Sesquiterpene lactone containing Mexican Indian medicinal plants and pure sesquiterpene lactones as potent inhibitors of transcription factor NF-κB

Peter M. Bork[a], M. Lienhard Schmitz[1,b], Michaela Kuhnt[2,a], Claudia Escher[b], Michael Heinrich[a,*]

[a]*Institute of Pharmaceutical Biology, Schänzlestr. 1, Albert-Ludwigs University, 79104 Freiburg, Germany*
[b]*Institute of Biochemistry and Molecular Biology, H. Herderstr. 7, Albert-Ludwigs University, 79104 Freiburg, Germany*

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Abstract The potential inhibitory effect of 54 Mexican Indian medicinal plants on the activation of transcription factor NF-κB was studied. Band-shift experiments identified the ethanolic leaf extracts of *Artemisia ludoviciana* ssp. *mexicana*, *Calea zacatechichi*, and *Polymnia maculata* (all rich in sesquiterpene lactones) as inhibitors of NF-κB down to a concentration of 25 μg/ml. The sesquiterpene lactones isohelenin and parthenolide prevented NF-κB activation completely as low as 5 μM. Treatment of HeLa cells with leaf extract of *A. ludoviciana* ssp. *mexicana*, isohelenin and parthenolide prevented the induction of transcription on the IL-6 promoter. These experiments identify the eudesmanolide and germacranolide type of sesquiterpene lactones as potent non-antioxidant inhibitors of NF-κB. All plants active in the NF-κB assay also showed a delay in the onset of capillary reactions of the allantois membrane in a physiological model for anti-inflammatory activity — the HET-CAM assay.

Key words: Transcription factor NF-κB; Mixe Indian traditional medicine, Mexico; Anti-inflammatory; HET-CAM assay; Sesquiterpene lactone; Asteraceae

1. Introduction

NF-κB is a transcription factor consisting either of homo- or heterodimers of various subunits [1]. The five distinct DNA-binding subunits share an N-terminal homology region responsible for DNA-binding, dimerization and nuclear translocation. In its inactivated state it is bound to inhibitory IκB subunits. Stimulation of cells with many pathogenic or inflammatory agents leads to the increase of reactive oxygen intermediates (ROIs) and to the inducible phosphorylation and ubiquitinilation of IκB, which is prerequisite for its proteolytical degradation by the proteosome. The dimer enters the nucleus, binds to its recognition sequence in promoter regions of its target genes and induces their transcription [2]. Among these are important mediators of the immune and inflammatory response, including inflammatory cytokines such as IL-6, cell adhesion molecules, immunoreceptors, hemotopoetic growth factors, acute phase proteins, and transcription factors [1]. Triggering of the inflammatory response by NF-κB is achieved by the induced expression of the inflammatory cytokines TNF-α, IL-2, IL-6, IL-8, granulocyte/macrophage colony-stimulating factor and granulocyte colony-stimulating factor [3]. These and many more gene products stimulate the immune response and contribute to the activation of macrophages, monocytes and the T-cell response. The important role of NF-κB during inflammatory events is also evident from knock-out experiments and reveals this transcription factor as a relevant target for potential anti-inflammatory agents [3]. Also the anti-inflammatory glucocorticoids are able to repress NF-κB activity by a molecular cross-talk mechanism involving the glucocorticoid receptor [4]. Therefore compounds inhibiting NF-κB are potentially of great interest for developing lead structures for the treatment of acute and chronic inflammation such as Crohn's disease or chronic arthritis.

A potential source for NF-κB inhibitors (or stimulators) are medicinal plants used in indigenous medical sytems. This study is based on plants used in the indigenous medicine of the lowland Mixe (Oaxaca, Mexico; [5–7]). Therefore the medicinal plant extracts were tested for their potential effects on NF-κB. In addition, the hen egg test-chorioallantoic membrane (HET-CAM assay) using 10-day-old incubated hens' eggs was employed to study physiological anti-inflammatory effects of 19 of the 54 plants. This assay detects inhibition of the permeability of capillary systems and/or capillary stabilizing actions [8].

2. Materials and methods

2.1. Materials

2.1.1. Botanical documentation. Voucher specimens of all plants were collected and are deposited at the National Herbarium of Mexico (MEXU) in México, D.F., the Herbarium of the 'Instituto Mexicano del Seguro Social' (IMSSM) in México, D.F and the 'Institut für Pharmazeutische Biologie' in Freiburg, Germany (collection numbers Heinrich 1-350). Plants were identified by comparison with authentic specimens (for details on (ethno-)botanical data see [5]). Some plants (marked [b] in Table 1) are available to Mexican indian communities through a system of herb markets [9]. Two plant extracts are included which are not used by the Mixe, but in other areas (*Hyptis mutabilis*: malaria, stomach pain flatulence [10]; *Polymnia maculata*: malaria, fever; Heinrich, unpublished fieldnotes).

*Corresponding author. Fax: (49) 761-203-2803.

[1]*Current address:* DKFZ, Department of Immunochemistry, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany.

[2]*Current address:* Sandoz Research Institute, Brunnerstr. 59, 1235 Wien, Austria.

Abbreviations: AMLE, *Artemisia ludoviciana* ssp. *mexicana* leaf extract; ASA, acetylsalicylic acid; CZLE, *Calea zacatechichi* leaf extract; EMSA, electrophoretic mobility shift assay; HET-CAM, hen egg test-chorioallantoic membrane, NF-κB; PDTC, ammonium pyrrolidinedithiocarbamate; PMA, phorbol 12-myristate 13-acetate; PMLE, *Polymnia maculata* leaf extract; PMSF, phenylmethylsulfonyl fluoride; ROIs, reactive oxygen intermediates; SDS, sodium dodecyl sulfate; SL, sesquiterpene lactone

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2.1.2. Biochemical material. Poly dIdC and TNF-α were obtained from Boeringer-Mannheim (Germany), Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS) and penicillin/streptomycin from Gibco BRL, hygromycin from Calbiochem (USA), parthenolide (97%) from Aldrich (Germany), sterile filters (0.45 μm) from Schleicher and Schuell (Germany) and pMEB4 from Invitrogen (USA). All other chemicals were either from Sigma or Roth.

2.2. EMSA

Lyophilized, ethanolic (96%) extracts of plants [11] were prepared. For all assays between 10 and 40 g of air-dried plant material was extracted once with simmering ethanol (96%) and twice with ethanol (70%). The resulting combined extracts were concentrated, freeze-dried, redisolved in DMSO (final concentration in medium ≤0.5%) and then suspended in DMEM containing 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin (final concentration of extracts: 20, 50 or 100 μg/ml). Disolved extracts tested by EMSA were filtrated through sterile filters. Only extracts which showed no cytotoxicity after 2 h of incubation at 50 μg/ml were further analyzed for their effects on the activity of NF-κB. Approximately 5×10^5 HeLa cells (ATCC CCL2) were grown overnight on 10 cm dishes at 37°C and 5% CO_2. One hour prior to stimulation of cells the medium was replaced by the one containing the dissolved plant extracts. Subsequently the cells were stimulated with 50 ng/ml of the NF-κB activator phorbol 12-myristate 13-acetate (PMA) for 20 min. The HeLa cells were washed twice with TBS buffer (25 mM TRIS-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.1 mM $MgCl_2$). After addition of 1 ml of TBS buffer the cells were harvested with a rubber policeman and transferred into Eppendorf tubes. The HeLa cells were pelleted by centrifugation at 3000 rpm for 3 min at 4°C. The pellet was resuspended in TOTEX buffer (20 mM Hepes/KOH, pH 7.9, 0.35 M NaCl, 20% (v/v) glycerol, 1% (v/v) NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM dithiothreitol (DTT)) and incubated on ice for 30 min. The samples were carefully vortexed every 10 min. The cell debris was pelleted by centrifugation with 14000 rpm at 4°C for 10 min. Equal amounts of supernatant protein were tested for DNA binding activity of NF-κB (or AP-1 or Oct-1) by EMSA as described [12]. The radical scavenger ammonium pyrrolidinedithiocarbamate (PDTC) was used as a positive control.

The oligonucleotides used were (binding site underlined):

AP-1	5′-CGCTTGATGAGTCAGCCGGAA-3′
	3′-GCGAACTACTCAGTCGGCCTT-5′
NF-κB:	5′-AGTTGAGGGGACTTTCCCAGGC-3′
	3′-TCAACTCCCCTGAAAGGGTCCG-5′
Oct-1	5′-TGTCGAATGCAAATCACTAGAA-3′
	3′-ACAGCTTACGTTTAGTGATCTT-5′

2.3. Transactivation assay

HeLa cells were stably transfected with a *luciferase* reporter gene controlled by the IL-6 promoter (a kind gift of M. Merola, Verona) and the pMEB4 selection plasmid with the $CaPO_4$ method as described [13]. Two days after transfection the medium was changed and hygromycin was added at a concentration of 200 μg/ml. Another 4 weeks later the cell clones were isolated and tested for the inducibility of luciferase expression after stimulation with TNF-α/PMA. Clone number 97 showed a good inducibility and was used to test transcriptional effects of the extracts and pure compounds described in this study. 10^5 cells of the HeLa clone 97 were pre-incubated for 1 h with the extracts as specified in the figure legend. Subsequently, the

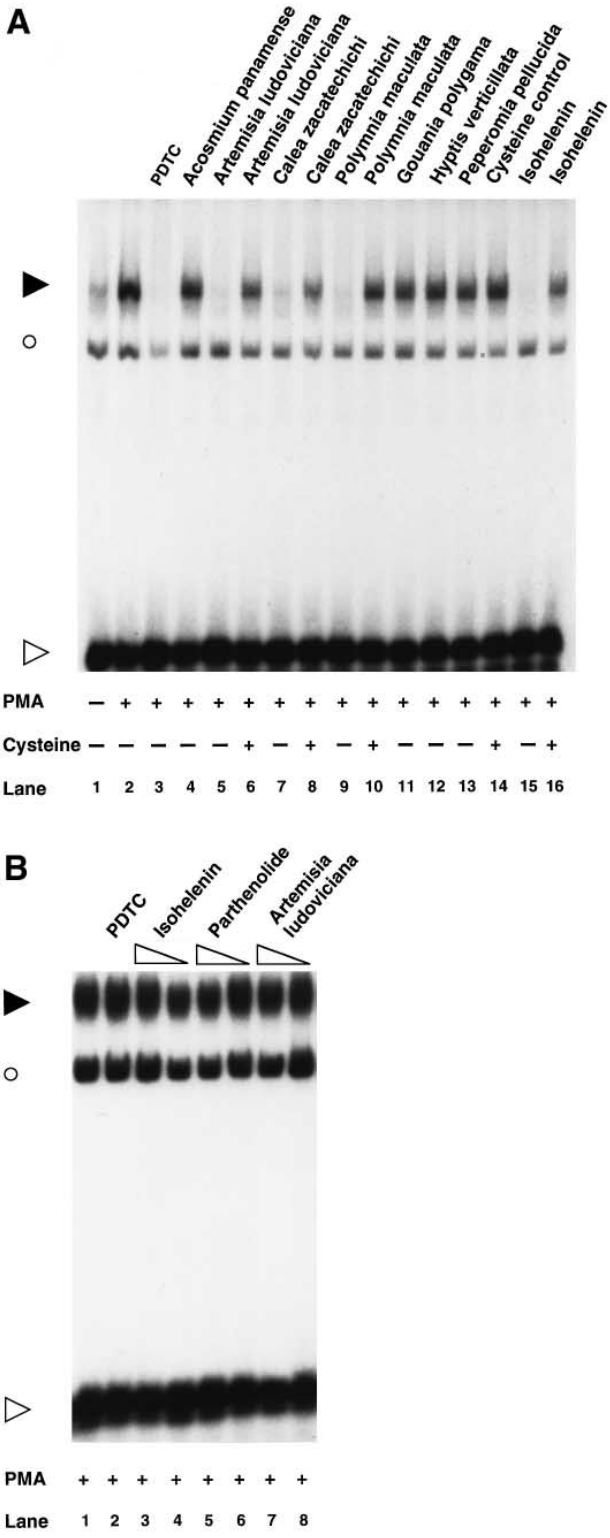

Fig. 1. Effects of medicinal plant extracts and SLs on the activation of transcription factors NF-κB and AP-1. A: Effects of medicinal plant extracts (100 μg/ml) and isohelenin (20 μM) (with and without the addition of 1 mM cystein in case of the 5 active samples) on NF-κB. HeLa cells were pre-incubated with the indicated plant extracts and subsequently stimulated with PMA as indicated. EMSA was performed by incubating equal amounts of nuclear protein extracts with a ^{32}P-labelled oligonucleotide containing a NF-κB site. An autoradiogram of a typical experiment is shown. ▷, Unbound DNA oligonucleotide; ○, position of a constitutively binding protein; ▶, position of the NF-κB–DNA complex. For inhibiting the activity the crude extracts and isohelenin were pre-incubated for 8 h with 1 mM cysteine. B: Effects of AMLE (50 μg/ml, 100 μg/ml), isohelenin (5 μM, 10 μM) and parthenolide (5 μM, 10 μM) on AP-1. HeLa cells were pre-incubated with AMLE or pure compounds, stimulated with PMA and further tested as described in (A), except that an oligonucleotide encompassing an AP-1 binding site was used for EMSA. The symbols are as described in (A); ▶, position of the AP-1–DNA complex.

Table 1
Medicinal plants from the Mixe ethnobotanical collection tested for inhibitory activity on NF-κB

Species	Family	Plant parts	Relevant uses
Acacia cornigera L.[a]	Mimosaceae	LE	strong skin infections
Acalypha alopecuroides Jacq.[a]	Euphorbiaceae	AP	skin infections
Acosmium panamense (Benth.) Yakov[a]	Fabaceae s.str.	BA	stomachache, malaria, hemorrhage
Agastache mexicana (Kunth) Lint. and Epl.[b]	Lamiaceae	AP	various gastrointestinal, nervous and cardio-vascular complaints
Annona muricata L.	Annonaceae	BA	diarrhoea
Annona reticulata L.	Annonaceae	BA	diarrhoea
Artemisia ludoviciana ssp. *mexicana* Nutt.[a]	Asteraceae	LE	stomachache, vomiting
Asclepias curassavica L.	Asclepiadaceae	AP	purgative
Begonia heracleifolia Schlecht. and Cham.[a]	Begoniaceae	LE	gonorrhoea
Buddleja americana L.	Loganiaceae	LE	stomachache
Byrsonima crassifolia (L.) Kunth[a]	Malpighiaceae	BA	diarrhoae
Calea urticifolia (Mill.) D.C.[a]	Asteraceae	LE	stomachache
Calea zacatechichi Schlecht[a]	Asteraceae	LE	skin infections
Capraria biflora L.	Scrophulariaceae	AP	itching skin, skin and vaginal infections
Castela texana (Torr et Gray) Rose	Simaroubaceae	WO	stomachic
Castilleja tenuifolia Benth.[a,b]	Scrophulariaceae	AP	skin infections
Cecropia obtusifolia Nert.	Cecropiaceae	LE	'diabetis'
Chaptalia nutans (L.) Pollak[a]	Asteraceae	LE	skin infections
Chenopodium graveolens Willd.[a]	Chenopodiaceae	AP	intestinal parasites
Cissampelos pareira L.	Menispermataceae	RO	dysentery
Cordia curassavica (Jacq.) Roem. and Schlecht.	Boraginaceae	LE	fever, hemorrhage, colds
Desmodium incanum D.C.	Fabaceae	LE	blood in the urin
Dracocephalon moldavica L.[b]	Lamiaceae	AP	see *Agastache mexicana*
Eugenia acapulcensis Steud.	Myrtaceae	LE	dysentery
Fraxinus uhdei (Wenzig) Lingel.[b]	Oleaceae	LE	fever
Gouania polygama (Jacq.) Urban[a]	Rhamnaceae	LE	skin infections
Guazuma ulmifolia Lam.	Sterculiaceae	BA	diarrhoea, hemorrhage
Hippocratea excelsa Kunth[a,b]	Hippocrateaceae	BA	hemorrhage
Hyptis mutabilis (L.C. Rich) Briq.	Lamiaceae	LE	–
Hyptis verticillata Jacq.[a]	Lamiaceae	LE	skin infections, gastrointestinal pain
Justicia spicigera Schldl.[a,b]	Acanthaceae	AP/LE	general stimulant, diarrhoea
Leonurus japonicus Houtt	Lamiaceae	LE	pain in the 'lower part of the belly'
Loeselia mexicana Brand[b]	Polemoniaceae	LE/FL	fever, purgative
Ludwigia octovalvis (Jacq.) Raven[a]	Onagraceae	LE	skin infections, insect bites
Malvaviscus arboreus Cav.	Malvaceae	AP	diarrhoea, labour problems
Miconia albicans (Sw.) Triana[a]	Melastomataceae	LE	skin and mouth infections
Mimosa tenuiflora Willd.	Mimosaceae	BA	skin burns
Muntingia calabura L.	Sterculiaceae	BA	fever
Pavonia schiedeana Steudel	Malvaceae	LE	fever
Peperomia pellucida (L.) Kunth[a]	Piperaceae	AP	fungal skin infections
Pluchea symphytifolia (Miller) Gilis	Asteraceae	LE	ear and skin infections, stomachache, diarrhoea, difficult menstruation
Polymnia maculata Cav.	Asteraceae	LE	–
Psidium guayava L.	Myrtaceae	BA	diarrhoea
Quercus oleoides Schlecht. and Cham.	Fagaceae	BA	diarrhoea
Russelia sarmentosa Jacq.	Scrophulariaceae	LE	stomachache
Satureja macrostema Bricq. [b]	Lamiaceae	AP	gastrointestinal problems, bathes for women in labour
Sida acuta Burm.	Malvaceae	AP	toothache, itching skin, fever
Sida rhombifolia L.	Malvaceae	AP	toothache, itching skin, fever
Sinningia incarnata (Aublet) D. Dehnh.	Gesneriaceae	TU	dysentery
Smilax lanceolata L.	Smilacaceae	RO	hemorrhage
Spondias purpurea L.	Anacardiaceae	BA	diarrhoae (esp. in children)
Tithonia diversifolia (Hemsl.) Gray	Asteraceae	LE	skin infections, fever
Tournefortia glabra L.	Boraginaceae	LE	skin infections
Waltheria americana L.[a,b]	Sterculiaceae	AP	skin afflictions, diarrhoea

Plant parts used: AP, aerial parts; BA, bark; FL, flowers; FR, fruit; LE, leaves; RO, root/rootstock; SE, seed; ST, stem; TU, tubers; WO, wood.
[a]Tested in the HET-CAM assay (see text).
[b]Plants sold on Mexican markets and thus available to the community (ethnobotanical data based on [9,10]).

expression of the IL-6 promoter was induced by treatment of cells with 2000 U/ml human TNF-α and 20 ng/ml PMA. After 7 h of stimulation the cells were harvested and the luciferase activity was measured as described [13].

2.4. HET-CAM

The HET-CAM assay utilizes hen eggs after 10 days of incubation [8]. Extracts and compounds were dissolved or suspended in 0.9% NaCl with 5% DMSO and injected into the egg white. Two hours later the shell was carefully opened on the spot where the gas bulb is situated. The inner egg membrane was carefully removed and 0.3 ml of 0.5% SDS (sodium dodecyl sulfate) was applied on the chorioallantoic membrane to induce capillary reactions (injection, haemorrhage, lysis). The activity of the test samples in stabilizing the capillary membrane is expressed as the time difference in percent compared to untreated controls (isotonic NaCl solution) until pathological reactions (injection of blood into the vessels of the allantois membrane and beginning of hemorrhage) are recorded. The statistical signifi-

cance ($P \leq 0.05$) of the results was ascertained (Student's t-test). Samples with values ≥20% change in comparison to control values are considered to possess promising activity. Each series of six eggs was repeated at least twice.

3. Results and discussion

All 54 plants included in this study are listed in Table 1. Most of the plants are reportedly used for illnesses associated with inflammatory processes, but species with other uses were also included. All plants available from this ethnobotanical collection were tested for their inhibiting activity on NF-κB activation. HeLa cells were pre-incubated with plant extracts at a concentration of 100 μg/ml prior to stimulation with PMA. Band-shift experiments with total cell proteins revealed that only the leaf extracts of *Artemisia ludoviciana* ssp. *mexicana* (AMLE), *Calea zacatechichi* (CZLE) and *Polymnia maculata* (PMLE) negatively interfered with the activation of NF-κB (positive control: 100 μM PDTC; Fig. 1A) and were active down to an concentration of 25 μg/ml (data not shown). Since it is known that these three Compositae are rich in sesquiterpene lactones (SL) [14–16] it seemed likely that this group of compounds is responsible for the observed inhibitory effects. The lactone ring carrying an exomethylene group forms a reactive Michael-system which is a nucleophilic target, e.g., for cystein [15]. SLs thus react with cystein which leads to a covalent modification of the oxomethylen group. The addition of cystein to the crude extract led to a complete loss in its inhibitory activity (Fig. 1A, see lanes 6, 8, 10 and 14), suggesting that SLs contribute to the inhibitory activity of the plant extract. To directly demonstrate that SLs inhibit the activation of NF-κB, a germacranolide (parthenolid) and an eudesmanolide (isohelenin) were tested. Both substances prevented the activation of this transcription factor as low as 5 μM (data only shown for parthenolid, Fig. 2). In a control experiment the addition of 1 mM cystein also abolished the inhibitory effects of parthenolide and isohelenin (>95%) (data only shown for isohelenin, Fig. 1A, lane 16). Santonine, an SL which lacks the exomethylene group, did not interfere with NF-κB activation even at a concentration of 100 μM (Fig. 2). Since most of the described inhibitors of NF-κB have antioxidative properties (for review see [17]) it is noteworthy that the chemical structure of isohelenin and parthenolide does not confer radical-scavenging activity. Future studies will show which of the multiple steps in the NF-κB activation cascade is affected by the SLs investigated. None of the plants was cytotoxic at 50 μg/ml and 2 h of incubation. All extracts active in the NF-κB assay were additionally tested for cytotoxic effects at 100 μg/ml and 6 h incubation. None of the three extracts displayed cytotoxic activity. In order to ensure the specificity of the inhibitory activities, the potential effects of the plant extracts and pure compounds active in the NF-κB-assay were tested on the transcriptions factors AP-1 and OCT-1. As shown in Fig. 1B isohelenin, parthenolide and AMLE did not influence DNA-binding of the inducible transcription factor AP-1. Also CZLE and PMLE did not affect DNA binding of AP-1 (data not shown). Similarly, the binding of the constitutively active transcription factor OCT-1 remained unchanged (data not shown).

We next tested the potential effects of the identified inhibitors isohelenin, parthenolide and AMLE crude extract on the transcription of the inflammatory cytokine IL-6, which is mainly regulated by NF-κB. HeLa cells were stably transfected with a construct harboring the IL-6 promoter fused to the *luciferase* reporter gene. The strong induction of IL-6

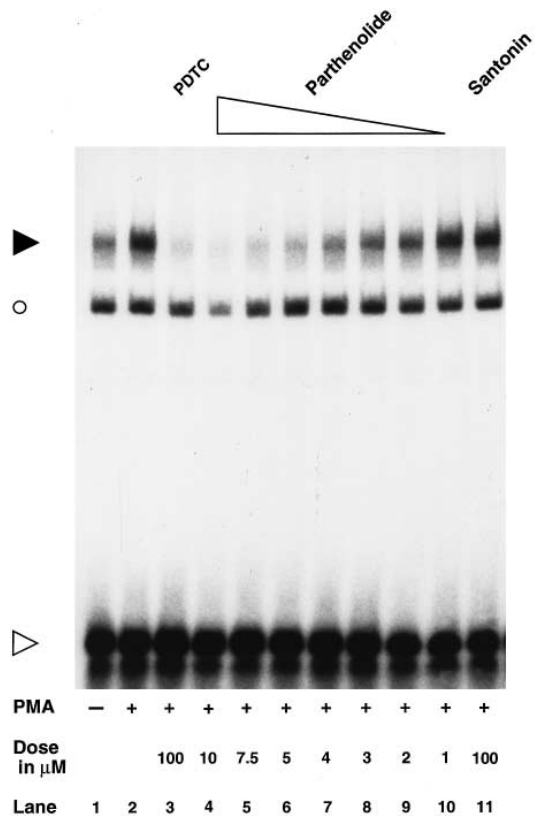

Fig. 2. The sesquiterpene lactone parthenolide inhibits activation of NF-κB, while santonin is inactive. HeLa cells were pre-incubated with various amounts of parthenolide or 100 μM santonin and tested for PMA-mediated activation of NF-κB by EMSA. A typical autoradiogram is shown (for explanation of the symbols see Fig. 1).

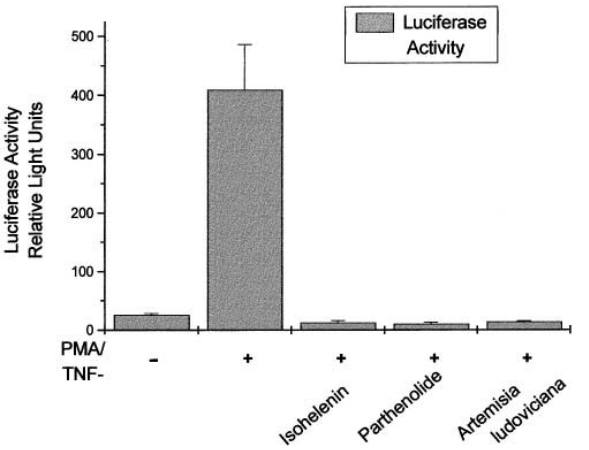

Fig. 3. Sesquiterpene lactones and medicinal plant extracts inhibit the activation of IL-6 transcription. HeLa cells stably transfected with the IL-6 promoter fused to the *luciferase* gene were pre-incubated with isohelenin, parthenolide and AMLE for 1 h. Subsequently transcription of the *IL-6* gene was induced upon addition of 2000 U/ml TNF-α and 20 ng/ml of PMA. Seven hours later cells were harvested and the luciferase activity was measured. Mean values of three independent experiments are shown; the bars indicate the standard deviation.

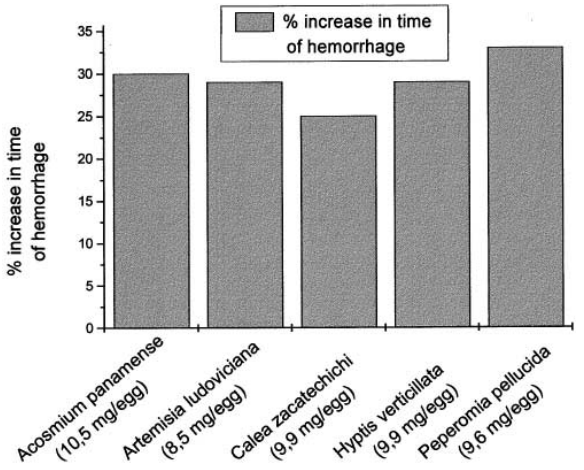

Fig. 4. The HET-CAM assay using 10-day-old incubated hen eggs detects inhibition of the permeability of capillary systems and/or capillary stabilizing actions on the chorioallantoic membrane. The figure shows the delay in onset of the pathological phenomena (injection and hemorrhage) for those extracts which exerted an effect of ≥20% (hemorrhage) as compared to untreated controls. The statistical significance ($P \leq 0.05$) of the raw data was ascertained (Student's *t*-test [7]). The figures in parentheses give the amount of extract applied (in mg/egg). All other extracts ($n = 14$) showed much lower activity.

transcription by stimulation of the stably transfected HeLa cells with PMA and TNF-α was completely suppressed by pre-incubation of cells with the inhibitors (Fig. 3). This proves that the three inhibitors also prevent the expression of inflammatory target genes of NF-κB.

Some plant extracts including two of those which showed inhibitory effects on NF-κB were tested for their anti-inflammatory effects in the more physiological HET-CAM assay. Fig. 4 shows the data for extracts which delayed the onset of the beginning of hemorrhage by ≥20% in the HET-CAM assay. Notably, both AMLE and CZLE which were active in the NF-κB assay also retarded the onset of pathological reactions in the HET-CAM assay significantly. This shows that the anti-inflammatory effects of these two taxa can at least partially be assigned to the inhibition of NF-κB.

Also extracts of *Acosmium panamense*, *Hyptis verticillata*, and *Peperomia pellucida* delayed the onset of the pathological effects (injection of blood into the vessels of the allantois membrane and beginning of hemorrhage) significantly. The molecular mechanism of their anti-inflammatory activity remains to be elucidated since those four plants did not interfere with the activity of NF-κB. *H. verticillata* crude extract and sideritoflavone isolated from this extract showed inhibitory effects on cyclooxygenase. In the case of the other three species the mode of action and active compounds are not yet known. 8 species showed an effect between −5% and +10% in the HET-CAM assay, which was statistically insignificant and six species showed a weak to moderate delay (10–20%) in the start of hemorrhage (data not shown). The positive controls rutoside (1.5 mg) and acetylsalicylic acid (ASA, 1.5 mg) showed a delay in the beginning of hemorrhage of 40% and 16%, respectively.

4. Conclusion

The identification of several Asteraceae as potent inhibitors of the transcription factor NF-κB shows that the assay is a useful new tool for evaluating plant extracts for potential anti-inflammatory activity. Of particular interest is a comparison with the results of the HET-CAM assay. The HET-CAM assay detects inhibitory effects of plant extracts and pure compounds on a broad array of pathophysiological inflammatory effects. All samples tested so far, which were active in the NF-κB assay, were also active in the HET-CAM test. In case of the two Asteraceae the delay of the pathological effects observed may therefore well be due to an inhibition of the transcription factor NF-κB. Since different types of sesquiterpene lactones showed an inhibitory effect down to similar molar concentrations, this effect seems to be characteristic for many of the sesquiterpene lactones with a lactone ring and an exomethylene group. Further studies are required to determine the structure–activity relationship. The inhibitory effect of SLs on NF-κB activation also gives an explanation for the continued use of SL-containing drugs (like *Tanacetum parthenium* and *Arnica montana*) as anti-inflammatoric remedies in popular and officinal medicine.

Until now approximately 80 plants used in Mexican Indian traditional medicine were evaluated in the NF-κB assay ([12] and this study) and four taxa inhibited the activation of NF-κB. The ethnobotanical [6,18] approach therefore provides useful pharmacological leads if it is combined with biochemical or physiological methods. Phytochemical studies of some of the active plants are currently in progress in order to isolate the active constituent(s).

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