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Antiaging Effects of Limonene in Ageing-Induced HaCaT Cells

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Abstract

Limonene, obtained from citrus oils such as orange, grapefruit, and lemon, is widely used in cosmetics as a scenting agent. It is regarded as safe because it is not mutagenic, carcinogenic, or nephrotoxic to people and has low human toxicity. This study aims to show the beneficial effects of limonene against hydrogen peroxide (H_2O_2) induced ageing in human keratinocytes (HaCat) cell line. For this purpose, the concentration value at which H_2O_2 can cause ageing in HaCaT cells was determined as 200 µM by the MTT method. Then, cytotoxic effects of limonene (1, 10, 25, 50, 100, 250 and 500 µg/mL) alone and in aging-induced cells were determined by the MTT method; possible antigenotoxic effects were determined by alkaline single-cell gel electrophoresis (Comet) method. According to the results obtained, limonene, an ingredient in cosmetics, has protective effects against skin aging in HaCaT cells.

Keywords

senescence, hydrogen peroxide, cytotoxicity, genotoxicity, MTT assay

Introduction

Limonene (d-limonene, l-limonene, and dl-limonene) is a monoterpene, a lemon-scented compound found in the essential oils of aromatic plants (Figure 1). This compound, obtained from citrus oils such as orange, grapefruit and lemon, is widely used in cosmetics, foods, drugs, pesticides and industrial solvents (Kim *et al.*, 2013). There are two optical isomers of limonene, d- and l-limonene, as well as a racemic combination (Vieira *et al.*, 2018). D-limonene, the main active form of limonene, has many therapeutic activities such as anticancer, antidiabetic or anti-inflammatory (Anandakumar *et al.*, 2021). In addition, studies on monoterpenes such as limonene with the 2,2-diphenyl-1-picryl-hydrazine-hydrate (DPPH) free radical method have shown that these compounds have antioxidant activity (Graßmann, 2005; Junior *et al.*, 2009; Roberto *et al.*, 2010).

D-limonene prevents damage to cell membranes by inhibiting free radicals and lipid peroxidation and stimulates carcinogenic metabolizing enzymes (cytochrome P-450) that convert chemical carcinogens into less toxic compounds and prevent their reaction with DNA (Hajizadeh *et al.*, 2019).

Limonene is regarded as safe because it is not mutagenic, carcinogenic, or nephrotoxic to people and has low human toxicity (Vieira *et al.*, 2018). A study in humans and guinea pigs reported that limonene was not sensitizing, and in a study in mice, it was very weakly sensitizing compared to the local lymph node test (Letizia *et al.*, 2003; Roberts *et al.*, 2007; Natsch *et al.*, 2019).

Although d-limonene is described as a low-toxicity chemical, it can tend to oxidize and form hydroperoxides when exposed to air for long periods of time. These compounds have been found to be potent skin sensitizers in animal tests (Kern et al., 2014). Studies have reported that metabolites have an irritating effect on the skin (Christensson *et al.*, 2008; Kim *et al.*, 2013). It has also been reported that the limonene compound has the potential for skin irritation or sensitization (Okabe *et al.*, 1990; Karlberg *et al.*, 1991; Kim *et al.*, 2013).

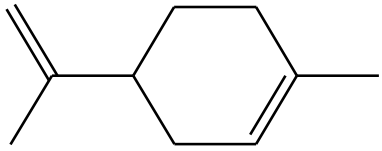

Figure 1. Chemical structure of limonene

Ageing, which is the main cause of many degenerative diseases, appears most clearly with cumulative changes in skin structure, function, and appearance, such as increased wrinkles, laxity, and abnormal pigmentation. Skin ageing is a complex and poorly understood process that is affected by both the internal ageing process and various external environmental factors that accelerate ageing (Gu *et al.*, 2020). Examples of these factors are environmental factors such as ultraviolet radiation, smoking, lifestyle differences, and pollution. All these factors stimulate the formation of reactive oxygen species (ROS) in the body, and thus cause oxidative stress, resulting in the deterioration of body functions (Kurfurst *et al.*, 2010; Kohl *et al.*, 2011). Skin ageing causes many undesirable consequences such as disruption of the dermal extracellular matrix and loss of skin barrier function, loss of antimicrobial function (Wu *et al.*, 2021).

Parameters such as hydrogen peroxide (H_2O_2) are used in studies to examine the effect of oxidative stress and the responses of skin cells (*Kurfurst et al.*, 2010). ROS, such as hydrogen peroxide, cause oxidative stress, disrupting cell metabolism and redox balance. Although cells use antioxidant mechanisms (such as superoxide dismutase (SOD), glutathione peroxidase and catalase (CAT)) to maintain balance, lipid peroxidation, DNA damage and protein denaturation may occur as a result of excessive ROS formation (Hahn *et al.*, 2017).

Hydrogen peroxide (H_2O_2) acts directly by oxidative mechanisms or by converting to hydroxyl (OH) via the Fenton reaction (Kurfurst *et*

al., 2010). As a result of the overproduction of H_2O_2 in human dermal fibroblasts, antioxidant defense mechanisms are impaired (Hahn *et al.,* 2017). Because H_2O_2 induces free radical formation and lipid peroxidation, it is frequently used to induce oxidative stress and cellular senescence in the *in vitro* tests (Liu *et al.,* 2022). The aim of this study was to evaluate the cytotoxic and genotoxic effects of limonene, an active ingredient in cosmetic products, against hydrogen peroxide (H_2O_2) induced aging in human keratinocytes (HaCat) cell line.

Material and methods

Chemicals

The following suppliers provided the compounds used in the study: Dulbecco's Modified Eagles Medium (DMEM) and Phosphate Buffered Saline (PBS) purchased from Wisent Bioproducts (Quebec, Canada); trypsin-EDTA used from Biological Industries (Beit-Haemek, Israel); Fetal Bovine Serum (FBS) and penicillin-streptomycin supplied from Capricorn Scientific (Ebsdorfergrund, Germany); 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), low melting point agarose (LMA), normal melting point agarose (NMA), dimethyl sulfoxide (DMSO), ethidium bromide (EtBr), phosphate-buffered saline (PBS) tablets, N-Lauroylsarcosine sodium salt, Tris HCl, and Triton X-100 purchased from Sigma-Aldrich (Munich, Germany).

Cell Culture

All cell culture studies were performed under a sterile working cabinet. HaCaT (passage number: 16) cells were transferred to horizontal cell culture flasks using Dulbecco's Modified Eagle Medium (DMEM) (with high glucose and L-glutamine containing 10% FBS and 1% Penicilline-Streptomycin). An incubator with 95% humidity and 5% carbon dioxide (CO_2) at 37°C for 24 hours was used for incubation of cells. The flasks were examined under a microscope to check the saturation and contamination status of the cell culture medium.

Induction of Aging

The HaCaT cell suspension was diluted with 1 x 10^5 cells/200 µL medium in each well, and cells were seeded into 96-well plates. H_2O_2 was used to induce ageing in HaCaT cells. For this purpose, different concentrations of H_2O_2 (50 µM, 100 µM, 200 µM, 300 µM, 400 µM) were applied to the cells for 24 hours and the cytotoxic effects of these solutions determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Bacanlı et al., 2022). For the determination of cell viability, after 24 hours of exposure in an incubator at 37°C with 5% CO_2, H_2O_2 solutions were removed. Furthermore, after adding 0,5 mg/mL MTT solution to each well, it was incubated for 3 hours. MTT solutions were removed following incubation, and DMSO solution was then added to each well. The color intensity in the wells was measured at 570 nm after the plates were shaken horizontally for 10 minutes to mix them. Calculations were made about cell viability and 50% inhibitory concentrations (IC_{50}) at various incubation periods. One percent of DMSO in DMEM was used as a negative control. The lowest concentration of H_2O_2 solution, which significantly reduces cell viability, was used to induce ageing in the cells.

Determination of Effects of Limonene against Aging

After determining the H_2O_2 concentration for inducing senescence in cells, HaCaT cells were seeded in 96-well plates at 1 x 10^5/200 µL medium in each well, and cells were incubated with a solution of 200 µM H_2O_2 for 24 hours. In order to determine the effects of limonene on ageing, the cells were treated for 1 hour with limonene solutions (1, 10, 25, 50, 100, 250 and 500 µg/mL) prepared in the medium with 1% DMSO at

different concentrations while this period continued. MTT method was used to determine cell viability as described in Section 2.3.

Determination of Genotoxicity

After the determination of the IC_{50} values of limonene in Section 2.3, the effects of limonene on genotoxicity in HaCaT cells where ageing was induced at concentrations below the IC_{50} value were determined by the alkaline single cell gel electrophoresis (Comet) method. Comet assay was performed by *Collins et al.* (2023) protocol and MIRCA guidelines by Moller et al. (2020). Cells were counted on the Neubauer counting slide and seeded at 3 x 10^5/well concentration in 24-well plates. Cells were exposed to 200 µM H_2O_2 for 24 hours at 37°C in an incubator with 95% humidity and 5% CO_2. In order to determine the effects of limonene on ageing induced genotoxicity, the cells were treated for 1 hour with limonene solutions (1, 10, 25, 50 and 100 µg/mL) prepared in the medium with 1% DMSO at different concentrations while this period continued.
After cells were incubated, 75 µL of cell suspension was mixed with 150 µL of 0.5% LMA dissolved at 37°C ± 0.5°C and spread on slides coated with 1% NMA. DMEM (containing 1% DMSO) was used as negative control. The slides were covered with coverslips and incubated at 4°C for 2-10 minutes to solidify the agar. The slides were separated from their coverslips and placed in the chalets, cold lysis solution (2.5 M NaCl, 100 mM EDTA, 100 mM Tris, 1% sodium sarcosinate, pH 10.0 with Triton-X-100 and 10% DMSO) was poured on them and kept at 4°C for at least 1 hour.
Then, the slides were taken from the chalets and placed in the electrophoresis tank with the agar spread parts facing up. Cold electrophoresis solution (1 mM sodium EDTA and 300 mM NaOH, pH 13.0, 4°C) was poured into the electrophoresis tank and the slides were kept for 20 minutes without applying any current (denaturation process). After this procedure, electrophoresis was applied for 20 minutes by applying 25 V and 300 mA 1 V/cm current at 4°C. After electrophoresis, the slides were returned to the chalets and kept in PBS solution for 10 minutes (neutralization). Then, the slides were left to dry by soaking in 50%, 75% and 99% ethanol solutions, respectively, for 5 minutes. Ethidium bromide (EtBr) solution (20 µg/mL) was used for staining. Evaluation of cells was performed under a fluorescent microscope at 100 cells per slide. For this purpose, the computer program "Perspective Instruments COMET Assay IV Analysis System, UK" was used. The degree of DNA damage was assessed using the tail density (%) descriptor.

Results and Discussion

Determination of Effects of Limonene against Aging

The results of the cytotoxic effects of limonene in HaCaT cells by MTT assay are shown in Figure 1. It was observed that in the same cell line, H_2O_2 produced more cytotoxic effects in the concentration range of 200-300 µM, while limonene produced more cytotoxic effects in the concentration range of 100-200 µg/mL. After exposure to H_2O_2, it was observed that the applied limonene produced more cytotoxic effects in the concentration range of 100-200 µg/mL. Limonene reduced H_2O_2-induced cytotoxicity at all concentrations except for low concentrations (1 and 5 µg/mL).
In a study investigating the cytotoxicity effects of D-limonene on human liver cancer (HepG2) cells in the concentration range of 1-400 µM, it was observed that cell viability decreased depending on the concentration, with IC_{50} value in the concentration range of 200-400 µM (Hajizadeh *et al.*, 2019). In another study, investigating the cytotoxicity of d-limonene, lung cancer cells (A549 and H1299) were exposed to 0.5 and 0.75 mM d-limonene for 24, 48, and 72 hours, and cell viability was determined by The Cell Counting Kit-8 assay.

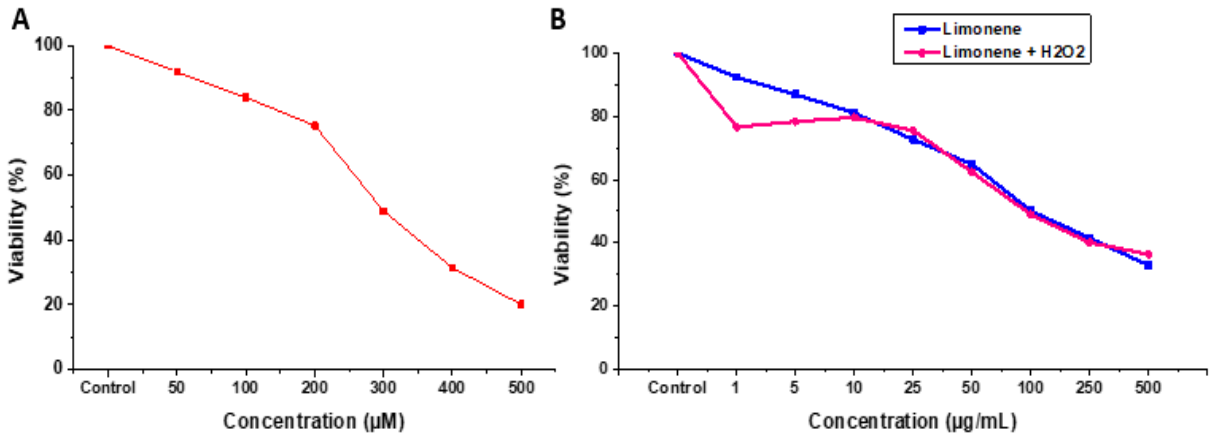

Figure 2. Viability of HaCaT cells after H_2O_2 (A); limonene and limonene + H_2O_2 exposure (B)

That study resulted with the cell viability decrease due to the increase in concentration in both cell lines, while the cytotoxic effect was very high at 0.75 mM concentration. Another study showed that d-limonene inhibited the growth of lung cancer cells (Yu *et al.*, 2018).

Another study investigating the cytotoxicity of D-limonene showed that after 48 hours of exposure to colon cancer (LS174T) cells, it suppressed the viability of cells and caused apoptotic cell death in a dose-dependent manner (Jia *et al.*, 2013). In another study, cytotoxicity, and apoptosis in human lymphatic endothelial cells (HLEC) were investigated and demonstrated a protective effect of D-limonene from H_2O_2-induced oxidative stress. It was observed that d-limonene increased cell viability by reducing ROS formation and suppressed apoptosis by inhibiting caspase activation. This shows that D-limonene effectively protects cells from H_2O_2-induced oxidative stress (Bai *et al.*, 2016). In our study, it was observed that the IC50 values of limonene in the HaCat cell line were in the concentration range of 100-200 µg/mL. After exposure to H_2O_2, the IC_{50} values of limonene were in the concentration range of 100-200 µg/mL.

Limonene treatment seemed to reduce the cytotoxicity caused by H_2O_2 at all concentrations except for low concentrations. In addition, in our study, cell viability decreased due to the increase in limonene concentration. In other studies investigating the cytotoxicity of an essential oil containing 70% limonene and linalyl acid, it was observed that the percentage of viability of human neuroblastoma (SH-SY5Y) cells decreased depending on the concentration (Berliocchi *et al.*, 2011; Vieira *et al.*, 2018). The results of our study are in agreement with the data on the cytotoxic effects of limonene.

Determination of Genotoxicity

The results of the genotoxic and antigenotoxic effects of limonene in HaCaT cells by the alkaline Comet assay are shown in Figure 3. According to the data obtained from the experiments, it was observed that all applied limonene concentrations (2, 5, 10, 25 and 50 µg/mL) caused similar levels of DNA damage. In addition, DNA damage increased with the increase in the limonene concentration. No significant increase in DNA damage was observed when compared to the negative control. In addition, limonene treatment

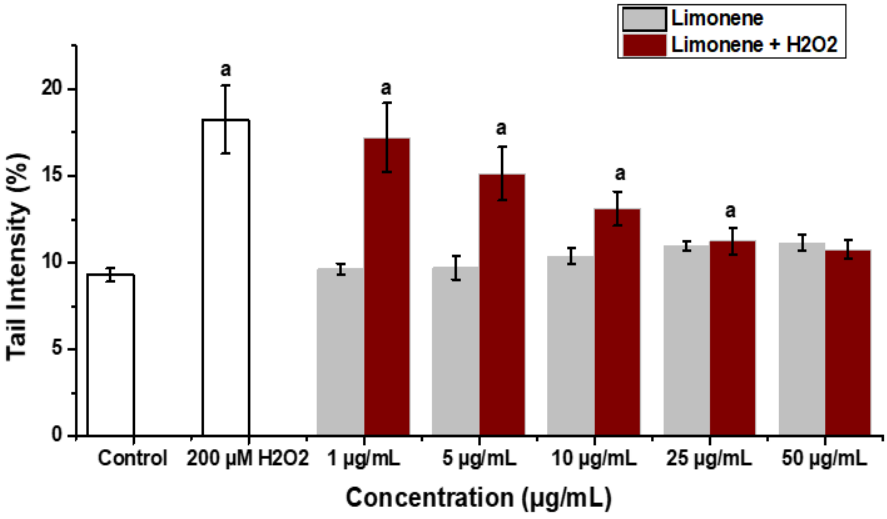

Figure 3. Antigenotoxic effects of limonene against H_2O_2 induced aging in HaCaT cells

at all concentrations studied appeared to reduce DNA damage caused by H_2O_2. In a study using four different *S. typhimurium* strains (TA98, TAIOO, TA1535 or TA1537) investigating the genotoxicity of D-limonene, no mutagenic effects were observed in the compound. Moreover, induction of sister chromatid exchange of the compound was not observed in cultured Chinese hamster ovary cells (Sun, 2007).

In a study investigating the cytotoxicity and genotoxicity of limonene, there was no effect on cell viability up to 100 µM in Hamster Chinese lung (V79) cells, but afterwards, cell viability decreased below 50% at a concentration of 1000 µM. As a result of genotoxicity studies, DNA damage did not show a significant increase at low concentrations (500-5000 µM), but increased at high concentrations (10000 µM). However, DNA damage induced by H_2O_2 was decreased at the studied concentrations (Bacanlı et al., 2015). When compared to the negative control (1% DMSO) in our study, HaCaT cells exposed to limonene at various concentrations (50–2000 M) did not result in genotoxic effects at any of the studied concentrations. On the other hand, treatment with limonene at all investigated concentrations appeared to decrease the genotoxic effects produced on by H_2O_2. The results of our study are consistent with previous data on the genotoxic and antigenotoxic effects of limonene.

Conclusion

The positive effects of limonene, which is an active ingredient in many cosmetic products, on skin ageing caused by H_2O_2 have been demonstrated in our study. Based on the data obtained in our study, it is predicted that cosmetic products containing limonene in different forms may be beneficial against skin ageing. All of the data in our study were obtained *in vitro*. Therefore, further *in vivo* and *in vitro* studies are needed to understand the antiaging properties of limonene.

Conflict of Interest

None to declare.

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References

Anandakumar, P., Kamaraj, S., & Vanitha, M. K. (2021). D-limonene: A multifunctional compound with potent therapeutic effects. *Journal of Food Biochemistry*, *45*(1), e13566.

Bacanlı, M., Başaran, A. A., & Başaran, N. (2015). The antioxidant and antigenotoxic properties of citrus phenolics limonene and naringin. *Food and chemical Toxicology*, *81*, 160-170.

Bacanlı, M., Secerli, J., Karayavuz, B., Erdem, O., & Erdoğan, H. (2022). Is a non-cytotoxic and non-genotoxic novel bioinspired dipeptide structure synthesis possible for theragnostic applications?. *Drug and Chemical Toxicology*, 1-9.

Bai, J., Zheng, Y., Wang, G., & Liu, P. (2016). Protective effect of D-limonene against oxidative stress-induced cell damage in human lens epithelial cells via the p38 pathway. *Oxidative Medicine and Cellular Longevity*, *2016*.

Berliocchi, L., Ciociaro, A., Russo, R., Cassiano, M. G. V., Blandini, F., Rotiroti, D., ... & Corasaniti, M. T. (2011). Toxic profile of bergamot essential oil on survival and proliferation of SH-SY5Y neuroblastoma cells. *Food and chemical toxicology*, *49*(11), 2780-2792.

Christensson, J. B., Johansson, S., Hagvall, L., Jonsson, C., Börje, A., & Karlberg, A. T. (2008). Limonene hydroperoxide analogues differ in allergenic activity. *Contact dermatitis*, *59*(6), 344-352.

Collins, A., Møller, P., Gajski, G., Vodenková, S., Abdulwahed, A., Anderson, D., ... & Azqueta, A. (2023). Measuring DNA modifications with the comet assay: A compendium of protocols. *Nature Protocols*, 1-61.

Graßmann, J. (2005). Terpenoids as plant antioxidants. *Vitamins & Hormones*, *72*, 505-535.

Gu, Y., Han, J., Jiang, C., & Zhang, Y. (2020). Biomarkers, oxidative stress and autophagy in skin aging. *Ageing research reviews*, *59*, 101036.

Hahn, H. J., Kim, K. B., An, I. S., Ahn, K. J., & Han, H. J. (2017). Protective effects of rosmarinic acid against hydrogen peroxide-induced cellular senescence and the inflammatory response in normal human dermal fibroblasts. *Molecular Medicine Reports*, *16*(6), 9763-9769.

Hajizadeh, M. R., Maleki, H., Barani, M., Fahmidehkar, M. A., Mahmoodi, M., & Torkzadeh-Mahani, M. (2019). In vitro cytotoxicity assay of D-limonene niosomes: an efficient nano-carrier for enhancing solubility of plant-extracted agents. *Research in pharmaceutical sciences*, *14*(5), 448.

Hajizadeh, M. R., Maleki, H., Barani, M., Fahmidehkar, M. A., Mahmoodi, M., & Torkzadeh-Mahani, M. (2019). In vitro cytotoxicity assay of D-limonene niosomes: an efficient nano-carrier for enhancing solubility of plant-extracted agents. *Research in pharmaceutical sciences*, *14*(5), 448.

Jia, S. S., Xi, G. P., Zhang, M., Chen, Y. B., Lei, B. O., Dong, X. S., & Yang, Y. M. (2013). Induction of apoptosis by D-limonene is mediated by inactivation of Akt in LS174T human colon cancer cells. *Oncology reports*, *29*(1), 349-354.

Junior, M. R. M., e Silva, T. A. R., Franchi, G. C., Nowill, A., Pastore, G. M., & Hyslop, S. (2009). Antioxidant potential of aroma compounds obtained by limonene biotransformation of orange essential oil. *Food Chemistry*, *116*(1), 8-12.

Karlberg, A. T., Boman, A., & Melin, B. (1991). Animal experiments on the allergenicity of d-limonene—the citrus solvent. *The Annals of occupational hygiene*, *35*(4), 419-426.

Kern, S., Granier, T., Dkhil, H., Haupt, T., Ellis, G., & Natsch, A. (2014). Stability of limonene and monitoring of a hydroperoxide in fragranced products. *Flavour and Fragrance Journal*, *29*(5), 277-286.

Kim, Y. W., Kim, M. J., Chung, B. Y., Bang, D. Y., Lim, S. K., Choi, S. M., ... & Lee, B. M. (2013). Safety evaluation and risk assessment of

d-limonene. *Journal of Toxicology and Environmental Health, Part B*, *16*(1), 17-38.

Kohl, E., Steinbauer, J., Landthaler, M., & Szeimies, R. M. (2011). Skin ageing. *Journal of the European academy of dermatology and venereology*, *25*(8), 873-884.

Kurfurst, R., Favıer, A., & Sauvaıgo, S. (2010). Study of fibroblast gene expression in response to oxidative stress induced by hydrogen peroxide or UVA with skin aging. *European Journal of Dermatology*, *20*(3), 308-20.

Letizia, C. S., Cocchiara, J., Lalko, J., & Api, A. M. (2003). Fragrance material review on linalool. *Food and Chemical Toxicology*, *41*(7), 943-964.

Liu, X., Xing, Y., Yuen, M., Yuen, T., Yuen, H., & Peng, Q. (2022). Anti-Aging Effect and Mechanism of Proanthocyanidins Extracted from Sea buckthorn on Hydrogen Peroxide-Induced Aging Human Skin Fibroblasts. *Antioxidants*, *11*(10), 1900.

Møller, P., Azqueta, A., Boutet-Robinet, E., Koppen, G., Bonassi, S., Milić, M., ... & Langie, S. A. (2020). Minimum Information for Reporting on the Comet Assay (MIRCA): recommendations for describing comet assay procedures and results. *Nature protocols*, 15(12), 3817-3826.

Natsch, A., Nägelin, M., Leijs, H., van Strien, M., Giménez-Arnau, E., Vey, M., & Gonzalez, C. (2019). Exposure source for skin sensitizing hydroperoxides of limonene and linalool remains elusive: an analytical market surveillance. *Food and Chemical Toxicology*, *127*, 156-162.

Roberto, D., Micucci, P., Sebastian, T., & Anesini, C. (2010). Antioxidant activity of limonene on normal murine lymphocytes: relation to H_2O_2 modulation and cell proliferation. *Basic & clinical pharmacology & toxicology*, *106*(1), 38-44.

Roberts, D. W., Patlewicz, G., Kern, P. S., Gerberick, F., Kimber, I., Dearman, R. J., ... & Aptula, A. O. (2007). Mechanistic applicability domain classification of a local lymph node assay dataset for skin sensitization. *Chemical research in toxicology*, *20*(7), 1019-1030.

Sun, J. (2007). D-Limonene: safety and clinical applications. *Alternative medicine review*, *12*(3).

Vieira, A. J., Beserra, F. P., Souza, M. C., Totti, B. M., & Rozza, A. L. (2018). Limonene: Aroma of innovation in health and disease. *Chemico-Biological Interactions*, *283*, 97-106.

Vieira, A. J., Beserra, F. P., Souza, M. C., Totti, B. M., & Rozza, A. L. (2018). Limonene: Aroma of innovation in health and disease. *Chemico-Biological Interactions*, *283*, 97-106.

Wu, Y., Cao, K., Zhang, W., Zhang, G., & Zhou, M. (2021). Protective and Anti-Aging Effects of 5 Cosmeceutical Peptide Mixtures on Hydrogen Peroxide-Induced Premature Senescence in Human Skin Fibroblasts. *Skin Pharmacology and Physiology*, *34*(4), 194-202.

Yu, X., Lin, H., Wang, Y., Lv, W., Zhang, S., Qian, Y., ... & Qian, B. (2018). D-limonene exhibits antitumor activity by inducing autophagy and apoptosis in lung cancer. *OncoTargets and therapy*, *11*, 1833.