Wound healing activity of Sweetgum oil (Liquidambar orientalis L. balsam): Characterization of its mechanism of action on HaCaT human keratinocyte cells and possible responsible active constituents

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Abstract

The wound healing activity of sweetgum oil being a product of *Liquidambar orientali*s endemically grown in Turkey was tested in cell culture and its mechanism of action was first investigated in this study. By using extracts of sweetgum oil belonging to two different local producers in Muğla, experiments were conducted with human derived HaCaT keratinocyte cells lines. For the investigation of possible active constituents of sweetgum oil samples, IT-TOF/MS spectroscopic analyses were applied. Titrated *Centella asiatica* extract, a standard wound healing drug, was used as a positive control to compare the efficacy of sweetgum oil. Initially, MTT assay was applied to determine the non-toxic concentration of sweetgum oil and a possible proliferative activity in HaCaT keratinocyte cells. Real-time cell analysis system for proliferation and cell migration detection; scratch test and real-time polymerase chain reaction (RT-PCR) to check collagen type-1 alpha-1 mRNA expression. In MTT assay, non-toxic concentrations were determined as 0.1, 0.01 and 0.001 µg/mL. According to the real-time cell analysis system results, a significant increase in cell proliferation and migration was observed at 0.001 µg/mL concentrations. In scratch assay, it was observed that the distance on both sides of the wound was significantly closed at 0.001 µg/mL concentrations. Effects on HaCaT cells were time-dependent in nature. According to the Real-Time PCR result, no increase in collagen type-1 alpha-1 mRNA expression was observed. From these findings, it was concluded that sweetgum oil is effective in wound healing, and phenylpropyl cinnamate seems to be the main active ingredient for this effect.

1. Introduction

There is a growing understanding that wound care is costly. In the USA, Healthcare expenditures on wound care are estimated to be between $28.1 billion and $96.8 billion for the patient population alone. Approximately 15% of healthcare clients have at least one type of wound or wound-related infection, and care largely occurs in the outpatient setting (Law et al., 2020). While small and simple wounds are generally ignored in daily life and do not require treatment, except antiseptics' applications, large and complicated wounds require medical attention. In cases such as wounds concomitant to comorbid diseases like diabetes (Vatankhah et al., 2017), obesity (Frasca & Strbo, 2022), renal failure (Maroz & Simman, 2014), vasculitis (Rawlings et al., 2016), varicose veins (Robson et al., 2001), cancer (Zarchi, 2015) etc., bedsores (Hajhosseini, 2020), surgical wounds (Franz et al., 2000) and burns (Oryan et al., 2017), healing of wounds becomes difficult by delays, and then, medical intervention becomes inevitable. The world population has been increasingly consisting of older individuals. It has been long known that tissue regeneration and wound healing are impaired in the elderly (Bonifant &Holloway, 2019). Taking into consideration of the aging world, wound care and therefore its wound healing effect is becoming increasingly important in terms of health economics and maintaining quality of life, so that cheaper and efficacious modalities for wound care will gain importance in the future. On the other hand, various wound healing agents from plant sources have been used widely for a long time. There are some articles that provide excellent reviews of the use of herbal materials in wound care (Pazyar et al., 2014; Vitale et al., 2022). Among these, medicinal plants like Aloe vera (Budovsky et al., 2015), *Calendula officinalis* (Budovsky et al., 2015, Givol et al., 2019), *Centella asiatica* (Budovsky et al., 2015, Korkmaz et

al., 2000), *Hypericum perforatum* (Budovsky et al., 2015, Öztürk et al., 2007, Dikmen, 2011), *Gentiana lutea* (Budovsky et al., 2015, Öztürk et al., 2006) have used traditionally for centuries and/or clinically along with experimental investigations on their efficacies and mechanisms of actions at cellular or molecular levels. Turkish sweetgum oil or sytrax liquidus is an oleoresin (balsam) obtained from the wounded trunk of the *Liquidambar orientalis* Mill. (Hamamelidaceae). *L. orientalis* tree., known with the local names as 'Sığla ağacı' or 'Günlük ağacı' in Turkey, it is an endemic plant exhibiting a local distribution on eastern Mediterranean region particularly at the south-western coastal district of Turkey (Köycegiz, Fethiye, Marmaris and Urla). Sytrax liquidus mainly consists of resin alcohols presenting both free and combined with cinnamic acid which constitutes approximately 30–45% of the total weight (Hafızoğlu et al., 1982). In Turkish folk medicine, sweetgum oil has been used as a cure for various skin problems, i.e. chaps on lips, wounds and burns, beside of other curative purposes, such as peptic ulcers, nocturnal enuresis, parasitic infections, antiseptic or expectorant (Baytop,1999, Gürbüz et al., 1999). However, wound healing activity of this balsam, in connection with its mechanism of action and the substances in its composition, has not been subject to any experimental studies to date, although its several other types of biological activities such as antibacterial, antiviral, antifungal, antihypertensive and anticonvulsant have also been reported previously (Lingbeck, 1982). Hence, this study was designed to characterize wound healing activity of sweetgum using HaCaT human keratinocyte cell line as an experimental model with sweetgum oil extracts whose chemical analyses were made. To best of our knowledge, this study is the first one that investigate possible wound healing activity of sweetgum oil on keratinocyte cell cultures together with its phytochemical composition.

2. Materials and methods

2.1. Plant Material

In this study, sweetgum oil samples that produced by injuries on the trunk of *L. orientalis* was purchased from two different local producers in Marmaris (Muğla, Turkey) in order to investigate wound healing activities *in vitro*. Sweetgum oil samples were purchased from local producers from Köyceğiz and Marmaris districts of Muğla. Titrated extract of *Centella asiatica* (TECA; Bayer, Turkey), which has a proven wound healing effect, was used as a positive control. They were kept at 4°C until analysis. Stock solutions of TECA, sweet gum oil samples, which were dissolved in DMSO giving concentrations of 200 mg/mL were encoded as TECA, SO-1 (Köyceğiz origin) and SO-2 (Marmaris origin), respectively. All stock solutions were sterilized in an autoclave at 121ºC, 1.5 atm/Hg for 20 minutes and diluted in medium (DMEM) freshly to obtain different final concentrations in cell culture. When the same procedure repeated with DMSO alone as vehicle, which was added to cell culture in a final volume not exceeding 10 µL.

2.2. Cell Culture

For cell culture; human keratinocyte cell line (HaCaT) was purchased from Cell Line Services (CLS, Germany). HaCaT keratinocyte cells were cultured in 20% fetal bovine serum (FBS), 1% penicillin-

streptomycin, 1% L-glutamine and 1% sodium pyruvate containing DMEM medium, at 37°C, in %95 humidified incubator with 5% CO_2 (Thermo Scientific, Massachusetts, USA). HaCaT keratinocyte cells are adherent type cells. The area of the surface on which they are located limits growth, so they must be passaged for certain periods of time. In our study, after the cell density reached a certain level on the surface to which they adhered, they were separated enzymatically and cultivated.

Before cell counting, human HaCaT keratinocyte cells proliferating in the flasks were washed with PBS. They were treated with 2X trypsin-EDTA solution to remove adherent cells adhering to the surface of the flask. The cells were kept in the incubator for approximately 3–5 minutes to allow the cells to be removed from the flask to which they adhered. To neutralize the effect of trypsin, medium was immediately added to the non-adherent cells and the medium-cell mixture was transferred to an appropriate centrifuge tube. After centrifugation at 1200 rpm for 5 minutes, the supernatant was discarded and 1mL of 20% FBS (fetal bovine serum) was added onto the remaining pellet; DMEM medium containing 1% penicillin-streptomycin and 1% L-glutamine was added and pipetting was performed. After pipetting, cells were counted with the help of a cell counter device (Cedex XS, Innovatis,USA) before seeding into 96-well plates (approximately 10000 cells per well). During the cell counting process, the cells were stained with Trypan blue solution and the counts were performed.

HaCaT keratinocyte cells were stained with Trypan blue solution and counted each time before applying MTT, proliferation and migration determination in Real Time Cell Analysis System (RTCA-DP), scratch experiment and Real-Time PCR methods.

2.3. MTT Cytotoxicity Test

To find non-cytotoxic concentrations of the compounds, cell viability was determined by MTT (3,4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay (Hansen et al., 1989; Van Meerloo, 2011). HaCaT cells were inoculated for 24h in 96-well plates, with the density of 10.000 cells per well. Stock solution of sweetgum oil was dissolved in DMSO (dimethyl sulfoxide) and the stock solution was diluted to the required concentrations by using DMEM medium. A two-step concentration screening strategy was developed using the MTT test to investigate the possible cytotoxic effect of sweetgum oil on HaCaT keratinocyte cells. Accordingly, in the first step, the toxic concentration starting point was determined for SO-1 and SO-2 extracts by using wider concentration ranges (0.01, 0.10, 1.00, 10.00 and 100.00 µg/mL) with a geometric concentration increase factor of 10. In the second step, non-toxic concentrations were more precisely determined for both extract samples by using narrower concentration ranges (0.39, 0.78, 1.56, 3.13, 6.25, 12.20, 25.00 and 50.00 µg/mL) with a geometric concentration increase factor of 2. The same protocol was repeated for the positive control TECA using the same concentrations. After adding SO-1, SO-2 and TECA concentrations to growth media, cells were incubated in CO_2-incubator for 24h at 37°C. At the end of incubation time, MTT solution was added to each well and incubated for 3h at 37°C, then the purple MTT-formazan crystals were dissolved by adding 100 µL DMSO to each well. Absorbance values were measured of with ELISA reader (at 540nm). During experiment, each group was performed

in 8 wells and absorbance measurements were repeated 3 times and all data were recorded as mean ± standard deviation of mean (S.D.). Data were analyzed by one-way ANOVA with Tukey's post-hoc

2.4. Determination of COL1A1 mRNA expression levels by RT-PCR method

In this research method, applying TaqMan technique cDNA synthesis from total RNA and amplification of the COL1A1 gene (collagen gene) from template cDNA were performed. GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) was used as the housekeeping gene. The main steps of the method are summarized in Fig. 1. The application steps of the Real Time-PCR method in the research are given under the headings below.

2.4.1. Total RNA isolation

After incubation of HaCaT cells with SO-1, SO-2 and TECA concentrations, total RNA was isolated using the total RNA isolation robot, MagNA Pure Compact (Roche). Incubated with 0.001, 0.01and 0.1 µg/mL concentrations of SO-1, SO-2 and TECA for 24 and 48 hours. Total RNA was isolated from HaCaT keratinocyte cells. Main RNA isolation steps are given as follows: After the incubated cells were removed, they are centrifuged. After wash with 2 mL PBS, they were centrifuge again. 220 µL of lysis buffer were added, pipetted, so the cell membranes were disrupted. They were transferred to the sample tube of the robot as 200 µL. 50 µL of RNA was isolated from the robot within 30 minutes. The isolated RNAs were sent to the nanodrop 2000 spectrophotometer device (Thermo Scientic, Massachusetts, USA) for quantification. RNA to be stored for further studies was stored in a -20°C deepfreeze or a -80°C liquid nitrogen tank.

RNA yield was determined by spectrophotometric measurement at 260 nm and 280 nm optical density using the Nanodrop device. Before proceeding with the production of cDNA from RNA, the amounts of RNA were equalized. For this, measurement RNA results taken from the nanodrop device were used. Then, an equal amount of RNA (100 ng/sample) from each sample was used for the PCR step for cDNA synthesis. For HaCaT cells, RNA Nanodrop results for 48h are given in Table 1.

2.4.2. cDNA synthesis

In order to examine the mRNA expression of genes, the total RNA obtained must first be converted into complementary DNA (cDNA). After RNA isolation, cDNA was obtained by reverse transcription (RT) reaction using the 'Transcriptor High Fidelity cDNA Synthesis Kit' (Roche Diagnostics, Germany).

For this process, oligo (dT) primers, which are primers specific for the small poly (A) pool in the entire total RNA pool, were used. 100 ng total RNA (1000 / nanodrop RNA result), 1 µL Oligo (dT) per sample was added to each PCR tube and the total volume was made up to 11.4 µL with distilled water. Each sample tube was heated at 65°C for 10 minutes in a thermal cycler to denature the template-primer mixture. Following reagents added the contents of the tube in order: 4 µL Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer, 0.5 µL protector RNase inhibitor (20U), 2 µL dNTP

(deoxynucleotide mix) mixture (10mM each), 1 µL DTT (5mM), 12.5 µL Transcriptor High Fidelity Reverse Transcriptase (10U) was added to make the total volume 20 µL. Then, the tubes were incubated in the PCR Thermal Cycler device at 55°C for 30 minutes and at 85°C for 5 minutes. The obtained cDNAs were used for amplification in Real Time-PCR.

2.4.3. Determination of mRNA expressions by RT-PCR (Real time PCR)

Effects of 0.001, 0.01 and 0.1 µg/mL concentrations of SO-1, SO-2 and TECA was investigated on RT-PCR COL1A1 mRNA expression of HaCaT keratocyte cell lines. The RT-PCR method was applied in a single step using the kit and primers suitable for the device. The obtained cDNAs were optimized and amplified in the LightCycler® 480 RT-PCR device, using a TaqMan primer and PCR kit specific to the COL1A1 gene, in 3 replicates from each sample, according to the kit protocol. GAPDH was used as the housekeeping gene.

The reagents loaded into 96 clear plates compatible with Light Cycler PCR 480 are given in Table 1, respectively.

Table 1
Reagents added to Lightcycler PCR compatible clear plates

Order	Chemical	Amount
1	H_2O	4 µL
2	Prob Master	10 µL (LightCycler® 480 Probe Master kit REF: 04 707 494 001 for COL1A1)
3	Primer (Taqman)	1 µL (COL1A1 / GAPDH)
4	cDNA	5 µL

This prepared mixture was loaded into a 96-well plate separately for COL1A1 and GAPDH. After the loading process, the 96 plate was placed in the Light Cycler 480 device and the quantitative measurement amounts of the samples were determined. Using the Using 'Advanced Relative Quantification' program for results obtained, the effects of sweetgum oil extracts (SO-1 and SO-2) and TECA on COL1A1 expression were compared.

2.5. Xcelligence Real-Time Cell Analyse System

With this system, both proliferation and migration of cells in the culture medium can be measured, thus these parameters were determined in HaCaT keratinocytes in the present study. So, kinetic information about cell proliferation were assayed using the Real-Time Cell Analyser (RTCA-DP). The RTCA-DP system measures the electrical impedance across the high-density electrode array coating the bottom of the well and converts the impedance values to a Cell Index (CI) [26]. Background of the E-plates (16 wells) were measured in 100 µl medium. Afterwards 100 µl of HaCaT cell suspension was added (10,000

cells/well). Plates were incubated for 30 min at room temperature and E-plates were placed into the instrument and impedance was measured 1h intervals over 24 hours. Non-toxic concentrations of sweetgum oils (0.001, 0.01 and 0.1 µg/mL) which determined by MTT results were then added to the wells and impedance monitoring continued for another 48 hours. Dose-response curves at 48h were generated.

2.6. Scratch Wound Healing Technique

The spreading and migration capabilities of HaCaT keratinocyte cells were evaluated using a scratch wound healing assay which measures the expansion of a cell population on surfaces. The cells were seeded into 6-well plates at a concentration of 1×10^6 cells per well and cells were grown in complete media until cells were at approximately 85 to 95% confluent. Then, a linear wound was generated in the monolayer with a sterile 100 µL plastic pipette tip.

Any cellular debris was removed by washing the wells with phosphate buffer saline (PBS) and following washing growing cells were treated with concentrations of sweetgum oil samples (0.001, 0.01 and 0.1 µg/mL), which containing fresh medium and incubated for 48h at 37°C with 5% CO_2. Three representative images from each well of the scratched areas were photographed at 0h ,24h, 48h with Leica DM 300 inverted microscope and to estimate the relative migration of the cells the images were analysed with Leica LAS Image Analysis programme by measuring the scratched area (Fronza et al., 2009). The experiments were performed at least in duplicate.

2.7. Chemical analyses of sweetgum oil samples

Qualitative and quantitative analyses of the elements in the sample sweetgum oils (SO-1 and SO-2) used in this study were carried out. The samples were placed in water and methanol and kept in an ultrasonic bath for 10 minutes, then the mixtures were filtered through 0.22 µm pore diameter PTFE or CA syringe tip filters and analysed with the Shimadzu brand IT-TOF/MS high-resolution mass spectroscopy (HRMS) system (Shimadzu, Kyoto, Japan). was done. It was analysed in positive and negative modes with the electronspray ionization (ESI) technique. Acetonitrile and water mixture containing 0.1% Formic acid as mobile phase was used as a gradient. MS terms are as follows; high voltage probe, -3.5 kV (3.5 kV for Negative ESI); nebulizing gas flow rate, 1.5 L/min; CDL temperature, 200°C; heat block temperature, 200°C; drying gas pressure, 200 KPa. TOF detector voltage is 1.6 kV. The LC part consists of two LC-20AD dual pumps, DGU-20A3R degasser unit, CTO-10ASvp column oven, SIL-20AC autosampler and SPD-M20A PDA detector. Shim-Pack FC-ODS (150 x 2 mm, 2 µm) C 18 HPLC column was used in the analyses where reverse phase chromatographic separation was performed. Water/Acetonitrile mixture containing 0.1% Formic acid was mixed as a gradient and used as the mobile phase. Data were processed using LCMSsolution (v. 3.80) software. Screening was carried out using the drugbank (drugbank.ca) online database and literature information, using the masses of the peaks densely detected in the PDA or MS spectra.

3. Results

3.1. Determination of non-toxic concentrations in MTT test

In the first step of cytotoxic concentration screening in HaCaT cells, it was determined that 0.001, 0.01 and 0.1 µg/mL concentrations of SO-1 and SO-2 concentration ranges were not toxic. Even mild proliferative effects were observed at some SO-1 and SO-2 concentrations during this screening procedure. During this initial screening concentration range for cyctotoxic activities of sweetgum oil extracts were estimated between 10.0 and 100.0 µg/mL. Cytotoxic (antiproliferative) actions of SO-1 and SO-2 extracts at the concentration of 100.0 µg/mL obvious and statistically significant (for both extracts $p < 0.05$). In the first stage of the cytotoxic concentration screening in HaCaT cells, it was determined that the concentration ranges of 0.001, 0.01, 0.1 µg/mL SO-1 and SO-2 were not toxic (Figure-2A). Even mild proliferative actions were observed at some SO-1 and SO-2 concentrations (0.01 and 0.1 µg/mL) during this screening process. Increase in the proliferation of HaCaT cells for 0.01 µg/mL of SO-2 extract was statistically significant ($p < 0.01$), while that for the same concentration of SO-1 extract was too small to be significant. At the concentration of 0.1 µg/mL, both SO-1 and SO-2 extracts still exhibited proliferative actions, but they were statistically insignificant (Figure 2A). Similar profile of action in MTT test was also observed for TECA. However, none of its concentrations exerted a significant proliferative action, while its 100.0 µg/mL concentration was found to have a highly significant antiproliferative (cytotoxic) action ($p < 0.005$) (Figure-2A).

In the second step of cytotoxic concentration screening in HaCaT cells, seven concentrations of SO-1 and SO-2 extracts applied in the culture media in a range of 0.39 and 25.0 µg/mL was found to be non-toxic. Particularly, some concentrations (0.39, 0.78, 1.56 µg/mL) of SO-2 extract exhibited a slight and not statistically significant proliferative action. For both sweetgum oil extracts, cytotoxic concentrations in HaCaT cultures were established to be 50.0 µg/mL, since SO-1 and SO-2 extracts showed statistically significant antiproliferative actions ($p < 0.05$) on these cells as measured by MTT method. For TECA, similar results were obtained on cultured HaCaT keratinocyte cells. Unlike sweetgum extracts, significant and concentration-dependent cytotoxic actions of TECA on these cells were observed at µg/mL concentrations of 6.25 ($p < 0.05$), 12.50, 25.00 ($p < 0.01$), 50.00 and 100.00 ($p < 0.005$) (Fig. 2B).

3.2. Determination of COL1A1 mRNA expression levels by RT-PCR

Table 2 shows RNA nanodrop results for HaCaT keratinocyte cells for 48 h. As a result of the analysis performed by RT-PCR, it was observed that COL1A1 mRNA expression levels did not change in HaCaT keratinocyte cell lines with SO-1, SO-2 and TECA at concentrations of 0.001, 0.01 and 0.1 µg/mL. Analysis results were created in detail using Software Release LCS480 version 1.5.0.39. (Fig. 3).

Table 2
For HaCaT cells, RNA Nanodrop results for 48h

Extracts	Concentrations (µg/mL)	Absorbance
SO-1	0.001	351,6
	0.010	372,6
	0.100	289,3
SO-2	0.001	340,7
	0.010	417,1
	0.100	274,7
TECA	0.001	329,4
	0.010	379,5
	0.100	463
Control		610,4

3.3. Xcelligence Real-Time Cell Analyse System

According to RTCA-DP analysis performed for 72 hours, a significant increase in cell proliferation and migration was observed at 0.001 µg/mL concentrations in a time-dependent manner. RTCA-DP analysis graphs for SO-1, SO-2 and TECA are given in Fig. 4. These results seem to be consistent with the results obtained from MTT experiments.

According to the RTCA-DP analysis performed during the 72-hour period, a significant increase in the migration of HaCaT cells was observed with SO-1, SO-2 and TECA extracts at the concentration of 0.001 µg/mL in a time-dependent manner. Migration graphs and their slope histograms related to the effects of SO-1, SO-2 and TECA on HaCaT cells are shown in Figs. 5,6,7, respectively. Both graphs and slope histograms clearly indicate increased migrations of HaCaT keratinocyte cells at applied concentrations of SO-1, SO-2 and TECA.

3.4. Scratch Wound Healing Technique

When evaluated cell migration with a model of scratch assay, keratinocyte cells treated with non-toxic concentrations of sweetgum oil samples and TECA extracts, proliferated and migrated from wound edges within 48h. Figures 8,9,10 show wound closure in the scratched area consisting cultured HaCaT cells, in which obvious wound healing effects were depicted with different concentrations of SO-1, SO-2 and TECA, respectively. While all tested extracts exhibited time-dependent actions in scratch wound technique, the highest activity was observed at 0.001 µg/mL concentrations.

3.5. Chemical analyses of sweetgum oil samples

Table 3 summarizes the IT-TOF/MS high-resolution mass spectroscopy analyses of sweetgum samples (SO-1 and SO-2). Accordingly, considering the relative intensity values of SO-1 and SO-2 samples, the main component of the samples appears to be phenylpropyl cinnamate (82.1% and 69.5%, respectively). As a phytoconstituent, it is followed by cinnamyl cinnamate with an intensity value of 9.3% and 19.6%. Other phytochemicals in SO-1 and SO-2 samples include benzyl alcohol, cinnamaldehyde, cinnamic acid and *p*-hydroxy cinnamic acid.

TABLE 3. IT-TOF/MS high-resolution mass spectroscopy analyses of sweetgum samples, SO-1 and SO-2.

Compound	Ionization Type	Adduct	Mass Calculated	Mass Found	Intensity for SO-1 (%)	Intensity for SO-2 (%)
Cinnamyl cinnamate	Positive	H	265.1223	265.1226	9.3	19.6
Phenylpropyl cinnamate	Positive	H	267.1380	267.1383	82.1	69.5
Benzyl alcohol	Positive	Na	131.0528	131.0499	2.1	3.1
Cinnamaldehyde	Positive	H	133.0648	133.0670	2.9	6.2
Cinnamic acid	Positive	H	149.0597	149.0584	0.2	0.9
p-hydroxy cinnamic acid	Negative	H	164.0401	163.0187	3.4	0.7

4. Discussion

As a product of an endemic tree that grows only in Turkey, sigla oil has been used for medicinal purposes from ancient times to the present day in the treatment of itchy dermatological diseases such as fungal infections and scabies, as a mucolytic in asthma, bronchitis, and other respiratory disorders, in haemorrhoids, in the treatment of stomach ulcers, in intestinal diseases such as dysentery, as a wound healer, and in the prevention of wound-related infections (Lingbeck, 1982).

At the present time, there are only a limited number of studies on the wound healing effect of sweetgum oil. All of the previous studies on the wound healing effects of sweetgum oil have been carried out in animals using *in vivo* experimental wound models. It has been reported in various studies that sweetgum oil exhibits wound healing properties by epithelisation and its antibacterial effects contributes to the healing process (Sağdıç et al., 2005, Öçsel et al., 2012).

In one of these studies, the effects of sweetgum oil on burn wound healing were investigated in comparison with silver sulfadiazine in an experimental burn wound model on rats. According to the results obtained, sweetgum oil has been stated significantly accelerated burn wound healing. Additionally, in histopathological evaluations, it has been reported that parameters such as inflammation,

collagen accumulation, angiogenesis, granulation tissue formation and epithelialization, which are the stages of wound healing, increased in the sweetgum oil and silver sulfadiazine groups compared to the control groups. However, the results have been found to be similar in the two groups (Yanik, 2016). In another study, the effectiveness of ointments containing sweetgum oil and collagenase on wound healing has been evaluated in an experimental burn wound model in rats. According to the results of the research, it has been stated that the wound healing time is shorter and the wound surface area is statistically smaller in the groups where ointments containing sweetgum oil and collagenase were applied compared to the control and sham groups (Deniz, 2010).

In the present study, the effect of sweetgum oil on wound healing was evaluated in HaCaT human keratinocyte cells *in vitro*, which are suitable for mechanism of action studies. As measured by the MTT method, non-toxic concentrations were determined as 0.001, 0.01 and 0.1 µg/mL. It was concluded that this concentration range is safe in terms of cytotoxicity. According to real-time cell analysis (RTCA-DP) performed for 72 hours, a significant time-dependent increase in cell proliferation/migration was observed at concentrations of 0.001 µg/mL. According to scratch analysis results, at the end of the 48 hours, it was observed that the distance on both sides of the wound was significantly closed at the same concentrations in a time-dependent manner as a result of cell migration. In our study, no change was also observed in COL1A1 mRNA expression levels with SO-1, SO-2 and TECA to HaCaT keratocyte cell lines at 1, 10 and 100 ng/ml concentrations. This result tells us that the pathways used by sweetgum in creating its effects are not through increasing intracellular collagen synthesis. Confirming our results, (Öçsel et al., 2012), who employed porcine wound model, have reported that sweetgum oil has no effect on both fibroblastic activity and collagen density.

Phytochemical content analysis has been carried out by many researchers on sweetgum oil. One study reported the detailed chemical composition of sweetgum oil and its main components were α-pinene, benzaldehyde, β-pinene, benzyl alcohol, acetophenone, 1-phenyl-1-ethanol, hydrocinnamyl alcohol, *trans*-cinnamyl aldehyde, *trans*-cinnamyl alcohol, b-caryophyllene and styrene (Lee et al., 2009), (Gürbüz et al.,2013) showed that the main components of sweetgum oil are styrene, cinnamyl alcohol, α-pinene. It has also been reported that the styrene content in the essential oil of sweetgum is only 1.56% and consists mainly of resin alcohols, both free and combined with cinnamic acid, which is approximately 30–45% (Park, 2014). In another study, hydro-distilled essential oils obtained from sweetgum oil were analysed by GC and GC-MS and the main components were (E)-ethyl cinnamate, torreyol and cinnamyl alcohol (Aşkun, 2021). In the studies conducted, it is thought that the different ratios of the components in the structure of sweetgum oil arise from the analysis methods used and the method and time of obtaining sweetgum oil. Results of our study are in general accordance with previous ones in terms of qualitative composition of sweetgum samples used. Phenylpropyl cinnamate was found as main constituent both in SO-1 and SO-2 samples. Therefore, this compound seems like as the main responsible phytoconstituent for the wound healing activity of sweetgum oil, as observed in in HaCaT human keratinocyte cells *in vitro*. However, it is possible that other constituents like cinnamyl cinnamate, benzyl alcohol and cinnamaldehyde contribute to the wound healing.

5. Conclusion

In conclusion, both sweetgum samples seem to be effective on wound healing mainly through keratinocyte migration and proliferation resulting in epithelisation of wound area. SO-1 extract at the concentration of 1 ng/mL was found to be more effective on cell proliferation and migration than SO-2 extract at the same concentration and the positive control *Centella asiatica* extract. Results of phytochemical analyses of two samples indicated that phenylpropyl cinnamate may be responsible component of sweetgum oil for the wound healing activity. However, further studies should be performed especially with main constituent(s) at the same experimental conditions. Overall, it is suggested that sweetgum oil may be a potential therapeutic for wound repair by improving healing process as an alternative modality.

Declarations

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Author contributions

methodology, M.D.; software, S.L.; validation, M.D.,Y.Ö.; formal analysis, M.D.; investigation, B.E.; resources, S.Ö.; data curation, M.D.; writing—original draft preparation, M.Y.; Y.Ö.; writing—review and editing, M.Y.; Y.Ö.; visualization, S.L.; supervision, M.D.; Y.Ö.; project administration, M.Y.; B.E funding acquisition, M.Y. The authors confirm that no paper mill and artificial intelligence was used.

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Conflict of interest

There are no conflicts of interest to disclose from any author listed on this publication.

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Figures

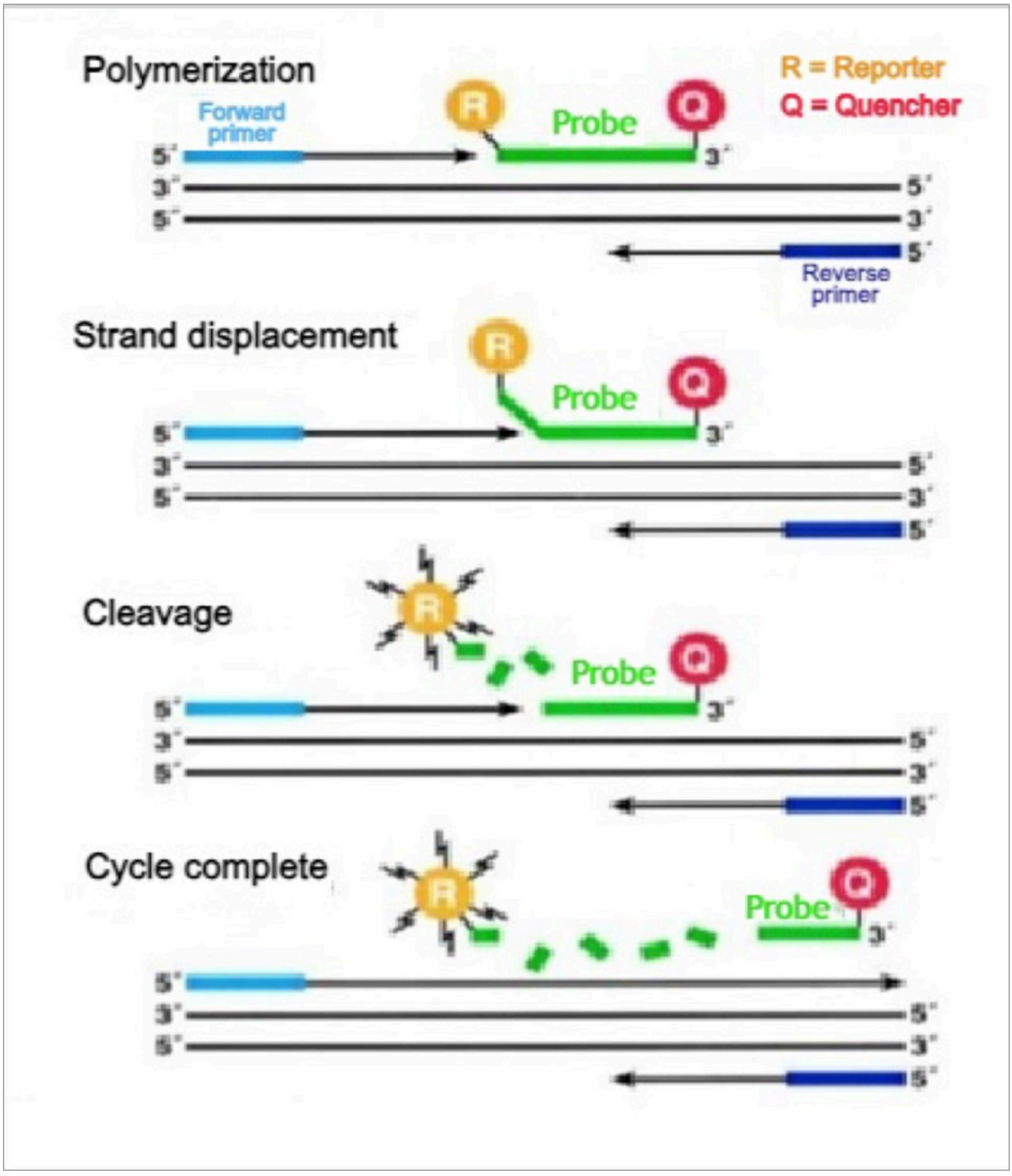

Figure 1

TaqMan technique stages

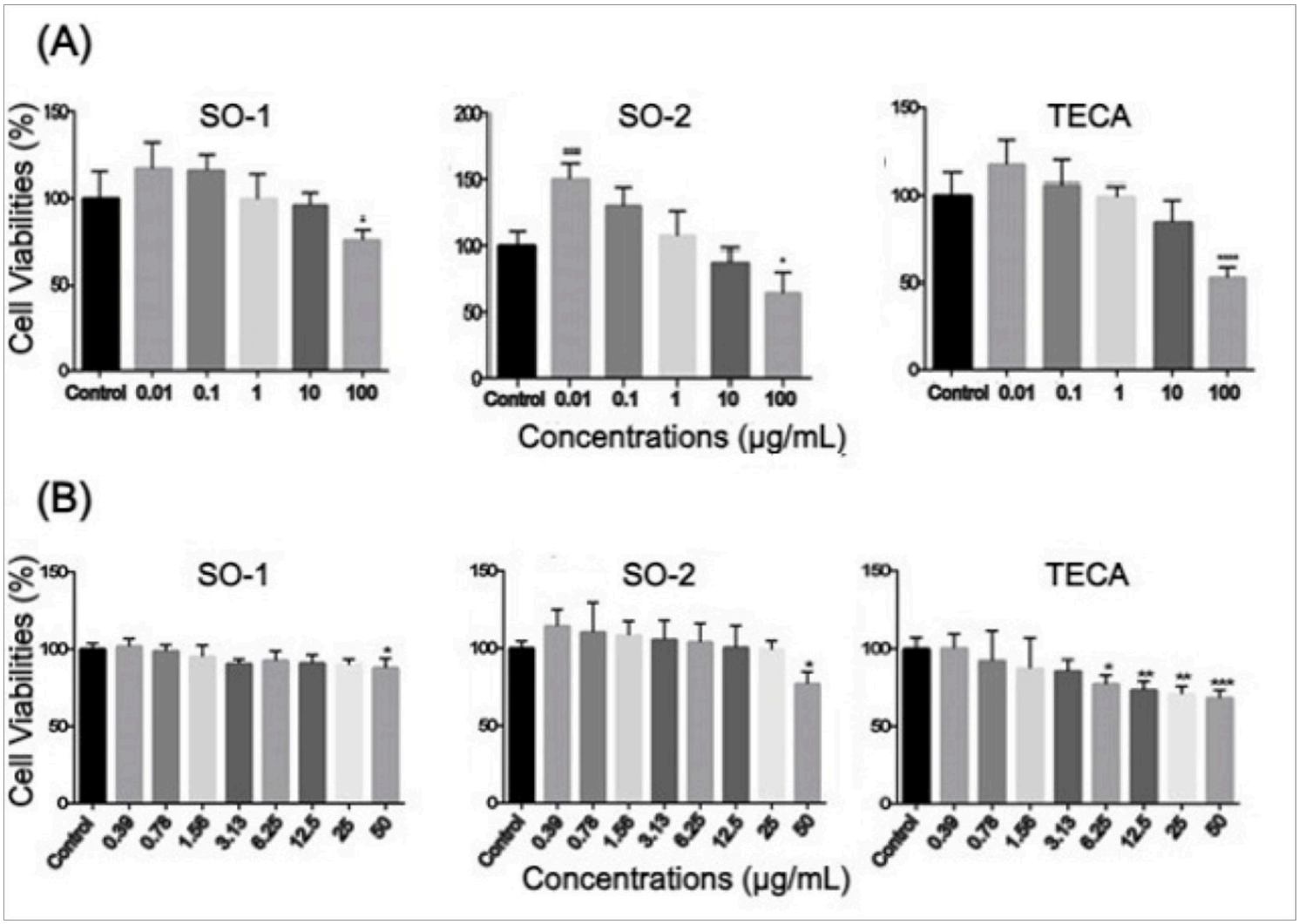

Figure 2

MTT cell viability result graphs at different concentration ranges: (A) Concentrations of 100, 10, 1, 0.1, 0.01 µg/mL (n=6 for each case), (B) Concentrations of 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 µg/mL (n=6 for each case; *p<0.05, **p<0.01, ***p<0.005, statistical significance versus control).

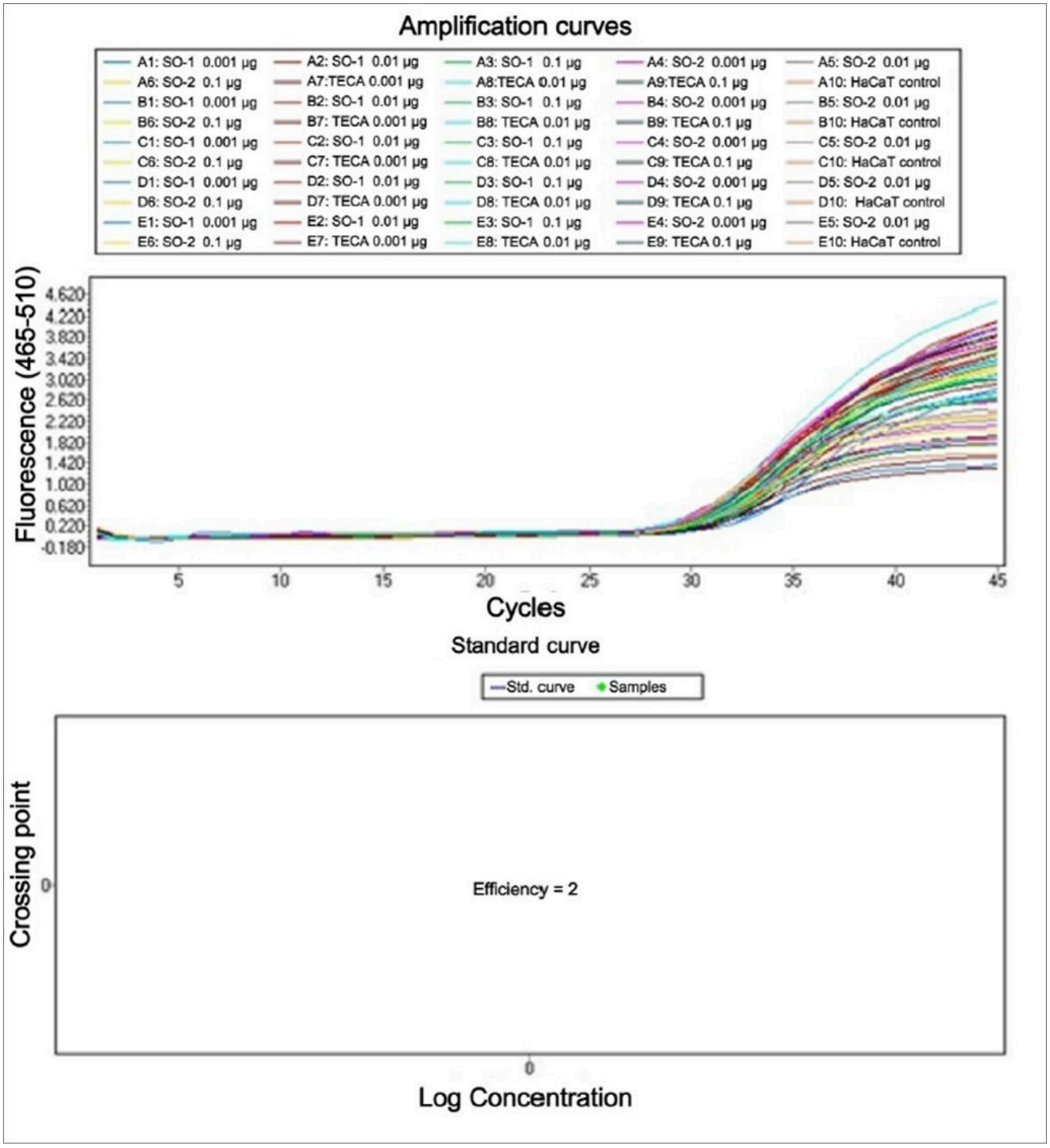

Figure 3

Amplification curve of COL1A1 gene in HaCaT keratinocyte cells incubated with SO-1, SO-2 and TEKA at concentrations of 0.001, 0.01, 0.1 µg/mL for 24 hours.

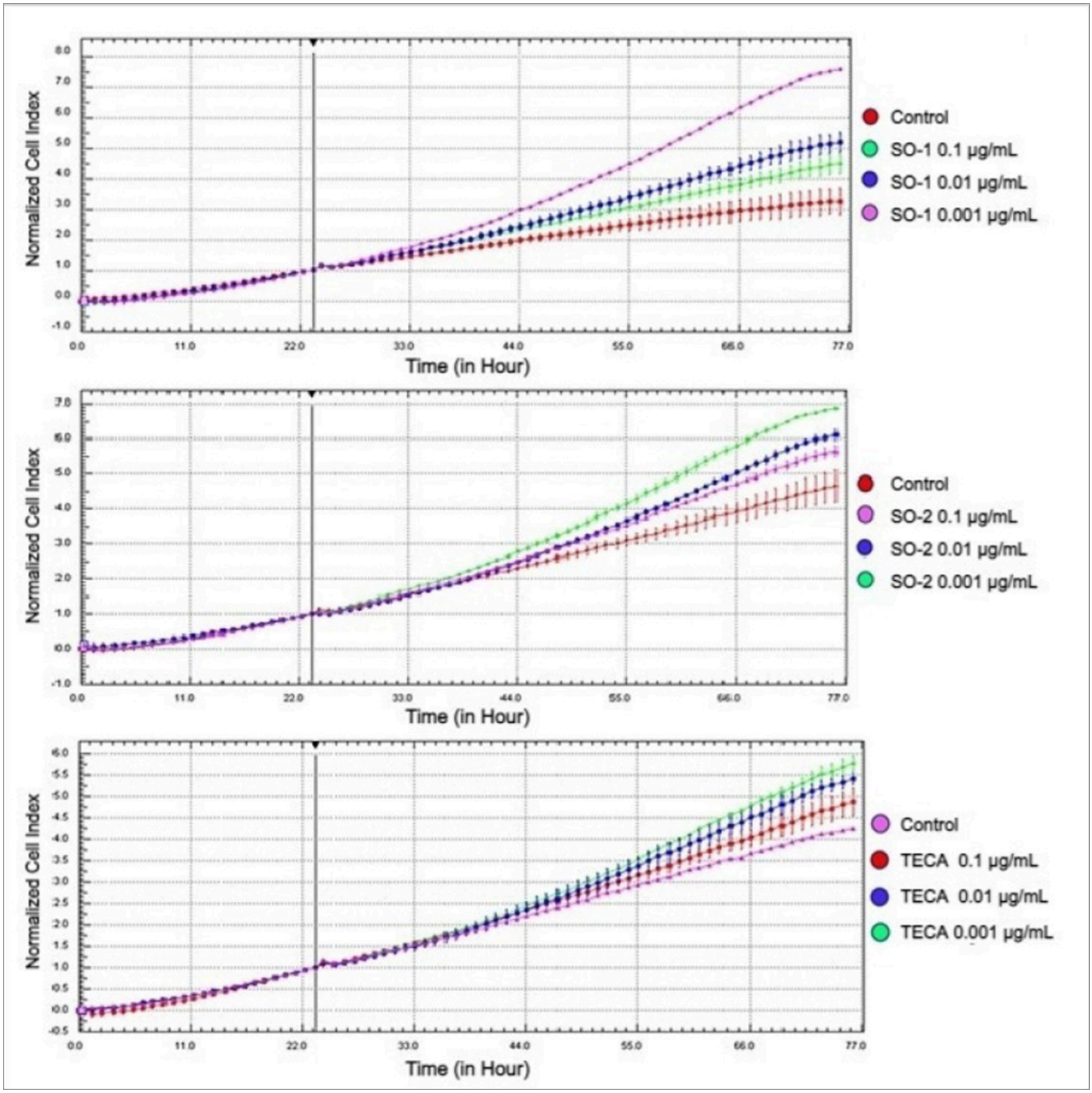

Figure 4

Results of RTCA-DP cell proliferation assays for SO-1, SO-2 and TECA (n=6 for each case).

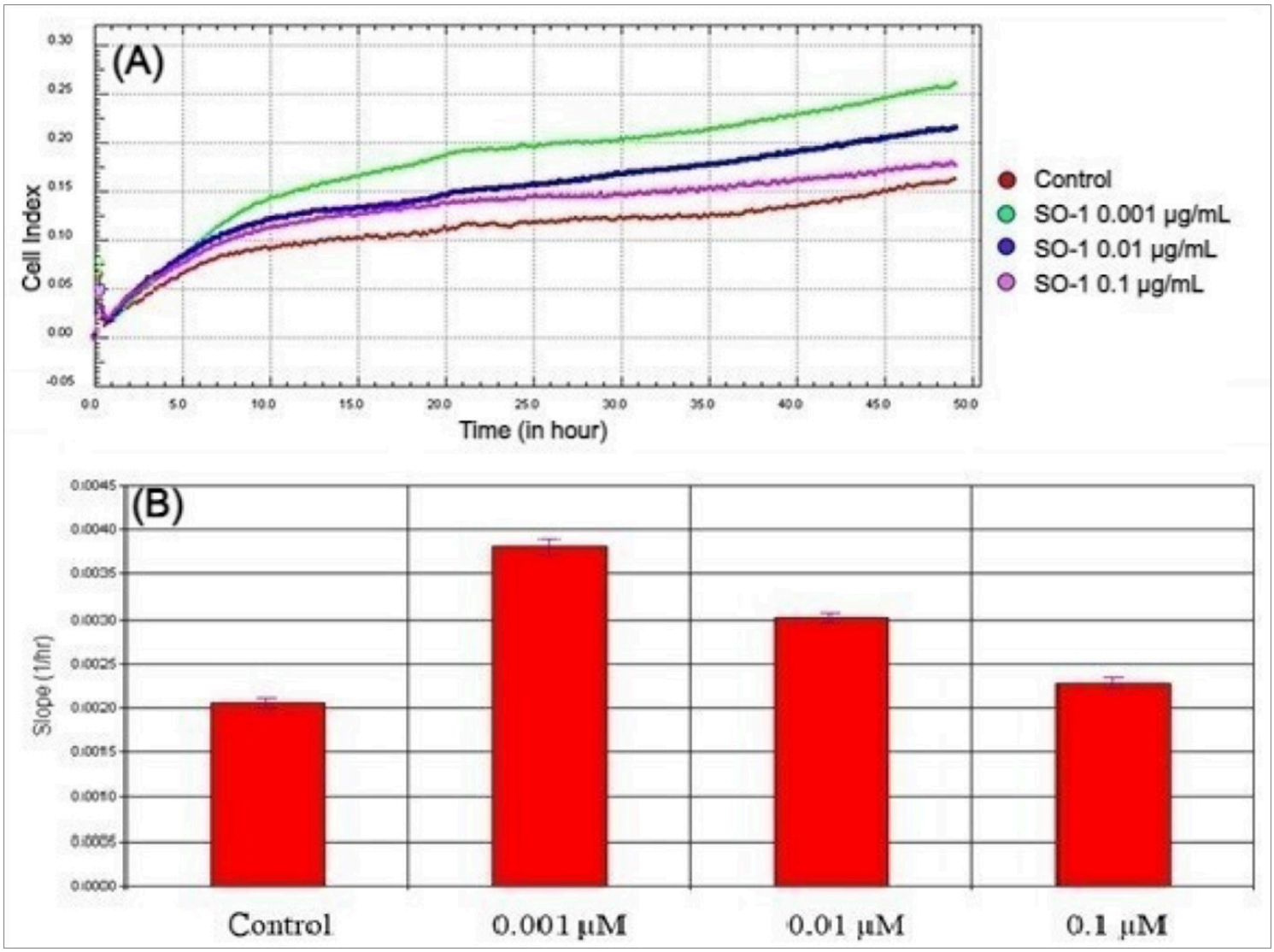

Figure 5

(A) Graph of time-dependent migration effect of sweetgum oil (SO-1) concentrations on HaCaT keratinocyte cells in RTCA-DP (n=6); (B) Slope values of SO-1 concentrations in HaCaT keratinocyte cells in the RTCA-DP system after 72 hours (n=6; mean ± standard deviation)

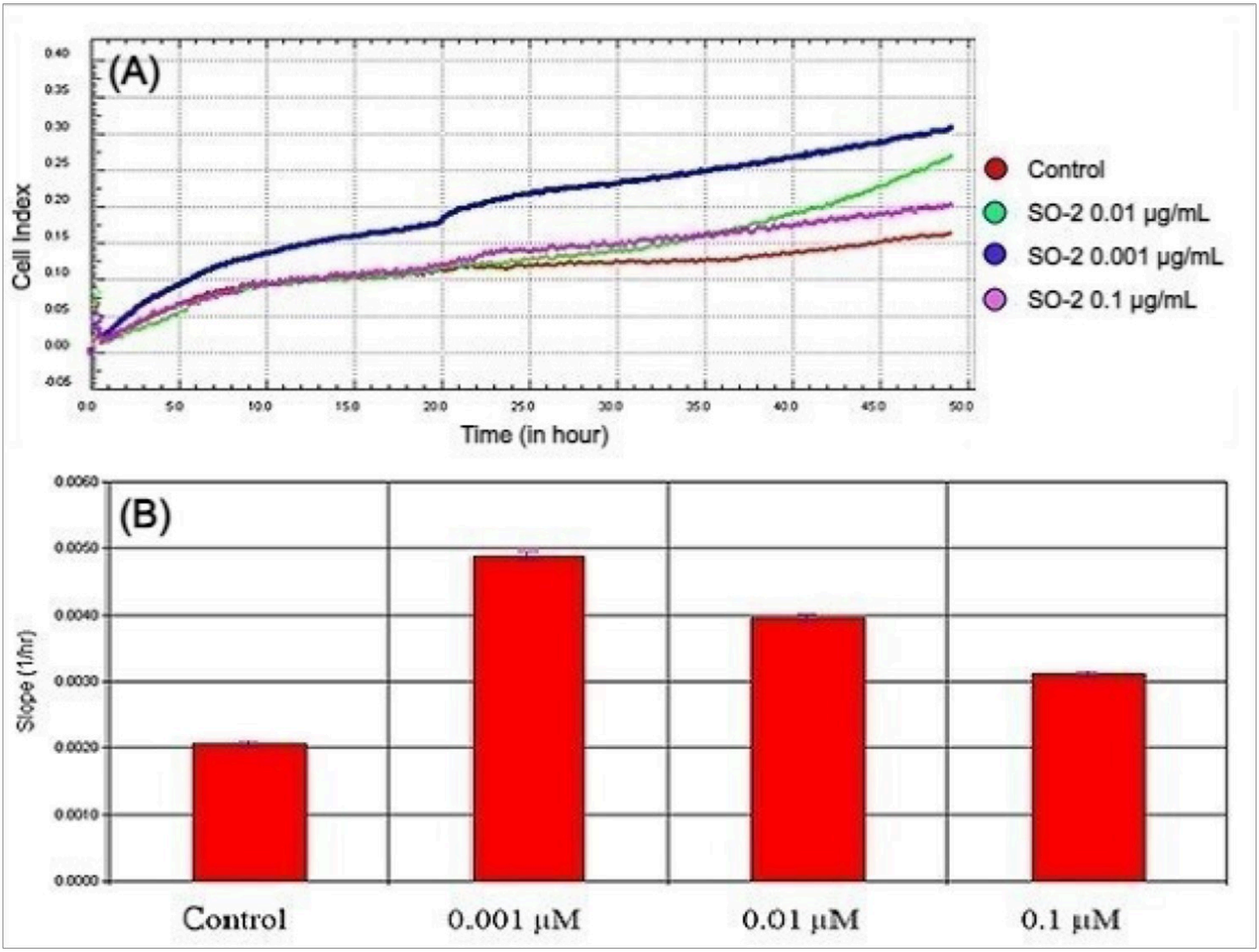

Figure 6

(A) Graph of time-dependent migration effect of sweetgum oil (SO-2) concentrations on HaCaT keratinocyte cells in RTCA-DP (n=6); (B) Slope values of SO-2 concentrations in HaCaT keratinocyte cells in the RTCA-DP system after 72 hours (n=6; mean ± standard deviation)

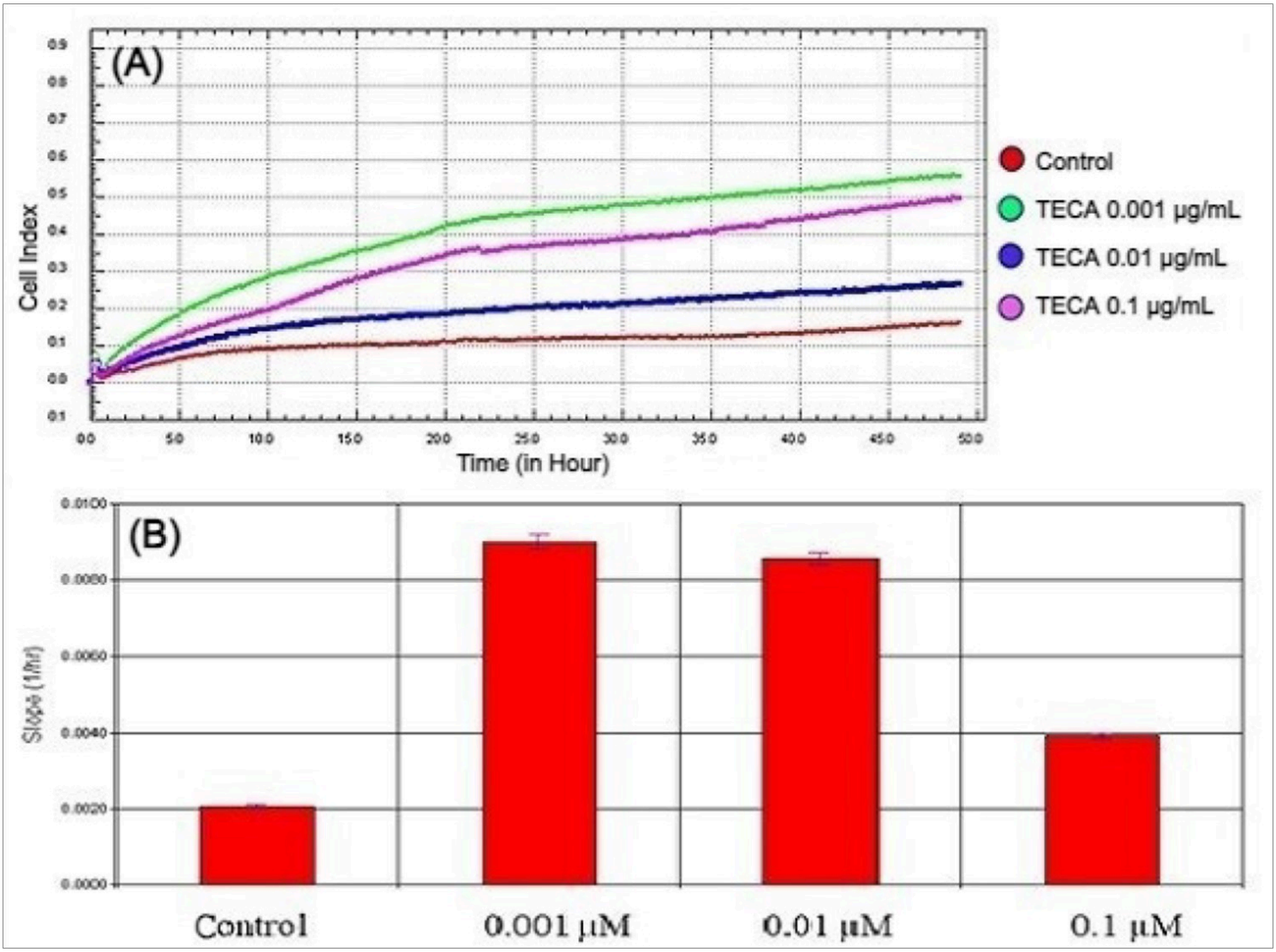

Figure 7

(A) Graph of time-dependent migration effect of TECA concentrations on HaCaT keratinocyte cells in RTCA-DP (n=6); (B) Slope values of TECA concentrations in HaCaT keratinocyte cells in the RTCA-DP system after 72 hours (n=6; mean ± standard deviation)

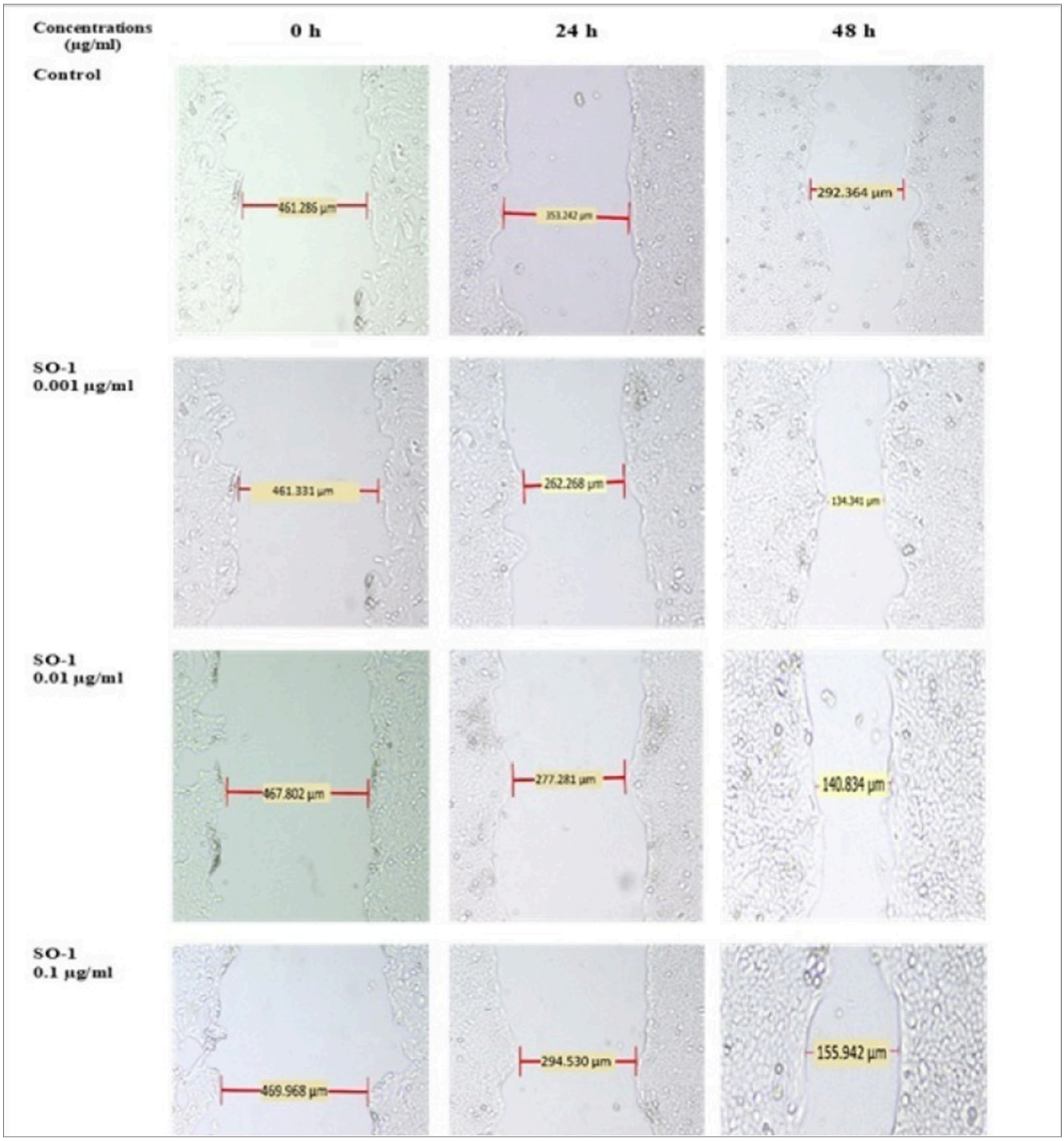

Figure 8

Photomicrographs of scratch assay 24h and 48h after the application of different concentrations of SO-1.

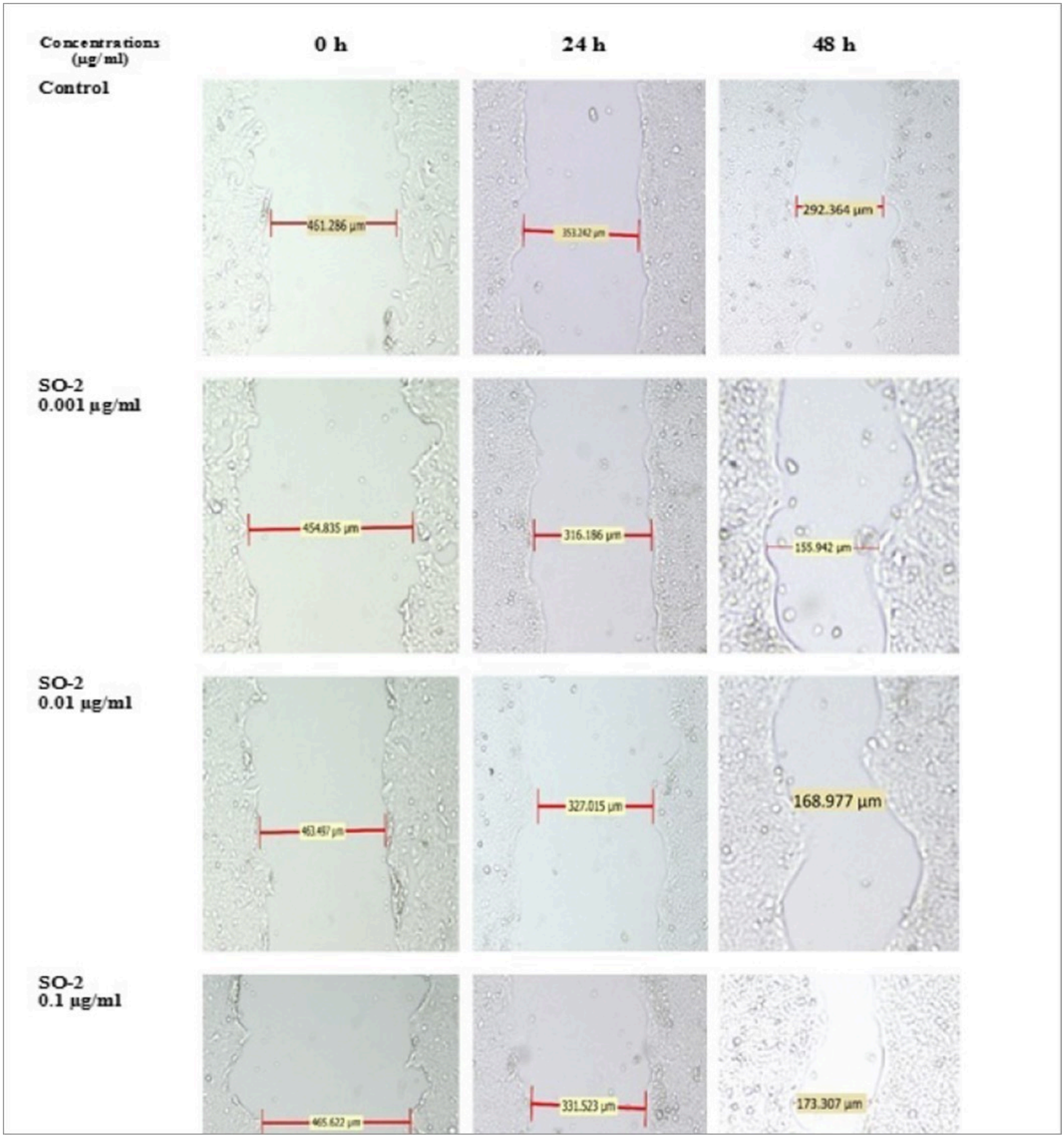

Figure 9

Photomicrographs of scratch assay 24h and 48h after the application of different concentrations of SO-2.

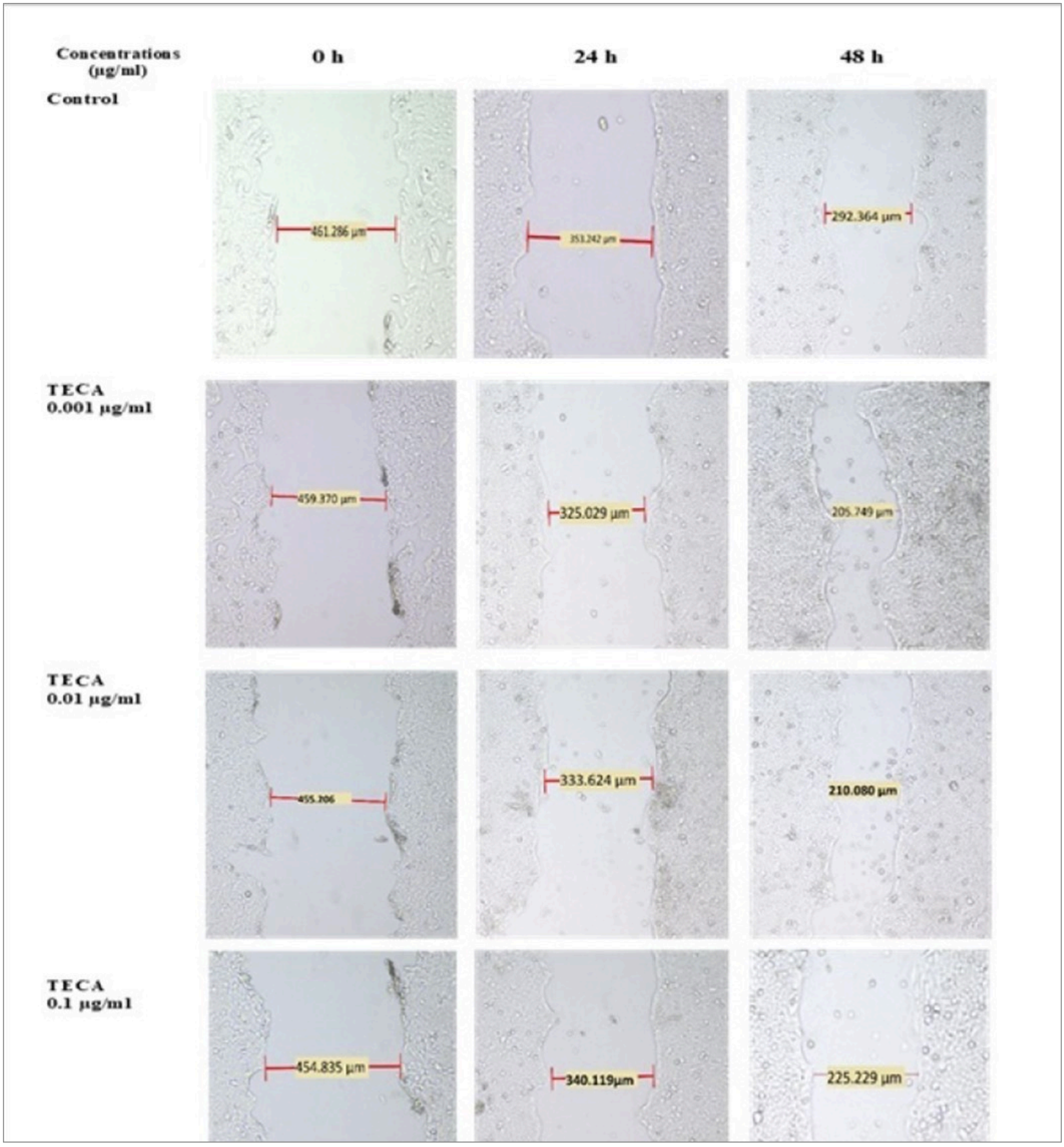

Figure 10

Photomicrographs of scratch assay 24h and 48h after the application of different concentrations of TECA.