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Phytochemical composition of *Verbascum stachydifolium* Boiss & Heldr. var. *stachydifolium* growing in Türkiye and *in vitro* analysis of wound healing activity

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Abstract: This study aimed to investigate the phenolic content, antioxidant activity, cytotoxicity and the *in vitro* wound healing activity of methanolic and aqueous extracts of *Verbascum stachydifolium* Boiss & Heldr. var. *stachydifolium*. Total phenolic and flavonoid contents and antioxidant activity were measured using spectrophotometry-based methods. Quantitative analysis of the selected phenolics was performed by HPLC. The cytotoxic effects of the extracts on L929 mouse fibroblast cells were evaluated by the MTT assay. The migration of treated fibroblast cells was assessed by the cell scratch assay. The expressions of type I collagen, FGF7, TGF-β1 and VEGF were evaluated by qRT-PCR and ELISA. The HPLC-based analysis revealed the presence of different phenolic compounds at varying amounts and high antioxidant activities were detected. The cytotoxicity assay results indicated that the methanolic and aqueous extracts did not exhibit any cytotoxic effect on fibroblast cells when used up to 500 μg/mL concentration. Fibroblast migration was stimulated to the highest degree by the aqueous extract obtained by maceration as observed in the scratch assay at 60.4% closure. The molecular mechanism of the wound healing activity involves the upregulation of the analyzed genes.

Keywords: *Verbascum stachydifolium*; wound healing; plant extract; antioxidant

Abbreviations: high-performance liquid chromatography (HPLC), HPLC-diode array detector (HPLC-DAD), dimethylsulfoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffer saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), enzyme-linked immunosorbent assay (ELISA), quantitative real-time polymerase chain reaction (qRT-PCR), titrated *Centella asiatica* extract (TECA), the herbarium of Gazi University (GAZI), the limit of detection (LOD), the limit of quantitation (LOQ), relative standard deviation (RSD%), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), vascular endothelial growth factor (VEGF), transforming growth factor-beta 1 (TGF-β1), fibroblast growth factor 7 (FGF7), analysis of variance (ANOVA), human umbilical vein endothelial cells (HUVEC), mouse fibroblast cell (L929).

INTRODUCTION

About 60% of the world's population and 60-90% of the human population in developing countries use traditional medicinal plants in primary health care [1]. In recent years, there has been an increase in the use of herbal and natural products in the management and treatment of various disease states among people in developed countries, including the US, Europe, and Japan. Ethnomedicinal uses of natural products, including medicinal plants, also provide a basis for the isolation and discovery of new bioactive molecules. [1]. In traditional medicine, many plants have ethnomedicinal use for treating wounds. The wound healing activities of various plant extracts have also been demonstrated by scientific research using *in vitro* and *in vivo* methods [1-4]. Moreover, research on the wound healing properties of plant extracts has led to the production of certain commercial products, the best-known being titrated *Centella asiatica* (Gotu Kola) plant extract (TECA), which is used as an active ingredient of medical ointments (e.g., Madecassol®) prescribed to treat wounds and burns [1,4].

Verbascum genus is represented by 411 taxa in Türkiye, 197 of which are endemic. *Verbascum stachydifolium* Boiss & Heldr. var. *stachydifolium* (Mullein) is an endemic plant species of Türkiye distributed

in central Anatolia [5]. The aboveground parts of the plant (stem, leaf, flower) that belong to the genus *Verbascum* are used for medical purposes [6] with some uses having scientific support [3,7,8]. The wound healing properties of the methanolic extract of *V. stachydifolium* have been investigated using *in vivo* linear incision and circular excision wound models, which revealed its remarkable wound healing activity [3]; however, to the best of our knowledge, no additional study has been carried out and there is no detailed information regarding the antioxidant activity, cytotoxicity and chemical constituents of this endemic plant species.

Investigations into the chemical constituents and biological activities of medicinal plants have led to the discovery of alternative drugs and methods of wound treatment. The aim of this study was to assess the wound healing potential of *V. stachydifolium* var. *stachydifolium* extracts by *in vitro* methods at the molecular level, along with an investigation of its phytochemical content. We examined the phytochemical content with a special emphasis on phenolic compounds. The antioxidant activity, cytotoxicity, *in vitro* wound healing activity and its effect on the expression of collagen and selected cytokines, were investigated. The methanolic and aqueous extracts obtained by maceration and the Soxhlet method were compared.

MATERIALS AND METHODS

Plant material

Field studies to collect plant material were carried out in the central regions of Türkiye by the authors. *V. stachydifolium* var. *stachydifolium* was collected from steppes, wheat fields (986 m a.s.l.) between Cihanbeyli and Konya (38°13'25.20"N, 32°44'56.11"E, 10.07.2017). Plants were collected from a single population to minimize the compositional variation. One of the collected plant specimens was given the herbarium number (BK 1268) and deposited in the herbarium of Gazi University (GAZI, also the herbarium code). The aerial parts of the plants were dried in the shade and ground in a commercial Waring blender. The ground plant material was kept in dark at room temperature until use.

Extraction procedure

Extracts of *V. stachydifolium* var. *stachydifolium* were obtained by maceration (60 g plant material was used) and Soxhlet extraction (20 g was used) techniques. Water and methanol were used as solvents with volumes of 600 mL for maceration and 150 mL for Soxhlet. The ground plant materials were macerated three times at room temperature on a magnetic stirrer and the extracts were filtered through Whatman grade no.1 filter paper. Methanol was evaporated using a rotary vacuum evaporator (Heidolph-Rotary VV2000 Schwabach, Germany) at 40°C. Water extracts were frozen at -20°C and lyophilized in a freeze-dryer (Christ Gamma 2-16 LSCplus, Germany). The plant extracts were stored in the dark at 4°C. Four different extracts were prepared as follows: *V. stachydifolium* var. *stachydifolium* maceration with methanol (VMM), maceration with water (VWM), Soxhlet extraction with methanol (VMS), Soxhlet extraction with water (VWS).

Determination of total phenolic content

The total phenolic content was determined based on the Folin-Ciocalteu method [9]. Gallic acid was used as a reference compound to obtain the standard calibration curve. Briefly, 0.5 mL of extracts (1 mg/mL), 2.5 mL of 1:10 diluted Folin-Ciocalteu reagent and 2 mL sodium carbonate solution (7.5% w/v) were mixed in separate tubes and incubated for 15 min at 45°C. Blank, standards and samples were transferred to cuvettes and measured using a UV/VIS spectrophotometer at 765 nm wavelength (Lambda 25, Perkin Elmer, Waltham, MA, USA). Each sample was measured in triplicate and mean values were used. Results were presented as mg/g gallic acid equivalents (mg GAE/g).

Determination of total flavonoid content

The total flavonoid content was determined by the aluminum chloride colorimetric method as described [10]. Briefly, 0.5 mL of extract solutions (0.5 mg/mL) was mixed with 0.1 mL of 10% aluminum chloride hexahydrate, 0.1 mL of 1 M potassium acetate and 2.8 mL of deionized water. After incubation at room temperature for 40 min, standards and samples were transferred to cuvettes and the absorbance of the reaction mixture was measured at 415 nm against a blank

using a UV/VIS spectrophotometer. Rutin was used as a standard compound and results were calculated as mg/g rutin equivalents (mg RUE/g). Each sample was measured in triplicate and mean values were used.

Determination of total antioxidant capacity

Total antioxidant capacity was determined as described [11]. Briefly, 0.3 mL extract (1 mg/mL), and 3 mL of reagent solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate were incubated at 95°C for 90 min. Standards and samples were transferred into cuvettes and the absorbance of the solution was measured at 695 nm using a UV/VIS spectrophotometer after cooling at room temperature. The calibration curve was plotted using ascorbic acid and the antioxidant activity was calculated as the equivalent of ascorbic acid (EAA). Standards and samples were measured in triplicate and mean values were used.

2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay

The DPPH free radical scavenging assay was performed based on the original method by Blois [12] with some modifications. Briefly, the extracts were prepared in concentrations of 15.62, 31.25, 62.5, 125 and 250 µg/mL. Three mL of each concentration was mixed with 1 mL of 0.1 mM DPPH solution freshly prepared in methanol. The tubes were incubated in the dark at room temperature for 30 min and then measured at 517 nm using a UV/VIS spectrophotometer (Perkin Elmer, Lambda 25). Solvent without extract was used as a negative control and ascorbic acid was used as a positive control. The color change of the purple DPPH solution to yellow/light-yellow indicates antioxidant activity, and the percent inhibition values of each extract were calculated using the following equation:

$$\text{Inhibition (\%)} = ((A_{control} - A_{blank}) - (A_{sample} - A_{blank})) \times 100 / (A_{control} - A_{blank})$$

where $A_{control}$ is the absorbance, optical density (OD) of the negative control and A_{sample} is the absorbance of ascorbic acid or extract samples, and A_{blank} is the absorbance of blank. IC_{50} values were calculated with inhibition rates using a four-parameter logistic regression model after plotting sigmoidal curves. Standards and samples were measured in triplicate and mean values were used for the calculations.

HPLC analysis and quantification

The chemical contents of the extracts were analyzed by the reversed-phase high-performance liquid chromatography-diode array detector (HPLC-DAD) method, which is widely used for screening phytochemicals in plant extracts [10]. The reference compounds were selected mainly from among phenolics, which are common plant secondary metabolites. We analyzed 15 reference compounds in *V. stachydifolium* by matching the retention times of reference standards with the sample chromatograms. Chromatograms were recorded at 8 different wavelengths, 210, 260, 270 and 320 nm, that were chosen for the analyses according to the maximum absorbances of the reference peaks. For quercitrin and 4-hydroxybenzoic acid, 260 nm were used, and gallic acid was detected at 270 nm. For catechin, epicatechin, vanillic acid, syringic acid, rutin, quercetin, apigenin and kaempferol, 210 nm was used. Chlorogenic acid, caffeic acid, p-coumaric acid and sinapic acid were analyzed at 320 nm. Extracts were prepared at 1 mg/mL concentration for HPLC analyses. All the standards and samples were filtered through a 0.45-µm polytetrafluoroethylene membrane, measured in triplicate and mean values were used.

Chromatographic separation was performed using a C18 column (Agilent Poroshell 120 SB-C18, 2.7 µm, 4.6×10 mm; PerkinElmer, MA, United States) on an HPLC instrument equipped with a DAD (Agilent 1220 Infinity, PerkinElmer). Column temperature was set at 30°C, a flow rate of 0.8 mL/min was used and 20 µL of standard or sample was injected. Reversed-phase separation was achieved using a gradient method with mobile phases A (deionized water acidified with 0.1% trifluoroacetic acid (TFA)) and B (acetonitrile acidified with 0.1% TFA). The gradient was applied as follows: 0-1 min 95% A, 2-30 min A 95% to 50%, 31-35 min A 50% to 5%, 36-37 min A 5%, 38-39 min A 5% to 95%, A 95% for 1 min. The limit of detection (LOD) and the limit of quantitation (LOQ) values were calculated for each reference according to the Eurachem Guide, 2nd ed. [13].

Cell culture

The L929 (ATCC® CCL-1™) mouse fibroblast cell line (*Mycoplasma*-free) was obtained from the Republic of Türkiye Ministry of Agriculture and Forestry, Şap Institute (Ankara, Türkiye) and used for *in vitro* experiments. The cells were grown in 25 cm^2 or 75 cm^2 cell culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 4 mM L-glutamine in a humidified incubator with 5% CO_2 at 37°C. The growth medium was routinely changed every 2nd day and cells were passaged after reaching 80-90% confluence (at a split ratio 1:3) using trypsin-EDTA. Cells between the 4th and 6th passages were used in all the experiments.

Cell viability assay

Cell viability was determined using a modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures the mitochondrial reductase activity of viable cells [14]. After confluency, the cells were seeded into 96-well tissue culture plates in 200 µL of growth medium at a concentration of 10^4 cells/mL and maintained until reaching confluency (80-90%). Plant extracts were prepared in concentrations of 31.25, 62.5 125, 250 and 500 µg/mL in the growth medium and 200 µL of the prepared extracts were added to the wells. Only solvent was added to negative control wells. After 18, 24 and 48 h of incubation, 50 µL of MTT solution was added to the wells. Then all the solutions were removed and DMSO was added. The plates were incubated in the dark for 30 min and the absorbance was measured at 570 nm (Epoch™ Microplate Spectrophotometer, Biotek, Winooski, VT, USA). The experiment was carried out in quadruplicate and mean values are presented.

Cell scratch assay

The migration capabilities of L929 mouse fibroblasts were assessed using a cell scratch wound healing assay, which measures the expansion of the cells on surfaces [15]. The cells were seeded into 48-well tissue culture dishes at a concentration of 2×10^4 cells/mL and cultured until forming nearly confluent cell monolayers. All the extracts were prepared at a concentration of 62 µg/mL in the growth medium and a linear "wound" was generated on the cell monolayer with a sterile 200 µL plastic pipette tip. Any cellular debris was removed by washing the wells with phosphate buffer saline (PBS). After this step, 300 µL of medium containing plant extracts was added and incubated for 24 h at 37°C with 5% CO_2. The solvent (methanol or water) without extract was added to the negative control wells. Cells were visualized under an inverted microscope. Three representative images from different parts (upper, middle and bottom parts of the well) of the scratched area from each replicate well were digitally photographed at 0 (the beginning) and 24 h to calculate the relative migration of cells.

The area between the scratch edges was calculated by image processing using ImageJ software. First, the edges of the cells were contoured and then the cell-free area in between was calculated based on pixels. The mean values of three photographs from the same well were used for each replicate well. The closure rate was calculated with these values using the following formula:

$$\text{SCR (Scratch Closure Rate)} = [(\text{Area}_{t0} - \text{Area}_{t24}) / \text{Area}_{t0}] \times 100$$

where Area_{t0} is the calculated area value at 0 h and Area_{t24} is the area value at 24 h. The experiment was performed in triplicate (three different replicate wells) and mean values are presented.

RNA isolation and cDNA synthesis

For gene expression experiments, cells were incubated with the plant extract (62 µg/mL VWM in growth medium) for 24 h. The extract was substituted with sterile deionized water to obtain a negative control sample. The experiment was carried out in 3 separate flasks. RNA isolation was performed using a commercial kit (Thermo Fisher Scientific GeneJET RNA purification kit, Waltham, MA, USA) in accordance with the protocol provided by the manufacturer. RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA). The cDNA synthesis was carried out using a commercial kit (GeneAll Hyperscript First Strand Synthesis Kit, Lisbon, Portugal) following the instructions of the manufacturer. Samples were stored at -80°C.

Quantitative real-time PCR (qRT-PCR)

TaqMan gene expression assay (primer-probe set) was used (Thermo Fisher Scientific, 4331182) for qRT-PCR experiments using cDNA as a template following the manufacturer's instructions. The reaction was performed in a real-time PCR instrument (Bioneer, Exicycler 96, Kew East, AU). The glyceraldehyde phosphate dehydrogenase (GAPDH) gene was used for the normalization and normalized transcript levels were calculated by the $2^{-\Delta\Delta Ct}$ method [16].

The expression levels of vascular endothelial growth factor-A (VEGF-A), fibroblast growth factor 7 (FGF7), transforming growth factor beta 1 (TGF-β1) and collagen type I were compared with the control. TaqMan primer-prob set IDs are as follows: Mm00801666_g1 for COL1A1 (collagen type I) gene, Mm00433291_m1 for FGF7, Mm00437306_m1 for VEGF, Mm01178820_m1 for TGF-β1, and Mm99999915_g1 for GAPDH. The master mix was purchased from Bioline, Meridian Bioscience, OH USA (SensiFAST™ Probe No-ROX Kit-Cat No. 86005).

Determination of protein levels by the ELISA method

For the enzyme-linked immunosorbent assay (ELISA) experiments, the same conditions were applied as for RNA isolation, but the media were collected from each well instead. VEGF protein amount analysis was performed with a Duoset ELISA-mouse VEGF kit (R&D Systems Minneapolis, MN, USA) following the protocol provided by the manufacturer. In addition to this kit, an Ancillary Reagent Kit 2 of the same brand was used for sample preparation. TGF-β1 analysis was performed using the Duoset ELISA-mouse TGFβ1 kit with Ancillary Reagent Kit 1. The amount of extracellular collagen was determined using Mouse Col1 (Collagen Type 1) ELISA kit (Elabscience, Houston, TX, USA) in accordance with the provided protocol. The amount of FGF7 protein was determined using the ELISA assay kit FGF7 obtained from Cloud-Clone Corp., Katy, TX, USA.

Statistical analysis

All the values used in statistical analyses were obtained from at least three replicates and the results were expressed as the mean±standard deviation. Statistical significance between groups was determined by one-way analysis or variance (ANOVA) followed by Tukey's test for post-hoc comparison. Mean values were considered statistically different if $P<0.05$.

RESULTS

Total phenolic and flavonoid contents

The calculated total phenolic and flavonoid contents of different extracts of *V. stachydifolium* var. *stachydifolium* are shown in Table 1. The total phenolic content was the highest in VMM with 104.45 mg GAE/g, followed by VMS with a value of 77.79 mg GAE/g. The phenolic contents of VWM and VWS were 63.35 and 41.41 mg GAE/g, respectively. The total flavonoid content was the highest in VMS with 52.65 mg RU/g, followed by VMM with 42.08 mg RUE/g. VWS and VWM contained 19.55 and 13.22 mg RUE/g total flavonoids, respectively. These results indicate that the methanolic extracts contained phenolic compounds in higher amounts compared to the aqueous extracts.

Table 1. Total phenolic and flavonoid contents, total antioxidant capacity, and diphenyl-2picrylhydrazyl (DPPH) scavenging activity of the extracts (maceration with methanol (VMM), maceration with water (VWM), Soxhlet with methanol (VMS), Soxhlet with water (VWS)).

Extracts	Total Phenolic Content (mg/g GAE)	Total Flavonoid Content (mg/g RUE)	Total Antioxidant Capacity (mg/g AAE)	DPPH Scavenging Activity (IC_{50} values, μg/mL)
VMS	77.79±0.29^{d}	52.65±0.86^{e}	215.46±0.61^{e}	31.41±0.12^{d}
VMM	104.4 ±0.78^{e}	42.08±0.94^{c}	212.36±0.89^{f}	31.08±0.07^{e}
VWS	41.41±1.87^{f}	19.55±0.00^{d}	175.82±0.71^{g}	85.80±0.10^{f}
VWM	63.35±1.40^{g}	13.22±0.77^{f}	119.27±0.41^{h}	113.83±0.11^{g}
Ascorbic acid	–	–	–	1.49±0.01^{h}

Values are the means of three replicates±standard deviation. The differences between the mean values with different letters in the same column are statistically significant ($P<0.05$).

Antioxidant activity

Antioxidant activity was assessed using two different methods, DPPH free radical scavenging and phosphomolybdenum assays. The calculated IC_{50} values of the DPPH assay performed for different extracts varied between 31.08 and 113.83 µg/mL (Table 1). In the phosphomolybdenum assay, the total antioxidant capacity was expressed as mg ascorbic acid equivalents per g of dry extract and the values varied between 119.27 and 215.46 (Table 1). The methanolic extracts expressed better antioxidant activity than the aqueous extracts whereas the extraction method did not affect the activity significantly.

Chemical constituents as revealed by HPLC analysis

In the present study, the methanolic and aqueous extracts of *V. stachydifolium* var. *stachydifolium* were analyzed for 15 different secondary metabolites (mainly phenolics) by the HPLC method and the results are presented as mg/g dry weight (DW) for each substance (Table 2). The coefficient of determination (R^2) values of linear regression of the calibration curves, the limit of detection (LOD) and the limit of quantitation (LOQ) of the method were calculated. Representative chromatograms showing the identified compounds are presented in Fig. 1. In all the extracts analyzed, all the selected reference compounds were identified but chlorogenic acid, vanillic acid, epicatechin, p-coumaric acid, sinapic acid, apigenin and kaempferol were detected in very small amounts. Gallic acid and quercetin were the two compounds present in the highest amounts in the extracts, ranging between 7.71-7.78 and 2.90-3.64 mg/g DW, respectively. The amounts of compounds varied between different extracts depending on the solvent (methanol or water) rather than the extraction method. Gallic acid was not detected in VMM.

Table 2. Comparison of the secondary metabolite content (mg/g DW mean values) of *V. stachydifolium* var. *stachydifolium* based on the reference compounds analyzed with the LOD/LOQ values of the method for each reference measurement.

	R_t (min)	VMS	VMM	VWS	VWM	LOD/LOQ
Gallic acid	2.57	7.78±0.00	—	7.75±0.02	7.71±0.01	0.008/0.02
4-hydroxybenzoic acid	7.37	0.60±0.00*	0.53±0.05	0.35±0.12	1.03±0.01	0.08/0.29
(+)-Catechin	8.13	0.67±0.03	0.74±0.17	0.68±0.05	—	0.04/0.14
Chlorogenic acid	8.51	1.09±0.00	0.92±0.00	—	—	0.005/0.01
Vanillic acid	8.87	0.55±0.29*	0.54±0.24*	0.66±0.32*	1.17±0.56	0.28/0.93
Caffeic acid	9.21	0.77±0.03	0.77±0.00	0.76±0.05	1.08±0.34	0.08/0.28
Syringic acid	9.65	1.33±0.01	1.65±0.21	1.56±0.12	1.12±0.09	0.14/0.49
(-)-Epicatechin	10.04	+	+	+	+	0.11/0.39
p-Coumaric acid	11.77	0.37±0.07	0.49±0.17	0.44±0.07	1.11±0.05	0.06/0.22
Rutin	13.07	3.59±1.34	3.15±0.57	2.03±0.12	1.90±0.02	0.35/1.17
Sinapic acid	13.24	1.42±0.62	1.28±0.53	0.62±0.25	0.25±0.00*	0.15/0.51
Quercitrin	15.13	2.03±0.76	1.98±0.73	1.53±0.37	1.65±0.28	0.10/0.35
Quercetin	19.23	3.64±0.13	3.47±0.08	3.24±0.03	2.90±0.10	0.46/1.54
Apigenin	21.90	+	+	+	+	0.05/0.19
Kaempferol	22.40	1.12±0.36	0.78±0.02	—	—	0.08/0.27

Rt: retention time of the standard, LOD: limit of detection, LOQ: limit of quantitation, N/A: not applicable for that sample (maceration with methanol (VMM), maceration with water (VWM), Soxhlet with methanol (VMS), Soxhlet with water (VWS)). Values are the means of three replicates±standard deviation. + – peak detected but equal or <LOD; * – the estimated concentration is >LOD, <LOQ.

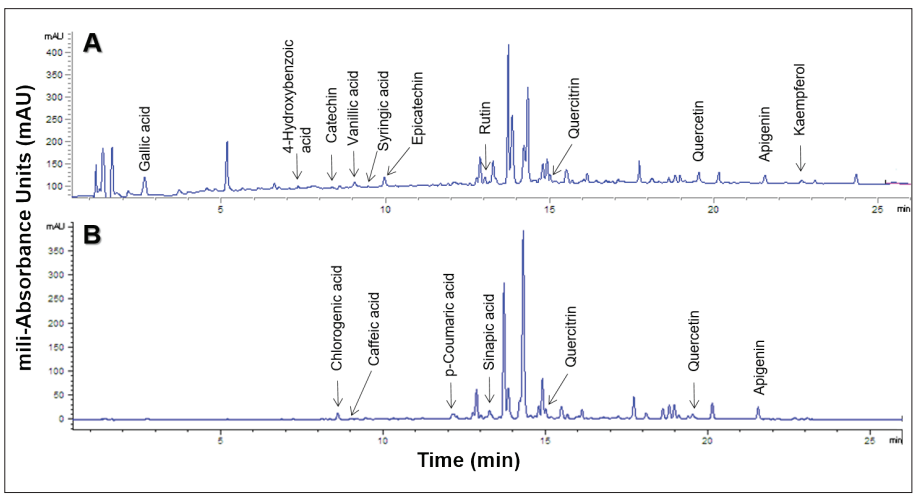

Fig. 1. HPLC chromatogram of *Verbascum stachydifolium* var. *stachydifolium* methanolic extract obtained by Soxhlet (VMS). **A** – 210 nm; **B** – 320 nm.

In vitro cytotoxicity and wound healing activity

Wound healing activity was assessed using the well-established *in vitro* cell scratch assay, which is a widely used method to assess the

wound healing activity of plant extracts [7]. Prior to the cell scratch wound healing assay, we investigated the potential cytotoxic effect of the extracts on the L929 mouse fibroblast cell line since reduced levels of cell proliferation can affect the results. Moreover, toxicity assessment is an important parameter for the evaluation of the use of herbal extracts in pharmaceutical preparations.

None of the extracts showed significant cytotoxicity on mouse fibroblast cells at 18 and 24 h at the highest concentrations (250 and 500 µg/mL), except VMM, which reduced cell viability at 500 µg/mL but showed no significant cytotoxicity at the concentration of 250 µg/mL. None of the extracts, except VMM, reduced cell viability by 50% so the IC_{50} values were not calculated, and the % cell viability values of the two highest concentrations (250 and 500 µg/mL) are provided in Table 3 instead. The extracts exhibited a low level of cytotoxicity at 48 h at the highest concentrations. No significant difference was observed between the extracts obtained using different solvents and extraction methods.

Table 3. Cell viability percentages obtained by the MTT assay after treatment with the highest extract concentrations (250 and 500 µg/mL).

	250 µg/mL			500 µg/mL		
Extracts	**18 h**	**24 h**	**48 h**	**18 h**	**24 h**	**48 h**
VMS	97.4%	94.5%	84.3%	96.5%	93.8%	72.5%
VMM	94.3%	94.4%	88.3%	14.4%	8.2%	6.9%
VWS	115.4%	88.8%	78.3%	103.3%	88.7%	77.7%
VWM	97.1%	94.2%	79.1%	95.9%	91.5%	49.6%

Maceration with methanol (VMM), maceration with water (VWM), Soxhlet with methanol (VMS), Soxhlet with water (VWS)).

The *in vitro* cell scratch assay was used to assess the wound healing potential of plant extracts, and the area closure percentages were calculated for qualitative comparison. Our results indicated that VWM significantly increased ($P<0.05$) fibroblast migration with a 60.4% closure rate compared to the negative control (Fig. 2B). However, it is also possible that other extracts may be more potent at higher concentrations. Representative images in Fig. 2A show the induction in fibroblast migration. The highest closure percentage was observed for the aqueous maceration extract.

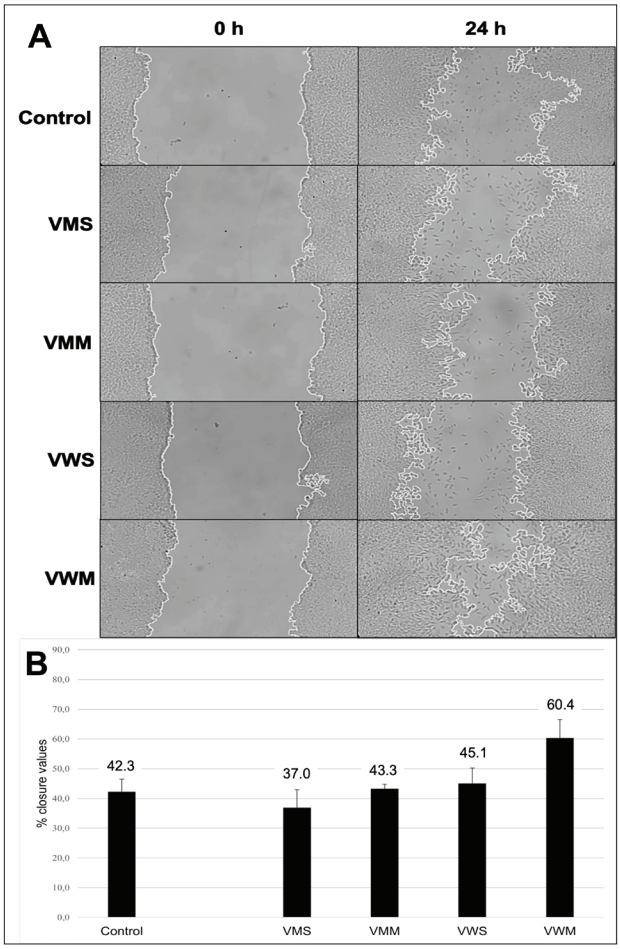

Fig. 2. Scratch test assay. **A** – Representative image showing the effect of *V. stachydifolium* var. *stachydifolium* (62 µg/mL) on mouse dermal fibroblast (L929) migration in a wound scratch test assay. Magnification (4×). **B** – Graphic showing scratch assay closure percentages of *V. stachydifolium* var. *stachydifolium* (62 µg/mL, 24h) on mouse dermal fibroblast (L929) migration in a wound scratch test assay. Results showed that VWM significantly increased ($P< 0.05$) fibroblast migration compared to the negative control.

Gene expression levels as determined by qRT-PCR

Total RNA amounts were between 64.1-111.7 ng/µL in the control group and between 91.1-128.2 ng/µL in the VWM group. When 62 µL/mg of VWM extract was applied to L929 cells, mRNA (cDNA) levels were quantified using the TaqMan gene expression assay, the values were normalized with GAPDH and the resulting expression value of the control group was fixed to 1 for comparison, it was observed that the gene expression levels of collagen I, TGF-β1 and VEGF were increased 2.2-, 2.1- and 1.9-fold, respectively, while a 4.8-fold increase was detected for FGF7 (Fig. 3).

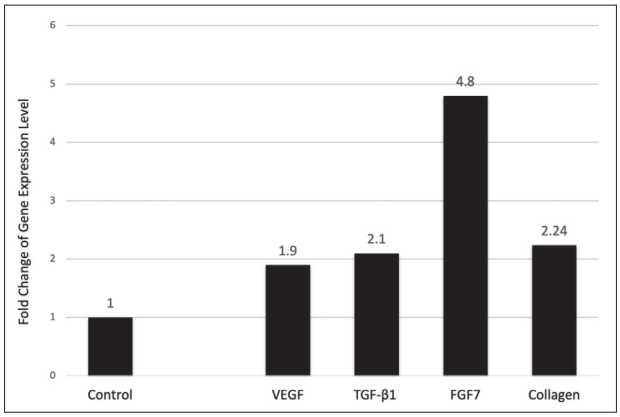

Fig. 3. Relative change in mRNA levels/expression in L929 cells treated with 62 μg/mL VWM extracts.

Protein quantities as determined by ELISA

The amounts of proteins that are the products of the genes of interest were determined by the ELISA method. Although the protein amounts were relatively low in some samples, the calculated concentrations against the blank were above the lower limit of detection of the commercial kits used. According to the results (Table 4), the amounts of all the proteins were increased with the most prominent increases observed for collagen I and FGF7.

Table 4. Protein concentrations in L929 cells treated with VWM extracts.

	Control (μg/mL)	**VWM** (μg/mL)
VEGF	81.04	101.59
TGF-β1	23.19	34.02
Collagen	0.005	0.29
FGF7	0.19	3.29

Maceration with water (VWM), vascular endothelial growth factor (VEGF), transforming growth factor-beta 1(TGF-β1), fibroblast growth factor 7 (FGF7), mouse fibroblast cell (L929).

DISCUSSION

There is strong evidence in the literature that wound healing and antioxidant activities are correlated with the amount/number of phenolic compounds produced in plants [1,17]. Therefore, special emphasis was put on these molecules in the present study. Previous studies have shown that methanolic extracts are rich in phenolic compounds [18,19], corroborating our results. There are many studies on saponins and glycosides of species belonging to the genus *Verbascum* [8,20-22] but there are only a few studies on their phenolic compounds [23-25]. There is no information about the phytochemical components of *V. stachydifolium* in the literature, but the phenolic contents of some other members of the genus have been studied and the results of the present study are generally in accordance with findings published previously [19,24]. The aqueous extract of *V. phlomoides* (mullein), one of the best-known medicinal plants in the genus, was found to contain 4.18% total phenolics expressed as GAE according to the Folin-Ciocalteu method [24]. We determined the percentages to be 4.14% and 6.33% after the aqueous Soxhlet and maceration extraction procedures, respectively. Another study has compared the total phenolic and flavonoid contents of *V. nigrum* L. and *V. thapsus* L. with *V. phlomoides* [19]; the authors found the total phenolic and flavonoid contents of the methanolic extracts to be between 85.0-135.0 mg GAE/g and 37.2-53.4 mg RUE/g, respectively. The values of the aqueous extracts ranged between 51.3-74.1 mg GAE/g and 10.2-20.0 mg RUE/g, respectively. The phenolic contents of *V. pinetorum* (Boiss.) O. Kuntze [26] and *V. antiochium* Boiss. & Heldr. [27] were determined to be 42.45 and 92.71 mg GE/g, respectively. Our results show that *V. stachydifolium* is a good source of polyphenols when compared to other species in the genus.

There are no data in the literature on the antioxidant activity of *V. stachydifolium*, but studies have shown that different species of *Verbascum* possess antioxidant properties [21,26,27]. The free radical (DPPH) scavenging activity of *V. macrurum* was reported as 67 μg/mL [28]. Our results are consistent with those in the literature. Antioxidants are capable of inhibiting or delaying the oxidation process in cells and blocking the harmful effects of reactive oxygen species (ROS); these properties make them comparatively popular as they play a role in the prevention of different diseases such as atherosclerosis, cancer and neurodegenerative diseases [29]. Antioxidant activity is also important for the wound healing activity of plant extracts [1,17]. The results of this study showed that the antioxidant activity of *V. stachydifolium* is close to that of other members of the genus when compared with the data in the literature. Antioxidant activities detected in this study are probably due to the synergistic effects of different phytochemicals, including phenolic compounds, which contribute to the wound healing effects. Antioxidant activity and total

phenolic content levels showed a correlation between the different extracts examined herein. This result is expected since it is well-documented that the antioxidant activity of plants is mainly due to the phenolic compounds they contain [18,30].

There is no information about the chemical constituents of *V. stachydifolium* in the literature, but a number of secondary metabolites were isolated and identified in other members of the genus. Studies on different *Verbascum* species resulted in the identification of various secondary metabolites including iridoid glycosides (e.g., aucubin, ajugol, catalpol and their derivatives), phenylethanoid glycosides (e.g., verbascosides), flavonoid glucosides (e.g., apigenin 7-glucoside, luteolin 7-glucoside), neolignan glucosides, saponins (e.g., verbascosaponins,), phenolic acids (e.g., vanillic, hydroxybenzoic, p-coumaric, hydroxycinnamic and ferulic acids) and flavonoids (e.g., apigenin, rutin, kaempferol, tamarixetin 7-rutinoside) [20,22,23,31,32]. Studies on the polyphenolic content of *Verbascum* species are limited. In a study of the polyphenol content of the *V. phlomoides* L. water extract, reverse-phase HPLC-DAD analysis was used and chlorogenic (12.3 mg/g), caffeic (39.9 mg/g), ferulic (29.6 mg/g), rosmarinic acids (14.9 mg/g) and quercetin (17.2 mg/g) were determined as major phenolics [24], while apigenin and aucubin were detected in traces. All these phenolics were also detected in our study of *V. stachydifolium* extracts by HPLC, except rosmarinic acid and aucubin (they were not screened), but the amounts are much lower. The highest amounts were determined for gallic acid and quercetin. Our results indicate that *V. stachydifolium* contains several phenolic compounds that contribute to its biological activities. According to our HPLC analysis, the number/amount of the compounds differed between the extracts, depending on the solvent type (methanol or water) rather than on the extraction method. Gallic acid was not detected in the VMM extract and kaempferol was detected only in VMS and VMM extracts. We used water as one of the extraction solvents since decoction, infusion and maceration using water are common methods used for preparing herbal extracts, and our results revealed that aqueous extracts also contained effective amounts of phenolics compared to the methanolic extract.

The methanolic extract of *V. speciosum* Schrad. showed cytotoxicity in the L929 mouse fibroblast cell line, human umbilical vein endothelial cells (HUVEC) and primary human fibroblasts at concentrations higher than 400 μg/mL after 24, 48 and 72 h [7]. In another study, the cytotoxic effects of 15 taxa of *Verbascum* sp. (not including *V. stachydifolium*) on different cancer and non-cancerous normal cells (Vero, African green monkey kidney) were investigated, and cytotoxic activity of some species against certain cancer cell lines was detected, whereas the activity was not significant at 100 μg/mL concentration [33]. Consistent with previous studies, our results also indicate that different extracts of *V. stachydifolium* did not show significant cytotoxicity against mouse fibroblast cells, which is a valuable property for pharmaceutical preparations.

The *in vivo* wound healing activities of 13 *Verbascum* species using linear incision and circular excision wound models were investigated previously and the best results were obtained with the methanolic extracts of *V. splendidum*, *V. stachydifolium* and *V. uschackense*, justifying their use in Turkish folk medicine [3]. No additional information is available in the literature regarding either wound healing and cytotoxic activity or the chemical composition of *V. stachydifolium*. The cell scratch assay, performed in the present study, is a widely used *in vitro* wound healing model to assess the activity of compounds and natural products [34]. Our results confirmed the wound healing activity of the studied species and showed that its aqueous extract also has a wound healing effect. The wound healing activity of the methanolic extract of *V. speciosum* was also shown in *in vitro* studies using L929 mouse fibroblasts, primary human fibroblast cells and the HUVEC cell line; according to these results, a concentration of 200 μg/mL increased the closure rate in L929-HUVEC coculture and collagen type I expression [7]. We obtained a similar result with the aqueous extract of *V. stachydifolium* var. *stachydifolium* and at a lower concentration.

Within a few days after wound formation, the proliferation and migration of fibroblasts in the area around the wound occur. Growth factors and cytokines secreted locally in the wound area are of great importance during this process [35]. The main sources of these cytokines are macrophages, neutrophils, platelets, keratinocytes and fibroblast cells. Examples of major factors responsible for wound healing include TGF

alpha and beta, platelet-derived growth factor (PDGF), VEGF, tumor necrosis factor-alpha (TNF-α), epidermal growth factor (EGF), insulin-like growth factor (IGF) and the FGF gene family [36,37]. In the present study, the expression/secretion levels of collagen I and the cytokines TGF-β, VEGF and FGF7 were investigated by qRT-PCR and ELISA. In this way, we obtained information about the molecular mechanisms of the wound healing activity of the studied species. Our results showed that VWM increased the expression of all the genes at mRNA and protein levels, with the most prominent results obtained for FGF7 and collagen I.

Fibroblast migration and proliferation play an important role in the wound healing process, and these events are regulated by both paracrine and autocrine cytokines. Fibroblasts are the main collagen-producing/secreting cells, which is a very important event for proper wound healing. The different cytokines mentioned above have been found to increase collagen synthesis in cultured fibroblast cells. The increase in collagen synthesis is also a factor that accelerates the wound healing process, and it has been shown that various plant extracts possessing wound healing activity increase collagen synthesis [1,38]. The *C. asiatica* extract, which is well known for its wound healing properties, has also been shown to increase collagen synthesis in fibroblast cells [38].

Proteins belonging to the FGF family have very important roles in cell proliferation, differentiation, migration and vascularization. There are 23 well-characterized known members of the FGF family. Of these, the more important ones in wound healing are FGF2, FGF7 and FGF10. Different types of FGFs are synthesized by keratinocytes, endothelial cells, smooth muscle cells, chondrocytes, mast cells and fibroblasts. These are generally paracrine cytokines with receptors in the cells they affect [39]. *In vitro* studies have shown that FGF7 promotes epithelialization by stimulating keratinocyte migration and proliferation, so it has an important role in wound healing. It is also mitogenic for vascular endothelial cells and increases VEGF synthesis [39]. Moreover, FGF7 stimulates the synthesis of urokinase-type plasminogen activator (a serine protease necessary for revascularization) in endothelial cells [40]. The wound healing effect of FGF7 has also been demonstrated using *in vivo* animal models and in clinical studies [41]. Because of these properties, FGF7 is among the most important cytokines in wound healing.

TGF-β also plays role in the wound healing process by interfering with different mechanisms such as vascularization, epithelialization and connective tissue regeneration [39,42]. TGF-β1 is synthesized and released by platelets, macrophages, keratinocytes and fibroblasts in scar tissue [42-49]. Studies have shown that TGF-β1 levels increase in scar tissue following wound formation and that TGF-β1 stimulates VEGF secretion from diverse cells. TGF-β1 also plays a role in collagen production during the remodeling phase [39,50,51].

One of the major roles of VEGF in wound healing is the stimulation of vascularization. The revascularization that takes place during the wound healing process includes several steps. The most important ones are vasodilation, basement membrane degradation, endothelial cell migration and proliferation. Subsequently, capillary tube formation takes place, and a new basement membrane is formed [52]. The effects of VEGF-A on wound healing have been demonstrated in previous studies [39,48,49,52-56], which have shown that VEGF-A plays an important role in wound healing by increasing endothelial cell migration and proliferation during the revascularization process, and its expression increases in the early stages of wound healing [39,52,55,57]. VEGF is also important in epithelialization and collagen synthesis [52]. In addition, *in vivo* studies have shown that VEGF-A levels increased in acute wounds [58].

CONCLUSIONS

Our results show that the endemic plant *Verbascum stachydifolium* var. *stachydifolium* contains phenolic compounds and possesses antioxidant and wound healing activities. The antioxidant activity, phenolic content and cytotoxicity of this taxon as well as the molecular mechanism underlying its wound healing potential were investigated for the first time. HPLC analysis showed that the extracts contained phenolic compounds, but their amounts were not too high. Our results support the use of the *in vitro* scratch assay for the initial screening of natural products to assess their wound healing potential to reduce animal use. Herbal extracts exhibit their wound healing effects at the molecular level usually by increasing collagen synthesis or

affecting the expression of cytokines. This effect was also remarkable in the present study. In light of the results, *V. stachydifolium* var. *stachydifolium* deserves further investigation aiming to isolate and identify the active compounds, including not only phenolics but also other secondary metabolites such as glycosides and saponins responsible for its wound healing activity, by bioactivity-guided characterization studies.

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REFERENCES

1. Agyare C, Bekoe EO, Boakye YD, Dapaah SO, Appiah T, Bekoe SO. Medicinal Plants and Natural Products with Demonstrated Wound Healing Properties. In: Alexandrescu V, editor. Wound Healing - New insights into Ancient Challenges. London: InTechOpen; 2016. p. 487-535. https://doi.org/10.5772/63574
2. Süntar I, Akkol EK, Keleş H, Oktem A, Başer KHC, Yeşilada E. A novel wound healing ointment: A formulation of Hypericum perforatum oil and sage and oregano essential oils based on traditional Turkish knowledge. J Ethnopharmacol. 2011;134:89-96. https://doi.org/10.1016/j.jep.2010.11.061
3. Süntar I, Tatlı II, Küpeli Akkol E, Keleş H, Kahraman Ç, Akdemir Z. An ethnopharmacological study on Verbascum species: From conventional wound healing use to scientific verification. J Ethnopharmacol. 2010;132:408-13. https://doi.org/10.1016/j.jep.2010.08.004
4. Maver T, Maver U, Stana Kleinschek K, Smrke DM, Kreft S. A review of herbal medicines in wound healing. Int J Dermatol. 2015;54:740-51. https://doi.org/10.1111/ijd.12766
5. Güner Adil, Aslan S, Ekim T, Babaç T. List of Turkish Flora (Vascular Plants). Nezahat Gökyiğit Botanical Garden and Flora Research Society; 2012.
6. Ozkan G, Kamiloglu S, Ozdal T, Boyacioglu D, Capanoglu E. Potential use of Turkish medicinal plants in the treatment of various diseases. Molecules. 2016;21:1-32. https://doi.org/10.3390/molecules21030257
7. Demirci S, Doğan A, Demirci Y, Şahin F. In vitro wound healing activity of methanol extract of Verbascum speciosum. Int J Appl Res Nat Prod. 2014;7:37-44.
8. Akdemir Z, Kahraman Ç, Tatlı II, Küpeli Akkol E, Süntar I, Keles H. Bioassay-guided isolation of anti-inflammatory, antinociceptive and wound healer glycosides from the flowers of Verbascum mucronatum Lam. J Ethnopharmacol. 2011;136:436-43. https://doi.org/10.1016/j.jep.2010.05.059
9. Singleton VL, Orthofer R, Lamuela-Raventós RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol. 1999;299:152-78. https://doi.org/10.1016/S0076-6879(99)99017-1
10. Kaptaner İğci B, Aytaç Z. An investigation on the in vitro wound healing activity and phytochemical composition of Hypericum pseudolaeve Robson growing in Turkey. Turk J Pharm Sci. 2020;17:610-9. https://doi.org/10.4274/tjps.galenos.2019.80037
11. Aliyu AB, Ibrahim MA, Musa AM, Musa AO, Kiplimo JJ, Oyewale AO. Free radical scavenging and total antioxidant capacity of root extracts of Anchomanes difformis ENGL. (ARACEAE).Acta Pol Pharm. 2013;70:115-21.
12. Blois MS. Antioxidant determinations by the use of a stable free radical. Nature. 1958;181:1199-200. https://doi.org/10.1038/1811199a0
13. Magnusson B, Örnemark U. The Fitness for Purpose of Analytical Methods A Laboratory Guide to Method Validation and Related Topics. 2nd ed. Teddington: Eurachem; 2014. 70 p.
14. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55-63. https://doi.org/10.1016/0022-1759(83)90303-4
15. Felice F, Zambito Y, Belardinelli E, Fabiano A, Santoni T, di Stefano R. Effect of different chitosan derivatives on in vitro scratch wound assay: a comparative study. Int J Biol Macromol. 2015;76:236-41. https://doi.org/10.1016/j.ijbiomac.2015.02.041

16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402-8. https://doi.org/10.1006/meth.2001.1262
17. Agar OT, Dikmen M, Ozturk N, Yilmaz MA, Temel H, Turkmenoglu FP. Comparative studies on phenolic composition, antioxidant, wound healing and cytotoxic activities of selected Achillea L. species growing in Turkey. Molecules. 2015;20:17976-8000. https://doi.org/10.3390/molecules201017976
18. Alali FQ, Tawaha K, El-Elimat T, Syouf M, El-Fayad M, Abulaila K, Nielsen SJ, Wheaton WD, Falkinham JO. Antioxidant activity and total phenolic content of aqueous and methanolic extracts of Jordanian plants: an ICBG project. Nat Prod Res. 2007;21:1121-1131. https://doi.org/10.1080/14786410701590285
19. Mihailovic V, Kreft S, Benkovic ET, Ivanovic N. Chemical profile, antioxidant activity and stability in stimulated gastrointestinal tract model system of three Verbascum species. Ind Crops Prod. 2016;89:141-51. https://doi.org/10.1016/j.indcrop.2016.04.075
20. Akdemir ZŞ, Tatli II, Bedir E, Khan IA. Neolignan and phenylethanoid glycosides from Verbascum salviifolium boiss. Turk J Chem. 2004;28:621-8.
21. Kahraman C, Akdemir ZS, Tatli II. Promising Cytotoxic Activity Profile, Biological Activities and Phytochemical Screening of Verbascum L . Species. Med Aromat Plant Sci Biotechnol. 2012;6:63-75.
22. Turker AU, Camper ND, Gürel E. High-Performance Liquid Chromatographic Determination of a Saponin from Verbascum. Biotechnol Biotechnol Equip. 2004;18:54-9. https://doi.org/10.1080/13102818.2004.10819230
23. Akdemir ZS, Tatlı II, Bedir E, Khan IA. Antioxidant Flavonoids from Verbascum salviifolium Boiss. FABAD J Pharm Sci. 2003;28:71-5.
24. Grigore A, Colceru-Mihul S, Litescu S, Panteli M, Rasit I. Correlation between polyphenol content and anti-inflammatory activity of Verbascum phlomoides (mullein). Pharm Biol. 2013;51:925-9. https://doi.org/10.3109/13880209.2013.767361
25. Klimek B, Olszewska MA, Magdelena T. Simultaneous determination of flavonoids and phenylethanoids in the flowers of Verbascum densiflorum and V. phlomoides by high-performance liquid chromatography. Phytochem Anal. 2010;21:150-6. https://doi.org/10.1002/pca.1171
26. Ozcan B, Esen M, Caliskan M, Mothana RA, Cihan AC, Yolcu H. Antimicrobial and antioxidant activities of the various extracts of Verbascum pinetorum Boiss. O. Kuntze (Scrophulariaceae). Eur Rev Med Pharmacol Sci. 2011;15:900-5.
27. Caliskan B, Ozcan M, Yilmaz M. Antimicrobial and Antioxidant Activities of Various Extracts of Verbascum antiochium Boiss. (Scrophulariaceae). J Med Food. 2010;13:1147-52. https://doi.org/10.1089/jmf.2009.0213
28. Aligiannis N, Mitaku S, Tsitsa-Tsardis E, Catherine, Harvala Ioannis, Tsaknis Stavros L, Serko H. Methanolic Extract of Verbascum macrurum as a Source of Natural Preservatives against Oxidative Rancidity. J Agric Food Chem. 2003;51:7308-12. https://doi.org/10.1021/jf034528
29. Liu Z, Ren Z, Zhang J, Chuang C-C, Kandaswamy E, Zhou T, Zuo Li. Role of ROS and Nutritional Antioxidants in Human Diseases. Front Physiol. 2018;9:477. https://doi.org/10.3389/fphys.2018.00477
30. Skerget M, Kotnik P, Hadolin M, Ri A, Simoni M, Zeljko Kne. Phenols, proanthocyanidins , flavones and flavonols in some plant materials and their antioxidant activities. Food Chem. 2005;89:191-8. https://doi.org/10.1016/j.foodchem.2004.02.025
31. Tatlı İİ, Schuhly W, Akdemir ZS. Secondary Metabolites from Bioactive Methanolic Extract of Verbascum pycnostachyum Boiss. & Helder Flowers. Hacettepe Uni J Fac Pharm. 2007;27:23-32.
32. Armatu A, Bodırlau R, Nechıta CB, Nıculaua M, Teaca CA, Ichım M, Spiridon I. Characterization of biological active compounds from Verbascum phlomoides by chromatography techniques. I. Gas chromatography. Rom Biotechnol Lett. 2011;16:6297-304.
33. Tatlı İ, Akdemir Z. Cytotoxic Activity on Some Verbascum Species Growing in Turkey. Hacettepe Uni J Fac Pharm. 2006;26:77-85.
34. Fronza M, Heinzmann B, Hamburger M, Laufer S, Merfort I. Determination of the wound healing effect of Calendula extracts using the scratch assay with 3T3 fibroblasts. J Ethnopharmacol. 2009;126:463-7. https://doi.org/10.1016/j.jep.2009.09.014
35. Gill SE, Parks WC. Metalloproteinases and their inhibitors: Regulators of wound healing. Int J Biochem Cell Biol. 2008;40:1334-47. https://doi.org/10.1016/j.biocel.2007.10.024
36. Tsirogianni AK, Moutsopoulos NM, Moutsopoulos HM. Wound healing: Immunological aspects. Injury. 2006;37:S5-S12. https://doi.org/10.1016/j.injury.2006.02.035
37. Martin P. Wound Healing-Aiming for Perfect Skin Regeneration. Science. 1997;276:75-81. https://doi.org/10.1126/science.276.5309.75
38. Maquart F-X, Bellon G, Gillery P, Wegrowski Y, Borel J-P. Stimulation of Collagen Synthesis in Fibroblast Cultures by a Triterpene Extracted from Centella asiatica. Connect Tissue Res. 1990;24:107-20. https://doi.org/10.3109/03008209009152427
39. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. Wound Repair Regen. 2008;16:585-601. https://doi.org/10.1111/j.1524-475X.2008.00410.x
40. Niu J, Chang Z, Peng B, Xia Q, Lu W, Huang P, Tsao MS, Chiao PJ. Keratinocyte growth factor/fibroblast growth factor-7-regulated cell migration and invasion through activation of NF-kappaB transcription factors. J Biol Chem. 2007;282:6001-11. https://doi.org/10.1074/jbc.M606878200
41. Robson MC, Phillips TJ, Falanga V, Odenheimer DJ, Parish LC, Jensen JL, Steed DL. Randomized trial of topically applied repifermin (recombinant human keratinocyte growth factor-2) to accelerate wound healing in venous ulcers. Wound Repair Regen. 2001;9:347-52. https://doi.org/10.1046/j.1524-475x.2001.00347.x
42. Frank S, H bner G, Breier G, Longaker MT, Greenhalgh DG, Werner S. Regulation of Vascular Endothelial Growth

Factor Expression in Cultured Keratinocytes. J Biol Chem. 1995;270:12607-13. https://doi.org/10.1074/jbc.270.21.12607
43. Ashcroft GS, Dodsworth J, Boxtel E Van, Tarnuzzer RW, Horan MA, Schultz GS, Ferguson MW. Estrogen accelerates cutaneous wound healing associated with an increase in TGF-β1 levels. Nat Med. 1997;3:1209-15. https://doi.org/10.1038/nm1197-1209
44. Lee H-S, Kooshesh F, Sauder D, Kondo S. Modulation of TGF-beta 1 production from human keratinocytes by UVB. Exp Dermatol. 1997;6:105-10. https://doi.org/10.1111/j.1600-0625.1997.tb00155.x
45. Wu L, Yu YL, Galiano RD, Roth SI, Mustoe TA. Macrophage Colony-Stimulating Factor Accelerates Wound Healing and Upregulates TGF-β1 mRNA Levels through Tissue Macrophages. J Surg Res 1997;72:162-9. https://doi.org/10.1006/jsre.1997.5178
46. Mani H, Sidhu GS, Kumari R, Gaddipati JP, Seth P, Maheshwari RK. Curcumin differentially regulates TGF-beta1, its receptors and nitric oxide synthase during impaired wound healing. Biofactors. 2002;16:29-43. https://doi.org/10.1002/biof.5520160104
47. Werner S, Krieg T, Smola H. Keratinocyte-Fibroblast Interactions in Wound Healing. Journal of Investigative Dermatology. 2007;127:998-1008. https://doi.org/10.1038/sj.jid.5700786
48. Chen L, Tredget EE, Wu PYG, Wu Y. Paracrine Factors of Mesenchymal Stem Cells Recruit Macrophages and Endothelial Lineage Cells and Enhance Wound Healing. PLoS One. 2008;3:1886. https://doi.org/10.1371/journal.pone.0001886
49. Anitua E, Pino A, Orive G. Plasma rich in growth factors promotes dermal fibroblast proliferation, migration and biosynthetic activity. J Wound Care. 2016;25:680-7. https://doi.org/10.12968/jowc.2016.25.11.680
50. Kane CJM, Hebda PA, Mansbridge JN, Hanawalt PC. Direct evidence for spatial and temporal regulation of transforming growth factor beta 1 expression during cutaneous wound healing. J Cell Physiol. 1991;148:157-73. https://doi.org/10.1002/jcp.1041480119
51. Colwell AS, Phan T-T, Kong W, Longaker MT, Lorenz PH. Hypertrophic scar fibroblasts have increased connective tissue growth factor expression after transforming growth factor-beta stimulation. Plast Reconstr Surg. 2005;116:1387-91. https://doi.org/10.1097/01.prs.0000182343.99694.28
52. Bao P, Kodra A, Tomic-Canic M, Golinko MS, Ehrlich HP, Brem H. The Role of Vascular Endothelial Growth Factor in Wound Healing. J Surg Res. 2009;153:347-58. https://doi.org/10.1016/j.jss.2008.04.023
53. Brown BLF, Yeo KT, Berse B, Yeo T, Senger DR, Dvorak HF. Expression of Vascular Permeability Factor (Vascular Endothelial Growth Factor) by Epidermal Keratinocytes during Wound Healing. J Exp Med. 1992;176:1376-9. https://doi.org/10.1084/jem.176.5.1375
54. Nissen NN, Polverini PJ, Koch AE, Volin M V, Gamelli RL, DiPietro LA. Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. Am J Pathol. 1998;152:1445-52.
55. Chakroborty D, Sarkar C, Lu K, Bhat M, Dasgupta PS, Basu S. Activation of Dopamine D1 Receptors in Dermal Fibroblasts Restores Vascular Endothelial Growth Factor-A Production by These Cells and Subsequent Angiogenesis in Diabetic Cutaneous Wound Tissues. Am J Pathol. 2016;186:2262-70. https://doi.org/10.1016/j.ajpath.2016.05.008
56. Lafosse A, Dufeys C, Beauloye C, Horman S, Dufrane D. Impact of Hyperglycemia and Low Oxygen Tension on Adipose-Derived Stem Cells Compared with Dermal Fibroblasts and Keratinocytes: Importance for Wound Healing in Type 2 Diabetes. PLoS One. 2016;11:e0168058. https://doi.org/10.1371/journal.pone.0168058
57. Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Perruzzi CA, Detmar M. Stimulation of Endothelial Cell Migration by Vascular Permeability Factor/Vascular Endothelial Growth Factor through Cooperative Mechanisms Involving the abf3 Integrin, Osteopontin, and Thrombin. Am J Pathol. 1996;149:293-305.
58. Shukla A, Dubey MP, Srivastava R, Srivastava BS. Differential Expression of Proteins during Healing of Cutaneous Wounds in Experimental Normal and Chronic Models. Biochem Biophys Res Commun. 1998;244:434-9. https://doi.org/10.1006/bbrc.1998.8286