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Effect of Various Extracts Obtained From Bee Pollen on L929 Fibroblast Cell Proliferation

Arı Poleninden Elde Edilen Çeşitli Ekstrelerin L929 Fibroblast Hücre Proliferasyonu Üzerine Etkisi

İrfan ÇINAR
Çiğdem SEVİM
Kastamonu Univercity, Faculty of Medicine, Department of Pharmacology, Kastamonu, Türkiye

ABSTRACT

Objective: Since ancient times bee pollen has been considered a good source of bioactive substances and energy, so its effects on tissue regeneration has been seen and evaluated. Considering the increasing demand for healthy and natural foods in recent years, it is unsurprising that bee pollen has been attracting commercial interest, making it one of the most widely consumed food supplements. The substances contributing to tissue regeneration are essential for skin health and cosmetology. Cell culture studies have gained importance in tissue regeneration studies in recent years. Fibroblast cell line is frequently used in control studies, especially on the efficacy and toxicity of cosmetic products and chemical substances. In light of these data, we investigated the effects of bee pollen extracts on L929 fibroblast cells.
Methods: Different concentrations of Türkiye Bee pollen extract obtained with different solvents (hexane, dichloromethane, methanol, methanol + water, acetone, water) were applied to L929 fibroblast cells. The effects of pollen on cell proliferation were examined time-dependent by real-time cell counting system Xcelligence.
Results: In our studies, all bee pollen extracts in the fibroblast cell line increased cell proliferation. The methanol extract was especially observed to improve L929 cell proliferation by dose-dependent.
Conclusion: This study concluded that bee pollen extracts obtained at various concentrations and by different extractions do not cause any toxic effects on fibroblasts but significantly affect proliferation. From these results, bee pollen contributes to fibroblast proliferation and maybe a new target in cosmetics and developing drugs. However, more detailed work is needed to determine which pollen component is practical.

Keywords: L929, Fibroblast, Bee Pollen, Proliferation

ÖZ

Amaç: Antik çağlardan beri arı poleni iyi bir biyoaktif madde ve enerji kaynağı olduğu için doku rejenerasyonundaki etkileri görülüp değerlendirilmiştir. Son yıllarda sağlıklı ve doğal gıdalara olan artan talep dikkate alındığında, arı poleninin çok tüketilen gıda takviyelerinin içeriğine girmesi ve ticari ilgiyi çekmesi şaşırtıcı değildir. Doku rejenerasyonuna katkısı olan maddeler cilt sağlığı ve kozmetoloji açısından önem arz etmektedir. Son yıllarda doku rejenerasyonu çalışmalarında hücre kültür çalışmaları önem kazanmaktadır. Özellikle kozmetik ürünlerde ve kimyasal maddelerin etkinlik ve toksisiteleri ile ilgili kontrol çalışmalarında Fibroblast hücre hattı sıkça kullanılmaktadır. Biz de bu bilgiler ışığında arı poleni ekstrelerinin L929 fibroblast hücreleri üzerine etkilerini araştırdık.
Yöntemler: Çalışmada Türkiye arı poleninin farklı çözücülerden (hekzan, diklorometan, metanol, metanol+su, aseton, su) elde edilen ekstreleri farklı konsantrasyonlarda L929 hücrelerine uygulanmıştır. Polenin hücre proliferasyonu üzerine etkileri Xcelligence gerçek zamanlı hücre sayım sistemi ile zaman bağımlı olarak incelenmiştir.
Bulgular: Yaptığımız çalışmalarda Fibroblast hücre hattında bütün Arı poleni ekstrelerinin hücre proliferasyonunu artırdığı görülmüştür. Özellikle metanol ekstresinin doza bağımlı olarak L929 hücre proliferasyonunu iyileştirdiği gözlenmiştir.
Sonuç: Sonuç olarak bu çalışmada çeşitli konsantrasyonlarda ve çeşitli ekstraksiyon yöntemleriyle elde edilmiş arı poleni ekstraktlarının fibroblastlara herhangi bir toksik etki oluşturmadığı aksine proliferasyonunda önemli etkilerde bulunduğu gösterilmiştir. Bu sonuçlardan yola çıkarak da arı poleninin fibroblast proliferasyonuna katkıda bulunarak gerek kozmetik alanında gerekse ilaç geliştirmede yeni bir hedef olabileceği öne sürülebilir. Ancak polenin hangi bileşeninin etkili olduğunun belirlenmesi için daha detaylı çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: L929, Fibroblast, Arı poleni, Proliferasyon

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Sorumlu Yazar/Corresponding author: Çiğdem Sevimli
E-mail: cigdemsevim@kastamonu.edu.tr

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Introduction

Bee products were used in medicine in the ancient world (Egypt, Greece, China). Today, bee products (propolis, honey, royal jelly, beeswax, bee pollen) are recognized as alternative medicines and their application refers to complementary and alternative medicine (Zizic, Vukovic et al. 2013). Bee pollen, commonly called "life-giving dust", results from the agglutination of flower pollen with nectar and saliva of honey bees and is used as food for all developmental stages in the hive. The composition of bee pollen varies depending on biogeographic (regional) origin, ecological habitat and even seasonality. Recently, bee pollen has been promoted as a valuable apitherapeutic product due to its potential therapeutic value. Bee pollen is used apitherapeutically as it exhibits a range of actions such as antifungal, antimicrobial, antiviral, anti-inflammatory, immunostimulatory and local analgesic, and also facilitates the granulation process of burn wound healing (Almaraz-Abarca, Campos et al. 2004).

Wound healing is a dynamic reaction whose intact course allows restoring the continuity and functionality of damaged skin as a result of dynamic cooperation between many molecular factors (Velnar, Bailey et al. 2009, Pereira, Lima-Ribeiro et al. 2012, Sinno and Prakash 2013). The process consists of 4 specific phases to progress smoothly and move from one to the next. The durations of the specific healing stages depend on the type of damage and the possible coexistence of additional intervening factors, i.e., the size and location of the damage, the blood supply of the wound edges, the cleanliness of the wound, the degree of microbiological contamination of the wound, the presence of necrotic tissue and appropriately treated healing management in combination with other factors (Velnar, Bailey et al. 2009, Gethin 2012, Pereira, Lima-Ribeiro et al. 2012, Reinke and Sorg 2012).

The L929 type of these cells, which play a role in the proliferation phase of wound healing, especially by contributing to collagen synthesis, are the most preferred cells for the evaluation of wound healing, especially in experimental cell culture studies. Again, cell culture studies have gained importance in tissue regeneration studies in recent years. In the light of this information, we investigated the effects of bee pollen extracts on L929 fibroblast cells.

Methods

Mixed pollen samples collected by honey bees from different plants were obtained from beekeepers in the Erzurum (Erzurum, Türkiye) region and dried in the laboratory at 40- 45°C and pulverized in the mill. In contrast to routine methods, ultrasonic extraction method was used in this study. Solutions (hexane, dichloromethane, methanol, methanol+water, acetone, water) were added. The extraction steps were carried out and the solvents were removed in a rotary evaporator at 40°C and the extracts obtained were stored at +4°C until the study. L929 cells were treated at concentrations of 200, 100, 50, 25, 10, 5 µg/ml.

Cell Culture

Thawing and Culturing L929 Cells

L929 cell line was used in our study. The L929 cell line was removed from the cryotube in a liquid nitrogen tank and kept in a water bath at 37°C for a short time to thaw. The thawed cells were transferred to a T75 cm^2 flask. The L929 cell line was cultured in an incubator at 95% humidity, 5% CO_2 and 37°C using standard RPMI 1640 (20% FBS, 1% PSA, 2mmol Glutamine) medium. After 24 hours, xCELLigence system plates were seeded with 5000 cells in each well and extracts were applied 24 hours later. Up to 72 hours, the effects of bee pollen on cell proliferation were examined with xCELLigence system. At the end of 72 hours, the results were taken and the effects at 24, 48 and 72 hours were analyzed.

Proliferation studies

MTT (Cell Viability Test)

MTT assay method is a method in which the amount of cell proliferation is determined based on colorimetric measurement of enzymatic activity due to the reduction of formazone dyes or MTT. Thus, the cytotoxic or proliferative effects of any therapeutic agent on the cell can be determined. It is based on the colorimetric determination of the color change that occurs in cells incubated with MTT agent. The color change occurs due to the reduction of tetrazolium salt in the mitochondria of active cells by formazone salts colored with yellow. The absorbance value of these compounds is proportional to their metabolic activity. 10 µl of the prepared thiazolyl blue tetrazolium bromide solution was added to 100 µl of medium and cell mixture in 96% well plate wells and left to incubate in the incubator for 4 hours. After 4 hours of incubation, the medium on the surface of the cells in 96-well plate wells was removed with a pipette. 100 µl of MTT solvent solution was added and kept in the incubator overnight (18 hours). The incubated cells were measured with a microplate reader spectrophotometer (Epoch Microplate

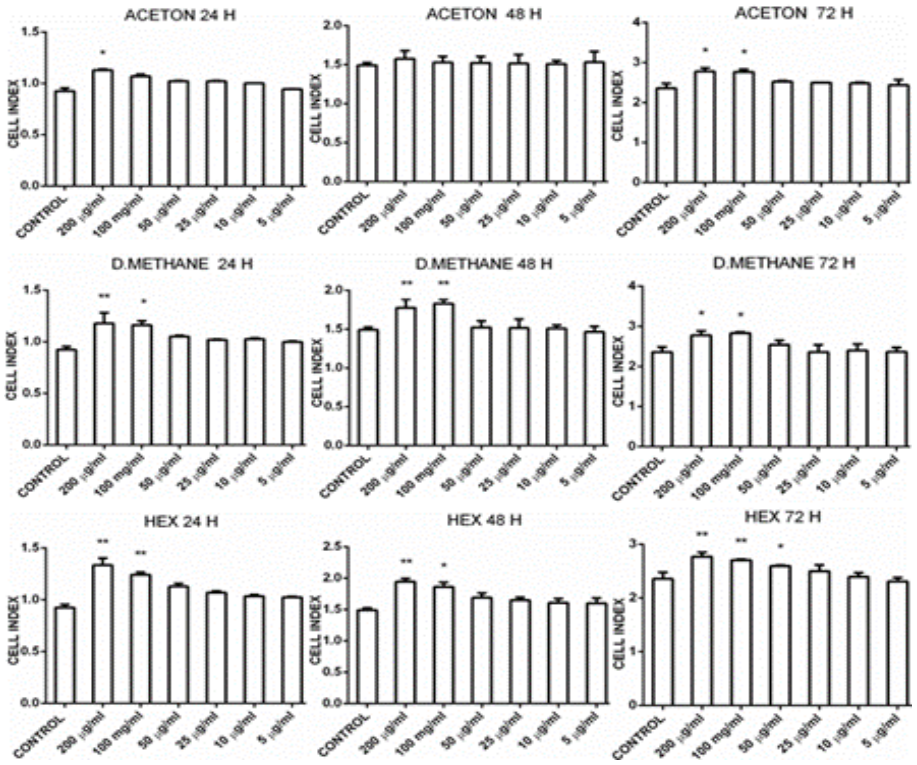

Figure 1: Effects of pollen extracts in Acetone, D. Methane and Hexane solutions; 200, 100, 50, 25, 10 and 5 µg/ml doses on cell proliferation in L929 cell line at 24, 48 and 72 hours.

Spectrophotometer, BioTek, USA) at 620 nm absorbance value in 3 replicates.

Real Time PCR analyzes

Determination of Gene Expression in cell lines

Cells were seeded at 200000/well in 6-well plates and incubated at 37° C in a humidified environment containing 5% CO2. Cells were removed from 6-well plates by trypsinization method, homogenized in Tissue Lyser II (Qiagen) (350 µl RLT buffer per 1*105 cells) and RNA extraction was performed in QIAcube RNA isolation device as recommended by the manufacturer.

Reverse Transcriptase Reaction and cDNA Synthesis

cDNA synthesis was performed from total RNA using the High Capacity cDNA Reverse Transcription Kit enzyme. Each reaction was performed with 10µl RNA and cDNA synthesis was performed with Veriti 96 Well Thermal Cycler (Applied Biosystem) according to the following temperature values and cDNA amount was measured by nano drop spectrophotometry (EPOCH Take3 Plate, Biotek) and stored at -20˚C until the day of analysis.

Real-time quantitative PCR

TGF-β1 (Rn00572010_m1) gene was quantified using Taq Man Gene Expression Master Mix kit. Amplification and quantification were performed on a StepOne Plus Real Time PCR System (Applied Biosystems). For 100 ng cDNA, TGF-β1 gene and ACTB (Rn00667869_m1) as housekeeping gene (Applied Biosystems) were pipetted and run for 40 cycles. Ct values were automatically converted to delta delta Ct in the device and the findings obtained as a result of our studies.

Statistical Analysis

For statistical analysis, all data were calculated using Microsoft Excel program and the results obtained were shown as mean±standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test. $P<0.05$ was considered significant.

Results

Pollen extracts dissolved in acetone, dichloromethane and hexane solvents were applied to L929 cell line at 200, 100, 50, 25, 10, 5 µg/ml doses and cell viability was evaluated at 24, 48 and 72 hours. It was observed that cell viability increased at 24 and 72 hours at 200 µg/ml dose of pollen extract applied in acetone solvent ($p<0.05$). In addition to this increase, a significant increase was also found in the 100 µg/ml dose group at 72 hours ($p<0.05$). It was observed that 200 and 100 µg/ml dose groups of pollen extract applied in dichloromethane solvent significantly increased

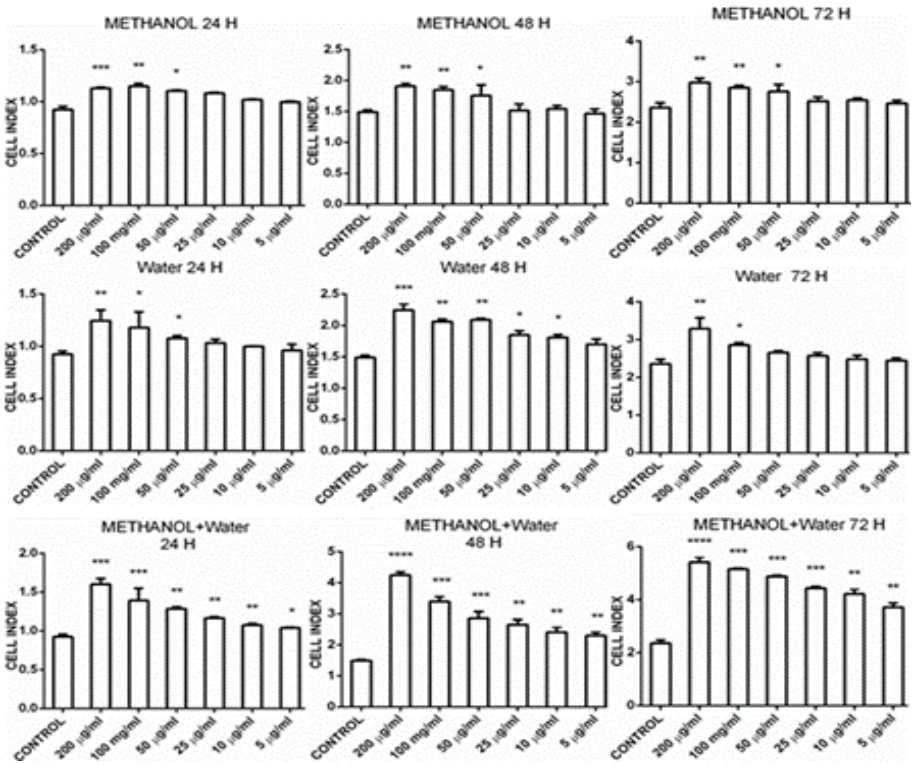

Figure 2: Effects of methanol, water and methanol+water solutions of pollen extracts on cell proliferation in L929 cell line at 24, 48 and 72 hours at doses of 200, 100, 50, 25, 10 and 5 µg/ml.

cell proliferation at 24, 48 and 72 hours. When these increases are analyzed, the significance levels of 200 µg/ml and 100 µg/ml dose groups at 24 hours are p<0.005 and p<0.05, respectively, compared to the control.

At 48.hour, the significance level of both dose groups was p<0.005 compared to the control, while it was p<0.05 at 72.hour. It was observed that 200 and 100 µg/ml dose groups of pollen extract applied in hexane solvent significantly increased cell proliferation at 24, 48 and 72 hours. When these increases are analyzed, the significance levels of 200 µg/ml and 100 µg/ml dose groups are p<0.005 and p<0.05, respectively, at 48th hour compared to the control.

At 24 and 72 hours, the significance level of both dose groups is p<0.05 compared to the control. In addition to these dose groups, it was observed that cell proliferation was significantly increased in the 50µg/ml dose group at 72 hours compared to the control (p<0.05) (Figure 1).

Pollen extracts dissolved in methanol, water and methanol+water solutions were applied to L929 cell line at 200, 100, 50, 25, 10, 5 µg/ml doses and cell viability was evaluated at 24, 48 and 72 hours. It was observed that pollen extract applied in methanol solvent increased cell proliferation in 200, 100, 50 µg/ml dose groups. At 24, 48 and 72 hours, it was found that 200 µg/ml dose group increased cell proliferation at p<0.001, 100 µg/ml dose group increased cell proliferation at p<0.005, 50 µg/ml dose group increased cell proliferation at p<0.05 level of significance. It was observed that 200, 100 and 50 µg/ml dose groups of pollen extract applied in water solvent significantly increased cell proliferation at 24 hours (p<0.005 and p<0.05). At 48.hour, 200, 100, 50, 25 and 10 µg/ml dose groups significantly increased cell proliferation (p<0.001, p<0.005 and p<0.05). At 72 hours, 200 and 100µg/ml dose groups significantly increased cell proliferation (p<0.005 and p<0.05). When methanol+water combination was performed, it was found that cell proliferation increased in all dose groups at all 3 time points (p=0.00, p<0.001, p<0.005 and p<0.05) (Figure 2).

When pollen extracts were dissolved in methanol + water combination and applied to L929 cell line, it was analyzed that TGF-β1 mRNA level increased significantly in 200, 100, 50 and 25 µg/ml dose groups compared to the control group. While the 200 and 100 µg/ml dose groups were found to be statistically significant at p<0.001, the 50 µg/ml group was found to be p<0.005 and the 25 µg/ml group was found to be p<0.05 (Figure 3).

Discussion

Solvents are required to dissolve both drugs and extracts used for experiments. Almost all solvents have the potential to be toxic to cells in vitro, the difference between them being their concentration (Stammati, Zampaglioni et al. 1997, Forman, Kás et al. 1999). Although it is quite easy to investigate the toxicity or therapeutic effects of water-soluble substances, it is sometimes necessary to use toxic solvents to dissolve substances that do not dissolve in water well enough or do not dissolve in water at all (Forman, Kás et al. 1999). Under these conditions, the efficacy of the test compound may be misinterpreted. For this reason, the effect of the solvent should be evaluated in the studies and possible risks should be evaluated at this point and it is important not to cover the effect of the material to be tested. In a study with HeLa S3 cell line, cells were exposed to DMSO, methanol, ethanol, acetone, isooctane and hexane in the concentration range of 0.1-7.5% and their changes were examined. It was found that 1% (v/v) concentration of DMSO suppresses cell proliferation and 2% (v/v) concentration has a toxic effect on cells (Shier 1988, Forman, Kás et al. 1999). In another study with PC12 cell line, cells were treated with different concentrations of methanol, ethanol, acetone, and glycerol and their toxicity was investigated. It was found that 20ml/L ethanol treatment decreased cell viability by 60% at 24 hours, acetone by 20% and ethanol by 15% (Fengyan 2014). In our study, when methanol+water combination was used as solvent, it was found to increase cell proliferation in all dose groups (200, 100, 50, 25 and 10 µg/ml) at all 3 time points ($p=0.00$, $p<0.001$, $p<0.005$ and $p<0.05$). The other solvents we used, acetone, dichloromethane, hexane and water, increased cell proliferation at different time periods in 200 and 100 µg/ml dose groups. It was determined that the most effective group among all solvents was the methanol+water combination.

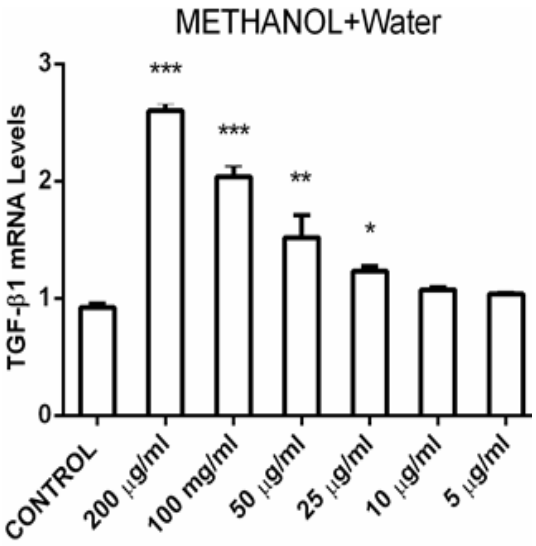

Figure 3: TGF-β1 mRNA level in L929 cell line at 200, 100, 50, 25, 10 and 5 µg/ml doses from methanol+water solutions of pollen extracts.

Pollen is a highly diverse plant product rich in biologically active substances. Pollen grains of different plant species contain 200 substances. The basic chemical substances include proteins, amino acids, carbohydrates, lipids and fatty acids, phenolic compounds, enzymes and coenzymes as well as vitamins and bioelements (Komosinska-Vassev, Olczyk et al. 2015). Pollen is a substance with a high anti-inflammatory effect and its efficacy can be compared with drugs such as naproxen, phenylbutazone or indomethacin (Pascoal, Rodrigues et al. 2014). Bee pollen shows bioactivity with its phenolic acids and polyphenols in the form of flavonoids (Nguyen, Nguyen et al. 2020). Analysis of bee pollen using LC-MS/MS showed that it contains 23 phenolic compounds (such as 2,5-dihydroxybenzoic acid, protocatechuic acid and kaempferol) and 42 free amino acids (Bayram, Gercek et al. 2021). Thermal skin damages are treated by applying therapeutic preparations as well as surgical methods. Recently, the therapeutic effect of standardized, pharmacologically active fractions obtained from bee products has started to be used. These agents are called apitherapeutic agents. They help to re-establish the balance of the skin barrier (Campos, Bogdanov et al. 2008, Rzepecka-Stojko, Pilawa et al. 2012). Whether various bee products are effective in wound healing and their interactions with each other have been investigated. Propolis and honey have been shown to be very effective in wound healing in rats (Peršurić and Pavelić 2021). In an in vitro study, it was found that this duo increased the migration, proliferation and viability of dermal fibroblasts in a dose-dependent manner (Ebadi and Fazeli 2021).

Transforming growth factor (TGF)-β belongs to a family of multifunctional peptides known to have five isoforms. In addition to its effects on cell proliferation, differentiation, adhesion, migration, ECM production, TGF-β is also a potent inducer of many components of the ECM, including collagen, fibronectin and cell surface integrins. TGF-β increases the levels of protease inhibitors while decreasing the synthesis of collagenase from matrix components. In studies, it has been found to accelerate wound healing as a result of local injection (Sankar, Mahooti-Brooks et al. 1996). In the period of 6-24 months after the first injury, the final stage of the wound is passed by remodeling the tissue. During this remodeling period, newly synthesized type I collagen begins to replace type III collagen. The production of most of the collagen and fibronectins, which are the basic components of the extracellular matrix, is also stimulated by PDGF and TGF-β1. In our study, it was found that 200, 100, 50, and 25 µg/ml dose groups increased TGF-β1 expression. This

suggests that bee pollen may be effective in increasing collagen production in L929 cells, especially in the 200 and 100 µg/ml dose groups during the proliferation phase of wound healing. The study provides a basis for expanding the study by performing it on animals and supporting it with different pathway analyses, as well as providing support for the use of bee pollen.

Conclusion and Recommendations

As a result, in this study, it was shown that bee pollen extracts obtained at various concentrations and by various extraction methods did not cause any toxic effect on fibroblast cells, on the contrary, they had significant dose-dependent effects on their proliferation. Although the effect occurred in all solvents, the most effective proliferation was observed in methanol + water solution. Based on these results, it can be said that bee pollen may be a new target in both wound healing and drug development in this field by contributing to fibroblast proliferation. In addition to all these effects, it can be suggested that bee pollen can be used as new therapeutic agents for wound healing because of its easy accessibility, low risk of side effects and low cost.

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