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Exploration of the wound healing potential of *Helichrysum graveolens* (Bieb.) Sweet: Isolation of apigenin as an active component

Ipek Süntar [a], Esra Küpeli Akkol [a,*], Hikmet Keles [b], Erdem Yesilada [c], Satyajit D. Sarker [d]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Etiler 06330, Ankara, Turkey
[b] Department of Pathology, Faculty of Veterinary Medicine, Afyon Kocatepe University, Afyonkarahisar 03200, Turkey
[c] Faculty of Pharmacy, Yeditepe University, Atasehir 34755, Istanbul, Turkey
[d] Department of Pharmacy, School of Applied Sciences, University of Wolverhampton, MA Building, Wulfurna Street, Wolverhampton WV1 1LY, United Kingdom

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ABSTRACT

Ethnopharmacological relevance: In Turkish traditional medicine, the flowers of *Helichrysum graveolens* (Bieb.) Sweet (Asteraceae) have been used for the treatment of jaundice, for wound-healing and as a diuretic.

Aim of the study: In order to find scientific evidence for the traditional utilization of this plant in wound-healing, the effect of the plant extract was investigated by using *in vivo* and *in vitro* experimental models. Then through bioassay-guided fractionation procedures active wound-healing component(s) was isolated and its possible role in the wound-healing process was also determined.

Material and methods: The linear incision and the circular excision wound models were applied in order to evaluate *in vivo* wound-healing potential of *Helichrysum graveolens*. Anti-inflammatory and antioxidant activities, which are known to involve in wound-healing process, were also assessed by the Whittle method and the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging assay, respectively. The total phenolic content of the crude extract and solvent fractions was estimated to find correlation between the phenolic content and the antioxidant activity. Combined application of the chromatographic separation techniques on sephadex and silica gel columns, and bioassay techniques have yielded the active wound-healing principle of *Helichrysum graveolens*. Moreover, *in vitro* inhibitory effect of active principle on hyaluronidase, collagenase and elastase enzymes were investigated to explore the activity pathways.

Results: The 85% methanol (MeOH) extract of *Helichrysum graveolens* flowers displayed significant wound-healing, anti-inflammatory and antioxidant activities. Then the crude extract was partitioned by successive solvent extractions, in increasing polarity, to give five solvent fractions. Among the solvent fractions, the ethyl acetate (EtOAc) fraction exerted the highest activity. The EtOAc fraction was further subjected to chromatographic separations to yield active constituent and its structure was elucidated to be apigenin by spectrometric methods. Further *in vivo* and *in vitro* assays revealed that apigenin was one of the components responsible for the wound-healing effect of the plant remedy and also found to possess significant anti-inflammatory, antioxidant, anti-hyaluronidase and anti-collagenase activities.

Conclusion: Present study supported the traditional use of *Helichrysum graveolens* flowers for wound-healing and through bioassay-guided fractionation procedures from the crude extract apigenin was isolated as one of the active components.

1. Introduction

There are approximately 600 species of *Helichrysum* Mill. (family: Asteraceae) particularly distributed in South Africa, Australasia and Eurasia. The South African species have been used against infectious and rheumatic diseases, headache, cold, liver diseases and for wound-healing (Lall et al., 2006; Van Wyk et al., 2008). Similar folkloric utilizations have also been recorded in the other parts of the world, such as *Helichrysum stoecheas* for the treatment of bronchitis in Portugal, and *Helichrysum litoreum* and *Helichrysum italicum* against respiratory and inflammatory diseases in Italy (Barros et al., 2010).

There are 16 *Helichrysum* species growing wild in Turkey and all species are widely consumed as tea due to their medicinal properties. Infusion prepared from the capitulums of *Helichrysum plicatum* has been used for wound-healing and for the treatment of

* Corresponding author: Tel.: +90 312 2023185; fax: +90 312 2235018.
E-mail address: esrak@gazi.edu.tr (E. Küpeli Akkol).

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stomachache, jaundice, intestinal problems, as well as diuretic to pass kidney stones, and as urinary antiseptic. In the ethnobotanical records similar utilizations were also reported for *Helichrysum graveolens*, which is also a widespread species in Turkey (Sezik et al., 1991; Yesilada et al., 1995).

Phytochemical studies on various *Helichrysum* species have revealed that they contain mainly flavonoids, phloroglucinols, pyrones and terpenic compounds and exert a wide range of biological activities including antimicrobial, antioxidant, anti-inflammatory, sedative, antidiabetic and cytotoxic (Lourens et al., 2008).

In the present study, the primary objective was to evaluate the wound-healing potential of *Helichrysum graveolens*, a traditional wound-healing remedy in Turkey. Afterwards by using bioassay-guided procedures it is aimed to isolate of the active constituent(s) and suggest the possible mechanisms.

2. Materials and methods

2.1. Plant material

The flowers of *Helichrysum graveolens* (Bieb.) Sweet were collected from Uludağ (Bursa), Zone 1, in July, 2009. The voucher specimen of the plant was authenticated by Prof. Dr. Hayri Duman from Gazi University, Department of Biology, Faculty of Science and Art, Ankara and a specimen of the plant (GUE 2977) was deposited in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara, Turkey. The aerial parts were shade-dried and ground using a blender.

2.2. Extraction, fractionation and isolation procedures for the bioassays

Dried and powdered flowers (350 g) of *Helichrysum graveolens* were extracted with 85% MeOH (7.5 L) and evaporated to dryness to give "Hg–MeOH" (121.8 g; yield: 34.8%). The residual dried extract was then dissolved in MeOH/H_2O (9:1) (400 ml) and partitioned with *n*-hexane (20 × 500 ml). Combined *n*-hexane extracts were evaporated under reduced pressure to give "Hg-hexane" fraction (11.6 g; yield: 9.5%). After removal of methanol from the remaining extract and dilution with distilled H_2O to 400 ml, the extract was successively partitioned with dichloromethane (CH_2Cl_2) (20 × 500 ml), EtOAc (20 × 500 ml) and finally with *n*-butanol (BuOH) saturated with water (20 × 500 ml). Each solvent fraction was evaporated to dryness under reduced pressure to give "Hg–CH_2Cl_2" (2.1 g; yield: 1.7%), "Hg–EtOAc" (17.8 g; yield: 14.6%) and "Hg–BuOH" (15.7 g; yield: 12.9%) fractions, respectively. The final aqueous phase was also evaporated to dryness and designated as "Hg–R–H_2O" (36.7 g; yield: 30.1%).

2.2.1. Fractionation of Hg–EtOAc by chromatographic techniques and isolation of the active constituent

By following bioassay-guided procedures, Hg–EtOAc, the active solvent fraction, was further fractionated by chromatographic techniques. Hg–EtOAc (15 g) was first subjected to separation on a Sephadex LH-20 (Sigma-Aldrich 095K1220) column using MeOH as an eluent. The eluates were combined depending on their thin layer chromatographic (TLC) profile as follows: Hg–Fr.A (0.80 g), Hg–Fr.B (12.35 g) and Hg–Fr.C (0.12 g). The most active fraction Hg–Fr.B (10 mg) was further subjected to silica gel (Sigma-Aldrich 28.862-4) column chromatography using $CHCl_3$ and $CHCl_3$:MeOH (80:20); $CHCl_3$:MeOH (70:30); $CHCl_3$:MeOH (60:40) and MeOH as eluents. Three fractions namely Hg–Fr.B_1 (7.20 mg); Hg–Fr.B_2 (0.95 mg); Hg–Fr.B_3 (0.37 mg) were obtained.

2.2.2. Structure elucidation of the active compound Hg–Fr.B_1

Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR) and Mass Spectral (MS) techniques were employed for the structure elucidation of the silica gel column fraction obtained from the eluate $CHCl_3$:MeOH (80:20) (Hg–Fr.B_1**)** (7.20 mg). NMR spectra were recorded on a Bruker spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) instrument, and using methanol d4 as the solvent. Fourier transform mass spectroscopy **(**FT-MS) analyses were performed using a Finnigan spectrometer. The structure of **1** was determined as apigenin (4′,5,7-trihydroxyflavone) by comparison of the spectroscopic data with the previous reports (Ersöz et al., 2002).

2.3. Determination of total phenolic content of the crude extract and solvent fractions

Total phenolic contents of the methanolic extract and solvent fractions were quantified by using the reference methods involving the Folin–Ciocalteu reagent and gallic acid as reference (Spanos and Wrolstad, 1990). An aliquot of extract solution (100 μl) containing 1 mg extract was taken into a volumetric flask, distilled water and the Folin–Ciocalteu reagent (5 ml) were added and flask was shaken thoroughly. Sodium carbonate (4 ml) was added and the mixture was allowed to stand for 2 h with intermittent shaking at room temperature. Then absorbance was measured at 765 nm. The same procedure was applied to reference gallic acid solutions prepared in different concentrations (0.05 mg/ml; 0.1 mg/ml; 0.15 mg/ml; 0.25 mg/ml and 0.5 mg/ml) to obtain the standard curve.

2.4. Pharmacological experiments

2.4.1. In vivo biological activity tests

2.4.1.1. Animals. Male Sprague–Dawley rats (160–180 g) and Swiss albino mice (20–25 g) purchased from the animal breeding house of Saki Yenilli (Ankara, Turkey) were used in the experiments.

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 internationally accepted issues considering the animal experimentation and biodiversity rights (Gazi University Ethical Council Project Number: G.U.ET-08.037).

2.4.1.2. Wound-healing activity. For the assessment of wound-healing activity by using the incision and the excision wound models, an ointment prepared with the test materials was topically applied onto the wounded area on the dorsal part of test animals. The test ointments were prepared by mixing either extracts/solvent fractions/column fractions or purified compounds with a mixture of ointment base consisting of glycol stearate/propylene glycol/liquid paraffin (3:6:1) in a mortar thoroughly. Treatments were started immediately after the production of wound by daily application of the test ointments on the wounded area. The control group animals were topically treated with blank vehicle base [glycol stearate/propylene glycol/liquid paraffin (3:6:1) mixture], while the animals in negative control group were not treated with any product. Madecassol® (Bayer, 00001199) ointment (0.5 g) was applied topically as the reference drug.

2.4.1.2.1. Linear incision wound model. Animals, six rats in each group, were anaesthetized with 0.05 cm^3 Xylazine (2% Alfazine®) and 0.15 cm^3 Ketamine (10% Ketasol®). The dorsal part of each rat was shaved and the area was cleaned with 70% alcohol. Two linear-paravertebral incisions in 5 cm length were made with a sterile blade through the shaved skin at a distance of 1.5 cm from the dorsal