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Original Article

Wound Healing Effects of Frankincense and Myrrha on Adult Human Dermal Fibroblasts (HDFa)

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ABSTRACT

Introduction: Oleogum resins extracted from Boswellia sacra (Frankincense) and *Commiphora myrrha* (Myrrha) have been traditionally used to facilitate wound healing and address skin injuries. Moreover, they have anti-inflammatory, antioxidant, and antimicrobial effects. Therefore, we hypothesized that their combination can be effective in wound healing. In this study, we evaluated the effects of methanol extracts from two oleogum resins, Boswellia sacra (Frankincense) and *Commiphora myrrha* (Myrrha), as well as their combination on cell migration promotion and wound healing in human dermal fibroblast cells (HDFa).

Methods: The methanol extracts of B. sacra (BS) and *C. myrrha* (CM) and their combination were tested to determine their optimum cytoprotective concentrations using the AlamarBlue assay. The level of reactive oxygen species (ROS) was also evaluated using a DCFDA detector. To assess cell migration promotion and wound healing properties of the extracts, a scratch wound closure assay was performed in HDFa cells and the images were analyzed using ImageJ software. Western blot analysis was employed to detect the activation of fibroblast migration-associated protein extracellular signal-regulated kinase (ERK).

Results: Using the viability assay, the optimum non-cytotoxic concentrations of the extracts (10 and 20 µg/ml) were chosen to evaluate their wound healing effects on HDFa cells. BS, CM and BC at 10 and 20 µg/ml significantly reduced H_2O_2-induced ROS levels compared to the control. In the scratch assay, BS and BC, both at 10 µg/ml, could significantly reduce the average wound width compared to the control. Western blot analysis showed that CM significantly increased the pERK/ERK ratio compared to the control.

Conclusion: These findings suggest the beneficial effects of both frankincense and myrrh, as well as their combination, in improving proliferation, migration, and thecwound healing process in HDFa.

Keywords:
Frankincense
Myrrh
Wound Healing
Persian Medicine
Western blotting

Introduction

Skin accounts for approximately 16% of an adult's body weight and consists of two primary layers: the inner (dermis) and the outer (epidermis) (Muniandy et al., 2018). Due to its constant exposure to the external environment, the skin is highly sensitive and vulnera-

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ble to environmental damage, such as burns and wounds (Pereira and Bartolo, 2016). The detachment of cells and disruption of anatomical integrity in different tissues is called a wound, which can result from physical, chemical, thermal, microbial, and immunological damages (Maver et al., 2015). Incomplete and inappropriate wound healing can lead to chronic and infectious wounds, adversely affecting the quality of life. In addition, the treatment of chronic wounds requires extended hospital care and the use of more expensive wound care products (Pereira and Bartolo, 2016). The process of wound healing typically occurs in three steps: The hemostatic and inflammation phase, the proliferation and migration phase, and tissue remodeling (Guo et al., 2021). The hemostatic phase begins immediately after an injury to prevent bleeding and the spread of microorganisms in the body. The inflammatory phase overlaps with hemostasis and involves the release of significant amounts of inflammatory cytokines, proteases, reactive oxygen species (ROS), cationic peptides, and growth factors, all contributing to wound healing (Cañedo-Dorantes and Cañedo-Ayala, 2019). The second stage of wound healing, occurring 2-10 days after the injury, involves cell proliferation and the migration of different cell types to the wound area. Simultaneously, angiogenesis begins to replace newly formed vessels with a fibrin matrix and granulation tissue (Pereira and Bartolo, 2016). Along with keratinocytes, fibroblasts migrate to the wound area and are transformed into myofibroblasts by macrophages. These new cells aid in closing the wound edges and, with the help of fibroblasts, form the extracellular matrix responsible for scar formation (Budovsky et al., 2015; Pereira and Bartolo, 2016). In the final stage, the inflammatory response decreases, and all cells involved in the previous stages are replaced by collagen and extracellular matrix proteins (Budovsky et al., 2015), gradually transforming the damaged tissue into healthy tissue (Pereira and Bartolo, 2016).

Herbal medicines have been used for centuries to promote wound healing (Budovsky et al., 2015). Plants with antioxidant, anti-inflammatory, and antimicrobial properties are widely used in the management and treatment of wounds (Fahimi et al., 2015). Persian Medicine (PM) is a rich source of natural medicines for the treatment of various diseases, especially skin problems. Among the natural products known for their wound healing effects are frankincense and myrrh oleo-gum-resins, which have received significant attention in Traditional Persian Medicine (TPM) texts (Jorjani, 1976).

Frankincense is a transparent and fragile oleo-gum-resin extracted from *Boswellia sacra* Flueck (Burseraceae). Frankincense consists of resin (60-80%), which is a mixture of terpene compounds, gum (6-30%), composed of a mixture of polysaccharides (hexose and pentose sugars), and essential oil (5-9%) (Almeida-da-Silva et al., 2022). Frankincense essential oil and extract exihibit pharmacological activities, including immune system modulation, antimicrobial, antioxidant, wound healing, and anti-inflammatory properties (Prakash et al., 2014). It also stimulates the growth and proliferation of skin cells, making it useful in repairing cuts, wounds, eczema, burns, acne, and pimples (Han et al., 2017). Frankincense can reduce redness and skin sensitivity and is also used for infectious wounds. The main active ingredient in frankincense is the 5-ringed triterpene compound called boswellic acid ($C_{32}H_{50}O_4$), which exerts significant anti-inflammatory effects by inhibiting the 5-lipoxygenase enzyme pathway (Almeida-da-Silva et al., 2022). In addition, boswellic acid has been shown to inhibit the formation of leukotrienes. Frankincense essential oil has demonstrated antimicrobial activity against several microorganisms, including fungi and bacteria (Di Stefano et al., 2020). A combination of frankincense methanolic extract with clarithromycin has also shown synergistic effects against *P. auroginosa* and *S. aureus* (Fahimi et al., 2015). It has been shown that frankincense possesses an anti-inflammatory effect, similar to that of Non-steroidal anti-inflammatory drugs (NSAIDs), without causing long-term gastrointestinal side effects. Another compound in frankincense extract is icnesole, a diterpene with cytotoxic and anti-tumor properties (Hamidpour et al., 2013; Hasson et al., 2011).

Myrrh is a reddish-brown oleo-gum-resin obtainrd from *Commiphora myrrha* (Nees) Engl. (Burseraceae), considered one of the oldest remedies in human history, dating back to 2800 BC according to the medical texts of ancient Egypt. Myrrh contains essential oil, water-soluble gum, and alcohol-soluble resin (Su et al., 2012; Shalaby and Hammouda, 2014). Myrthanol A, a monoterpenoid alcohol found in myrrh essential oil, possesses stronger anti-inflammatory activity than hydrocortisone (Maver et al., 2015; Shalaby and Hammouda, 2014; Basil et al., 2016). Myrrh and frankincense are traditionally used in combination to offer synergistic analgesic, an-

ti-inflammatory, and blood flow-stimulating effects (Su et al., 2012; Su et al., 2015a).

The migration and proliferation of fibroblasts at the wound area have a crucial role in the formation of granular tissue. Among various signaling pathways, extracellular signal-regulated kinase (ERK)1/2 and its phosphorylated form (p-ERK) are known as important mitogenic factors in cell types that regulate cell survival, differentiation, proliferation, migration, and motility (Fujiwara et al., 2014; Ranzato et al., 2010). An increase in the pERK/ERK ratio indicates higher levels of pERK and, subsequently, higher expression of genes involved in cell proliferation.

Herein, we aimed to investigate the wound healing properties and the relevant mechanisms of action of methanol extracts from oleo-gum-resins extracted from *B. sacra* and *C. myrrha,* as well as their combination, on adult human dermal fibroblasts (HDFa).

Material and Method

Plant Material

Frankincense and myrrh oleo-gum-resins were purchased from a reliable local supplier and authenticated by Mrs. Mitra Suzani in the Herbarium of the School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

Preparation of the Extracts

Frankincense and myrrh oleo-gum-resins (1g) were powdered and then macerated in 50 ml of methanol for 24 hours. The solution was subsequently filtered, and the solvent was evaporated to obtain the dry extract. The obtained extract was stored at -20 ˚C until use.

The extraction yield was calculated using the following equation:

Yield %= (W1/W2)×100

Here, W1 represents the weight of the resulting extract, and W2 is the weight of the oleo-gum-resins used for the extraction process.

Cell culture

HDFa, prepared from General Pasteur Institute Cell Bank with NCBI code C646, were cultured in flasks containing high glucose Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% Fetal bovine serum (FBS), 2 mM glutamine, 100 UI/ml penicillin, and 100 µg/ml streptomycin. The cultures were maintained at 37˚C with 5% CO_2. To ensure cell culture survival, the culture medium was refreshed every 2-3 days (Alvarez-Suarez et al., 2016). All procedures were approved by the ethics committee of Mashhad University of Medical Sciences (Approval ID: IR.MUMS.PHARMACY.REC.1398.106).

Frankincense and myrrh were used as the intervention groups: H_2O_2 and Allantoin were used as positive controls in viability analysis and scratch assays, respectively. Untreated cells received an equal amount of solvent and served as the negative control.

Viability assay

A viability assay was performed in order to choose the optimal concentrations of the extracts. Briefly, HDFa cells were cultured in a 96-well plate at a density of 7×10^3 cells/well in enriched DMEM medium for 24 hours (Syarina et al., 2015). The culture medium was then replaced with 100 µl of serial concentrations ranging from 5 to 160 µg/ml of methanol extracts of frankincense (BS) and myrrh (CM), as well as their combination (B.C.). The cells were incubated for 24 hours. As a positive control, some cells were treated with 1 mM H_2O_2 for 1 and 2 hours. Then, all wells were washed once with PBS, and immediately 100 µl of culture medium containing 10% Alamar blue dye was added to the plates before further incubation. After approximately 4 hours, the absorption of all wells was measured at 570^{nm} and 600^{nm} using an ELISA reader.

Detection of ROS

An ROS assay was performed to assess the ability of BS, CM, and BC to reduce ROS levels. HDFa cells were cultured in a 96-well plate at a density of 6×10^3 cells/well and incubated at 37˚C with 5% CO_2 for 24 hours. Then, the medium in each well was replaced with 100 µl of 10 and 20 µg/ml of each extract, and the plate was further incubated for 48 hours. The contents of the wells were then removed, and 100 µl of DCFH_DA reagent, prepared by diluting 5 µl of the reagent in 5 ml of PBS, was added to the wells. The plate was incubated in the dark for 30 minutes. Finally, the fluorescence intensity of samples was measured using a Microplate Reader at an excitation wavelength of 485^{nm} and an emission of 530^{nm}. The percentage of ROS was calculated according to the following formula:

$$\text{Rate of reactive oxygen species \%} = \left(\frac{\text{fluorescence intensity of each sample}}{\text{Average fluorescence intensity of control}}\right) \times 100$$

In Vitro Scratch Assay

To investigate the effect of BS, CM, and BC on the migration of HDFa cells into the scratch area, a cell scratch assay was performed. HDFa cells were seeded in a 24-well plate (2×10^4 cells/well) to achieve the desired cell density. A small linear scratch was made in the middle of each well using a sterile 100 µl plastic pipette tip, and cell debris was rinsed twice with PBS. Subsequently, 10 and 20 µg/ml of BS, CM, and BC were added to the plates before incubating them at 37°C with 5% CO_2. Images of a specific portion of the scratch margin were captured at 0 hours and 24 hours. Allantoin-treated cells served as the positive control, while untreated cells served as the negative control. Each step and imaging were repeated three times, and the average wound closure was determined by measuring the width of the wound and analyzing it with Image J software (National Institute of Health, Bethesda, MD, USA).

Western Blot Analysis

Western blotting was conducted to assess the pERK and ERK (the pivotal proteins in the wound healing assay) ratio. Whole cellular lysate was prepared by gently shaking fibroblasts with a buffer containing 0.9% NaCl, Tris-HCl (20 mM, pH 7.6), triton X-100 (0.1%), 1 mM phenylmethylsulphonylfluoride (PMSF), leupeptin (0.01%), and a cocktail of protease inhibitors. Electrophoresis was conducted on a 12% acrylamide SDS/PAGE gel. ERK and phospho-ERK antibodies were employed to detect the proteins through Western blot analysis. The proteins were electrophoresed, transferred to PVDF membranes, blocked with 5% skimmed milk, and incubated for 12 hours at 4°C with the respective antibody solution (1:1000 v/v). Membranes were then probed with their respective secondary antibodies (1:2000 v/v). The antibody-reactive bands were visualized by enhanced chemiluminescence reagents. The band densities were calculated with UV Band software (Alvarez-Suarez et al., 2016; Raeiszadeh et al., 2018).

Statistical Analysis

Statistical analysis and visualization of the results were conducted using GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, CA). Means ± SD of all data were compared to the corresponding control using one-way ANOVA, followed by Bonferroni post hoc analysis. Significance was defined as $P \leq 0.05$.

Results

Extraction Yield

The extraction yields for CM and BC were found to be 8.68 % w/w and 44.1 % w/w, respectively.

The cytotoxic effects of the methanol extracts of frankincense and myrrh on HDFa cells viability

As shown in figure1, BS, CM, and BC (equal amounts) did not show any cytotoxicity at 10 and 20 µg/ml. Therefore, these concentrations were selected for further evaluation.

The effects of the methanol extracts of frankincense and myrrh on ROS production level

ROS levels in the cells treated with H_2O_2 were significantly increased compared to untreated cells. As shown in figure 2, ROS levels were significantly decreased in HDFa cells treated with BS, CM, and BC (10 and 20 µg/

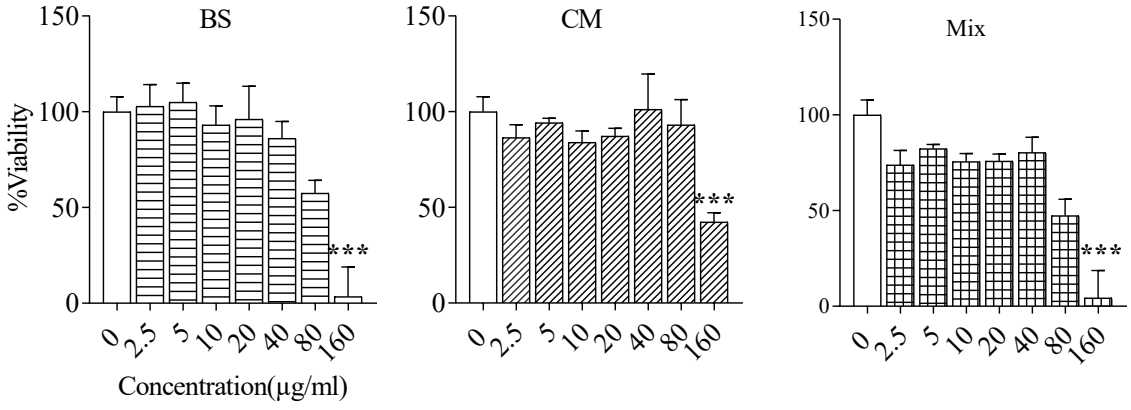

FIGURE 1. Effects of BS, CM, and BC (10 and 20 µg/ml) on HDFa cell viability. Results are presented as mean ± SD of three evaluations. **P*<0.05, ***P*<0.01, and ****P*<0.001 indicate levels of significance compared to the control group.

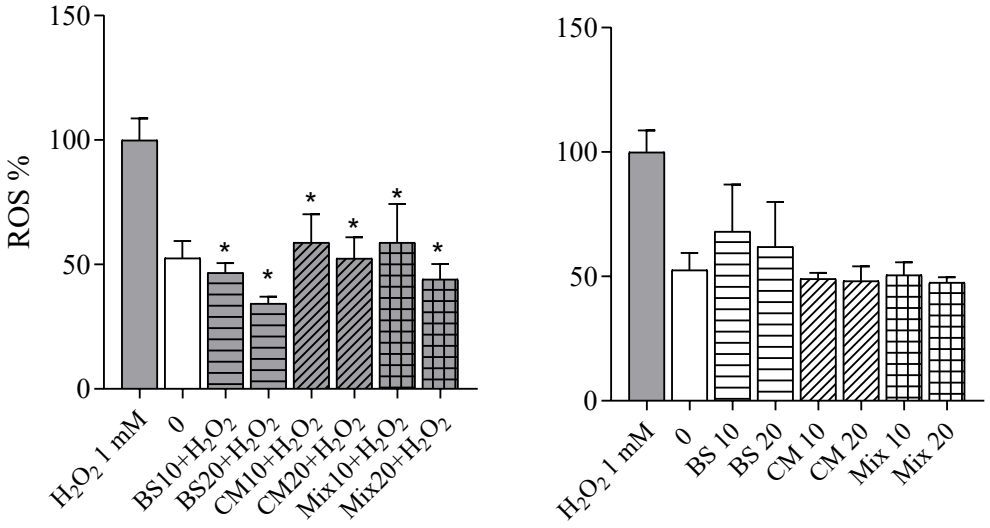

FIGURE 2. Effects of BS, CM, and BC (10 and 20 µg/ml) on ROS production. Results are presented as mean ± SD of three evaluations. **P*<0.001 indicates significant differences compared to H_2O_2-treated cells.

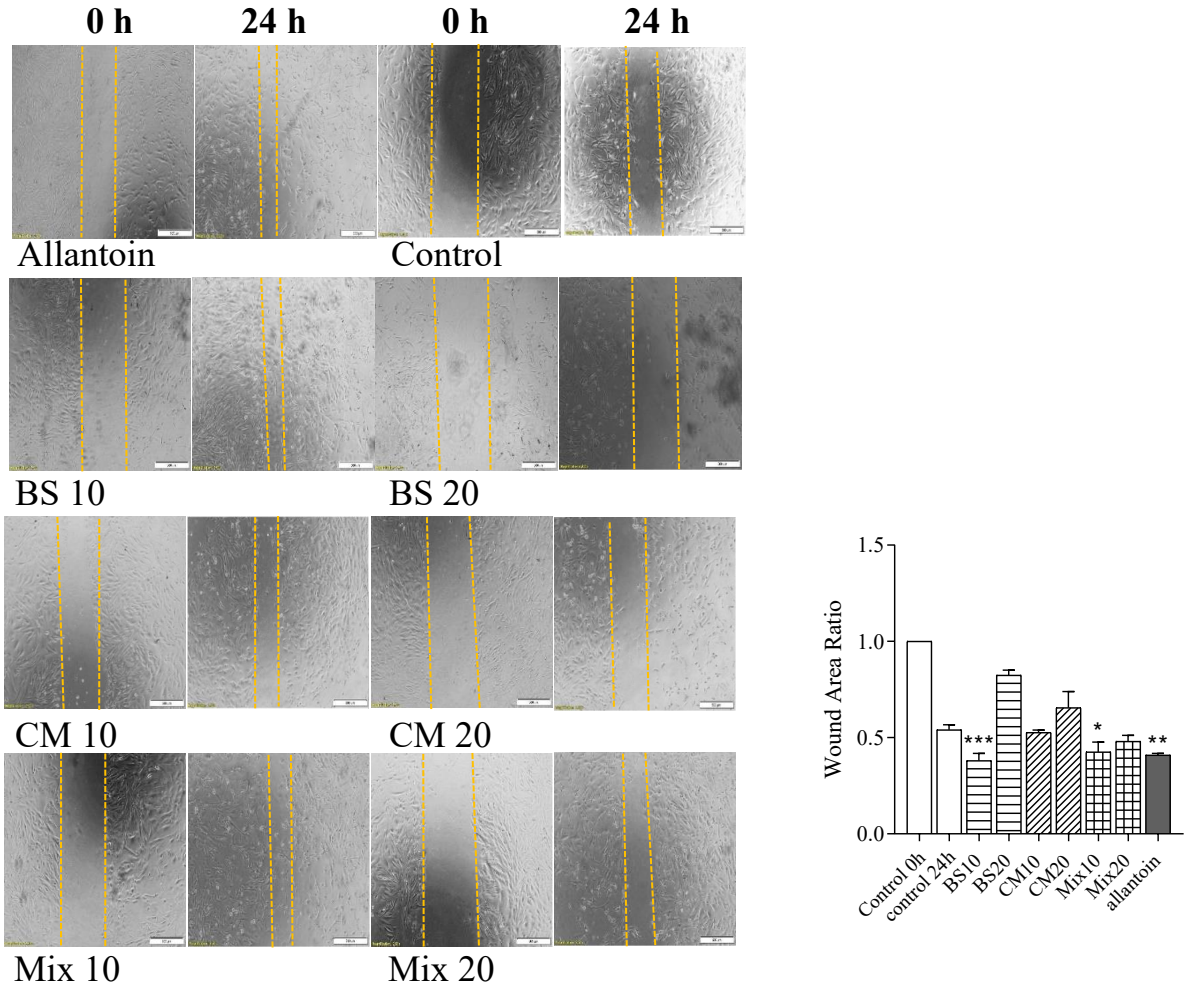

FIGURE 3. Effect of BS, CM, BC, and allantoin on tissue repair, migration, and wound closure in HDFa cells. A. Representative images illustrating the migration of HDFa cells into the scratch wound before and after 24 hours of exposure. B. Effects of BS, CM, BC, and allantoin on scratch wound closure in HDFa cells. Results are presented as mean±SD of three evaluations. **P*<0.05, ***P*<0.01, and ****P*<0.001 indicate levels of significance compared to the control at 24 hours. Frankincense and myrrh were used in the intervention groups; H_2O_2 and Allantoin served as positive controls in viability analysis and scratch assay, respectively. Untreated cells, receiving an equal amount of solvent, were used as the negative control.

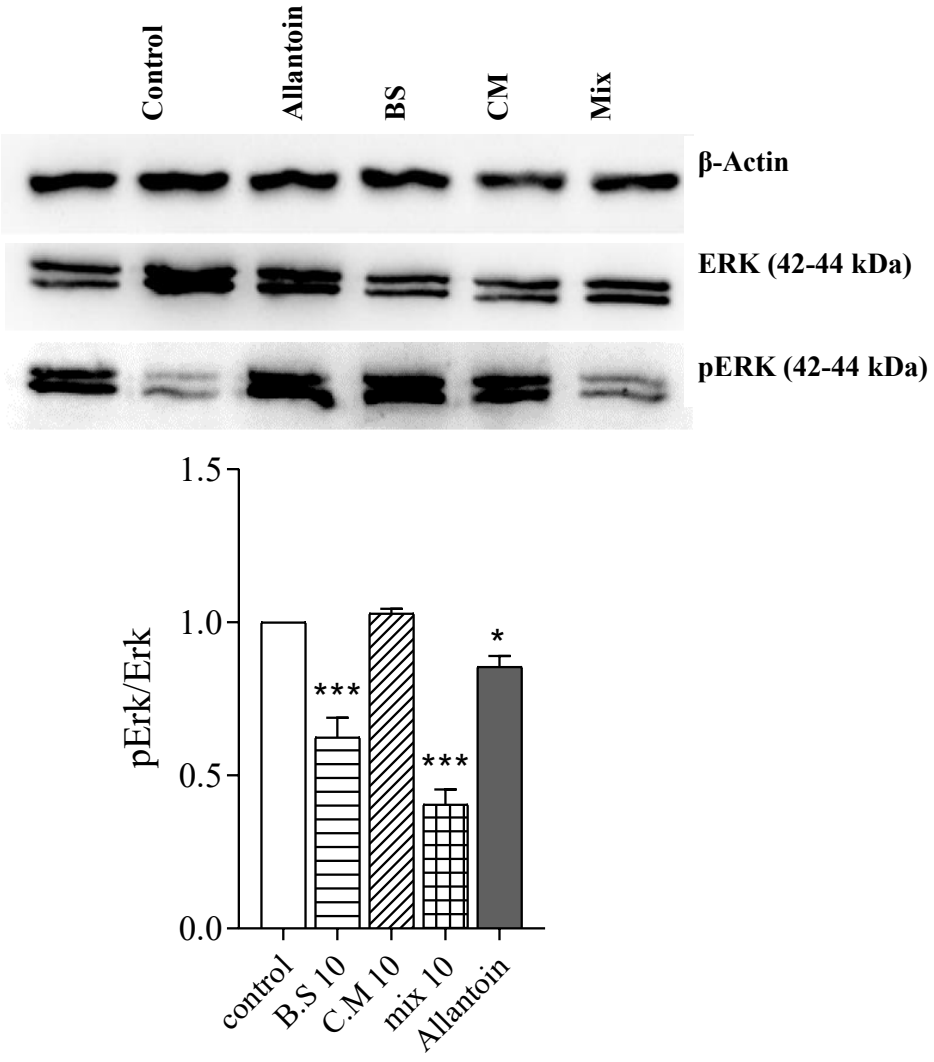

FIGURE 4. Protein expression levels of pERK and ERK in response to exposure to BS, CM, and BC at 10 µg/ml. Results are presented as mean ± SD of three evaluations. *P<0.05 and ***P<0.001 indicate significant differences compared to the control group. Frankincense and Myrrh were used in the intervention groups; H_2O_2 and allantoin served as positive controls in viability analysis and the scratch assay, respectively. Untreated cells, receiving an equal amount of solvent, were used as the negative control.

ml) in the presence of H_2O_2, compared to H_2O_2-treated cells.

The Effects of the methanol extracts of frankincense and myrrh on cell proliferation and migration

As shown in figure 3, allantoin (as positive control), as well as BS and BC (10 µg/ml), significantly reduced the wound area in HDFa cells after 24 hours compared to the control.

The Effects of methanol extracts of frankincense and myrrh on the expression level of ERK and pERK

As shown in figure 4, only CM at 10 µg/ml could significantly increase the ratio of pERK/ERK, even more than allantoin and similar to the untreated control cells.

Discussion

In vitro wound healing assessment is used to observe cell migration in two dimensions and is highly dependent on the second phase of wound healing, involving the migration and proliferation of fibroblasts and keratinocytes (Jonkman et al., 2014; Syarina et al., 2015; Gottrup et al., 2000; Liang et al., 2007).

In our study, exposure of HDFa to H_2O_2-induced oxidative stress resulted in a remarkable decline in cell viability, increased levels of apoptosis, and intracellular ROS production. To determine the optimal concentrations for the subsequent assays, we evaluated the cytotoxic effects of BS, CM, and BC on HDFa cells. Remarkably, BS, CM, and BC, all at equal consecrations, demonstrated no cytotoxicity at 10 and 20 µg/ml. These

concentrations were subsequently chosen for further investigation. In agreement with our results, Rashan et al., also reported no cytotoxic effects for lower concentrations of *B. sacra* oleo-gum-resin extract in HDFa cells (Rashan et al., 2021). Additionally, in immortalized human keratinocytes (HaCaT), acetyl-11-keto-β-boswellic acid (AKBA), the major component of frankincense, has been shown to enhance cell viability and alleviate UVA-induced apoptosis, ROS production, and malondialdehyde (MDA) levels, while increasing the expression of superoxide dismutase (SOD). Moreover, AKBA could modulate the expression of inflammatory molecules such as COX-2, nuclear factor-κB (NF-κB), and NADPH oxidase 1 (NOX1) (Yang et al., 2017).

To investigate the antioxidant effects of BS, CM, and BC, ROS levels were measured using DCFDA reagent. ROS levels were significantly reduced in HDFa cells treated with BS, CM, and BC (10 and 20 µg/ml), both in the presence and absence of H_2O_2, compared to the control group. These results suggest that both the extracts and their combination have the potential to reduce ROS level. In line with our results, previous research demonstrated that frankincense aqueous and hydroalcoholic extracts (30 and 300 µg dwt/mL, respectively) were able to reduce over 60% of H_2O_2-mediated ROS generation in the THP-1 cell line (Kokkiripati et al., 2011). In addition, three furanosesquiterpenoids extracted from *C. erythraea*, a species close to *C. myrrha,* exhibited potent antioxidant activities (with EC_{50} values ranging from 4.28 to 1.08 mg/mL) as well as anti-inflammatory effects, leading to a reduction in the edematous response by 26–32% at a concentration of 0.3 µmol/cm^2 (Fraternale et al., 2011).

The cell scratch assay was conducted to assess the effect of BS, CM, and BC on fibroblast migration to the scratched area. Our results showed that the average wound width in the allantoin-treated group was significantly smaller than in the control group after 24 hours, which was predictable due to the known wound-healing properties of allantoin. Both BS and BC, at a concentration of 10 µg/ml, also reduced the average wound width after 24 hours compared to the control group. However, CM appeared to increase fibroblast migration to the wound area and decrease the wound wide, but this effect was not significant, possibly due to the relatively short duration (24 hours) of the scratch assay. In agreement with our results, Jahandideh et al., reported wound healing effects of a poly herbal paste (PHP) containing *Aloe vera* gel, *C. myrrha,* and *B. carteri* (a close species to *B. sacra*) oleo-gum-resins using a rat excision wound model. Their results showed a significant wound healing effect in PHP 10%-treated animals 7 days after treatment ($p<0.05$). In addition, PHP 10% demonstrated significantly greater wound healing effects compared to the control, tetracycline, and paste base-received animals on days 2, 14, and 21 ($p<0.05$). Notably, on 14th day, PHP 40% demonstrated significantly more healing activity when compared to the tetracycline (control) and paste base-treated animals ($p<0.05$). The study observed fewer inflammatory cells, greater re-epithelialization with remarkable neovascularization, and increased antioxidant activity in PHP 10%-treated animals (Jahandideh et al., 2017). In a clinical trial by Faraji et al., the efficacy and safety of sitz-baths containing myrrh and frankincense on episiotomy wound healing were investigated. Subjects received either a 10-minute bath of myrrh or frankincense extract (intervention group) or a betadine bath (control group) twice a day for a week. The results showed that myrrh significantly improved episiotomy wound healing compared to frankincense or betadine on days 2, 7, and 14 (Faraji et al., 2021). In another clinical trial conducted by Vaziri et al., the effects of a 5% gel containing *B. carteri* oleo-gum-resin (Frankincense) and a hydrocolloid dressing on the improvement of pressure ulcers in hospitalized patients were compared for five weeks. The ulcers were assessed using the Pressure Ulcer Scale for Healing (PUSH). It was shown that the ulcer size score significantly decreased in the Frankincense gel-treated group compared to the control ($P<0.014$). Moreover, Transforming Growth Factor (TGF)-β1 and TGF-β3 levels were higher in Frankincense-received patients (Vaziri et al., 2021).

The study by Tsung-Jung et al., showed that a herbal ointment containing frankincense and myrrh extracts (200, 20, and 2 µg/mL) which is used to treat diabetic foot ulcer infection could stimulate cell proliferation, migration, and angiogenesis (Ho et al., 2016).

The wound healing effects of these extracts may be contributed to their anti-inflammatory properties. Su et al., reported that a combination of frankincense and myrrh extracts significantly protected against adjuvant-induced arthritis (AIA) in rats. This combination also improved elevated levels of inflammatory cytokines including TNF-α and IL-2, PGE2, NO, and MDA,

by regulating the MAPK signaling pathway (Su et al., 2015b).

As suggested by previous studies, it is essential to understand the mechanisms behind the wound healing effects of BS, CM, and BC. To investigate this, we conducted a Western blot analysis to explore the potential involvement of the ERK signaling pathway in cellular proliferation, differentiation, migration and wound healing induced by theses extracts.

The Western blot analysis revealed that, in H_2O_2-induced cells, only CM at 10 µg/ml significantly increased the pERK/ERK ratio compared to the control. This indicates the pivotal role of ERK1/2 activation in the effects of CM on cell proliferation, migration, and the wound healing process. In contrast, BS and BC did not activate ERK1/2 within the 24-hour study period, however, it is important to note that ROS inhibition and wound healing, as demonstrated through the scratch assay, were conducted over 48 hours. This suggest that BS and BC might improve cellular proliferation, differentiation, and migration through signaling pathways other than ERK1/2. Multiple pathways including MAPK, phosphoinositide 3-kinase (PI3Ks), Ras, and PKC, are involved in cell migration and could be responsible for the wound healing effects of frankincense (Lee et al., 2018). While the wound healing effects of the tested extracts have been previously investigated in vivo, no mechanistic approach was performed. To the best of our knowledge, this is the first study to assess the involvement of ERK in HDFa cell proliferation, migration, and the wound healing process induced by these extracts. Subsequent research can delve into the effects of frankincense on these signaling pathways. The findings of this study suggest that a combination of BS and BC methanol extracts can be effective in the wound healing process. This opens the door to the development of local wound healing formulations and the investigation of their effects through human studies and clinical trials. Moreover, this combination may be beneficial in the repair and healing of cuts, wounds, eczema, burns, acne, and pimples.

A limitation of our study was the absence of identifying the primary active ingredients in both extracts and their combination. This information would have been valuable in understanding how these ingredients contribute to the wound healing activities. Moreover, further in vivo investigations into the mechanisms underlying the wound healing effects of the tested extracts would greatly enhance the support for our findings.

Conclusion

Our findings demonstrate that BS, CM, and BC can significantly reduce ROS levels both in the presence and absence of H_2O_2 when compared to the control group. Additionally, they are effective in reducing the average wound width. Notably, CM significantly enhances the pERK/ERK ratio, highlighting the essential role of ERK1/2 phosphorylation in the positive effects of CM on cell migration, proliferation, and the wound healing process.

These results strongly indicate the beneficial effects of frankincense, myrrh, and their combination in enhancing proliferation, migration, and the wound healing process in HDFa.

Conflict of interest

The authors declare no conflicts of interest.

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Ethical approval

All procedures were approved the ethics committee of Mashhad University of Medical Sciences. Approval ID: IR.MUMS.PHARMACY.REC.1398.106.

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