# An investigation on the in vitro wound healing activity and phytochemical composition of Hypericum pseudolaeve Robson growing in Turkey

# Türkiye'de yayılış gösteren Hypericum pseudolaeve Robson türünün in vitro yara iyileştirme aktivitesi ve fitokimyasal kompozisyonu üzerine bir araştırma

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GİRİŞ ve AMAÇ: Bu çalışma, Hypericum pseudolaeve Robson türünün iki farklı yöntemle elde edilmiş metanol ve su ekstrelerinin in vitro yara iyileştirici etkisi ile sitotoksisitesi, antioksidan aktivitesi ve seçilmiş fitokimyasallarını araştırmayı amaçlamıştır.
YÖNTEM ve GEREÇLER: Toplam fenolik ve flavonoit içerikleri spektrofotometri tabanlı yöntemler kullanılarak ölçülmüştür. Ekstrelerin L929 fare fibroblast hücreleri üzerindeki sitotoksik etkileri MTT deneyi ile değerlendirilmiştir. Ayrıca, bitki uygulanmış fibroblast hücrelerinin göçü, bir in vitro yara iyileşme modeli olan hücre çizik yöntemi ile değerlendirilmiştir. Ek olarak, türün kimyasal içeriği Yüksek Performanslı Sıvı Kromatografisi (YPSK) ile belirlenmiştir.
BULGULAR: Sitotoksisite deneyinin sonuçları, metanol ve su ekstrelerinin, 500 µg/mL konsantrasyonuna kadar kullanıldığında fibroblast hücreleri üzerinde herhangi bir sitotoksik etkiye sahip olmadığını göstermiştir. Fibroblast göçü 62 µg/mL konsantrasyonunda su ekstreleri uygulandığında negatif kontrole göre anlamlı derecede artış göstermiştir. Ekstreler iyi derecede antioksidan aktivite göstermiştir ve YPSK analizi ile 16 madde tespit edilmiştir. En fazla bulunan bileşik epikateşin olarak belirlenmiştir.
TARTIŞMA ve SONUÇ: Bu çalışma Hypericum pseudolaeve ekstrelerinin, yara iyileşme potansiyeline sahip olduğunu ve çeşitli önemli antioksidan fenolikler içerdiğini göstermiştir. Bu türle ilgili aktif bileşiklerin izole edilmesi ve tanımlamasını amaçlayan daha fazla araştırma yapılmalıdır.
**Anahtar Kelimeler:** Yara iyileşmesi, bitki ekstresi, antioksidan, fenolikler, YPSK

INTRODUCTION: This study was aimed to investigate the in vitro wound healing effects of the methanolic and aqueous extracts of Hypericum pseudolaeve Robson obtained by two different methods as well as its cytotoxicity, antioxidant activity and selected phytochemical constituents.
METHODS: Total phenolic and flavonoid contents were measured using spectrophotometry based methods. Cytotoxic effects of the extracts on L929 mouse fibroblast cells were evaluated by MTT 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 Performance Liquid Chromatography (HPLC).
RESULTS: The results of 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 negative control. Extracts showed good antioxidant activity and 16 phytochemical compounds were detected by HPLC, with the highest amount for

epicatechin.
DISCUSSION AND CONCLUSION: Our results showed that H. pseudolaeve extracts have wound healing potential and contain several important antioxidant phenolic compounds. This species deserve further investigation aiming to isolate and identify the active compounds.
**Keywords:** Wound healing, plant extract, antioxidant, phenolics, HPLC

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## 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 damages, burn, infections or chronic illnesses which disrupt normal skin anatomy and function. They cause the 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 have also important role by degrading extracellular matrix components such as collagen, elastin and fibrin. But their activity must 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, marine environment, fungi, and other microorganisms) having potential to heal acute and chronic wounds especially in patients with the metabolic disorder 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 anti-inflammatory properties.[2]

Hypericum genus 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 Robson is grouped under Hirtella (Drosanthe) section and distributed in central and eastern Anatolia in Turkey. The genus Hypericum, especially H. perforatum (St. John's Worth), is one of the most widely used medicinal plants for depression and its wound healing effects have been shown with both ethnobotanical and functional studies.[2,3,11–13] Common ethnobotanical preparation method of the genus Hypericum sp. for wound healing is maceration of the aerial parts in olive oil under direct sunlight for at least four weeks.[11] However, there is no data in the literature regarding the ethnobotanical usage and wound healing potential of H. pseudolaeve to the best of authors' knowledge and also there is limited information on the chemical constituents of the species.

The aim of this study is 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 a 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 for the future studies searching for alternative drugs.

## MATERIALS AND METHODS

### Chemical compounds

Standards used for High Performance Liquid Chromatography (HPLC) analyses (except hypericin), thiobarbituric acid and dimethylsulfoxide (DMSO) were purchased from Sigma; hypericin from Santa Cruz Biotechnology; Diphenyl-2picrylhydrazyl (DPPH) was from Aldrich; Folin-Ciocalteu reagent, ascorbic acid, sodium carbonate, potassium acetate, aluminium chloride hexahydrate and sulfuric acid were from Merck; sodium phosphate from Riedel-de Haën; ammonium molybdate was from Fluka; Dulbecco's modified Eagle's medium (DMEM) from Gibco; fetal bovine serum (FBS), phosphate buffer saline (PBS), L-glutamine and penicillin/streptomycin were from PAN Biotech; trypsin/EDTA from Biological Industries; 2h-tetrazolium,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) was from Fisher Scientific.

### Plant materials

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

### Extraction procedure and determination of the yield

Extracts of H. pseudolaeve were obtained by maceration or soxhlet extraction method using methanol or water as solvents. After extractions, 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 freezed at -20°C and lyophilized by a freze-dryer thereafter (Christ Gamma 2-16 LSC). The plant extracts were stored in the dark at 4°C until studied. Finally, four different extracts were prepared and abbreviated throughout the paper as follows: H. pseudolaeve maceration with methanol (HMM), maceration with water (HWM), soxhlet with methanol (HMS), soxhlet with water (HWS). Extraction efficiencies of the plant materials were calculated by 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 Folin-Ciocalteu method.[14] Gallic acid was used for the reference compound to obtain a standard curve (10 different concentrations were used between 10-100 µg/mL). Briefly, 0.5 mL of extracts (1 mg/mL) was mixed with 2.5 mL of a 1:10 diluted Folin-Ciocalteu reagent, 2 mL sodium carbonate solution (7.5% w/v) and allowed to stand 15 minutes at 45°C. Blank, standards and samples were transferred to cuvettes and read by using a UV/VIS spectrophotometer (Perkin Elmer, Lambda 25) at 765 nm wavelength. 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 the total flavonoid content

The total flavonoid content was determined by the aluminium chloride colorimetric method. Briefly, 0.5 mL of the extract solutions (0.5 mg/mL) was mixed with 0.1 mL of 10%

aluminium 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, 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 (Perkin Elmer, Lambda 25). Rutin was used as a standard compound at 8 different concentrations between 10-80 µg/mL 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 the total antioxidant capacity

0.3 mL 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). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then blank, standards and samples were transferred into cuvettes and measured at 695 nm using a UV/VIS spectrophotometer (Perkin Elmer, Lambda 25) after cooling to room temperature. Calibration graphics was plotted using ascorbic acid as a standard at the concentrations between 3.9 and 500 µg/mL obtained by 2-fold serial dilution and the antioxidant activity is calculated as the equivalents 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 extracts were prepared in concentrations of 15.62, 31.25, 62.5, 125 and 250 µg/mL for this assay. A volume of 3 mL extract of each concentration was mixed with 1 mL of the 0.1 mmol/L DPPH solution prepared in methanol. The tubes were incubated in the dark at room temperature for 30 minutes and then read at 517 nm by using a UV/VIS spectrophotometer (Perkin Elmer, Lambda 25). Solvent without extract was used as negative control and ascorbic acid was used as a positive control. Effect of the antioxidant capacity was observed as the color change of purple DPPH to yellow/light-yellow and % inhibition values of each extract were calculated by 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 (O.D.) of the negative control, $A_{sample}$ is the absorbance of ascorbic acid or extracts. $IC_{50}$ values were calculated with inhibition rates using a four-parameter logistic regression model after plotting sigmoidal curves. Each of the standards and the samples were measured in triplicate and mean values were used for the calculations.

HPLC analysis and quantification

Chemical contents of the extracts were analyzed by reversed-phase High Performance Liquid Chromatography-Diode Array Detector (HPLC-DAD) method. The reference compounds were selected mainly from phenolics which 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 PFTE membrane, measured in triplicate and mean values were used.

Chromatographic separation was performed on C18 column (Agilent Poroshell 120 SB-C18, 2.7 µm, 4.6 ×10 mm) using Agilent 1220 Infinity HPLC equipped with DAD. The column temperature was set at 30 °C, flow rate was 0.8 mL/min and 20 µL standard or sample was injected to column. The reversed-phase separation was achieved by 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%, A 95% for one minute. 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

L929 (ATCC® CCL-1™) mouse fibroblast cell line (Mycoplasma-free) was obtained from "Republic of Turkey Ministry of Agriculture and Forestry Şap 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 medium supplemented with 10% FBS and 1% penicillin-streptomycin and 4 mM L-glutamine at 37 °C incubator with 5% $CO_2$ and subcultured after reaching 80–90% confluence using trypsin-EDTA. Cells in all experiments were used between 4th and 6th passages.

Cell viability was determined by using a modified colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, which measures the mitochondrial reductase activity of viable cells.[16] Cells grown in 96-well plates were treated with plant extracts at the concentrations of 31.25, 62.5 125, 250 and 500 µg/mL in the growth medium. Solvent alone (methanol or water) was added to negative control wells. After 18, 24 and 48 hours of incubation, MTT solution was added into the wells. Then all the solutions were removed and DMSO was added to dissolve the formazan crystals. The plates were incubated for 30 minutes 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 by 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 $2\times10^4$ cells/mL and cultured until forming nearly confluent cell monolayers. 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 phosphate buffer saline (PBS). After that, growth medium the containing plant extracts (62 µg/mL) was added and incubated for 24 h. Solvent (methanol or water) without the extract was added to negative control wells. Cells were visualized under the inverted microscope. Three representative images from different parts (up, middle and bottom parts of the well) of the scratched area for each replicate wells 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 by 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 wells. The closure rate was calculated with these values by using the following formula:

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

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.

Statistics

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 analysis or variance (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, 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 the extracts obtained with soxhlet extraction method. When solvents are 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], a special emphasis was put 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 was 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 content was found highest in HMS with 123.40 mg RUE/g and lowest in HWM with 21.79 mg RUE/g values. When the extraction solvents are compared, we observed that methanolic extracts had more phenolic substances than aqueous extracts. Our results are consistent with the 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 the considerable amount of the phenolic compounds in H. pseudolaeve consisted of the flavonoids, corroborating the previous reports on other Hypericum species.[22–26] Wound healing and anti-depressant 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 activies of Hypericum sp. Methanolic extract of H. pseudolaeve was previously studied in terms of total phenolics and flavonoids and antioxidant activity[23], but we still preferred 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 only methanol maceration extract while we present the results of both methanol and aqueous extracts obtained by two different methods, in a comparatively manner. According to the results of the present study, total phenolic and flavonoid contents were found to be much higher than those reported previously. This result 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 as 191 mg GAE/g with the same method used in the present study.[30] When we compare our results with those in the literature, it can be stated that the total phenolic and flavonoid amount of H. pseudolaeve are noteworthy and close to 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). 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 DPPH scavenging assay.[22,23,26,31,32] Eroglu Ozkan et al.[23] reported 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 the technical variation or calculation model of inhibitory concentration, since the authors did not state the model of the response curve (linear or sigmoidal). Geographical variation can be another issue as discussed above. Antioxidant activity of a flavonoid-rich extract of H. perforatum, a well-known medicinal plant of the genus, was previously studied by using DPPH assay and its $IC_{50}$ value was reported as 10.63 µg/mL.[22] When we compare our results with those in the literature, we concluded that H. pseudolaeve has a good antioxidant capacity among the other members of the genus, which is close to H. perforatum. Antioxidant activity

is important in the wound healing activity of plant extracts and generally listed as one of the properties which a good wound healing agent should possess.[2,4,7,19]

Chemical constituents revealed by HPLC analysis

In the present study, H. pseudolaeve 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 shows the compounds identified. Coefficient of determination ($R^2$) values of linear regression of the calibration curves (calculated values were 0.9803 for quercetin and between 0.9935-0.9999 for other compounds), LOD and LOQ values of the method were acceptable (Table 3). According to HPLC analysis of the present study, epicatechin was detected as the main compound among the references we used with an amount of 14.46-21.35 mg/g DW in all extracts. Apigenin was not detected in H. pseudolaeve extracts. The amount of the compounds varied especially depending on the solvent, rather than the extraction method. As an exception, epicatechin concentration was found to be significantly higher in HWM than 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 among humans and found that aqueous extracts of H. pseudolaeve also contain a considerable amount of phenolics. However, hypericin, a naphtodianthrone molecule was not detected in the aqueous extracts.

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] 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, making their results not comparable with ours. But 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, 4-hydroxybenzoic acids and quercitrin were not included in 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 which is beneficial for cardiovascular and neuropsychological health.[39] Moreover, the active fraction of H. perforatum with a 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.

Chemical constituents of Hypericum species are analyzed also for their chemotaxonomical importance. Secondary metabolites such as quercetin, quercitrin, hyperoside and hypericin were considered as 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 the members belonging to the sect. 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 Hypericum genus.

In vitro cytotoxicity and wound healing activity

We assessed the wound healing activity by using a well-established in vitro cell scratch assay, which is a widely used method to assess the wound healing activity of the plant extracts.[40–44] To the best of our knowledge, this is the first report regarding the wound healing activity of the species studied. Before performing wound healing assay, we investigated the potential cytotoxic effect of the extracts on 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 the 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 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 sp. also showed that their extracts did not show a significant cytotoxic effect on fibroblasts, which makes them safer for topical applications.[45,46]

Cell scratch assay using the skin cells such as fibroblasts and keratinocytes is a widely used method as an in vitro wound healing model which 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.

In a study by Fronza et al.[40], the wound healing activity of H. perforatum oil was investigated by cell scratch assay using 3T3 mouse fibroblast cell line. They found that 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 as a potent wound healing natural product.[11] 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 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 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 their 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 Hypericum genus and considered as one of the major antidepressant component 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 found to be very low in H. pseudolaeve (0.0023%) in a previous study.[23]

The most active extract in cell scratch assay was found as 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 which showed 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 amount 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 have important role in the wound healing potential of Hypericum genus.

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 a 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 is a common form of preparation of the medicinal plants among humans, also have a good activity similar to methanolic extracts. Our results also showed that in vitro scratch assay can be used for initial screening studies aiming to assess the wound healing potential of Hypericum sp. 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 wound healing activity of the genus Hypericum. Hypericum pseudolaeve accumulates this flavonoid in high amount compared with the other members of the genus and deserves further investigation aiming to isolate and identify the active compounds.

## ACKNOWLEDGMENTS

Authors are grateful to Gazi University Projects of Scientific Investigations for financial support to this study (Project number: 05/2017-16). Bahar KAPTANER İĞCİ acknowledges to The Scientific and Technological Research Council of Turkey (TÜBİTAK) for the support under 2211/C National Scholarship Programme for PhD students in priority areas. Special thanks are given to Nevşehir Hacı Bektaş Veli University Science and Technology Research and Application Center for HPLC facilities, and Prof. Dr. Aslıhan KARATEPE (Nevşehir Hacı Bektaş Veli University Department of Chemistry), Prof. Dr. Ceyda Sibel KILIÇ (Ankara University Faculty of Pharmacy, Laboratory of Pharmaceutical Botany) and Prof. Dr. Belma ASLIM (Gazi University Laboratory of Biotechnology) for providing access to laboratory infrastructure, Prof. Dr. Hilal ÖZDAĞ (Ankara University Biotechnology Institute) and Prof. Dr. Aykut ÖZKUL (Ankara University Faculty of Veterinary Medicine and Biotechnology Institute) for their helps obtaining cell line. We also thank Ümmügülsüm TÜKENMEZ EMRE and Kübra TAN for their technical assistance during initial cell passaging, Derya ÇİÇEK (Ankara University Faculty of Pharmacy) for her helps during maceration extraction and Dr. Naşit İĞCİ (Nevşehir Hacı Bektaş Veli University Department of Molecular Biology and Genetics) for his assistance during HPLC analyses and critical review of the manuscript.

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**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

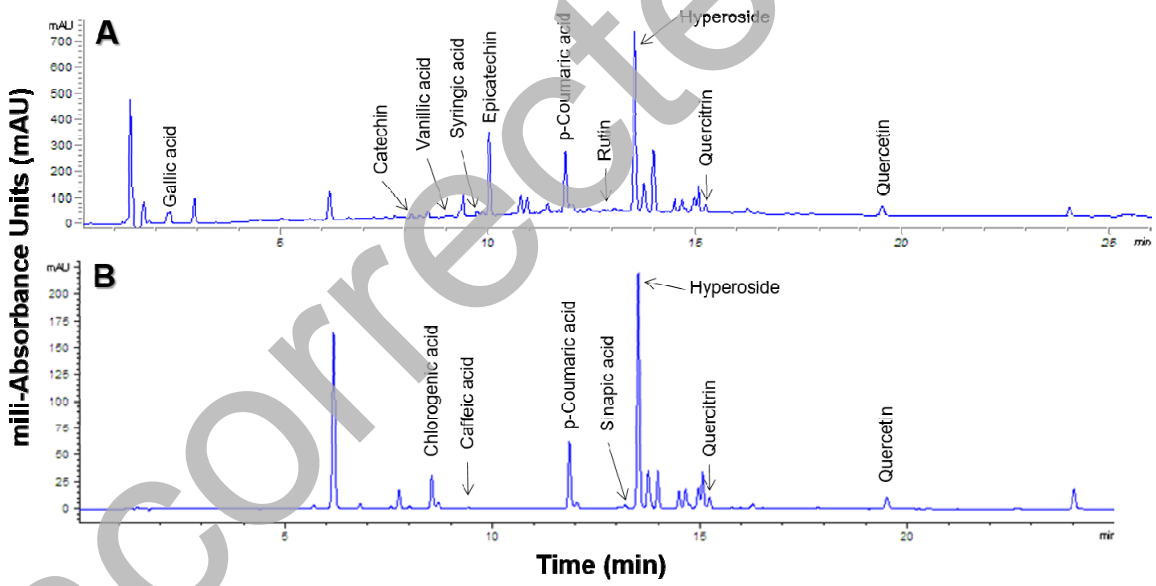


**Figure 2.** Representative HPLC chromatogram of Hypericum pseudolaeve methanolic extract obtained by maceration **A.** 210 nm wavelengths **B.** 320 nm wavelengths

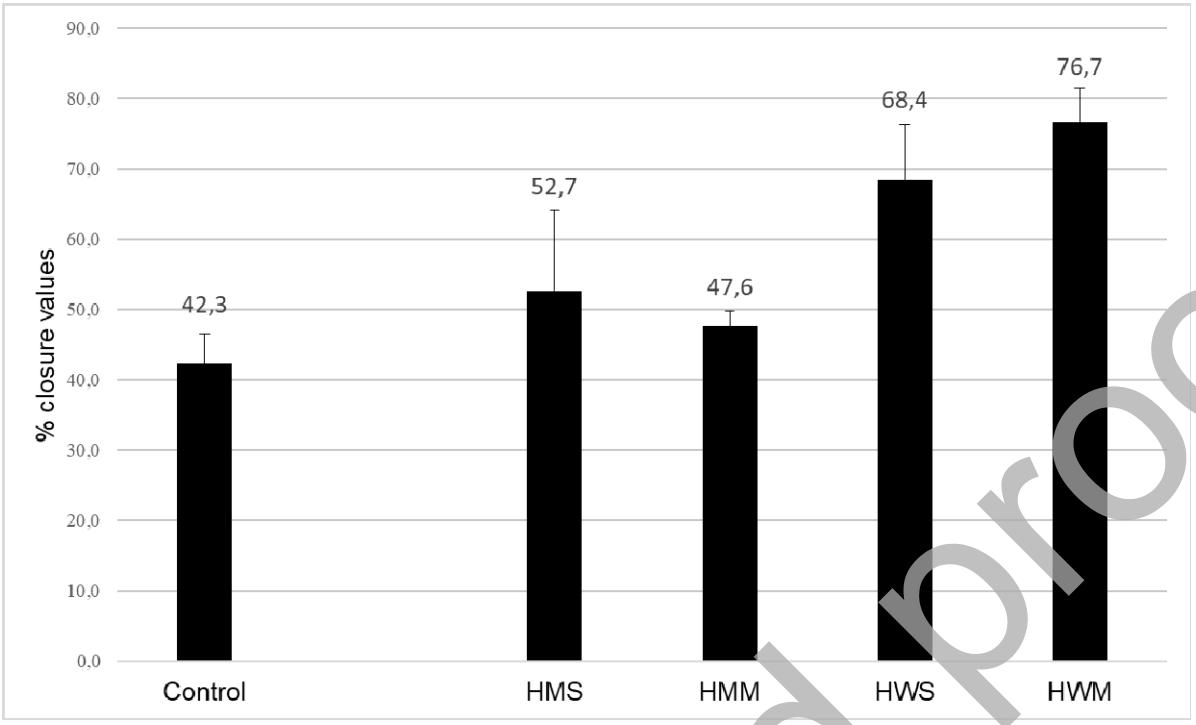


**Figure 3.** Graphic showing scratch assay closure percentages of H. pseudolaeve (62 µg/mL, 24h) on mouse dermal fibroblast (L929) migration in a wound scratch test assay. Mean values of three replicate wells were expressed with standard error bars. Results showed that HWM and HWS significantly increased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

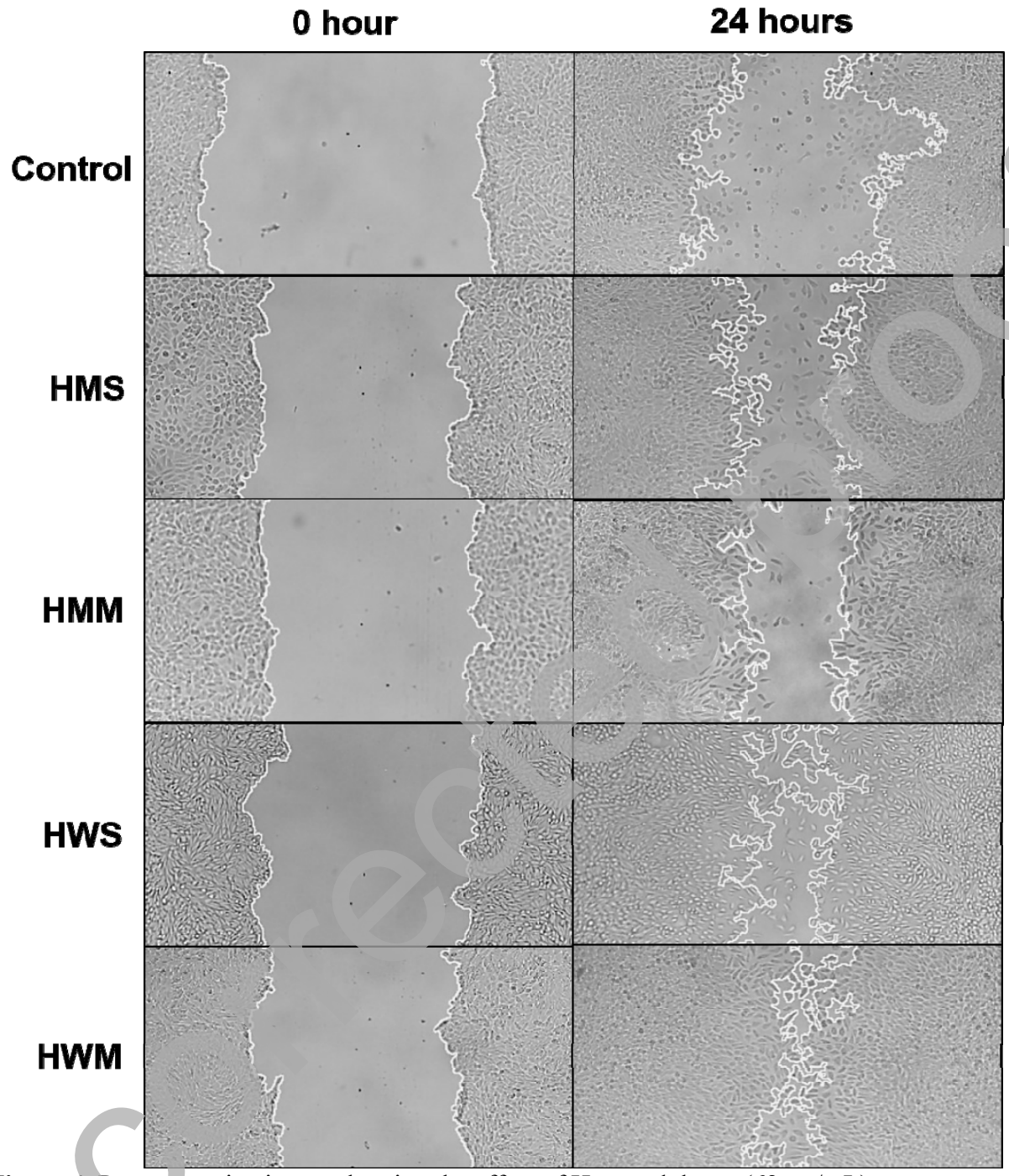


**Figure 4.** Representative image showing the effect of H. pseudolaeve (62 µg/mL) on mouse dermal fibroblast (L929) migration in a wound scratch test assay. Magnification (4x)



**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 |

**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 ($IC_{50}$ values, µg/mL)** |
|---|---|---|---|---|
| **HMS** | $177.21 \pm 1.48^{a}$ | $123.40 \pm 2.61^{a}$ | $290.70 \pm 1.03^{a}$ | $14.32 \pm 0.13^{a}$ |
| **HMM** | $127.50 \pm 0.48^{b}$ | $114.2 \pm 0.45^{b}$ | $318.67 \pm 0.00^{b}$ | $14.68 \pm 0.07^{b}$ |
| **HWS** | $123.48 \pm 0.29^{c}$ | $44.32 \pm 1.10^{c}$ | $247.84 \pm 0.41^{c}$ | $13.04 \pm 0.03^{c}$ |
| **HWM** | $123.03 \pm 1.95^{c}$ | $21.79 \pm 0.17^{d}$ | $243.91 \pm 0.41^{d}$ | $13.29 \pm 0.10^{c}$ |
| **Ascorbic acid** | – | – | – | $1.49 \pm 0.01^{d}$ |

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 ($p < 0.05$).

**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. $R_t$: retention time of the standard, LOD: limit of detection, LOQ: limit of quantitation, N/A: not applicable for that sample

| | $R_t$ (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** | 13.0 | 210 | 1.72±0.1 | 2.04±0.15 | 1.75±0.0 | 1.53±0.0 | 0.35/1.17 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 7 | | 1 | | 2 | 5 | |
| **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

**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 % |