RESEARCH ARTICLE / *Araştırma Makalesi*

Antiviral Activity and Wound Healing Potential of a Traditionally Used Herbal Oil Blend

Geleneksel Kullanıma Sahip Bitkisel Bir Yağ Karışımının Yara İyileşmesine Etkileri ve Antiviral Aktivitesi

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Abstract

Objective	Complementary and alternative medicine (CAM) has an increasing usage in the last decades throughout the world. Herbal medicine, the most preferred CAM, is used in the treatment of various disorders by approximately eighty percent of the world's population. In this study, it was aimed to determine the antiviral activity and wound healing potential of a special herbal oil blend prepared from *Olea europaea*, *Nigella sativa* and *Rosmarinus officinalis* that has been used for centuries in Middle and East Anatolia.
Materials and Methods	The nontoxic concentration of herbal blend was determined by MTS assay. This concentration was investigated for its wound healing potential using in vitro scratch assay on HaCaT cells. A scratch was made on cultured keratinocyte cell layer and the herbal blend was added to medium. Pictures of cells were taken at different time points. The antiviral activity was determined using Adenovirus type 5 and Poliovirus type 1 on HEp-2 cells. Virus titer was calculated by Spearman-Karber method.
Results	The nontoxic concentration of the blend was found to show statistically higher wound healing rate in compare to control group at the end of 12, 24 and 36 hours. According to antiviral efficacy test, four log of reduction in virus titer was seen, which meant that the mixture was quite effective against the viruses used in the study.
Conclusion	The study shows that the special herbal blend speed up wound healing rate and it also has antiviral activity.
Keywords	*Olea europaea; Nigella sativa; Rosmarinus officinalis*; antiviral; wound healing

Öz

Amaç	*Modern tıp yöntemlerine ek olarak kullanılan tedavi yöntemlerini tanımlayan Geleneksel, Tamamlayıcı ve Alternatif tıp (GTAT) kullanımında son yıllarda dünya genelinde bir artış meydana gelmiştir. En sık kullanılan GTAT yöntemlerinden biri olan bitkisel tıp dünya nüfusunun yaklaşık yüzde sekseni tarafından çeşitli rahatsızlıkların tedavisinde kullanılmaktadır. Bu çalışmada, geleneksel tıpta çok yönlü etkileriyle bilinen Olea europaea, Nigella sativa ve Rosmarinus officinalis bitkilerinden özel olarak hazırlanan, Orta ve Doğu Anadolu'da yüzyıllardır kullanılan bir yağ karışımın antiviral etkisinin ve yara iyileşme sürecine katkısının araştırılması amaçlanmıştır.*
Gereç ve Yöntemler	*Çalışmanın ilk aşamasında, yağ karışımının hücrelere toksik olmayan dozu MTS testi ile belirlendi. Belirlenen dozda yağ karışımının in vitro scratch (çizik) testi kullanılarak HaCaT hücrelerinde yara iyileşmesine etkisini incelendi. Bunun için, kültüre edilmiş keratinosit hücre tabakasında çizik oluşturulduktan sonra yağ kombinasyonu uygulandı ve farklı zaman aralıklarında hücrelerin fotoğrafları çekildi. Yağ karışımının antiviral etkinliği ise HEp-2 hücrelerinde Poliovirüs tip 1 ve Adenovirüs tip 5 kullanılarak araştırıldı. Virüs titresi Spearman-Karber metodu kullanılarak hesaplandı.*
Bulgular	*Toksik etkisi olmadığı belirlenen %0,005'lik yağ kombinasyonu uygulanan grupta yara kapanma hızının kontrol grubu ile karşılaştırıldığında 12., 24. ve 36. saat sonunda istatistiksel olarak önemli derecede fazla olduğu tespit edildi. Antiviral etkinlik analizi sonucunda yağ karışımının virüs titresinde en az dört log azalmaya neden olarak Poliovirüs tip 1 ve Adenovirüs tip 5 virüslerine karşı oldukça etkili olduğu ortaya kondu.*
Sonuç	*Çalışma sonuçları yağ karışımının yara iyileşmesini hızlandırıcı etkisi olduğunu ve antiviral aktiviteye sahip olduğunu göstermiştir.*
Anahtar Kelimeler	*Olea europaea; Nigella sativa; Rosmarinus officinalis; antiviral; yara iyileşmesi*

INTRODUCTION

Complementary and alternative medicine (CAM) describes a set of treatment methods used in addition to or in place of modern medical treatment. CAM applications are widely used all over the world and in all age groups. It is reported that the frequency of use is twice as high in diseases such as various types of cancer, multiple sclerosis, rheumatological diseases, HIV infection and asthma.[1] In recent years, it has been observed that there has been an increase in the use of CAM all over the world, both for the protection of health and the treatment of various diseases. In addition to its use among the public, CAM has become very popular in the biomedical literature. This popularity includes many factors; easy access to complementary and alternative treatment products, the belief of the society that alternative medicine is effective, unmet health needs, sociocultural characteristics, behaviors and attitudes.[2]

Herbal medicine is one of the most commonly used CAM methods all over the world. It is estimated that eighty percent of the world's population still uses herbal products to protect their health and treat various ailments.[3] In particular, herbal therapies are frequently used in the treatment of diseases such as cancer and migraine, chronic diseases such as diabetes, high blood pressure, bronchial asthma, epilepsy, and common diseases such as upper respiratory tract infections.[4] In recent years, herbal therapy has gained popularity again due to factors such as the serious side effects of synthetic drugs, the inability to fully heal many chronic diseases with modern medicine methods, and the thought that natural products will be effective and harmless.[5]

Olea europaea (O. europaea, olive) is a plant whose strong antioxidant effect has been proven by various studies[6], besides, it has antimicrobial properties.[7] It has been shown to have versatile biological effects such as antithrombotic, anti-inflammatory, hypocholesterolemic, antimicrobial and antiviral properties.[8] It has also been stated that olive is used as hypotensive, hypoglycemic, antihelmentic, antiseptic and also against hair loss.[9] Scientific studies with *Nigella sativa (N. sativa)* have shown that it has anticarcinogenic[10], antitumoral[11], antiulcerogenic[12], antibacterial[13], anti-inflammatory, analgesic[14], antioxidant[15], hypoglycemic[16] and immune system booster effects.[17] Similarly, *Rosmarinus officinalis L. (R. officinalis, rosemary)* is a plant whose antibacterial, antioxidant, antiviral, and immunogenic effects have been shown by several studies, and it also has hemorrhagic, antiseptic, stomach-healing and carminative effect.[18] In addition, essential oils and extracts obtained from *R. officinalis* have been shown to possess number of important biological activities such as anticancer, antimicrobial, anti-HIV.[19] So far, there have been a number of studies conducted to investigate these plants individually, but their effectiveness as a combination has not been searched. This is the first study demonstrating the antiviral activity and wound healing effect of the specially prepared oil blend from *O. europaea, N. sativa* and *R. officinalis*, which are used in the treatment of many ailments in Central and Eastern Anatolia for many years.

MATERIAL and METHODS

Cell lines and viruses

HaCaT cells (CVCL-0038) were used for cell viability and scratch assay experiments. HEp-2 cells (ATCC CCL-23) were used for antiviral activity tests. HaCaT cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA) whereas HEp-2 cells were maintained in Minimum Essential Medium (MEM; Sigma-Aldrich, USA). Both of the media were supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin Amphotericin (PSA). The cultures were maintained at 37 °C in a humidified incubator with 5% CO2.

Adenoid 75 strain of Human Adenovirus Type 5 (ATCC VR-5) and Chat strain of Human Poliovirus Type 1 (ATCC VR-1562) viruses were used for determining antiviral activity of the oil blend.

Preparation of herbal oil blend

The oil combination prepared from O. europaea, N. sativa and *R. officinalis* was used for the experiments (NBV, Konya, Turkey). The mixture was prepared using one unit of O. europaea oil, two units of *N. sativa* and two units of *R. officinalis* oils. *N. sativa* and *R. officinalis* oils were obtained by distillation method, whereas *O. europaea* was obtained by cold press extraction method. Due to its dominant and intense nature, one unit of olive oil was used while two units from other oils were used. *O. europaea* oil was obtained from its fruits, *N. sativa* oil was from seeds and *R. officinalis* oil was from the leafs.

Analysis of cell viability

After the oil blend was diluted with Tween 80 (Merck, Darmstadt, Germany) 10%, 5%, 0.5%, 0.05% and 0.005% concentrations were prepared using DMEM. Cell viability was determined by MTS assay. Briefly, HaCaT cells were seeded on 96-well culture plates at a density of 3×10^3 cells/well. After adherence, the cells were treated with increasing concentrations of herbal oil blend for 24, 48 and 72 h. At the end of the incubation period, 10 µl of MTS reagent (Sigma-Aldrich, USA) was added to the wells with 100 µl medium. After 3 hours of incubation at 37°C, the absorbance of each well was measured at 490 nm using an ELISA plate reader. The most effective concentration was selected for further experiments.

In vitro scratch assay

The effect of oil blend on the proliferation/migration capabilities of HaCaT cells was assessed using a scratch wound healing assay. The cells were seeded into 12-well tissue culture dish at a concentration of 1×10^5 cells/well. After incubation for 24 hours at 37°C, a linear wound was generated in the monolayer with a sterile plastic pipette tip. The wells were washed by PBS in order to remove any cellular debris. The medium containing 0.005% of oil combination was added to the wells. At the end of 0, 12, 24 and 36 hours, images from the scratched areas were photographed to estimate the relative migration of cells. The data were analyzed using Image-J software. The experiments were performed in triplicate.

Preparation of infected HEp-2 cell cultures

After incubation in MEM for 24 hours, passage of HEp-2 cells at 1:4 in culture flasks was infected by Poliovirus Type 1 (PV-1) and Adenovirus Type 5 (AV-5) to prepare viral stocks. Cytopathic effect (CPE) of the stocks was controlled by microscopy. At 50–75% CPE, the cells were added to 10% FBS and then frozen at -80 °C. After a freeze-thaw cycle, supernatant of the infected cells was collected by centrifugation for 30 minutes at 3300 rpm and 4°C. The debris was discarded and the supernatant was used as virus stocks for further experiments. To measure virus titer, HEp-2 cells were seeded into 96-well plates at a density of 2×104 cells/well and incubated at 37 °C for 24 hours. Each individual sample was serially diluted from 10−1 to 10−9 in 10-fold increments. Each dilution was inoculated into HEp-2 cells and incubated for 3 days at 37 °C. PV-1 and AV-5 titers in the cell culture were calculated by Spearman–Karber method.[20]

Antiviral effects of oil blend on Poliovirus and Adenovirus HEp-2 cells were treated with 0.005% of oil combination for 5 and 60 minutes. The cells were then seeded into 96-well plates at a density of 2×10^4 cells/well in MEM with 2% FBS and incubated at 37 °C. After PV-1 and AV-5 were serially diluted from 10^{-1} to 10^{-9} in 10-fold increments, diluted viruses were treated with 0.005% of oil combination for 5 and 60 minutes. Each dilution was inoculated into HEp-2 cells and incubated for 72 hours at 37°C. PV-1 and AV-5 titers in the cell culture were calculated by Spearman-Karber Method.[20]

Statistical Analysis

GraphPad Prism 9.1.0 (La Jolla, CA, ABD) was used to perform statistical analysis. One-way analysis of variance (ANOVA) followed by Tukey's test was used. P values less than 0,05 were considered as statistically significant.

RESULTS

Cytotoxic effect of oil combination

The cytotoxic effect of oil blend on the proliferation of HaCat cells was evaluated to determine the suitable concentration and exposure time for further experiments. MTS assay performed 24, 48 and 72 hours after the cells were incubated with different concentrations of oil combinations showed that the 0.005% combination increased cell viability statistically significantly ($p<0,05$) at each time point. The viability of HaCat cells was statistically significantly reduced at 0.05% and higher concentrations of oil blend ($p<0,05$). Therefore, 0.005% concentration of the blend was chosen for further analysis to evaluate its wound healing potential and antiviral activity. Figure 1 shows the effect of increasing oil combination concentrations on cell viability.

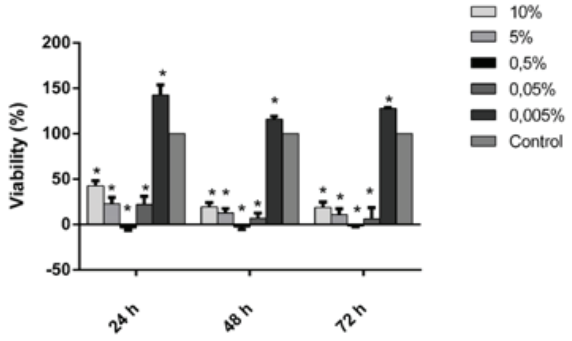

Figure 1. HaCat cell viability at different concentrations of the oil combination ($p<0.05$)

Evaluation of the wound healing effect of oil blend

The effect of the 0.005% concentration of oil combination on wound healing was evaluated using an in vitro scratch assay. The scratch assay, one of several in vitro assays used for wound healing, is a valuable and inexpensive tool for obtaining initial insights into how various compounds may affect new tissue formation. The assay covers the second phase of wound healing characterized by proliferation and migration of either keratinocytes or fibroblasts.[21] It was observed that the scratch was almost completely closed after 24 hours in the oil treated group, while in the control group it was not closed yet after 24 hours. Thus, the oil treatment restored the cells to a confluent or near-confluent state within 24 hours, in contrast to the control cells. At the end of 36 hours, it was seen that the closure of the scratch, which was completely closed in the treated group, was not completed in the control group (Figure 2).

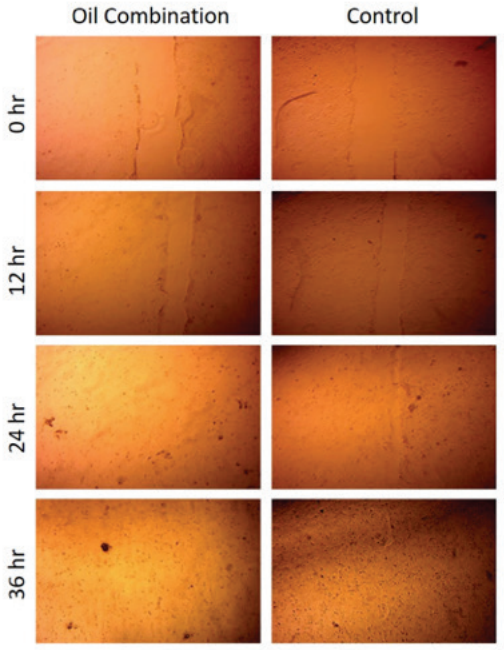

Figure 2. In vitro scratch assay images of 0.005% oil blend treated and control group. The images were taken by invert microscope immediately after the scratch was made and after 12, 24 and 36 hours

The images taken from the scratched areas of cells were analyzed using Image-J software to measure the rate of closure of the wounds. By measuring the closure amount of the scratches from the beginning to the 36th hour, the % migration rates of the cells were calculated and the graphs showing the percentage of wound closure were drawn as depicted in figure 3. It was observed that from the 12th hour, the oil blend increased the wound closure rate statistically significantly compared to the control group ($p<0,05$).

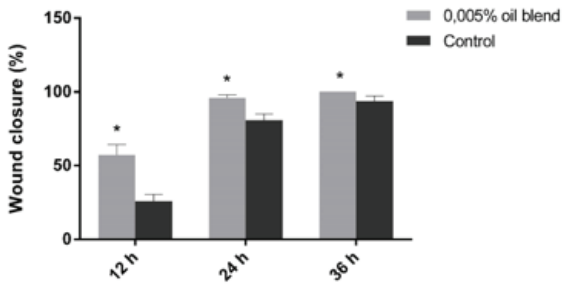

Figure 3. The rate of wound closure in oil blend treated group in compare to control cells at the end of 12, 24 and 36 hours ($p<0.05$)

Assessment of the antiviral activity of oil combination

After a serial dilution, collected viral stocks were inoculated into HEp-2 cells and the titers of the stocks were calculated. Initial PV-1 and AV-5 viral titers were determined as 5.2 log(10)/ml and 5.5 log(10)/ml, respectively. Viruses were exposed to nontoxic concentration of oil combination for 5 and 60 minutes. After 72 hours of incubation, the antiviral effects were determined by the 50% tissue culture-infected dose (TCID50) and the virus-induced cell death was recorded by observing cell morphology under inverted light microscope. Table 1 shows the antiviral test results after exposure of oil combination at 72 hours. The test revealed that the oil blend showed similar antiviral effect against PV-1 and AV-5. Both viral titers were decreased at least 4.0 log(10)/ml in the treated groups. Rate of decline in virus titers between wells were calculated by observing cell deaths under microscope. PV-1 virus titer decreased to 1.2 and 1.0 log(10)/ml when they were treated for 5 and 60 minutes, respectively. AV-5 virus titer decreased to 1.5 log(10)/ml when they were treated for both 5 and 60 minutes. The combination showed the highest antiviral activity against PV-1 (4.2 log(10)/ml) when treated for 60 minutes. Based on US Environmental Protection Agency (EPA) guidelines, when cytotoxicity is evident, it has to be shown at least a 3-log reduction in virus titer beyond the cytotoxic level. Thus, it has been clearly demonstrated that the oil blend has antiviral activity against both viruses.

Table 1. Antiviral test results of oil combination against adenovirus type 5 (AV-5) and poliovirus type 1 (PV-1).

Chat strain of Human Poliovirus Type 1 (ATCC VR1562)	Reference virus	Oil combination	
Virus titera	5.2	5 min	60 min
Oil blend treated virus titer[b]		1.2	1.0
The rate of decline in virus titer[c]		4.0	4.2
Adenoid 75 strain of Human Adenovirus Type 5 (ATCC VR5)	Reference virus	Oil combination	
Virus titera	5.5	5 min	60 min
Oil blend treated virus titer[b]		1.5	1.5
The rate of decline in virus titer[c]		4.0	4.0

a Logarithmic TCID50 value of virus per milliliter

b Logarithmic TCID50 value of oil blend treated virus at different times

c Logarithmic TCID50 ratio between virus titer and treated viral titer

DISCUSSION

Herbal extracts have been used in wound healing for centuries. Some plants are used in the treatment of fresh wounds, while others are used in the treatment of chronic wounds.[22] When ancient medicine is examined, it is seen that olive oil (*O. europaea*) is used in the preparation of medicines such as ointments and in the treatment of wounds and burns.[23] Various studies showing the effectiveness of *O. europaea* on wound healing, has pointed out its usage as a treatment on skin ulcers and inflammatory wounds. Koca et al. investigated the wound-healing effect of n-Hexane and aqueous extracts prepared from the leaves of *O. europaea* using an in vivo wound model. The findings of the study showed the great healing potential of n-Hexane extract. As a result of the analysis of the aqueous extract, secoiridoid oleuropein (4.6059%) was determined as the major active ingredient.[24]

Studies conducted to examine the effect of *N. sativa* on wound healing have revealed that the plant has this potential.[25] Yaman et al. compared the effects of *N. sativa* and silver sulfadiazine on the healing of burn wounds in rats and revealed that *N. sativa* significantly accelerated wound healing compared to other groups.[26] Abu-Zinadah, on the

other hand, investigated the contribution of *N. sativa* oil to wound healing in rabbits with a burn model and found that it accelerated the healing process.[27] *R. officinalis* is among the plants used in the treatment of various wounds. Especially in Jordanian folk medicine, this plant is frequently used in wound treatment. Abu-Al-Basal investigated the curative effect of the aqueous extract and essential oil of *R. officinalis* on diabetic BALB/c mice. As a result of his research, it was revealed that essential oil is the most effective in the healing of diabetic wounds. Thus, this study provided scientific evidence for the traditional use of *R. officinalis* in wound healing.[28] The effects of each of the three plants individually have been revealed in various studies. In this study, the effect of the combination was demonstrated for the first time. Results of the study revealed that the mixture accelerated wound healing statistically significantly. It could be speculated that the blend is more effective than the use of these plants individually, yet needs to be proven by further detailed studies.

With the determination that sixty percent of the diseases in developed countries are caused by viral infections, studies on the development of active drugs against viruses have accelerated and many plants have been tested to detect their antiviral activities.[29] Many studies have shown that *O. europaea* has antiviral properties.[30] Lea-Huang et al., showed that the olive leaf has high antiviral properties against Human Immunodeficiency Virus type 1 (HIV-1).[31] Micol et al. investigated the efficacy of oleuropein, obtained from olive leaves, against viral haemorrhagic septicaemia virus (VHSV) and showed that oleuropein inhibited the infectivity of the virus.[32] Yamada et al. proved that a small phenolic compound called hydroxytyrosol found in olive leaves and fruit inactivates Newcastle disease virus and influenza A viruses including H1N1, H3N2, H5N1, and H9N2 subtypes.[33]

In studies with a variety of viruses to determine the antiviral activity of N. sativa, it has been revealed that this plant and its seeds are quite effective against viruses[34]. Salem and Hossain examined the antiviral effect of oil obtained from *N. sativa* seeds in their study using murine cytomegalovirus (MCMV) as a model and showed that the oil has a very strong antiviral effect against MCMV.[35] Zaher et al. showed that *N. sativa* seeds have antiviral activity against Infectious Laryngo-Tracheitis Virus (ILTV).[36] *R. officinalis* is one of the many other plants whose antiviral properties have been investigated. In a study, where eigthteen plants were tested to determine their antiviral activity against Herpes simplex virus (HSV), the essential oil of *R. Officinalis* has been showed to have partial effect against this virus.[37] The antiviral effect of *R. officinalis* essential oil on HSV was also shown by the study of Minami et al.[38] In addition, carnosol and carsonic acid, components of *R. officinalis* extract, have been shown to have efficacy in preventing the transmission of HIV virus.[39]

Based on the results of studies proving the antiviral effects of *O. europaea, N. sativa* and *R. Officinalis*, in the current study, the antiviral effectiveness of the oil combination prepared from these three plants was demonstrated using AV-5 and PV-1. Even though the antiviral activity of these plants have been investigated on some viruses, the antiviral effect of the oil blend on these two viruses was studied for the first time in this study. The analyzes revealed that the oil combination caused at least 4 log reductions in the titer of the virus, with a significant statistical difference compared to the control group. This result demonstrated the highly potent antiviral activity of the mixture.

CONCLUSION

As a result, the oil combination prepared from *O. europaea, N. sativa* and *R. officinalis* with a special ratio (1:2:2) accelerates wound healing significantly. The oil blend, which has been traditionally used in Central Anatolia for centuries, also exhibits strong antiviral properties. Considering these effects, it is concluded that the herbal blend could be used as an agent for wound healing or viral infections. It may also be used as a disinfectant. Future in vivo studies will further strengthen these data and contribute to the ex-

pansion of the use of the combination and its inclusion in treatment regimens.

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Conflicts of Interest

None declared.

Declaration of Contribution

The authors confirm contribution to the paper as follows: EŞ, AÖ and SY designed the study; SY and BA performed the experiments; SY, BA and EŞ interpreted the data; SY and BA scanned the literatüre; SY wrote the manuscript in consultation with EŞ, AÖ and FŞ.

Ethics Committee Approval

Ethics approval was not required for this study.

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