diseases in the digestive system and threaten people with the food chain. The second objective is to convert the gastrointestinal tract of animals in favor of positive microorganisms and to ensure that the host can benefit from the nutrients in the feed at the highest level (Nir and Şenköyülü, 2000; Yavuz, 2001).

Addition of herbal extracts to poultry feeds provides benefits such as weight gain, higher egg yield and better feed conversion efficiency, killing of pathogenic microorganisms in the digestive system starting in the mouth, increasing the flavor of feed, increasing the secretion of digestive juices, increasing effectiveness of digestive enzymes, promoting the immune system, providing low-cholesterol animal products, increasing protein synthesis by stimulating the production of higher quality and lean meat, and establishing a cleaner and healthier environment by binding ammonia (Kutlu and Görgülü, 2001; Gill, 1999).

![Cell Viability](image)

**Figure 1.** Distributions of mean cell viability (%) between the different experimental groups

**Conclusion**

We had previously examined the density of blood and lymphatic capillaries, the length and density of jejunal villi, and the effect of tissue fatty acid composition and shelf life by adding orange shell oil to broiler feed at different levels (Erhan and Bölükbaşı, 2017; Erhan and Bölükbaşı Aktaş, 2017). In this study, we wanted to move the topic one step further and examine whether or not orange peel oil has antiproliferative effects on the immortalized human keratinocyte non-tumorigenic cell line (HaCaT).

When the results of this study (Table 1 and Figure 1) are examined, it is seen that orange peel oil has a toxic effect on the non-tumorigenic cell line (HaCaT). When compared with the control group, it appears that all doses have a lethal impact on the cells. However, in our previous studies (Erhan and Bölükbaşı, 2017) we found that orange shell oil increased
the number of villi in the jejunum of the small intestine and the number of goblet cells where absorption was achieved.

In this cell culture study, we observed the killing effect of orange shell oil in the cells. However, in the previous study, adding it to the ration and allowing it to enter the metabolism increased the number of villus and goblet cells. Thus, I assume that orange shell oil has different effects in the metabolism. I recommend this subject to be studied by comparing it in both live animals and cell-culture studies.

Combining the findings of this study with the data of previous studies, the areas where orange peel oil can be used may be as follows:

i- As an auxiliary agent in cancer prevention and cancer treatments due to its anti-cancer effects,

ii- Natural anti-oxidant effect,

iii- Anti-allergic, anti-inflammatory, and antimicrobial effects,

iv- The impact of controlling pathogen microorganisms,

v- Use as food and beverage aroma,

vi- The use of medicines as spice ingredients in the pharmaceutical industry to hide unpleasant flavors, its use in perfumes and cosmetics,

vii- Finally, based on the results of this study, it is estimated that orange peel oil can be used as a preservative to prevent bacterial growth in animal feed and human food. To determine its protective effect in feeds, it is recommended to perform feed toxicity studies after adding orange shell oil.

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