

Anti-inflammatory and immune-regulatory mechanisms prevent contact hypersensitivity to *Arnica montana* L.

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Abstract: Sesquiterpene lactones (SL), secondary plant metabolites from flowerheads of *Arnica*, exert anti-inflammatory effects mainly by preventing nuclear factor (NF)- κ B activation because of alkylation of the p65 subunit. Despite its known immunosuppressive action, *Arnica* has been classified as a plant with strong potency to induce allergic contact dermatitis. Here we examined the dual role of SL as anti-inflammatory compounds and contact allergens *in vitro* and *in vivo*. We tested the anti-inflammatory and allergenic potential of SL in the mouse contact hypersensitivity model. We also used dendritic cells to study the activation of NF- κ B and the secretion of interleukin (IL)-12 in the presence of different doses of SL *in vitro*. *Arnica* tinctures and SL potently suppressed NF- κ B activation and IL-12 production in dendritic cells at high concentrations, but had immunostimulatory effects at

low concentrations. Contact hypersensitivity could not be induced in the mouse model, even when *Arnica* tinctures or SL were applied undiluted to inflamed skin. In contrast, *Arnica* tinctures suppressed contact hypersensitivity to the strong contact sensitizer trinitrochlorobenzene and activation of dendritic cells. However, contact hypersensitivity to *Arnica* tincture could be induced in acutely CD4-depleted MHC II knockout mice. These results suggest that induction of contact hypersensitivity by *Arnica* is prevented by its anti-inflammatory effect and immunosuppression as a result of immune regulation in immunocompetent mice.

Key words: *Arnica* – contact dermatitis – inflammation – NF- κ B – sesquiterpene lactone

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Introduction

Arnica montana, a traditional medicinal plant, is known for its anti-inflammatory effects. Extracts of flowerheads are used to treat inflammatory conditions such as bruises, sprains, gingivitis and rheumatic complaints. The secondary plant metabolites that mediate these anti-inflammatory effects are mainly sesquiterpene lactones (SL) of the pseudoguaianolide type such as helenalin, 11 α ,13-dihydrohelenalin and their ester derivatives (for structures see Fig. 1a). These terpenoids constitute about 0.6% of the dry mass of the flowerheads (1,2). Interestingly, flowerheads of *Arnica* growing in Spain mostly contain the chemically less reactive 11 α ,13-dihydrohelenalin derivatives, whereas the more reactive helenalin derivatives dominate in flowerheads from Central Europe (Fig. 1a) (2).

SL exert their anti-inflammatory activity mainly by alkylation of cysteine 38 in the p65 subunit of the transcription

factor nuclear factor (NF)- κ B, thereby preventing its activation (3,4). NF- κ B-regulated genes comprise genes for the pro-inflammatory cytokines interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α and the chemokines CXCL8 (IL-8) and CXCL10. Inflammation is pivotal both for the sensitization to contact allergens as well as for elicitation of the allergic response (5,6). The activation of the innate immune system in the skin triggers activation of keratinocytes and dendritic cells (DC). Epidermal Langerhans cells and dermal DC then migrate to the skin draining lymph nodes. The DC present hapten-modified major histocompatibility complex (MHC)-associated peptides thereby activating allergen-specific T cells (5,7) and, at the same time, induce skin-specific homing receptors enabling the T cells to migrate to the inflamed skin in the elicitation phase (8–10).

SL are considered as classical haptens because of their alkylation of amino acids by a Michael-type addition as a

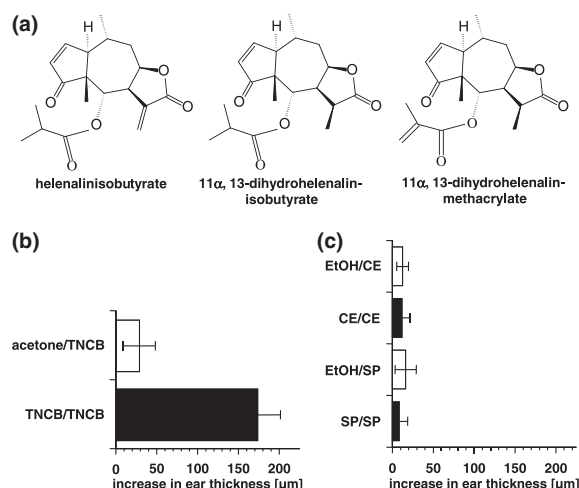


Figure 1. Sesquiterpene lactones fail to induce contact hypersensitivity. (a) Structures of the investigated sesquiterpene lactones. (b, c) Ear swelling reactions to 2,4,6-trinitrochlorobenzene (TNCB) and *Arnica* preparations. C57BL/6 mice were sensitized either with acetone or 7% TNCB/acetone as a positive control (b), or with ethanol (EtOH) or undiluted Central European (CE) or Spanish (SP) *Arnica* tincture (c), respectively, on day 0. Ear challenge was performed with 1% TNCB or undiluted tinctures on day 5. Results are expressed as the ear swelling at 24 h postchallenge from groups of three mice $\pm$ SD.

result of their α,β -unsaturated carbonyl structural elements (11). Protein modification by SL could result in the generation of immunogenic hapten determinants for recognition by T cells as shown for the contact sensitizer 2,4,6-trinitrochlorobenzene (TNCB) (1,5,6) explaining their potential to induce allergic contact dermatitis (ACD) (12). Previous studies using the guinea pig skin erythema test classified *Arnica* as a plant with strong contact sensitizing potency (12). Because of the many limitations of this test (13), the well known anti-inflammatory effects and the low clinical incidence of ACD to *Arnica* as supported by clinical studies (6,14,15), we have reinvestigated the skin sensitizing potency in the mouse, which is now the standard animal model for assessment of this parameter (13,16). Recent data comparing chemokines involved in human and murine irritant and ACD have, again, validated the use of the mouse contact hypersensitivity (CHS) model for the study of ACD (17). We have studied the discrepancy between anti-inflammatory and pro-allergic effects of alcoholic tinctures prepared from *A. montana* of Spanish (SP) or Central European (CE) origin and of pure SL of the helenalin or 11 α ,13-dihydrohelenalin type in the mouse CHS model.

We found that *Arnica* tinctures and SL are only weak contact sensitizers because of their low potency to induce skin inflammation. Furthermore, we provide evidence for a crucial role of CD8⁺ T cells as effector cells of CHS to these weak allergens and a role for CD4⁺ cells in prevention of CHS in immunocompetent mice. These studies reveal that the potent anti-inflammatory effects of SL are

dominant *in vivo* and fail to overcome immune regulation which prevents CHS.

Materials and methods

Animals

C57BL/6 (B6), Balb/c and T cell receptor (TCR) transgenic P14 mice (18) were provided by the breeding facility of the University Medical Center Freiburg. Female C57BL/6 mice (6–8 weeks old) were purchased from Charles River Laboratories (L'Arbresle, France). C57BL/6 mice with a mutation in the I-A^b gene (MHC class II knockout (KO) (19) were provided by Christophe Benoist and Diane Mathis (Harvard Medical School, Boston, USA). Heterozygous KO mice were backcrossed for more than eight generations to C57BL/6 and used to generate the offspring of the present study. All experimental procedures were in accordance with the University Medical Center Freiburg, INSERM and national guidelines on animal welfare and have been approved.

Media, chemicals and antibodies

RP-10 consisted of RPMI 1640 (Gibco, Invitrogen Life Technologies, Karlsruhe, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 2 mM of L-glutamine (Gibco), 25 mM of HEPES buffer (Gibco), 50 μ g/ml of penicillin-streptomycin (Gibco) and 10 μ M of 2-mercaptoethanol (Sigma, Deisenhofen, Germany). 2,4-dinitrofluorobenzene (DNFB) was from Sigma and 2,4,6-TNCB was from VeZerf Laborsynthesen GmbH, Idar-Oberstein, Germany.

Anti-CD8 α (53-6.7) and anti-TCR V α 2 (B20.1) were from BD PharMingen (Heidelberg, Germany) and were used as Fluoresceinisoithiocyanate (FITC), Phycoerythrin (PE) or Biotin conjugates. Biotinylated antibodies were revealed by Streptavidin-Cy-Chrome[®] (BD PharMingen). The rat anti-mouse CD4 B cell hybridoma GK1.5 was obtained from ATCC (Rockville, MD, USA). Hybridomas were grown as ascites in pristane-primed Swiss-nude mice at INSERM.

Materials for testing CHS

11 α ,13-dihydrohelenalinisobutyrate and -methacrylate were isolated from flower heads of *A. montana* from the SP chemotype and helenalinisobutyrate from the CE chemotype as described (20). Identity was confirmed by nuclear magnetic resonance (NMR) and mass spectroscopy (MS) analysis, and purity was evaluated by gas and thin layer chromatography analyses (Figs S1 and S2). The concentrations used in the CHS experiments for helenalinisobutyrate [1.2 mM = 39.89 mg/100 ml tincture or ethanol (EtOH) and 12 mM], 11 α ,13-dihydrohelenalinmethacrylate (2.12 mM = 69.93 mg/100 ml tincture or EtOH and 21.2 mM) and for 11 α ,13-dihydrohelenalinisobutyrate

(2.12 and 21.2 mM) corresponded to the same molar concentrations contained in the respective undiluted or 10-fold diluted tinctures.

'SP' *Arnica* tincture was prepared from flowers of *A. montana*, SP chemotype, and contained mainly 11 α ,13-dihydrohelenalin esters (0.70 mg/ml calculated as 11 α ,13-dihydrohelenalinmethacrylate) (2). 'CE' *Arnica* tincture was prepared from *A. montana* flowers, CE chemotype, by percolation according to the European Pharmacopoeia 1997 and contained mainly helenalin esters (0.83 mg/ml calculated as helenalinisobutyrate).

CHS and mouse ear swelling test

CHS was tested in C57BL/6 and Balb/c mice. Mice were sensitized epicutaneously on day 0 by application of 100 μ l of TNCB (7%) in acetone, CE and SP tincture (undiluted), helenalinisobutyrate (1.2 and 12 mM), dihydrohelenalinmethacrylate (2.12 and 21.2 mM) and dihydrohelenalinisobutyrate (2.12 and 21.2 mM), all in EtOH, on shaved abdominal skin. Control mice received acetone or EtOH. Mice were challenged on day 5 with 20 μ l of 1% TNCB or 20 μ l of the *Arnica* tinctures or SL applied on both ears. Ear thickness was measured prior to ear challenge with a thickness gage (Mitutoyo, Leonberg, Germany) and repeated 24 h later.

We tested CHS to weak sensitizers in C57BL/6 mice or MHC class II KO mice treated with anti-CD4 (21). Anti-CD4 monoclonal antibody (mAb; 20 μ g GK1.5 in 200 μ l of phosphate-buffered saline, PBS) was given intraperitoneally on days -3, -2, -1 before and on day +4 after the last sensitization (day 0). CD4 depletions > 98% were observed in all experiments. In the MHC II KO experiments sensitization was done with 50 μ l of undiluted tinctures on three consecutive days (days -2, -1 and 0). Ear challenge was performed on days 5 and 6 with 25 μ l of tincture. Ear thickness was measured prior to and on consecutive days after ear challenge.

Lipopolysaccharide (LPS) stimulation of dendritic cells

DC were incubated for 48 h with 1 μ g/ml of LPS from *Escherichia coli* (Sigma) in the presence or absence of graded concentrations of the CE or SP *Arnica* tincture. LPS was added to the cells 1 h before tincture treatment. IL-12p70 production was determined by enzyme-linked immunosorbent assay (ELISA) from supernatants.

Electrophoretic mobility shift assay (EMSA)

Mouse bone marrow-derived DC (BM-DC) were prepared as described (22). For EMSA, cells were plated in small petri dishes (BD Falcon, Heidelberg, Germany) at a density of 7×10^5 – 1×10^6 cells in 5 ml of starved medium on the day before. The starved medium consisted of Dulbecco's modi-

fied Eagle's medium (DMEM) with 100 IU/ml penicillin and 100 μ g/ml streptomycin without FCS. A lower basal stimulation is obtained under these starving conditions.

The assays were carried out as previously described (3). However, [^{32} P]-labelled oligonucleotide was replaced by [^{33} P]-labelled one. Signals were detected and quantified by densitometry using a PhosphorImager.

Flow cytometry

Cells (5×10^5 – 1×10^6) were stained for 20 min with 0.5–1 μ g of biotinylated mAb in 100 μ l of PBS/2% FCS/0.02% NaN₃. Fluorescence Activated Cell Sorter (FACS) buffer on ice. Cells were washed thrice in FACS buffer and FITC-, PE-conjugated secondary antibodies as well as streptavidin-Cy-Chrome® (BD PharMingen) were added for 20 min on ice. Samples were washed thrice and resuspended in 200 μ l of 1% paraformaldehyde. Data were acquired on a FAC-Scan instrument and analysed using CellQuest Software (BD Biosciences).

ELISA

ELISA was performed as described (22). Mouse IL-12 ELISA was performed according to manufacturers' instructions using OptEIA ELISA Sets from BD Biosciences. Plates were measured in an MWGt Sirius HT-TRF instrument (MWG, Ebersberg, Germany) and concentrations were calculated using BIO-TEK KC⁴ v3.1 software (Bio-Tek Instruments GmbH, Bad Friedrichshall, Germany).

Adoptive P14 T cell transfer and skin homing assay

The assay was performed as described (8). Exactly 1.5×10^6 spleen cells of P14 mice in 200 μ l of PBS were injected intravenously into C57BL/6 mice on day 0. DC were incubated for 1 h in RP-10 with 1 μ M of peptide GP33 (23) (BioChip Technologies GmbH, Freiburg, Germany) at 37°C. After three washes with PBS, 3×10^5 DC in 100 μ l of PBS were used for intracutaneous injection into two sites of the shaved abdominal skin on days 0 and 2 after P14 T cell transfer. On day 9 after adoptive P14 transfer, skin inflammation was induced by ear painting with 20 μ l of 1% TNCB in acetone, *Arnica* tinctures or SL in EtOH. Ear sheets were prepared 24 h later and incubated overnight in RP-10. Emigrated cells were counted, stained with antibodies for the expression of CD8 α and the transgenic P14 TCR V α 2 and were analysed by flow cytometry.

Statistical analysis

Statistical analysis was performed by using the Origin 7.0 software (North Hampton, MA, USA). Data are reported as means $\pm$ SD and analysed using an independent Student's *t*-test (two groups). A *P*-value < 0.05 is considered statistically significant.

Results

SL and tinctures from *Arnica montana* fail to induce CHS in mice

We used the mouse CHS model to test the potential of *Arnica* tinctures to induce CHS. According to our established protocol (8,24,25), C57BL/6 mice were treated with undiluted CE or SP *Arnica* tinctures on the abdomen on day 0 and challenged 5 days later by painting the same tinctures on the ears (Fig. 1c). As a positive control, mice were treated with the strong contact sensitizer TNCB, which induced a strong ear swelling response (Fig. 1b). In contrast, no ear swelling was induced by *Arnica* tinctures (Fig. 1c) or SL (data not shown). Of note, CHS could also not be induced in Balb/c mice excluding mouse strain-specific effects (data not shown).

Dose-dependent effects of SL on NF- κ B activation

To determine if the known anti-inflammatory effect and the failure of CHS could be due to deficient activation of NF- κ B in DC and resulting in an absence of pro-inflammatory cytokines like IL-12, we performed EMSA studies (Fig. 2a–c). The NF- κ B EMSA was performed with helenalin- and 11 α ,13-dihydrohelenalinisobutyrate as well as 11 α ,13-dihydrohelenalinmethacrylate using bone marrow-derived DC from C57BL/6 mice (Fig. 2a–c). We found a dose-dependent inhibition of NF- κ B DNA binding activity as determined by densitometry. In each case, murine DC exhibited a strong activation in the absence of the stimulus which does not allow studying the effect of the respective SL alone. However, when using human DC and, exemplarily, helenalinisobutyrate, a dose-dependent activation or inhibition of NF- κ B DNA binding was observed (Fig. 2d). Similar effects were noticed on measurement of NF- κ B activation by human HaCaT keratinocytes (Fig. 2e–g). These data reveal a dual effect of SL: inhibition of NF- κ B DNA binding at high concentrations and activation of NF- κ B DNA binding at low concentrations.

SL and tinctures from *Arnica montana* are weak inducers of skin inflammation

As the irritant effect of contact sensitizers is crucial for CHS induction (5,6), we next tested the pro-inflammatory potential of the tinctures and the pure SL by quantifying the inflammation-dependent immigration of skin-homing CD8⁺ effector T cells into treated skin in our previously established skin homing assay (8). CD8⁺ P14 T cells with a transgenic lymphocytic choriomeningitis (LCMV) GP33-specific TCR (18) were transferred into C57BL/6 mice. They were then activated and induced to express skin homing receptors by intracutaneous injection of BM-DC pulsed with their specific antigenic peptide GP33 (8). Ears were

painted with TNCB, *Arnica* tinctures or pure SL 9 days after P14 transfer to induce skin inflammation and recruitment of the activated skin-specific P14 T cells. After 24 h, ear sheets were prepared and incubated in medium for 24 h. The emigrated cells were counted and characterized by flow cytometry to detect and quantify the TCR transgenic P14 T cells (Fig. 3). In contrast to TNCB, the number of emigrated P14 T cells was not increased over vehicle controls when pure SL were used. *Arnica* tinctures induced P14 T cell infiltration which, however, was about fivefold lower compared with that induced by TNCB. These data show that the tinctures have a low potential for induction of skin inflammation compared with the strong contact sensitizer TNCB while the pure SL did not show any significant effects above solvent control.

Arnica tinctures suppress LPS-induced DC activation

The potent anti-inflammatory activity of the tinctures observed *in vivo* may prevent appropriate activation of DC, which is a crucial step in the initiation of CHS (5,6). In order to analyse if *Arnica* tinctures can interfere with Toll-like receptor (TLR)-dependent DC activation, BM-DC were stimulated with the TLR4 ligand LPS in the absence or presence of graded concentrations of the *Arnica* tinctures. A dose-dependent inhibition of LPS-induced IL-12p70 production by the DC was observed for the CE tincture (Fig. 4a). Similar effects were observed on treatment with the SP tincture, however at higher doses (about threefold for half-maximal inhibition) (Fig. 4b). A similar dose-dependent inhibition of IL-12p70 production was observed in human DC treated analogously with LPS, interferon (IFN)- γ and SL (data not shown).

Tinctures from *Arnica montana* decrease TNCB-induced CHS

To test whether the known anti-inflammatory effect of the tinctures is superposing a pro-allergic inflammatory effect in mice, we pretreated the ear skin of TNCB-sensitized mice with the tinctures 7 h before ear challenge with TNCB. We observed that this pretreatment significantly decreases the ear swelling response to TNCB (Fig. 5a). Notably, the decrease induced by the SP tincture was higher than that induced by the CE tincture. As SL are the effective compounds in the tinctures these results demonstrate a potent anti-inflammatory activity of the SL that counteracts their pro-inflammatory effect *in vivo*. In order to imitate the clinically relevant situation of an application of undiluted tinctures to inflamed skin, mice were treated repeatedly with the strong contact sensitizer DNFB to induce skin inflammation. Then, the inflamed skin was treated with undiluted *Arnica* tinctures followed by ear challenge. We did not detect a significant ear swelling

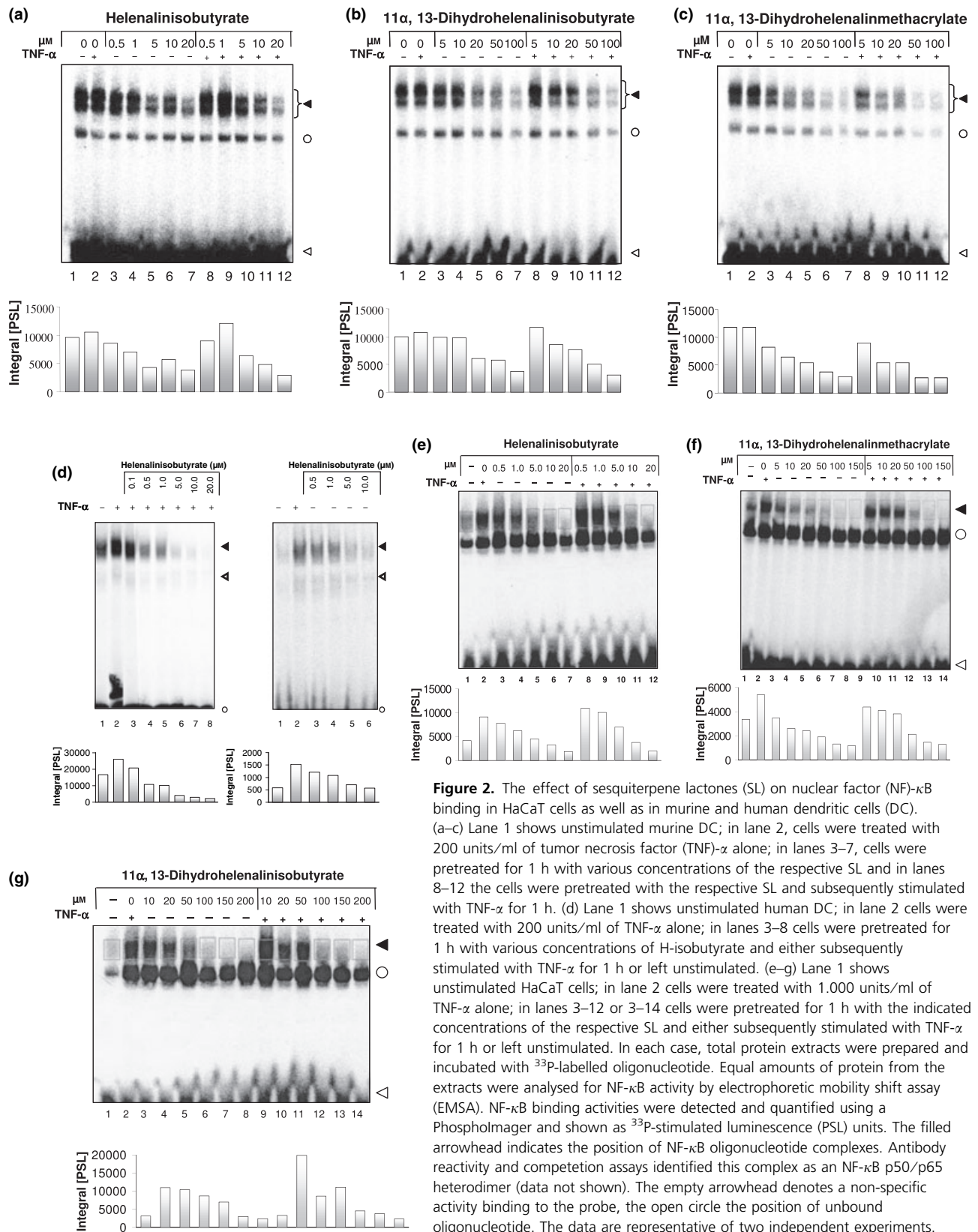


Figure 2. The effect of sesquiterpene lactones (SL) on nuclear factor (NF)- κ B binding in HaCaT cells as well as in murine and human dendritic cells (DC). (a–c) Lane 1 shows unstimulated murine DC; in lane 2, cells were treated with 200 units/ml of tumor necrosis factor (TNF)- α alone; in lanes 3–7, cells were pretreated for 1 h with various concentrations of the respective SL and in lanes 8–12 the cells were pretreated with the respective SL and subsequently stimulated with TNF- α for 1 h. (d) Lane 1 shows unstimulated human DC; in lane 2 cells were treated with 200 units/ml of TNF- α alone; in lanes 3–8 cells were pretreated for 1 h with various concentrations of H-isobutyrate and either subsequently stimulated with TNF- α for 1 h or left unstimulated. (e–g) Lane 1 shows unstimulated HaCaT cells; in lane 2 cells were treated with 1.000 units/ml of TNF- α alone; in lanes 3–12 or 3–14 cells were pretreated for 1 h with the indicated concentrations of the respective SL and either subsequently stimulated with TNF- α for 1 h or left unstimulated. In each case, total protein extracts were prepared and incubated with ^{32} P-labelled oligonucleotide. Equal amounts of protein from the extracts were analysed for NF- κ B activity by electrophoretic mobility shift assay (EMSA). NF- κ B binding activities were detected and quantified using a PhosphorImager and shown as ^{32} P-stimulated luminescence (PSL) units. The filled arrowhead indicates the position of NF- κ B oligonucleotide complexes. Antibody reactivity and competition assays identified this complex as an NF- κ B p50/p65 heterodimer (data not shown). The empty arrowhead denotes a non-specific activity binding to the probe, the open circle the position of unbound oligonucleotide. The data are representative of two independent experiments.

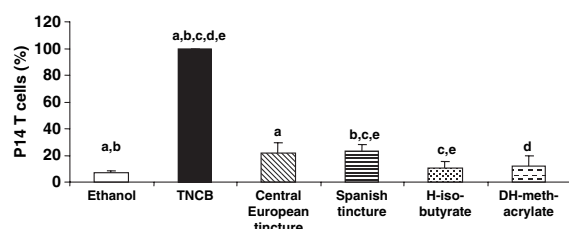


Figure 3. Skin inflammation induced by 2,4,6-trinitrochlorobenzene (TNCB), *Arnica* tinctures and sesquiterpene lactones (SL). P14 T cells with a transgenic LCMV GP33-specific TCR were transferred intravenously into C57BL/6 mice on day 0 and activated *in vivo* by intracutaneous injection of GP33-pulsed dendritic cells (DC) on days 0 and 2. On day 9, TNCB (1%), Central European (CE; undiluted), Spanish (SP; undiluted), H-isobutyrate (1.2 mM) or DH-methacrylate (2.12 mM) and ethanol (EtOH), respectively, were applied on ear skin. Ear sheets were prepared 24 h later and incubated in the media for 24 h. The emigrated cells were counted and P14 T cells were identified by flow cytometry. The results show the relative amount of emigrated P14 T cells with the value for TNCB set as 100% which corresponds to 30.000–115.000 emigrated P14 T cells per ear depending on the experiment. Data present the average of three independent experiments with three or two mice per group. Values with significance level of $P < 0.05$ are marked with the same letter.

response (Fig. 5b). These data argue for a strong anti-inflammatory effect of the tinctures that dominates *in vivo*.

CE but not SP tincture causes CHS in acutely CD4-depleted mice

Based on the previous observation that CHS to weak contact sensitizers such as α -hydroxycinnamaldehyde (HCA) and eugenol involved in fragrance allergy can be induced in CD4 T cell-depleted mice (21), we wanted to test whether this is also the case for *Arnica*. As compared with wild-type mice, CHS was induced in acutely CD4-depleted MHC II KO mice by the CE (Fig. 6a) but not by the SP tincture (Fig. 6b). These data reveal a crucial role for CD8⁺ T cells in induction, and for CD4⁺ T cells in the prevention of CHS to *Arnica*.

Discussion

In this study, we have demonstrated that tinctures obtained from two different chemotypes of *A. montana* or purified SL are unable to induce CHS in mice with an intact immune system. These compounds have dual effects: anti-inflammatory effects mainly because of alkylation of NF- κ B p65 (3,4,6) and pro-inflammatory and pro-allergic effects as a result of NF- κ B activation and hapten modification of proteins to generate antigenic T cell determinants (5,7). We were able to show *in vitro*, both in human keratinocytes and human and mouse DC, that the balance between these opposing effects is dose-dependent. At high concentration, the NF- κ B inhibitory effect, equal to an anti-inflammatory effect, is dominant, while the NF- κ B activating effects such as pro-inflammatory and pro-allergic

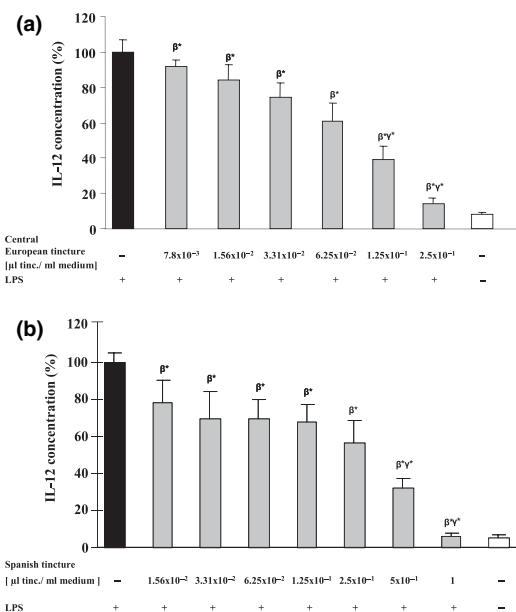


Figure 4. Suppression of lipopolysaccharide (LPS)-induced dendritic cell (DC) activation by *Arnica* tinctures. Bone marrow (BM)-derived DC from C57BL/6 mice (a, b) were treated for 48 h with the indicated concentrations of Central European (a) or Spanish tincture (b) in combination with 1 μ g/ml of LPS. Interleukin (IL)-12 p70 production was measured in supernatants by enzyme-linked immunosorbent assay. IL-12 concentrations are expressed in per cent with the amount of IL-12 induced by LPS only set as 100%. The absolute amount of IL-12 induced by LPS corresponded to 500–750 ng/ml depending on the experiment. The results represent the average of two independent experiments with triplicate values per group $\pm$ SD. Values with significance to the positive control are marked with β and with significance to the former with γ . Values for β and γ are significant at $P < 0.05$; β^* and γ^* at $P < 0.005$ (b, d).

effects prevail at low concentration. In the case of the dihydrohelenalin derivatives, approximately 10-fold higher concentrations were needed. This is most likely because of their weaker chemical reactivity. Our findings underline previous observations that contact allergens modulate NF- κ B activation (26,27). In the case of SL, we know the functional molecular target, at least for the inhibitory effect (3,4). For other contact allergens and also irritants these targets will have to be identified in order to better understand their mechanisms of action and to develop new therapeutic strategies in ACD (6).

Interestingly, topically applied immunosuppressive corticosteroids, which also partly exert their anti-inflammatory effect through targeting NF- κ B are also able to induce ACD (28,29). In clinical patch tests, a positive skin reaction often becomes manifest only after 4–7 days rather than after 48 h as observed for most contact sensitizers. This may be explained by similar dose-dependent effects as observed by us for SL *in vitro*. Thus, at early time points and high concentrations the anti-inflammatory effect is dominant, while, most likely due to a

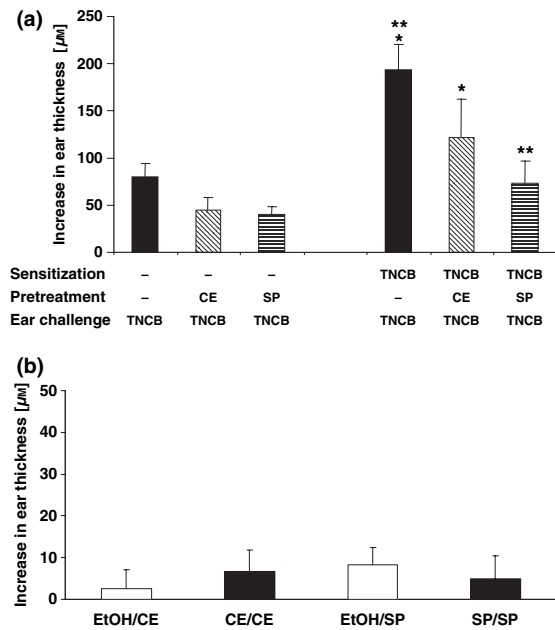


Figure 5. Co-application of tinctures with strong contact sensitizers. (a) C57BL/6 mice were sensitized with acetone or 7% 2,4,6-trinitrochlorobenzene (TNCB)/acetone on day 0 and challenged on day 5. The indicated groups were pretreated with Central European (CE) or Spanish (SP) tincture 7 h before ear challenge with 1% TNCB/acetone. Results are expressed as the ear swelling at 24 h postchallenge for groups of three mice $\pm$ SD. Differences between the indicated groups (*, **) are significant at $P < 0.05$. (b) The shaved abdomen of C57BL/6 mice was treated with 0.5% 2,4-dinitrofluorobenzene (DNFB) in acetone/olive oil (4:1) on days -2 and -1 to induce a massive inflammation. On day 0, the mice were abdominally treated with the undiluted tinctures. Ear challenge was performed with the same tinctures on day 5. Results are expressed as the ear swelling at 24 h postchallenge for groups of three mice $\pm$ SD. The observed differences between the groups were not significant.

decrease in concentration of the compounds in skin over time, the pro-allergic effect becomes visible at later time points. In our *in vivo* experiments, however, we did not observe such kinetic dose-dependent effects: ear swelling responses were absent at any time after elicitation in immunocompetent mice.

The clinical situation that is often the cause for CHS to *Arnica* preparations is the repeated application of undiluted tinctures to inflamed skin. When we tried to imitate these conditions *in vivo*, even daily treatment of mice with the undiluted tinctures for 2 weeks (data not shown) or the induction of potent skin inflammation by topical application of the strong contact sensitizers TNCB (data not shown) or DNFB prior to sensitization or elicitation with the tinctures or SL failed to result in CHS. Also lower concentrations of the tinctures that stimulated NF- κ B activation *in vitro* were unable to induce CHS. Furthermore, the tinctures induced only a weak skin inflammation and recruitment of skin-homing effector T cells. They also sig-

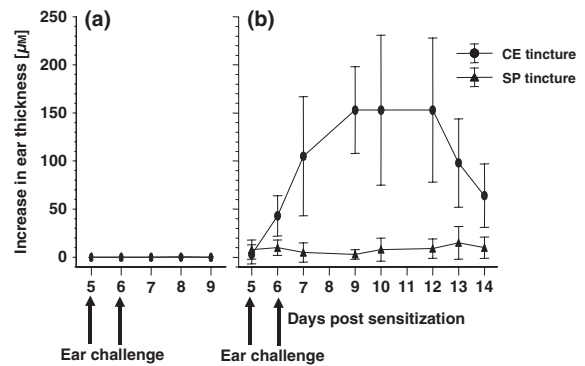


Figure 6. Induction of contact hypersensitivity (CHS) to Central European *Arnica* tincture in acutely CD4-depleted mice. C57BL/6 (a) or anti-CD4-depleted major histocompatibility complex (MHC) class II KO mice (b) were sensitized on days -2, -1 and 0 with the Central European (CE) or Spanish tincture (SP). Elicitation by ear challenge was performed on days 5 and 6 and the ear swelling response was measured at the indicated time points. Data show the ear swelling from groups of three (WT) and four mice (CD4-depleted MHC II KO) $\pm$ SD. The data are representative of two independent experiments.

nificantly suppressed CHS to TNCB *in vivo* and LPS-induced IL-12 production by DC *in vitro*. IL-12 drives the development of IFN- γ -producing effector T cells that are required for CHS. The higher immunosuppressive activity of the SP tincture on TNCB-induced CHS may be because of its weaker chemical reactivity resulting in a better bio-availability (30). The fact that, in contrast to the tinctures, the SL did not inhibit TNCB-induced CHS may be explained by their poor skin penetration, which is much improved in the tinctures (31). These findings underline the potent anti-inflammatory effects of *Arnica* also in the mouse model. The pivotal inflammatory response in CHS is counteracted by potent anti-inflammatory effects of SL. Thus, the weak skin inflammation induced by *Arnica* tinctures may prevent efficient activation of DC in the skin. Consequently, priming of T cells in the draining lymph nodes in the sensitization phase of CHS and the recruitment of effector cells to inflamed skin following elicitation might be significantly impaired as shown in our skin homing assays. Interestingly, we have previously shown that SL can also inhibit irritant contact dermatitis induced by croton oil (32,33). These findings suggest that similar mechanisms are involved in the initiation of skin inflammation in allergic and irritant contact dermatitis.

Moreover, we observed that CHS induction to the weak allergens from *Arnica* is actively prevented by CD4⁺ immunoregulatory cells (34,35) as shown for HCA and eugenol (21). The mechanisms of immune regulation of CHS to *Arnica* are currently under investigation and are, therefore, not addressed in this study. The fact that only CE tincture was able to induce CHS in acutely CD4-depleted mice may be because of the more efficient generation of immuno-

genic hapten determinants by the more reactive helenalin derivatives.

Our studies indicate that SL and tinctures from *Arnica* flowerheads and, most likely also from many other plants of the Asteraceae family, should be classified as weak contact allergens. Their failure to induce CHS in healthy and inflamed skin may be due to a potent anti-inflammatory effect that counteracts and significantly decreases the essential irritant effect and due to efficient immunoregulation by CD4⁺ cells. Our findings support the concept that the development of CHS to strong and weak contact sensitizers is mediated by CD8⁺ effector T cells and crucially depends on the balance between effector T cells and immunosuppressive regulatory T cells. It is a future challenge to elucidate the mechanisms that govern the regulation of this delicate balance by different contact allergens and to confirm the mechanism also in human models.

Our data demonstrate the crucial role of the innate immune response that results in inflammation in CHS (36,37). Studies in mice as well as clinical studies demonstrate a strong potential of a variety of steroidal and non-steroidal anti-inflammatory drugs that reduce or prevent sensitization or elicitation of CHS following topical or systemic application. Examples are indomethacin, acetylsalicylic acid (38), α -Melanocyte stimulating hormone-related peptides (39), inhibitors of phosphodiesterase 4 (40) or phospholipase 2 (41) or cannabinoid receptor agonists (42). Interestingly, quinacrine inhibited DC migration in the CHS model by inhibiting NF- κ B activation and production of TNF- α , IL-1 β and the chemokine CCL21 (43). Such compounds act on different components of the allergic response. The results from such studies are promising and indicate that anti-inflammatory drugs will be efficient in the treatment of various inflammatory skin diseases such as ACD.

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Supplementary material

The following supplementary material is available for this article online:

Figure S1. GC-chromatogram (total ionen current) of “Central European” *Arnica* tincture. The zoomed intervall contains the sesquiterpene lactones as labelled.

Figure S2. GC-chromatogramm (total ionen current) of “Spanish” *Arnica* tincture. The zoomed intervall contains the sesquiterpene lactones as labelled. Signals of dihydrohelanalin derivates show a double peak due to an intramolecular conversion in the injector.

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Supporting information

GC-Chromatograms of the Arnica tinctures used in these studies. Preparation followed according the official guideline of the European Pharmacopoea. Analysis is described in Willuhn and Leven, 1991.

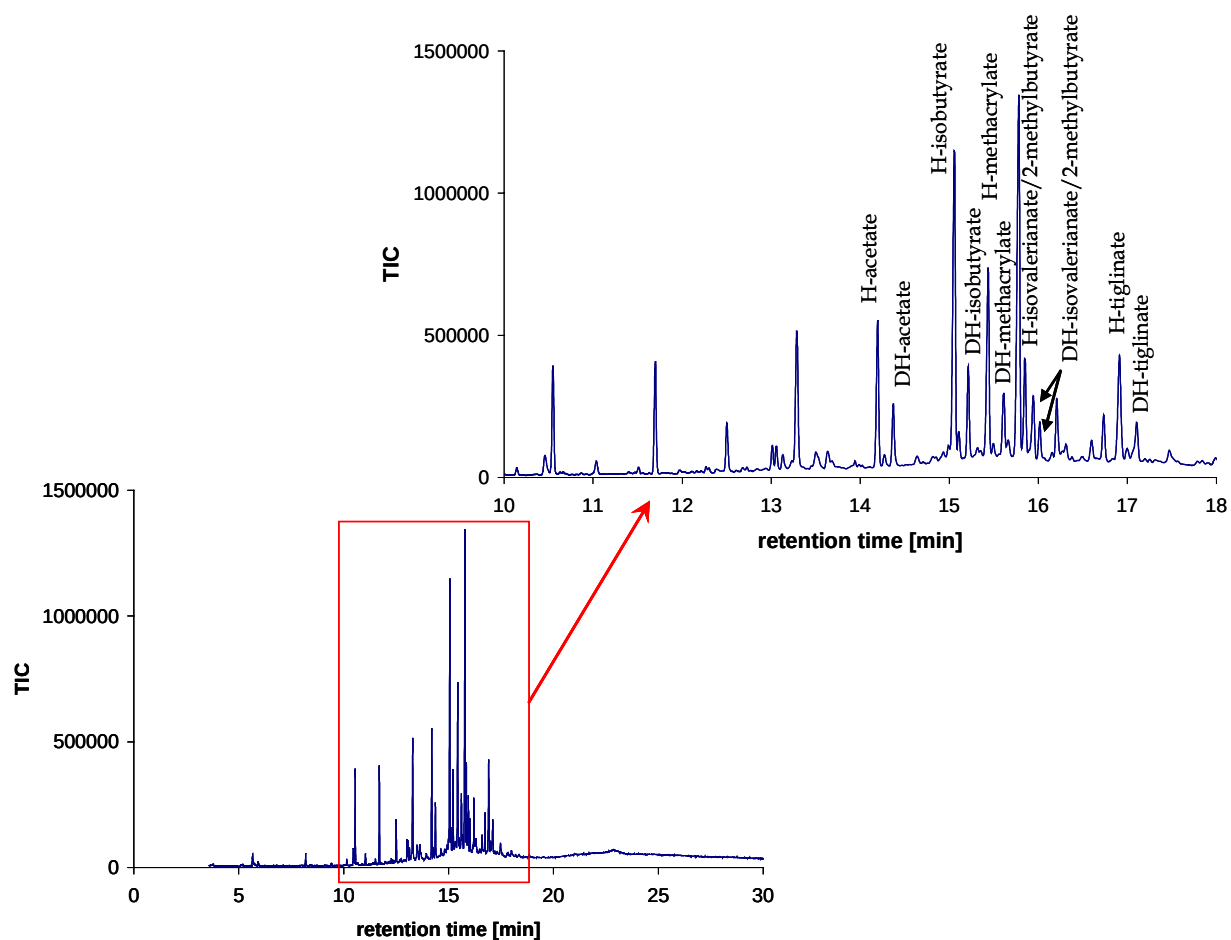


Figure S1. GC-chromatogram (total ion current) of „Central European“ Arnica tincture. The zoomed intervall contains the sesquiterpene lactones as labelled.

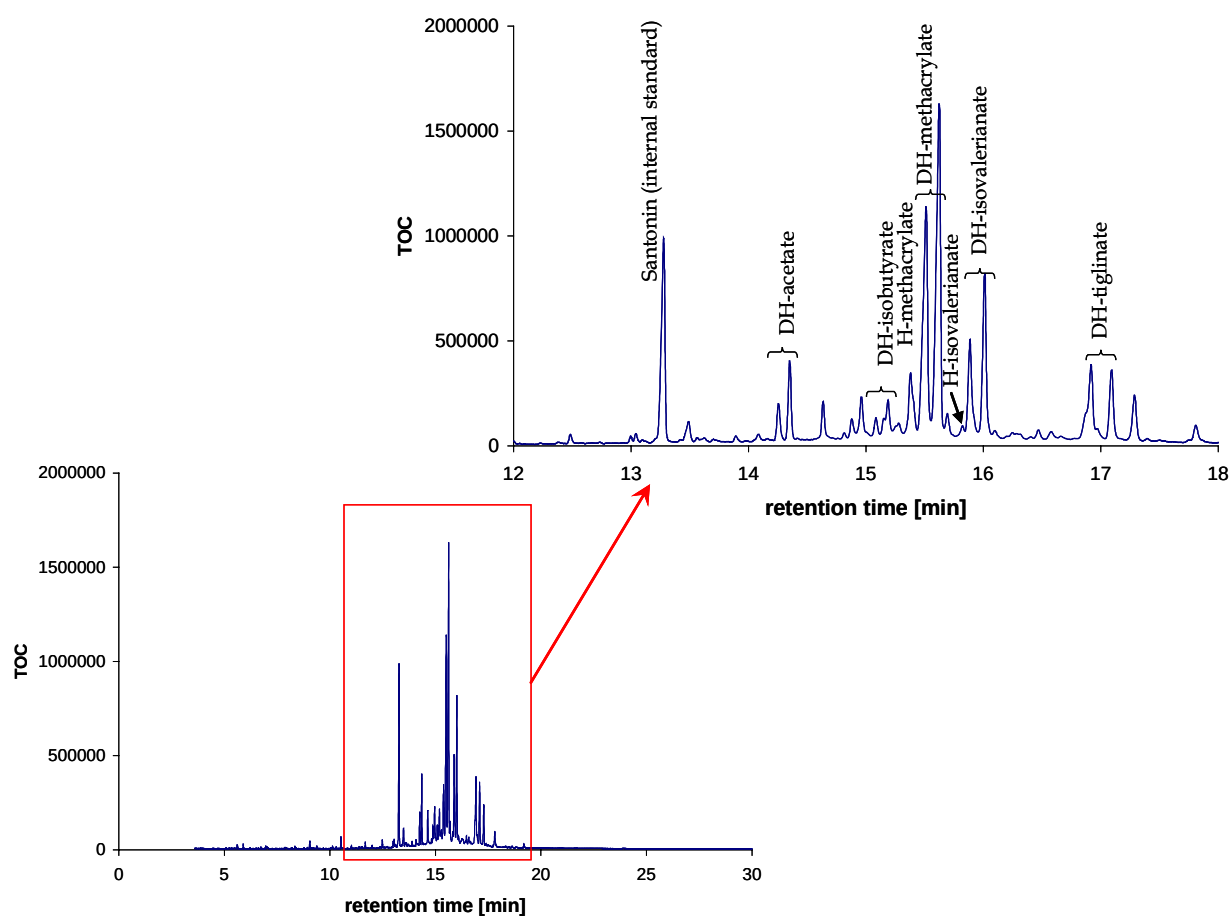


Figure S2. GC-chromatogramm (total ionen current) of „Spanish“ Arnica tincture. The zoomed intervall contains the sesquiterpene lactones as labelled. Signals of dihydrohelenalin derivatives show a double peak due to an intramolecular conversion in the injector.