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ORIGINAL ARTICLE

An Investigation on the *In Vitro* Wound Healing Activity and Phytochemical Composition of *Hypericum pseudolaeve* N. Robson Growing in Turkey

Türkiye'de Yayılış Gösteren *Hypericum pseudolaeve* N. Robson Türünün *In Vitro* Yara İyileştirme Aktivitesi ve Fitokimyasal Kompozisyonu Üzerine Bir Araştırma

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ABSTRACT

Objectives: The aim of this study was to investigate the in vitro wound healing effects of the methanolic and aqueous extracts of *Hypericum pseudolaeve* N. Robson obtained by two different methods as well as its cytotoxicity, antioxidant activity, and selected phytochemical constituents.

Materials and Methods: Total phenolic and flavonoid contents were measured using spectrophotometry-based methods. The cytotoxic effects of the extracts on L929 mouse fibroblast cells were evaluated by and 2h-tetrazolium,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Moreover, migration and spreading of the treated fibroblast cells were assessed by cell scratch assay as an *in vitro* wound healing model. In addition, the chemical content of the species was determined by high pressure liquid chromatography (HPLC).

Results: The results of the cytotoxicity assay indicated that the methanolic and aqueous extract did not have any cytotoxic effect on fibroblast cells at concentrations up to 500 µg/mL. Fibroblast migration was significantly increased by 62 µg/mL concentration of the aqueous extracts compared to the negative control. The extracts showed good antioxidant activity and 16 phytochemical compounds were detected by HPLC, with the highest amount for epicatechin.

Conclusion: The results showed that *Hypericum pseudolaeve* extracts have wound healing potential and contain several important antioxidant phenolic compounds. This species deserves further investigation aiming to isolate and identify the active compounds.

Key words: Wound healing, plant extract, antioxidant, phenolics, HPLC

ÖZ

Amaç: Bu çalışmanın amacı, *Hypericum pseudolaeve* N. Robson türünün iki farklı yöntemle elde edilmiş metanolik ve su ekstrelerinin *in vitro* yara iyileştirici etkisi ile sitotoksisitesini, antioksidan aktivitesini ve seçilmiş fitokimyasallarını araştırmaktır.

Gereç ve Yöntemler: Toplam fenolik ve flavonoit içerikleri spektrofotometri-temelli yöntemler kullanılarak ölçülmüştür. Ekstrelerin L929 fare fibroblast hücreleri üzerindeki sitotoksik etkileri 2h-tetrazolium,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide yöntemi ile değerlendirilmiştir. Ayrıca, uygulana yapılmış fibroblast hücrelerinin göçleri ve yayılmaları *in vitro* yara iyileşme modeli olarak kullanılan hücre çizik yöntemi ile değerlendirilmiştir. Ayrıca, türün kimyasal içeriği yüksek basınçlı sıvı kromatografisi (HPLC) ile belirlenmiştir.

Bulgular: Sitotoksisite deneyinin sonuçları, metanolik ve sulu ekstrelerinin, 500 µg/mL konsantrasyona kadar kullanıldığında fibroblast hücreleri üzerinde herhangi bir sitotoksik etkiye sahip olmadığını göstermiştir. Fibroblast göçü 62 µg/mL konsantrasyonunda sulu ekstreler uygulandığında negatif kontrole göre anlamlı derecede artış göstermiştir. Ekstreler iyi derecede antioksidan aktivite göstermiştir ve HPLC analizi ile içerinde en yüksek düzeyde epikateşin olmak üzere 16 fitokimyasal bileşik tespit edilmiştir.

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Sonuç: Sonuçlar *Hypericum pseudolaeve* ekstrelerinin, yara iyileşme potansiyeline sahip olduğunu ve çeşitli önemli antioksidan fenolik bileşikler içerdiğini göstermiştir. Bu türle ilgili aktif bileşiklerin izole edilmesi ve tanımlamasını amaçlayan daha fazla araştırma yapılmalıdır.

Anahtar kelimeler: Yara iyileşmesi, bitki ekstresi, antioksidan, fenolikler, HPLC

INTRODUCTION

Recent estimations show that approximately 6 million people suffer from chronic wounds worldwide. Wounds related to diabetes, gastric disorders, and duodenal ulcers and due to injuries such as cuts and burns continue to have serious impacts on the life quality of patients.[1] Wounds are generally caused by a cut or an opening in the skin as a result of physical damage, burns, infections, or chronic illnesses that disrupt normal skin anatomy and function. They cause loss of the connective tissue underlying the skin and integrity of the epithelial tissue. Chronic and delayed acute wounds are the most difficult to heal. Wound healing is a dynamic process involving the stages of inflammation (0-3 days), cellular proliferation (3-12 days), and remodeling (3-6 months), where cell-cell and cell-matrix interactions take place.[1] In the wound healing process, collagenase and elastase enzymes also play important roles by degrading extracellular matrix components such as collagen, elastin, and fibrin. However, their activity must be balanced by inhibition mechanisms and prolonged overexpression of these enzymes may cause impaired wound healing.[1] In recent years, the search for alternative and powerful remedies from nature (plants, animals, the marine environment, fungi, and other microorganisms) having potential to heal acute and chronic wounds especially in patients with metabolic disorders has increased considerably.[2] In folk medicine worldwide, many plants have traditional use for treating wounds. Wound healing activities of various plant extracts have also been demonstrated by scientific research using *in vitro* and *in vivo* methods.[2-8] Wound healing agents exert their effects by induction of keratinocyte differentiation and proliferation, stimulation of fibroblast proliferation and migration, increasing collagen formation, and exhibiting antioxidant, antimicrobial and antiinflammatory properties.[2]

The genus *Hypericum* is represented by 484 taxa from 36 taxonomic sections in the world according to the recent review of the genus[9] and by 119 taxa in Turkey, 49 of which are endemic.[10] *Hypericum pseudolaeve* N. Robson is grouped under the section *Hirtella* (*Drosanthe*) and distributed in central and eastern Anatolia in Turkey. The genus *Hypericum*, especially *Hypericum perforatum* (St. John's wort), is one of the most widely used medicinal plants for depression and its wound healing effects have been shown in both ethnobotanical and functional studies.[2,3,11-13] A common ethnobotanical preparation method for *Hypericum* spp. for wound healing is maceration of the aerial parts in olive oil under direct sunlight for at least 4 weeks.[11] However, there are no data in the literature regarding the ethnobotanical usage and wound healing potential of *H. pseudolaeve* to the best of the authors' knowledge and there is limited information on the chemical constituents of the species.

The aim of this study was to assess the wound healing potential of *H. pseudolaeve* extracts by *in vitro* methods, as well as to investigate their cytotoxicities, antioxidant activities, and phytochemical compositions with special emphasis on phenolic compounds. Moreover, we compared methanolic and aqueous extracts obtained by maceration and Soxhlet methods. Investigation on the chemical constituents and biological activities of this plant could be helpful in future studies searching for alternative drugs.

MATERIALS AND METHODS

Chemical compounds

The standards used for the high performance liquid chromatography (HPLC) analyses (except hypericin), thiobarbituric acid, and dimethylsulfoxide (DMSO) were purchased from Sigma; hypericin was from Santa Cruz Biotechnology; 2,2-diphenyl-1-picrylhydrazyl (DPPH) was from Aldrich; Folin-Ciocalteu reagent, ascorbic acid, sodium carbonate, potassium acetate, aluminum chloride hexahydrate, and sulfuric acid were from Merck; sodium phosphate was from Riedel-de Haën; ammonium molybdate was from Fluka; Dulbecco's modified Eagle's medium (DMEM) was from Gibco; fetal bovine serum (FBS), phosphate buffer saline (PBS), L-glutamine, and penicillin/streptomycin were from PAN Biotech; trypsin/ethylenediaminetetraacetic acid (EDTA) was from biological Industries; and (2h-tetrazolium,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Fisher Scientific.

Plant materials

Field studies were carried out by the authors in Nevşehir Province in Turkey for the collection of plant material. *H. pseudolaeve* was collected from dry igneous metamorphic slopes or steppes (1434 m, a.s.l.) between Ortahisar and Nevşehir on 06.06.2017 (Figure 1). Plant samples of a single population were used in the studies to minimize compositional variation. One of the collected plants was given a herbarium number (BK 1265) and deposited in the Herbarium of Gazi University. The aerial parts of the plants were dried in the shade and powdered with a commercial blender (waring). The powdered plant material was kept in the dark at room temperature until used.

Extraction procedure and determination of the yield

Extracts of *H. pseudolaeve* were obtained by maceration or Soxhlet extraction using methanol or water as solvents. After extraction, Whatman grade no.1 filter paper was used for the filtration procedure. Methanol was evaporated using a rotary vacuum evaporator (Heidolph-Rotar VV2000) at 40°C. Water extracts were frozen at -20°C and lyophilized by a freeze-dryer thereafter (Christ Gamma 2-16 LSC). The plant extracts were stored in the dark at 4°C until studied. Finally, four different

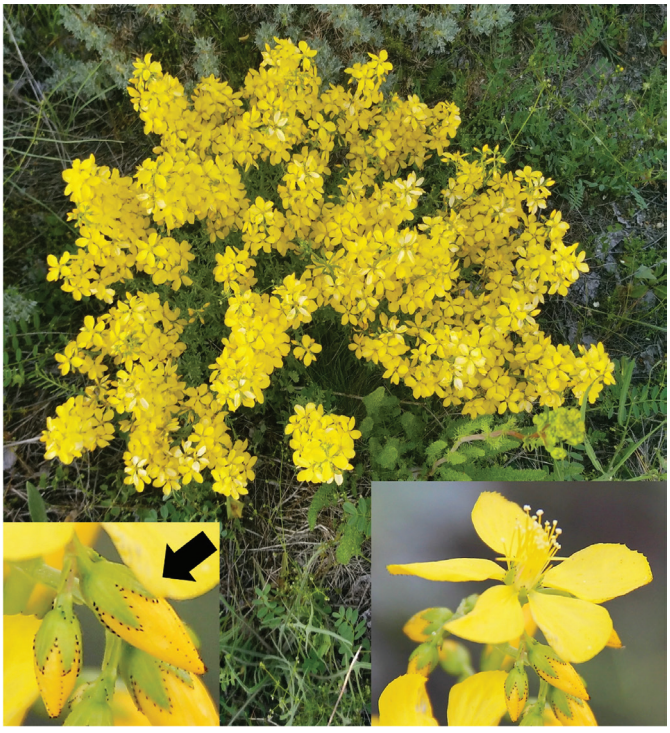

Figure 1. Photographs from the field of *Hypericum pseudolaeve*. Arrow indicates the dark secretion glands on the edges of the sepals and petals of *H. pseudolaeve*

Photographs: Bahar Kaptaner İğci

extracts were prepared and are abbreviated throughout the paper as follows: *H. pseudolaeve* maceration with methanol, maceration with water (HWM), soxhlet with methanol (HMS), and soxhlet with water (HWS). The extraction efficiencies of the plant materials were calculated using the following formula and expressed as percentages (%):

Percentage efficiency (w/w)=(weight of the dried extract, g)/(weight of dry plant material measured before the extraction process, g) ×100

Determination of total phenolic content

Total phenolic content was determined by the Folin-Ciocalteu method.[14] Gallic acid was used for the reference compound to obtain a standard curve (10 different concentrations were used between 10 and 100 µg/mL). Briefly, 0.5 mL of extracts (1 mg/mL) was mixed with 2.5 mL of 1:10 diluted Folin-Ciocalteu reagent and 2 mL of sodium carbonate solution (7.5% w/v) and allowed to stand for 15 min at 45°C. Blank, standards, and samples were transferred to cuvettes and read using a ultraviolet-visible spectroscopy (UV-VIS) spectrophotometer (PerkinElmer, Lambda 25) at 765 nm wavelength. Each sample was measured in triplicate and mean values were used. The results were presented as mg/g gallic acid equivalents (mg GAE/g).

Determination of the total flavonoid content

The total flavonoid content was determined by the aluminum chloride colorimetric method. Briefly, 0.5 mL of the extract solutions (0.5 mg/mL) was mixed with 0.1 mL of 10% aluminum chloride hexahydrate, 0.1 mL of 1 mol/L potassium acetate, and 2.8 mL of deionized water. After incubation at room temperature for 40 min, the blank, standards, and samples were transferred to cuvettes and the absorbance of the reaction mixture was measured at 415 nm against a blank by a UV-VIS spectrophotometer (PerkinElmer, Lambda 25). Rutin was used as a standard compound at 8 different concentrations between 10 and 80 µg/mL and the results were calculated as mg/g rutin equivalents (mg RUE/g). Each sample was measured in triplicate and mean values were used.

Determination of the total antioxidant capacity

First, 0.3 mL of extract (1 mg/mL) was mixed with 3 mL of the reagent solution (0.6 M sulfuric acid, 28 mmol/L sodium phosphate, and 4 mmol/L ammonium molybdate). Next, the tubes containing the reaction solution were incubated at 95°C for 90 min. Then the blank, standards, and samples were transferred into cuvettes and measured at 695 nm using a UV-VIS spectrophotometer (PerkinElmer, Lambda 25) after cooling to room temperature. Calibration graphics was plotted using ascorbic acid (AA) as a standard at concentrations between 3.9 and 500 µg/mL obtained by 2-fold serial dilution and the antioxidant activity was calculated as the equivalents of AA (EAA). The standards and samples were measured in triplicate and mean values were used.

2,2-Diphenyl-1-picrylhydrazyl free radical scavenging activity assay

The extracts were prepared in concentrations of 15.62, 31.25, 62.5, 125, and 250 µg/mL for this assay. First, 3 mL of extract of each concentration was mixed with 1 mL of the 0.1 mmol/L DPPH solution prepared in methanol. Next, the tubes were incubated in the dark at room temperature for 30 min and then read at 517 nm using a UV-VIS spectrophotometer (PerkinElmer, Lambda 25). Solvent without extract was used as a negative control and AA was used as a positive control. The effect of antioxidant capacity was observed as the color change of purple DPPH to yellow/light-yellow and % inhibition values of each extract were calculated using the following equation:

Inhibition (%)=[($A_{control}$ — A_{blank}) — (A_{sample} — A_{blank})]×100/($A_{control}$ — A_{blank}),

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of AA or extracts. Inhibitory concentration (IC_{50}) values were calculated with inhibition rates using a four-parameter logistic regression model after sigmoidal curves were plotted. Each of the standards and the samples were measured in triplicate and mean values were used for the calculations.

High performance liquid chromatography analysis and quantification

The chemical contents of the extracts were analyzed by reversed-phase HPLC-diode array detector (DAD) method. The reference compounds were selected mainly from phenolics that are common in plants as secondary metabolites. Chromatograms were recorded at 8 different wavelengths and 210, 260, 270, and 320 nm were chosen for the analyses

according to the maximum absorbances of reference peaks. All the standards and samples were filtered through 0.45-µm polytetrafluoroethylene membrane, measured in triplicate, and mean values were used.

Chromatographic separation was performed on a C18 column (Agilent Poroshell 120 SB-C18, 2.7 µm, 4.6x10 mm) using an Agilent 1220 Infinity HPLC system equipped with a DAD. The column temperature was set at 30°C, flow rate was 0.8 mL/min, and 20 µL of standard or sample was injected into the column. The reversed-phase separation was achieved using a gradient method with mobile phases A (deionized water acidified with 0.1% TFA) and B (acetonitrile acidified with 0.1% TFA). 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%, and A 95% for 1 min. As method validation parameters, limit of detection (LOD) and limit of quantitation (LOQ) values were calculated for each reference according to the Eurachem guide, 2nd edition.[15]

Cell culture and cell viability assay

The L929 (ATCC® CCL-1™) mouse fibroblast cell line (*Mycoplasma*-free) was obtained from the Republic of Turkey Ministry of Agriculture and Forestry Foot and Mouth Disease Institute (Ankara, Turkey) and used for *in vitro* experiments. The cells were grown in 25-cm^2 or 75-cm^2 cell culture flasks in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin and 4 mM L-glutamine at 37°C in an incubator with 5% CO_2 and subcultured after reaching 80-90% confluence using trypsin-EDTA. Cells in all experiments were used between the 4th and 6th passages.

Cell viability was determined using a modified colorimetric MTT assay, which measures the mitochondrial reductase activity of viable cells.[16] Cells grown in 96-well plates were treated with plant extracts at concentrations of 31.25, 62.5 125, 250, and 500 µg/mL in the growth medium. Solvent alone (methanol or water) was added to the negative control wells. After 18, 24, and 48 h of incubation, MTT solution was added to the wells. Then all the solutions were removed and DMSO was added to dissolve the formazan crystals. The plates were incubated for 30 min and then read at 570 nm (Epoch™ Microplate Spectrophotometer, Biotek, Winooski, VT, USA). The experiment was carried out in quadruplicate and mean values were used.

Cell scratch wound healing assay

The migration capabilities of L929 mouse fibroblasts were assessed using a cell scratch *in vitro* wound healing assay, which measures the expansion of a cell population on surfaces. The cells were seeded into 48-well tissue culture dishes in the growth medium at a concentration of 2x10^4 cells/mL and cultured until nearly confluent cell monolayers formed. Then 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 PBS. After that, growth medium containing plant extracts (62 µg/mL) was added followed by incubation for 24 h. Solvent (methanol or water) without the extract was added to the negative control wells. The cells were visualized under an inverted microscope. Three representative images from different parts (top, middle, and bottom parts of the well) of the scratched area for each replicate well were digitally photographed at 0 (the beginning) and 24 h to calculate the relative migration of cells.[17]

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

$$\text{Sclosure rate}=[(\text{Area}_{t0} - \text{Area}_{t24})/\text{Area}_{t0}] \times 100,$$

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 were used.

Statistical analysis

All the results were obtained from at least three replicates and expressed as mean ± standard deviation. Statistical significance between groups was determined by One-Way ANOVA followed by Tukey's test for *post hoc* comparison. Mean values were considered statistically different if $p<0.05$.

RESULTS AND DISCUSSION

Plant extract yield and total phenolic and flavonoid contents

Extract yield was calculated and presented as percentage efficiency (Table 1). The yields of the extracts obtained with maceration were significantly higher than those of the extracts obtained with the soxhlet extraction method. When the solvents were compared, we found that methanolic extracts showed better yields than aqueous extracts (Table 1).

Since the correlation between the wound healing activity and antioxidant properties of plants was reported for some species in the literature,[2,7,18,19] special emphasis was placed on the phenolic compounds in the present study. Phenolics are among the most studied and important phytochemicals and there is a strong relationship between the phenolic content and antioxidant activity of plants.[2,14,19-21] The calculated total phenolic and flavonoid contents of the extracts of *H. pseudolaeve* are shown in Table 2. The results showed that the total phenolic content was highest in HMS with 177.21 mg GAE/g and lowest in HWM with 123.03 mg GAE/g values. Similarly, total flavonoid

Table 1. Amount of the total extracts and percentage efficiency of the extraction yield

Extracts	Dry plant material before extraction (g)	Extract after extraction (g)	Yield efficiency percentage (%)
HMS	20	5.89	29.45
HMM	60	24.05	40.08
HWS	20	2.22	11.10
HWM	60	10.25	17.08

HMS: Soxhlet with methanol, HMM: Maceration with methanol, HWS: Soxhlet with water, HWM: Maceration with water

Table 2. Total phenolic and flavonoid content, total antioxidant capacity and DPPH scavenging activity of the extracts

Extracts	Total phenolic content (mg/g GAE)	Total flavonoid content (mg/g RUE)	Total antioxidant capacity (mg/g AAE)	DPPH scavenging activity (IC50 values, µg/mL)
HMS	177.21±1.48[a]	123.40±2.61[a]	290.70±1.03[a]	14.32±0.13[a]
HMM	127.50±0.48[b]	114.2±0.45[b]	318.67±0.00[b]	14.68±0.07[b]
HWS	123.48±0.29[c]	44.32±1.10[c]	247.84±0.41[c]	13.04±0.03[c]
HWM	123.03±1.95[c]	21.79±0.17[d]	243.91±0.41[d]	13.29±0.10[c]
Ascorbic acid	-	-	-	1.49±0.01[d]

Values are the means of three replicates ± standard deviation. [a,b,c,d]The differences between the mean values with different letters in the same column are statistically significant ($p<0.05$), HMS: Soxhlet with methanol, HMM: Maceration with methanol, HWS: Soxhlet with water, HWM: Maceration with water, DPPH: 2,2-diphenyl-1-picrylhydrazyl

content was highest in HMS with 123.40 mg RUE/g and lowest in HWM with 21.79 mg RUE/g values. When the extraction solvents were compared, we observed that methanolic extracts had more phenolic substances than aqueous extracts. Our results are consistent with those of previous studies, which have shown that methanol extracts are richer in terms of phenolic content.[20,21] In the present study, it was found that a considerable amount of the phenolic compounds in *H. pseudolaeve* consisted of flavonoids, corroborating the previous reports on other *Hypericum* species.[22-26] Wound healing and antidepressant activities of the members of this genus have been associated with phenolic compounds such as hyperoside and epicatechin in some of the previous studies.[11,27] Therefore, phenolic content is important for the biological activities of *Hypericum* spp.

Methanolic extract of *H. pseudolaeve* was previously studied in terms of total phenolics and flavonoids and antioxidant activity[23], but we decided to present our results since we studied a different population. It is a well-known phenomenon that accumulation of phytochemicals in plants shows variation depending on the geographic region, season, phenological stage, and habitat properties.[28,29] Additionally, the previous study reported the results of methanol maceration extract only, while we present the results of both methanol and aqueous extracts obtained by two different methods in a comparative manner. According to the results of the present study, total phenolic and flavonoid contents were much higher than those reported previously. This shows that different geographical populations of *H. pseudolaeve* could vary in the accumulation of phenolic compounds. Moreover, in a study on the methanolic extract of *H. perforatum*, a widely used medicinal plant, its total phenolic content was found to be 191 mg GAE/g by the same method used in the present study.[30] When we compare our results with those in the literature, it can be seen that the total phenolic and flavonoid amounts of *H. pseudolaeve* are noteworthy and close to those of *H. perforatum*.

Antioxidant activity

According to the results of DPPH and total antioxidant capacity (phosphomolybdenum) assays, methanolic extracts expressed slightly better antioxidant activity than aqueous extracts, whereas the extraction method did not affect the activity significantly (Table 2). The antioxidant activities of several *Hypericum* species (including *H. perforatum*, *H. thymbrifolium*, *H. spectabile*, *H. scabrum*, *H. triquetrifolium*, *H. scabroides*, *H. lysimachioides*, *H. retusum*, and *H. pseudolaeve*) have been published using various methods including the DPPH scavenging assay.[22,23,26,31,32] Eroglu Ozkan et al.[23] reported the DPPH radical scavenging activity (expressed as EC_{50} values in mg/mL) of *H. pseudolaeve* methanolic extract as 0.916 mg/mL (916 µg/mL). We obtained approximately sixty times lower IC_{50} values for *H. pseudolaeve* extracts, ranging between 13.04 and 14.68 µg/mL. Such a difference may be observed due to technical variation or the calculation model of IC_{50}, since the authors did not state the model of the response curve (linear or sigmoidal). Geographical variation can be another issue, as discussed above. The antioxidant activity of a flavonoid-rich extract of *H. perforatum*, a well-known medicinal plant of the genus, was previously studied by DPPH assay and its IC_{50} value was reported as 10.63 µg/mL.[22] When we compared our results with those in the literature, we concluded that *H. pseudolaeve* has good antioxidant capacity among the other members of the genus close to that of *H. perforatum*. Antioxidant activity is important in the wound healing activity of plant extracts and generally listed as one of the properties that a good wound healing agent should possess.[2,4,7,19]

Chemical constituents revealed by HPLC analysis

In the present study, *H. pseudolaeve* plants growing in Turkey were analyzed for 17 different secondary metabolites (mainly phenolics) and the results were presented as mg/g DW (Table 3). Representative chromatograms in Figure 2 show the compounds identified. The coefficient of determination (R^2) values of linear regression of the calibration curves (calculated values were 0.9803 for quercetin and 0.9935-0.9999 for other compounds) and the LOD and LOQ values of the method were acceptable (Table 3). According to HPLC analysis of the present study, epicatechin was the main compound among the references we used, with 14.46-21.35 mg/g DW in all extracts. Apigenin was not detected in *H. pseudolaeve* extracts. The amounts of the compounds varied especially depending on the solvent, rather than the extraction method. As an exception, epicatechin concentration was significantly higher in HWM than in all the other extracts and quercitrin was also higher in maceration extracts. Moreover, kaempferol was detected only in HMS. The amount of chlorogenic acid was slightly higher in aqueous extracts, while p-coumaric acid and hyperoside (the second major compound) were higher in methanolic extracts. We used water as one of the extraction solvents since decoction is a widely used method and found that aqueous extracts of

Table 3. Comparison of the secondary metabolite content (mg/g DW mean values) of *H. perforatum* based on the reference compounds analyzed, with the LOD/LOQ values of the method for each reference measurement

	R_t (min)	Wavelength (nm)	HMS	HMM	HWS	HWM	LOD/LOQ
Gallic acid	2.57	270	7.79±0.01	7.73±0.02	7.87±0.00	8.00±0.04	0.008/0.02
4-hydroxybenzoic acid	7.37	260	+	0.10±0.04*	+	0.16±0.05*	0.08/0.29
(+)-Catechin	8.13	210	1.48±0.29	1.36±0.09	1.85±0.32	1.47±0.26	0.04/0.14
Chlorogenic acid	8.51	320	2.83±0.03	3.02±0.01	4.25±0.07	4.84±0.27	0.005/0.01
Vanillic acid	8.87	210	+	0.37±0.06*	+	+	0.28/0.93
Caffeic acid	9.21	320	0.77±0.02	0.71±0.00	0.82±0.05	1.33±0.11	0.08/0.28
Syringic acid	9.65	210	1.05±0.13	1.00±0.01	1.91±0.04	1.39±0.14	0.14/0.49
(-)-Epicatechin	10.04	210	14.79±0.16	14.49±0.08	14.46±0.07	21.35±0.07	0.11/0.39
p-Coumaric acid	11.77	320	3.37±0.02	2.60±0.01	0.95±0.04	0.08±0.07*	0.06/0.22
Rutin	13.07	210	1.72±0.11	2.04±0.15	1.75±0.02	1.53±0.05	0.35/1.17
Sinapic acid	13.24	320	0.53±0.03	0.44±0.00*	0.26±0.01*	-	0.15/0.51
Hyperoside	13.42	210	9.28±0.04	8.27±0.04	3.76±0.03	0.49±0.10*	0.23/0.77
Quercitrin	15.13	260	5.75±0.62	8.07±0.71	5.55±0.21	9.21±0.50	0.10/0.35
Quercetin	19.23	210	5.44±0.39	4.76±0.99	3.81±0.08	2.46±0.10	0.46/1.54
Apigenin	21.90	210	-	-	-	-	0.05/0.19
Kaempferol	22.40	210	0.28±0.01	-	-	-	0.08/0.27
Hypericin	33.59	590	+	-	-	-	2.49/8.30

*Values are the means of three replicates ± standard deviation. +: Peak detected but equal or <LOD and asterisk indicates that estimated concentration is >LOD, <LOQ
R_t: Retention time of the standard, LOD: Limit of detection, LOQ: Limit of quantitation, N/A: Not applicable for that sample, HMS: Soxhlet with methanol, HMM: Maceration with methanol, HWS: Soxhlet with water, HWM: Maceration with water

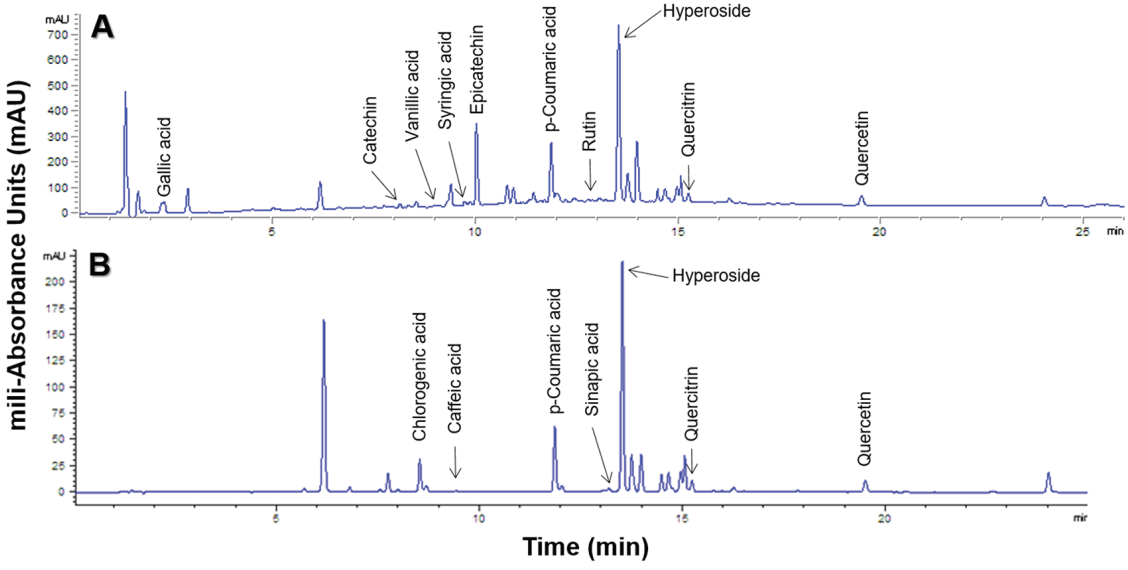

Figure 2. Representative HPLC chromatogram of *Hypericum pseudolaeve* methanolic extract obtained by maceration A. 210 nm wavelengths B. 320 nm wavelengths
HPLC: High performance liquid chromatography

H. pseudolaeve also contain considerable amounts of phenolics. However, hypericin, a naphthodianthrone molecule, was not detected in the aqueous extracts.

The chemical constituents of some *Hypericum* species were previously analyzed by chromatographic methods. In general, the secondary metabolites identified in *H. pseudolaeve* in the present study are in agreement with the previously published literature records.[11,23,25,33-38] The chemical composition of *H. pseudolaeve* from Turkey was studied by HPLC recently.[23] The authors gave yield (%) values for each compound and did not

specify the calculation, preventing comparison of their results with ours. However, even with this situation, it is observable that the amounts of some compounds show variation compared to our data. These results show that the amounts of the specific phenolic compounds may vary between different geographical populations of *H. pseudolaeve*. Moreover, catechin; epicatechin; vanillic, caffeic, syringic, p-coumaric, sinapic, gallic, and 4-hydroxybenzoic acids; and quercitrin were not included in the aforementioned study. Epicatechin was not included by Eroglu Ozkan et al.[23] while this compound was detected as one of the major constituents of *H. pseudolaeve* in the present study. Here we presented the results of all the reference compounds analyzed in the present study and provided more detailed and extended information on the chemical constituents of the species.

As a prominent result of this study, *H. pseudolaeve* was found to contain a high amount of epicatechin compared to published data of the other species of the genus. Epicatechin is an important antioxidant flavonoid that is beneficial for cardiovascular and neuropsychological health.[39] Moreover, the active fraction of *H. perforatum* with wound healing activity was also found to contain epicatechin in a previous study.[11] Our results show that *H. pseudolaeve* contains several phenolic compounds contributing to its biological activities.

The chemical constituents of *Hypericum* species are also analyzed for their chemotaxonomical importance. Secondary metabolites such as quercetin, quercitrin, hyperoside, and hypericin were considered useful biomarkers for chemotaxonomic analyses.[25] Chemical profiling can provide additional data for taxonomic classifications based on morphology and genetics. Our results are in concordance with previously published data reporting the chemical constituents of members belonging to the section *Drosanthe*.[25] As an exception, we detected caffeic acid in low amounts, whereas this compound was not found in the other members of the section.[25] Our results provide additional data for the chemotaxonomy of the genus *Hypericum*.

In vitro cytotoxicity and wound healing activity

We assessed wound healing activity using a well-established *in vitro* cell scratch assay, which is a widely used method to assess the wound healing activity of plant extracts.[40-44] To the best of our knowledge, this is the first report on the wound healing activity of the species studied. Before performing 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 may affect the results. Moreover, toxicity assessment is also an important parameter for the quality control of pharmaceutical preparations.

None of the extracts showed significant cytotoxicity or reduced the cell viability by 50% on mouse fibroblast cells at 18 and 24 h at the highest concentrations of 250 and 500 µg/mL. However, a low level of inhibition (not more than 22%) was observed after 48 h of treatment (Table 4). We observed no significant difference in cytotoxic effects depending on the extraction solvent or extraction method. Similar studies on different species of *Hypericum* also showed that their extracts did not show a significant cytotoxic effect on fibroblasts, which makes them safer for topical applications.[45,46]

The cell scratch assay using skin cells such as fibroblasts and keratinocytes is a widely used method as an *in vitro* wound healing model that provides information about the activity of compounds and natural products.[40] In the present study, we used this assay and calculated the area closure percentages for comparison. Our results showed that HWM (76.7%) and HWS (68.4%) significantly increased ($p<0.05$) fibroblast migration compared to the negative control (Figure 3) at the tested concentration. Representative images in Figure 4 clearly show the induction of fibroblast migration.

Table 4. Cell viability percentages obtained by 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**
HMS	99.2%	91.9%	81.6%	94.9%	79.6%	78.0%
HMM	93.3%	92.5%	78.7%	91.2%	87.2%	78.6%
HWS	95.5%	90.5%	81.5%	97.2%	91.8%	79.7%
HWM	109.5%	93.1%	86.6%	112.1%	91.9%	83.9%

HMS: Soxhlet with methanol, HMM: Maceration with methanol, HWS: Soxhlet with water, HWM: Maceration with water, MTT: (2h-tetrazolium,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

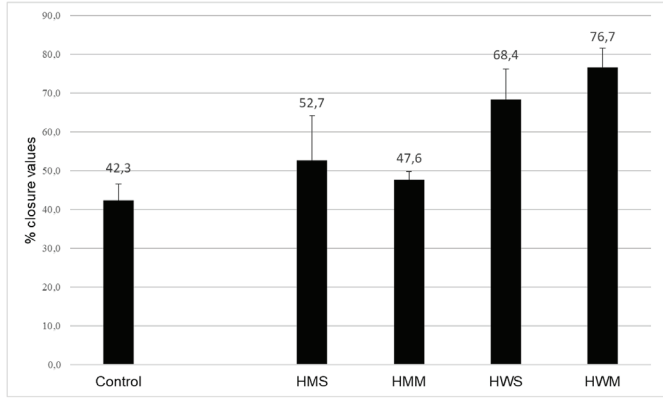

Figure 3. Graphic showing scratch assay closure percentages of *Hypericum pseudolaeve* (62 µg/mL, 24 h) on mouse dermal fibroblast (L929) migration in a wound scratch test assay. Mean values of three replicate wells were expressed with standard error bars. Results showed that HWM and HWS significantly increased ($p<0.05$) fibroblast migration compared to the negative control

HWM: Maceration with water, HWS: Soxhlet with water

In a study by Fronza et al.[40] the wound healing activity of *H. perforatum* oil was investigated by cell scratch assay using the 3T3 mouse fibroblast cell line. They found that the prepared oil was cytotoxic at concentrations higher than 0.5 µg/mL. However, wound healing activity of *H. perforatum* was shown by *in vivo* wound models and suggested it to be a potent natural wound healing product.[11] The results of the present study showed for the first time that *H. pseudolaeve*, a plant species distributed mainly in central and eastern Anatolia, has potential wound healing activity. The phytochemicals of *H. pseudolaeve*

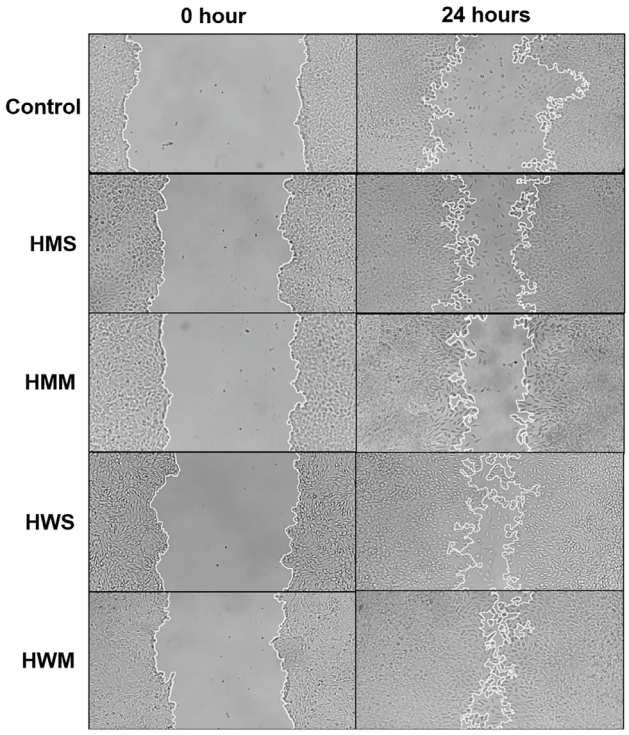

Figure 4. Representative image showing the effect of *Hypericum pseudolaeve* (62 µg/mL) on mouse dermal fibroblast (L929) migration in a wound scratch test assay. Magnification (4x)

HMS: Soxhlet with methanol, HMM: Maceration with methanol, HWS: Soxhlet with water, HWM: Maceration with water

and their synergistic actions are responsible for its biological activities.

Süntar et al.[11] investigated the wound healing potential of *H. perforatum* using *in vivo* wound models and detected hypericin, hyperoside, and rutin in the active fraction. These molecules were also detected in *H. pseudolaeve* in the present study. Previously published studies showed that hypericin has a broad range of molecular functions and biological activities, including the inhibition of protein kinase C and $CD8^+$ T-cell mediated cytotoxicity and antiviral activity.[12] Some limited studies also showed that purified hypericin may have wound healing potential.[47] According to our results, aqueous extracts were more potent regarding cell migration but we did not detect hypericin in these extracts and its concentration was low in methanolic extracts. Its contribution to the wound healing process should be investigated with more detailed studies using purified hypericin. Hyperforin (a phloroglucinol derivative) is another typical compound of the genus *Hypericum* and is considered one of the major antidepressant components of *H. perforatum*. Hyperforin also has other biological effects including antibacterial, antioxidant, anticancer, and anticyclooxygenase-1 activities.[12,48] However, hyperforin was not detected in the active fraction of *H. perforatum* by Süntar et al.[11] and the amount of hyperforin was very low in *H. pseudolaeve* (0.0023%) in a previous study.[23]

The most active extract in the cell scratch assay was HWM, which contains a significantly higher amount of (-)-epicatechin compared to the other extracts according to our HPLC analysis, indicating a correlation between the activity and the amount of this phenolic compound. In a study by Süntar et al.[11] epicatechin was identified in the active fraction of *H. perforatum* extract, showing remarkable wound healing activity. Wound healing activity of pure epicatechin gallate was reported previously.[49] These results suggest (-)-epicatechin as an important biologically active secondary metabolite of the genus *Hypericum*. The amounts of chlorogenic acid and quercitrin also show correlation with the *in vitro* wound healing activities of *H. pseudolaeve* extracts; HWM was found to contain the highest amounts of these compounds according to our HPLC analysis. Chlorogenic acid and quercitrin were also shown to have wound healing potential.[50,51] Our results and the data in the literature show that phenolic compounds may play important roles in the wound healing potential of the genus *Hypericum*.

Study limitations

Crude methanolic and aqueous extracts of *H. pseudolaeve* were investigated for their *in vitro* wound healing activity, antioxidant activity, and phytochemical content. Crude extracts showed promising results and this plant can be used for further investigations aiming to isolate active molecules.

CONCLUSION

Our results showed that *H. pseudolaeve* has potential wound healing activity and contains several important antioxidant phenolic compounds, as well as hypericin and hyperoside, which may be associated with its wound healing activity. We found that aqueous extracts, which are a common form of preparation of medicinal plants, also have good activity similar to methanolic extracts. Our results also showed that an *in vitro* scratch assay can be used for initial screening studies aiming to assess the wound healing potential of *Hypericum* spp. Using such *in vitro* tests will reduce animal use. The results of the present study, together with those in the literature, highlight that (-)-epicatechin is one of the possible contributors to the wound healing activity of the genus *Hypericum*. *H. pseudolaeve* accumulates this flavonoid in high amounts compared with the other members of the genus and deserves further investigation aiming to isolate and identify the active compounds.

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