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PII: S0378-8741(15)30141-0
DOI: http://dx.doi.org/10.1016/j.jep.2015.09.015
Reference: JEP9738

To appear in: *Journal of Ethnopharmacology*

Received date: 11 June 2015
Revised date: 1 September 2015
Accepted date: 13 September 2015

Cite this article as: G. Brusotti, F. Andreola, G. Sferrazza, P. Grisoli, A. Merelli, F.S Robustelli della Cuna, E. Calleri, G. Nicotera, P. Pierimarchi and A. Serafino, *In vitro* evaluation of the wound healing activity of *Drypetes klaine* stem bark extracts, *Journal of Ethnopharmacology* http://dx.doi.org/10.1016/j.jep.2015.09.015

In vitro evaluation of the wound healing activity of Drypetes klainei stem bark extracts

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Abstract

Ethnopharmacological relevance: Drypetes klainei Pierre ex Pax is used in Cameroon by Baka Pygmies in the wound healing process and for the treatment of burns.

Aim of the study: To validate the traditional use of Drypetes klainei Pierre ex Pax stem bark extracts through the evaluation of their antimicrobial properties and their ability to improve wound healing process in fibroblast cell cultures.

Materials and methods: The antimicrobial properties of Drypetes klainey extracts were evaluated against Staphylococcus aureus ATCC 6538, Streptococcus pyogenes ATCC 19615, Escherichia coli ATCC 10536, Candida albicans ATCC 10231, on the basis of the minimum inhibitory concentration (MIC) and the minimum bactericidal-fungicidal concentration (MBC-MFC) by the macrodilution method. The extracts abilities to

accelerate wound healing were studied on murine and human fibroblasts in terms of cell viability and migration (scratch wound-healing assay).

Results: All the extracts were non-toxic against the selected microorganisms at the tested concentrations, and significantly improve wound healing process in vitro, compared to untreated controls. However, the defatted methanol extract was active at lower concentrations, compared to the water extract.

Conclusions: The ability of both water and defatted methanol extracts to accelerate scratch wound closure in fibroblast cultures may support the traditional use of Drypetes klainey stem bark in the treatment of skin lesions (such as burns) even if no antimicrobial activity was evidenced.

Keywords: Drypetes klainei Pierre ex Pax, bark extract, wound healing, cell proliferation

1. Introduction

The plants of the genus Drypetes (Putranjivaceae) are widely distributed worldwide. The genus comprises about 200 species, which have a long history of herbal usage in African traditional medicine, particularly in Kenya and Cameroon. Several studies have been reported concerning the chemical and pharmacological properties of some Drypetes species. An overview of the published works reveals that secondary metabolites such as phenols, flavonoids and terpenoids play important biological roles in Drypetes genus. In D. roxburghii, used in traditional medicine for cold, fever and rheumatism, flavonoids and phenolic compounds seem to be responsible for the claimed biological activity (Sundharshan et al., 2010). Terpenoids have been identified in D. tessmanniana, D. chevalieri and D. inaequalis as the bioactive compounds responsible

for antibacterial (Kuete et al., 2010), antileishmanial (Wansi et al., 2007) and antimicrobial (Awanchiri et al., 2009) activities, respectively. The anti-inflammatory and analgesic actions of the crude extract and friedelane derivatives from D. molunduana were thoroughly investigated by Wandji (Wandji et al., 2000) and Chungag-Anye (Chungag-Anye et al., 2001, 2000); antocyanins, flavonoids and tannins, found in extracts and fractions of D. gossweileri, support its fungicidal activity, as reported in 2011 by Ngouana et al.

Drypetes klainei is traditionally used by Baka Pygmies in Cameroon in the wound healing process and for the treatment of burns. The stem bark is directly applied on the skin injury as fine powder or orally consumed as water macerated. In spite of the broad data reported on the genus Drypetes, to our knowledge no data are available on D. klainei. Thus, following our research field on the validation of traditional uses of Baka pygmies medicinal plants (Ngueyem et al., 2008; Brusotti et al., 2011, Cesari et al., 2015, 2013), the aim of this work is to validate the traditional use of Drypetes klainei in the wound healing process and in the treatment of burns.

2. Experimental

*2.1.*Plant Material

The stem bark of Drypetes klainei was collected in Cameroon in July 2011 in the camps of Abing. The plant was identified at the National Herbarium of Yaoundé by the Cameroonian botanist Mr Victor Nana. A voucher specimen (no. BWPV 10) has been deposited at the Department of Drug Sciences of the University of Pavia. Bark was dried in the dark, in a ventilated room at 25-30 °C, then grounded and the powder stored at -20 °C.

2.2 Extraction procedure

According to the traditional use, the first extraction was carried out in water. The dried powder (10 g) was macerated in distilled water (100 ml) for 3 h and the crude extract obtained was frozen and lyophilized (water extract, WE, yield 2.4 % on a dry mass basis).

A second extraction was carried out on a new batch of stem bark. 50 g of dried powder were suspended in n-hexane (500 ml) in a round bottom flask equipped with a condenser. The mixture was sonicated for 15 min and then refluxed for 60 min, filtered, re-suspended in fresh n-hexane (500 ml) and refluxed for further 60 min. The procedure was repeated for 3 times, the fractions collected and the solvent removed under vacuum (yield 0.15 % on a dry mass basis). The same 50 g were successively extracted, following the procedure described above, with dichloromethane (3 × 500 ml), ethyl acetate (3 × 500 ml) and MeOH (3 × 500 ml); the solvent removal afforded 0.06 %, 0.15 % and 1.12 % of dried extracts on a dry mass basis, respectively. All dried extracts were stored at -20 °C until biological tests.

2.3. Microorganisms

The following strains were used for testing the antimicrobial activity of the crude extracts: Staphylococcus aureus ATCC 6538, Streptococcus mutans ATCC 25175, Streptococcus pyogenes ATCC 19615, Escherichia coli ATCC 10536, Candida albicans ATCC 10231. Bacteria were cultured in Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK) at 37 °C and in Todd Hewitt Broth (THB, Oxoid, Basingstoke, UK) at 37 °C in the presence of 5 % CO_2 for S. pyogenes. The bacteria cultures were

centrifuged at 3000 rpm for 20 min to separate cells from broth and then suspended in phosphate buffered saline (PBS, pH 7.3). The suspension was diluted to adjust the number of cells to 1×10^7–1×10^8 CFU/ml. Candida albicans was grown in Potato Dextrose Broth (PDB) (DIFCO, Detroit, MI, USA) for 24 h at 25 °C. The yeast culture was centrifuged at 3000 rpm for 20 min to separate cells from broth and then suspended in PBS (pH 7.3). The suspension was diluted to adjust the number of cells to 1×10^7–1×10^8 CFU/ml.

2.4. Evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal-fungicidal concentration (MBC-MFC).

All the extract were dissolved in 10 % dimethyl sulfoxide (DMSO) aqueous solution at a concentration of 40 mg/ml. These solutions were used in the determination of the antimicrobial activity against the reference strains.

MICs and MBCs-MFC were determined by twofold serial broth dilution method in Iso-Sensitest broth (ISB, Oxoid, Basingstoke, UK) according to Clinical and Laboratory Standards Institute (CLSI, 1999). The starting inoculum was 1 x 10^7 CFU/ml. Concentrations of plant extracts were tested in the range 20–4000 µg/ml. Solvent blanks were included.

The MIC was the lowest D. klainei extract solution concentration inhibiting observable microbial growth after 24 h incubation at 37 °C, (CLSI, 2009). The MBC-MFC was the lowest concentration resulting in > 99.9 % reduction of the initial inoculum after 24 h incubation at 37 °C. All experiments were performed in triplicate (CLSI, 1999). Stock standard solutions of ampicillin and of amphotericin B were used as a positive control.

2.5 Cell cultures and treatment

The mouse embryonic fibroblast cell line NIH 3T3 (3T3), obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), was grown in Dulbecco's Minimal Essential Medium (DMEM), supplemented with 10 % heat-inactivated Fetal Bovine Serum (FBS), L-glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 µg/ml). All media and supplements for 3T3 maintenance were obtained from Hyclone (Logan, UT, USA). The adult human dermal primary fibroblasts HDFa, obtained from Gibco Invitrogen (Carlsbad, CA, USA), were grown in Medium 106 supplemented with the Low Serum Growth Supplement (LSGS, Invitrogen). Cells were maintained at 37 °C, in a humidified atmosphere of 5 % CO_2, and passaged after being detached from culture flasks with 0.05% trypsin and 0.002% EDTA solution.

Exponentially growing cells were seeded at a density of $4 \times 10^4/cm^2$ and were maintained in culture for 24 h prior to treatments. Thereafter, cells were treated with increasing doses of water extract (WE) or defatted methanol extract (DME), ranging from 3 to 400 µg/ml, and maintained in culture for a period ranging from 24 h to 48 h. Controls consisted of cells cultured in basal medium (untreated controls).

2.6 Cell viability assay and cell cycle analysis

For the determination of extract cytotoxicity on murine fibroblasts, cells were seeded at a density of $4x10^4$ cells/cm^2 onto T25 culture flasks and allowed to adhere for 24 h. Thereafter, extracts were added to culture medium and cells were allowed to grow for additional 24 h. Cell viability was determined based on the Trypan blue dye exclusion method. Treated cultures were also analysed at 24 h by phase contrast microscopy to evaluate the presence of morphological changes indicative of cell toxicity (i.e.,

vacuolization, detachment from the substrate, presence of cellular debris), as compared to the untreated control.

The effect of the treatments on cell cycle was evaluated by cytofluorimetric analysis of DNA content after propidium iodide (PI) staining. Total DNA content was measured using the FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

2.7 In vitro scratch wound-healing assay

Murine 3T3 and human HDFa fibroblasts were seeded in 6-well plates (8×10^5 cells/well) and grown until reached a confluence of 90-95%, in the proper culture conditions described above. The scratch wound assay was performed as previously described (Rodriguez et al., 2005). Briefly, in the middle of cell monolayer, a scratch was made by a P10 pipette tip, to mimic a wound, and cell debris were removed by washing with fresh medium. The wound was exposed to increasing doses (3, 6, 12, 25 and 50 µg/ml) of WE or DME for 24-48 h at 37 °C in a humidified atmosphere of 5 % CO_2. Fibroblasts grown in extract-free medium were used as control. Scratch wound closure was analysed in two modalities: i) under the phase contrast microscope Motic AE31 (Motic, Milan, Italy) equipped with a digital CCD camera, by acquiring two digital images at time 0 (T0) and at time 24 h (T24) after wounding (static imaging): in order to identify the same scratched area at T0 and at T24, each well was marked under the cover-plate surface by drawing a circle around the area of interest; ii) by time-lapse imaging in bright field, carried out using the July Br Cell Movie Analyser (NanoEnTek, Seoul, Korea). The static imaging modality was used in preliminary dose-response experiments, to select the most effective dose/s to be used in the time-lapse experiments. In the static imaging modality, the closure of the scratch was quantified, as

recently described (Felice et al., 2015), by measuring the difference between the wound width at T0 and T24, using the ImageJ processing software [http://rsbweb.nih.gov/ij/]. Scratch Closure Rate (SCR) was calculated as described by Felice et al., 2015 using the following formula:

$$\mathrm{SCR} = \left[\frac{(\mathrm{At0} - \mathrm{At24})}{\mathrm{At0}}\right] \mathrm{x100}$$

where At0 = scratch area at time 0; At24 = scratch area at time 24 h. Results were reported as mean of three independent experiments ± SD.

The doses of WE and DME, selected from the preliminary dose-response experiments, were then tested for their ability of accelerating wound healing in vitro, by time-lapse imaging in bright field. By this modality we analysed the dynamic of scratch wound closure in treated and untreated murine 3T3 and human HDFa fibroblasts. Cells were monitored for 24 h (3T3) and 48 h (HDFa) of culture in absence and in presence of WE or DME, by acquiring 1 frame / 5 min. During the acquisition, the wound healing rate was automatically calculated by the July Br Cell Movie Analyser and recorded as percentage of confluence in function of time. From the quantitative data of the wound healing dynamic, we calculated the wound healing index (WH) using the formula:

$$\mathrm{WH\ index} = \left(\frac{\mathrm{CoeffLT\ x\ Final\ \%\ Confluence}}{\mathrm{Time\ delay}}\right)$$

where CoeffLT = slope coefficient of linear trend-line equation (directly correlated to the speed of wound closure); Time delay = time at which the first migratory movement is recorded by the instrument (inversely correlated to promptness of the effect). The linear trend-line equation for each curve was obtained directly by the Excell software.

On frames at T0 and T24 from the time-lapse acquisitions, the SCRs were also obtained as described above.

2.8 Phytochemical screening

A phytochemical screening was performed on all active extracts by Thin Layer Chromatography (TLC, Merck Kieselgel 60 F_{254} and RP-18 F_{254} S), according to the procedure described in the TLC atlas Plant Drug Analysis (Wagner et al., 1984) and by using appropriate tests (Chaudhari et al., 2006).

2.9 HPLC analysis

HPLC was used for the metabolite profiling of the extracts. Chromatographic experiments were performed with an Agilent 1100 liquid instrument (Palo Alto, CA, USA) equipped with an Agilent 1100 variable-wavelength detector. The system was connected to a HPLC ChemStation. (Revision A.04.01). Reverse phase chromatography analyses were carried out with a Agilent Technologies LiChrospher 100 RP-18 column (5 µm) 250-4, using a step gradient system of 0.04 % TFA in H_2O (solvent A) and MeOH (solvent B), as follow: 5 % to 30 % of B in 12 min, 30 % to 41 % of B in 25 min, 41 % to 60 % of B in 40 min, 60 % to 100 % of B in 10 min; flow rate: 0.7 ml/min. The UV absorbance was measured at 225 nm.

2.10. Statistical analysis

Statistical analysis were conducted using the two-tailed Student's t test and a P value threshold of ≤ 0.05. All data were presented as mean ± SD.

3. Results and discussion

D. klainei is traditionally used in Cameroon in the wound healing process and for the treatment of burns (Brisson, 1999). The stem bark is directly applied on the skin injury as fine powder or orally assumed as water macerated.

In order to validate the traditional use, the stem bark extracts have been investigated for:

i) their potential antimicrobial activity against pathogenic microorganisms involved in soft tissue infections such as Staphylococcus aureus ATCC 6538, Streptococcus pyogenes ATCC 19615, Escherichia coli ATCC 10536, Candida albicans ATCC 10231;

ii) their ability to accelerate wound healing process in murine and human fibroblasts, in terms of cell viability and migration (scratch wound assay).

The first extraction of D. klainei stem bark was carried out in water (water extract, WE) to reproduce the Pygmies Baka traditional preparation. The defatting procedure applied to the second batch of stem bark removes lipidic compounds, such as chlorophyll and other waxy substances, which may hinder the extraction of bioactive polar components and reduce the extracts solubility in bioassay water medium. Moreover, as previously reported (Ngueyem et al., 2008; Brusotti et al., 2012), methanol allows to obtain extracts with composition similar to WE but with higher amount of secondary metabolites, often associated with higher biological activity.

Although none of the extracts showed activity against the selected micro-organisms (data not shown), the WE and the defatted methanol extract (DME) were tested and compared for their ability to promote scratch wound closure in fibroblast cultures. Wound healing is a complex and dynamic process in which proliferation and migration

of fibroblasts play an essential role in orchestrating wound repair (Darby et al., 2014). For this reason, fibroblast cultures were used as in vitro model for our study.

To determine the highest non-toxic concentrations of both extracts, we firstly assessed the effect of WE – that reproduces the Baka Pygmies traditional preparation - on cell viability and growth of murine fibroblasts. To this purpose, 3T3 cells were treated for 24 h with 12, 25, 50, 100, 200, 400 µg/ml of water extract and analysed for cell morphology and viability (Fig. 1). No significant cytotoxic effects were recorded at concentration lower than 50 µg/ml (Fig. 1b), as also confirmed by the absence, below this concentration, of morphological changes indicative of cellular toxicity, such as vacuolization or loss of cell adhesion from the substrate, that were conversely observed at concentration equal or higher than 50 µg/ml (arrows in Fig. 1 a). Moreover, 25 µg/ml of WE had the highest effect on cell growth between T0 and 24 h (Fig. 1 c).

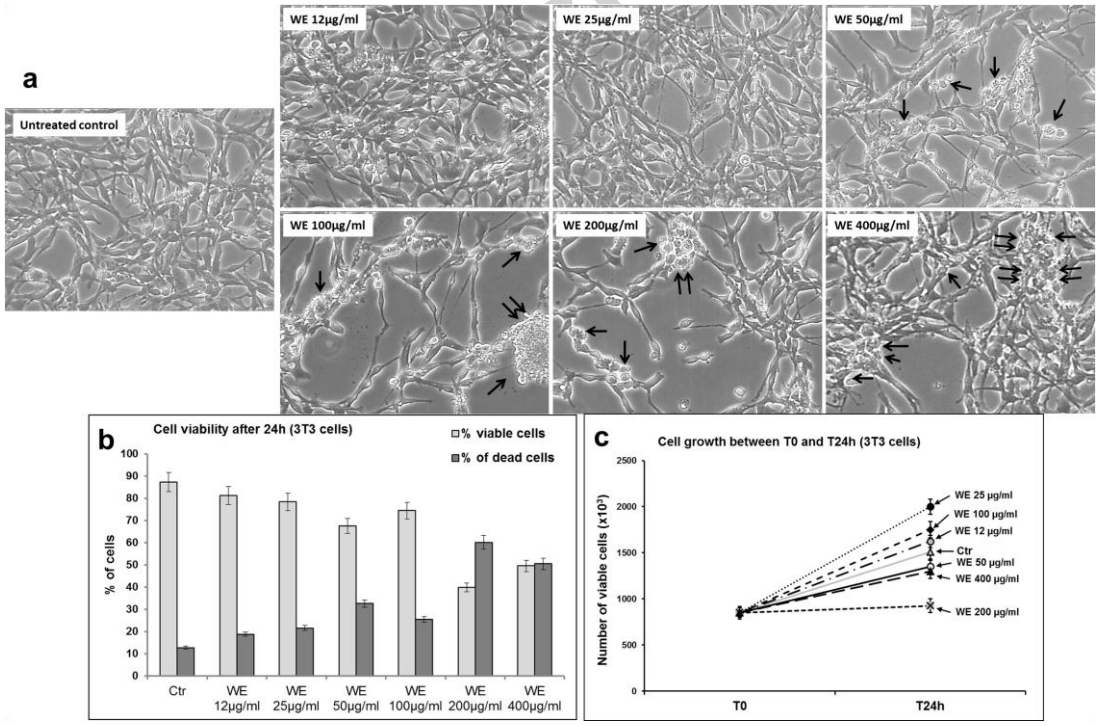


Fig. 1. Effect of water extract (WE) from D. klainei stem bark on viability and growth of murine fibroblasts. a) Phase contrast microscopy of 3T3 cells treated for 24 h with 12, 25, 50, 100, 200, 400

µg/ml of WE, compared with the untreated control; arrows point to clusters of cells showing morphological changes indicative of cellular toxicity. Original Magnification: 20 x. b, c) Cell viability (b) and growth (c), evaluated by Trypan blue dye exclusion method, in untreated controls and cells treated for 24 h with 12, 25, 50, 100, 200, 400 µg/ml of WE.

The comparative analysis of the effect of increasing concentrations (3, 6, 12, 25, 50, 100 µg/ml) of WE and DME on 3T3 cell viability and growth (Fig. 2), revealed that DME not only positively affected cell viability and growth at lower concentrations (from 3 µg/ml), compared to WE (from 12 µg/ml), but also exhibited a marked dose-dependent effect up to 12 µg/ml, that resulted the highest non-toxic concentration for DME. This suggests that in DME the component/s responsible for the biological activity of the traditional preparation could be present in higher amount than in WE.

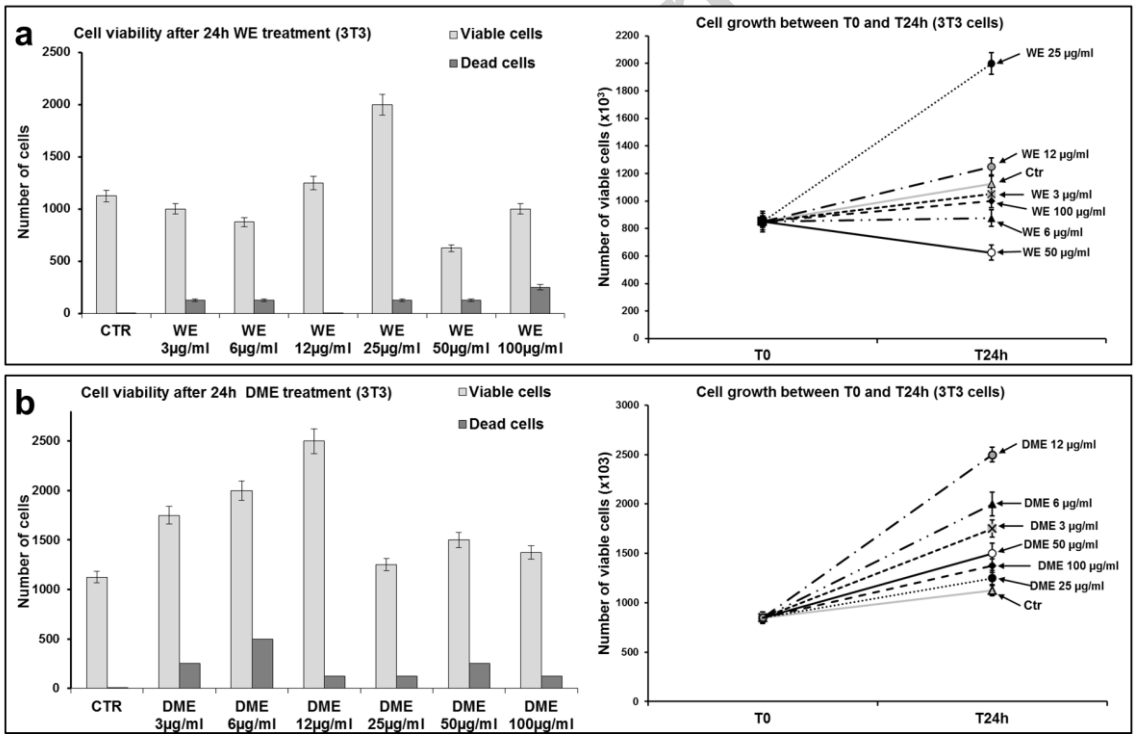
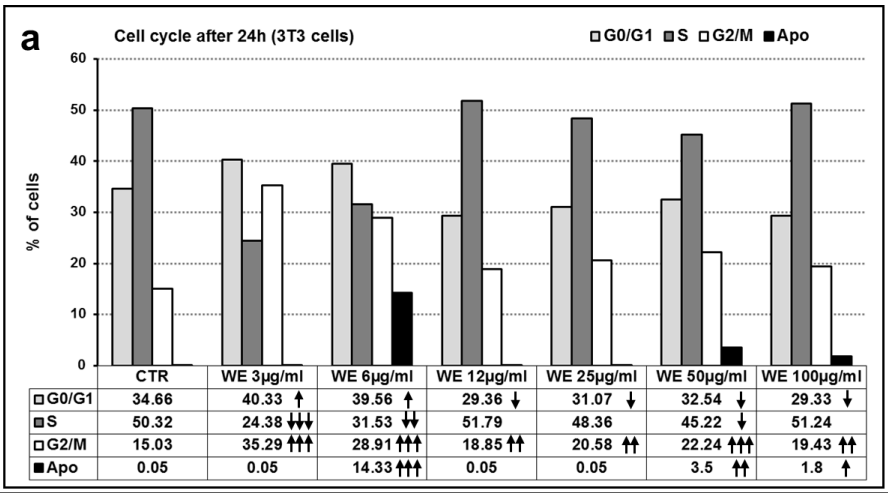


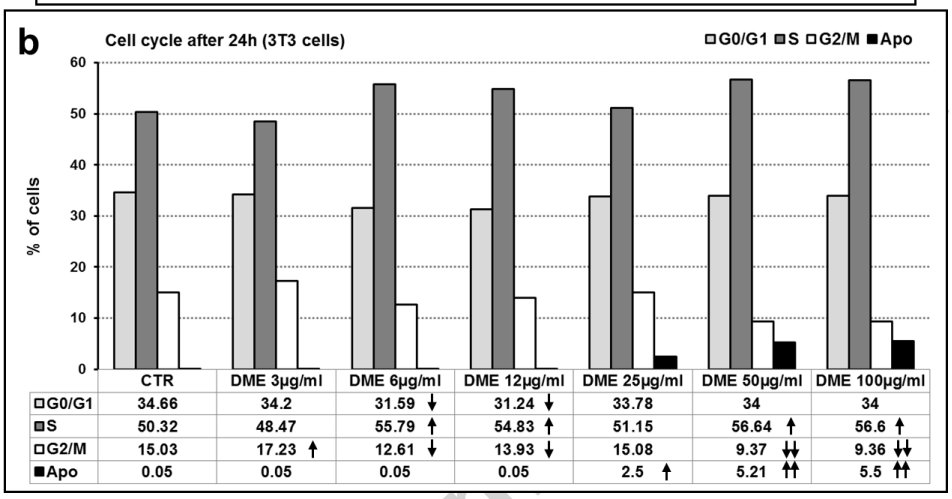
Fig. 2. Comparative analysis of the effect of increasing concentrations of WE and DME from D. klainei stem bark on 3T3 cell viability and growth. Cell viability (left panels) and growth (right

panels), evaluated by Trypan blue dye exclusion method, in untreated controls and cells treated for 24 h with 3, 6, 12, 25, 50, 100 µg/ml of WE (a) and DME (b).

Cytofluorimetric analysis of cell cycle performed after 24 h of treatment, confirmed the toxicity of WE and DME used at concentrations equal or higher than 50 µg/ml and 25 µg/ml, respectively - as demonstrated by the presence of apoptotic cells at these concentrations - and the higher efficacy and lower toxicity of DME compared to WE (Fig. 3). In detail, the concentrations of 12 and 25 µg/ml, for WE, and 3, 6, 12 µg/ml, for DME, induced an increment of the percentage of cells in S phase - the synthesis phase, when DNA replication occurs - and/or in G2/M phase - the premitotic/post-synthetic (G2) and mitosis (M) phase, when cell division occurs. This results suggested an increase in DNA synthesis and mitotic activity in 3T3 cells during cell migration before wound closure, and were consistent with the higher proliferation ability recorded at the same doses by the viability assay (Fig. 2).



	CTR	WE 3µg/ml	WE 6µg/ml	WE 12µg/ml	WE 25µg/ml	WE 50µg/ml	WE 100µg/ml
G0/G1	34.66	40.33 ↑	39.56 ↑	29.36 ↓	31.07 ↓	32.54 ↓	29.33 ↓
S	50.32	24.38 ↓↓↓	31.53 ↓↓	51.79	48.36	45.22 ↓	51.24
G2/M	15.03	35.29 ↑↑↑	28.91 ↑↑↑	18.85 ↑↑	20.58 ↑↑	22.24 ↑↑↑	19.43 ↑↑
Apo	0.05	0.05	14.33 ↑↑↑	0.05	0.05	3.5 ↑↑	1.8 ↑



	CTR	DME 3µg/ml	DME 6µg/ml	DME 12µg/ml	DME 25µg/ml	DME 50µg/ml	DME 100µg/ml
G0/G1	34.66	34.2	31.59 ↓	31.24 ↓	33.78	34	34
S	50.32	48.47	55.79 ↑	54.83 ↑	51.15	56.64 ↑	56.6 ↑
G2/M	15.03	17.23 ↑	12.61 ↓	13.93 ↓	15.08	9.37 ↓↓	9.36 ↓↓
Apo	0.05	0.05	0.05	0.05	2.5 ↑	5.21 ↑↑	5.5 ↑↑

Fig. 3. Comparative analysis of the effect of increasing concentrations of WE and DME from D. klainei stem bark on cell cycle of murine fibroblasts. Cytofluorimetric analysis was performed on 3T3 cells after 24 h of treatment with 3, 6, 12, 25, 50, 100 µg/ml of WE (a) and DME (b), and in the untreated controls. Arrows indicate percent of modifications vs the untreated control ≥ 5 % (lightly modified ↑), ≥ 20 % (modified ↑↑) or ≥ 40 % (highly modified ↑↑↑)

Conversely, the lower doses tested for WE (3 and 6 µg/ml) induced a block of cells in G2/M phase and a decrement of the S phase, with the presence of a high percentage of apoptotic cells recorded for 6 µg/ml (Fig. 3 a), that could justify the lack of effect on cell proliferation and the lower viability recorded at these concentrations by the viability assay (Fig. 2 a). Even if we have no experimental data for explain this "paradoxical" effect (increased toxicity at lower doses), we can speculate that it could be ascribable to

some other component/s – different from the component/s responsible for wounding activity - present in the whole WE extract that, when diluted, negatively affect cell survival. In any case, this effect was not evidenced under DME treatment at the tested concentrations (Fig. 2 b).

To conclusively validate the traditional use of stem bark extracts, the ability of WE and DME in accelerating wound healing was studied on murine and human fibroblasts by in vitro scratch wound-healing assay. In preliminary dose-response experiments, scratch wound closure was analysed by static imaging, by acquiring two digital images at time 0 (T0) and at time 24 h (T24) after wounding, as described in the method section (Fig. 4). The comparative analysis of the effect of increasing concentrations (3, 6, 12, 25 µg/ml) of WE and DME on the murine cell line 3T3, showed that, while for WE treatment the wound healing process was significantly ($P = 0.023$) accelerated only by the 25 µg/ml, all the concentrations used for DME resulted significantly active on the healing process ($P \leq 0.05$), compared to the control (Fig. 4). Time-lapse imaging of scratch wound closure during the 24/48 h of exposure to 12 and 25 µg/ml of WE or DME (Supplementary Videos 1 - 6) confirmed the in vitro efficacy of both extracts in accelerating wound healing process in murine fibroblast cell line 3T3 (Fig. 5 and Supplementary Fig. S1 a) as well as in human primary fibroblasts HDFa (Fig. 6, Supplementary Fig. S1 b and Supplementary Fig. S2). In particular, in 3T3 cells, WE 25 µg/ml and DME 12 µg/ml incremented the wound healing process of 1.66-fold and 1.35-fold, respectively, vs the untreated control (WH index = 6.47 and 5.26, respectively, vs WH index 3.9 for control; Fig. 5 b).

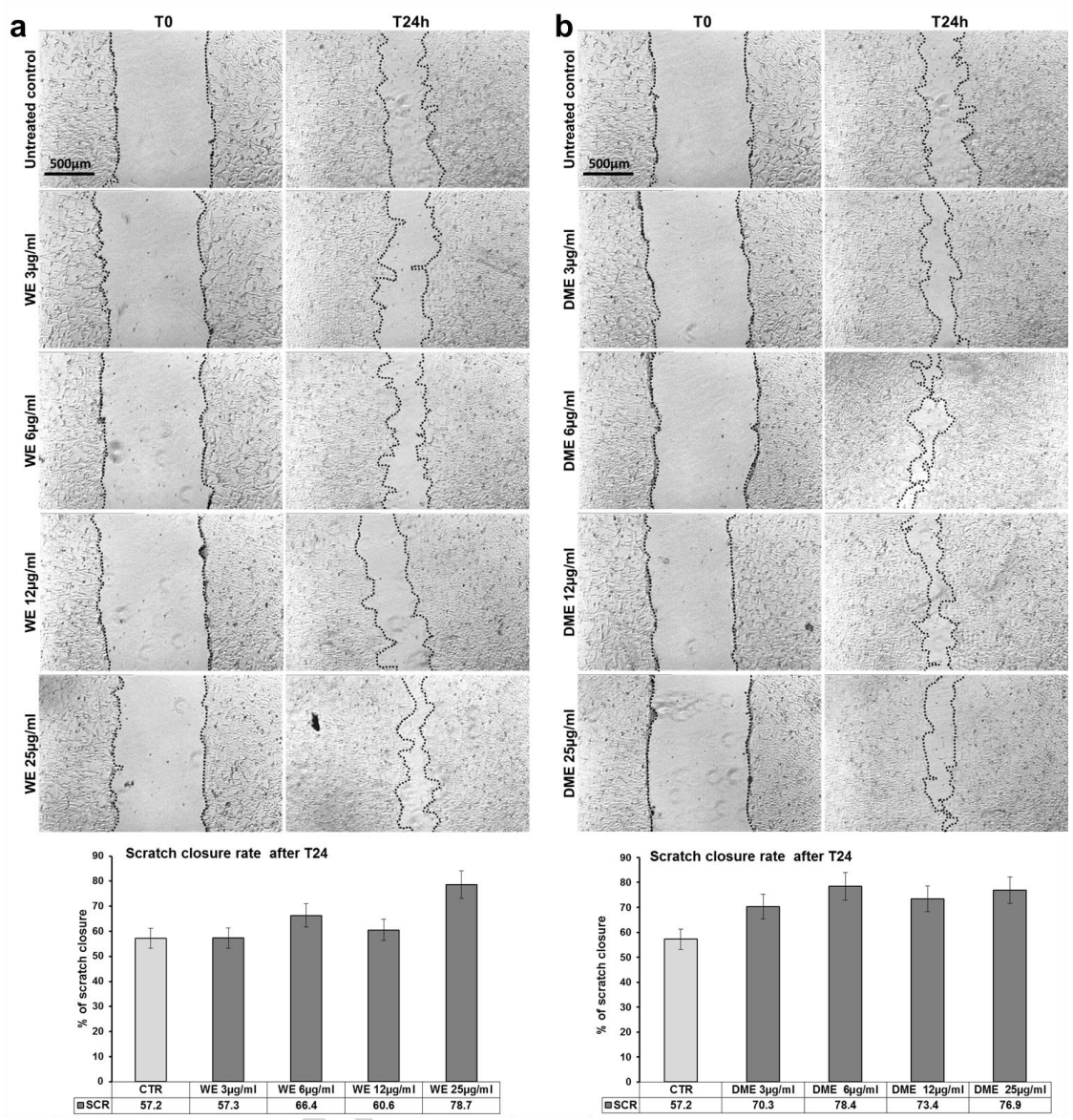
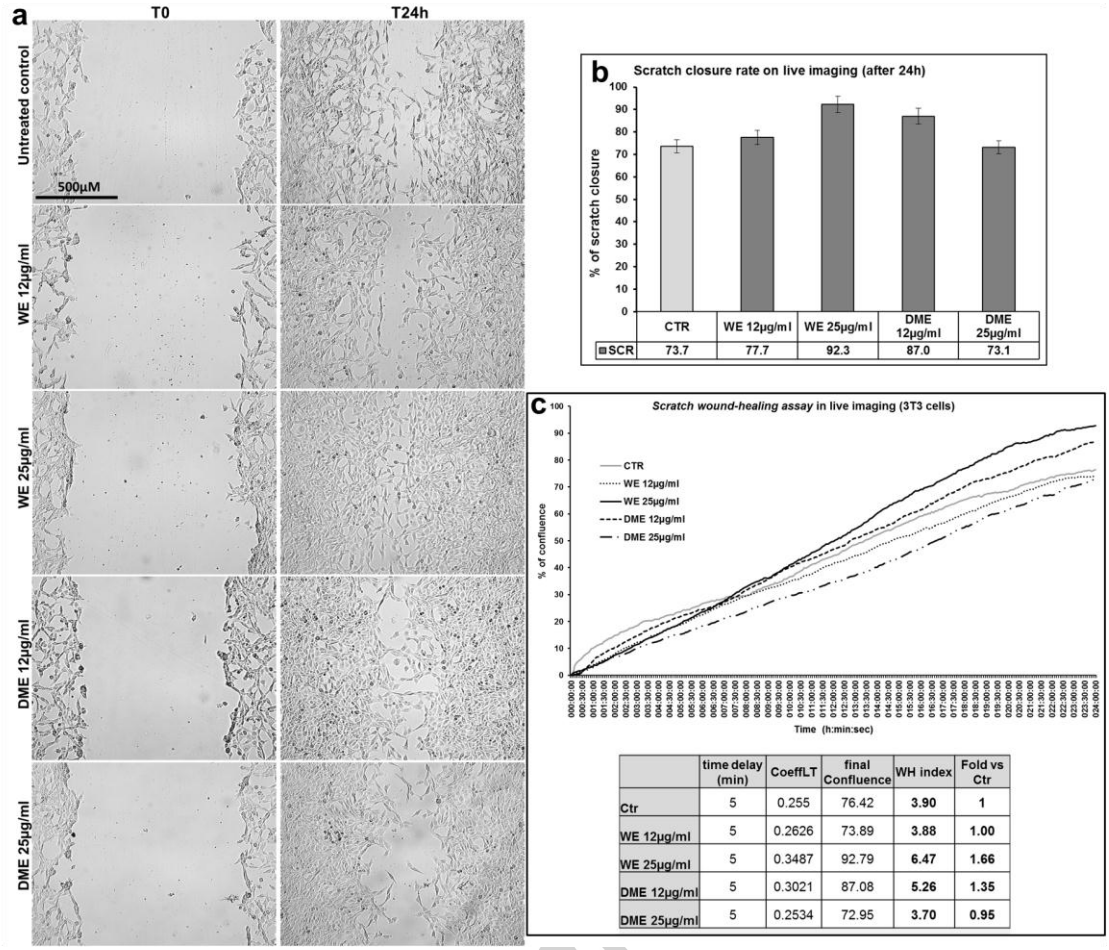


Fig. 4. Effect of increasing concentrations of WE and DME from D. klainei stem bark on scratch wound closure (static imaging) in 3T3 cell monolayer. Phase contrast microscopy (top panels) of 3T3 cells before (T0) and after 24 h (T24) of treatment with 3, 6, 12, 25 µg/ml of WE (a) and DME (b), compared to the untreated control. The bar graphs of scratch closure rates (SCR) at T24, calculated as described in the method section, were also reported in the bottom panels.

Significance SCR vs control (P value threshold of ≤ 0.05):

a) WE: 3 µg/ml: P = 0.96; 6 µg/ml: P = 0.091; 12 µg/ml: P = 0.353; 25 µg/ml: P = 0.023

b) DME: 3 µg/ml: P = 0.05; 6 µg/ml: P = 0.023; 12 µg/ml: P= 0.036; 25 µg/ml: P = 0.026



	time delay (min)	CoeffLT	final Confluence	WH index	Fold vs Ctr
Ctr	5	0.255	76.42	**3.90**	**1**
WE 12µg/ml	5	0.2626	73.89	**3.88**	**1.00**
WE 25µg/ml	5	0.3487	92.79	**6.47**	**1.66**
DME 12µg/ml	5	0.3021	87.08	**5.26**	**1.35**
DME 25µg/ml	5	0.2534	72.95	**3.70**	**0.95**

Fig. 5. Comparative analysis in time-lapse imaging of wound healing ability of WE and DME on murine fibroblast cell line 3T3. a) Time-lapse imaging in bright field on living 3T3. Cells were monitored for 24 h of culture in absence (left panels) and in presence (right panels) of 12 and 25 µg/ml of WE or DME, by acquiring 1 frame / 5 min. During the acquisition, the wound healing rate was automatically recorded as percentage of confluence in function of time (c). From the quantitative data of the wound healing dynamic, we calculated the wound healing index (WH) as describe in the method section (table in panel c). CoeffLT = slope coefficient of linear trend-line equation (directly correlated to the speed of wound closure); Time delay = time at which the first migratory movement is recorded by the instrument (inversely correlated to promptness of the effect). On frames at T0 and T24 from the time-lapse acquisitions, the SCRs were also obtained (b).

Significance SCR vs control (P value threshold of ≤ 0.05):

WE: 12 µg/ml: P = 0.13; 25 µg/ml: P = 0.009

DME: 12 µg/ml: P = 0.016; 25 µg/ml: P = 0.739

In HDFa cells, which being primary cultures have a growth rate lower than 3T3, the wound healing process was incremented by WE and DME of 5.89-fold and 1.77-fold, respectively, vs the untreated control (WH index = 0.075 and 0.023, respectively, vs WH index 0.013 for control) after 24 h (Fig. 6), and these increments were preserved after 48 h, when cells were near confluence in both treated and untreated cultures (Supplementary Fig. S1 b and S2).

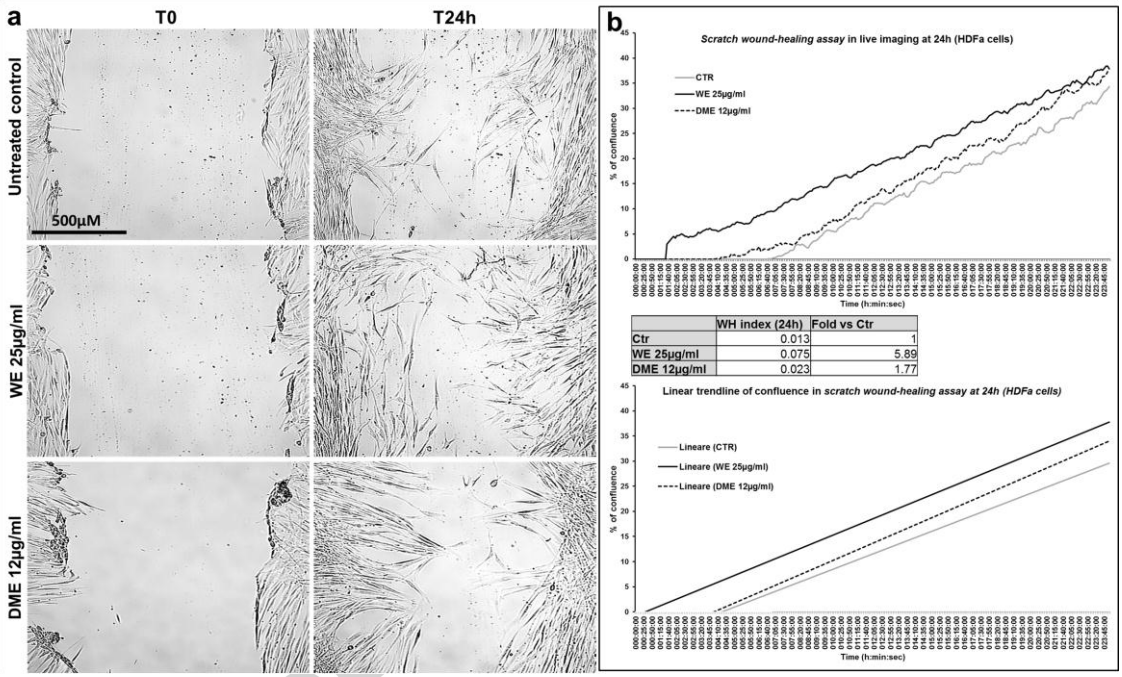


Fig. 6. Comparative analysis in time-lapse imaging of wound healing ability of WE and DME on primary human fibroblasts (HDFa). a) Time-lapse imaging in bright field on living HDFa during the first 24 h of culture. Cells were monitored for 48 h of culture in absence (left panels) and in presence (right panels) of 25 and 12 µg/ml of WE or DME, respectively, by acquiring 1 frame / 5 min. During the acquisition, the wound healing rate was automatically recorded as percentage of confluence in function of time (top panel in b). From the quantitative data of the wound healing dynamic, we calculated the wound healing index (WH) at 24 h (top panel and table in b) and 48 h (Supplementary Figure S2), as describe in the method section. The linear trend-line equation for each curve, from which the WH was calculated, was obtained directly by the Excell software (bottom panel in b).

A preliminary phytochemical screening on the active extracts highlighted the presence of polyphenols, flavonoids and triterpenoids, in accordance with the chemical composition reported for other related species (Ngouana et al., 2011; Ng'ang'a et al., 2011¸Wandji et al. 2003). The comparison between the HPLC fingerprint of the traditional preparation (WE) and the DME, allowed to identify some common zones in the chromatogram profiles (Fig. 7). As already observed in our previous works (Cesari et al., 2015, 2013), these findings suggest that the presence of most of the same secondary metabolites in WE and DME, but with differences in the biological activity observed, might be ascribed to the too low percentage of the active compound/s within the crude WE and/or to the presence of interfering compounds (Butler, 2004).

These preliminary results may support the traditional use of Drypetes klainey stem bark in the treatment of wound healing and burns.

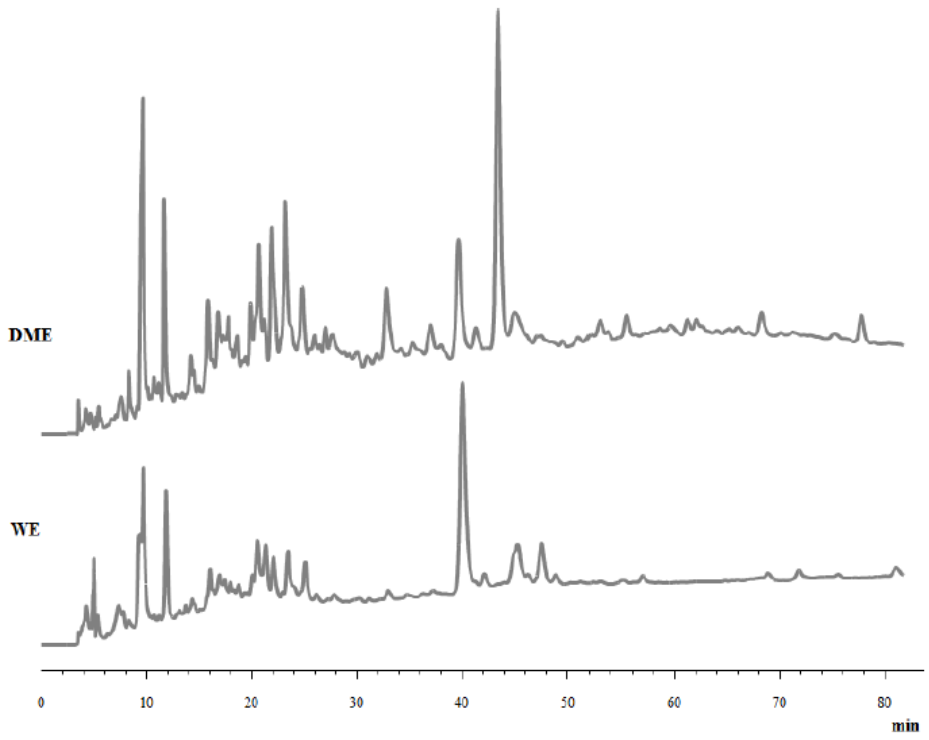


Fig. 7. Comparison between the HPLC fingerprint of D. klainei traditional preparation (WE) and the most active extract (DME)

Conclusion

Herbal preparations have been used for centuries as the main therapeutic tool. The increasing use of herbal plants led to extensive researches and great investments by multinational companies on herbal drugs and preparations. These products are regulated by European Parliament that established a special category of "traditional herbal medicinal products" in order to validate the safety and quality of the products by physico-chemical, biological and microbiological assays (Directive 2004/24/EC of the European Parliament and of the Council)

This is the first report on the biological properties of Drypetes kalinei stem bark. Even if both WE and DME are able to stimulate scratch wound closure in murine fibroblast cell line 3T3 as well as in human primary fibroblasts HDFa, the defatted methanol extract exhibited higher in vitro efficacy in accelerating wound healing process as compared with the water extract. A preliminary phytochemical screening, together with the acquisition of the chromatographic profiles, allowed to assume that DME and WE have a similar qualitative composition which may justify the traditional use in the treatment of wound healing and burns. Further studies are now focused on DME in order to identify the bioactive compounds responsible for the enhancement of the fibroblasts migration rate in vitro.

Acknowledgements

The authors are grateful to the "Cameroon National Herbarium of Yaoundé, Cameroon" for the technical support and to the Baka tribe where the samples were collected and who accepted to deliver their traditional knowledge. We also thank Arianna Colini Baldeschi (graduating student) for contributing to cell culture

maintenance. This work was partially supported by IFT-CNR (National Research Council of Italy).

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Figure captions

Fig. 1. Effect of water extract (WE) from D. klainei stem bark on viability and growth of murine fibroblasts. a) Phase contrast microscopy of 3T3 cells treated for 24 h with 12, 25, 50, 100, 200, 400 µg/ml of WE, compared with the untreated control; arrows point to clusters of cells showing morphological changes indicative of cellular toxicity. Original Magnification: 20 x. b, c) Cell viability (b) and growth (c), evaluated by Trypan blue dye exclusion method, in untreated controls and cells treated for 24 h with 12, 25, 50, 100, 200, 400 µg/ml of WE.

Fig. 2. Comparative analysis of the effect of increasing concentrations of WE and DME from D. klainei stem bark on 3T3 cell viability and growth. Cell viability (left panels) and growth (right panels), evaluated by Trypan blue dye exclusion method, in untreated controls and cells treated for 24 h with 3, 6, 12, 25, 50, 100 µg/ml of WE (a) and DME (b).

Fig. 3. Comparative analysis of the effect of increasing concentrations of WE and DME from D. klainei stem bark on cell cycle of murine fibroblasts. Cytofluorimetric analysis was performed on 3T3 cells after 24 h of treatment with 3, 6, 12, 25, 50, 100 µg/ml of WE (a) and DME (b), and in the untreated controls. Arrows indicate percent of modifications vs the untreated control ≥ 5 % (lightly modified ↑), ≥ 20 % (modified ↑↑) or ≥ 40 % (highly modified ↑↑↑).

Fig. 4. Effect of increasing concentrations of WE and DME from D. klainei stem bark on scratch wound closure (static imaging) in 3T3 cell monolayer. Phase contrast microscopy (top panels) of 3T3 cells before (T0) and after 24 h (T24) of treatment with 3, 6, 12, 25 µg/ml of WE (a) and DME (b), compared to the untreated control. The bar graphs of scratch closure rates (SCR) at T24, calculated as described in the method section, were also reported in the bottom panels.

Significance SCR vs control (P value threshold of ≤ 0.05):

a) WE: 3 µg/ml: P = 0.96; 6 µg/ml: P = 0.091; 12 µg/ml: P = 0.353; 25 µg/ml: P = 0.023

b) DME: 3 µg/ml: P = 0.05; 6 µg/ml: P = 0.023; 12 µg/ml: P = 0.036; 25 µg/ml: P = 0.026

Fig. 5. Comparative analysis in time-lapse imaging of wound healing ability of WE and DME on murine fibroblast cell line 3T3. a) Time-lapse imaging in bright field on living 3T3. Cells were monitored for 24 h of culture in absence (left panels) and in presence (right panels) of 12 and 25 µg/ml of WE or DME, by acquiring 1 frame / 5 min. During the acquisition, the wound healing rate was automatically recorded as percentage of confluence in function of time (c). From the quantitative data of the wound healing dynamic, we calculated the wound healing index (WH) as describe in the method section (table in panel c). CoeffLT = slope coefficient of linear trendline equation (directly correlated to the speed of wound closure); Time delay = time at which the first migratory movement is recorded by the instrument (inversely correlated to promptness of the effect). On frames at T0 and T24 from the time-lapse acquisitions, the SCRs were also obtained (b).

Significance SCR vs control (P value threshold of ≤ 0.05):

WE: 12 µg/ml: P = 0.13; 25 µg/ml: P = 0.009

DME: 12 µg/ml: P = 0.016; 25 µg/ml: P = 0.739

Fig. 6. Comparative analysis in time-lapse imaging of wound healing ability of WE and DME on primary human fibroblasts (HDFa). a) Time-lapse imaging in bright field on living HDFa during the first 24 h of culture. Cells were monitored for 48 h of culture in absence (left panels) and in presence (right panels) of 25 and 12 µg/ml of WE or DME, respectively, by acquiring 1 frame / 5 min. During the acquisition, the wound healing rate was automatically recorded as percentage of confluence in function of time (top panel in b). From the quantitative data of the wound healing dynamic, we calculated the wound healing index (WH) at 24 h (top panel and table in b) and 48 h (Supplementary Figure S2), as describe in the method section. The linear trendline equation for each curve, from which the WH was calculated, was obtained directly by the Excell software (bottom panel in b).

Fig.7. Comparison between the HPLC fingerprint of D. klainei traditional preparation (WE) and the most active extract (DME)

Supplementary Materials

Supplementary Fig. S1. Comparative analysis in time-lapse imaging of wound healing ability of WE and DME on murine fibroblast cell line 3T3 and on primary human fibroblasts HDFa. Linear trend-line graphs of the percentage of confluence in function of time.

Supplementary Fig. S2. Comparative analysis in time-lapse imaging of wound healing ability of WE and DME on primary human fibroblasts (HDFa), monitored for 48 h of culture in absence and in presence of the extracts.

Supplementary Video 1. Time-lapse imaging of wound healing in murine fibroblast cell line 3T3, monitored for 24 h of culture in absence of D. klainei stem bark (untreated control), by acquiring 1 frame / 5 min.

Supplementary Video 2. Time-lapse imaging of wound healing in murine fibroblast cell line 3T3, monitored for 24 h of culture in presence of 25 µg/ml of WE, by acquiring 1 frame / 5 min.

Supplementary Video 3. Time-lapse imaging of wound healing in murine fibroblast cell line 3T3, monitored for 24 h of culture in presence of 12 µg/ml of DME, by acquiring 1 frame / 5 min.

Supplementary Video 4. Time-lapse imaging of wound healing in primary human fibroblasts (HDFa), monitored for 48 h of culture in absence of D. klainei stem bark (untreated control).

Supplementary Video 5. Time-lapse imaging of wound healing in primary human fibroblasts (HDFa), monitored for 48 h of culture in presence of 25 µg/ml of WE, by acquiring 1 frame / 5 min.

Supplementary Video 6. Time-lapse imaging of wound healing in primary human fibroblasts (HDFa), monitored for 48 h of culture in presence of 12 µg/ml of DME, by acquiring 1 frame / 5 min.