

Asparagus Officinalis: An Herbal Candidate for an Intraoral Healing Mouthwash

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(Submitted: 12 September 2020 – Revised version received: 01 February 2021 – Accepted: 021 February 2021 – Published online: Winter2021)

Objectives Asparagus officinalis (*A. officinalis*) extract has several bioactive ingredients. This study assessed the healing effects of *A. officinalis* methanolic extract.

Methods In this experimental study, after preparing the methanolic extract of *A. officinalis* with a concentration of 100µg/mL, its bioactive ingredients were determined using high-performance liquid chromatography (HPLC) and then its cytotoxicity was assessed using the methyl thiazolyl tetrazolium (MTT) assay. Five experimental groups with 25 samples were assessed as follows: (I) human gingival fibroblast (HGFs) cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM), (II) same as group I but with 10 µg/mL methanolic extract of *A. officinalis*, (III) same as group I but with 25µg/mL methanolic extract of *A. officinalis*, (IV) same as group I but with 50 µg/mL methanolic extract of *A. officinalis*, and (V) same as group I but with 100µg/mL methanolic extract of *A. officinalis*. Cell motility in the control group and group V was examined quantitatively using the cell scratch assay at 24 h. We used one-way ANOVA and t-test to analyze the cytotoxicity of *A. officinalis* extract and the motility of HGFs, respectively.

Results The MTT assay showed no significant difference in cell viability among the experimental groups ($P=0.07$). A remarkable cellular wound closure equal to 60.85% was noted after 24 h.

Conclusion The methanolic extract of *A. officinalis* with a concentration of 100 µg/mL showed significant healing effects on an experimental scratch setup of HGFs.

Keywords Wound Healing; Fibroblasts; Asparagus Plant; Herbal Medicine

Introduction

Intra-oral mucosal healing has been one of the most critical issues in dentistry and regenerative medicine. Several systemic diseases and local factors such as trauma, and fungal and viral infections can induce or aggravate intra-oral wounds and ulcers. These factors can exert their adverse effects via several mechanisms. Physical or chemical damage to the epithelial cells, local inflammation induction, bacterial load increase resulting in acute or chronic infections, viral invasion to the epithelial cells, and any change in cell cycle are among the etiologic or exacerbating factors for intra-oral wounds.^{1, 2} Surgical wounds are among other types of wounds to be considered. Nowadays, many surgical processes can only be done via intra-oral incisions which would require further healing in patients. Multiple cell types are involved in the wound healing process, such as fibroblasts that have a moderate proliferative rate necessary for tissue re-organization and regeneration.³ On a broader perspective, oral ulcers can decrease the quality of life in addition to causing pain and discomfort for the patients. They can impair nutrition, complicate oral hygiene, and impair speech. Malignancies can also cause life-threatening ulcers.⁴ Aside from the etiology of oral ulcers, different interventions have been suggested to decrease oral ulcer symptoms such as using immunosuppressive medications, anti-inflammatory drugs,

topical anesthetic agents, and analgesics.^{5, 6} One major goal of regenerative medicine is to accelerate oral wound healing.

Mouthwashes play an important role in oral hygiene and decreasing oral bacterial load. Herbal mouthwashes have been widely investigated for their antibacterial⁷, antiviral⁸, and anti-fungal⁹ efficacy, and treatment of oral malodor and oral mucositis.¹⁰ *Asparagus officinalis* (*A. officinalis*) is among the herbal species with medicinal properties. This plant is a subgroup of *Asparagaceae* genus, which is currently used as an oral supplement worldwide. This plant is rich in vitamins A, B1, B2, C, and E and minerals such as Mg²⁺, Zn²⁺, P³⁻, Ca²⁺, Fe²⁺, Mn²⁺, folic acid, asparagusic acid, and steroidal saponins.¹¹ Considering the biological functions of *A. officinalis*, it is plausible to assess its healing effects.

The main biological functions of the active ingredients of *A. Officinalis* are as follows: anti-oxidative activity, immune modulation, fibroblast proliferation, collagen deposition and synthesis, anti-glycation, and induction of angiogenic pathways.^{12, 13} There are also several minor ingredients in *A. officinalis* extract such as calcium, steroidal saponins, anthocyanins, quercetin, carotenoids, β-carotene, Fe, Mg, Se, and vitamin A¹⁴, which are usually considered as active molecular regulators in homeostasis and wound healing.¹⁵ There is evidence supporting the contribution of these components to proliferative and

resolution phases of wound healing.^{11, 16} Quercetin, another compound in *A. officinalis*, is involved in reduction of inflammation through protein kinase B and microtubule-associated protein kinase pathways.¹⁷ Looking at its bioactive profile, we can suggest *A. officinalis* as a suitable choice to be investigated with regard to its healing effects. This study assessed the healing effects of *A. officinalis* methanolic extract.

Methods and Materials

Firstly, the methanolic extract of *A. officinalis* stem (4-month dried) was prepared using the wetting technique. For this purpose, 20 L of methanol was used to wet the stem parts of the plant, leaving them to stir for 3 days for maximum extraction. The obtained extract was then filtrated using filter papers and injected into an industrial spinning evaporator machine (Hiedolf, Germany) at 40°C; afterwards, the solvent was repeatedly added to the plant extract. Finally, 164 g of methanolic extract was obtained.

High-performance liquid chromatography (HPLC) was done to develop a chemical index for the prepared methanolic extract. A Waters HPLC apparatus consisting of a separation module (2695; Waters, USA) and a PDA detector (996; Waters, USA) was used for HPLC analysis. We used auto sampler injector for chromatography. The chromatographic assay was performed on a 15 cm×4.6 mm pre-column, and Eurospher 100-5 C18 analytical column provided by Waters (Sun fire) reversed-phase matrix (3.5 μm) (Waters). Elution was done in a gradient system with methanol as the organic phase (solvent A) and distilled water (solvent B). The flow-rate (1 mL/min. peaks) was monitored at 195–400 nm wavelength. The injection volume was 20 μL and the temperature was maintained at 25°C.

Next, a cell cytotoxicity test was performed. After determining the highest non-toxic concentration of *A. officinalis* extract, the cell scratch assay was done using the concentration found in the previous step. Human gingival fibroblasts (HGFs) were purchased (with a cell line number of IBRC C10459) and cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin in a humid environment with 5% CO₂ at 37°C for 24 h. According to a previous study on the cytotoxic effects of *A. officinalis*, we chose the following concentrations to examine the cytotoxicity: 10, 25, 50, and 100 μg/mL (18). Our five experimental groups (n=5) were as follows: (I) HGFs cultured in high-glucose Dulbecco's modified Eagle's medium, (II) same as group I but with 10 μg/mL methanolic extract of *A. officinalis*, (III) same as group I but with 25 μg/mL methanolic extract of *A. officinalis*, (IV) same as group I but with 50 μg/mL methanolic extract of *A. officinalis*, and (V) same as group I but with 100 μg/mL methanolic extract of *A. officinalis*. To do the cytotoxicity test, we performed a methyl thiazolyl tetrazolium (MTT) assay for each concentration of the

extract. HGFs were first treated with methanolic extracts for 24 and 48 h; then, 100 μL of the MTT solution (5 mg/mL) was added to each well of a 96-well plate followed by an incubation for 3 h. After removing the MTT solution from the wells, 100 μL of dimethyl sulfoxide solution was added to each well. After 15 min, the plate was transferred to an ELISA-reader and its absorbance was measured at 570 nm. The cell viability was determined using the following formula:

$$\text{Cell viability (\%)} = (\text{OD experiment} / \text{OD control}) \times 100$$

OD experiment: optical density of the treatment group

OD control: optical density of the control group

To prepare an appropriate collagen coating for HGFs to attach to, we used the human plasma. A blood sample was taken from a volunteer who verbally consented to participate, sitting still for 30 min in an upright position. In the next step, the blood sample was fractionated (5418R; Eppendorf, Germany) at 2200 rpm for 15 min. The supernatant (plasma) was poured into the culture plates; then, 0.05 mL of calcium chloride (CaCl₂) was added to each plate as a coagulant with a concentration of 2 M (Sigma, Saint Louis, USA).

After preparing the collagen coating, the extra plasma solution in the plates was discarded and the cultures were fixed by 3 mL of bovine albumin serum with a concentration of 2 mg/mL at 37°C for 1 h. Afterwards, we washed the plates with PBS and added 4 mL of DMEM to them; the control group was ready as such. The cultured cells in DMEM containing *A. officinalis* methanolic extract with a concentration of 100 μg/mL were washed with trypsin twice and mixed with serum-containing medium, after the cells reached 90% confluence. The floating cells were transferred on the collagen coating using a p200 pipette; next, the plates were gently shaken and incubated for 6 h at 37°C, allowing the HGFs to form a cellular monolayer. A scratch was then created in the cellular monolayer by a p200 pipette tip and the cellular debris was removed by washing the plates with 1 mL of DMEM. Next, the media in the plates were replaced with 5 mL of DMEM solution containing the methanolic extract of *A. officinalis* with a concentration of 100 μg/mL. The exact place of the cellular scratch was determined by a scratch on the exterior bottom surface of the plates. The outer scratch was placed at the lower border of the substage, and the first cellular image was taken at the initiation time by a phase-contrast microscope. The cellular plates were incubated for 24 h at 37°C; afterwards, the sub stage was adjusted similarly and a second phase-contrast microscope image (Interlabs, Ambala cantt, India) was taken. Finally, HGFs were fixed using 1 mL of 2% formalin solution for each plate at 37°C. The extra formalin was washed using 1 mL of saline solution and the HGFs were stained with Giemsa staining technique; a final phase-contrast cellular image was taken at the end. All the steps in our study are shown in Figure 1. This study was approved by the Ethics Committee of the Research Institute

of Dental Sciences-Shahid Beheshti University of Medical Sciences with the code number

IR.SBMU.RIDS.REC.1396.527 in 2018.

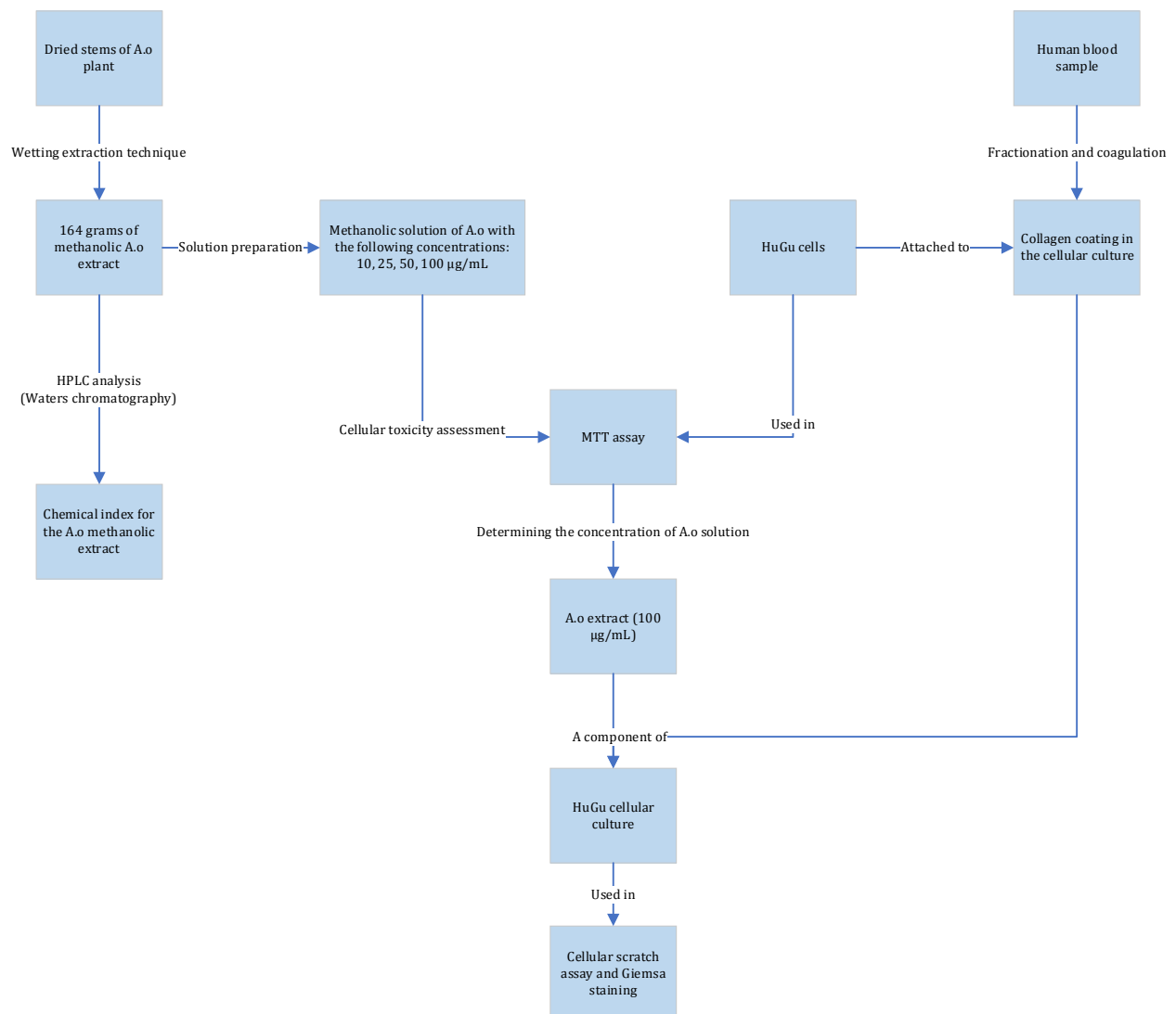


Figure 1-Workflow of the materials used, and methods applied in this study
A. o: *Asparagus officinalis*

To quantify the cell scratch assay result, we used the following formula¹⁹:

$$\text{Wound closure (\%)} = \frac{At=0h - At=\Delta h}{At=0h} \times 100$$

At=0h: Surface of the wound immediately after creating the cell scratch

At=Δh: Surface of the wound “h” hours after creating the cell scratch

Data acquisition and integration for the HPLC analysis were performed by Millennium32 software. Based on a previously approved protocol, all the measurements were made using “Image J” application (V. 1.52e, Wayne Rasband, USA).¹⁹

Results

Regarding the results of HPLC analysis, the most abundant

and biologically notable components existing in our methanolic *A. officinalis* extract are demonstrated in Figure 2. Phenolic compounds and especially flavonoids, catechins, vitamins and a range of other bioactive compounds were present in *A. officinalis* extract. Methanolic extract of *A. officinalis* showed a high biocompatibility profile even in group five with maximum concentration.

A) 3,4 DHB with a concentration of 71.09 ppm in *A. officinalis* methanolic extract (100 µg/mL)

B) Catechin with a concentration of 64.57 ppm in *A. officinalis* methanolic extract (100 µg/mL).

3,4 DHB: 3,4-Dihydroxybenzoic acid; T: time in minutes; C: concentration in ppm

A. o: *Asparagus officinalis*.

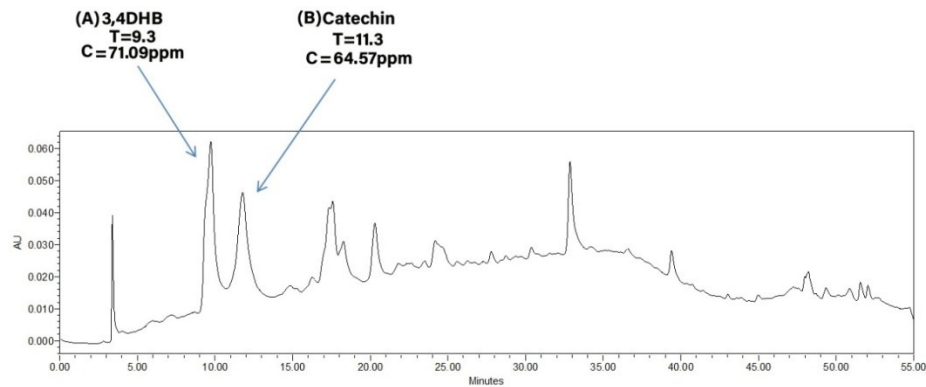


Figure 2- Results of HPLC analysis for the methanolic extract of *Asparagus officinalis*

According to the results of one-way ANOVA, 24- and 48-hMTT assays for the cytotoxicity assessment showed no significant difference in cell viability among the experimental groups ($P=0.07$, Table 1).

Considering the results of the cell scratch assay, the cellular wound closure percentage after 24 h was 60.85%. Paired sample t-test showed a significant difference in wound closure between groups one and five after 24 h ($P<0.001$). According to the results of t-test, wound closure in the control group was not significant after 24 h ($P=0.86$). Cell

motility is illustrated in Figures 3 and 4.

Table 1- Quantitative results of 24- and 48-hMTT assays

A. officinalis concentration ($\mu\text{g/mL}$)	Relative cell viability after 24 h	Relative cell viability after 48 h
0	1	1
10	1.27	0.84
20	1.46	0.94
50	1.64	0.84
100	1.35	0.96

A. officinalis: *Asparagus officinalis*.

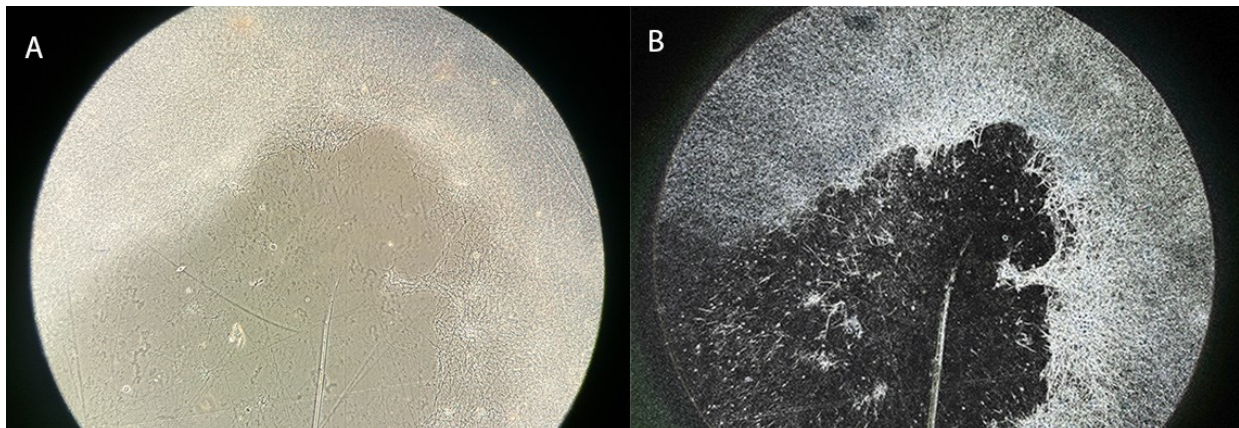


Figure 3- Phase contrast image of cell motility in the control group after 24 hours. HGFs were cultured on a collagen cellular base. Scratch was made by a p200 pipette tip. (A). No cellular nucleus is seen in the phase-contrast original image. (B). Sharpening, edge enhancement, and contrast enhancement were done using ImageJ software. Cellular motility was observable in this image, but it was not statistically significant.

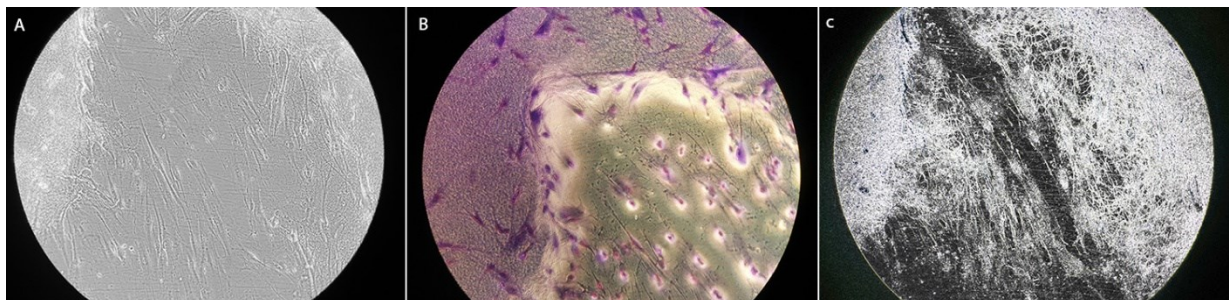


Figure 4- Phase contrast image of cell motility in group five after 24 h. HGFs were cultured on a collagen cellular base. Scratch was made by a p200 pipette tip. (A). Multiple cellular nuclei are seen in the phase-contrast original image. (B). Alizarin red staining was done on HGFs and the cells were fixed using 1 mL of 2% formalin solution for each plate at 37°C. (C) Sharpening, edge enhancement, and contrast enhancement were done using ImageJ software. Cellular motility was notable and statistically significant.

Discussion

In this study, we evaluated the healing effects of methanolic extract of *A. officinalis* in an experimental setting. As of now, there is no herbal mouthwash with confirmed healing effects in the literature, and considering the trend towards the consumption of herbal products, the need for appropriate herbal products for this purpose is obvious. One of the best candidates is *A. officinalis*, a species with several bioactive compounds and a history of usage as an oral supplement.²⁰ The stem part of *A. officinalis* plant was used to prepare a methanolic extract and investigate its healing effects. By evaluating the most concentrated and most biologically active elements in *A. officinalis* extract, we were able to further suggest the healing pathways in which this plant may play a role. Based on HPLC analysis, protocatechuic acid (PCA; 3,4-dihydroxybenzoic acid [3,4, DHB]), and epicatechingallate (ECG) were the two main ingredients present in the methanolic extract. These two can be considered as the main active compounds in *A. officinalis* extract.

Controversial results regarding the cytotoxicity of *A. officinalis* extract provokes a discussion about dose-dependence and time-dependence of the cytotoxic effects of *A. officinalis* methanolic extract. Since there was no significant wound closure in the control group after 24 h ($P=0.86$), the healing can not be merely attributed to the proliferation of HGFs. This finding substantiates the promotive wound closure effects of *A. officinalis* extract. After doing the cellular tests, a search in Kyoto encyclopedia of genes and genome (KEGG) pathways and investigating the possible cellular pathways responsible for the bioactivities of *A. officinalis* extract seemed indispensable. *A. officinalis* might exert its healing effects through several intracellular pathways that inhibit inflammation, induce collagen accumulation and cross-linkage, and enhance angiogenesis (20). Catechins found in *officinalis* are classified as flavonoids which generally have antioxidant and collagen and extracellular matrix synthetic characteristics *A.*²¹ The catechin in our *A. officinalis* extract, ECG, induces angiogenesis via the vascular growth factor as well.²²

PCA is considered as an active component of some traditional Chinese herbal medicines and is present in considerable amounts in *A. officinalis* extract. Its mechanism of action is mainly based on its antioxidant activity, including inhibition of generation and the scavenging effect of free radicals, metal chelation, and up regulating antioxidant enzymes like glutathione reductase.²³ Other biological aspects seem to affect the activity of inducible isoenzyme of cyclooxygenase and nitric oxide synthase, glucose transporters and inflammatory cytokines. Accordingly, the following cellular mechanisms are regulated in the wound healing process: insulin-like activity, angiogenesis, inhibition of pro-inflammatory cells' motility, and fibroblastic proliferation.^{23, 25} These pathways are depicted in more details in the appendices.

Steroidal saponins are minor bioactive components in this plant with antioxidant, anti-hepatotoxicity, antibacterial, anti-cancer, and wound healing effects.¹¹ *A. officinalis* is also rich in minerals like iron (Fe), zinc (Zn), magnesium (Mg), and manganese (Mn). Zinc, magnesium, and selenium (Se) have antioxidant activities.^{11, 15} To date, no significant healing effects have been reported for herbal mouthwashes in clinical and in-vivo studies; although, some herbal extracts have shown promising results. Nicolas et al. examined the healing effects of *Calendula officinalis* extract. Based on the results of their study, ethanolic extract of *Calendula officinalis* can regulate the motility of keratinocytes via activating NF- κ B and increasing the level of IL-8 cytokine.²⁶ These effects are in line and comparable with the healing effects of ECG, PCA, anthocyanin, carotenoids, steroidal saponins, and linoleic acid in *A. officinalis* extract.

There are two compounds in *A. officinalis* with angiogenic inductive effects: ECG and quercetin. ECG acts as a VEGF-inductive factor while quercetin acts through PKB and MAPK pathways. In a study by Liu et al, angiogenic characteristics of an herbal mixture named NF3 was evaluated; it increased the endothelial cell migration and regulated tubular systems intra- and inter-cellularly.²⁷ Torabi et al. evaluated the healing effects of *Thymus caramanicus* extract and essence in *Jalas* mice. The results showed significant healing effects, which were attributed to carvacrol, the main bioactive compound in *Thymus caramanicus*.²⁸ This compound is not present in *A. officinalis*; whilst, its antioxidant and anti-inflammatory effects are in line with the inhibitory effects of ECG against COX-2 enzyme which leads to a drop in thromboxane A2 and platelet receptor levels in fibrinogen.²¹ Nirmala and Karthiyayini aimed to measure the healing effects of alcoholic and aqueous extracts of *Achillea millefolium* L. in intraoral wounds of *Wistar* rats and showed a significant increase in wound contraction rate, skin tear resistance, and dry and wet weight of granulation tissue.²⁹ These effects are similar to the effects of vitamin A and β -carotene on cross-linkage and accumulation of collagen in the resolution phase of wound healing.

Based on our results, the methanolic extract of *A. officinalis* with a concentration of 100 μ g/mL showed significant healing effects on HGFs in an experimental setup. This extract can be introduced as an appropriate candidate to produce a healing mouth wash. There are also some limitations in using *A. officinalis* products such as the presence of cytotoxic compounds in specific parts of the plant such as the fruits or the seeds. Compounds like protodioscin and methyl protodioscin in the seeds, and steroids and asparagosides in the root section of the plant have shown significant cytotoxic effects. Inhibitory effects on cyclooxygenase-2 enzyme and side effects of this pathway on the cardiovascular system as well as decreasing the prostacyclin production should also be considered while consuming this herbal product.¹¹

Conclusion

Overall, the methanolic extract of *A. officinalis* showed significant healing effects on HGFs. Therefore, we can suggest this herbal species as a suitable candidate to produce a healing mouth wash for intraoral wounds. To further reinforce this statement, further *in vivo* studies are recommended.

Conflict of Interest

No Conflict of Interest Declared ■

Funding

This study did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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How to cite:

Erfan Shamsoddin, Davood Bashash, Malek Zarei, Behzad Houshmand. *Asparagus Officinalis*: An Herbal Candidate for an Intraoral Healing Mouthwa. *J Dent Sch* 2020;38(1):31-36