Araştırma Makalesi – Original Article

# *In vitro* Investigation of Wound Healing, Cytotoxic, Antioxidant Properties of Shed Snake Skin (*Dolichophis caspius)* Aqueous Extract*

## Dökülmüş Yılan Derisi (*Dolichophis caspius*) Sulu Ekstresinin Yara İyileştirici, Sitotoksik ve Antioksidan Özelliklerinin *In Vitro* Araştırılması

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**ABSTRACT**

**Purpose:** As cold-blooded reptiles, snakes seasonally shed dead skin cells due to thickening of the skin and removing harmful parasites during growth. *Dolichophis caspius* is a popularly known snake in Türkiye that sheds its skin at certain times of the year. The focus of this study was to determine the total phenolic and total flavonoid contents, as well as the antioxidant activity, of the aqueous extracts of *D. caspius* shed skin and to investigate the effects of these extracts on cytotoxicity and wound healing.

**Method:** The shed snake skins were extracted using distilled water. Then, the total phenolic contents, the total flavonoid contents, and the antioxidant activity were evaluated through the Folin-Ciocalteu method, the aluminum-chloride colorimetric assay, and the DPPH method, respectively. The cytotoxic activity was determined by MTT assay and the effect on *in vitro* wound healing was determined by scratch assay.

**Findings:** Total phenolic content, total flavonoid content, and antioxidant activity of the extract were calculated as 681±46 µg GAE/g, 442.35±13 µg CE/g, and 13.06%, respectively. $IC_{50}$ values were determined as 2972±179 µg/mL and 2657±128 µg/mL for dermal fibroblast cells (BJ cell line) and melanoma cells (SK-MEL-30 cell line) in 72 hours. In comparison to the control group, it was found that the extracts at the studied doses do not have effective wound closure rates in BJ cells.

**Conclusion:** It has been determined that shed snake skins will have promising results in cytotoxicity and wound healing studies thanks to their potentially active biomolecules**.**

**Keywords:** *Dolichophis caspius,* Shed snake skin, Wound healing, Cytotoxicity, Antioxidant activity

**ÖZ**

**Amaç:** Soğukkanlı sürüngenler olan yılanlar, büyüme sırasında derilerinin kalınlaşması ve deri üzerindeki zararlı parazitlerin uzaklaşması amacıyla mevsimsel olarak ölü deri hücrelerini dökerler. Türkiye'de popüler olarak bilinen bir yılan olan *Dolichophis caspius* yılın belirli zamanlarında deri dökmektedir. Bu çalışmada, dökülmüş *D. caspius* derisinin sulu ekstraktlarının toplam fenolik ve toplam flavonoid içerikleri ile antioksidan aktivitesinin belirlenmesi ve bu ekstraktların sitotoksisite ve yara iyileşmesi üzerindeki etkilerinin belirlenmesi amaçlanmıştır.

**Yöntem:** Dökülmüş yılan derileri distile su kullanılarak ekstrakte edilmiştir. Toplam fenolik içeriği, toplam flavonoid içeriği ve antioksidan aktivitesi sırasıyla Folin-Ciocalteu yöntemi, alüminyum-klorür kolorimetrik deneyi ve DPPH yöntemi ile değerlendirilmiştir. Sitotoksik aktivite MTT testi ile, *in vitro* yara iyileşmesi üzerindeki etki çizik testi ile belirlenmiştir.

**Bulgular:** Ekstraktın toplam fenolik içeriği, toplam flavonoid içeriği ve antioksidan aktivitesi sırasıyla 681±46 µg GAE/g, 442.35±13 µg CE/g ve %13.06 olarak hesaplanmıştır. $IC_{50}$ değerleri BJ dermal fibroblast hücreleri ve SK-MEL-30 melanoma hücreleri için 72. saatte sırasıyla 2972±179 µg/mL ve 2657±128 µg/mL olarak bulunmuştur. Çalışılan dozlarda BJ hücrelerindeki yara kapanma oranları kontrol grubu ile karşılaştırıldığında ekstraktların yara iyileşmesini hızlandırıcı bir etkiye sahip olmadığı görülmektedir.

**Sonuç:** Dökülmüş yılan derilerinin barındırdıkları potansiyel aktif biyomoleküller sayesinde sitotoksisite ve yara iyileştirme çalışmalarında umut verici sonuçlar doğuracağı tespit edilmiştir.

**Anahtar Kelimeler:** *Dolichophis caspius,* Dökülmüş yılan derisi, Yara iyileşme, Sitotoksisite, Antioksidan aktivite

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## Introduction

Dead skin cells are shed by many living beings during their growth and development processes. In humans, this process occurs in pieces, whereas reptiles such as snakes shed their skin in a single piece.[1] The outer layer of snake skin is rigid and durable, while the inner layer is flexible.[2] The multi-layered membrane formed by snake skin is composed of bioactive keratin and lipids, providing natural mechanical support.[3] These cold-blooded, reptilian creatures shed their skin at certain times of the year. As they grow and the skin thickens, it becomes too small for them to manage. Furthermore, because they have eye caps instead of eyelids, they experience vision and mobility problems due to their thick skin, and removing harmful parasites is also necessary. This skin sheath of dead cells, which encases their bodies as a single layer, begins to compress them. They then slide through the skin by rubbing against hard surfaces, such as rocks.[1] On the other hand, snakes excrete certain secretions when searching for a mate or food. These creatures, trapped within their skin sheaths during molting, also release numerous secretions and pheromones when stressed. These skin sheaths combine with these skin secretions.[4]

The waste skin separated from the snake's body can be consumed by humans orally.[5,6] This consumption dates back to ancient Chinese medicine.[6] It is considered a treatment for many diseases such as skin disorders, hypertension, open wounds, glaucoma, hemorrhoids, cancer, and epilepsy in different societies.[3,6] The safety profiles of these naturally occurring substances used in alternative medicine vary depending on their use; and the potential toxicity of these products can differ significantly depending on the dose of use.[3,5,6] According to a case report, a 36-year-old male patient was admitted to Muğla Sıtkı Koçman Training and Research Hospital in 2020. The patient complained of itching, urticaria, and mild shortness of breath. Despite treatment, the urticaria did not disappear. As a result, the patient stated that he purchased and consumed snake skin with his meals for 10 days, as it was recommended as a suitable treatment for warts.[6] In the Eastern Anatolia region, shed snake skin is ground into powder, added to soups, or mixed with honey and fed to patients having warts. Another alternative is to mix unsalted butter and apply it externally to the wart. Some other method is to burn shed snake skins and smoke the warty areas.[7]

*Dolichophis caspius*, commonly known as Bozyörük, is a widespread snake species in southeastern Europe as well as in Türkiye. It typically measures between 140-180 cm and is not poisonous.[8] It is active throughout the day and is also very important in controlling agricultural pests and insects. This species, which is active during the day, usually sheds its skin in spring and summer. Depending on the growth rate and age of the snake, this shedding can occur more than once a year.[9]

Free radicals cause many diseases in humans, such as atherosclerosis, cancer, arthritis, and central nervous system damage. Due to the depletion of natural antioxidants in the immune system, it may be necessary to consume antioxidants as free radical scavengers. Natural compounds, such as phenolic compounds, can be effective in antioxidant activity. Flavonoids are a group of polyphenolic compounds with properties such as free radical scavenging and anti-inflammatory effects.[10] Tragically, cancer remains the leading cause of mortality globally. For a long time, commonly used approaches to cancer treatment such as radiation therapy and chemotherapy have had significant limitations. In this context, it is important to discover new biologically active compounds and determine their cytotoxicity on cells through *in vitro* methods.[11]

Regeneration and tissue repair are cellular and molecular processes that occur to repair damaged tissue.[12] Wound healing, a normal process in the human biological system, consists of four main programmed steps: hemostasis, inflammation, proliferation, and remodeling. The first step of hemostasis is the formation of a fibrin clot and vascular constriction following injury. Wounded tissue secretes growth factors such as transforming growth factor-beta (TGF-β), epidermal growth factor (EGF), and platelet-derived growth factor (PDGF). For the wound to heal properly, these four steps must occur in the correct order and at the

appropriate time. In the process of wound healing, macrophages play a crucial role. They are also responsible for stimulating and clearing apoptotic cells. In this way, they prevent inflammation. T-lymphocytes, macrophages, and many other factors influence these processes. Age, stress, diabetes, medications, smoking, and diet also play an important role.[13-15] Wound healing is essential for the skin to regain its former barrier function. During wound healing, cellular proliferation and migration from the wound edges facilitate re-epithelialization, a crucial step that ensures the restoration of epidermal integrity and wound closure. Wound healing analyses are used to study wound repair mechanisms and investigate treatments for healing. Many healing models have been successfully applied in recent years.[16] The scratch assay is one of these wound healing models. It is a very low-cost but effective technique for monitoring the rate of wound healing. Essentially, it involves creating a scratch in the cell layer and examining the cell migration that occurs to close this scratch.[17]

In this study, the naturally shed skin of *D. caspius*, obtained from Bursa, was used. The aim of the study was to investigate the total phenolic and total flavonoid contents, antioxidant activity, cytotoxic effect, and wound healing property of aqueous extracts of shed Bozyörük snake skin. The ground shed of snake skins was extracted using distilled water with the aid of ultrasound to increase the yield. Cytotoxic activities on BJ and SK-MEL-30 cells were determined by the MTT assay, and the effect on wound healing was assessed by the *in vitro* scratch assay. In this study, the cytotoxic activity and wound healing potential of the aqueous extracts of Bozyörük snake skin were investigated for the first time.

## Material and Methods

### Procurement and grinding of shed snake skin

The shed snake skin of the *Dolichophis caspius* species was used in the study. This study did not involve any harm or physical restriction to live snakes at any stage of the process. The snake skins were collected after the natural shedding process occurred. These skins were obtained from the Bursa region. Afterwards, they were identified by Prof. Dr. Hikmet Sami Yıldırımlar. Since the collected skins were obtained dry, they were ground into powder in a mortar and pestle without any further processing. They were stored in the refrigerator (-20°C) until further processing steps were performed.

### Aqueous extraction of shed snake skin

The obtained ground shed snake skins were extracted in distilled water by ultrasound-assisted extraction for 40 minutes. Then, precipitation was performed by centrifugation at 4000 rpm for 15 minutes. The upper part was taken to use in experimental processes.

### Total phenolic contents of aqueous extract of shed snake skin

For the determination of the total phenolic content in the aqueous extracts obtained from shed snake skin, 7.5 mL of distilled water was added to a 100 mL flask. Then, 1 mL of the extract and 5 mL of Folin-Ciocalteu was added and shaken. After three minutes, 10 mL of saturated sodium carbonate ($Na_2CO_3$) was added, and the solution was made up to 50 mL with distilled water. The solution was kept in the dark for 100 minutes in order to prevent it from being affected by light. Absorbance was then measured at 720 nm via a spectrometer. Total phenolic content was expressed in terms of gallic acid equivalent (µg GAE/g). The assay was performed in triplicate. [18]

### Total flavonoid contents of aqueous extract of shed snake skin

The aluminum-chloride colorimetric assay, as reported by Molan and Mahdy, was used to determine total flavonoid content. 25 µL of the extract was mixed with 100 µL distilled water and 7 µL 5% $NaNO_2$ in 96-well microplates. The mixture was then kept at room temperature for 5 minutes. At the end of the time, 7 µL of

10% $AlCl_3$ was added to the mixture and incubated for 5 minutes. Then, 50 µL of 1M NaOH and 60 µL of distilled water were added to the mixture. Absorbance was measured at 490 nm in a microplate reader. Total flavonoid content was expressed in terms of catechin equivalent (µg CE/g). The assay was performed in triplicate. [19]

**Antioxidant activity of aqueous extract of shed snake skin**

Antioxidant activity was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. Initially, 150 µL of methanol and 3.8 mL of DPPH were sequentially added to 50 µL of the extract in test tubes. After vortexing, the tubes were incubated in the dark for 30 minutes. Finally, absorbance values were read in a spectrophotometer at 515 nm. For comparison, BHA (Butylated hydroxyanisole) was used. The results were expressed as % inhibition using the following formula[20]: % inhibition=$(A_{blank}-A_{sample})/A_{blank}*100$

**Determination of the cytotoxic activity of shed snake skin aqueous extract**

The cytotoxic effects of aqueous extracts obtained from shed snake skin of *D. caspius* on dermal fibroblast cells (BJ cells) and melanoma cells (SK-MEL-30 cells) were determined in a 96-well plate by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Thus, the cytotoxic activities of shed snake skin extracts were examined on both healthy skin cells and cancerous melanoma cells. Complete medium contained 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% L-glutamine, and DMEM medium. Cells cultured in the flask were removed from the surface with trypsin, and cell counting was performed under an inverted microscope using trypan blue stain and a hemocytometer. 100 µl of $10^4$ cells were seeded in each well of 96-well plates and incubated in an incubator at 37°C and 5% $CO_2$ for 24 hours. Seven different concentrations of the extract, ranging from 156.25 to 10000 µg/mL, were prepared and applied to the cells at the end of incubation. Control wells were treated with 0.01% substance solvent. Cells were incubated for 24, 48, and 72 hours. At the end of the incubation period, 10 µl of 5 mg/mL MTT/PBS reagent was added to the cells and incubated in an incubator at 37°C and 5% $CO_2$ for 3.5 hours. The formazan crystals formed on the live cells at the bottom of the wells were dissolved by adding 100 µl DMSO. The absorbance values of the wells were measured with a spectrophotometer at a wavelength of 570 nm.[21] At the end of the MTT assay, the cytotoxicity was determined based on the absorbance values read at a 570 nm wavelength. % viability values were calculated by proportioning to the control. Cytotoxicity levels were calculated with the following formula: Cytotoxicity level = (absorbance of test well / absorbance of control well) x 100

The concentration showing a 50% cytotoxic effect compared to the control was considered as the cytotoxic dose ($IC_{50}$). Each dose was performed in triplicate, and the GraphPad Prism program was used to create dose-% viability graphs. Data obtained from cytotoxicity assays are expressed as the mean (±) and standart deviation from three independent values. Percentage viability values of determined concentrations statistically compared to the control group within the same time interval, and $IC_{50}$ values were statistically compared at different time intervals within the same cell line. A one-way analysis of variance (ANOVA) was performed to determine differences between groups. Statistical analyses were performed using GraphPad Prism (Version 8.4.2, GraphPad Software, San Diego, CA, USA) software. A $p<0.05$ value was considered statistically significant.

**Determination of *in vitro* wound healing property of aqueous extract of shed snake skin**

*In vitro* wound healing property of aqueous extracts from shed snake skin of *D. caspius* species on BJ dermal fibroblast cells was determined by scratch assay. The scratch assay is a commonly used method to measure cell migration parameters. A thin wound was created by scratching the confluent cells with a pipette tip, and the migration of cells at the wound edge into this gap, as well as the rate of wound closure, were examined.[22] After cell counting, 6-well plates were seeded with $3x10^5$ cells/well at the initial concentration. Cells were

then incubated in an incubator at 37°C and 5% $CO_2$ until they reached 100% confluence. Using a sterile micropipette tip, a linear line was drawn and wounds were created in the fibroblast cells. After washing, aqueous extracts at varying concentrations were applied to the cells. The scratches were imaged under an inverted microscope at certain times until they were closed. The images obtained were recorded and analyzed. Differences between groups were determined using one-way ANOVA compared to the control group. Statistical analyses were performed using GraphPad Prism (Version 8.4.2, GraphPad Software, San Diego, CA, USA) software. A $p<0.05$ was considered statistically significant.

## Results

### Total phenolic content, total flavonoid content, and antioxidant activity results

Total phenolic content and total flavonoid content were determined according to the Folin-Ciocalteu method and the aluminum-chloride colorimetric assay, respectively. In this regard, total phenolic content of shed snake skin aqueous extract was 681±46 µg GAE/g, while total flavonoid content was 442.35±13 µg CE/g. The antioxidant activity in terms of % inhibition of the aqueous extract was detected as 13.06%, while BHA as control was calculated as 97.09% according to DPPH method. Although the extract exhibited moderate total phenolic content and total flavonoid content values, its DPPH scavenging activity was lower than that of the synthetic antioxidant BHA. This inconsistency may be due to the presence of bound or inactive phenolic compounds in the extract. This reduces the efficiency of radical scavenging despite the high total content.

### Cytotoxicity activity results

The cytotoxic activity of aqueous extracts obtained from shed snake skin on BJ and SK-MEL-30 cells was investigated by MTT assay. Cells were treated with doses of 156.25, 312.5, 625, 1250, 1250, 2500, 5000, and 10000 µg/mL at three different timepoint (24h, 48h, and 72h). $IC_{50}$ values calculated as a result of MTT assay are given in ***Table 1***.

**Table 1.** $IC_{50}$ values for dermal fibroblast (BJ) and melanoma (SK-MEL-30) cells

| Time | Dermal fibroblast (BJ) cells | Melanoma (SK-MEL-30) cells |
|---|---|---|
| **24 hours** | >10000 µg/mL | 5511±263 µg/mL |
| **48 hours** | 5527±432 µg/mL | 2888±118 µg/mL |
| **72 hours** | 2972±179 µg/mL | 2657±128 µg/mL |

Cytotoxicity assays performed on BJ cells revealed a time-dependent decrease in $IC_{50}$ values. At 24h, none of the tested concentrations reached the $IC_{50}$ threshold, whereas at 48h the $IC_{50}$ was determined to be 5527±432 µg/mL. A further reduction was observed at 72h, where the $IC_{50}$ value decreased to 2972±179 µg/mL. Statistical comparison of $IC_{50}$ values across different time points demonstrated significant differences between 24h and 48h, between 48h and 72h, as well as between 24h and 72h ($p < 0.01$), indicating a progressive enhancement of cytotoxic activity in a time-dependent manner. In SK-MEL-30 melanoma cells, $IC_{50}$ values demonstrated a significant time-dependent reduction. At 24h, the $IC_{50}$ was calculated as 5511±263 µg/mL, whereas it markedly decreased to 2888±118 µg/mL at 48h, and further to 2657±128 µg/mL at 72h. Statistical analysis revealed a highly significant difference between 24h and 48h ($p < 0.001$), as well as between 48h and 72h ($p < 0.001$). Moreover, the most pronounced difference was observed between 24h and 72h ($p < 0.0001$). These findings indicate that the cytotoxic effect of the treatment on SK-MEL-30 cells intensifies progressively with exposure time, particularly within the first 48h, and continues to increase significantly up to 72h.

Graphs showing cell % viability values after 24, 48, and 72 hours of treatment with the extracts are shown in ***Figure 1*** (for BJ cells) and ***Figure 2*** (for SK-MEL-30 cells). Shed snake skin aqueous extract reduced cell viability

in a dose- and time-dependent manner for both cell lines. The extracts were found to reduce cell viability more in the melanoma cell line. The cytotoxic effect in BJ cells increased in a dose-dependent and time-dependent manner. According to the 24h viability results, viability remained around 90% at low concentrations (156.25 µg/mL), and a significant decrease ($p < 0.05$) was detected compared to the control group. In all other concentrations at 24h, although cell viability did not decrease below 50%, a statistically significant decrease was determined compared to the control group ($p < 0.01$). In the 48-hour viability results, the significant decrease ($p < 0.01$) observed between concentrations of 156.25-5000 µg/mL, increased at a concentration of 10000 µg/mL, where cell viability fell below 50% ($p < 0.001$). The most significant results in BJ cells were obtained after 72 hours. Viability decreased to 60% at concentrations between 156.25-2500 µg/mL ($p < 0.01$), to around 45% at 5000 µg/mL ($p < 0.001$) and around 25% at 10000 µg/mL ($p < 0.001$). This demonstrates that the cytotoxic effect of the extract is both dose- and time-dependent.

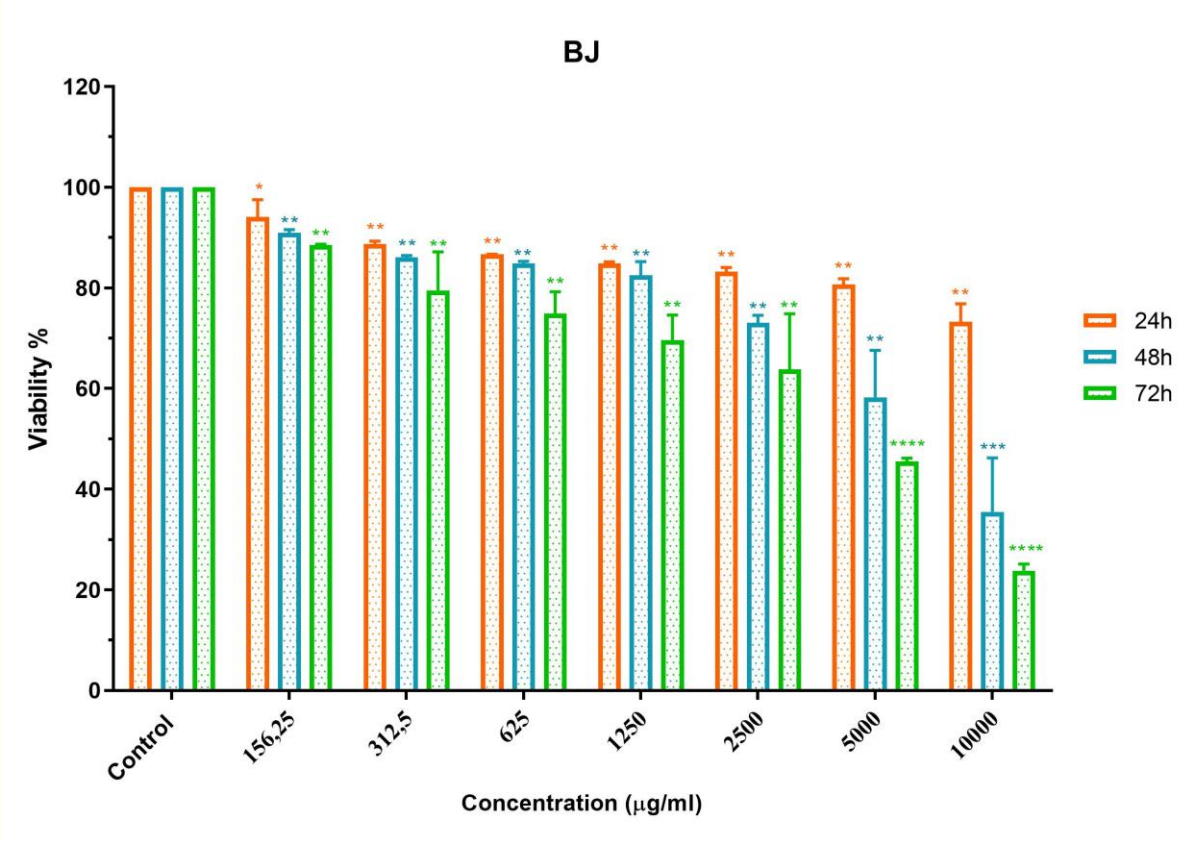


**Figure 1.** Cell viability graph determined by MTT assay after treatment of snake skin aqueous extracts with BJ cells (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$)

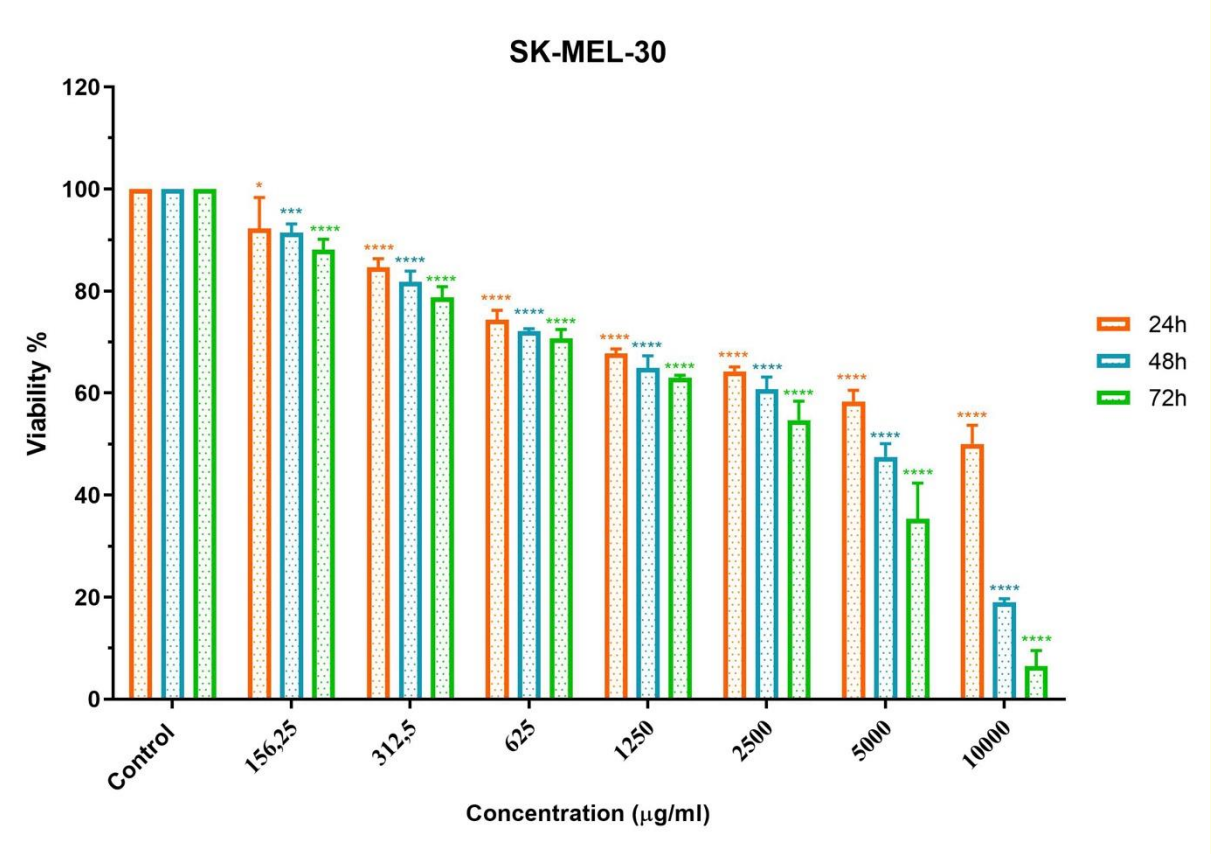


**Figure 2.** Cell viability graph determined by MTT assay after treatment of snake skin aqueous extracts with SK-MEL-30 cells (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$)

The extract exhibited a more significant cytotoxic effect on SK-MEL-30 cells compared to BJ cells. A decrease in cell viability were observed at 48 and especially 72h. Therefore, the extract affected SK-MEL-30 cells in a dose-dependent and time-dependent manner. At a concentration of 156.25 µg/mL, cell viability was found to be 95% ($p < 0.05$) at 24h, 97% ($p < 0.001$) at 48h, and 87% ($p < 0.0001$) at 72h. A statistically significant decrease was observed at all other time points and all doses ($p < 0.0001$). Notably, viability values at 72h showed a significant decrease to 35% at 5000 µg/mL and 6% at 10000 µg/mL.

**Wound healing test results**

The wound healing potential of aqueous extracts derived from shed snake skin was evaluated on BJ dermal fibroblast cells using a scratch assay. The images obtained are given in ***Figure 3***. Aqueous extracts of shed snake skin at doses of 25 µg/mL and 50 µg/mL were applied to the cells. There was also a control group. Comparison of wound closure rates with the control group revealed that the extracts did not exhibit a significant accelerating effect on wound healing. In the next set of experiments, the dose can be lowered, and new experiments can be conducted.

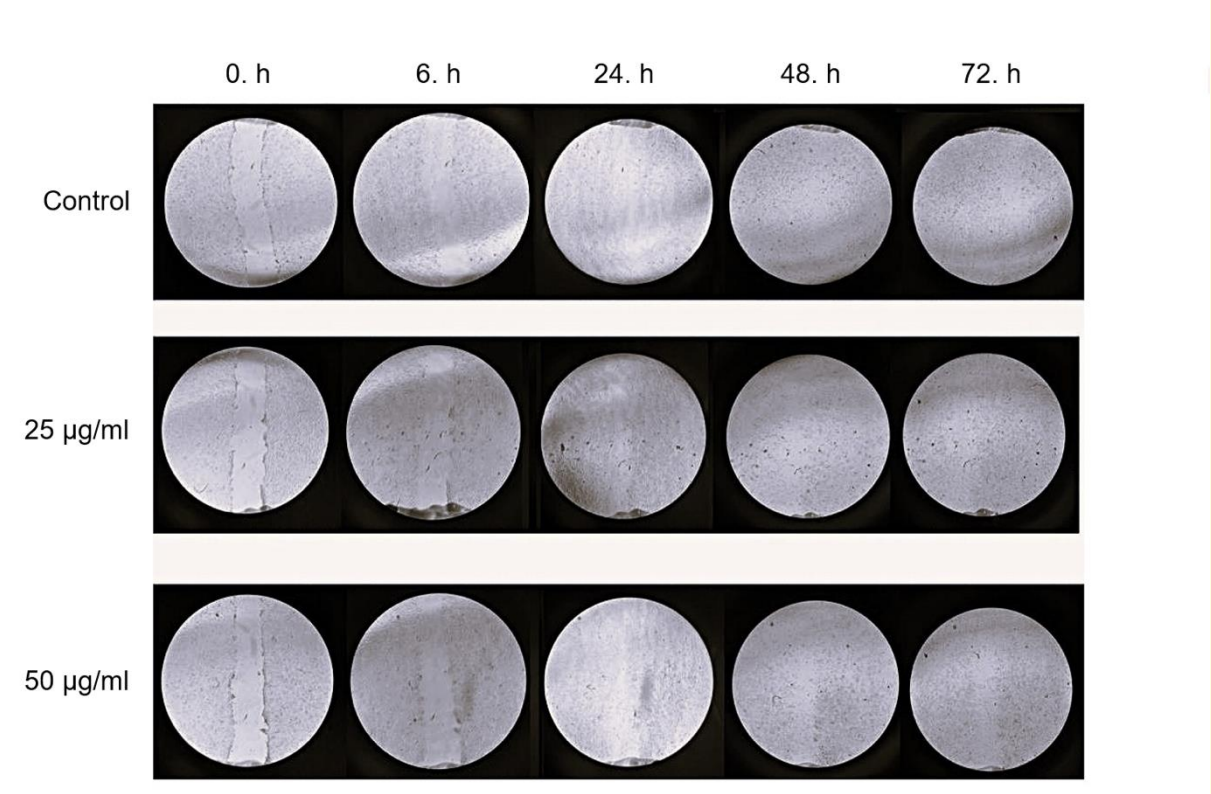


**Figure 3.** Scratch assay results

The effect of snake skin extracts (25 and 50 µg/mL) on wound closure rate was evaluated over a 72h period at specific time intervals. At 6th hours, wound closure in the control group reached 12% compared to the initial (0h) measurement, whereas 25 µg/mL and 50 µg/mL extracts resulted in 11% and 9% closure, respectively. At this time point, no statistically significant difference was observed between the treatment groups and the control ($p > 0.05$). Wound closure rates at 24h were calculated as 88% for the control group, 86% for the 25 µg/mL extract treated group, and 83% for the 50 µg/mL extract treated group, with no significant intergroup differences identified ($p > 0.05$). By 48 hours, the cells had reached confluence.

**Discussion**

Substantial financial resources are allocated annually for the discovery and development of novel therapeutic agents to address a broad and diverse range of diseases. Of the 252 essential chemicals listed by the World Health Organization for pharmaceutical use, 11% are of plant origin and 8.7% of animal origin. For this reason, studies aimed at identifying therapeutic components using traditional methods, particularly in cancer, have gained significant importance in recent years.[7] Research to date shows that snake skin is not just biological waste, but a valuable medicinal resource containing chemical compounds with numerous beneficial biological activities.[3]

Snakes shed their skin at certain times of the year due to the growth of their bodies. Traditionally, it is said that this shed skin is consumed orally by humans without dosage control for the curing of such illnesses as warts and cancer. It is essential to determine the content of this biological material and contribute to scientific research. Considering the studies, Sakalli and Çömelekoğlu analyzed the elemental contents of shed skins from *Dolichophis jugularis* and *Malpolon insignitus* species collected from Kahramanmaraş, Türkiye. They found a total of 21 macro- and microelements, including magnesium, titanium, manganese, iron, and

nickel. They stated that these results have the potential to synthesize new drugs for the treatment of warts. The authors state that this study is the first to perform elemental analysis on shed snake skin belonging to the species *D. jugularis and M. insignitus.*[7] In a study, Andonov et al. used gas chromatography to examine the chemical differences between male and female individuals, live and dead skin cells, and pre- and post-reproductive periods of *Vipera ammodytes* snake. Apolar, fat-soluble compounds secreted for different purposes in skin secretions were determined. 88 compounds were detected in the skin secretions of 13 different snake samples. Samples taken from snakes were soaked in n-hexane for 24 hours. Gas chromatography revealed the presence of alkane and phenol compounds, particularly 2-pentacosanone and 2-heptacosanone, in most samples.[4] Ball determined the fatty acid composition of nonpolar lipids by examining the shed skins of female and male corn snakes (*Elaphe guttata guttata*). For this purpose, hexane, dichloromethane, and methanol extractions were performed, and information was obtained regarding whether the shed snake skin based on lipids could provide sexual information related to the breeding season.[23] These studies were generally detected contents of the shed snake skin following the chromatography methods, but they were unable to measure any biological activity compared to our study. However, the primary objective of our study is to determine the amounts of phenolic (681±46 µg GAE/g) and flavonoid (442.35±13 µg CE/g) compounds using spectrophotometric methods that may play a role in antioxidant activity, cytotoxic effects, and wound healing capacity processes.

Studies on the biological activities of shed snake skins are quite limited. Sinmez and colleagues investigated the immunomodulatory effects of different *Elaphe sauromates* shed skin extracts obtained by using methanol, hexane, and chloroform on murine lymphocytes and T cells. Additionally, the protein concentration, cytotoxicity, and anticancer activities of the extracts in SK-MEL-30 (human melanoma) and L929 (mouse fibroblast) cell lines were determined using the MTT method. After 24-h incubation, $IC_{50}$ values of chloroform, methanol, and hexane extracts were calculated as 1.6 mg/mL, 1.2 mg/mL, and 0.7 mg/mL for SK-MEL-30 cells. It was found that hexane extract at low concentrations has a superior anti-proliferative effect. In our study, water, an environmentally friendly solvent, was selected, and the $IC_{50}$ values for these cells were measured as 5511±263 µg/mL based on a 24-h treatment. Researchers have suggested that the potential use of extracts *in vivo* disease models should be further investigated.[3] Mukherjee et al. reported in their study that the estrous cycle, cytokine profile, and ovary and uterus histopathology of Swiss albino mice affected specific doses of shed snake skin extract.[24] Also, the negative impact of shed snake skin (*Naja naja*) on the male reproductive system was studied *in vivo* on Swiss albino mice and shed snake skin has been highlighted as a potential treasure trove of bioactive compounds.[25] Park et al. have highlighted studies indicating that crude peptides, extracts, venom and sera isolated from other reptiles apart from snakes can successfully inhibit the proliferation of several cancer cells.[26] Turtles, lizards, and snakes, like other reptiles, are exposed to high levels of radiation or heavy metals, feed on contaminated food, live in unhealthy habitats, and yet continue to survive. Jeyamogan et al. have stated that cancer is rare in these species due to the presence of anticancer molecules or mechanisms. Here, Asian water monitor lizard, python and turtle sera on prostate cancer cells (PC3), Henrietta Lacks cervical adenocarcinoma cells (HeLa), human breast adenocarcinoma cells (MCF7), and human keratinised skin cells (HaCat). They also performed liquid chromatography-mass spectrometry for molecular characterization. The findings revealed that reptile sera, unlike bovine sera, eliminated the viability of HeLa, PC3 and MCF7 cells.[27] In a study conducted by Derakhshani et al., recombinant cytotoxin-II obtained from snake venom was investigated in terms of signaling pathways and used in the MTT assay to evaluate the viability of SK-MEL-3 and HFF-2 cells. The $IC_{50}$ value was found to be lower in melanoma cells (17.7 µg/mL) than in non-tumour cells (24.76 µg/mL).[28] Although there are currently many cancer studies conducted using different components of snakes, it is

necessary to determine the safe dosage range for the medical use of shed snake skin material, which has been studied to a limited extent.

Wound healing models are usually realized in a two-dimensional, single-layer cell format. The basic principle is to damage the combined cell layer and create a cell-free zone for the cells to repair this area. The most common is the scratch technique. In this technique, the two-dimensional cell layer is scratched with a pipette, and the healing process is observed under an inverted microscope.[16] The stages of the healing process must be well understood in order to recognize the factors that affect and complicate wound healing. Several factors influence healing, including infection, abnormal bacteria, pressure, and edema.[29] One of the main causes of non-healing wounds is an out-of-control inflammatory response. The need for materials to support the wound-healing process is increasing daily.[30] Keratin-based biomaterials are a useful platform for studying appropriate wound healing. Snake skin, composed of keratin and lipids, is among the most prevalent natural materials. Vakilian and colleagues conducted a study on the wound-healing effects of the shed skin of two different Omani snakes, which was investigated in rats and yielded positive results related to enhanced wound closure.[31] The researchers demonstrated that shed snake skin extracts can directly or indirectly aid in the healing of acute wounds. They do this by helping to release inflammatory cytokines, which leads to fibroblast expansion, epithelialisation, neovascularisation, and granulation tissue formation. Additionally, researchers have indicated that more studies are required to assess the efficacy of wound healing using shed skins from different species of snake.[3] Research on shed snake skin and its effect on wound healing is quite limited, but snake venoms are being evaluated as material in various wound healing studies due to the proteins and peptids they contain.[32,33]

This study is based on the seasonal shedding of a snake species commonly found in our geographical region, as well as the traditional use of shed snake skin in the treatment of various diseases. The data obtained suggest that shed snake skins, which are typically considered waste and can be collected without causing any adverse effects on the animal, can be incorporated into controlled studies on cancer treatment and wound healing. The biological activity potential of the aqueous extract of shed snake skin is reported here for the first time, providing fundamental evidence for its future pharmaceutical use. However, the shed skin content of the snake species used in the study needs to be determined in detail. The current findings have certain limitations, such as *in vitro* experimental design, the use of a single cell line, low antioxidant activity, and the inability to obtain meaningful results in wound healing. Future studies should include more cell line trials, *in vivo* models, and optimized formulations.

**Conclusion**

The skin of shed snakes is used in folk medicine, cosmetics, and pharmacological studies. Therefore, it should be emphasized that natural bio-products may not always be safe, and they should not be used without control. Consumption the shed skin by people through traditional methods, especially when the dosage is unknown, can cause serious problems. It is important to determine dosages. In this study, $IC_{50}$ values were found to be 2972±179 and 2657±128 µg/mL at 72 hours for dermal fibroblast (BJ) cells and melanoma (SK-MEL-30) cells, respectively. The extracts did not exhibit any wound-healing-promoting effect at the doses studied. The presence of phenolic compounds such as flavonoids in snake skin has shown promising results in antioxidant and cytotoxic activity studies. Although the aqueous extract has not demonstrated measurable improvement in wound healing under current conditions, further studies using *in vivo* models may better elucidate its potential in tissue repair.

**Conflict of Interest**

The authors report there are no competing interests to declare.

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