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RESEARCH ARTICLE

Molecular effect of Silver nanoparticles on wound healing activity of Salvia officinalis extract in adult mice

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ABSTRACT

Objective(s): This study assessed how Silver nanoparticles (Ag NPs) and hydroalcoholic extract of *Salvia officinalis* (Sage) influence the expression levels of the vascular endothelial growth factor (*VEGF*) and matrix metalloproteinase 2 (*MMP2*) genes, which play a role in wound healing.

Methods: An excision wound was induced on the back of the 48 adult male mice. Wound treatments done with AgNPs and *Salvia officinalis* extract in separate animal groups for 14 days. On two weeks after treatment, the wound skin tissue was removed and gene expression analysis was done by real-time polymerase chain reaction.

Results: The results showed that the expression of both target genes (*VEGF* and *MMP2*) in the wound skins treated with 0.05% Ag NPs increased significantly compared with the wound skin of control. The expression of *VEGF* gene increased significantly in the wound tissues treated with Sage extract compared with the Vaseline group, but the expression of *MMP2* gene didn't change significantly. The expression of two target genes increased significantly in the wound tissues treated with 0.5% Sage extract plus 0.05% Ag NPs in comparison to the wound tissues treated with 0.5% Sage extract alone. The expression of the two target genes did not significantly differ in the wound tissues treated with Sage extract and Ag NPs compared to those treated with 0.05% Ag NPs alone.

Conclusions: Based on the results above, it can be concluded that the combination of hydroalcoholic extract of sage and low doses of Ag NPs exhibits significant healing activity and could serve as a viable option for wound healing management.

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INTRODUCTION

Nanotechnology, or nanomedicine, is an emerging and vital field in medical applications, providing advanced therapies for various diseases, including wound healing. Nanotechnology has emerged as a promising approach for wound healing. For example, wound dressings coated with metal nanoparticles demonstrated excellent antibacterial properties and significantly contributed to the wound healing process (1). Nanoparticles (1 to 100 nm in size) like silver, copper, cerium, zinc oxide, and carbon-based materials have shown antibacterial, anti-inflammatory, and cell proliferation properties that are beneficial for wound healing (1, 2). Additionally, nano-drug delivery systems have been created to improve the therapeutic effectiveness of medications, sustain drug release, and accelerate wound healing processes (3). These nano-drug delivery systems can attach bioactive molecules to the affected area, facilitating a conducive environment for cell migration, proliferation, and angiogenesis, thereby improving the overall healing quality of wounds (3). Metal nanoparticles have exhibited excellent bactericidal activity against multidrug-resistant bacteria (3). Nanoparticles have unique properties such as high strength, shape-shifting

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ability, and a high strength-to-weight ratio. Silver nanoparticles are utilized as an antimicrobial agent in wound dressings and have proven effective against microbial infections (4). The use of silver in wound dressings has demonstrated benefits that extend beyond its antimicrobial properties, such as anti-inflammatory effects and the promotion of tissue regeneration (5). Silver nanoparticles have attracted attention for their improved antimicrobial properties compared to traditional silver forms. These nanoparticles have a larger surface area, which increases their interaction with microorganisms and results in a more effective antimicrobial action (6). One of the major challenges related to the use of Ag NPs in wound dressings is the development of bacterial resistance to these nanoparticles. Continuous research is needed to develop new combinations of nanomaterials, antibiotics, and polymers to overcome this issue (7).

Plant-based compounds have shown promising results in increasing the rate of wound healing by stimulating fibroblast proliferation, collagen synthesis, and re-epithelialization, which are crucial steps in the wound repair process (8). *Salvia officinalis*, also known as common Sage, is a perennial, evergreen subshrub that belongs to the mint family Lamiaceae and is native to the Mediterranean region (9). *Salvia officinalis* contains various phytochemicals that have been studied for their potential effects on pain and inflammation. These compounds include phenolic acids, flavonoids, terpenoids, and essential oils, which have demonstrated anti-inflammatory and analgesic properties (10, 11).

The skin is the largest organ in the human body, and it serves several important functions. A skin wound is characterized by a disturbance in the normal structure and function of the tissue. Various causes lead to the formation of different types of wounds. These include surgery, trauma, pathological changes in the body, and pressure (12). Wound healing is a dynamic and natural biological process that encompasses several phases: hemostasis, inflammation, proliferation, and remodeling. Many studies have highlighted the potential of plant-based remedies in enhancing these phases and expediting the healing of wounds (12). Mouse models remain valuable in skin wound healing research because they effectively mimic the human wound healing process, including various cell types, environmental cues, and paracrine interactions (13). The wound healing process is influenced by various factors, such as growth factors, cytokines, chemokines, and the extracellular matrix. The controlled release and interaction of growth factors and cytokines are fundamental in the intricate process of wound healing (14, 16). The interplay of growth factors and cytokines in wound healing is crucial for the various phases of the healing process. These molecules help regulate cell growth, migration, and differentiation, ultimately leading to effective wound closure and tissue repair (15, 16). Advancements in understanding the roles of growth factors and cytokines have enabled researchers to devise strategies for delivering these factors effectively to promote and expedite the process of tissue regeneration, thereby enhancing wound healing outcomes (14).

VEGF-A, also known as VEGF, (Vascular endothelial growth factor) is a potent angiogenic factor that was first described as an essential growth factor for vascular endothelial cells (17). VEGF also plays a role in normal physiological functions such as bone formation, hematopoiesis, wound healing, and development. In wound healing, VEGF plays a key role in forming new blood vessels and attracting immune cells to the injury site (18). The expression patterns of VEGF-A mRNA are closely associated with blood vessel proliferation during embryonic development and wound healing (19). The matrix metalloproteinase (MMP) family of proteins, including over 25 enzymes, are zinc-dependent endopeptidases that play a crucial role in degrading various components of the extracellular matrix (ECM). These enzymes can process or degrade a wide range of targets, such as proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, growth factors, cell surface receptors, and structural ECM proteins (20, 21). MMPs are involved in regulating numerous biological processes, including cell behavior, tissue morphogenesis, wound repair, inflammatory diseases, and more (21). They are known to influence cell proliferation, migration, differentiation, angiogenesis, apoptosis, and host defense (21). The MMP family is tightly regulated through mechanisms like transcription, secretion, activation, inhibition, localization, and clearance, highlighting their importance in various physiological and pathological processes (20, 21).

Sage extract has demonstrated anti-inflammatory, antimicrobial, and antifungal properties, making it a potential treatment for infected wounds. Silver nanoparticles have shown promise in wound healing by potentially accelerating

the process through reducing inflammation and promoting tissue formation. Nanoparticles have been demonstrated to affect the expression of genes that play a role in wound healing, highlighting their potential role in modulating cellular responses and promoting the healing of wounds (21). This indicates that nanoparticles may significantly regulate the genetic mechanisms involved in wound healing, potentially improving the effectiveness of wound healing therapies. In this study, we seek to assess the molecular impact of silver nanoparticles and sage extract on the gene expression of *VEGF* and *MMP2*, which play a crucial role in the wound healing process.

MATERIALS AND METHODS

Plant material and chemicals

The powdered plant extract of Sage (*Salvia Officinalis*) was purchased from an Agro-industry and medicinal plants processing company (Soha jissa, Iran). According to the Company data sheet, HPLC chromatogram analysis indicates the presence of phenolic components in the *Salvia officinalis* extract namely the Rosmarinic acid as the major phenolic compound (Figure 1).

The solvents used purchased from Merck and all chemicals purchased from Sigma. Ag NPs (20 nm) purchased from US Research Nanoparticles, Inc. (Houston, USA). TEM image of Silver nanoparticles provided by Company have been shown in Figure 2.

Ointment processing

The simple ointment base was Vaseline. For plant test ointments, appropriate amounts of Sage plant extract (0.5% and 5.0% (w/w) added to the simple ointment base. For the nanoparticle test ointments, 0.05% and 0.1% of powdered Ag NPs

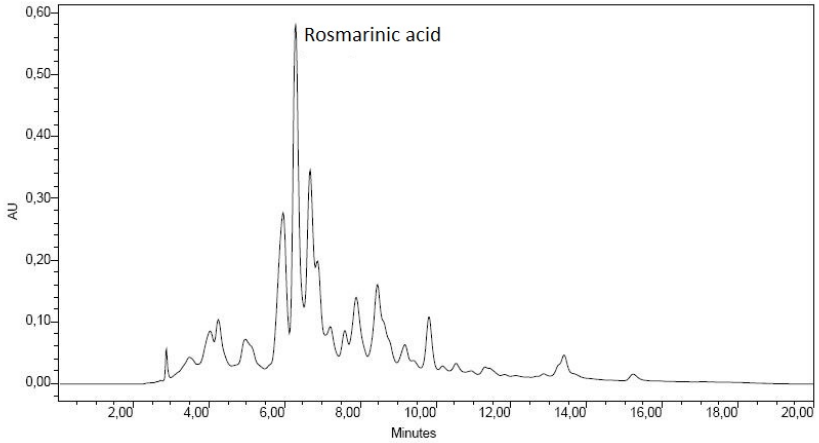

Fig. 1. HPLC chromatogram of sage extract

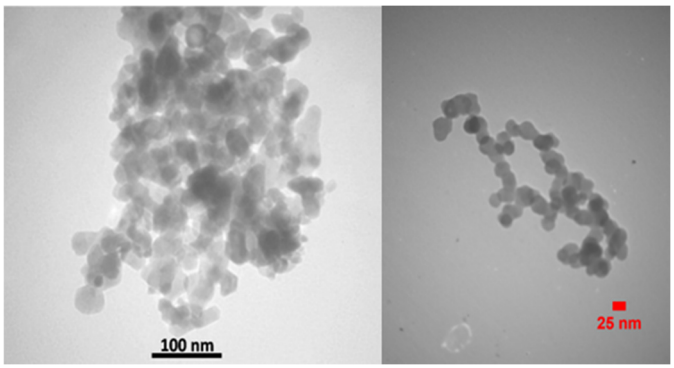

Fig. 2. TEM images of Ag NPs used in this study

added to the simple ointment base.

A combination ointment was also prepared by adding 0.5% Sage extract plus 0.05% Ag NPs to the simple ointment base. The wound areas treated with the ointments or reference drug (1.0% phenytoin) topically once a day, depending upon group assignment for 14 days.

Animal groups and experimental protocol

In this experimental study, 48 male mice, with a weight range of 25-30 g, purchased from Jondishapour Ahvaz University of Medical sciences. All animal experimentations conducted by institutional guidelines for animal care and use, which adhered to the international principles of Laboratory Animal Care (NIH publication #85-23, revised in 1985). This study was authorized by the Medical Ethics Committee of Shahid Chamran University of Ahvaz with the number IR.SCU. REC.1402.063

The animals transferred to the animal house and kept in cages at a controlled temperature of 22±2 °C and humidity 50±5 % under a 12h/12h dark/light cycle. They had free access to food and water. After a week the mice randomly divided into eight groups of six animals. The experimental design of the treatment is detailed in Table 1.

Wound induction and Animal treatment

All mice anesthetized with Ketamine (50 mg/kg) and Xylazine (10 mg/kg), administered intraperitoneal. Briefly, the hairs of the mice shaved, the exposed skin area was cleaned with 70% ethanol, and a full-thickness skin wound (1 cm in diameter) was created on the back of each mouse (Figure 3).

As shown in Table 1 the mice randomly divided into eight groups of six animals and treated at time zero with a low and high concentration of Ag NPs, low and high concentration of Sage extract, Ag NPs plus Sage extract, and Vaseline as control). In all groups, treatments applied with topical ointment as described in Table 1. After 14 days all animals euthanized with chloroform. The wound skin tissues excised and stored at -70 ˚C.

Gene expression analysis

Total RNA was extracted from the skin wound

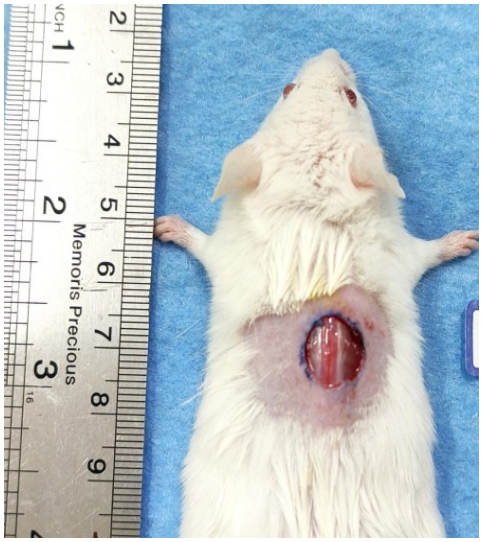

Fig. 3. An excision wound with a 1 cm diameter was created on the back of the mouse

Table 1. Grouping and number of treated mice

Groups	Negative control	Vehicle control	Test					Positive control
Animal Treat	Non-treated	Vaseline	Sage ext. 0.5%	Sage ext. 5.0%	Ag NPs 0.05%	Ag NPs 0.1%	Sage ext. 0.5%+Ag NPs 0.05%	Phenytoin 1.0%
Number	6 mice	6 mice	6 mice	6 mice	6 mice	6 mice	6 mice	6 mice

tissue and stored at −70 °C using the Favorgen Total RNA purification kit (Favorgen-Taiwan). RNA samples quantified by spectrophotometer (Nanodrop 2000, Germany). RNA samples exhibiting an A260/A280 ratio of more than 1.8 used for the cDNA synthesis. PrimeScript ™ RT reagent kit (Takara Bio Inc., Japan) was used to synthesize cDNA according to the reverse transcription method. The specific primers of the *GAPDH* gene (as an endogenous standard) and *VEGF* and *MMP2* genes (as target genes) designed by primer 3 (Table 2).

Quantitative real-time polymerase chain reaction (PCR) was done using SYBR PremixExTaq II (Tli RNaseH Plus) kit (Takara BioInc., Japan) on Lava96 Real-time PCR Detection System (DaanGene Co. Ltd) to determine relative amounts of target genes against standard gene. The thermal profile was 50 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 30 s. Real-time PCR was performed in duplicate for every cDNA. Expression in wound tissues was treated with plant extract, silver nanoparticles, and the both of them compared with the control (Vaseline-treated tissues) after normalization with *GAPDH*.

Statistical Analysis

Statistical analysis was done using the GraphPad Prism (Version 8) software. One-way analysis of variance (ANOVA) was employed to compare means in different groups with each other. The Least Significant difference analysis was used at a probability level of $P<0.05$ to determine differences in treatment levels.

RESULTS

A mouse wound healing model with a 1 cm diameter excision wound was used to evaluate the impact of the Ag NPs, Sage extract, Ag NPs plus Sage extract, and Vaseline (Control) on the wound treatment process. Wound healing was analyzed daily and selected wound images on the day of zero, 7, and 14 are shown in Figure 4. The results showed that the wound treated with Sage extract 0.5%, Ag NPs 0.1%, and Sage 0.5% + Ag NPs 0.05% healed at a faster rate on days 7 and 14, indicating these components accelerated the wound healing in these groups (Figure 4).

We assessed the relative expression levels of the *VEGF* and *MMP2* genes in skin tissues that were treated with sage extract (0.5%, 5.0%), Ag

Table 2. Primers used in real-time PCR

Primer	Sequence (5'>3')	GenBank ACC	Product length
Gapdh-F	ATGACTCTACCCACGGCAAG	NM_001289726.1	
Gapdh-R	CTGGAAGATGGTGATGGGTT		
mmp2-mice-F	CAAGGATGGACTCCTGGCACAT	NM_008610	139 bp
mmp2-mice-R	TACTCGCCATCAGCGTTCCCAT		
vegf-mice-F	CTGCTGTAACGATGAAGCCCTG	NM_001025250	119 bp
vegf-mice-R	GCTGTAGGAAGCTCATCTCTCC		

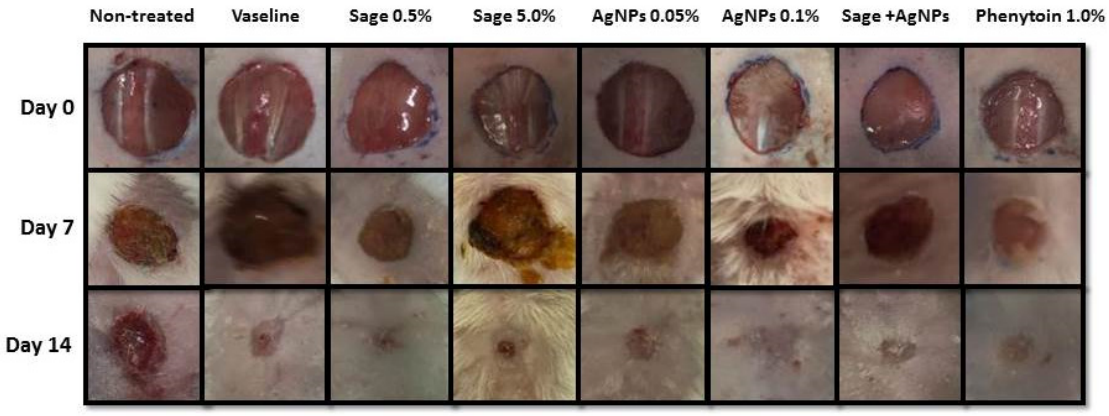

Fig. 4. The effect of treatments on 8 experimental animal groups. The progress of wound healing was digitally photographed on days 0, 7, and 14.

NPs (0.05%, 0.1%), and Sage extract 0.5% plus Ag NPs 0.05%. Our findings indicated a significant ($p < 0.01$) increase in VEGF transcript expression the wounds treated with Sage extract compared to the non-treated and Vaseline groups (Figure 5). Also our results showed the level of *VEGF* transcript expression was significantly ($p < 0.001$) increased in the excision wounds treated with Ag NPs (0.05 and 0.1%) compared with the non-treated and Vaseline groups. The comparison between the composition of Ag NPs (0.05%) and Sage (0.5%) compared to Ag NPs (0.05%) did not show a significant difference (P= 0.99).

The level expression of *MMP2* transcript increased significantly ($P < 0.05$) in the wound treated with Sage extract compared with the non-treated group, but no significant change was seen in *MMP2* mRNA expression in the wounds treated with Sage extract compared with the Vaseline group. (P= 0.97) (Figure 6). Also our results showed the level of *MMP2* transcript expression was significantly ($p < 0.001$ and $P > 0.01$) increased with Ag NPs (0.05 and 0.1%) compared with the non-treated and Vaseline groups, but the comparison between the composition of Ag NPs (0.05%) and Sage (0.5%) compared to Ag NPs (0.05%) did not show a significant difference (P= 0.79). The drug phenytoin (1.0%) had the most pronounced effect on the expression of both target genes (Figure 5, 6).

It is worth noting that increasing the concentration of Sage extract from 0.5% to 5.0% led to a significant upregulation in the expression of both *VEGF* and *MMP2* genes ($P< 0.05$) (Figure 7A, B). While increasing the concentration of Ag NPs from 0.05% to 0.1% resulted in a significant reduction in the *MMP2* gene expression (Figure 6).

The mRNA expression of both target genes (*VEGF* and *MMP2*) in the wound tissues treated with Sage extract plus Ag NPs increased significantly ($P < 0.05$) in comparison to the wound tissues treated with Sage extract alone (Figure 7A, B). These results show that sage in the presence of Ag NPs shows a better healing effect by increasing the expression of two target genes.

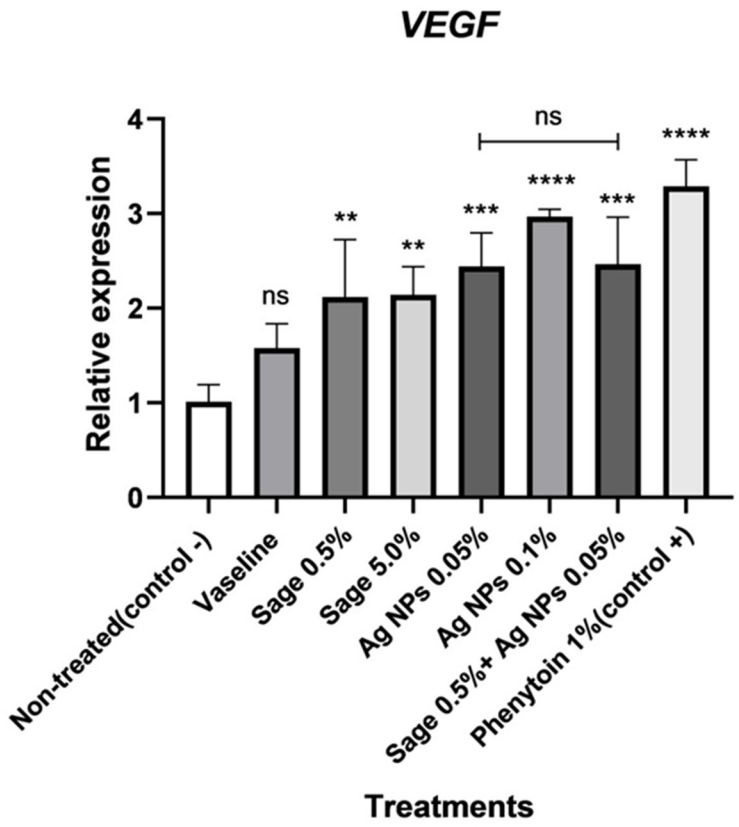

Fig. 5. Changes in the relative expression of *VEGF* gene in mouse wound skins treated with Sage extracts and Ag NPs after 14 days. (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. control group, ns. no significant

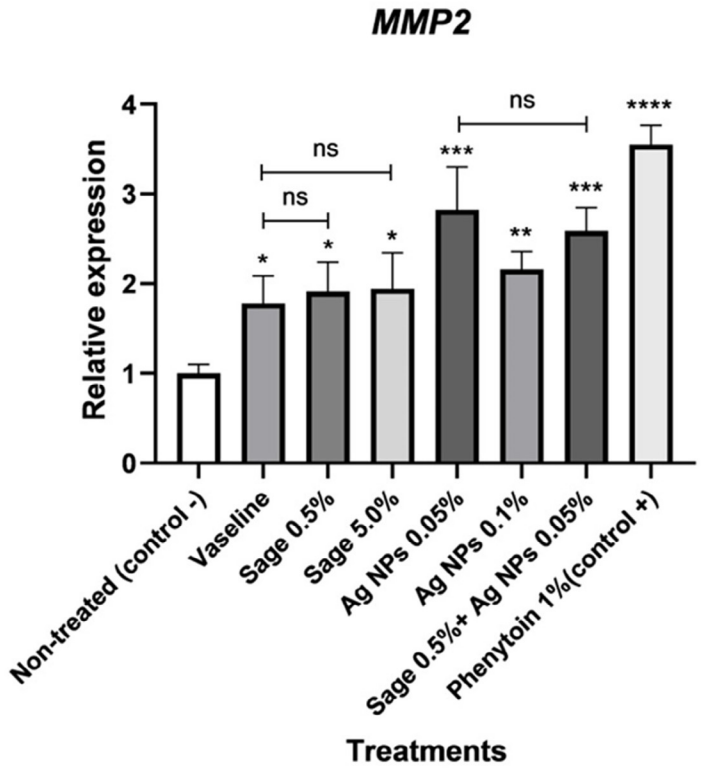

Fig. 6. Changes in the relative expression of *MMP2* gene in mouse wound skins treated with Sage extracts and Ag NPs after 14 days. (*P < 0.05, **P<0.01, ***P < 0.001, ****P < 0.0001 vs. control group, ns. no significant)

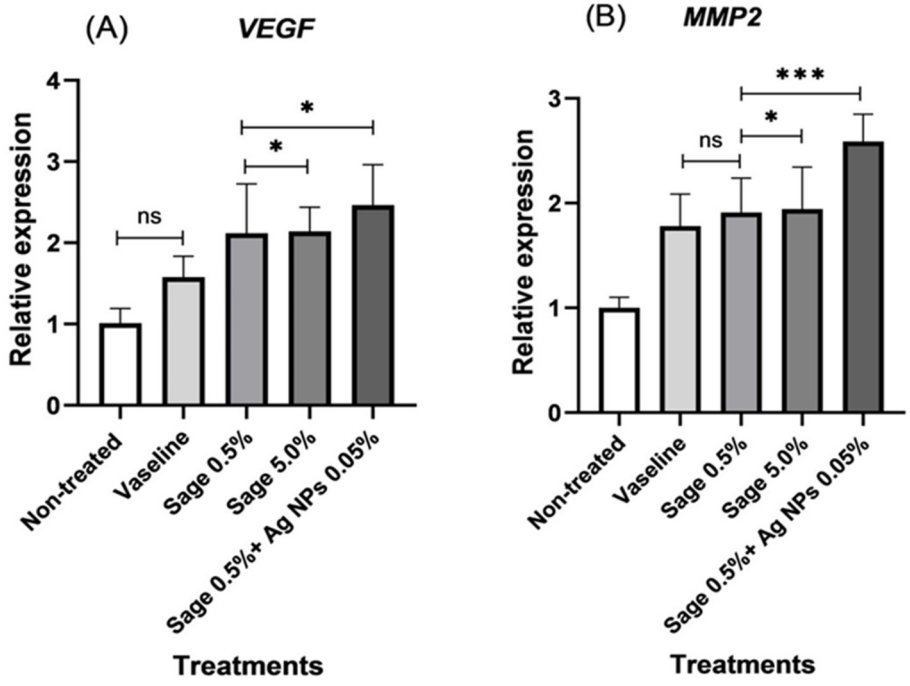

Fig. 7. Relative expression of *VEGF* (A) and *MMP2* (B) genes in different concentrations of Sage extract in the presence of Ag NPs. (*P<0.05, ***P < 0.001 vs control, ns. no significant)

DISCUSSION

In the present study, we evaluated the impact of Ag NPs and *Salvia officinalis* (Sage) extract on the expression levels of *VEGF* and *MMP2* genes during the wound healing process. Our results indicated that sage extract accelerated wound healing by enhancing the expression of *VEGF*, while silver nanoparticles further boosted this process by increasing the expression of both *VEGF* and *MMP2*. Also, Sage extract showed a better healing effect in the presence of Ag NPs than alone.

Some study indicates that Ag NPs can improve the antibacterial and wound- healing effects of plant-derived extracts. Paladini and Pollini (2019) found that Ag NPs synthesized using Azadirachta indica (AI) extract (AI-Ag NPs) had potent antibacterial activity and also promoted wound-healing in in vivo experiments (22). The authors suggest that AI-Ag NPs able to disrupt bacterial cells while also actively participating in wound-healing processes through free-radical scavenging and modulation of inflammatory cytokines (22). Therefore, the available evidence indicates that Ag NPs can enhance the wound- healing properties of plant extracts, likely through a combination of improved antimicrobial effects and direct stimulation of wound- healing processes like re-epithelialization and collagen deposition.

The combination of Ag NPs with plant extracts can provide a synergistic effect, resulting in better wound healing outcomes and enhanced antimicrobial activity (23). Liu et al. (2021) modified silver nanoparticles with Aloe Vera, which not only enhanced the antibacterial properties of the nanoparticles but also imparted the ability to promote cell proliferation and migration (24).

Many studies have shown the green synthesis of Ag NPs using the Sage and assessed the activity of these nanoparticles in the wound healing process. Baharara et al. (2017) evaluated the antioxidant and anti-inflammatory activity of green synthesized Ag NPs using Salvia officinalis extract and showed that AgNPs increased IL-8 and TNF-α genes expression in MCF-7 cells (25).

Ag NPs at low concentrations demonstrate a wide range of antibacterial activity while remaining non-cytotoxic to cells at bactericidal levels. Mohanty et al. (2012) found that Ag NPs possess strong antibacterial properties and are non-cytotoxic to macrophages at bactericidal concentrations. Furthermore, Ag NPs serve as a promising template for developing antibacterial agents aimed at targeting bacterial colonization and addressing drug resistance. (26).

Lin et al. (2016) demonstrated that silver-containing dressings not only decreased bacterial load during the inflammatory phase but also enhanced wound healing in the proliferation phase by stimulating collagen-1 production (27).

Our findings revealed that the mRNA expression levels of the *VEGF* and *MMP2* genes in wound tissue treated with Ag NPs were significantly higher compared to the control groups. However, when the concentration of Ag NPs was increased from 0.05% to 0.1%, there was a notable decrease in *MMP2* gene expression. This decline may be attributed to the cytotoxic effects of Ag NPs, as several studies have shown that Ag NPs possess cytotoxic properties. (28-30).

Previous research has demonstrated increased transcription and secretion of *VEGF* in full-thickness skin wound models (31). This rise in *VEGF* expression is essential for facilitating wound healing, as it promotes angiogenesis, collagen deposition, and epithelialization, particularly in full-thickness wounds.

Loo et al. (2022) demonstrated that Ag NPs, particularly chitosan-based nanoparticles, can enhance *VEGF* mRNA expression in the wound tissues, indicating their potential to accelerate the healing process. The upregulation of *VEGF* by Ag NPs highlights its role in enhancing tissue repair and promoting the closure of non-healing wounds, especially in conditions like arterial occlusive disease and diabetes (32).

Liu et al. (2010) showed that the use of silver nanoparticles promotes the healing process by directly stimulating the expression of collagen and specific growth factors, which facilitates re-epithelialization, vasculogenesis, and the deposition of collagen fibers (33).

Matrix metalloproteinase-2 (MMP2) is crucial in various processes related to wound healing, including angiogenesis, inflammation, and fibrosis. Elevated levels of *MMP2* expression have been observed after skin injury, indicating its involvement in the healing cascade (34).

Our results showed that the Sage extract had a relative impact on the expression of MMP2 gene. It appears that various other factors involved in wound healing also influenced the

expression of this gene.

Several studies indicate that the expression and activity of matrix metalloproteinases (MMPs)

significantly increase upon injury, with evidence supporting elevated gene expression during wound repair. Following injury, MMPs are secreted by inflammatory cells to cleanse the wound from damaged extracellular matrix (ECM) and tissue (35, 36). Our results align with previous studies that have demonstrated the upregulation of *MMP2* expression and activity in response to skin injury, highlighting the essential role of *MMP2* in processes critical for wound- healing.We also demonstrated that the level of *VEGF* mRNA expression significantly increased in excision wounds treated with *Salvia officinalis* extract. *Salvia officinalis* has been shown to accelerate the wound healing process, as evidenced by studies demonstrating its beneficial effects on wound repair. The topical administration of *Salvia officinalis* essential oil has been found to promote wound healing by modulating the expression of pro-inflammatory cytokines, growth factors, and antioxidant properties (37, 7).

CONCLUSION

According to the above results, it can be concluded that the combination of hydroalcoholic extract of sage in presence of low doses of Ag NPs exhibits significant healing activity and could be used as a candidate for wound healing management. By evaluating the efficacy, safety, and potential benefits of plant-derived nanomaterials and biomaterials in wound healing, this study can provide a valuable approach to optimize wound care and healing strategies.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

IR.SCU.REC.1402.063

AVAILABILITY OF DATA AND MATERIAL

Not applicable.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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