



## Determination of the wound healing effect of *Calendula* extracts using the scratch assay with 3T3 fibroblasts

M. Fronza<sup>a</sup>, B. Heinzmann<sup>b</sup>, M. Hamburger<sup>c</sup>, S. Laufer<sup>d</sup>, I. Merfort<sup>a,\*</sup>

<sup>a</sup> Department of Pharmaceutical Biology and Biotechnology, University of Freiburg, Freiburg, Germany

<sup>b</sup> Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, Santa Maria, Brazil

<sup>c</sup> Department of Pharmaceutical Sciences, Institute of Pharmaceutical Biology, University of Basel, Switzerland

<sup>d</sup> Department of Pharmaceutical/Medicinal Chemistry, University Tübingen, Germany

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### ABSTRACT

**Pharmacological relevance:** Presentation of the scratch assay as a convenient and inexpensive *in vitro* tool to gain first insights in the wound healing potential of plant extracts and natural compounds.

**Aim of the study:** The present study deals with the optimization of the scratch assay which can be used as an *in vitro* model for quantification of fibroblast migration to and proliferation into the wounded area. It is suitable for the first evaluation of the wound re-epithelialization potential of crude herbal extracts, isolated compounds and pharmaceutical preparations. As a proof of concept three preparations from traditional medicinal plants were investigated.

**Materials and methods:** Swiss 3T3 albino mouse fibroblasts were used in monolayers and platelet derived growth factor as positive control. Hexane and ethanolic extracts from *Calendula officinalis* and *Matricaria recutita*, *Hypericum* oil as well as the triterpenoids faradiol myristate and palmitate were studied. To differentiate between proliferation and migration antimetabolic mitomycin C was added.

**Results:** Both extracts of *Calendula officinalis* stimulated proliferation and migration of fibroblasts at low concentrations, e.g. 10 µg/ml enhanced cell numbers by 64.35% and 70.53%, respectively. Inhibition of proliferation showed that this effect is mainly due to stimulation of migration. Faradiol myristate and palmitate gave comparable stimulation rates at an almost 50 µg/ml concentration, indicating that they contribute partially, but not most significantly to the wound healing effects of *Calendula* preparations. Extracts from *Matricaria recutita* were only moderately active. *Hypericum* oil was cytotoxic at concentrations higher than 0.5 µg/ml.

**Conclusions:** The scratch assay in the present form can be used as a promising scientific approach and platform to differentiate between plant extracts known for their wound healing and their anti-inflammatory properties.

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### 1. Introduction

Upon injury of adult mammalian skin, complex and intricate processes are initiated to restore the function and integrity of the damaged tissues. Wound healing involves inflammation as well as the formation and remodelling of new tissue (Martin, 1997; Martin and Leibovich, 2005; Schafer and Werner, 2007; Gurtner et al., 2008). Processes, such as migration of keratinocytes of the injured epidermis and hair follicles followed by proliferation of these cells at the wound edge, are observed in the beginning of new tissue formation. This phase is also called re-epithelialization.

Redifferentiation of keratinocytes finally occurs to restore the barrier function. Additionally, fibroblasts are important in the repair of the injured dermis. These cells proliferate to expand and migrate into the wound area, synthesize new extracellular matrix (ECM), as well as express thick actin bundles as myofibroblasts (Schafer and Werner, 2007; Gurtner et al., 2008).

Since ancient times, people have used plants and preparations thereof to accelerate the wound healing process (Reuter et al., 2009; Schmidt et al., 2009). Often their use is merely based on tradition, without any scientific evidence of efficacy and little knowledge about putative active compounds or their mode of actions. As wound healing is a complex biological process several *in vitro* and *in vivo* assays are available. Among these, the scratch assay has been proven as a valuable and inexpensive tool to obtain first insights into how plant preparations or their isolated compounds can positively influence formation of new tissue (van Horsen et al., 2006; Liang et al., 2007).

\* Corresponding author at: Department of Pharmaceutical Biology and Biotechnology, University of Freiburg, Stefan-Meier-Strasse 19, 79104 Freiburg, Germany. Tel.: +49 761 203 8373; fax: +49 761 203 8383.

E-mail address: [irmgard.merfort@pharmazie.uni-freiburg.de](mailto:irmgard.merfort@pharmazie.uni-freiburg.de) (I. Merfort).

When wounded or scratched, cell monolayers respond to the disruption of cell–cell contacts by increasing the concentration of growth factors and cytokines at the wound edge, thus initiating proliferation and migration of different cell types, such as keratinocytes and fibroblasts. When performing a scratch assay, an artificial gap, a so-called “scratch” is typically created in a cell monolayer with a sharp object such as a pipette tip or syringe needle. The assay is carried out on individual coverslips or in a multi-well plate. The monolayers recover and wound healing occurs in a process that can be observed over time. The wound heals in a patterned fashion – cells polarize toward the wound, initiate protrusion, migrate, and close the wound. Progression of these events can be monitored by manually imaging samples fixed at different time points, or by time-lapse microscopy (Liang et al., 2007).

The present study aims to optimize the scratch assay for quantitative determination of fibroblast migration to and proliferation into the wounded monolayer, and to evaluate its suitability for testing crude extracts of medicinal plants, isolated compounds and pharmaceutical preparations. Swiss 3T3 fibroblasts were used, and platelet derived growth factor (PDGF) served as positive control. For a rapid and reproducible evaluation, manual counting was replaced by a computational method. This improved scratch assay was applied to three herbal preparations used externally in traditional medicine: hexane and ethanolic extracts of *Calendula officinalis* and *Matricaria recutita* as well as a commercially available oil from *Hypericum perforatum*. Preparations of *Calendula*, also known as marigold, are used as a wound healing remedy, but with a largely unknown mode of action (Brown and Dattner, 1998; Basch et al., 2006; Leach, 2008); extracts of *Matricaria*, better known as chamomile, are used for their anti-inflammatory effects (Brown and Dattner, 1998; Reuter et al., 2009), and *Hypericum* oil is used as a traditional wound healing remedy which is supposed to reduce scar formation (Wichtl and Loew, 2008).

## 2. Materials and methods

### 2.1. Cell lines, chemicals and biochemicals

Swiss 3T3 albino mouse fibroblasts (Cell Line Service, Appenheim, Germany) was kindly supplied by Dr. J. Orth (Institute of Experimental Pharmacology and Toxicology, University of Freiburg, Germany) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin, at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> (all Gibco-BRL, Netherlands). Collagen solution, type I from rat tail was purchased from Sigma–Aldrich Chemie GmbH, Germany. Mitomycin C was from Roth, Germany. Prolong Gold antifade reagent with 4',6-diamino-2-phenylindole (DAPI) and platelet derived growth factor-BB (PDGF-BB) were from Invitrogen, Germany.

### 2.2. Plant material and isolated compounds

Flowers from *Calendula officinalis* and *Matricaria recutita* were provided by Heinrich GmbH & Co., Schwebheim, Germany. *Hypericum perforatum* oil was purchased from a local pharmacy shop, Germany. Faradiol myristate acid ester and faradiol palmitate acid ester were isolated from *Calendula* flowers (Hamburger et al., 2003). Stock solutions were prepared in dimethyl sulfoxide (DMSO).

### 2.3. Preparation of extracts

Flowers of the respective plants were powdered. 10 g were extracted in Soxhlet apparatus using first hexane and subsequently ethanol (150 ml each). The solvent was removed by a vacuum evaporator at 40 °C and lyophilized. The following amounts were

obtained: *Calendula*: 1.29 g hexane and 1.89 g ethanolic extract; *Matricaria recutita*: 0.65 g hexane and 1.12 g ethanolic extract. The extracts were stored in sealed vials at –20 °C before used for further analysis.

### 2.4. Scratch assay

The spreading and migration capabilities of Swiss 3T3 fibroblasts were assessed using a scratch wound assay which measures the expansion of a cell population on surfaces. The cells were seeded into 24-well tissue culture dishes containing coverslips pre-coated with collagen type I (40 µg/ml) for 2 h at 37 °C, at a concentration of  $3 \times 10^5$  cells/ml and cultured in medium containing 10% FBS to nearly confluent cell monolayers. Then, a linear wound was generated in the monolayer with a sterile 100 µl plastic pipette tip. Any cellular debris was removed by washing the coverslips with phosphate buffer saline (PBS). DMEM medium with dimethyl sulfoxide (0.25%) (control group), platelet derived growth factor (2 ng/ml) (as positive control), the crude extracts (1 and 10 µg/ml), the commercial *Hypericum perforatum* oil (0.5 µg/ml) and the isolated compounds (10 and 50 µg/ml) was added to a set of 3 coverslips per dose and incubated for 12 h at 37 °C with 5% CO<sub>2</sub>. The cells were fixed with 4% paraformaldehyde for 15 min and stained with 4',6-diamino-2-phenylindole (DAPI) overnight. Three representative images from each coverslip of the scratched areas under each condition were photographed to estimate the relative migration cells. The data were analysed using CellC software (Selinummi et al., 2005). The experiments were performed at least in duplicate.

### 2.5. Statistical analysis

Statistical evaluation was carried out with the Origin Scientific Graphing and Analysis Software, version 7.0 or with Microsoft Office Excel 2007. Data are expressed as the mean ± S.E.M. Significant differences between the treated groups and the control were determined by the Student's *t*-test, at a level of  $P < 0.05$ .

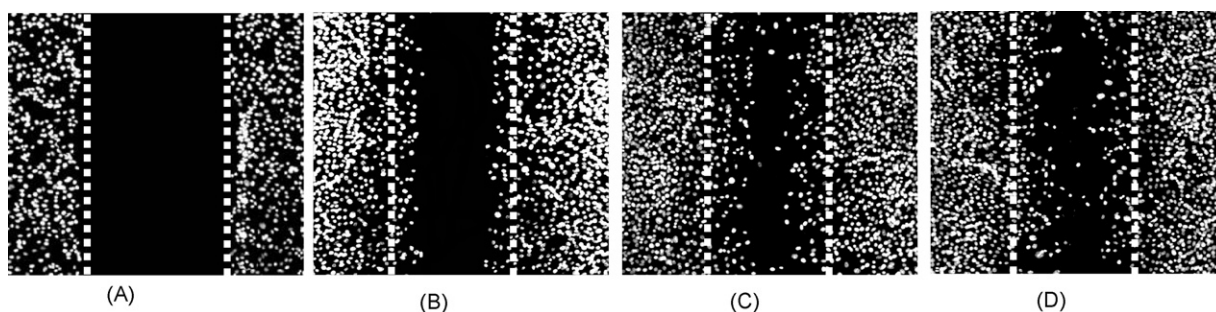
## 3. Results and discussion

### 3.1. Improvement of the scratch assay

To estimate the wound re-epithelialization potential of an extract or compound and to have a quality control for the assay, a standard is required. A number of growth factors and cytokines have been reported to affect fibroblast motility directly or indirectly. Since the role of PDGF in wound healing is best characterized, PDGF was taken as positive control. This growth factor stimulates production of matrix proteins in fibroblasts, including fibronectin, collagen, and hyaluronic acid as well as synthesis of collagenases. *In vivo*, direct application of PDGF induces increased formation of granulation tissue in skin wounds and wound breaking strength, thereby improving the rate of healing (Hosang et al., 1989; Kiritsy et al., 1993; Li et al., 2004).

To find the best concentration for the positive control, fibroblasts were stimulated with concentrations of PDGF in the range of 0.5–15 ng/ml. Quantification was by staining of cell nuclei with 4',6-diamino-2-phenylindole (DAPI), photographs were taken and analysed using CellC software (Selinummi et al., 2005). Low concentrations of 0.5, 1 and 2 ng/ml resulted in a good correlation between dose and an increased number of fibroblasts in the denuded area compared to the control (Fig. 1 and Fig. S1). In contrast, higher concentrations of 4 and 15 ng/ml induced lesser migration. Therefore, 2 ng/ml of PDGF was selected as positive control.

To elucidate the optimal treatment time, cellular proliferation and migration of fibroblasts were studied after 8, 10, 12 and 14 h of incubation using 2 ng/ml of PDGF. As shown in Fig. S2, an incubation



**Fig. 1.** Fluorescent microscope image to evaluate wound healing *in vitro* in the scratch assay using a confluent monolayer of 3T3 fibroblasts. Cell migration into the wound was observed in response to an artificial injury. A single representative area is shown immediately after the wounding (A) as well as control (B), 2 ng/ml PDGF (C) and 10 µg/ml of hexane extract of *Calendula* (D) after 12 h incubation.

time of 14 h resulted in the highest number of migrated cells in the denuded area. However, density of the cells was too high for them to be counted and distinguished unambiguously due to clusters or aggregates. A 12-h incubation time allowed for a more accurate measurement.

DMSO was used as a solvent for extracts and isolated compounds. Studies with different concentrations of DMSO (0.2–1.5%) showed toxic effects at concentrations above 1%, whereas lower concentrations (<0.5%) DMSO exhibited negligible effects. Therefore, only DMSO concentrations smaller than 0.5% were used in further experiments.

### 3.2. Evaluation of the wound healing effect of preparations from traditional medicinal plants

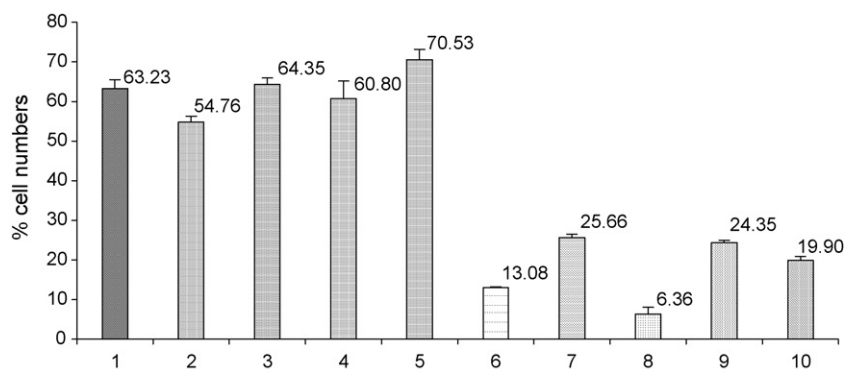
The effect of two different preparations, a hexane and an ethanolic extract, from *Calendula officinalis* flowers on fibroblasts migration and proliferation were investigated under the established conditions. As shown in Fig. 2, the hexane extract of *Calendula* increased cell numbers to 54.76% ± 1.59 and 64.35% ± 1.60 at a 1 and 10 µg/ml concentration. The same concentrations of the ethanolic extract gave slightly higher cell numbers of 60.80% ± 4.36 and 70.53% ± 2.64, respectively. Comparing the results with the control group, a significant difference ( $P < 0.05$ ) was observed in all tested concentrations. The activity of the *Calendula* extracts in promoting healing was similar to that of PDGF at a concentration of 2 ng/ml. Both treatments restored the cells to a confluent or near-confluent state within 18 h, in contrast to the control cells (data not shown).

The hexane and ethanolic extracts prepared from *Matricaria recutita* flowers were investigated under the same conditions

(Fig. 2), resulting in a much lower migration and proliferation activity with values of 13.08% ± 0.18 and 25.66% ± 0.86 for 1 and 10 µg/ml of the hexane extract and 6.36% ± 1.67 and 24.35% ± 0.60 for the respective concentrations of the ethanolic extract. The changes were not statistically significant ( $P < 0.05$ ).

Analysis of a commercial pharmaceutical preparation of *Hypericum perforatum* oil was also carried out (Fig. 2). Only a concentration of 0.5 µg/ml was studied and gave a higher cell number by 19.90% ± 1.05 (not significant;  $P < 0.05$ ). Higher concentrations were cytotoxic.

Altogether, results obtained with these traditional medicinal plants show that the scratch assay is a convenient and inexpensive first tool to differentiate between extracts which are known for their wound healing activity, such as *Calendula* extracts, and extracts which have an anti-inflammatory activity, such as *Matricaria* extracts. The scratch assay covers the second phase of wound healing characterized by proliferation and migration of either keratinocytes or fibroblasts (Martin, 1997; Schafer and Werner, 2007; Gurtner et al., 2008). Consequently, *Hypericum* oil, which is used as a wound healing remedy to avoid scar formation failed to increase the cell number at therapeutically relevant doses in the scratch assay due to its antiproliferative activity. Although the scratch assay cannot substitute *in vivo* studies as a final proof for efficacy in wound healing, this study confirms its usefulness for gaining first insights into the potential of an extract or compound to repair injured dermis. Moreover, our results with *Calendula* extracts are in line with the assumption that *Calendula* preparations stimulate granulation and increase collagen metabolism at the wound site (Kloucek-Popova et al., 1982; Rao et al., 1991; Brown and Dattner, 1998). Fibroblasts, which are involved in granulation and collagen metabolism, are stimulated by *Calendula* extracts resulting in



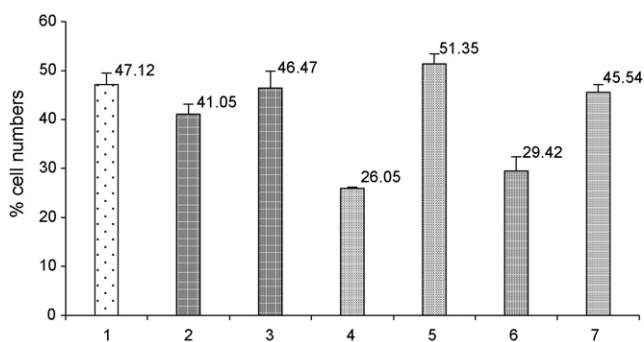
**Fig. 2.** Effect of preparations from *Calendula officinalis*, *Matricaria recutita* and *Hypericum perforatum* oil on the migratory and proliferative activities of fibroblasts in the scratch assay after 12 h of incubation (37 °C; 5% CO<sub>2</sub>) in DMEM medium supplemented with 10% FBS: (1) PDGF (2 ng/ml); (2 and 3) hexane extract of *Calendula officinalis* (1 and 10 µg/ml); (4 and 5) ethanolic extract of *Calendula officinalis*; (6 and 7) hexane extract of *Matricaria recutita* (1 and 10 µg/ml); (8 and 9) ethanolic extract of *Matricaria recutita* (1 and 10 µg/ml); (10) *Hypericum perforatum* oil (0.5 µg/ml). Data are expressed as percent of cell numbers in the wounded area compared to the control. Bars represent the mean ± S.E.M. of three experiments.

proliferation and migration within the wound site (Gurtner et al., 2008).

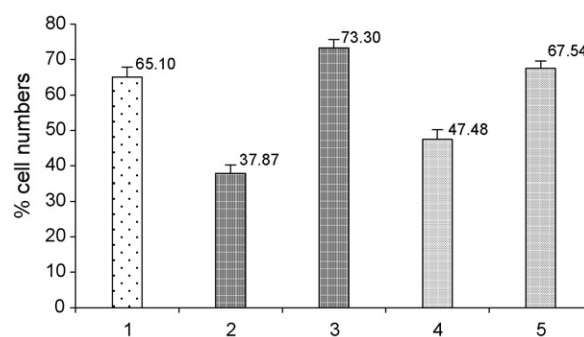
The hexane extract from *Calendula* flowers exhibited the most promising effects in the scratch assay, as a high activity at low concentrations was shown. Efficacy of *Calendula* extracts has been previously demonstrated in some *in vivo* studies (Kloucek-Popova et al., 1982; Rao et al., 1991), and also in a phase III trial in the prevention of acute dermatitis during irradiation for breast cancer (Pommier et al., 2004). However, despite some further trials summarized in (Leach, 2008) the use of *Calendula* containing herbal products is mainly based on folk medicine, and profound mechanistic studies are missing (Leach, 2008). This prompted us to extend our studies on wound healing effects using the hexane extract of *Calendula*.

The hexane extract of *Calendula* increases the population of fibroblasts in the scratched area due to the immigration of cells and/or by proliferation of the migrated cells. To differentiate between these two distinct processes, mitomycin C (5  $\mu\text{g/ml}$ ) was applied to the wounded monolayer cultures of fibroblasts together with either PDGF (2 ng/ml) or the hexane extract from *Calendula* (1 and 10  $\mu\text{g/ml}$ ). Addition of mitomycin C blocks mitosis and allows to discriminate between stimulation of migration and proliferation (Schreier et al., 1993). The cell numbers decreased to about 41.05% and 46.47%, respectively, when *Calendula* extracts were applied, and a similar decrease to 47.12% was also observed with PDGF (see Fig. 3, bars 1–3). Hence, the wound healing effect in the “scratch” might be due to proliferation as well as migration with a dominance of the latter effect. Interestingly, the result with PDGF does not agree with studies in literature where PDGF exclusively stimulated migration (Schreier et al., 1993). As the proliferation effect is not so pronounced, this discrepancy may be explained by differences in the experimental conditions.

The active components of *Calendula*'s anti-inflammatory activity are thought to be mainly triterpenoids, in particular faradiol monoesters (Della Loggia et al., 1994). Compounds responsible for the wound healing effects are still unknown (Matysik et al., 2005). Carotinoids have been discussed but they failed to affect the function of human fibroblasts (Schneider et al., 1991). To investigate whether the triterpene monoesters from *Calendula* do not only possess an anti-inflammatory activity but stimulate also fibroblast proliferation and migration, faradiol palmitate and faradiol myristate were studied under the same conditions. When tested at 10  $\mu\text{g/ml}$  (15.31  $\mu\text{M}$ ) and 50  $\mu\text{g/ml}$  (76.55  $\mu\text{M}$ ) faradiol myristate showed an increase in cell number of  $37.87\% \pm 2.32$  and  $73.30\% \pm 2.43$ , respectively. Similarly, faradiol palmitate increased



**Fig. 3.** Effect of PDGF, hexane extract of *Calendula officinalis* and triterpene esters on the migratory activities of fibroblasts in the scratch assay in the presence of 5  $\mu\text{g/ml}$  of antimetabolic mitomycin C after 12 h of incubation (37 °C; 5% CO<sub>2</sub>) in DMEM medium supplemented with 10% FBS: (1) PDGF (2 ng/ml); (2 and 3) hexane extract of *Calendula officinalis* (1 and 10  $\mu\text{g/ml}$ ); (4 and 5) faradiol myristate (10 and 50  $\mu\text{g/ml}$ ); (6 and 7) faradiol palmitate (10 and 50  $\mu\text{g/ml}$ ). Data are expressed as percent of cell numbers in the wounded area compared to the control. Bars represent the mean  $\pm$  S.E.M. of three experiments.



**Fig. 4.** Effect of the triterpene esters faradiol myristate and faradiol palmitate on the migratory and proliferative activities of fibroblasts in the scratch assay after 12 h of incubation (37 °C; 5% CO<sub>2</sub>) in DMEM medium supplemented with 10% FBS: (1) PDGF (2 ng/ml); (2 and 3) faradiol myristate (10 and 50  $\mu\text{g/ml}$ ); (4 and 5) faradiol palmitate (10 and 50  $\mu\text{g/ml}$ ). Data are expressed as percent of cell numbers in the wounded area compared to the control. Bars represent the mean  $\pm$  S.E.M. of three experiments.

the number of fibroblasts to  $47.48\% \pm 2.66$  and  $67.54\% \pm 2.07$  in the scratched area when tested at the same concentrations. The difference was significant when compared to the control ( $P < 0.05$ ) (see Fig. 4). Again, studies were carried out to determine to which extent proliferation contributed to the enhancement of fibroblasts in the denuded area after the scratch. Combined incubation of the antimetabolic mitomycin C (5  $\mu\text{g/ml}$ ) with faradiol myristate or faradiol palmitate (10 and 50  $\mu\text{g/ml}$ , respectively) revealed a similar decrease in cell numbers as previously seen for the hexane extract of *Calendula* (see Fig. 3). Taken together, these results show that triterpenes may positively influence the wound healing effect of *Calendula* extract by stimulating the proliferation and, to a higher extent, the migration of fibroblasts. The hexane extract showed higher activity in the scratch assay than the isolated triterpenes when both were tested at 10  $\mu\text{g/ml}$  (see Figs. 2 and 4). This indicates that the triterpenes contribute to the wound healing effect of *Calendula* extracts, however other yet unknown compounds may also exert a similar effect. The results reported here corroborate the potential of herbal extracts as topical medicines for wound healing.

#### 4. Conclusions

Our findings demonstrate that the scratch assay is a convenient and inexpensive method that gives robust and reproducible results for the proliferation as well as the migration of fibroblasts in an artificial wounded area. Thus, this method can be used to gain first insights in the wound healing potential of phytomedicines and their isolated compounds. This was successfully shown with *Calendula*, a traditional medicinal plant known for its wound healing properties. In addition, we report for the first time that anti-inflammatory triterpene esters also contribute to the proliferation and migration of fibroblasts. Both effects are prerequisites for wound granulation and re-epithelialization. However, further compounds likely contribute to the wound healing activity of *Calendula* flowers. Our results can be a starting point for further studies aiming at the elucidation of the molecular processes and signalling pathways underlying proliferation and migration of the fibroblasts induced by *Calendula* preparations.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jep.2009.09.014.

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