The Effects of *Adiantum capillus-veneris* on Wound Healing: An Experimental *In Vitro* Evaluation

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**ABSTRACT**

**Background:** A lot of remedies, mostly plant based, were mentioned in the Persian old pharmacopoeias for promoting of burn and wound healing and tissue repairing. The efficacy of most of these old remedies is unexplored till now. *Adiantum capillus-veneris* from Adiantaceae family is one of them that was used to treating of some kinds of chronic wounds.

**Methods:** Methanol extract was fractionated to four different partitions that is, hexane, ethyl acetate, n-butanol, and aqueous. The potential of *A. capillus-veneris* fractions in wound healing or prevention of chronic wounds were evaluated through angiogenesis and fibroblast proliferation, in addition to *in vitro* tests for protection against damage to fibroblasts by oxygen free radicals.

**Results:** The aqueous part of *A. capillus-veneris* promoted significant angiogenesis (*P* < 0.05) through both capillary-like tubular formations and proliferation of endothelial cells *in vitro*. In addition, in the tests for protection against damage to fibroblasts by oxygen free radicals, aqueous and butanol fractions showed significant protective effects in the concentrations 50, and 500 µg/ml (*P* < 0.05) in comparison with a control group. In the toxicity testing, it showed weak irritation in the Hen’s egg test chorioallantoic membrane (CAM) bioassay at the vascular level on the CAM of the chicken and no significant cytotoxicity in the MTT assays on normal human dermal fibroblasts.

**Conclusions:** Angiogenic effects and protective effects against oxygen free radicals suggested aqueous partition of *A. capillus-veneris* local application for prevention of late-radiation-induced injuries after radiation therapy and healing of external wounds similar to bedsores and burns.

**Keywords:** *Adiantum capillus-veneris*, angiogenesis, fibroblast proliferation, hen’s egg test chorioallantoic membrane test, Iranian traditional medicine, wound healing

**INTRODUCTION**

Many pharmacological studies are done on wound healing,
but still there are many problems in clinics with prevention or treatment of open wounds like diabetic ulcers, bedsores and burns or tissue injuries after radiation therapy. Wound healing is a complex cascade of cellular and biochemical processes leading to the restoration of structural and functional of injured tissues. It involves several cellular and biochemical overlapping phases including hemostasis, inflammation, proliferation, and tissue remodeling.[1,2] In the hemostasis, platelet derived growth factors, Insulin like growth factor, and transforming growth factor beta activates angiogenesis, and also cause keratinocyte and fibroblast proliferation.[2] Although there is considerable interest in the application of angiogenic or fibroblast growth factors, a simpler alternative is interested. Medicinal plants are used for years in different countries for wound healing, and are preferred because of their low toxicity and their availability. Many remedies, mostly plant based, were mentioned in the Persian old pharmacopoeias of this system for promoting of burn and wound healing and tissue repairing. Distinguished Persian scientists such as Avicenna, Rhazes, Jorjani, and Aghili were familiar with these kinds of wound herbal treatments and spreading and classified them for treating of different kinds of wounds such as abrasion, punctured, cut, perforated, chronic, purulent, and septic wounds.[3,4] They were named those remedies under different old Farsi and Arabic terms like “Monbete Lahm” and “Ghorooh khabiseh”. These old terms has referred to some topical remedies that accelerate physiological sprouting of new vessels and tissues from preexisting tissues and modulate blood movements to the affected areas. The efficacy of most of these old remedies is unexplored until now. The aim of this study is to evaluate the potential of Adiantum capillus-veneris, as one of the plants used in Iranian traditional medicine,[3] for wound healing through standard in vitro assays on angiogenesis, fibroblast proliferation assay, and in vitro assay for protection against damage by free reactive radicals to fibroblasts [Figure 1]. It is reported in Qrabadin-e Kabir, one of the pharmaceutical manuscripts in Persian medicine written by Aghili Khorasani, as topical application for treatment of open wounds.[3]

**METHODS**

**Plant material**

Aerial parts of *A. capillus-veneris* L. (Adiantaceae) was collected in the summer time of 2012 from Semirom waterfall, Isfahan, Iran. Plant material
was identified by Dr. Mustafa Ghanadian, and a voucher specimen (nos. 1661) deposited in the Herbarium of Isfahan University of Medical Sciences, Iran.

**Preparation of plant extracts**

The shade-dried aerial parts of the plant material (100 g) was macerated 3 days with methanol (600 cc × 3), at room temperature. Filtration and in vacuum evaporation of the solvent resulted in a green gum (8.3 g), which using liquid-liquid extraction, was partitioned through a separating funnel between n-hexane and methanol ethanol. The defatted methanol extract was evaporated and dissolved in H₂O to make a suspension and then partitioned with different solvents such as ethyl acetate and n-butanol, respectively. Four different fractions, that is, hexane (A1, 2.3 g), ethyl acetate (A2, 0.8 g), n-butanol (A3, 6.4 g), and aqueous (A4, 0.5 g) parts were obtained which were evaporated to dryness under vacuum and stored in the refrigerator at −20°C.

**Cell preparation**

Human umbilical vein endothelial cells (HUVECs) and normal human dermal fibroblast line (AGO 1522) were obtained from a national cell bank of Pasteur, Tehran, Iran and were cultured according to the supplier’s instructions. The HUVECs were cultured in Dulbecco's modified Eagles medium containing 10% fetal bovine serum (FBS). The AGO 1522 cell line was cultured in Roswell Park Memorial Institute (RPMI) 1640 + FBS 10% medium. All cells were used between passages 5-6.5,6

**In vitro test for fibroblast MTT assay**

The fibroblast growth stimulation and the cytotoxic effects of the samples were examined using the MTT assay method. Sixth passages of cells were trypsinized, suspended in RPMI + FBS 10%, and centrifuged. Supernatant was discarded and re-suspended in the same medium to give a suspension of 1.8 × 10⁶ cells/ml. Fibroblast cells were seeded (4 × 10⁵ cells/well) in a 96 well plate, RPMI + FBS 10% (170 µl) was added and incubated in 5% CO₂ and 37°C for 24 h. About 20 µl of vehicle as a control or samples in different concentrations were added to impart a final concentration of 5, 50, 250, and 500 µg/ml.

After 24 h incubation, the medium was discarded from the cells, replaced with 50 µl RPMI + 10 µl MTT, and incubated for 3 h. The medium was then removed, 50 µl dimethyl sulfoxide (DMSO) added, and the optical density of cells measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader.6

**In vitro test for protection against damage to fibroblasts by oxygen free radicals**

The same culture of AGO 1522 (human dermal fibroblast) was used equally for the fibroblast growth stimulation assay. Sixth passages of cells were trypsinized, suspended in RPMI + FBS 10%, and centrifuged. Supernatant was discarded and re-suspended in the same medium to give a suspension of 4 × 10⁶ cells/ml. About 10 µl of Fibroblast cells were seeded (4 × 10⁵ cells/well) in a 96 well plate, RPMI + FBS 10% (150 µl) was added and incubated in 5% CO₂ and 37°C for 24 h. 20 µl of H₂O₂ (0.1 mM) was added simultaneously with 20 µl of vehicle as a control or samples. Cells were treated with different concentrations of the test samples (5, 50, 250, and 500 µg/ml) and incubated for 24 h. Control and negative controls consisted of the cells treated with hydrogen peroxide alone, and the untreated cells and catalase (250 IU/ml), a scavenger of H₂O₂, as a standard antioxidant control.6,7

The medium was then pulled out from the cells, replaced with 50 µl RPMI + 10 µl MTT, and incubated for 3 h. The medium was then removed, 50 µl DMSO added, and the optical density of cells measured at 570 nm using an ELISA reader.6

**In vitro test for human umbilical vein endothelial cells growth stimulation**

Human umbilical vein endothelial cells suspension (10 µl) was seeded in each well (5 × 10⁵ cells/well) and incubated in 5% CO₂ and 37°C for 24 h. About 20 µl of samples or control in different concentrations (5, 50, 500, and 1000 µg/ml) were added. Doxorubicin was used as positive control and wells without treatment as a control group. After 48 h treatment and incubation, the upper fluid of each well in the plate pulled out and then 10 µl of MTT + 50 µl RPMI were added. After 3 h, 50 µl DMSO was added to the wells and the optical density (OD) of the survival cell in each well were determined in a wavelength of 570 nm with ELAISA reader.4 This procedure repeated.
for 3 times and the average of the cell survival percentage was determined as follows:

\[
\text{Cell survival} \% = \frac{(\text{OD experimental group} - \text{OD blank group})}{(\text{OD control group} - \text{OD blank group})} \times 100.
\]

**Capillary-like tube formation assay**

Cells were seeded in 24-well Matrigel-coated plates and were treated with samples as is described below. The capillary-like tubular formations were considered for evaluating angiogenic or antiangiogenic effects of the test sample of endothelial cells on Matrigel base (Invitrogen, USA) as described before [my article]. Matrigel thawed on ice was added 100 µl/well of a 24 well plate, carefully and incubated for 30 min in 37°C allowing matrigel to form a jelly base with a flat surface. About 40 µl of HUVECs suspension (10⁵ cells/well) were seeded in each well. The wells were treated with 40 µl of test samples with the final concentrations of 5, 50, and 500 µg/ml per well at the same time. Blank or 10 ng/ml recombinant human vascular endothelial growth factor (VEGF) (R and D Systems, USA) alone served as a negative or positive control and culture medium added up to 300 µl. The plate was incubated for 24 h in 37°C and after incubation in vitro endothelial tube formation data were expressed as a percentage of the number of capillary-like tubes in treating wells to untreated control wells of triplicate readings. Capillary-like tube formation was discovered with a Nikon inverted microscope and the images recorded digitally using Angioquant analysis software (Tampere University of Technology, Tampere, Finland).[9]

**Statistical analysis**

The experiments are done in triplicates, and the results are presented as mean ± standard deviation. One-way analysis of variance followed by Dunnett’s post hoc comparison was used for multiple between-group comparisons. Analysis were performed with the statistical package SPSS version 18 (SPSS Inc., Chicago, IL, USA).

**RESULT AND DISCUSSION**

**In vitro test for fibroblast MTT assay**

Results are depicted in Figure 2 and show the viability, which is a reflection of cell counts. The effects did not show significant fibroblast proliferation (P < 0.05) or toxicity in aqueous or butanol fractions. Ethyl acetate and hexane fractions showed significant inhibitory effects on the growth of fibroblast cells at the higher

<table>
<thead>
<tr>
<th>Table 1: Irritation classification based on IS</th>
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<tr>
<td><strong>HET-CAM score range</strong></td>
</tr>
<tr>
<td>0-0.9</td>
</tr>
<tr>
<td>1-4.9</td>
</tr>
<tr>
<td>5-8.9 or 5-9.9</td>
</tr>
<tr>
<td>9-21 or 10-21</td>
</tr>
<tr>
<td>HET-CAM=Hen’s egg test chorioallantoic membrane, IS= Irritation score</td>
</tr>
</tbody>
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for 3 times and the average of the cell survival percentage was determined as follows:

\[
\text{Cell survival} \% = \frac{(\text{OD experimental group} - \text{OD blank group})}{(\text{OD control group} - \text{OD blank group})} \times 100.
\]
concentration (500 µg/ml) by 64.07 ± 2.81 and 76.55 ± 7.2%, with IC\textsubscript{50} values more than 500 µg/ml, suggesting that higher concentrations of these two factions are cytotoxic to fibroblast cells.

\textit{In vitro} test for protection against damage to fibroblasts by oxygen free radicals

Results for the fractions obtained from liquid–liquid partitioning are shown in Figure 3. It can be seen that the significant protective effect in comparisons with the control group treated with hydrogen peroxide alone, is shown by the aqueous fraction in the higher concentrations 50 and 500 µg/ml (P < 0.05).

\textit{In vitro} test for endothelial cell growth stimulation

The toxicity tests were done using HUVEC lines through standard MTT assay. Ethyl acetate and hexane fractions inhibit the growth of HUVECs by 35.4 ± 2.3, 76.4 ± 7.2% at 1000 µg/ml, respectively, with IC\textsubscript{50} values of 851.7 ± 53.4 and >1000 µg/ml. None of the fractions showed significant toxicities (P < 0.05) at the concentrations 5, 50, and 500 µg/ml. The aqueous fraction at concentrations 500 and 1000 µg/ml showed significant stimulatory effects (P < 0.05) on proliferation of endothelial cells by 134 ± 7.5 and 138 ± 8.3% [Figure 4].

\textit{Capillary-like tube formation assay}

Tube formation assay based on the ability of endothelial cells to form capillary-like tubular structures as seen in Figures 5 and 6, represents a quantitative model for studying inhibitors and activators of angiogenesis. Upon quantification...
of the capillary-tube-like structures using the angioquant software, aqueous fraction stimulates tube-similar structures at higher concentration of 500 μg/ml to 144.7 ± 14.4%. Butanol, ethyl acetate, and hexane fractions inhibited the size of tube-like structures at 500 μg/ml to 58.3 ± 3.3, 8.1 ± 0.6, and 4.3 ± 0.6%, significantly (P < 0.05). The IC_{50} values of the anti-angiogenetic effects of butanol, ethyl acetate, and hexane fractions were >500, 191.1 ± 7.5, and 85.3 ± 7.1 μg/ml, respectively.

**Hen’s egg test**

The HET-CAM bioassay was done to evaluate the potential irritation or toxicity induced by test samples at the vascular level of the CAM of the chicken [Figure 7]. In order to investigate the toxic effects of *A. capillus-veneris* aqueous fraction in the HET, the numerical time-dependent scores for lysis, hemorrhage, and coagulation were scored to give a single numerical value indicating the irritation potential of the test substance according to the criteria in Table 1, and mean irritation score was determined. The data are given in Table 2.

**DISCUSSION**

 Medicinal plants have been used for years in different countries as an alternative source of treatment for wound healing for wound healing and are chosen because of their low side effects and widespread availability.[10-12] In the present study, the potential of *A. capillus-veneris* in wound healing evaluated through angiogenesis and proliferation of endothelial cells in vitro. The aqueous partition of *A. capillus-veneris* promoted angiogenic effects through both capillary-like tubular formations and proliferation of endothelial cells in vitro. In addition, in the tests for protection against damage to fibroblasts by oxygen free radicals, aqueous and butanol fractions showed significant protective effects in comparison with a control group with no significant toxicity in the MTT assays on normal human dermal fibroblasts and with weak irritation in the HET-CAM bioassay at the vascular level on the CAM of the chicken.

Phytochemically, quercetin, and kampferol glycosides as kampferol-3-sulphate, kampferol-3-rhamnoglucoisde, quercetine-3-glucuronyl, and rutin are the main flavonoids isolated from polar fractions of *A. capillus-veneris* [13-15] which could be in part responsible for the aqueous and butanol fractions. In a confirming report about quercetin effects on dermal wound healing in rats, there was an increase in the proliferation of cells in quercetin treated groups.[16] In addition to flavonoids, saponin glycosides with triterpenoid hydroxyhopenone structure may be also incorporated in the angiogenesis effects.[17] On this point are some evidences on saponins to be efficacious in wound healing. For instance, asiaticoside saponins from *Centella asiatica* and damaran type saponins from *Panax ginseng* root showed angiogenic properties.[18,19]

In addition to proangiogenetic properties, the protection effects of *A. capillus-veneris* against damage to fibroblasts by reactive radicals suggested it for prevention of chronic wounds similar to those happened after radiation therapy and for treatment of bedsores. In

![Figure 6: Effect of the aqueous fraction of Adiantum capillus-veneris on capillary-like tube formation in human umbilical vein endothelial cells (HUVECs). Tube formation of HUVECs on Matrigel in the presence of three concentrations of (5, 50, and 500 μg/ml), and untreated cell as control.](image)

![Figure 7: (a) Normal, (b) chorioallantoic membrane (CAM) treated with an extract. The hen’s egg test CAM bioassay is used to evaluate the potential irritation or toxicity induced by test samples at the vascular level of the CAM of the chicken.](image)
the treatments for cancer by radiotherapy, radiation-damaged tissues usually lead to chronic wounds.\cite{20} It is reported that the progression of radiation-induced chronic wounds might be in part due to oxidative stress.\cite{21} Therefore, applying an antioxidant-based drug could reduce or even treat late-radiation-induced injuries.\cite{21} Reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radical are continuously produced after the initial injury, and thus may be responsible for chronic wound healing. Antioxidant activity of \textit{A. capillus-veneris} may be due to the presence of polyphenolics and flavonoids especially quercetin and kampfreol derivatives present in the aqueous and butanol fractions.\cite{22-24} Flavonoids are known for their antioxidant and cell protecting effects.\cite{22,23} However, more investigations are suggested be done on aqueous fraction of \textit{A. capillus-veneris} on cutaneous wound healing like cellular, and molecular action mechanisms via animal models.

**CONCLUSIONS**

Previously, multiple pharmacological properties like analgesic, antinociceptive, anti-implantation, and antimicrobial activities were reported from \textit{Adiantum} genus.\cite{17} Now, in the work outlined here wound healing properties are reported which might be explained in part by angiogenic properties of polar components present in the aqueous and butanol fractions.\cite{22,23} Flavonoids are known for their antioxidant and cell protecting effects.\cite{22,23} However, more investigations are suggested be done on aqueous fraction of \textit{A. capillus-veneris} on cutaneous wound healing like cellular, and molecular action mechanisms via animal models.

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