Christoph A. Klaas[1]
Gerd Wagner[2]
Stefan Laufer[2]
Silvio Sosa[3]
Roberto Della Loggia[3]
Ulrich Bomme[4]
Heike L. Pahl[5]
Irmgard Merfort[1]

# Studies on the Anti-Inflammatory Activity of Phytopharmaceuticals Prepared from *Arnica* Flowers[1]





**Abstract**

Phytopharmaceuticals prepared from flowerheads of *Arnica montana* of Spanish origin and of the new type "Arbo", which can be easily and economically cultivated, were studied for their capability to impair activation of the transcription factors NF-κB and NF-AT. Both proteins are responsible for the transcription of genes encoding various inflammatory mediators. Additionally, their influence on the release of the cytokines IL-1 and TNF-$\alpha$ were examined. The inhibitory activities correlate with their quantitative and qualitative content of sesquiterpene lactones (Sls). Moreover, it was shown that the inhibitory potency of 11$\alpha$,13-dihydrohelenalin derivatives being the main Sls in the Spanish flowers depend on their esterfication. Compounds with unsaturated acyl moieties, such as methacrylate and tiglinate, exhibited a stronger activity in the NF-κB EMSA as well as in the croton oil ear test in mice than the acetate derivative.

**Key words**
*Arnica montana* · Asteraceae · anti-inflammatory activity · sesquiterpene lactones · NF-κB · NF-AT · IL-1$\beta$ · TNF-$\alpha$ · mouse ear edema

**Abbreviations**

| | |
|---|---|
| Arbo tincture: | tincture prepared from *Arnica* flowerheads type Arbo |
| AP-1: | activator protein 1 |
| BSA: | bovine serum albumen |
| c.p.m.: | counts per minute |
| DTT: | dithiothreitol |
| EDTA: | ethylenediaminetetraacetic acid |
| EGTA: | ethylene glycol-bis($\beta$-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid |
| EMSA: | electrophoretic mobility shift assay |
| FCS: | fetal calf serum |
| HEPES: | *N*-[2-hydroxyethyl]-piperazine-*N′*-[2-ethanesulfonic acid] |
| IL: | interleukin |
| NP-40: | nonylphenoxypolyethoxyethanol |
| NF-AT: | nuclear factor of activated T cells |
| BPS: | phosphate buffered saline |
| PMA: | phorbol myristate acetate |
| PMSF: | phenylmethylsulfonyl fluoride |
| Poly (dI-dC): | polydeoxyinosinicdeoxycytidylic acid, double-stranded alternating copolymer |
| Spanish tincture: | tincture prepared from Spanish *Arnica* flowerheads |
| Sl: | sesquiterpene lactone |
| TNF-$\alpha$: | tumor necrosis factor alpha |

[1] Partly presented at the 3rd International Congress on Phytomedicine, 2000, Munich, Germany.

**Affiliation**
[1] Institute of Pharmaceutical Biology, University of Freiburg, Freiburg, Germany
[2] Institute of Pharmacy, University of Tübingen, Germany
[3] DEMREP, Università di Trieste, Italy
[4] Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, Freising-Weihenstephan, Germany
[5] Department of Experimental Anaesthesiology, University Hospital, Freiburg, Germany

**Correspondence**
Prof. Dr. Irmgard Merfort · Institute of Pharmaceutical Biology · Albert-Ludwigs-University · Stefan-Meier-Str. 19 · 79104 Freiburg · Germany · Phone: +49–761–203–8373 · Fax: +49–761–203–8383 · E-Mail: merfort@uni-freiburg.de

**Received** June 27, 2001 · **Accepted** October 7, 2001

**Bibliography**
Planta Med 2002; 68: 385–391 · © Georg Thieme Verlag Stuttgart · New York · ISSN 0032-0943

## Introduction

Phytopharmaceuticals prepared from *Arnica* flowers are externally used to treat a variety of different ailments. These are related to injuries and accidents, e.g., for haematomas, dislocations, sprains, bruising, edema associated with fractures, rheumatic muscle and joint complaints, inflammations of the mucous membranes of the mouth and throat, furuncles, inflamed insect bites, and surface phlebitis [1]. The secondary metabolites that mediate the anti-inflammatory effects of *Arnica* flowers are mainly sesquiterpene lactones (Sls) of the 10$\alpha$-methylpseudoguaianolide type like helenalin, 11$\alpha$,13-dihydrohelenalin and their ester derivatives (see Fig. **1**) ([1], and lit. cited therein). Recently, we made a contribution to the molecular mechanism by which they exert their anti-inflammatory effect. We have demonstrated that these Sls as well as *Arnica* tincture prepared from flowerheads of *A. montana* (Spanish chemotype) effectively interferes with the activation of NF-κB and that the total concentration of Sls is high enough to explain this effect [2], [3], [4]. Moreover, we could show that Sls also inhibit the transcription factor NF-AT [5].

The inducible transcription factors NF-κB and NF-AT are important regulators of the human immune response. NF-κB regulates the transcription of genes encoding, e.g., the cytokines IL-1$\beta$, IL-2, TNF-$\alpha$, adhesion molecules or enzymes like iNOS, cyclooxygenase-II and 5-lipoxygenase [6]. Components of the NF-AT family bind cooperatively with those of the AP-1 family to composite NF-AT:AP-1 sites [7] and play a major role in the control of activation and differentiation of T cells. These cells activate other cells of the immune system, thus maintaining or enhancing inflammatory processes [7]. In addition to NF-κB, NF-AT is involved in regulating the transcription of cytokines, such as TNF-$\alpha$, IL-2 or IL-4 in lymphocytes.

In continuation of our studies on the anti-inflammatory activity of sesquiterpene lactones, this paper is related to investigations on preparations from *Arnica* flowerheads as well as of some isolated Sls from these preparations. Two *Arnica* tinctures, are compared for their ability to inhibit DNA binding of the transcription factors NF-κB and NF-AT. One is prepared from flowerheads of the Spanish chemotype, the other from those of type "Arbo" which has been recently introduced in the market and has the advantage to be more easily and economically cultivated than the species from Spain [8]. Additionally, a commercially available *Arnica* preparation was studied as to the extent to which NF-κB inhibition occurs. The inhibitory activities were correlated with their qualitative and quantitative contents of Sls. In order to evaluate whether the production of cytokines decreases in correlation with the NF-κB inhibition results both *Arnica* tinctures were studied for their ability to influence the release of the cytokines IL-1$\beta$ and TNF-$\alpha$. Moreover, it was investigated whether the type of esterification within the dihydrohelenalins influence the potency to inhibit NF-κB. Finally, the *in vivo* mouse ear edema test was undertaken with two Sls in order to ascertain whether a correlation exists between the *ex vivo* and the *in vivo* results.

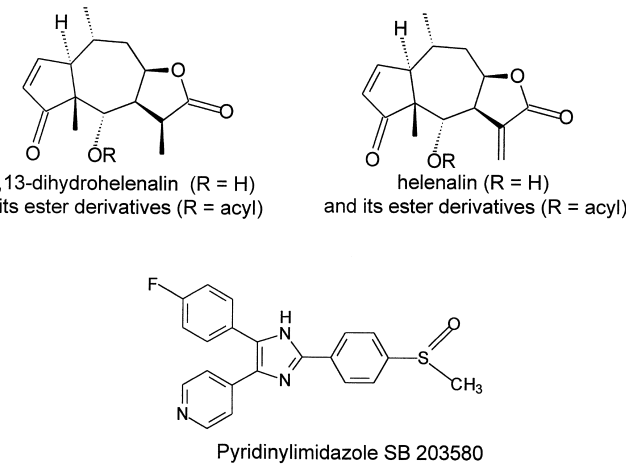


Fig. **1** Sesquiterpene lactones from flowerheads of *A. montana* and structure of SB203580.

## Materials and Methods

### Test compounds

*Arnica* tincture (German Pharmacopoeia, DAB) and ArnikaGel® were a gift from the Kneipp Company, Würzburg, Germany. *Arnica* tincture ("Arbo") was prepared from *Arnica montana* flowers type "Arbo" growing at Steinach (Bavaria, Germany) according to the German Pharmacopoeia. A voucher specimen from type "Arbo" is deposited at the Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, Freising-Weihenstephan, Germany.

11$\alpha$,13-Dihydrohelenalin acetate, 11$\alpha$,13-dihydrohelenalin tiglinate and 11$\alpha$,13-dihydrohelenalin methacrylate were isolated from flowerheads of *Arnica montana*, Spanish chemotype, as previously described [9]. Identity was confirmed by NMR and MS analysis, purity was evaluated by GC analysis.

10 mM stock solutions of the respective Sl were prepared in DMSO for the NF-κB assays. The tinctures as well as the ArnikaGel® were directly applied to the Jurkat T cells.

### HPLC analysis

10 g of the respective *Arnica* tincture were diluted with 50 ml $H_2O$. Santonin (Sigma, Deisenhofen, Germany) was added as an internal standard and the solution was heated for 30 min at 60 °C. Ethanol was removed by reduced pressure. The next steps were carried out as previously described [10]. The obtained values in mg/g were converted to mg/ml by using the correction factor of 0.868 (0.868 g = 1 ml tincture).

10 g of ArnikaGel® were diluted with 50 ml $H_2O$ and santonin was added as an internal standard. About 0.45 ml of aq. HCl (2.5 %), to a pH of 5.3, and 5 ml MeOH were added to destroy the gel structure. This solvent was heated 30 min at 60 °C and handled as described in [10]. Finally the obtained values were converted to mg/ml using the factor of 0.975 (0.975 g = 1 ml gel).

### Cell culture

Jurkat T cells were maintained in RPMI 1640 medium supplemented with 10 % fetal calf serum, 100 IU/ml penicillin and 100 $\mu$g/ml streptomycin (all Gibco-BRL, Groningen, Netherlands).

### NF-κB EMSA

Total protein extracts from Jurkat T cells were prepared using a high-salt detergent buffer (Totex: 20 mM Hepes, pH 7.9, 350 mM NaCl, 20% (v/v) glycerol, 1% (w/v) NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 1 mM PMSF, 1% aprotinin). Cells were harvested by centrifugation, washed once in ice-cold PBS (Sigma, Deisenhofen, Germany) and resuspended in four cell volumes of Totex buffer. The cell lysate was incubated on ice for 30 min, then centrifuged for 5 min at 13,000 rpm at 4 °C. The protein content of the supernatant was determined and equal amounts of protein (10 – 20 μg) added to a reaction mixture containing 20 μg BSA (Sigma, Deisenhofen, Germany), 2 μg poly (dI-dC) (Roche Diagnostics, Mannheim, Germany), 2 μl buffer D+ (20 mM Hepes, pH 7.9; 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.1% PMSF), 4 μl buffer F (20% Ficoll 400, 100 mM Hepes, 300 mM KCl, 10 mM DTT, 0.1% PMSF) and 100,000 c.p.m. (Cerenkov) of a $^{32}$P-labeled oligonucleotide, made up to a final volume of 20 μl with distilled water. Samples were incubated at room temperature for 25 min. NF-κB oligonucleotide (Promega, Mannheim, Germany) was labeled using γ-[$^{32}$P]-ATP (3000 Ci/mmol; Amersham Pharmacia Biotech, Freiburg, Germany) and T4 polynucleotide kinase (Promega, Mannheim, Germany).

### NF-AT EMSA

Nuclear cell extracts were prepared as previously described [11]. The protein content was determined and equal amounts of protein (10 μg) were added to the same reaction mixture as in the NF-κB EMSA. A oligonucleotide spanning the distal NF-AT site of the human GM-CSF promoter was used [12]. Sequence: 5′ - TTT CTC ATG GAA AGA TGA CAT A - 3′.

### Cellular assay of cytokine release inhibition, enzyme linked immunosorbent assay for TNF-α and IL-1β

*Arnica* tinctures were serially diluted in 70% ethanol (concentration range 10 – 0.001 μl/ml). Mononuclear cells were isolated from whole blood of healthy human donors by density gradient centrifugation. Cell samples were preincubated for 15 min (37 °C, 5% $CO_2$) with various concentrations of *Arnica* tincture (test samples) or 70% ethanol (control samples). Biosynthesis of cytokines was induced by cells stimulation with 1 μg/ml LPS (from *E. coli*, serotype 026:B6) for 4 h (37 °C, 5% $CO_2$). Cell reaction was terminated in an ice bath and cell samples were centrifuged. Concentrations of IL-1β and TNF-α were determined in supernatants using commercially available ELISA kits (Beckman Coulter, Krefeld, Germany). The anti-cytokine activity of each extract was calculated as percent reduction of cytokine concentration in test samples compared to control samples. Results are given as $IC_{50}$ values (μl/ml).

### Vitality test

The viability of cells which were treated with the respective sample for four hours was determined by trypan blue exclusion as described in [13].

### Croton oil-induced ear edema in mice

The *in vivo* anti-inflammatory activity of the sesquiterpene lactones was measured as inhibition of the croton oil-induced ear edema in mice, as previously described [14]. Cutaneous inflammation was induced by applying 15 μl of an acetonic solution containing 80 μg of croton oil to the inner surface of the right ear of male CD-1 mice (28 – 32 g, Harlan-Italy, S. Pietro al Natisone, Italy), anesthetized with ketamine-HCl (145 mg/kg intraperitoneally; Virbac, Milano, Italy). The test substances were dissolved in the croton oil solution. The left ear remained untreated since preliminary experiments had shown that acetone does not interfere with the inflammatory response. All experiments were uniformly started between 10:00 and 12:00 a.m. in order to avoid variations in the inflammatory response due to circadian fluctuations in the levels of corticosteroids. Six hours after induction of the inflammation, mice were sacrificed and a plug (6 mm Ø) was excised from both the treated and the untreated ears. The edema formation was quantified as the difference in weight between the two ear plugs. The anti-inflammatory activity was evaluated as percent inhibition of the edematous response in the animals treated with the test substances in comparison to the animals treated with the irritant alone. The experimental design was approved by the ethics committee of the University of Trieste.

### Statistical analysis

Values of HPLC analysis were obtained from three independent measurements, those of the EMSAs from two measurements. Graphical analysis on semi-logarithmic paper was used to calculate $IC_{50}$ values for the *Arnica* tinctures from percent reduction of cytokine release at various concentrations in one particular run. Experiments were carried out three/four times and the overall $IC_{50}$ value was calculated as the mean +/-SD of all three/four $IC_{50}$ s. The *in vivo* data were evaluated by the Student's *t*-test and significance was assumed for p values lower than 0.05.

## Results

To compare the inhibitory activity on the NF-κB DNA binding of different phytopharmaceuticals Jurkat T cells (5 – 6 × $10^5$ cells/ml) were incubated with various amounts of the respective *Arnica* tincture or the ArnikaGel® for 1 h. Cells were stimulated for 1 h with 200 U/ml TNF-α, and subsequently total cell extracts were prepared and analyzed for NF-κB DNA binding activity (see Fig. **2A** and **B**). Stimulation with TNF-α induced one novel DNA binding activity in Jurkat T cells (see Fig. **2**, lane 2). Antibody reactivity and competition assays identified this complex as an NF-κB p50/p65 heterodimer (data not shown). 5 μl/ml of the Arbo and 10 μl/ml of the Spanish tincture completely inhibited NF-κB DNA binding (see Fig. **2A**). ArnikaGel® was less active since a concentration of 50 μl/ml was necessary for a complete inhibition (see Fig. **2B**). In each case no cytotoxic effects could be observed.

The differences in the activity of the *Arnica* preparations correlate with their content and type of Sls (see Table **1**). The qualitative composition was determined by means of GC/MS analyses and by direct GC comparison with authentic compounds as far as available, the quantitative content was studied by HPLC analysis according to [10]. The Arbo tincture, yielding mostly helenalin derivatives was two fold more active in NF-κB inhibition than the Spanish tincture containing 11α,13-dihydrohelenalin esters. ArnikaGel®, with the lowest content of dihydrohelenalin derivatives, was less active.

In contrast to the results with the tinctures, it was recently shown that pure helenalin was about 20-fold more active than

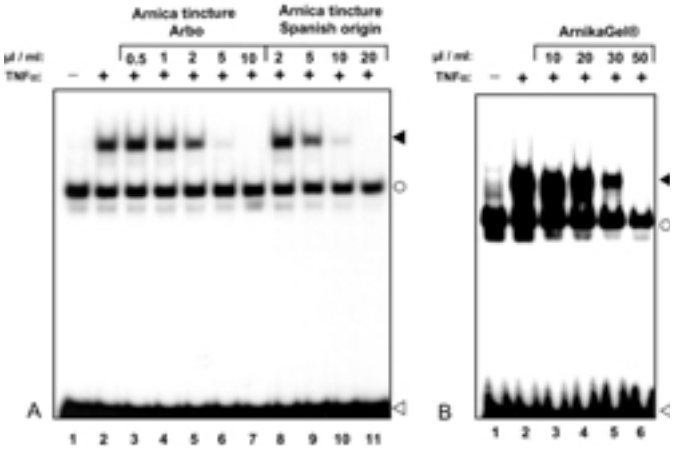


Fig. **2** **A** and **B** Effect of *Arnica* tinctures and ArnikaGel® on NF-κB DNA binding. Lane 1 shows unstimulated control cells; lane 2, cells treated with TNF-α alone. In the other lanes cells were pretreated for 1 h with the indicated amounts of *Arnica* tinctures or ArnikaGel®. Subsequently, cells were stimulated with 200 U/ml TNF-α for 1 h. Equal amounts of protein from cell extracts were analyzed for NF-κB activity by EMSA. A filled arrowhead indicates the position of NF-κB DNA complexes. The open circle denotes a non-specific activity binding to the probe and the open arrowhead shows unbound oligonucleotide.

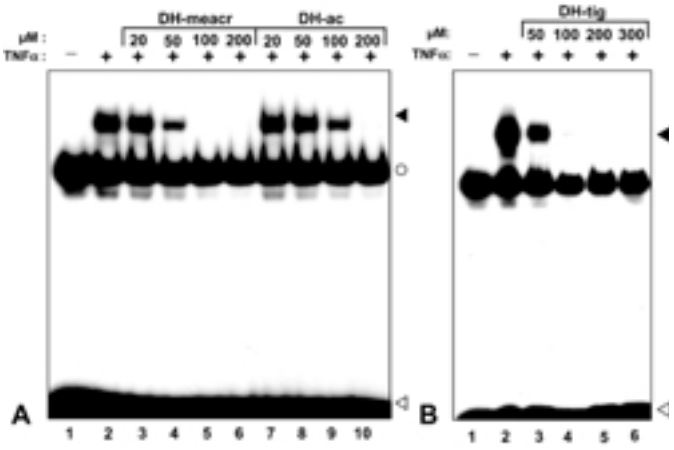


Fig. **3** **A** and **B**. Effects of 11α,13-dihydrohelenalin methacrylate, tiglinate and acetate, Sls isolated from flowerheads of *A. montana*, on NF-κB DNA binding. Lane 1 shows unstimulated control cells; lane 2, cells treated with TNF-α alone. In the other lanes cells were pretreated for 1 h with various concentrations of the indicated SL. Subsequently, cells were stimulated with 200 U/ml TNF-α for 1 h. Equal amounts of protein from cell extracts were analyzed for NF-κB activity by EMSA. A filled arrowhead indicates the position of NF-κB DNA complexes. The open circle denotes a non-specific activity binding to the probe and the open arrowhead shows unbound oligonucleotide.

pure 11α,13-dihydrohelenalin [3]. Therefore, we elucidated whether there might be differences in the inhibitory potency between dihydrohelenalin esters which possess different acyl moities and the unesterified compound. As shown in Fig. **3A** and **B**, 11α,13-dihydrohelenalin acetate also impaired NF-κB DNA binding at a 200 μM concentration and showed the same activity as the respective unesterified compound. However, the methacrylate and tiglinate, which are the dominant Sls in Spanish *Arnica* flowerheads, only required the concentration of 100 μM for complete NF-κB inhibition without showing any cytotoxic effects (see Fig. **3A** and **B**).

NF-κB is required for maximal transcription of various cytokines. *Arnica* preparations impair NF-κB DNA binding in an EMSA. To investigate whether signal transduction steps downstream of the transcription of cytokine genes are also inhibited, we evaluated whether the production of cytokines is also decreased by *Arnica* tinctures. We focused on IL-1 and TNF-α. Mononuclear cells from human blood were preincubated with the Spanish or Arbo tincture at various concentrations and subsequently stimulated with 1 μg/ml LPS from *Escherichia coli* for 4 h. Supernatants were collected and assayed in an ELISA. As cytokine release from mononuclear cells is mediated via a p38 MAP kinase dependent pathway, the pyridinylimidazole SB 203 580, a potent and selective inhibitor of p38 MAP kinase, was used as a positive control [15]. Both tinctures suppressed cytokine production in a concentration-dependent manner at low concentrations (see Fig. **4**). The Arbo tincture was more active. A concentration of 0.12 μl/ml was required to induce a 50 % inhibition ($IC_{50}$) of IL-1β. Higher concentrations were needed for suppression of TNF-α synthesis and

Table **1** Quantification of the phytopharmaceuticals for Sls by HPLC analysis (n = 3) using compound specific correction factors [10] and concentrations of phytopharmaceuticals from flowerheads of *A. montana* causing complete NF-κB or NF-AT inhibition. The amounts of the main Sls are separately calculated. Values are given in mg/ml. The total amount is related to the main SI occurring, in *Arnica* tincture from Spanish flowerheads and ArnikaGel® to 11α,13-dihydrohelenalin methacrylate, and in *Arnica* tincture from type Arbo flowerheads to helenalin tiglinate

| | ***Arnica* tincture *Spanish origin*** | **ArnikaGel®** | ***Arnica* tincture *Arbo*** |
|---|---|---|---|
| total content | 0.70 mg/ml | 0.22 mg/ml | 0.56 mg/ml |
| DH-ac | 0.04 | 0.01 | |
| DH-meacr | 0.22 | 0.05 | 0.02 |
| DH-tig | 0.10 | 0.03 | 0.05 |
| DH-i-val | 0.06 | 0.02 | |
| H-ac | | | 0.05 |
| H-i-but | | | 0.09 |
| H-tig | | | 0.08 |
| complete NF-κB inhibition | 10 μl/ml | 50 μl/ml | 5 μl/ml |
| complete NF-AT inhibition | 5 μl/ml | | 2 μl/ml |

an $IC_{50}$ value of 0.38 $\mu$l/ml was obtained. In contrast, concentrations of 0.24 and 1.78 $\mu$l/ml of the Spanish tincture were necessary for a half maximal suppression of IL-1$\beta$ and TNF-$\alpha$, respectively. The cell viability test by trypan blue exclusion was carried out to determine whether the inhibitory effects of the *Arnica* tinctures on cytokine release might be due to cytotoxic effects. Up to a concentration of 1 $\mu$l/ml no cytotoxicity was observed with Arbo tincture whereas 0.1, 1.0 and 10.0 $\mu$l/ml of the Spanish tincture all caused a mortality rate of about 4%, which can be neglected. Thus, it can be concluded that $IC_{50}$ values reported here are not influenced by cytotoxicity.

Having shown that dihydrohelenalin esters with unsaturated acyl moieties possess stronger inhibitory properties on NF-κB DNA binding in EMSA than those compounds esterified with saturated acids it was interesting to investigate whether this behavior could also be observed in *in vivo* inflammatory models. Therefore, the croton oil-induced mouse ear edema assay was used as an established model of acute inflammatory response [14]. The acetate and methacrylate of 11$\alpha$,13-dihydrohelenalin were tested at a dose of 1 $\mu$mol/cm$^2$, in comparison to the non-steroidal anti-inflammatory drug indomethacin (0.2 $\mu$mol/cm$^2$) which was used as a positive reference. The results obtained are given in Table **2**. 11$\alpha$,13-Dihydrohelenalin esterified with the unsaturated methacrylic acid induced 77% edema inhibition and exerted a stronger effect than the corresponding acetate derivative, which reduced the edematous response by 54%. As expected, 0.2 $\mu$mol/cm$^2$ of indomethacin reduced the edematous response by 44%.

Transcription of the cytokine TNF-$\alpha$ is regulated by the transcription factor NF-κB and NF-AT [7]. Therefore, the tinctures were also investigated for an influence on the activation of NF-AT. Jurkat T cells (5 – 6 × $10^5$ cells/ml) were incubated with tincture at

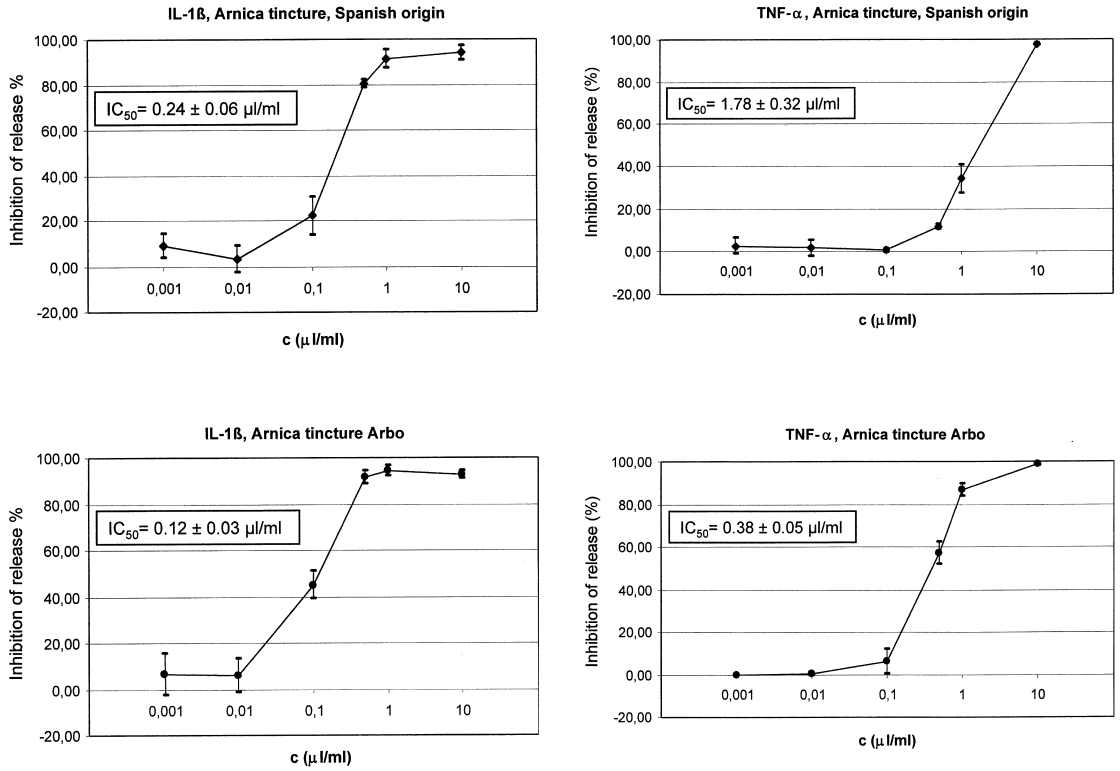


Fig. **4** Inhibitory effect of *Arnica* tinctures prepared from different *Arnica* flowers on IL-1$\beta$ and TNF-$\alpha$ synthesis in human mononuclear cells stimulated with LPS from *Escherichia coli* for 4 h. Supernatants were assayed in ELISAs. SB 203 580 was used as reference compound yielding $IC_{50}$ values of 0.08 + 0.06 $\mu$mol/l for IL-1$\beta$ inhibition and 0.19 + 0.06 $\mu$mol/l for that of TNF-$\alpha$. All values are the mean of three/four independent measurements.

Table **2** Effect of 11$\alpha$,13-dihydrohelenalin acetate and methacrylate from *Arnica* flowers on the croton oil-induced mouse ear edema. Indomethacin was used as reference compound

| *Substance* | *Dose ($\mu$mol/cm$^2$)* | *No. of animals* | *Edema (in mg) m ± S.E.* | *Edema Inhibition (%)* |
|---|---|---|---|---|
| control | – | 10 | 7.0 ± 0.3 | – |
| 11$\alpha$,13-dihydrohelenalin acetate | 1.0 | 10 | 3.2 ± 0.5* | 54 |
| 11$\alpha$,13-dihydrohelenalin methacrylate | 1.0 | 10 | 1.6 ± 0.3* | 77 |
| Indomethacin | 0.2 | 10 | 3.9 + 0.4* | 44 |

*$p < 0.001$ in the Student's *t*-test.

various concentrations for 1 h prior to stimulation with ionomycin and PMA for 3 h. Nuclear protein extracts were prepared and analyzed for NF-AT DNA binding activity in an EMSA using an oligonucleotide comprising the NF-AT binding sequence from the GM-CSF promoter [12]. Stimulation with ionomycin and PMA induced one novel DNA binding activity in Jurkat T cells (Fig. **5**, lane 2). Competition assays identified this complex as NF-AT (data not shown). NF-AT DNA binding was significantly reduced by pretreatment of Jurkat T-cells with 2 $\mu$l/ml of *Arnica* tincture from Spanish flowerheads, while 5 $\mu$l/ml completely prevented NF-AT DNA binding (see Fig. **5**, lanes 10 and 11). Arbo tincture was nearly twice as active. Here no NF-AT could be detected at a concentration of 2 $\mu$l/ml (see Fig. **5**, lane 5).

## Discussion

Herbal remedies have become increasingly popular in recent years. Therefore, it is not only important to prove their biological activity, but also to identify their active components and elucidate their molecular mechanism of action. This can already be applied to preparations from flowerheads of *Arnica montana* [2], [3], [4], whose most active compounds are known to be sesquiterpene lactones [1].

Here an *Arnica* tincture was prepared from *Arnica* flowerheads which originate from the easily and economically cultivated type "Arbo". For the first time its content and composition of Sls is described and correlated with its NF-κB inhibiting activity. Moreover, these results are compared to those obtained from the Spanish tincture. Each tincture should have its advantages. While the Spanish one presumably causes fewer side effects like contact dermatitis, for which compounds with an $\alpha$-methylene-$\gamma$-lactone ring are mainly responsible [1], the Arbo tincture possesses a stronger NF-κB inhibitory activity.

Moreover, three 11$\alpha$,13-dihydrohelenalin esters being the main occurring Sls in the Spanish tincture were studied for their inhibitory NF-κB DNA binding activity and new insights in structure-activity relationships were gained. Whereas we have previously proposed that the most important structural elements for a potent inhibitory activity are reactive centers, such as an exocyclic methylene group and an $\alpha$,ß-unsaturated carbonyl group [16], a further one, namely an $\alpha$,ß-unsaturated ester group has to be added. The methacrylate and the tiglinate of dihydrohelenalin were twice as active as dihydrohelenalin or its acetate. Studies are in progress to elucidate the basis for this stronger activity.

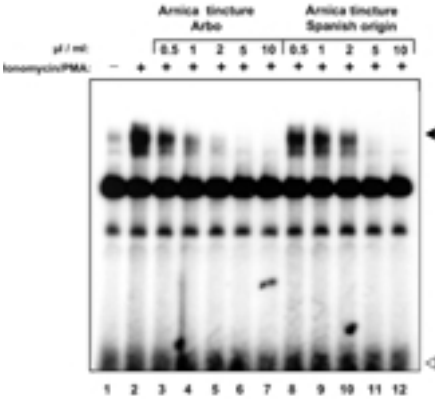


Fig. **5** Effect of *Arnica* tinctures on NF-AT DNA binding. Lane 1 shows unstimulated control cells. In lane 2 cells were treated with 2 $\mu$M ionomycin and 100 ng/ml PMA for 3 h. In the other lanes cells were preincubated with various amounts of *Arnica* tincture for 1 h. A filled arrowhead indicates the position of the NF-AT DNA complex. The open circle denotes a non-specific activity binding to the probe and the open arrowhead shows unbound oligonucleotide.

Both *Arnica* tinctures were studied for the first time on their influence on cytokine production. It was shown that their content and type of Sls correlate with their ability to inhibit cytokine release. Again, Arbo tincture inhibited IL-1 and TNF-$\alpha$ production more effectively than the Spanish tincture. Both cytokines are upregulated and/or disregulated in inflamed tissue and contribute directly or indirectly to the pathology in the synovial joint tissue [17]. IL-1 causes activation of T- and B-lymphocytes and induces chemotaxis of neutrophil granulocytes and other cells. It stimulates proliferation of fibroblasts and upregulation of COX-2, phospholipase $A_2$ and connective tissue degrading metalloproteinases, like collagenase. TNF-$\alpha$ has similar biological functions to those of IL-1 [17]. Our results are in accordance with recent reports demonstrating that Sls are able to inhibit the cytokines TNF-$\alpha$ and IL-1 ([18], and lit. cited therein), which are regulated by the transcription factor NF-κB.

A time-dependent cytokine production of IL-1 can also be observed in the croton oil-induced mouse ear edema [19]. For this reason, we compared the topical anti-inflammatory effect of 11$\alpha$,13-dihydrohelenalin acetate and methacrylate, showing that the latter compound was more active than the acetate derivative (77 and 54% oedema reduction, respectively, at a dosage of 1 $\mu$mol/cm$^2$). This result correlates well with that obtained in the NF-κB EMSA and shows again the influence of the unsaturated ester moiety.

TNF-$\alpha$, which is believed to be one of the main proinflammatory cytokines, has also binding sites in its promoter region for the transcription factor NF-AT. Therefore, we extended our studies on NF-AT and demonstrated that both tinctures were even more active in the NF-AT than in the NF-κB assay. For both, no NF-AT was detected at about half the concentration necessary for a complete NF-κB inhibition. Studies are in progress to elucidate how the effective ingredients of the tinctures, the Sls, inhibit the DNA binding activity of NF-AT. Preliminary studies suggest a different mechanism than that proved for cyclosporin and FK506 (unpublished data). These immunosuppressive drugs inhibit the phosphatase activity of calcium- and calmodulin-dependent calcineurin [20]. However, they also inhibit calcineurin activity in other tissues, which causes side effects, e.g. nephrotoxicity and neurotoxicity. Therefore, drugs which act in another way as cyclosporin and FK506 may be clinically preferable.

By inhibiting NF-κB and NF-AT and subsequently cytokine production *Arnica* tinctures act in a similar way as glucocorticoids, which also interfere with NF-AT and NF-κB–driven promoter activity [21], [22]. For inhibition of NF-κB a model was proposed in which glucocorticoids repress NF-κB-driven genes by interfering with the mechanistic interaction of p65 with the basal transcription machinery. Recently, we proved that Sls act directly on the p65 subunit, too [3]. It has been suggested that therapeutic agents directed at this protein might be useful, for instance in

the treatment of inflammatory bowel or rheumatic disease [6]. Phytomedicines may also be used successfully for the treatment of these inflammatory diseases, especially when their active compounds can be defined as is the case with preparations from *Arnica montana* flowers. However, it has also to be taken into account that the benefit of a therapy suppressing the activity of central mediators of the immune response, such as NF-κB and NF-AT, will depend on the delicate balance between suppressing inflammation and interfering with normal cellular functions.


## Acknowledgements

We thank Mrs. I. Hirschmüller-Ohmes for technical assistance in HPLC analysis and isolation of sesquiterpene lactones and Mrs. M. Weber for recording the GC/MS (both Institute of Pharmaceutical Biology, Freiburg). We are grateful to the Kneipp-Werke, Würzburg, Germany, for the supply of the Spanish *Arnica* tincture and ArnikaGel®.


## References


[1] Willuhn G. Arnica Flowers; Pharmacology, toxicology and analysis of the sesquiterpene lactones – their main active substances. In: Phytomedicines of Europe. Chemistry and biological activity. Lawson LD, Bauer R, Eds. ACS Symposium Series 691 Washington, DC: American Chemical Society, 1998: 118 – 32

[2] Lyß G, Schmidt TJ, Merfort I, Pahl HL. Helenalin, an anti-inflammatory sesquiterpene lactone from *Arnica*, selectively inhibits transcription factor NF-κB. Biological Chemistry 1997; 378: 951 – 61

[3] Lyß G, Knorre A, Schmidt TJ, Pahl HL, Merfort I. The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-κB by directly targeting p65. Journal of Biological Chemistry 1998; 273: 33 508 – 16

[4] Lyß G, Schmidt TJ, Pahl HL, Merfort I. Anti-inflammatory activity of *Arnica* tincture (DAB 1998) using the transcription factor NF-κB as molecular target. Pharmaceutical and Pharmacological Letters 1999; 9: 5 – 8

[5] Lyß G. Thesis. University Freiburg, 2000

[6] Tak PP, Firestein GS. NF-κB: a key role in inflammatory diseases. The Journal of Clinical Investigation 2001; 107: 7 – 11

[7] Serfling E, Berberich-Siebelt F, Chuvpilo S, Jankevics E, Klein-Hessling S, Twardzik T, Avots A. The role of NF-AT transcription factors in T cell activation and differentiation. Biochimica et Biophysica Acta 2000; 1498: 1 – 18

[8] von Raison J, Heilmann J, Merfort I, Schmidt TJ, Brock FE, Leven W, Bomme U, Bauer R. Arnika – Arzneipflanze mit Tradition und Zukunft. Zeitschrift für Phytotherapie 2000; 21: 39 – 54

[9] Willuhn G, Röttger PM, Wendisch D. Helenalin- und 11α,13-Dihydrohelenalinester aus Blüten von *Arnica montana*. Planta Medica 1984; 50: 35 – 7

[10] Willuhn G, Leven W. Zur qualitativen und quantitativen Analyse der Sesquiterpenlactone von Arnikablüten DAB 9. Pharmazeutische Zeitung - Wissenschaft 1991; 136: 32 – 9

[11] Schreiber E, Matthias P, Müller MM, Schaffner W. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Research 1989; 11: 6419

[12] Cockerill PN, Shannon MF, Bert AG, Ryan GR, Vadas MA. The granulocyte-macrophage colony-stimulating factor/interleukin 3 locus is regulated by an inducible cyclosporin A-sensitive enhancer. Proccceedings of the National Academy of Science 1993; 90: 2455 – 70

[13] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidmann J, Smith JA, Struhl editors. In: Current Protocols in Molecular Biology. J. Wiley and Sons, New York: 1998: 11.5.1

[14] Tubaro A, Dri P, Delbello G, Zilli C, Della Loggia R. The croton oil ear test revisited. Agents and Actions 1985; 17: 347 – 9

[15] Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, Strickler JE, McLaughlin MM, Siemens IR, Fisher SM, Livi GP, White JR, Adams JL, Young PR. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 1994; 372: 739 – 46

[16] Rüngeler P, Castro V, Mora G, Gören N, Vichnewski W, Pahl HL, Merfort I, Schmidt TJ. Inhibition of transcription factor NF-κB by sesquiterpene lactones – a proposed molecular mechanism of action and structure activity relationships. Bioorganic & Medinal Chemistry 1999; 7: 2343 – 52

[17] Scott DL, Willoughby DA, Blake DR. Molecular insights into rheumatoid arthritis. Molecular Aspects of Medicine 1991; 12: 341 – 94

[18] Koch E; Klaas CA, Rüngeler P, Castro V, Mora G, Vichnewski W, Merfort I. Inhibition of inflammatory cytokine production and lymphocyte proliferation by structurally different sesquiterpene lactones correlates with their effect on activation of NF-κB. Biochemical Pharmacology 2001; 62: 795 – 801

[19] Towbin H, Pignat W, Wiesenberg I. Time-dependent cytokine production in the croton oil-induced mouse ear oedema and inhibition by prednisolone. Inflammation Research 1995 (Supplement); 44: S160 – 1

[20] Schreiber SL, Crabtree GR. The mechanism of action of cyclosporin A and FK506. Immunology Today 1992; 13: 136 – 42

[21] Chen R, Burke TF, Cumberland JE, Brummet M, Beck LA, Casolaro V, Georas SN. Glucocorticoids inhibit calcium- and calcineurin-dependent activation of the human IL-4 promoter. Journal of Immunology 2000; 164: 825 – 32

[22] De Bosscher K, Vanden Berghe W, Vermeulen L, Plaisance S, Boone E, Haegeman G. Glucocorticoids repress NF-κB-driven genes by disturbing the interaction of p65 with the basal transcription machinery, irrespective of coactivator levels in the cell. Proceedings of the National Academy of Sciences 2000; 97: 3919 – 24