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Türk Hijyen ve Deneysel Biyoloji Dergisi

Investigation of the effect of *Moringa oleifera* extract on fibroblast degeneration caused by disinfectants used at different pH levels in the COVID-19 pandemic

COVID-19 pandemisinde farklı pH derecelerinde kullanılan dezenfektanların neden olduğu fibroblast dejenerasyonu üzerine *Moringa oleifera* ekstraktının etkisinin araştırılması

Yeşim YENİ[1] (ID), Sıdıka GENÇ[2] (ID), Ahmet HACIMÜFTÜOĞLU[1] (ID), Ali TAGHİZADEHGHALEHJOUGHİ[2] (ID)

ABSTRACT

Objective: The rapid spread of the coronavirus disease, which started in 2019, has caused it to become a global epidemic. To control the spread of the coronavirus epidemic, some prevention procedures such as wearing face masks, maintaining social distance and hand hygiene have begun to be implemented. Accordingly, the use of disinfectants in public places such as transportation and shopping has gained importance. However, studies have shown that long-term use of disinfectants with different pH values triggers the development of itching, redness, urticaria and even allergic rhinitis. The low saturated fatty acids and high amounts of olive oil found in *Moringa oleifera* (MO) leaves, flowers, green beans, seeds and seed oil are excellent sources of nutrients and vitamins. Today, MO is used in a variety of skin care applications. In addition, the leaf of MO also has antiretroviral, antimicrobial, antioxidant, antifungal properties. The aim of this study is to prevent toxicity against different pH changes by using MO plant.

Methods: In our study, a human fibroblast cell

ÖZET

Amaç: 2019 yılında başlayan koronavirüs hastalığının dünya çapında hızla yayılması, küresel bir salgın haline gelmesine neden olmuştur. Koronavirüs salgınının yayılmasını kontrol altına almak için yüz maskesi takma, sosyal mesafeyi koruma ve el hijyeni gibi bazı önleme prosedürleri uygulanmaya başlandı. Buna bağlı olarak ulaşım ve alışveriş gibi halka açık yerlerde dezenfektan kullanımı önem kazandı. Ancak araştırmalar, farklı pH değerlerine sahip dezenfektanların uzun süreli kullanımının kaşıntı, kızarıklık, ürtiker ve hatta alerjik rinit gelişimini tetiklediğini göstermiştir. *Moringa oleifera* (MO) yapraklarında, çiçeklerinde, yeşil fasulyelerde, tohumlarda ve tohum yağında bulunan düşük doymuş yağ asitleri ve yüksek miktarda zeytinyağı, harika besin ve vitamin kaynaklarıdır. Günümüzde MO, çeşitli cilt bakım uygulamalarında kullanılmaktadır. Ayrıca MO'nun yaprağı antiretroviral, antimikrobiyal, antioksidan, antifungal özelliklere de sahiptir. Bu çalışmanın amacı, MO bitkisi kullanılarak farklı pH değişimlerine karşı gelişen toksisitenin önlenmesidir.

Yöntem: Çalışmamızda insan fibroblast hücre

[1]Ataturk University, Faculty of Medicine, Department of Medical Pharmacology, Erzurum, Turkey
[2]Bilecik Seyh Edebali University, Faculty of Medicine, Department of Medical Pharmacology, Bilecik, Turkey

İletişim / Corresponding Author : Ali TAGHİZADEHGHALEHJOUGHİ
Bilecik Şeyh Edebali Üniversitesi, Tıp Fakültesi, Farmakoloji AD., Bilecik - Türkiye
E-posta / E-mail : alitgzd@gmail.com

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line was grown under the conditions specified by the manufacturer and inoculated into 96 well plates and a culture medium with different pH (5.0, 6.0, 7.0 and 8.0) was prepared and different doses of MO (20, 40, 80 and 160 µg/ml) were prepared. It is planned to eliminate the toxicity caused by using it for 24 hours. At the end of the study, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Glutathione Reductase (GR), and Lactate Dehydrogenase (LDH) tests were used.

Results: According to the results of our study, cell viability increased from %69 to %89 compared to positive control at pH 5.0. The vitality that decreased to %80 at pH 6.0 increased to 143 as a result of the application of the highest concentration of MO (160 µg/ml). While it increased from %79 to %115 at pH 7.0, the damage received by fibroblast cells at pH 8.0 was determined to be high (viability rate %66), and it was observed that this toxicity was removed, and the vitality was increased up to %93. All results were statistically evaluated and found to be significant (P<0.05 and P<0.01).

Conclusion: Considering the results obtained, MO is recommended to be used at doses of 80 and 160 µg/ml, as it has a protective effect. The LDH and GR results show a correlation with MTT.

Key Words: COVID-19, GR, LDH, *Moringa oleifera*, pH

hattı, üretici firma tarafından belirtilen koşullarda büyütülerek 96 kuyucuklu plaklara inoküle edildi; farklı pH'lı (5.0, 6.0, 7.0 ve 8.0) kültür ortamları hazırlanarak farklı dozlarda MO (20, 40, 80 ve 160 µg/ml) kutucuklara eklendi. 24 saat sonra 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Glutatyon Redüktaz (GR) ve Laktat Dehidrogenez (LDH) testleri kullanılarak elde edilen veriler kaydedildi.

Bulgular: Çalışmamızın sonuçlarına göre hücre canlılığı pH 5.0'te pozitif kontrole göre %69'dan %89'a çıkmıştır. pH 6.0'da %80'e düşen canlılık, MO'nun en yüksek konsantrasyonunun (160 µg/ml) uygulanmasıyla %143'e çıkmıştır. pH 7.0'de hücre canlılığı %79'dan %115'e yükselirken, pH 8.0'de fibroblast hücrelerinin aldığı hasarın yüksek olduğu (canlılık oranı %66) tespit edilmiş ve MO uygulaması ile hücre canlılığının %93'e kadar arttığı saptanmıştır. LDH ve GR sonuçlarının MTT ile korelasyon gösterdiği saptanmıştır. tüm sonuçlar istatistiksel olarak değerlendirilmiş ve anlamlı bulunmuştur (P<0.05 ve P<0.01).

Sonuç: Elde edilen verilere göre MO hücre canlılığı üzerinde koruyucu etkiye sahiptir. Bu etki 80 ve 160 µg/ml dozlarında maksimuma erişmektedir Dezenfektanların ciltte oluşturduğu istenmeyen etkilerin önlenmesi amacıyla MO kullanımı uygun bir seçenek gibi gözükmektedir. MO'nun bu amaçla güvenle kullanımı için klinik araştırmalara ihtiyaç vardır.

Anahtar Kelimeler: COVID-19, GR, LDH, *Moringa oleifera*, pH

INTRODUCTION

The exponential increase of cases affected by COVID-19 diseases in China was observed by the World Health Organization (WHO) and declared a global health emergency on January 30, 2020. The coronavirus spread between people through air and physical contact. In these circumstances, it has become important to search for materials and protective devices that can be used to control the spread of coronavirus diseases. There is no effective vaccine or treatment for COVID-19 that has yet to be introduced. However, the transmission of the virus has been suppressed by some prevention procedures such as wearing a face mask for self-protection, maintaining social distance and hand hygiene.

Accordingly, the use of disinfectants has gained importance in public places such as transportation and shopping, and the use of degenerate drugs with different chemical formulations has become widespread (1, 2).

Today, natural bioactive agents have been used for various skin care applications. These bioactive substances are compounds found in plants and certain foods such as fruits, vegetables, nuts, oils, and whole grains among the various bioactive agents, *Moringa oleifera* (MO) from the monogenic family has continued to be popular among people for good skin health (3, 4). The low saturated fatty acids and high amounts of olive oil found in MO leaves, flowers, green beans, seeds, and seed oil are a great source of nutrients and vitamins (5). In addition, MO's leaf has antiretroviral, antimicrobial, antioxidant, antifungal properties (6,7). Most of the studies reveal that the MO-derived extract inhibits the initiation of the viral replication cycle (8).

There are several articles on the extraordinary properties of this natural bioactive agent infused for wound healing and packaging applications (6, 9, 10, 11) MO was chosen because it exhibits antiviral activity against viral diseases. In addition, no report has been provided regarding the use of MO for disinfectant purposes. In this study, it was aimed to prevent the toxicity of the fibroblast cell line by using MO against different pH changes caused by increased disinfectant use with COVID-19.

MATERIAL and METHOD

Chemicals and Reagents

It was obtained from MO Solgar (U.K). All chemicals derived from Dulbecco Modified Eagles Medium (DMEM), Fetal bovine serum (FBS), phosphate buffer solution (PBS), antibiotic antimitotic solution (Penicillin / Streptomycin / Amphotericin B) (100 ×), L glutamine, and trypsin-EDTA has been. HCl, and NaOH were obtained from Sigma Aldrich (St. Louis, MO, USA).

Cell Culture

For the study, a fibroblast cell line (PCS-201-012), Ataturk University (Erzurum, Turkey) were obtained from medical pharmacology department. Briefly, the cell suspension was centrifuged at 1200 rpm for 5 minutes. Cells were resuspended in fresh medium DMEM, %10 FBS, and %1 antibiotic (penicillin, streptomycin, and amphotericin B), and cells were collected in a 25cm^2 flask. (Corning, USA), and stored in incubator (%5 CO_2; 37 °C). When %80 of the flask was covered with the cell, it was removed with Trypsin-Ethylene di amine tetra acetic acid (EDTA) (%0.25 trypsin-%0.02 EDTA), and planted in 96 well plates to be centrifuged (12).

pH Toxicity

DMEM medium was prepared as full medium and added to the cells by adjusting various pH values (5.0, 6.0, 7.0, and 8.0) with the use of HCl, and NaOH by pH meter.

MO Application

After the cells reached %85 confluence, MO of different concentration (20, 40, 80, and 160 µg/ml) was added to the corresponding wells, and the plates were left in the incubator for 24 hours.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) Test

The viability assessment for each group was determined with 12 wells. At the end of the experiment (after 24 hours of treatment) 10 µL of MTT solution was added to each well plate (1 mM final concentration). Plates were then incubated for 4 hours at 37 °C in a CO_2 incubator. After 4 hours, 100 µL of DMSO solution was added to each well to dissolve the formazan crystals. The density of the formazan crystals was read by the Multiskan ™ GO Microplate Spectrophotometer reader at a wavelength of 570 nm (13).

Lactate Dehydrogenase (LDH) Assay

LDH assay test was performed using a commercially available test kit from Cayman Chemical Co. Ltd, (Ann

Arbor, MI, USA). Briefly, the cell culture medium was centrifuged at 400 g for 5 min at the room temperature 100 µl of the supernatant was added to 100 µl of the reaction solution (LDH Assay Buffer, LDH Substrate Mix), and incubated with gentle shaking on an orbital shaker for 30 min at room temperature. Finally, the absorbance was read at 490 nm wavelength.

Glutathione Reductase (GR) Assay

In the activity measurement of the GR enzyme, the principle that the reacted NADPH gives maximum absorbance at 450 nm was used. The GR enzyme causes a decrease in NADPH in the reaction it catalyzes. Enzyme activity was determined by following this reduction spectrophotometrically at 450 nm (14).

Morphological Imaging

Morphological changes occurring in the cells were visualized with an inverted microscope (Leica Microsystems, Wetzlar, Germany). The images obtained were recorded with 20× magnification of the microscope.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey's HSD for posthoc comparisons using SPSS 22.0 software. $P<0.05$ and $P<0.01$ were accepted as the statistical threshold for each analysis.

RESULTS

MTT Assay

MTT analysis results are shown in Figure 1. Viability percentages of all groups were calculated by comparing with the positive control (pH application only) values and the control values were set as %100±4 viability. The lowest effect was seen in the MO 20 µg/ml group among the treatment groups compared to the pH control groups ($P>0.05$). In addition, it was observed that the survival rate in the MO (20, 40, 80 and 160 µg/ml) groups increased depending on the concentration (Figure 1). According to the results of our study, cell viability increased from %69±2.1 to 89±3.8 compared to positive control at pH 5.0. The vitality that decreased to %802.5 at pH 6.0 increased to 143±4.1 because of the application of the highest concentration of MO (160 µg/ml). While it increased from %79±1 to %115±2.8 at pH 7.0, the damage received by fibroblast cells at pH 8 was determined to be high (viability rate %66±1.3), and it was observed that this toxicity was removed, and the vitality was increased up to %93±2.82 ($P<0.05$ and $P<0.01$).

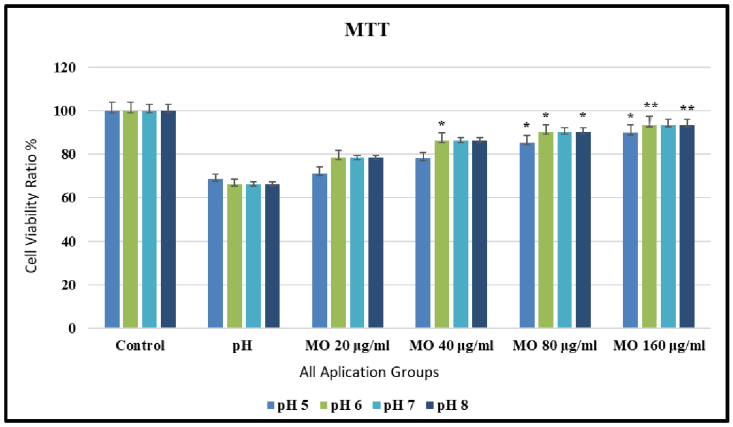

Figure 1. Different pH concentrations and MO treatments effects on fibroblast cells viability ratio. Cell viability rate changed depending on the pH change. Cell death caused by pH was evaluated statistically. *P<0.05, **P<0.001.

LDH Assay

Damage to the cell membranes is reflected as elevated LDH levels in the cell medium after the cells were exposed to MO (20, 40, 80 and 160 µg/ml) for 24h. The LDH activity of the control group was defined as %100, and the other groups were rated accordingly. Our results show that pH 8.0 was most toxic as indicated by the greatest amount of LDH activity in the media from the fibroblast cells in comparison to the other pH group. Figure 2 shows that combinations of MO treatment groups at all pH parameters reduce cytotoxicity in fibroblast cells in a time- and dose-dependent manner. Also, the high concentration MO groups (80 and 160 µg/ml) differed statistically from the pH control group (P<0.05), (P<0.01). A linear correlation was observed between LDH activity and cell viability.

GR Assay

GR levels in the cell medium after the cells were exposed to MO (20, 40, 80 and 160 µg/ml) were evaluated for 24h. The GR activity of the control group was defined as %100, and the other groups were rated accordingly. Figure 3 shows that combinations of MO treatment groups at all pH parameters increased GR level in fibroblast cells. Also, the high concentration MO groups (80 and 160 µg/ml) differed statistically from the pH control group (P<0.05 and P<0.01).

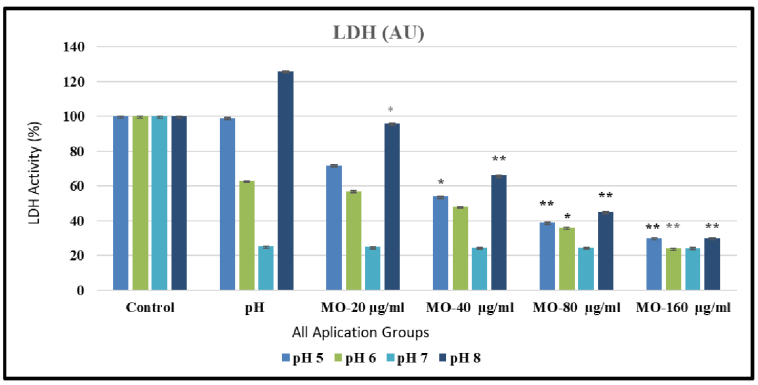

Figure 2. LDH activity (AU) was measured, and the result proportioned to control group. Depending on the pH change, the LDH level increased. On the other hand, there was a significant decrease in LDH level with MO, especially at 400 µg/ml concentration. *P<0.05, **P<0.001.

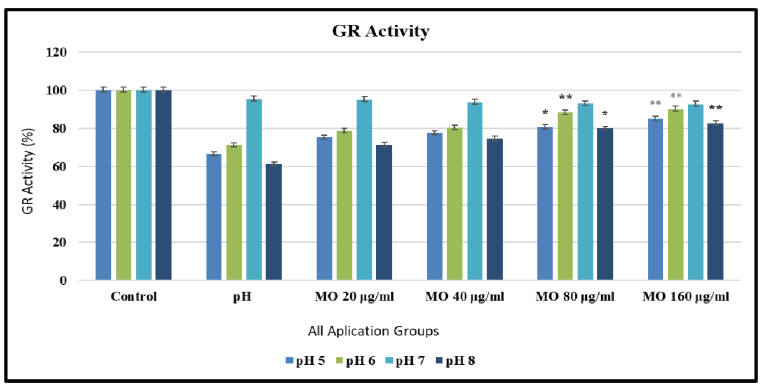

Figure 3. GR activity was measured, and the result proportioned to control group. Depending on the pH change, the GR activity decreased. On the other hand, there was a significant increase in LDH level with MO, especially at 400 µg/ml concentration. *P<0.05, **P<0.01.

Morphologic Determination of MO

Images of fibroblast cells obtained by inverted microscope are given in Figure 4. When images are examined, increased cell viability compared to pH controls indicates an improvement in the visuality of the cells, depending on the dose of MO.

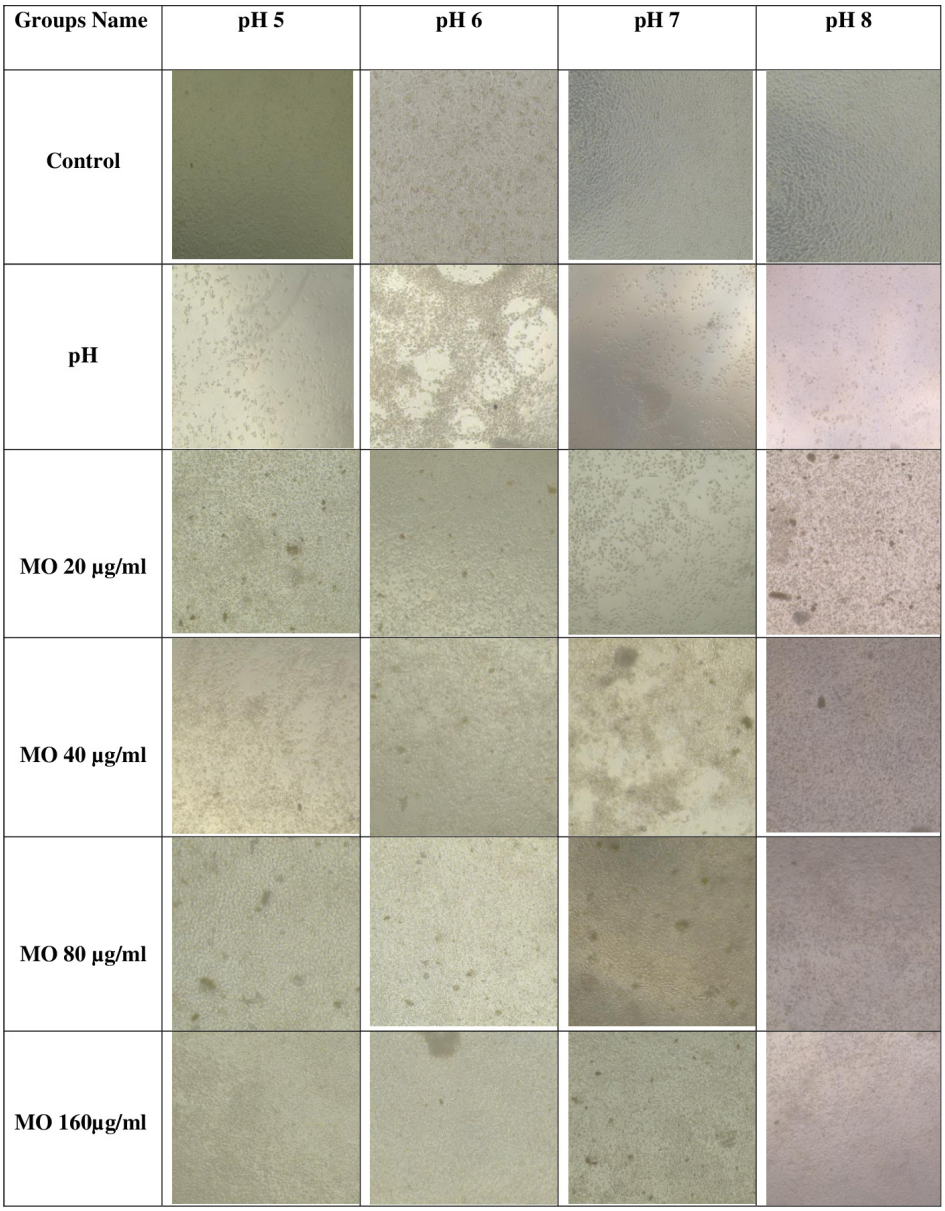

Figure 1. The effects of different pH concentrations and MO treatments on fibroblast cells.
Triangle : empty area,
Blue arrow: live cells,
Red arrow: dead cells.

DISCUSSION

MO is known as a valuable food source due to its high nutritional content and physiological properties (15). Extracts of MO leaves, seeds, and roots have been extensively studied for many potential uses including analgesic activity (16), antifertility (17), anti-tumor (18) hypotensive (19) and wound healing. In particular, sugar (such as L-arabinose, D-mannose, D-galactose, L-rhamnose and D-xylose) and fatty acid (auric acid, myristic acid, palmitic acid, arachidonic acid and oleic acid), which are among the main components of MO leaves, are pharmacologically active and have been shown to increase wound healing in many studies (20). Also, many researchers have shown that natural sugars such as D-mannose and D-glucose have a wide variety of antimicrobial functions (21, 22).

Hand hygiene is an important factor in reducing potentially disease-causing germs. Today, hand hygiene has been suggested as an important strategy among the measures taken against the rapidly spreading coronavirus. Accordingly, the number of hand disinfectants carried for instant hand hygiene application has increased. However, frequent use of hand disinfectants causes the skin to dry by cleaning the oils with natural antiviral activity on the skin surface (23). In addition, prolonged exposure to disinfectant agents may cause skin damage or hypersensitivity. Such exposures can cause immediate or delayed skin reactions. Highly concentrated solutions have caused severe chemical skin burns. The long-term use of these agents is thought to result in greater release of oxygen-derived free radicals, and increased tissue oxidative damage (24).

Oxidative attack on the biological system manifests itself at the cellular level on important biomolecules where lipid molecules and DNA molecules from the primary target are the ultimate target. Oxidative damage to DNA can have a variety of consequences, including cancer, age-related disorders, mutagenesis, and other human pathologies (25). It is known that foods of plant origin rich in antioxidants provide protection against such diseases (26-28). Therefore, it can be considered that antioxidant-rich plant extracts may protect cells against oxidative DNA damage.

The antioxidants found in plants improve wound healing by quenching free radicals and preventing cellular damage caused by free radicals. The redox properties of antioxidants can delay or prevent the onset of degenerative diseases. This property allows them to act as hydrogen donors or reducing agents, which improves the regeneration and organization of new tissue in wound healing. MO is a medicinal herb traditionally used for skin wounds, sore throats, and eye infections. Recently, the antioxidant activity property of MO leaves has been well demonstrated experimentally in both in vivo and in vitro models (29, 30).

The antioxidant property of MO was examined both in vitro and in vivo by Verma et al. MO when administered to rats, increased the antioxidant enzymes catalase and superoxide dismutase while decreasing lipid peroxidases. In addition, in a study involving the use of human dermal fibroblasts, Muhammad et al. showed that an aqueous extract of MO leaves significantly increased cell proliferation and viability as compared with untreated controls.

The MTT test provides a rapid and multifaceted method for evaluating cell viability (31). Many herbs and phytochemicals have been reported to have cytoprotective effects using the MTT test. In our in vitro study, it was observed that MO concentrations increased fibroblast proliferation in a dose-dependent manner, and also increased antioxidant levels by eliminating oxidative damage (30-33). After treatment with MO for 24 hours against toxicity applied at different pH, it was observed that the high concentration of Graviola extract (80 and 160 µg/ml) at pH 5.0, and 6.0 had a statistically significant positive effect on enhancing cellular viability and alleviating oxidative stress.

In conclusion; the demand for hand disinfectants will remain at the forefront until more effective anti-infection measures such as the SARS-CoV-2 vaccine

and the threat posed by the COVID-19 outbreak are achieved. If the use of disinfectants destroys viral agents, it also damages our skin cells. According to our results, skin cells suffer great damage during pH change. According to our study, MO has a strong protective effect on pH between 6.0 to 8.0 damage inductions. For this reason, we recommend the use of MO after the use of hand disinfectants.

AUTHOR CONTRIBUTIONS

Y. Yeni and S. Genc conducted the experiment and wrote the article. A. Taghizadehghalehjoughi and A. Hacimuftuoglu organized the design and writing of the study.

ETHICS COMMITTEE APPROVAL

* This study does not require Ethics Committee Approval.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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