

# *In vitro* Evaluation of the Effects of *Inula viscosa*'s Different Extracts on Wound Healing and Oxidative Stress in Mouse L929 Fibroblast Cell Line

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*Inula viscosa*'nın Farklı Ekstrelerinin Fare L929 Fibroblast Hücre Hattında Yara İyileşmesi ve Oksidatif Stres Üzerindeki Etkilerinin *In vitro* Değerlendirilmesi

## SUMMARY

This study evaluated the effects of extracts prepared using two different methods (decoction extraction and soxhlet ethyl acetate/hexane extraction) from *Inula viscosa* on H<sub>2</sub>O<sub>2</sub>-induced oxidative stress and wound healing model in mouse L929 fibroblast cells. The cytotoxic effect started to disappear statistically ( $p < 0.05$ ) at concentrations of Soxhlet ethyl acetate/hexane extracts (SoxEHE) in and below 0.1 mg/mL, while the same effect was observed at concentrations of decoction extracts (DE) in and below 0.2 mg/mL. Therefore, 0.2 and 0.02 mg/mL concentrations of DE, and 0.1 and 0.01 mg/mL concentrations of SoxEHEs were used. While cell migration was positively affected in all research concentrations, statistically significant results ( $p < 0.05$ ) were obtained from 0.2 mg/mL of DE and 0.1 mg/mL of SoxEHE extracts. Malondialdehyde (MDA) levels were found to be statistically ( $p < 0.05$ ) decreased, but COL1A1 levels were higher in cell lines treated with oxidative stress + extract than in the cell line treated only with H<sub>2</sub>O<sub>2</sub>, and reduced glutathione (GSH) levels were higher in cell lines only treated only with extract than in oxidative stress-induced cell lines. Consequently, it has been observed that the extracts have positive effects on migration and oxidative stress. Therefore, *I. viscosa* may serve as a new therapeutic agent for wound healing.

**Key Words:** Medicinal plants, extraction, scratch assay, cell proliferation, oxidative status

## ÖZ

Bu çalışmada *Inula viscosa*'dan iki farklı yöntem (dekoksasyon ekstraksiyonu ve soxhlet etil asetat/hexan ekstraksiyonu) kullanılarak hazırlanan ekstrelerin fare L929 fibroblast hücrelerinde H<sub>2</sub>O<sub>2</sub> kaynaklı oksidatif stres ve yara iyileşmesi modeli üzerindeki etkileri değerlendirilmiştir. Soxhlet etil asetat/hexan ekstrelerinin (SoxEHE) 0,1 mg/mL ve altındaki konsantrasyonlarında sitotoksik etki istatistiksel olarak kaybolmaya başlarken ( $p < 0,05$ ), aynı etki dekoksasyon ekstrelerinin (DE) 0,2 mg/mL ve altındaki konsantrasyonlarında da gözlenmiştir. Bu nedenle DE'lerin 0,2 ve 0,02 mg/mL konsantrasyonları ve SoxEHE'lerin 0,1 ve 0,01 mg/mL konsantrasyonları kullanılmıştır. Araştırmanın tüm konsantrasyonlarında hücre migrasyonu olumlu etkilenirken, 0,2 mg/mL DE ve 0,1 mg/mL SoxEHE ekstrelerinden istatistiksel olarak anlamlı sonuçlar ( $p < 0,05$ ) elde edilmiştir. Malondialdehit (MDA) düzeylerinin istatistiksel olarak ( $p < 0,05$ ) azaldığı ancak COL1A1 düzeylerinin oksidatif stres + ekstrakt uygulanan hücre hatlarında, sadece H<sub>2</sub>O<sub>2</sub> uygulanan hücre hattına göre daha yüksek olduğu ve indirgenmiş glutatyon (GSH) düzeylerinin oksidatif stresin kaynaklı hücre hatlarına göre yalnızca ekstreyle tedavi edilen hücre hatlarında daha yüksek olduğu görülmüştür. Sonuç olarak ekstrelerin migrasyon ve oksidatif stres üzerinde olumlu etkilerinin olduğu gözlemlenmiştir. Bu nedenle *I. viscosa* yara iyileşmesinde yeni bir terapötik ajan olarak hizmet edebilir.

**Anahtar Kelimeler:** Tıbbi bitkiler, ekstraksiyon, çizik testi, hücre proliferasyonu, oksidatif stres

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## INTRODUCTION

A wound is a deterioration of the cellular, anatomical, and functional continuity of tissue due to a physical, chemical, thermal, microbial, or immunological effect on living tissue. In contrast, wound healing is a biological process involving diverse biochemical and cellular mechanisms designed to restore the structural and functional integrity of injured tissues and enhance physiological conditions (Barku, 2019). Wound healing is a continuous and overlapping process characterized by hemostasis (considered the inflammation stage in the three-stage concept), inflammation, new tissue formation, and the tissue remodeling (Gurtner et al., 2008). Redox signaling and oxidative stress play essential roles in regulating normal wound healing and contribute to specific stages (Sen & Roy, 2008). The formation of reactive oxygen species (ROS) at low concentrations is essential not only for combating invasive microorganisms but also for cellular signaling in the wound healing process (Cano Sanchez et al., 2018). However, even if ROS production is essential for initiating wound repair, excessive ROS formation and uncontrolled oxidative stress in wound healing contribute to persistent and uncontrolled inflammation, which plays a vital role in the pathogenesis of chronic non-healing wounds (Barku, 2019). Problems related to wound healing can occur in the form of swift recovery (such as hypertrophy and keloid scars) (Gurtner et al., 2008), or these problems may be seen as delayed wound healing in especially diabetics or older people (Abe et al., 2000). The healing time of chronic non-healing wounds lasts on average 12 to 13 months and recurs in up to 60 to 70% of patients. If they are not treated, it can lead not only to a loss of function and a decline in quality of life but may also result in mortality (Frykberg & Banks, 2015). While various alternatives, ranging from topical applications (hydrogels, povidone-iodine solution, cadexomer iodine, etc.) to advanced treatment methods such as growth factors, extracellular matrix, and negative pressure wound therapy, exist in wound care (Frykberg & Banks, 2015), these methods sometimes prove insufficient or impractical. Therefore, the scien-

tific world has constantly been searching for effective methods both in practice and cost-wise.

Various herbal products have been used in wound treatment over the years, and these phytochemical compounds have been reported to fight infections, promote blood clotting, and accelerate the healing process. When these wound-healing plants are investigated, it has been claimed that several of them have the potential to increase wound healing owing to their high antioxidant properties (Barku, 2019). The genus *Inula*, belonging to the Asteraceae family, has more than a hundred species, found mainly in Africa, Asia (20 species are also found in China), and Europe, especially in the Mediterranean region (Seca et al., 2014). The *Inula* genus comprises a range of species that have demonstrated medical importance, substantiated by their use in traditional medicine, the biological properties exhibited by their extracts, and the isolation of pure secondary metabolites (Seca et al., 2015). *Inula viscosa* [*Dittrichia viscosa* (L.) Greuter] is used in folk medicine in the Mediterranean region for its anti-inflammatory, antipyretic, antiseptic, antiphlogistic, and balsamic activities, as well as for the treatment of lung and gastroduodenal disorders (Messaoudi et al., 2016). Antioxidant, antibacterial, antifungal, hypoglycemic, hypolipidemic, anticancer, antiparasitic, and phytotoxic effects have also been reported for *I. viscosa* extracts, and it has been argued that these effects originate from sesquiterpenoids, triterpenoids, and flavonoids (Mahmoudi et al., 2016). In the literature review, several reports on extracts and purified metabolites from *I. viscosa* have been identified. However, concerning wound healing, only a morphological study in rats has been encountered. Khalil et al. (Khalil et al., 2007) reported that only 10% aqueous extract of *I. viscosa* healed both the morphological and histological features of wounds. However, no physiopathological studies have shown the effects of different types of extracts obtained from *I. viscosa* on wound healing.

As a result, we examined the *in vitro* effects of different extracts of the *I. viscosa* plant on wound healing

in L929 cells with hydrogen peroxide-induced oxidative stress and levels of oxidative stress parameters such as malondialdehyde (MDA) and reduced glutathione (GSH), and COL1A1 gene expression, which have an effect on healing in this process.

## MATERIALS AND METHODS

### Preparation and extraction of plant material

The plants collected from Manisa/Akhisar were identified by Asst. Prof. Dr. Mustafa Kemal Altunoğlu (Kafkas University, Faculty of Sciences and Letter, Department of Biology, Kars), the aerial parts were dried in a dry place for two weeks. Dried plants were powdered using an electric blender.

### Ethyl acetate/hexane extraction

The plant extract was obtained using a Soxhlet extraction system. The conventional Soxhlet method, which requires minimal training, allows for getting more sample mass and eliminates the need for filtration after the leaching process (Luque de Castro & Priego-Capote, 2010). Briefly, forty (40) grams of the herb were weighed, wrapped in filter paper, and placed in a soxhlet apparatus. Ethyl acetate and n-hexane (3:1 v/v) were used as solvents. The system's temperature reached the optimum level, and the extraction process was terminated after three hours. The remaining solvent in the extract was vaporized at 60 °C in a rotary evaporator until removed entirely. Before testing, dilutions of the ethyl acetate/hexane extract (SoxEHE) (10, 1, 0.1, 0.01, and 0.001 mg/mL) were prepared.

### Extraction by method of decoction

In this study, a 2% extract of the plant material was prepared. Twenty grams of the plant were weighed and transferred to a teapot. After adding 1 liter of cold distilled water, the mixture was allowed to come to a boil. After boiling, it was waited for 10 minutes and allowed to cool to room temperature. It was filtered through filter paper to avoid residue (Üstü & Uğurlu, 2018). Dilutions (20, 2, 0.2, 0.02, and 0.002 mg/ml) were prepared from decoction extract (DE) immediately before test.

### L929 fibroblast cell proliferation and viability analysis

The L929 (mouse fibroblast cell, ATCC CCL-1) cell line was purchased from the American Type Culture Collection (ATCC, USA) and was used for the experiments. In the liquid nitrogen tank, the cell lines on the cryotube were removed from the tank and kept in a water bath for a short period to dissolve at 37°C. The dissolved cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum in 75 cm<sup>2</sup> plastic flasks. After 48 h, L929 cells were counted in DMEM containing 10% FBS at a density of 2x10<sup>5</sup> cells/well, and plated in a well plate with 96 wells, and incubated at 37°C in a humid atmosphere containing 5% CO<sub>2</sub>. For 24 h, the effects of DE concentrations (20, 2, 0.2, 0.02, and 0.002 mg/mL) and SoxEHE concentrations (10, 1, 0.1, 0.01, and 0.001 mg/mL) in L929 cells were investigated using the MTT method to determine toxic doses of decoction extracts (dissolved in pure water) and ethyl acetate (dissolved in 0.01% DMSO and applied to cells). After the appropriate doses were determined, the cells were planted again in a well plate with 96 wells. For 24 h, the cells were exposed to 2 mg/mL and 0.2 mg/mL decoction and 0.01 mg/mL and 0.001 mg/mL ethyl acetate extracts at different concentrations, and then 3 hours later, H<sub>2</sub>O<sub>2</sub> (0.75 mM) was added to the media (Sudsai et al., 2016). After the MTT method was applied to the cells, at the 24th hour, the absorbance was read in each well at 620 nm using a microtiter plate ELISA reader (Epoch Microplate Spectrophotometer, BioTek, USA). The assays were performed a minimum of three times for each repetition. The survival rates of L929 cells were analyzed by comparing them with those of the control wells.

### Migration test

The migration of L929 fibroblast cells was examined using the wound healing method. Briefly, L929 cells (2x10<sup>5</sup> cells/mL) in DMEM containing 10% FBS were seeded into each well of a 24-well plate and in-

cubated at 37°C and 5% CO<sub>2</sub>. When the cells formed a complete monolayer in the wells, a scratch was created horizontally in each well with a sterile pipette tip. Cellular debris was removed by washing with PBS and replaced with 2 mL of fresh medium without adding test samples. On day 0, images were taken using the Invitrogen Inverted Microscope, and the wells were photographed at 0, 12, and 24 h by keeping them in the incubator.

**Determination of oxidant/antioxidant parameters (MDA, GSH)**

L929 cells (2x10<sup>5</sup> cells/mL) were seeded in each well of 6-well plates (separately for the analysis of MDA and GSH) and incubated in a humid environment containing 5% CO<sub>2</sub> at 37°C. Cells were harvested from 6-well plates using a scraping method and stored at -80°C. Approximately 100 mg of cell lysate

from each group was homogenized with Tissue Lyser on ice in a specific homogenate buffer. The samples were then centrifuged. For biochemical studies, while MDA levels in supernatants were measured colorimetrically using the methods reported by Yoshioka et al. (1979), GSH levels were measured colorimetrically as described by Beutler et al. (1963).

**Gene expression analysis by RT-PCR**

Total RNA was extracted from actively growing cells using TRIzol Reagent (Sigma). This RNA was then treated with RQ1 DNase I (Promega). Reverse transcription (RT) was carried out following the manufacturer’s instructions (Fermentas) and involved using 1 unit of MMLV reverse transcriptase along with 5 µg of total RNA. Subsequently, the cDNA region corresponding to the COL1A1 protein was amplified using the F and R primers (Table 1).

**Table 1.** COL1A1 primer list.

Primer Name	Primer List	Tm
COL1A1F	GGCACTCCCGGACCTCAAG	64
COL1A1R	CGGTCACCGTTCTTGCCAG	62

**Statistical analysis**

Statistical analysis was conducted using Microsoft Excel, and the results were presented as the mean ± standard deviation. For data analysis, one-way variance analysis (ANOVA) was performed using the IBM 25.00 SPSS statistical program, followed by Tukey’s test. p < 0.05 was considered as significant.

**RESULTS AND DISCUSSION**

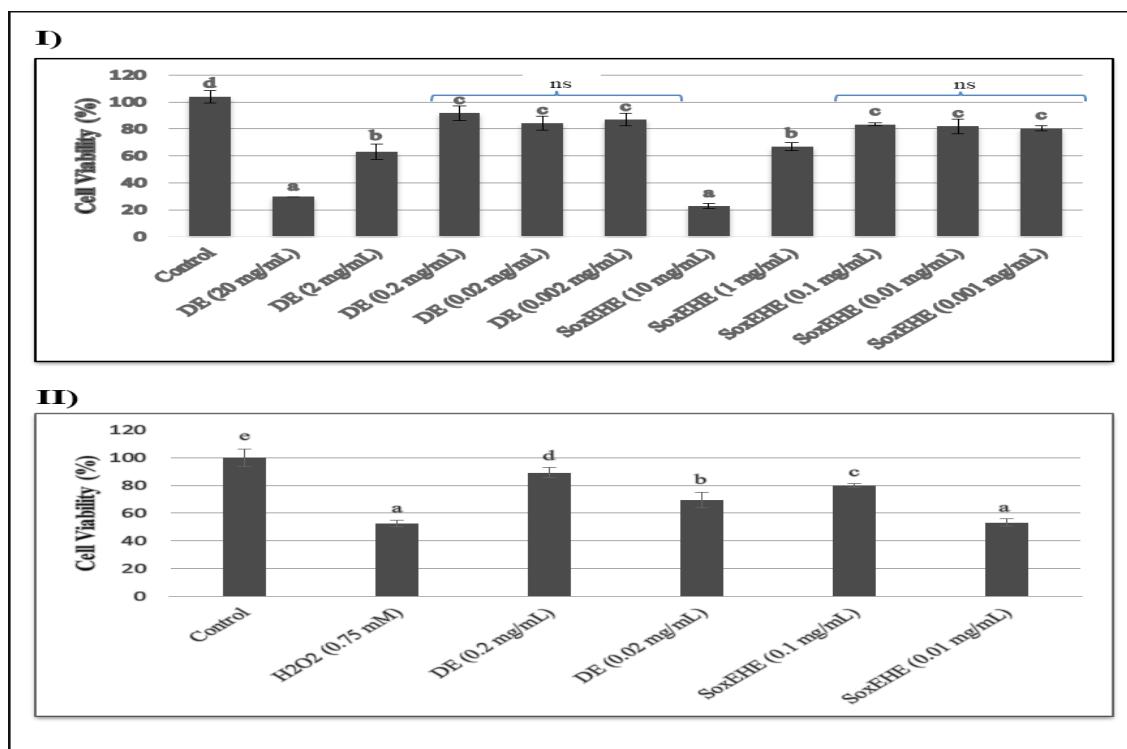
**Proliferation of L929 and viability analysis**

In this study, when the effects of decoction and ethyl acetate extracts on cell proliferation were evaluated separately, DEs demonstrated cytotoxic effects at 20 mg/mL and 2 mg/mL (p<0.05). At doses of 0.2 mg/mL and below, the cytotoxic effect in healthy fibroblast cells began to disappear (Figure 1). The efficacy of 0.2 mg/mL, 0.02 mg/mL, and 0.002 mg/mL were statistically similar (p>0.05). Therefore, we decided to use DE at concentrations of 0.2, and 0.02 mg/

mL in the later stages of our study. The cytotoxic effect of SoxEHes on healthy fibroblast cells at doses of 10 mg/mL and 1 mg/mL is shown in Figure 1 (p<0.05). This cytotoxic effect was observed to be eliminated at 0.1 mg/mL and lower doses. The effects of SoxEHE at doses of 0.1, 0.01, and 0.001 mg/mL were statistically similar (p>0.05). Therefore, SoxEHE was found to be effective at doses of 0.1 mg/mL, and 0.01 mg/mL.

**The effect of H<sub>2</sub>O<sub>2</sub> on L929 cells**

Analysis of Figure 1 revealed a notable reduction in cell viability in the presence of 0.75 mM H<sub>2</sub>O<sub>2</sub>, indicating a significant difference compared to the control group (p<0.05). The decoction extract significantly improved cell viability by protecting H<sub>2</sub>O<sub>2</sub> damage, especially at a dose of 0.2 mg/mL (p<0.05). Ethyl acetate/hexane extract maintained cell viability significantly against oxidative damage induced by H<sub>2</sub>O<sub>2</sub> at a dose of 0.1 mg/mL (p<0.05). Both extracts showed lower effectiveness at decreasing the doses.

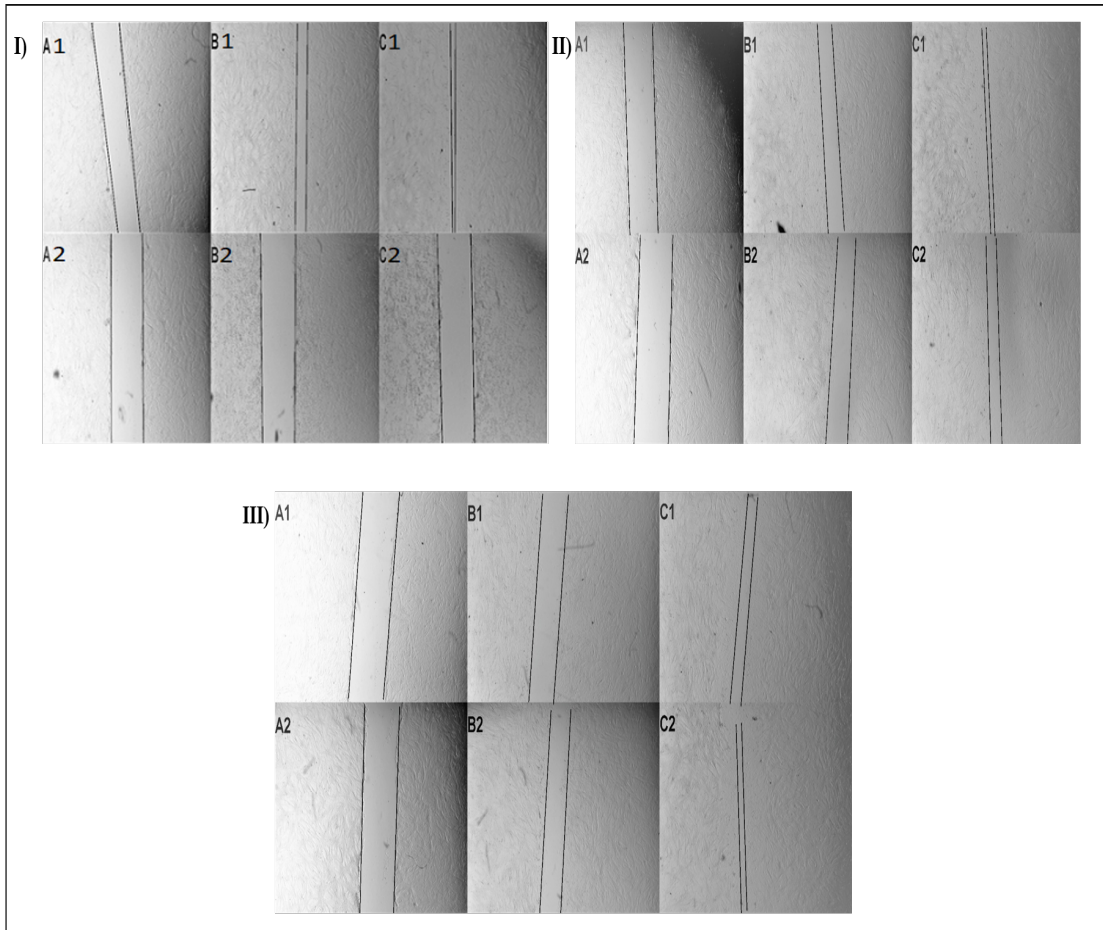


**Figure 1.** Determination of proliferative and cytotoxic doses of plant extracts (I) and indicating the protective effects of plant extracts against H<sub>2</sub>O<sub>2</sub>-induced damage (II) on L929 cells (if the letters in the columns are the same, the difference between them is statistically insignificant, p>0.05 ns: the groups included in the line are meaningless with each other, H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide, DE: Decoction extract, SoxEHE: Soxhlet ethyl acetate/hexane extract).

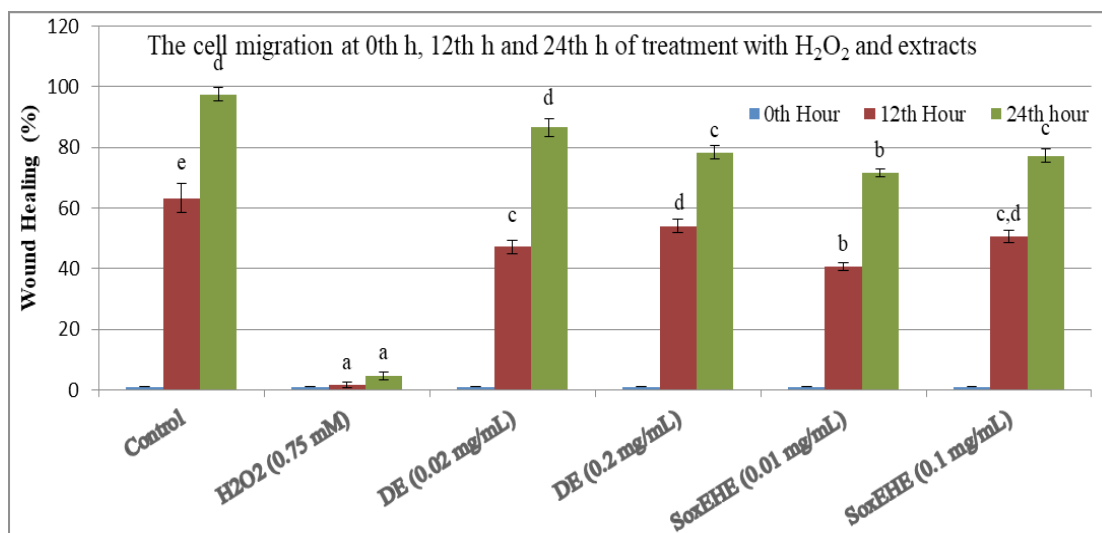
**Migration test results**

An *in vitro* migration test, known as the wound healing test, was conducted to assess how the plant extract influenced the movement of L929 cells toward the scratched area, often referred to as the injury site. According to the results, healthy fibroblast cells exhibited a nearly complete closure tendency by migrating to the wounded area within 24 hours (Figure 2.I; A1-C1). In L929 cells treated with only hydrogen peroxide, it was observed that cell migration was prevented, and this was statistically significant (p<0.05) in the

measurements made at the 12th and 24th hours (Figure 2.I; A2-C2). H<sub>2</sub>O<sub>2</sub> showed both a cytotoxic effect by creating oxidative damage and caused the wound to not heal by preventing cell migration. In both doses of DE applied, cell migration to the wounded area significantly increased. Unlike the MTT results, there was more healthy migration, especially at the dose of 0.02 mg/mL (p<0.05) (Figure 2.II; A2-C2). In groups where SoxEHE was applied, cell migration increased in both doses, while it significantly increased cell migration against H<sub>2</sub>O<sub>2</sub> at a dose of 0.1 mg/mL (Figure 2.III;A2-C2, 3).



**Figure 2.** Illustrating of the cell migration under a scratch assay (wound healing method), **I)** the migration of healthy cells (A1: 0<sup>th</sup> time, B1: 12<sup>th</sup> hour, C1: 24<sup>th</sup> hour) and the effect of H<sub>2</sub>O<sub>2</sub> application on the cell migration (A2: 0<sup>th</sup> time, B2: 12<sup>th</sup> hour, C2: 24<sup>th</sup> hour), **II)** the effect of DEs (A1: 0.02 mg/mL; 0<sup>th</sup> time, B1: 0.02 mg/mL; 12<sup>th</sup> hour, C1: 0.02 mg/mL; 24<sup>th</sup> hour, A2: 0.2 mg/mL; 0<sup>th</sup> time, B2: 0.2 mg/mL; 12<sup>th</sup> hour, C2: 0.2 mg/mL; 24<sup>th</sup> hour) and **III)** the effect of SoxEHEs (A1: 0.01 mg/mL; 0<sup>th</sup> time, B1: 0.01 mg/mL; 12<sup>th</sup> hour, C1: 0.01 mg/mL; 24<sup>th</sup> hour, A2: 0.1 mg/mL; 0<sup>th</sup> time, B2: 0.1 mg/mL; 12<sup>th</sup> hour, C2: 0.1 mg/mL ;24<sup>th</sup> hour) on cell migration against H<sub>2</sub>O<sub>2</sub>-induced damage.



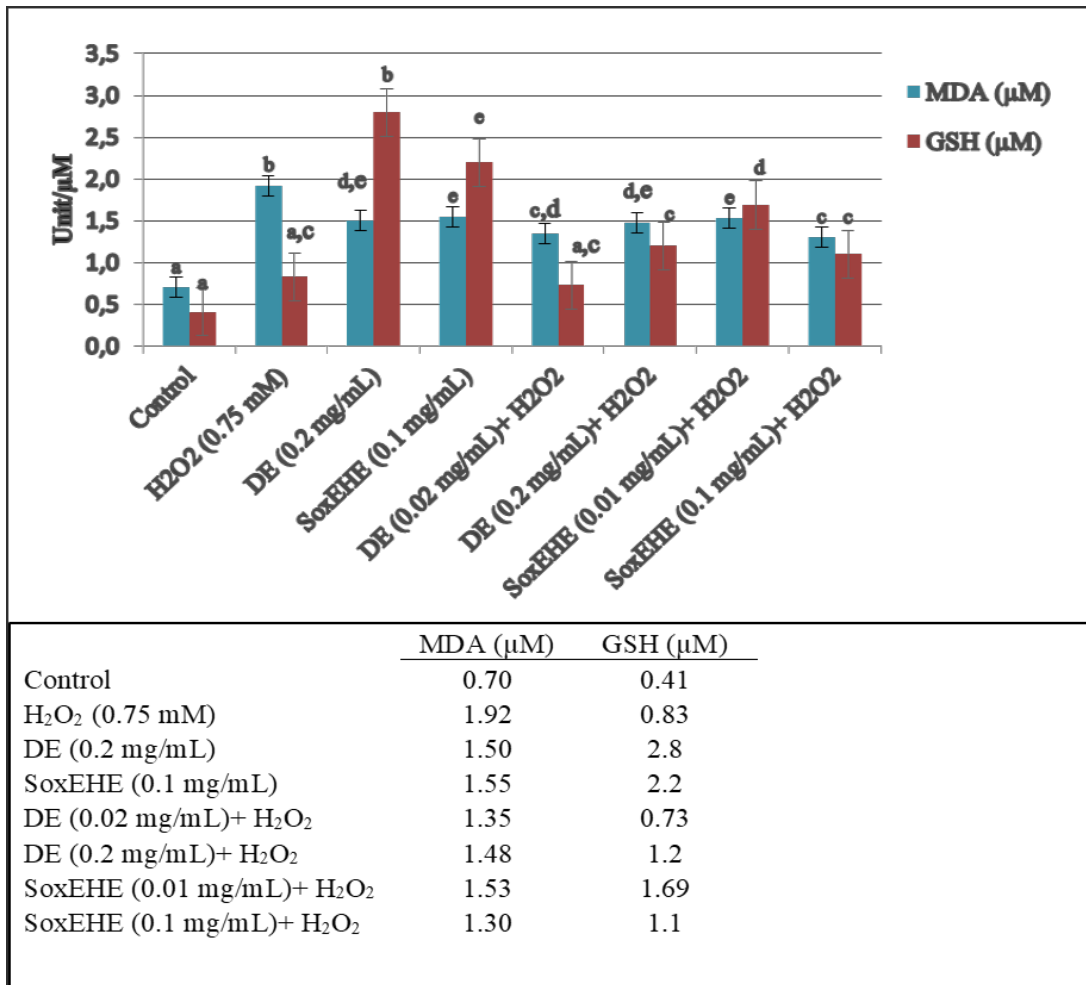
**Figure 3.** Demonstration of the effect of plant extracts on cell migration in an *in vitro* scratch assay (wound healing method) against damage induced by H<sub>2</sub>O<sub>2</sub> in L929 cells as % (If the letters in the columns are the same, the difference between them is statistically meaningless p>0.05, H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide, DE: Decoc-tion extract, SoxEHE: Soxhlet ethyl acetate/hexane extract).

### Results of MDA and GSH

When the levels of MDA as a signal of lipid per-oxidation were examined in the L929 cell line, it was observed that the highest level (p<0.05) was in the ox-idative stress-induced cell line compared to other cell lines (Fig. 4.). In the cell lines, where the effect of plant extract on oxidative stress was observed, MDA levels were significantly lower (p<0.05) compared to the cell line where only H<sub>2</sub>O<sub>2</sub> was applied, but they varied among themselves. The lowest MDA levels in these cell lines (except for the control cell line) were seen in DE (0.02 mg/mL) + H<sub>2</sub>O<sub>2</sub> and SoxEHE (0.1 mg/mL) + H<sub>2</sub>O<sub>2</sub> cell lines. These results indicated that 0.02 mg/mL concentration of DE and 0.1 mg/mL concentra-

tion of SoxEHE were effective on lipid peroxidation.

When the levels of the tripeptide antioxidant GSH were analyzed in all cell lines, statistically elevated levels (p<0.05) were observed exclusively in the ex-tracted cell lines. This finding indicated the effective-ness of plant extracts in enhancing antioxidant capa-city (Fig. 4.). The GSH levels of the plant extract and H<sub>2</sub>O<sub>2</sub>-treated cell lines decreased statistically (p<0.05) to almost the level of the cell line applied to H<sub>2</sub>O<sub>2</sub> alone (except SoxEHE (0.01 mg/mL) + the H<sub>2</sub>O<sub>2</sub> cell line) compared to only the plant extract used cell lines. This finding could be explained by GSH depletion during oxidative stress.



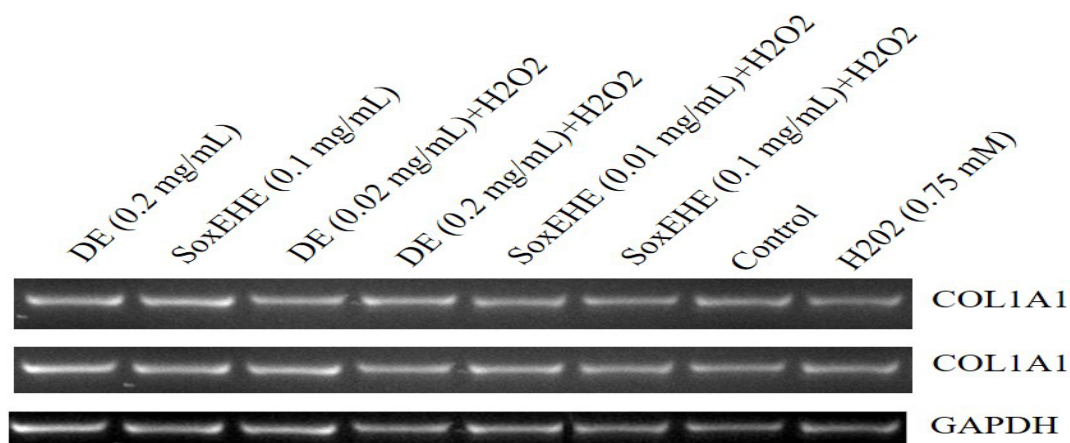
**Figure 4.** Effect of plant extracts on MDA and GSH levels in an *in vitro* wound healing model against damage caused by H<sub>2</sub>O<sub>2</sub> in L929 cells. (If the letters in the columns are the same, the difference between them is statistically insignificant  $p > 0.05$ , DE: Decoction extract, SoxEHE: Soxhlet ethyl acetate/hexane extract).

**mRNA distribution of COL1A1**

The mRNA expression levels of COL1A1 were investigated using RT-PCR. The increase in COL1A1 levels in these cell lines (except for the control cell line) were seen in DE (0.2 mg/mL) + H<sub>2</sub>O<sub>2</sub> and Sox-EHE (0.1 mg/mL). These results indicated that 0.2 mg/mL concentration of DE and 0.1 mg/mL concen-

tration of SoxEHE were effective on wound healing (Figure 5). The level of the GAPDH transcript was the same at all time intervals (Figure 5). When COL1A1 levels as collagen were analyzed in all cell lines, it was found that high levels were only present in the extracted cell lines, therefore, plant extracts were effective in improving collagen capacity (Figure 5).





**Figure 5.** mRNA distribution of COL1A1.

A large percentage of the human population worldwide tends to use herbal medicines because they find them more reliable and easier to access. They also realize synthetic drugs and their side effects. Throughout history, many plant extracts, mixtures, porridge, boiling, and pastes have been used in many countries for the treatment of diseases, cuts, wounds, and burns. Therefore, since ancient times, several herbs and plant-based strategies have been known for their important roles in wound healing and skin regeneration, as well as in their therapeutic application (Barku, 2019).

Scratch testing in fibroblast cell cultures is a method that is widely applied to identify components of wound healing that have been investigated (Liang et al., 2007). Oxidative stress induced by hydrogen peroxide serves as an alternative to assess the antioxidant activity in cells and to delay stress-induced wound healing (Pitz et al., 2016). In this study, *I. viscosa* extracts prepared using different methods were used to test the activity of fibroblast scratch wound healing in H<sub>2</sub>O<sub>2</sub>-induced L929 cells and the effects of H<sub>2</sub>O<sub>2</sub>-induced oxidative stress.

As cell proliferation and migration are crucial aspects of tissue formation in the wound healing process, it was essential to establish the non-toxic dosage of the extracts within the specific cell line under investigation for their therapeutic efficacy. Therefore, initi-

ally, the effects of the extracts on the proliferation and cell viability of L929 fibroblast cells were determined. It was determined that dilutions of 0.2 mg/mL and lower for DEs, and 0.1 mg/mL and lower for SoxEHEs were non-toxic, with cell viability exceeding 80%.

In a study in which the cell viability of *I. viscosa* extracts obtained by two different methods was examined using the XTT method, it was reported that 50 µg/mL decoction extract produced a cell viability of over 80% in the L929 fibroblast cell line (Hepokur et al., 2019). The results of this study are consistent with those of our research.

At the concentrations tested in our study, 20 mg/mL of DE and 10 mg/mL of SoxEHE were considered to have cytotoxic effects in the L929 fibroblast cell line (Figure 1). Therefore, it is plausible that extracts or purified phytochemicals obtained from the *Inula* genus may possess antiproliferative properties (Messaoudi et al., 2016; Talib et al., 2012).

Free radicals formed at the basal level during wound healing are necessary for cell signaling and the fight against invasive microorganisms. H<sub>2</sub>O<sub>2</sub> facilitates the recruitment of leukocytes to the wound site as a chemoattractant after injury formation, and also participates in the subsequent stages of healing. (Schäfer & Werner, 2008) in particular aged individ-

uals, patients with diabetes, and those treated with immunosuppressive drugs, chemo- or radiotherapy. The mechanisms underlying the impaired healing response are still poorly understood. Recent studies provided strong evidence for a role of oxidative stress in the pathogenesis of non-healing ulcers. Therefore, it is of major importance to identify and functionally characterize the factors involved in the generation and detoxification of ROS. When an excessive amount of free radicals is produced, a condition called oxidative stress occurs, which disrupts the structural integrity of the cell by damaging DNA, carbohydrates, proteins, and especially lipid structures in membranes. Therefore, wound healing is delayed, or the wound cannot heal (Shetty et al., 2008).

Oxidative stress originating from hydrogen peroxide is an alternative method for evaluating the antioxidant activity of extracts in cells (Jose et al., 2019). The determination of MDA, a technique used to measure oxidative stress, is based on the reaction of malondialdehyde formed through peroxidation of polyunsaturated fatty acids with thiobarbituric acid (Yoshioka et al., 1979). In the present study, MDA levels were higher in all groups compared to the control, but the highest level was only in the H<sub>2</sub>O<sub>2</sub>-treated cell line without statistical difference. This indicates that H<sub>2</sub>O<sub>2</sub> induced oxidative stress in this group. Increased ROS levels can inhibit cell migration and proliferation (Steiling et al., 1999). The absence of cell migration and proliferation at the 12th and 24th hours in the group treated with H<sub>2</sub>O<sub>2</sub> alone led to the consideration of oxidative stress and the cytotoxic effect of H<sub>2</sub>O<sub>2</sub>. The levels of MDA in the DE (0.2 mg/mL), SoxEHE (0.1 mg/mL), and extract + H<sub>2</sub>O<sub>2</sub> groups were not significantly different. In addition, the fact that cell migration and proliferation were close to the control in groups with oxidative stress and extract shows that the extracts have a positive effect against oxidative stress.

Antioxidants are protein systems designed to counteract the harmful effects of ROS by providing electrons. Therefore, they prevent the capture of elec-

trons from other important molecules such as DNA, proteins, and lipids (Dunnill et al., 2017). Antioxidants include antioxidant enzymes such as GSH reductase, GSH peroxidase, superoxide dismutase (SOD), catalase, and other endogenous free radical scavengers such as  $\alpha$ -tocopherol, ascorbic acid, and GSH (Arul et al., 2012). The H<sub>2</sub>O<sub>2</sub> effect is an interwoven healing process that is controlled by molecular antioxidants such as SOD, GPx, and phospholipid hydroperoxide glutathione peroxidase (Pitz et al., 2016). Glutathione is an important endogenous antioxidant. It functions as a cellular redox buffer and is crucial in protecting cells from the toxic effects of both endogenous and exogenous electrophilic compounds. (Schäfer & Werner, 2008) in particular aged individuals, patients with diabetes, and those treated with immunosuppressive drugs, chemo- or radiotherapy. The mechanisms underlying the impaired healing response are still poorly understood. Recent studies provided strong evidence for a role of oxidative stress in the pathogenesis of non-healing ulcers. Therefore, it is of major importance to identify and functionally characterize the factors involved in the generation and detoxification of reactive oxygen species (ROS).

In the study, GSH levels were the highest in the DE (0.2 mg/mL) and SoxEHE (0.1 mg/mL) groups compared to other groups. *In vitro* experimental studies have shown that the extract or purified phytochemical compounds obtained from *I. viscosa* have antioxidant activity (Danino et al., 2009; Mohti et al., 2020; Schinella et al., 2002). Although there were no statistical differences between the extract + H<sub>2</sub>O<sub>2</sub> groups in terms of GSH levels, there is a statistical decrease ( $p < 0.05$ ) in GSH levels compared to the DE (0.2 mg/mL) and SoxEHE (0.1 mg/mL) groups. The fact that the MDA levels were lower than those of the H<sub>2</sub>O<sub>2</sub> group suggests that GSH may have been depleted during migration. The functional importance of antioxidants such as glutathione, ubiquinones, uric acid, lipoic acid, vitamins E and C (ascorbic acid), carotenoids, and phenolic compounds in the wound repair process is suggested by their depletion in healing skin

wounds (Schäfer & Werner, 2008) in particular aged individuals, patients with diabetes, and those treated with immunosuppressive drugs, chemo- or radiotherapy. The mechanisms underlying the impaired healing response are still poorly understood. Recent studies provided strong evidence for a role of oxidative stress in the pathogenesis of non-healing ulcers. Therefore, it is of major importance to identify and functionally characterize the factors involved in the generation and detoxification of ROS. In a study in which the L929 fibroblast cell line was treated with the GSH blocker, buthionine sulfoximine, cell death occurred following GSH depletion, and GSH protected the cells from death (Zucker et al., 1997). In another study, wounds were reported to heal faster when GSH was applied topically to wounds in diabetic rats. These studies have shown that glutathione benefits wound repair (Mudge et al., 2002).

Fibroblasts, the primary cell type in the dermis, are responsible for the production and remodeling of the extracellular matrix during wound healing. Collagen, type III, alpha 1 (COL3A1) and Collagen, type I, alpha 1 (COL1A1) are well-established as major constituents of the extracellular matrix (ECM) found in the dermal tissues of the skin (McFarland et al., 2011). Beare et al. (2003) observed that the wounds of mice with a mutant collagen type-I gene (COL1A1<sup>tr</sup>) healed more slowly than those of wild-type mice. Hashimoto et al. (2020) reported that the COL3A1 gene was down-regulated on all surfaces throughout the cell culture. Their research did not reveal any significant variances in the expression of the COL1A1 gene among the three different surfaces in the cell culture. In the present study, we investigated the effect of this collagen (COL1A1) in wound healing. The increase in COL1A1 levels in these cell lines (except for the control cell line) were seen in DE (0.2 mg/mL) + SoxEHE (0.1 mg/mL). These results indicated that the concentration of 0.2 mg/mL for DE and 0.1 mg/mL for SoxEHE were effective in wound healing (Figure 5.). The level of the GAPDH transcript was the same at all time intervals (Figure 5.). When COL1A1 levels as collagen were analyzed in all

cell lines, it was found that high levels were only present in the extracted cell lines. Therefore, plant extracts were effectively improved collagen capacity (Figure 5). In another study, it was reported that COL1A1 and COL3A1 levels increased in parallel with the formation of the fibroblast cell layer (Wiegand et al., 2021).

According to the literature, the antioxidant, antibacterial, and other biological activities of *I. viscosa* are known to originate from sesquiterpenes, triterpenoids, and flavonoids. Mohti et al. (2020) reported that extracts obtained from *I. viscosa* exhibited strong antioxidant properties through various techniques, attributing this effect to the phenolic acid and flavonoid derivatives present in the extracts. Similarly, Mahmoudi et al. (2016) found that the methanolic extract of *I. viscosa* leaves possessed a phenolic profile, particularly rich in mono- and dicaffeoylquinic acids, indicating a high antioxidant capacity of the extract. In another study, Khalil et al. (2007) suggested that *I. viscosa* extract promoted the healing of excision wounds in mice, proposing that this effect could be attributed to the anti-inflammatory properties of inuviscolide, a sesquiterpene found in the extract. In our study, we think that the proliferative and antioxidative effects of *I. viscosa* extracts originate from these flavonoids and sesquiterpenes.

## CONCLUSION

Different extraction methods can unearth various phytochemicals in plants. Decoction extraction is less costly than Soxhlet extraction (in terms of chemicals and equipment). In our study, it was observed that extracts obtained from *I. viscosa* plant exhibited a positive effect on both migration and oxidative stress parameters in the L929 cell line, where oxidative stress occurs. In this context, *I. viscosa* may be a potential agent for the treatment of wounds. However, further investigation through preclinical and clinical studies is necessary to explore this in more detail.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHOR CONTRIBUTION STATEMENT

**AH:** Conceptualization; Formal analysis; Investigation; Methodology; Writing-original draft; Writing-review & editing. Data curation; Resources. **İÇ:** Conceptualization; Formal analysis; Investigation; Methodology; Resources. **MY:** Conceptualization; Formal analysis; Investigation; Methodology; Resources. **SH:** Investigation; Resources **MB:** Investigation; Resources. **CÖ:** Investigation; Methodology; Resources; Writing-review & editing.

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