Journal of Ethnopharmacology xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Investigations on the *in vivo* wound healing potential of *Hypericum perforatum* L.

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ARTICLE INFO

Article history:
Received 7 August 2009
Received in revised form 4 October 2009
Accepted 6 October 2009
Available online xxx

Keywords:
Anti-inflammatory activity
Excision wound
Flavonoids
Hypericaceae
Hypericum perforatum
Hypericum scabrum
Incision wound
Naphthoquinones
Tensiometer
Wound healing

ABSTRACT

Ethnopharmacological relevance: Olive oil extract of the flowering aerial parts of *Hypericum perforatum* L. (Hypericaceae) is a popular folk remedy for the treatment of wounds in Turkey.
Aim of the study: In order to prove the claimed utilization of the plant, the effects of the extracts and the fractions were investigated by using bioassay-guided procedures. For the wound healing activity assessment, *in vivo* excision and incision wound models were applied. For the anti-inflammatory activity, an *in vivo* model, based on the inhibition of acetic acid-induced increase in capillary permeability was used as well. Moreover, a parallel study was run on *Hypericum scabrum* L., which is a widespread species of the gender but not known as a folk remedy for wound healing, to provide a preliminary data to compare and emphasize the selection of correct plant species.
Results: Initial investigations proved that the olive oil extract of *Hypericum perforatum* has a significant wound healing effect on excision (5.1–82.6% inhibition) and circular incision (20.2–100.0% inhibition) wound models. In order to determine the active wound healing ingredient(s), aerial parts of the plant was extracted with ethanol, noteworthy wound healing activity profile was observed with the wound models; between 18.3% and 95.6% in excision model and from 13.9% to 100.0% inhibitions in incision model were determined. The ethanolic extract was then submitted to successive solvent extractions with *n*-hexane, chloroform and ethyl acetate (EtOAc). Each solvent extract was also applied on the same wound models, consequently, EtOAc subextract was found to be the most active one by inhibiting wounds between 17.9% and 100.0% in excision model, subsequently between 9.4% and 100.0% in incision model. However, all subfractions obtained from the EtOAc subextract using Sephadex LH-20 column chromatography showed wound healing activity not more than the whole EtOAc subextract, which revealed that a possible synergistic activity that might be questioned. Among the active Sephadex fractions, Fr. A further yielded hyperoside, isoquercitrin, rutin and (−)-epicatechin and Fr. B yielded hypericin as the major components. Moreover, a dose-dependent anti-inflammatory activity was found for the ethanol extract, EtOAc subextract and Sephadex fractions of *Hypericum perforatum*. These results suggest that anti-inflammatory activity of the active fractions might have a contributory role in the wound healing effect of the plant.
Conclusion: Results of the present study have proved that aerial parts of *Hypericum perforatum* possess remarkable wound healing and anti-inflammatory activities supporting the folkloric assertion of the plant in Turkish folk medicine. Flavonoids [hyperoside, isoquercitrin, rutin and (−)-epicatechin] and naphthoquinones (hypericins) were found as the active components of *Hypericum perforatum*. On the other hand, ethanol extract of *Hypericum scabrum* showed neither remarkable wound healing nor anti-inflammatory activity demonstrating the importance of correct plant species selection in therapeutic applications.

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1. Introduction

Several health benefits have been documented in official monographs for the aerial parts and the extracts of *Hypericum perforatum* L. (St. John's wort) that is a remedy against depressions, ulcers, dyspepsia, abdominal pains, burns, bacterial infections, migraine headaches, and sciatica (German Commission E, 1998; WHO Monographs, 1999; ESCOP, 2003).

The water extract (mainly decoctions, sometimes infusions) of the flowering aerial parts of *Hypericum perforatum* has been used as a remedy against various ailments including uro-genital inflammations, diabetes mellitus, neuralgia, heart diseases, gas-

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doi:10.1016/j.jep.2009.10.011

tritis, hemorrhoids and peptic ulcers in Turkish folk medicine (Yesilada et al., 1993, 1995). Moreover, it has been recorded that the olive oil macerate of the flowering herbs is a popular externally applied home-remedy for the rapid recovery of cuts and burns (Yesilada et al., 1993, 1995). The dried flowering parts were placed inside a jar of olive oil and kept about 4 weeks under sunshine. For the preparation of an efficient home-remedy, the key procedure is described as "*the content of the jar should be exposed to the heat of the sun and the breeze of the night*". A literature survey proved that the utilization of Hyperici oil for wound healing is not only known in Turkish folk medicine, but also practiced in all over the world. In fact, the preparation of the oily product was described as "sunlight maceration method" in the supplement of German Pharmacopoeia [DAB 6] (Maisenbacher and Kovar, 1992). Hyperforin was postulated as the active component responsible from the wound healing effect of this oily extract, based on chromatographical data only (Maisenbacher and Kovar, 1992).

The effect of ethanol extract of *Hypericum perforatum* flowering aerial parts on the ethanol-induced gastric lesions was previously investigated and a potent antiulcer activity was reported in mice (Yesilada and Gürbüz, 1998). One of the experimental studies on the wound healing activity of *Hypericum perforatum* was carried out by Öztürk et al. (2007) using chicken embryonic fibroblasts. They studied only the 50% ethanolic extract and obtained the positive results in the used *in vitro* model. *In vivo* wound healing activity of *Hypericum perforatum* was studied by Rao et al. (1991) using the similar experimental methods, however they administered the extract orally – not topically – to the groups of rats to assess the activity. A clinical study was also carried out by Lavagna et al. (2001) exploring the potential of a mixture of the oily extracts of *Hypericum perforatum* and *Calendula arvensis* on the epithelial reconstruction of surgical wounds. They obtained better results with the formulation than the control group. In spite of this common awareness, wound healing effect by topical application of *Hypericum perforatum* and its oily extract have not been investigated before by using such *in vivo* experimental wound models.

The aim of the present study is to evaluate the folkloric information on wound healing activity of the oily extract of *Hypericum perforatum* by means of *in vivo* excision and incision wound models as well as to find active component(s) through bioassay-guided procedures. In spite of widespread distribution of *Hypericum* species in Turkish flora (81 *Hypericum* species), only *Hypericum perforatum* has been used for wound healing in Turkish folk medicine. In order to find out a rational for that presumption, a parallel study was also run on the extract of another widespread species, *Hypericum scabrum* L.

2. Materials and methods

2.1. Plant materials

Hypericum scabrum L. flowering aerial parts were collected from roadside of Ankara-Kırsehir, about 2.5 km distance to Kırsehir in May 2006. *Hypericum perforatum* L. flowering aerial parts were collected from Ankara, Beypazarı, Gürsögüt in June 2006. The plants were identified by Professor Dr. Mecit Vural from the Department of Botany, Faculty of Art and Science, Gazi University. Voucher specimens (GUE-2462 and GUE-2463) were deposited in the Herbarium of Faculty of Pharmacy and Faculty of Science, at the Gazi University.

2.2. Drugs and chemicals

n-Butanol (Art.988, Merck), *n*-hexane (Art.4368, Merck), chloroform (Lab Scan), ethanol (Tekel), ethyl acetate (Art.864, Merck), Evans blue (Sigma, St. Louis, MO, USA), Hypericin (Sigma), indomethacin (Nobel), Ketamine (Ketamine HCl 10%, Bremer Pharma GMBH, Germany), Madecassol® (Bayer), non-absorbable natural surgical silk sutures (Carelife, 061211), olive oil (extra virgin, cold press, the acidity is less than 1%), Sephadex LH-20 [(25–100 μ), Sigma–Aldrich 095K1220], SiO_2 (Merck, Kieselgel 60, 60–230 mesh), TLC (silica gel aluminium plates, SiO_2 60 F_{254}, Merck) were used.

2.3. Extraction and fractionation

2.3.1. Preparation of extracts for preliminary activity testing

Two kinds of extracts were prepared from the plant material for the preliminary activity testing. Initially, an olive oil extract was prepared from the aerial parts of *Hypericum perforatum* as described in folk medicine for the assessment of wound healing activity. Following, the ethanolic extracts of the both plants were prepared and submitted to activity assessment, in order to apply the bioassay-guided procedures efficiently.

2.3.1.1. Preparation of olive oil extract. Dried and powdered aerial parts of *Hypericum perforatum* (50 g) was placed in a transparent glass jar containing olive oil (500 ml), the jar was kept under sunshine daily 12 h for 4 weeks in summer season. This extract was named as HP-Oo, used in following experiments without any further processing.

2.3.1.2. Preparation of ethanol extracts. A 100 g portion of the powdered *Hypericum perforatum* or *Hypericum scabrum* was extracted three times with 96% ethanol (2000 ml) at room temperature and evaporated to dryness under reduced pressure not exceeding 40 °C to give *Hypericum perforatum* (HPE) (yield: 29.93%) and *Hypericum scabrum* ethanolic extracts (HSE) (yield: 27.80%).

2.3.2. Extraction and fractionation procedures for the bioassays

Dried and powdered aerial parts (1000 g) of *Hypericum perforatum* was extracted with 96% EtOH and evaporated to dryness to give "HP-EtOH" (yield: 31.3%). The residual dried extract was then dissolved in 400 ml of methanol/H_2O (9:1) and this highly viscose extract transferred to a separator funnel and extracted with *n*-hexane (20 × 500 ml). Combined *n*-hexane extracts were evaporated under reduced pressure to give "HP-Hexane" (yield: 13.7%). The remaining methanol phase was evaporated and the residual methanol extract diluted with distilled H_2O to 400 ml and successively extracted subsequently with chloroform (20 × 500 ml) and ethyl acetate (20 × 500 ml). Each solvent extract was evaporated to dryness under reduced pressure to give "HP-$CHCl_3$" (yield: 1.4%) and "HP-EtOAc" (yield: 27.9%) respectively. The remaining aqueous extract was further extracted with *n*-butanol saturated with water (20 × 500 ml) and evaporated to dryness at 40 °C under reduced pressure to give "HP-BuOH" (yield: 21.7%). The final aqueous phase was also evaporated to dryness "HP-FH_2O (yield: 28.6%). (Yesilada and Küpeli, 2002). For the wound healing activity models the combined fractions HP-BuOH:HP-FH_2O (1:1) were tested as HP-RH_2O.

2.3.3. Fractionation of HP-EtOAc extract

According to the bioassay-guided procedures HP-EtOAc, the active extract was further fractionated by using chromatographical techniques. Three grams of HP-EtOAc was subjected to chromatographic separation on Sephadex LH-20 column using MeOH and MeOH:H_2O (1:1) as eluent systems. The eluents were combined depending on their TLC profile as follows: [Fr. 1–18; A] (yield: 1530 mg) and [Fr. 19–85; B] (yield: 660 mg).

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2.3.4. Chromatographic separation and isolation of the active constituents

Fraction A (1.2 g) was subjected to C_{18}-Medium Pressure Liquid Chromatography (C_{18}-MPLC, 130 g, Combi Flash Companion (Isco) Lincoln, Nebraska, USA) used with Redi step columns packed LiChroprep (C_{18}, 40–63 μm, Teledyne Isco) eluting with stepwise H_2O–MeOH gradient (5–90% MeOH) to give four subfractions, A_1–A_4. Rechromatography of Fr.A_3 (50 mg) by eluting with stepwise H_2O–MeOH gradient (20–60% MeOH) to obtain A_{3a-c}. Hyperoside (6 mg), isoquercitrin (7 mg) and rutin (4 mg) were separated from Fr.A_{3a} (40 mg) by SiO_2 CC ($CHCl_3$–MeOH–H_2O, 90:10:0 and 80:20:1). Fraction A_4 (116 mg) was separated by C_{18}-MPLC (43 g, 10–80% MeOH/H_2O, gradient) to obtain Fr.A_{4a} (15 mg) which was further applied to a SiO_2 column ($CHCl_3$–MeOH–H_2O, 90:10:1 to 70:30:3) to yield (−)-epicatechin (5 mg). The presence of hypericin in Fr.B was detected by TLC (mobile phase EtOAc–$CHCl_3$–HCOOH–gCH_3COOH–H_2O in the ratio of 75:30:10:10:8) using hypericin as reference substance.

2.3.5. Structure elucidation of the compounds

Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR) and Mass Spectral (MS) techniques were used for the structure elucidation of the compounds. NMR spectra were recorded on a Varian Mercury-400 spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) instrument, and using CD_3OD as solvent. ESI-MS analyses were performed using a Waters 2695 Alliance Micromass ZQ LC-MS spectrometer. The isolates were identified as hyperoside, isoquercitrin, rutin and (−)-epicatechin by comparison of their spectroscopic data with those of published values (Markham and Geiger, 1994).

2.4. Pharmacological experiments

2.4.1. Animals

Male Sprague–Dawley rats (160–180 g) and Swiss albino mice (20–25 g) were purchased from the animal breeding laboratories of Refik Saydam Central Institute of Health (Ankara, Turkey).

The animals were left for 3 days for acclimatization into animal room conditions and were maintained on standard pellet diet and water *ad libitum*. A minimum of six rats were used in each group for wound healing experiments, while 10 mice were used in anti-inflammatory studies. The present study was performed according to the international rules considering the animal experiments and biodiversity rights (Gazi University Ethical Council Project Number: G.U.ET-08.037).

2.4.2. Preparation of test samples for bioassay

For the anti-inflammatory test model, samples were given orally to test animals after suspending in a mixture of distilled water and 0.5% sodium carboxymethyl cellulose (CMC). The control group of animals received the same experimental handling as those of the test groups except that the drug treatment was replaced with appropriate volumes of the dosing vehicle. Indomethacin (10 mg/kg) in 0.5% CMC was used as a reference drug.

To evaluate wound healing activity by using incision and excision wound models, the olive oil extract was topically applied onto the wounded area on dorsal part of the test animals. However, for the preparation of test samples from extracts/subextract or fractions, each sample was mixed thoroughly in a mortar with a mixture of glycol stearate:propylene glycol:liquid paraffin (3:6:1) into an ointment form. Treatments were started immediately after the production of wound by daily application of the sample ointments on the wounded area. The control group animals were topically treated with blank vehicle, i.e., either olive oil or an ointment base consisting of glycol stearate:propylene glycol:liquid paraffin (3:6:1) mixture, while the animals in negative control group were not treated with any product. Madecassol® (0.5 g) was used topically as the reference drug.

2.4.3. Acetic acid-induced increase in capillary permeability

Effect of the test samples on the increased vascular permeability induced by acetic acid in mice was determined according to Whittle method (Whittle, 1964) with some modifications (Yesilada et al., 1988). Each test sample was administered orally to a group of 10 mice in 0.2 ml/20 g body weight. Thirty minutes after the administration, tail of each rat was injected with 0.1 ml of 4% Evans blue in saline solution (i.v.) and waited for 10 min. Then, 0.4 ml of 0.5% (v/v) AcOH was injected i.p. After 20 min. incubation, the mice were killed by dislocation of the neck, and the viscera were exposed and irrigated with distilled water, which was then poured into 10 ml volumetric flasks through glass wool. Each flask was made up to 10 ml with distilled water, 0.1 ml of 0.1N NaOH solution was added to the flask, and the absorption of the final solution was measured at 590 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA). A mixture of distilled water and 0.5% CMC was given orally to control animals, and they were treated in the same manner as described above.

2.4.4. Linear incision wound model

Animals, six rats in each group, were anesthetized with 0.15 cc Ketamine (Shetty et al., 2006), the hairs on the dorsal part of the rats were shaved and cleaned with 70% alcohol. Two 5 cm-length linear-paravertebral incisions were made with a sterile blade through the shaved skin at the distance of 1.5 cm from the dorsal midline on each side. Three surgical sutures were placed each 1 cm apart.

The ointments prepared with test samples, the reference drug (Madecassol®) or ointment base [glycol stearate:propylene glycol:liquid parafin (3:6:1)] were topically applied on the dorsal wounds in each group of animals once daily throughout 9 days. All the sutures were removed on the last day and tensile strength of previously wounded and treated skin was measured by using a tensiometer (Zwick/Roell Z0.5, Germany) (Suguna et al., 2002; Lodhi et al., 2006). Tensile strength was determined using the following equations (Saringat and Wasim, 1995):

$$\text{tensile strength} = \frac{\text{breaking force}}{\text{area}}$$

$$\text{area} = \text{thickness} \times \text{width}$$

2.4.5. Circular incision wound model

Each rat ($n = 6$) was anesthetized with 0.15 cc Ketamine and the back hair of each animal was shaved, then the area was disinfected with 70% ethanol. A circular incision of about 20 mm in diameter was made on the disinfected area of the skin surface and the skin carefully dissected out. The area was treated topically either with the test ointments or Madecassol® or blank ointment base (as control) once daily for 10 days. The progressive changes in the wounded area of each animal was measured on the 1st, 4th, 7th, 10th, 14th, and 17th day after the surgery under anesthesia by placing a transparent tracing paper over the wounded area and the borders of the healing area was traced. The tracing paper was then placed on a 1 mm^2 graph sheet and traced out. The squares representing the healed area were counted and recorded. The percentage wound healing on each measurement period was estimated. The rats were killed under anesthesia on 17th day, and the tissue samples from the healing wounds were removed and examined histopathologically (Chah et al., 2006).

2.4.6. Excision wound model

Back of six rats in each group was depilated and the areas were cleaned with 70% alcohol under Ketamine (0.15 cc) anesthesia.

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Table 1
Effect of the olive oil extract of *Hypericum perforatum* (HP-Oo) on linear incision wound model.

Groups	Force applied (N)	Area of skin (cm^2)	Tensile strength mean (N/cm^2) ± S.E.M.
Negative control	15.2 ± 6.5	0.36	42.2 ± 5.2
Vehicle (olive oil)	19.8 ± 4.3	0.37	53.5 ± 3.8**
HP-Oo	26.3 ± 5.2	0.38	69.2 ± 3.2***

Statistical evaluation of the experimental results was performed against the vehicle. HP: *Hypericum perforatum*; S.E.M.: standard error mean; N: newton.
** $p < 0.01$.
*** $p < 0.001$.

Excision wound was inflicted by cutting away a 500 mm^2 full thickness of skin from a predetermined area; the wound was left open. Then the test ointments, the reference ointment (Madecassol®) or blank ointment base for the purpose of control were topically applied till the wound was completely recovered. This model was used to monitor wound contraction and wound closure time. Wound contraction was calculated as percent reduction in wound area. The progressive changes in wound area were measured planimetrically by tracing the wound margin on graph paper every alternate day. The rats were killed under anesthesia on 21st day, immediately tissue samples were removed from the healed area for further histopathological examination (Saha et al., 1997).

2.4.7. Histopathology

Tissues were fixed in 10% formalin. After routine fixation procedures tissues were embedded in paraffin wax. Serial sections of paraffin embedded tissues of 5 μm thickness were cut. Haematoxylin and eosin stained preparations were examined under light microscope (Jagetia and Rajanikant, 2005). Ulceration, necrosis and epithelialization of skin tissues were evaluated using a scale up: – (not exist) and + (exist). Congestion, edema, polymorphonuclear leucocytes, mononuclear cells, fibroblasts and vascularization were also semi-quantitatively evaluated using a scale up: – (nil), + (weak), ++ (moderate) and +++ (remarkable).

2.4.8. Analysis of data

The data obtained from the tensile strength and wound area measurements were statistically analyzed using one-way analysis of variance (ANOVA), differences were considered significant at $P < 0.001$. Values were represented by mean ± S.E.M. (standard error mean). Chi-square test is used for the statistical evaluation of the ulceration, necrosis and epithelialization.

Table 2
Effect of the olive oil extract of *Hypericum perforatum* (HP-Oo) on excision wound model.

Days	Wound area (mm^2) ± S.E.M. (% contraction)[a]		
	Negative control	Vehicle (olive oil)	HP-Oo
1	505.9 ± 19.1	495.9 ± 18.7	421.0 ± 22.5 (15.1)
3	497.7 ± 17.3	477.4 ± 11.9	430.5 ± 31.9 (9.8)
5	479.3 ± 27.0	473.0 ± 19.8	418.8 ± 17.2 (11.5)
7	393.9 ± 20.1	360.2 ± 40.8	341.8 ± 19.0 (5.1)
9	360.9 ± 22.2	278.1 ± 44.1	221.4 ± 23.8 (20.4)
11	317.5 ± 10.0	169.9 ± 7.3	115.5 ± 20.3 (32.0)**
13	220.5 ± 26.1	90.9 ± 9.3	61.5 ± 11.9 (32.3)**
15	207.7 ± 12.7	71.2 ± 4.4	55.2 ± 10.3 (22.5)*
17	190.2 ± 9.6	50.1 ± 9.1	20.4 ± 7.6 (59.3)***
19	156.1 ± 17.9	28.3 ± 10.6	9.7 ± 2.9 (65.7)***
21	69.8 ± 9.5	23.5 ± 10.0	4.1 ± 1.3 (82.6)***

HP: *Hypericum perforatum*; S.E.M.: standard error mean.
[a] Statistical evaluation of the experimental results was performed against the vehicle.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 3
Effect of the olive oil extract of *Hypericum perforatum* (HP-Oo) on circular incision wound model.

Days	Wound area (mm^2) ± S.E.M. (% contraction)[a]		
	Negative control	Vehicle (olive oil)	HP-Oo
1	311.9 ± 21.5	276.0 ± 24.9	220.3 ± 12.8 (20.2)
4	300.4 ± 21.7	278.2 ± 3.5	180.2 ± 10.1 (35.2)*
7	262.9 ± 22.3	203.6 ± 27.4	151.2 ± 17.0 (25.7)
10	179.8 ± 16.8	167.8 ± 39.4	71.0 ± 9.0 (57.7)**
14	157.6 ± 18.4	70.7 ± 18.9	21.5 ± 1.6 (69.6)**
17	101.5 ± 13.3	15.3 ± 17.7	0.0 ± 0.0 (100.0)***

HP: *Hypericum perforatum*; S.E.M.: standard error mean.
[a] Statistical evaluation of the experimental results was performed against the vehicle.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

3. Results

In order to confirm the claimed utilization in folk medicine, the effect of olive oil preparation from the flowering aerial parts of *Hypericum perforatum* (HP-Oo) was investigated against various *in vivo* wound models. Excision and incision wound models were employed for the assessment of wound healing activity. The former model was used to evaluate the effect of the test samples on wound closure and contraction and consequently time of epithelialization. This is particularly important since the treated wounds should contract much faster (Swamy et al., 2007). The data obtained from circular incision model also provided supporting evidence on this subject. Alternatively, promotion of the tensile strength of a treated wound is an indication of wound healing possibly due to increase in collagen content and stabilization of the fibers. This may be well monitored by measurement of the tensile strength in the linear incision model through a tensiometer (Swamy et al., 2007).

In the tables have shown that olive oil itself – vehicle of the preparation – exerted some wound healing activity, however the activity of the *Hypericum perforatum* preparation was significantly higher. In order to avoid confusion, statistical evaluation was only performed between vehicle and test samples. As shown in Table 1, treatment with the oily preparation increased the tensile strength of the wounds that were induced by linear incision within 10 days. The effect was significantly greater when the results were compared to the negative control and the vehicle. Wound con-

Table 4
Effect of the ethanol extract and fractions from *Hypericum perforatum* and ethanol extract of *Hypericum scabrum* on linear incision wound model.

Groups	Force applied (N)	Area of skin (cm^2)	Tensile strength mean (N/cm^2) ± S.E.M
Negative control	15.4 ± 8.3	0.46	33.5 ± 11.7
Vehicle	17.8 ± 9.6	0.44	40.5 ± 8.5
HS-EtOH	18.3 ± 11.3	0.38	48.2 ± 10.2
HP-EtOH	29.9 ± 7.2	0.45	66.4 ± 5.1***
HP-Hexane	20.8 ± 9.5	0.39	53.3 ± 9.8
HP-$CHCl_3$	23.4 ± 7.7	0.40	58.5 ± 7.1*
HP-EtOAc	32.3 ± 3.1	0.46	70.2 ± 5.3***
HP-RH_2O	19.3 ± 8.8	0.37	52.2 ± 7.3
Fr.1–18	28.7 ± 6.2	0.46	62.4 ± 6.1**
Fr.19–85	30.4 ± 9.3	0.45	67.6 ± 7.9***
Madecassol®	36.6 ± 8.1	0.47	77.9 ± 8.5***

Statistical evaluation of the experimental results was performed against the vehicle. HS: *Hypericum scabrum*; HP: *Hypericum perforatum*; S.E.M.: standard error mean; N: newton.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

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Table 5
Effect of the ethanol extract and fractions from *Hypericum perforatum* and ethanol extract of *Hypericum scabrum* on excision wound model.

Days	Wound area (mm^2) ± S.E.M. (% contraction)[a]								
	Negative control	Vehicle	HP-EtOH	HS-EtOH	HP-Hexane	HP-$CHCl_3$	HP-EtOAc	HP-RH_2O	Madecassol®
1	461.0 ± 21.5	399.6 ± 11.6	326.5 ± 10.4 (18.3)	444.3 ± 40.9	408.3 ± 31.1	350.8 ± 47.1 (12.2)	306.2 ± 30.2 (23.4)	399.0 ± 33.1 (0.2)	375.0 ± 9.7 (6.2)
3	442.7 ± 17.3	395.4 ± 10.8	287.0 ± 18.9 (27.4)*	390.0 ± 27.8 (1.4)	438.9 ± 32.0	373.4 ± 55.0 (5.6)	324.6 ± 8.5 (17.9)	363.6 ± 35.4 (8.0)	332.5 ± 8.7 (15.9)
5	421.3 ± 19.1	339.4 ± 30.2	220.8 ± 18.4 (34.9)**	367.0 ± 16.7	367.1 ± 48.6	258.8 ± 35.1 (23.7)	218.4 ± 27.6 (35.6)**	269.3 ± 62.7 (20.7)	252.3 ± 18.3 (25.7)*
7	382.3 ± 11.0	199.0 ± 31.8	103.8 ± 8.7 (47.8)***	274.3 ± 31.9	217.1 ± 40.8	171.2 ± 40.2 (13.9)	129.4 ± 29.3 (34.9)*	187.8 ± 75.1 (5.6)	118.5 ± 7.5 (40.5)**
9	361.9 ± 13.2	170.4 ± 12.1	79.2 ± 4.6 (53.5)***	163.9 ± 19.2 (3.8)	138.1 ± 11.2 (18.9)	131.3 ± 14.1 (22.9)	59.05 ± 5.4 (65.3)***	147.8 ± 16.0 (13.3)	82.3 ± 5.4 (51.7)***
11	239.6 ± 12.0	151.2 ± 10.3	61.2 ± 4.2 (59.5)***	155.7 ± 24.8	149.3 ± 15.1 (1.3)	158.5 ± 21.0	49.0 ± 2.1 (67.6)***	136.0 ± 30.8 (10.1)	68.3 ± 3.8 (54.8)***
13	201.1 ± 32.3	114.8 ± 7.5	52.7 ± 2.7 (54.1)***	135.7 ± 38.6	96.9 ± 8.1 (15.6)	79.3 ± 4.9 (30.9)*	32.6 ± 1.1 (71.6)***	186.0 ± 4.9	59.7 ± 2.4 (47.9)**
15	183.2 ± 24.4	99.8 ± 6.2	41.1 ± 3.8 (58.8)***	128.0 ± 41.1	87.1 ± 5.1 (12.7)	69.4 ± 3.7 (30.5)*	27.5 ± 1.3 (72.4)***	157.2 ± 3.8	50.8 ± 3.3 (49.1)**
17	119.8 ± 10.5	66.7 ± 3.9	28.8 ± 1.7 (56.8)**	74.7 ± 21.4	45.3 ± 2.1 (32.1)	47.0 ± 4.1 (29.5)	16.2 ± 2.1 (75.7)***	56.1 ± 3.6 (15.9)	39.0 ± 0.4 (41.5)**
19	91.9 ± 9.9	39.0 ± 6.7	8.4 ± 2.7 (78.5)***	43.3 ± 5.5	31.2 ± 4.2 (20.0)	21.8 ± 5.8 (44.1)**	9.0 ± 1.1 (76.9)***	36.5 ± 3.5 (6.4)	8.8 ± 0.5 (77.4)***
21	75.9 ± 5.6	31.9 ± 3.7	1.4 ± 0.3 (95.6)***	35.8 ± 7.6	23.0 ± 2.6 (27.9)	19.0 ± 6.2 (40.4)*	0.0 ± 0.0 (100)***	20.1 ± 3.1 (36.9)	0.0 ± 0.0 (100)***

HS: *Hypericum scabrum*; HP: *Hypericum perforatum*; S.E.M.: standard error mean.
[a] Statistical evaluation of the experimental results was performed against the vehicle.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 6
Effect of the ethanol extract and fractions from *Hypericum perforatum* and ethanol extract of *Hypericum scabrum* on circular incision wound model.

Days	Wound area (mm^2) ± S.E.M. (% contraction)[a]								
	Negative Control	Vehicle	HP-EtOH	HS-EtOH	HP-Hexane	HP-$CHCl_3$	HP-EtOAc	HP-RH_2O	Madecassol®
1	327.5 ± 17.7	304.6 ± 6.2	262.3 ± 13.9 (13.9)	268.7 ± 14.3 (11.8)	269.4 ± 4.1 (11.6)	274.3 ± 4.6 (9.9)	276.0 ± 4.1 (9.4)	268.5 ± 2.9 (11.9)	263.2 ± 18.4 (13.6)
4	311.9 ± 18.0	289.6 ± 8.9	236.5 ± 12.0 (18.3)	259.7 ± 16.8 (10.3)	247.6 ± 4.6 (14.5)	227.2 ± 2.1 (21.5)	211.1 ± 5.1 (27.1)*	245.2 ± 5.2 (15.3)	204.8 ± 15.9 (29.3)*
7	300.8 ± 31.3	219.2 ± 25.4	159.2 ± 12.8 (27.4)*	216.7 ± 33.6 (1.1)	182.7 ± 23.2 (16.7)	177.5 ± 17.1 (19.0)	145.0 ± 20.6 (33.9)**	179.0 ± 25.4 (18.3)	164.7 ± 20.6 (24.9)*
10	191.6 ± 14.7	171.3 ± 7.6	84.3 ± 10.5 (50.8)***	158.3 ± 13.1 (7.6)	146.2 ± 3.6 (14.7)	133.7 ± 4.7 (21.9)	106.5 ± 5.6 (37.8)**	144.1 ± 4.4 (15.9)	93.2 ± 7.3 (45.6)***
14	177.8 ± 11.1	137.0 ± 26.7	45.2 ± 10.2 (67.0)***	105.0 ± 6.4 (23.4)	111.1 ± 20.4 (18.9)	100.8 ± 14.8 (26.4)	58.5 ± 18.2 (57.3)***	115.0 ± 24.7 (16.1)	25.0 ± 9.4 (81.8)***
17	103.0 ± 10.3	83.0 ± 2.9	0.0 ± 0.0 (100.0)***	58.7 ± 11.6 (29.3)	48.7 ± 2.0 (41.3)	29.5 ± 1.8 (64.5)*	0.0 ± 0.0 (100.0)***	49.6 ± 2.2 (40.2)	8.0 ± 4.3 (90.4)***

HS: *Hypericum scabrum*; HP: *Hypericum perforatum*; S.E.M.: standard error mean.
[a] Statistical evaluation of the experimental results was performed against the vehicle.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

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Table 7
Effect of the subfractions from HP-EtOAc on excision wound model.

Days	Wound area (mm^2) ± S.E.M. (% contraction)[a]				
	Negative control	Vehicle	Fr.1–18	Fr.19–85	Madecassol®
1	488.0 ± 22.9	467.5 ± 10.8	451.7 ± 21.6 (3.4)	341.7 ± 29.4 (26.9)	368.1 ± 16.9 (21.3)
3	456.8 ± 19.0	397.9 ± 14.3	369.3 ± 28.6 (7.2)	281.1 ± 20.1 (29.4)	349.5 ± 18.1 (12.2)
5	434.6 ± 26.1	361.8 ± 17.0	324.9 ± 22.3(10.2)	251.1 ± 19.4 (30.6)*	257.7 ± 11.2 (28.8)
7	301.3 ± 34.3	267.0 ± 19.5	208.5 ± 22.2 (21.9)	157.8 ± 11.1 (40.9)**	148.5 ± 9.5 (44.4)**
9	287.6 ± 23.1	149.9 ± 18.7	111.2 ± 23.1 (25.8)	84.2 ± 7.7 (43.8)***	63.9 ± 11.9 (57.4)***
11	217.3 ± 6.6	95.1 ± 6.9	73.9 ± 15.7 (22.3)	44.0 ± 7.8 (53.7)***	30.0 ± 12.8 (68.5)***
13	211.9 ± 17.3	88.5 ± 4.8	62.3 ± 29.4 (29.6)	36.9 ± 8.1 (58.3)***	21.0 ± 12.6 (76.3)***
15	180.2 ± 16.3	67.7 ± 16.0	46.4 ± 13.7 (31.5)**	24.8 ± 4.2 (63.4)***	19.8 ± 4.5 (70.8)***
17	109.6 ± 8.2	51.5 ± 4.9	33.1 ± 17.5 (35.7)*	17.8 ± 2.3 (65.5)***	8.7 ± 3.2 (83.1)***
19	81.5 ± 19.9	38.1 ± 10.7	21.6 ± 8.8 (43.3)**	11.7 ± 1.2 (69.3)***	3.3 ± 1.3 (91.3)***
21	62.3 ± 12.0	29.7 ± 7.7	15.6 ± 4.1 (47.5)**	8.4 ± 0.9 (71.7)***	0.0 ± 0.0 (100.0)***

S.E.M.: standard error mean.
[a] Statistical evaluation of the experimental results was performed against the vehicle.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 8
Effect of the subfractions from HP-EtOAc on circular incision wound model.

Days	Wound area (mm^2) ± S.E.M. (% contraction)[a]				
	Negative control	Vehicle	Fr.1–18	Fr.19–85	Madecassol®
1	341.5 ± 8.9	313.9 ± 21.8	258.8 ± 10.5 (17.6)	240.4 ± 18.8 (23.4)	271.7 ± 22.3 (13.4)
4	311.1 ± 19.1	277.5 ± 6.7	228.3 ± 7.7 (17.7)	238.9 ± 15.1 (13.9)	222.6 ± 13.2 (19.8)
7	300.9 ± 21.9	231.5 ± 20.0	163.8 ± 10.3 (29.2)*	141.2 ± 9.0 (39.0)*	155.9 ± 16.4 (32.7)*
10	273.6 ± 18.6	188.6 ± 13.1	151.9 ± 21.6 (19.5)	79.3 ± 5.7 (57.9)***	102.6 ± 8.8 (45.6)**
14	266.0 ± 8.9	171.6 ± 22.5	95.9 ± 14.4 (44.1)**	62.1 ± 8.2 (63.8)***	32.0 ± 8.1 (81.4)***
17	171.6 ± 15.7	98.7 ± 10.4	45.7 ± 8.9 (53.7)*	22.9 ± 6.6 (76.8)***	6.7 ± 5.3 (93.2)***

S.E.M.: standard error mean.
[a] Statistical evaluation of the experimental results was performed against the vehicle.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

tracting ability was measured every 2 days for excision model and every 3 days for circular incision model, for the evaluation of the effect of oily preparation on the experimentally induced wounds (Tables 2 and 3). The wound contracting ability of the oily preparation on excision and circular incision wound models was significantly greater than that of the negative control and the vehicle itself, with the contraction values increasing from 15.1% on 1st day to 82.6% on 21st day for excision and from 20.2% on 1st day to 100.0% on 17th day for incision models. These recent results have clearly demonstrated that the oily preparation of *Hypericum perforatum* has a remarkable effect on healing of wounds when the data compared to olive oil itself as a vehicle.

In order to subject bioassay-guided fractionation procedures, EtOH extract of *Hypericum perforatum* (HP-EtOH) was prepared and its effect on the same wound models was studied. As shown in Tables 4–6, HP-EtOH extract showed very similar wound healing activity profile against linear incision, excision and circular incision models of wound healing. HP-EtOH was then subjected to successive solvent extractions with hexane, chloroform and ethyl acetate. The effects of each solvent extracts and the remaining aqueous extract on the *in vivo* wound models are given in Tables 4–6. On the linear incision wound model, the tensile strength values of the wounds treated with HP-EtOAC was significantly higher than that of the vehicle group (Table 4). The wounds treated with HP-EtOAc exhibited significant activity with the contraction values between 23.4% (on the 1st day) to 100.0% (on the 21st day) on excision and between 9.4% (on the 1st day) and 100% (on the 17th day) on circular incision wound models (Tables 5 and 6).

The active subextract HP-EtOAc was further fractionated by using column chromatography on Sephadex LH-20 and eluted fractions were combined into two fractions [Fr.1–18] and [Fr.19–85] according to their TLC profile. Both the fractions showed significant wound healing activity on excision and circular incision models, however, the effect of the later [Fr.19–85] was more prominent (Tables 7 and 8).

The wound healing process includes an inflammation phase. In order to determine the possible contributory role of the anti-inflammatory components in the HP-EtOH extract, its subextracts and fractions on the inflammatory cell infiltration, which can be related with the wound healing, the Whittle method based on the inhibition of acetic acid-induced increase in capillary permeability was used. A dose-dependent inhibitory activity was observed for HP-EtOH extract up to the dose of 200 mg/kg with the highest inhibitory value of 40.9%, and then the activity drastically dropped in 400 mg/kg dose (Table 9). HP-EtOAc and its fractions also exerted significant and dose-dependent anti-inflammatory activity (Tables 10 and 11). The anti-inflammatory effect of the active extract and its fractions were quite comparable to the reference compound indomethacin.

On the histopathological examination of the wound sections, the negative control group exhibited wide area of ulcerations containing fibrinous exudates and inflammatory cells, mild degree of inflammation, vascularizations with congestions in dermis, which indicates the healing was not completed (Fig. 1). On the histopathological examinations of the tissue samples from HP-EtOAc extract treated group demonstrated that restoration of collagen fibers, fibroblasts and intact epidermis were obviously distinguished in

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Table 9
Inhibitory effect of the EtOH extracts from *Hypericum perforatum* (HP) and *Hypericum scabrum* (HS) on acetic acid-induced increased capillary permeability.

Material	Dose (mg/kg)	Evans blue concentration (μg/ml) ± S.E.M.	Inhibition (%)
Control		9.28 ± 0.62	
HP-EtOH	50	7.98 ± 0.65	14.0
	100	6.52 ± 0.50	29.8*
	200	5.48 ± 0.51	40.9***
	400	7.07 ± 0.46	23.8
HS-EtOH	50	8.55 ± 0.83	7.9
	100	7.87 ± 0.47	15.2
	200	8.67 ± 0.36	6.5
	400	7.56 ± 0.43	18.5
Indomethacin	10	5.13 ± 0.29	44.8***

HS: *Hypericum scabrum*; HP: *Hypericum perforatum*; S.E.M.: standard error mean.
* $p < 0.05$.
*** $p < 0.001$.

Table 10
Inhibitory effect of the fractions from *Hypericum perforatum* on the acetic acid-induced increased capillary permeability.

Material	Dose (mg/kg)	Evans blue concentration (μg/ml) ± S.E.M.	Inhibition (%)
Control		10.12 ± 0.71	
HP-Hexane	29.3	10.66 ± 0.65	–
	58.7	9.72 ± 0.89	3.9
HP-$CHCl_3$	3.0	13.29 ± 0.41	–
	6.0	9.86 ± 0.23	2.6
HP-EtOAc	59.8	7.83 ± 0.14	22.6*
	119.6	7.04 ± 0.11	30.4**
HP-*n*-BuOH	46.7	8.39 ± 0.57	17.1
	93.4	8.14 ± 0.69	19.6
HP-FH_2O	61.2	8.95 ± 0.78	11.6
	122.4	8.17 ± 0.91	19.3
Indomethacin	10.0	5.82 ± 0.44	42.5***

HP: *Hypericum perforatum*; S.E.M.: standard error mean.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 11
Inhibitory effect of the subfractions of HP-EtOAc on the acetic acid-induced increased capillary permeability.

Material	Dose (mg/kg)	Evans blue concentration (μg/ml) ± S.E.M.	Inhibition (%)
Control		10.06 ± 0.89	
Fr.1–18	85.3	7.83 ± 0.61	22.2
	167.1	6.15 ± 0.50	38.8**
Fr.19–85	36.0	8.17 ± 0.45	18.7
	72.1	6.83 ± 0.49	32.1*
Indomethacin	10.0	5.02 ± 0.31	50.0***

S.E.M.: standard error mean.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

the representing figure (Fig. 2). In excision wound model, re-epitelialization capacity of HP-EtOAc was the highest with a value of 100% among the extracts, fractions and the control groups. In Fr.19–85 treated group, although a mild inflammation was observed, intact epidermis and scarless tissue on the dermis were the indication of complete healing (Fig. 3). The tissue samples from the group treated with reference drug Madecassol®, also demonstrated complete healing with the observation of mature epidermis, mature hair follicles, fibroblasts in dermis (Fig. 4).

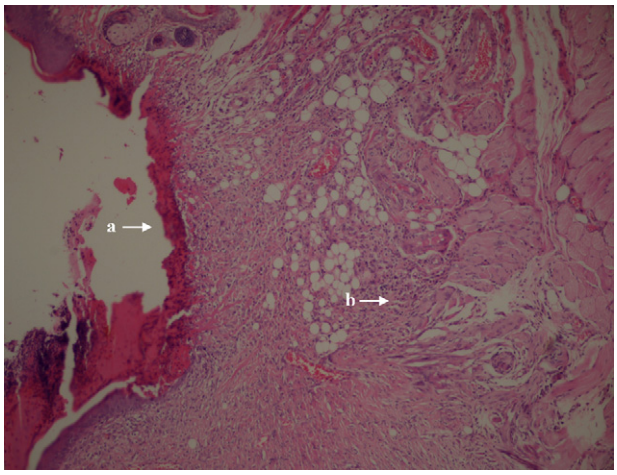

Fig. 1. Photograph of microscopic view of section on 21 day old wound tissue from negative control group (untreated). (a) Area of ulceration and (b) mixed type inflammatory cells.

Wound healing and anti-inflammatory experiments were run parallel with the ethanol extract of *Hypericum scabrum* (HS-EtOH) revealed that *Hypericum scabrum* was completely devoid of such activity against these bioassay models, which supported the ethnopharmacological information. The study design of all these experiments is summarized in Table 12.

4. Discussion

Although only the flowering aerial parts of *Hypericum perforatum* was used in European phytotherapy and Turkish folk medicine for wound healing, several other *Hypericum* species have also been reported to be used for wound healing outside these localities. The leaves of *Hypericum patulum* Thumb. and *Hypericum hookerianum* Wight and Arnott have been used in India for the same purpose. Mukherjee et al. investigated the wound healing effect of methanol extracts of these plants using *in vivo* excision and incision models in rats. Accordingly, they reported improvements in the wound contracting ability, tissue regeneration, tensile strength as well epithelialization time for both species (Mukherjee and Suresh, 2000; Mukherjee et al., 2000). However, we here tested the wound

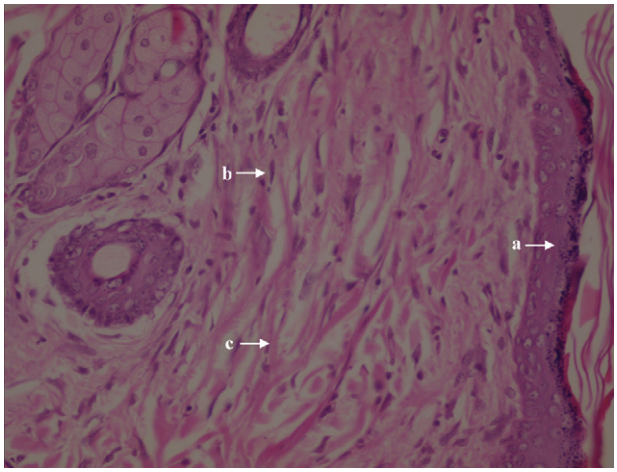

Fig. 2. Photograph of microscopic view of section on 21 day old wound tissue from group treated with HP-EtOAc-Fr. (a) Intact epidermis, (b) fibroblasts and (c) collagen fibers.

Table 12
Study design of the experimental models.

Main groups for all models	Experiment model	Animals in each group	Evaluation parameters	Time of the evaluation	Experiment duration	Investigations after the experiments
Negative control	Excision wound	6 rats	Wound contraction	Every alternate day	21 days	Histopathological assessment
Vehicle						
Extracts	Circular incision wound		Wound contraction	1st, 4th, 7th, 10th, 14th, 17th days	17 days	
Fractions	Whittle	10 mice	Inhibition of acetic acid-induced increase in capillary permeability	1st hour	1 h	–
Reference drug						

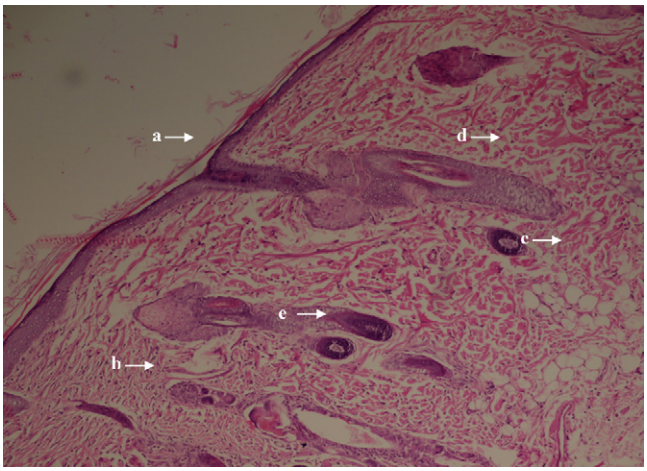

Fig. 3. Photograph of microscopic view of section on 21 day old wound tissue from group treated with Fr.19–85. (a) Intact epidermis, (b) fibroblasts, (c) collagen fibers, (d) edema and (e) hair follicle.

healing and anti-inflammatory activities of another widespread species, *Hypericum scabrum*, which has no related folkloric utilization and was found to be ineffective. These data indicate that the wound healing activity might be attributed only particular *Hypericum* species.

This investigation also provides new evidence on the active constituents of *Hypericum perforatum* on wound healing. Previously Maisenbacher and Kovar (1992) analyzed the major composition of the oily extract of *Hypericum perforatum* by TLC and HPLC. They found mainly hyperforin but no hypericin and postulated that hyperforin might be the active wound healing component of the plant. However, contrary to expectations, in the present study hypericin and flavonoids (hyperoside, isoquercitrin, rutin and (−)-epicatechin) were found in the active fractions, but no hyperforin. This might be due to extraction of hyperforin by the less polar solvents (hexane and chloroform) during the fractionation of extract. However, hyperforin containing hexane and chloroform subextracts were found to be inactive or only weakly active against the wound models. Since Maisenbacher and Kovar (1992) analyzed the oily extract, they detected hyperforin as the major component and supposed that it was the active component without testing by bioassays.

Moreover, several studies have been carried out for the evaluation of the anti-inflammatory effect of *Hypericum perforatum*.

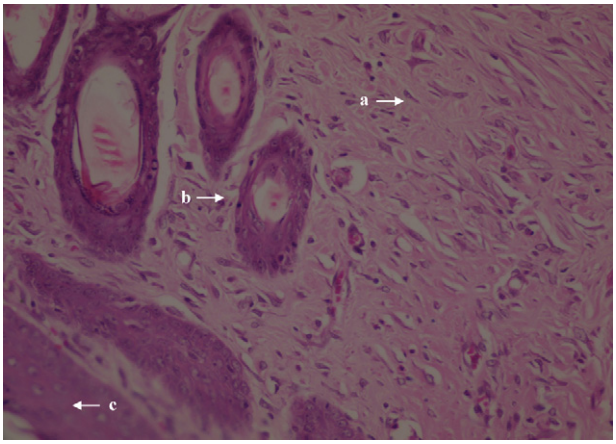

Fig. 4. Photograph of microscopic view of section on 21 day old wound tissue from group treated with Madecassol®. (a) Fibroblasts, (b) hair follicle and (c) intact epidermis.

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Menegazzi et al. (2006) studied *in vivo* anti-inflammatory activity of the methanol extract in carrageenan-induced pleurisy model and reported that the activity might be due to the prevention of nuclear factor-kappa B and STAT-3 activation. Hammer et al. (2007) provided further *in vitro* evidence that the flavonoids (quercetin and amentoflavone) along with pseudohypericin and hyperforin might be the major anti-inflammatory components. These components were demonstrated to inhibit the production of pro-inflammatory mediators such as prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10), but not enhancing inflammation-suppressing mediators. Hyperforin was found to act as a dual inhibitor of cyclooxygenase-1 and 5-lipoxygenase (Albert et al., 2002). The present study provides further evidence on the anti-inflammatory effect of *Hypericum perforatum* through evaluation of the inhibitory effects of the extracts and fractions on the acetic acid-induced increased vascular permeability.

On the other hand, previous studies have shown that *Hypericum* species possess significant antibacterial and antiviral properties (Andersen et al., 1991; Cecchini et al., 2007), which might be involved in the wound healing activity. As it is known, the first aim of the wound management is to keep the wound free of infections and complications. The agents, which helps to heal the wound as quick as possible, are always needed for the contribution of a rapid healing (Clark, 1991).

Wound healing is a natural response of injured skin, consists of complex interactive phases of inflammation, proliferation, and remodeling. The first response of the healing period is inflammation as a defence mechanism of the tissue, which provides a resistance to the microbial contaminations (Kondo, 2007). The anti-inflammatory activity of the plant extract reported here seems to be controversial to the wound healing process, especially in the first step of the phases. As a matter of fact, a long duration in the inflammatory phase causes a delay in healing process. In order to shorten the healing period as well as for minimal pain and scar, anti-inflammatory activity is required (Clark, 1991). Our results suggest that *Hypericum perforatum* shortens the inflammation period, and contributes a resistance against the infections. It probably provides a barrier against microbial contamination at the beginning of the wound healing period. Since, *Hypericum* species have active antibacterial principles such as hyperforin and flavonoids, which probably contributes a rapid and better wound healing (Herrera et al., 1996; Cecchini et al., 2007). Flavonoids are well known active antioxidant compounds responsible for the inhibition of the lipid peroxidation, which leds to prevention of the cell damage and increase in the viability of collagen fibrils (Getie et al., 2002; Shetty et al., 2008).

The results of the present study experimentally proved that the oily preparation of *Hypericum perforatum* is effective in wound healing as popularly practiced in Turkish folk medicine. Olive oil itself as a vehicle for the preparation was also shown to possess a remarkable wound healing activity, however the activity was significantly higher when added *Hypericum perforatum*. The active extract and its fractions enhanced re-epitelialization, whereas no effect was observed on the proliferation capacity on fibroblasts and new vessel formation. The activity might be due to enhancing the migration of fibroblasts and collagen deposition, which was evidenced with increased tensile strength. Further studies are warranted in order to explore the detailed mechanism of the wound healing effect of the plant.

Acknowledgement

This study was supported by the Scientific Research Project of Gazi University (02/2007-03).

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Please cite this article in press as: Süntar, I.P., et al., Investigations on the *in vivo* wound healing potential of *Hypericum perforatum* L. J. Ethnopharmacol. (2009), doi:10.1016/j.jep.2009.10.011

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Please cite this article in press as: Süntar, I.P., et al., Investigations on the *in vivo* wound healing potential of *Hypericum perforatum* L. J. Ethnopharmacol. (2009), doi:10.1016/j.jep.2009.10.011