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Jaceosidin protects L929 fibroblast cells by down-regulation of proinflammatory cytokines and attenuation of oxidative stress-induced impairment of cell proliferation and migration

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ABSTRACT

Aim: Oxidative stress has been a significant factor in wound-healing pathophysiology for a long time. Antioxidants, especially natural compounds, have recently been emphasized in instructions for wound healing treatments. Jaceosidin (JACE), a flavone derived from *Artemisia princeps*, is a potent antioxidant. This study aims to investigate JACE's anti-inflammatory and antioxidant properties and its capacity to improve the effects of in vitro wound healing.

Methods: Wound healing activities have been tested using cell proliferation and migration in vitro assays in the mouse fibroblast cell line L929. The concentration of hydrogen peroxide (H_2O_2-0.5 mM) has been used to induce the oxidative stress model. Tumor necrosis factor-alpha (TNF-α) and nuclear factor (NF-κβ) have been investigated as inflammatory indicators. Antioxidant activity has been checked using total antioxidant status (TAS) and total oxidant status (TOS) tests.

Results: JACE has significantly increased the proliferation of fibroblasts dose-dependent manner. It has enhanced the cell migration rate of fibroblasts compared with the H_2O_2 group. JACE at a concentration of 50 and 100 μM has significantly decreased TOS and oxidative stress index (OSI) levels and increased TAS levels. The anti-inflammatory mechanism of JACE has involved down-regulation of the mRNA expressions of the NF-κβ and TNF-α in a dose-dependent manner.

Conclusions: JACE has beneficial impacts on fibroblast viability and migration qualities through antioxidative actions and down-regulating proinflammatory cytokines through anti-inflammatory effects to promote wound healing. The present study shows that JACE may help to increase the range of available treatments for wound-healing by reducing inflammation and oxidative stress.

Key words: Wound-healing, jaceosidin, antioxidant, fibroblast L929 cells, oxidative stress, hydrogen peroxide.

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Introduction

Wound-healing is a major global health concern and a high economic burden for governments. In 2014, 8.2 million people in the USA received treatment for various types of wounds, which cost between $28.1 billion and $96.8 billion. The annual costs of wound-healing

treatment and its related complications are estimated to be €3 billion in the UK and $2.85 billion in Australia [1-3]. Therefore, developing new and effective treatments that support wound healing is necessary. Wound-healing is a complex biological process associated with activating various cells such as fibroblasts, endothelium, and blood cells and releasing many cytokines, chemokines, and growth factors responsible for restoring tissue integrity after injury [4-6]. This process is a distinct but overlapping sequence of events: an inflammatory phase (consisting of hemostasis and inflammation), a proliferative phase (consisting of granulation, contraction, and epithelialization), and a remodeling phase, which is concerned with limiting tissue damage and restoring the integrity and function of affected tissues [7, 8]. As the inflammatory stage of the wound-healing process continues, large numbers of cells are attracted to the injury site, and various inflammatory mediators are released [9]. Reactive oxygen species (ROS) are synthesized to contribute to the resolution of the inflammatory phase. Although the secretion of free radicals has a protective role, their overproduction causes dysregulation of the inflammatory response, leading to oxidative stress. Oxidative stress indicates a skewness of the balance between pro-oxidant/antioxidant homeostasis, which includes ROS production or defective antioxidant defense mechanisms. Thus, changes in oxidative stress and ROS status prolong the inflammatory phase of the repair or wound-healing process, impeding the recruitment of fibroblasts that must be attracted to the site to initiate the proliferative phase [10, 11]. Prolonged inflammatory phase with oxidative damage results in a chronic wound. Since ancient times, much research has been conducted to discover new plant-derived antioxidant compounds to prevent free radical damage. It is found that natural antioxidants of plant origin have essential roles in wound-healing [12, 13]. It is known that flavonoids, natural antioxidants of plant origin, can prevent or heal diseases caused by free radicals. Various plants containing flavonoids appear involved in wound-healing research [14-17].

Like many flavones, Jaceosidin (JACE) is protective against many diseases with its various pharmacological effects such as antioxidant, anti-inflammatory and anticarcinogenic. It is an active and potent flavone naturally found in the Japanese wormwood *Artemisia princeps* [18]. Research has shown that JACE induces apoptosis in various types of cancer, including glioblastoma cells [19, 20], endometrial cancer cells [21], breast cancer [22], and human ovarian cancer cells [23]. In addition, it reduces the formation of ROS and plays a role in the down-regulation of the expression of various cytokines [24]. JACE could also reduce the inflammatory response following LPS-induced sepsis and decrease complement levels by increasing antioxidant activity [25]. Based on the antioxidant and anti-inflammatory effects of JACE, a potent flavone, this study investigates the antioxidant and fibroblast proliferation parameters and molecular expression of proinflammatory cytokines against oxidative damage induced by H_2O_2.

Materials and metods

Reagents and cell culture: The L929 cell line, derived from mouse fibroblasts, was sourced from the American Type Culture Collection (ATCC, USA). The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco®, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Life Technologies Inc.), 100 U/mL penicillin, and 100 µg/mL streptomycin. Incubation occurred at 37°C in a

humidified atmosphere with 5% CO_2. Hydrogen peroxide (30%) and MTT were obtained from Merck and Apollo Scientific Company. JACE was purchased from Cayman Chemical.

Experimental design: Cells were divided into control, H_2O_2, and treatment groups. Control group cells with only medium; H_2O_2 group only treated cells with 0.5 mM H_2O_2. The treatment group was divided into three groups according to the dose of JACE. Treatment group (JACE + H_2O_2 group) cells were pretreated with 25, 50, and 100 μM JACE for 3 hours, followed by 0.5 mM H_2O_2. Based on the previous studies, the doses of H_2O_2 [15, 26] and JACE [27-29] were decided.

Cell viability and oxidative stress model induced by H_2O_2: L929 cells incubated in 75 cm^2 flasks were harvested 48 hours later by treatment with Trypsin-EDTA. Cells were counted as 2×10^5 cells in each well, and they were plated in 96 wells and incubated for 48 hours in DMEM containing 10% FBS in a humid atmosphere containing 5% CO_2 at 37 °C. At the end of the incubation, the cells were treated with different concentrations of JACE (25, 50, 100μM) for 24 hours. Three hours later, H_2O_2 (0.5 mM) was added, and cell viability was determined by the MTT test [30]. Each concentration was applied in triplicate. After 24, 48, and 72 hours of incubation, MTT solution was added to each well in a dark environment. After 4 hours of incubation, DMSO was added to each well to dissolve the purple formazan crystals and shaken to ensure complete dissolution. Absorbances were determined in a microplate reader ELISA reader (Epoch Microplate Spectrophotometer, BioTek, USA) at 540 nm spectrophotometric absorbance. Viability rates were analyzed by comparison with control wells. The H_2O_2-induced model was determined as previously described in the literature [15, 16].

Cell migration analysis through wound-healing assay: L929 cells were seeded in 6-well plates (2×10^5 cells/ml) in DMEM medium containing 10% FBS and incubated in an environment containing 5% CO_2 at 37°C. When the cells completely covered the bottom of the wells and became confluent, two horizontal scratches, right and left, were created in each well with a sterile pipette tip. Cellular wastes were cleaned by washing with PBS. 2 ml of fresh medium was added before applying the test samples. Microscope (Leica Inverted Microscope DMIL LED) images were taken from the cells on the first day. Then, images were taken under the microscope at 24, 48, and 72 hours to determine the migration of the incubated L929 cells.

An increase in the percent closed area means the cells are migrating, and the results are expressed as such. The migration test to be applied was done by the wound-healing procedure [31, 32].

Biochemical assay

Determination of TAS, TOS, and OSI: TAS and TOS levels were measured using the protocol specified by commercially available kits (Rel Assay) [33]. Trolox was used as a standard in the TAS tests, and the results were expressed as mmol Trolox equivalent/L. In TOS tests, H_2O_2 was used as a standard, and results were expressed as μmol H_2O_2 equivalent/L [34]. The ratio of TOS to TAS was accepted as OSI (arbitrary unit: AU) [33]. The OSI value was calculated according to the specified formula [35]:

$$OSI = \frac{\text{TOS (μmol H2O2 equiv./l)}}{\text{TAS (mmol Trolox equiv./l)}}$$

Molecular assay

Determination of gene expressions in L929 cell line: Cells were seeded in 6-well plates at $2\text{x}10^5$ cells/well and incubated at 37°C in a humid environment containing 5% CO_2. Cells

were then trypsinized, lifted from 6 well plates, and then homogenized in a Tissue Lyser II (Qiagen) device (350 µL of RLT buffer was added to $1x10^5$ cells). RNA samples were reverse transcribed into complementary DNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems), as previously described [36].

Quantification of gene expression using real-time PCR: Real-time PCR analysis was conducted to determine the TNF-α and NF-κB genes, by employing the StepOne Plus Real-Time PCR System technology (Applied Biosystems, Foster City, CA). cDNA synthesized from RNA extracted from L929 cells was utilized for the analysis. The amplification and quantification steps were performed using the StepOne Plus Real-Time PCR System (Applied Biosystems) instrument, employing the TaqMan Gene Expression Master Mix kit [37]. The housekeeping gene β-actin was employed as an internal control. Ct values were automatically transformed into $2^{-\Delta\Delta Ct,}$ and the outcomes were reported as fold changes relative to the expression observed in the control group [38].

Statistical analysis: Statistical analysis was conducted using GraphPad Prism 9 software. The results were presented as the mean ± standard deviation. Normality analysis was performed using the Shapiro-Wilk test. One-way analysis of variance (ANOVA) was utilized for data analysis, followed by the Tukey post hoc test with a statistical significance value ($p<0.05$).

Results

Effects of H_2O_2 and its combination with JACE on L929 viability: The H_2O_2 concentration was determined as 0.5 mM based on the previous studies [15, 26]. The effect of JACE against H_2O_2 on cell proliferation and viability was investigated at different concentrations (25, 50, and 100 µM) in the L929 mouse fibroblast cell line at 24, 48, and 72 hours using the MTT method. L929 fibroblast cells were treated with 0.5 mM H_2O_2 as a model study for oxidative stress and decreased cell viability in the H_2O_2 group (49.33±7.27%, p=0.000) after 24 hours of exposure. However, no significant increase in cell viability was observed in the

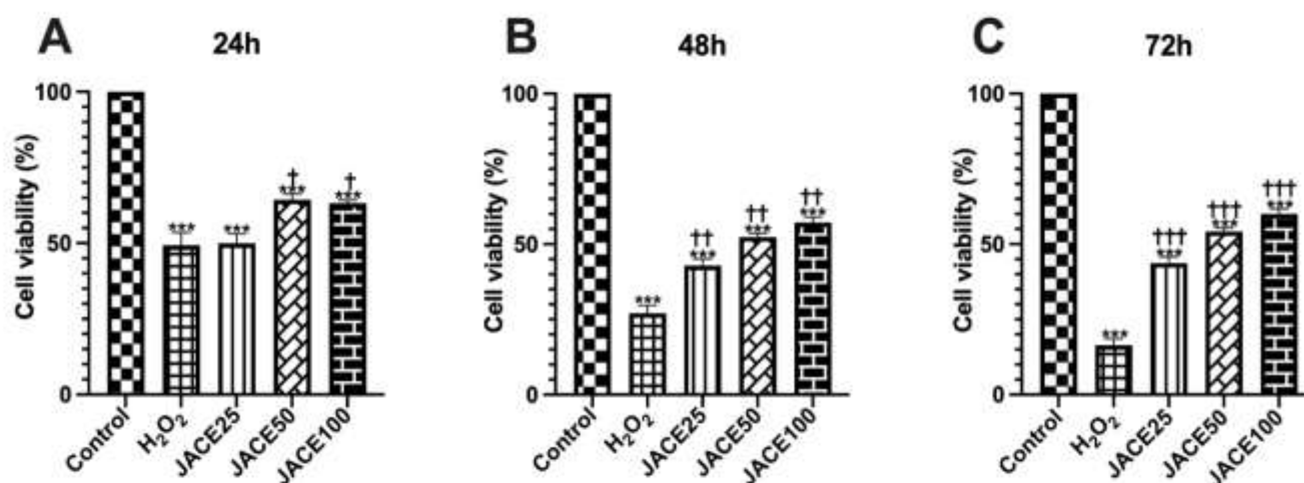

Figure 1. Effect of JACE and H_2O_2 on the viability of L929 fibroblast cells. **A**, 24 hours; **B**, 48 hours; **C**, 72 hours. Columns are shown as mean±SD. JACE: Jaceosidin. *p <0.05, ** p <0.01, and *** p<0.001 signs were used compared to the control group. †p<0.05, ††p<0.01, and †††p<0.001 signs were used in comparison with the H_2O_2 group.

groups given JACE (25, 50, 100 µM) within 24 hours, compared to the control group (p=0.000). However, a significant increase in cell viability was observed in the groups given JACE at 50 and 100 µM doses, compared to the H_2O_2 group (64.39±3.34, 63.17±1.45, respectively) (Figure 1A). H_2O_2 at 48 and 72 hours significantly inhibited L929 cells, compared to the control group (p=0.000). JACE, as shown in Figure 1B, 25, 50, and 100µM of JACE reduced the damage caused by 0.5 mM H_2O_2 and increased cell viability by 42.86±3.91%, 52.39±2.11%, and 57.22±3.22%, respectively, compared to the H_2O_2 group with 27.06±4.48%. 25, 50, and 100µg, JACE reduced the damage caused by 0.5 mM H_2O_2 and increased cell viability by 43.74±3.54%, 54.24±1.94% and 60.04±3.00 respectively, compared to the H_2O_2 group with 16.40±3.67% cell viability (Figure 1C). These results also indicated that JACE protects L929 cells against H_2O_2-induced damage.

Impacts of JACE on H_2O_2-induced oxidative damage and wound healing: To investigate whether JACE affects H_2O_2-damaged cell migration and to determine changes in cell migration ability, a standard in-vitro method, the Migration test (wound-healing test), was performed, and the rate of closed-scratch-wound was compared. The representative images of scratch wounds in L929 fibroblast cells at 0 h and 48 h post-injury were given in Figure 2A. The results showed that the wound closure rate plotted in the H_2O_2 group was significantly lower than in the control group (p=0.000) (Figure 2B). It was also observed that the treatment with 50 µM ($p<0.05$) and 100 µM ($p<0.001$) doses of JACE significantly increased the migration of fibroblast cells to the injured area.

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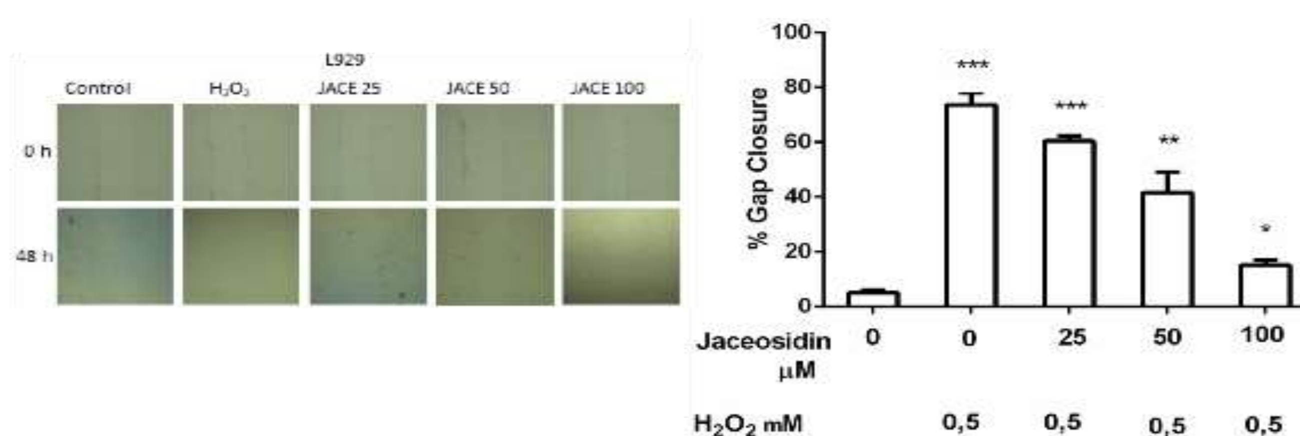

Figure 2. A: Microscope images of the effects of JACE at different concentrations (25, 50, 100 µg/mL) on the migration of L929 cells at 0 and 48 hours. **B:** Effects of JACE on the migration of L929 cells using the wound healing model (% gap closure). Columns are shown as mean±SD. JACE: Jaceosidin. *p <0.05, ** p <0.01, and *** p<0.001 signs were used compared to the control group.

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Restoration of TAS and TOS levels in H_2O_2-induced oxidative damage by JACE: The results revealed that H_2O_2 administration markedly increased the amount of TOS and OSI, in contrast, significantly decreasing the level of TAS compared to the control (Figure 3). However, TOS and OSI levels in the JACE25 group (25μM) remained higher. The amount of TAS was still relatively low compared to the control group. Also, 50μM and 100 μM JACE treatments significantly decreased the levels of TOS and OSI. The increase in TAS was also significant compared to the control group. In addition, there was no statistically significant difference in TOS and TAS levels in the JACE25 group compared to the H_2O_2 group. Also,

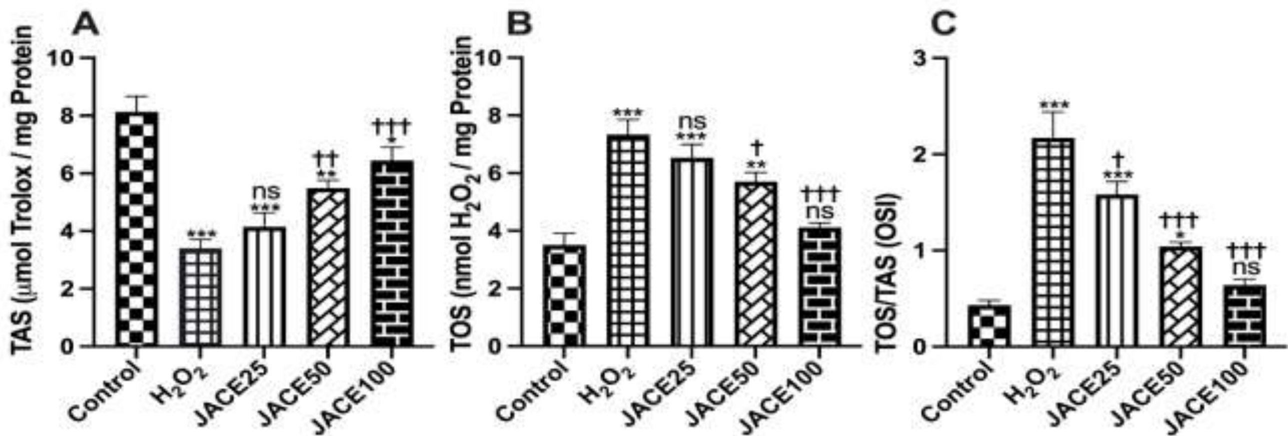

Figure 3. TAS (A), TOS (B), and OSI (C) levels in groups underwent H2O2-induced oxidative stress. Columns are shown as mean±SD. JACE: Jaceosidin. *p <0.05, ** p <0.01, and *** p<0.001 signs were used compared to the control group. †p<0.05, ††p<0.01, and †††p<0.001 signs were used in comparison with the H_2O_2 group. ns: non-significant.

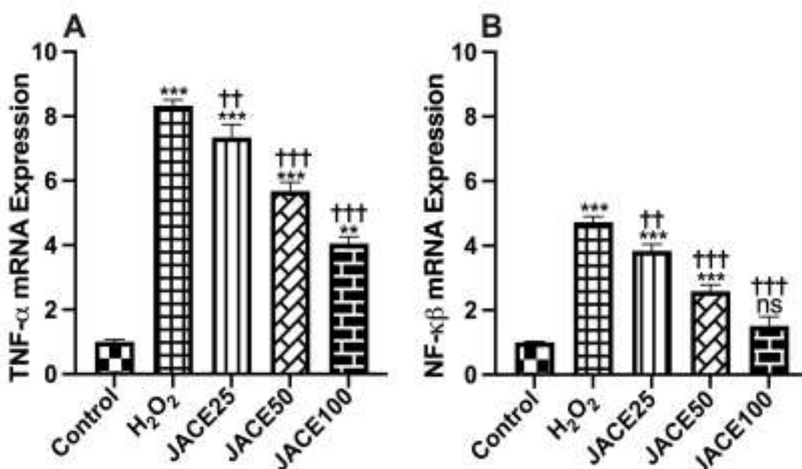

Figure 4. Relative TNF-α and NF-κβ gene expression levels.
Columns are shown as mean±SD. TNF-α: Tumor necrosis factor-alfa, NF-κβ: nuclear factor kappa beta, JACE: Jaceosidin.
*p <0.05, ** p <0.01, and *** p<0.001 signs were used compared to the control group. †p<0.05, ††p<0.01, and †††p<0.001 signs were used in comparison with the H_2O_2 group.
ns: non-significant.

treatment with doses of 50µM and 100µM JACE significantly increased the TAS level and decreased the TOS and OSI levels compared to the H_2O_2 group.

Effect of JACE on NF-κβ and TNF-α mRNA expressions in H_2O_2-induced oxidative damage: TNF-α and NF-κβ mRNA expression levels were examined using real-time PCR to determine whether JACE with different doses alleviation H_2O_2 -induced oxidative damage at doses in L929 cells (Figure 4). TNF-α and NF-κβ mRNA expression levels were shown to rise significantly due to H_2O_2-induced oxidative damage. However, JACE with 25, 50, and 100 µM administration reduced TNF-α and NF-κβ mRNA expression considerably, compared to the H_2O_2 group. In particular, the administration of JACE 100 µM reduced the NF-κβ mRNA expression level, similar to the level of the control group.

Discussion

This study shows that oxidative stress induced by H_2O_2 negatively impacts the proliferation and migration of L929 fibroblast cells. However, pretreatment with JACE alleviates this effect by reducing oxidative stress damage. This protective effect is achieved through the upregulation of TNF-α and NF-κβ and the regulation of biochemical parameters.

Wound-healing is a biological process characterized by inflammation, proliferation, and remodeling [7]. Today, it is known that many signaling pathways are activated in response to oxidative stress in regulating the wound-healing process [39]. Free radicals and ROS synthesis during normal wound-healing contribute to defense against pathogens and mediates critical intracellular pathways to resolve the inflammatory phase [10]. However, excessive amounts of free radicals and ROS increase oxidative stress and cause a decrease in fibroblasts, keratinocytes, and endothelial cells with their harmful effects on cell membranes, proteins, and nucleic acids [40, 41]. Therefore, antioxidants may be beneficial in removing excess ROS or suppressing their formation in preventing oxidative stress-induced cellular damage in wound-healing [10].

JACE, which has antioxidant and anti-inflammatory properties [23, 42], has many therapeutic effects that may act as scavenging free radicals by inhibiting NF-κβ activation and suppressing proinflammatory cytokines and the inducible nitric oxide expression [25]. It is an attractive option to prevent the harm brought on by oxidative stress due to its potent antioxidant activity.

One of the primary ROS intermediates, H_2O_2, reduces the production of intracellular reactive oxygen species and cell damage by acting as a direct oxidant that produces hydroxyl radicals. Therefore, applying exogenous H_2O_2 to a cell is particularly appealing to simulate oxidative stress. In our study, we first determined the effects of JACE with the combination of H_2O_2 on the viability of L929 cells at different concentrations. According to our findings, pretreatment with 50 and 100 µM JACE protected against oxidative damage from 0.5 mM H_2O_2 by inhibiting it. This result indicates that JACE has potent protective effects against oxidative damage-induced cell death. We hypothesized that JACE protects L929 cells from oxidative damage, possibly by altering intracellular ROS levels. Also, previous in-vitro studies [25, 43] support the hypothesis.

Fibroblast migration is a crucially critical phase in the development of tissue formation during wound healing. The wound-closure assay is a valuable method that mimics a wound in-vitro and assesses the rate of cell migration [44].

Additionally, it is regarded as a useful in-vitro method in the research community for understanding more about a compound's potential to heal a wound [45, 46]. According to the migration test findings in our study, JACE significantly increased the migration of fibroblast cells to the injured area at all doses. The wound area was almost entirely covered by 100µM amount of JACE.

Excessive amounts of free radicals and ROS in the wound area induce tissue oxidative stress, which results in harmful cellular damage [10]. Two main factors for evaluating redox balance in organisms are TAS and TOS. TOS defines as the total amount of oxidants in the sample as a whole [47]. While the amount of TAS was considerably raised in the current study, JACE therapy dramatically reduced the level of TOS. Furthermore, it reduced OSI levels [47], a gold standard for oxidative stress. Our findings demonstrate that JACE considerably aided in the healing of wounds by lowering the overproduction of ROS induced by H_2O_2. JACE ameliorated oxidative stress indicators in earlier research [25, 43, 48].

In the inflammatory stage of the wound-healing process, it was seen that several growth factors, proinflammatory cytokines, and chemokines elevated [49]. The transcription factor NF-κβ regulates the transcription of various inflammatory mediators involved in the inflammatory phase [50]. As a result, it appears that blocking the NF-κβ signaling pathway is a functional therapeutic approach for managing various inflammatory illnesses. The expression of various proinflammatory factors, such as TNF-α, increases in the early stage of wound healing [51, 52].

Furthermore, a study showed that ROS reduces angiogenesis by causing TNF-α induced inflammatory injury at the wounded site [52]. Hence, it may effectively treat skin disorders by lowering these proinflammatory cytokines' excessive production and activity. In our research, it was found that JACE treatment reduced the elevated TNF-α expression.

Conclusions

In sum, using cell-based assays, JACE effectively stimulated the proliferation and migration of fibroblasts, protected against oxidative damage by reducing the overproduction of free radicals, and suppressed the proinflammatory cytokines TNF-α and NF-κB expression. These results make JACE an excellent option for future applications in synthesizing and formulating natural-based drugs for wound-healing therapy.

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Conflict of interest: *The authors declare that they have no conflict of interest.*

Ethical statement: *Ethics committee decision was not taken as it was a cell culture laboratory study.*

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