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Research Paper

Effects of 20-hydroxyecdysone, *Leuzea carthamoides* extracts, dexamethasone and their combinations on the NF-κB activation in HeLa cells

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Abstract

Objectives The plant steroid 20-hydroxyecdysterone (20E) and 20E-containing extracts from *Leuzea carthamoides* (Willd.) DC are sold with claims of anabolic and immunomodulatory effects. Yet their effect on the activation of nuclear factor kappa B (NF-κB), a key player in immune response and cell fate, and their influence on the NF-κB-inhibiting activity of steroidal anti-inflammatory drugs is still unknown.

Methods The ability of 20E, Leuzea extracts and selected steroidal/non-steroidal anti-inflammatory drugs to influence the activation of NF-κB was explored using, as the experimental model, human cervical cancer HeLa-IL-6 cells stably transfected with an IL-6-bound reporter gene. Effects on cell viability and proliferation were monitored (MTT assay). HPLC-DAD was used to establish links between chemical patterns of Leuzea extracts and their bioactivities.

Key findings 20E inhibited NF-κB activation (IC50 31.8 μM) but was less active than other plant metabolites (xanthohumol 3.8 μM, withaferin A 1.4 μM). Leuzea extracts with high content in 20E had a fair activating effect, but in contrast, some extracts with low 20E content significantly inhibited NF-κB activation at IC50s ranging from 3.5 to 6.2 μg/ml. Combination tests confirmed that 20E does not explain the NF-κB modulation achieved by Leuzea extracts. The extracts but not 20E itself showed a significant modulation of the NF-κB inhibitory effect of dexamethasone.

Conclusions 20E is unlikely a major player in the NF-κB inhibitory effects displayed by some Leuzea extracts *in vitro*. If confirmed *in vivo*, caution should prevail towards marketed Leuzea extracts that are non-standardised or standardised on 20E only, since different starting materials and extracts may even cause opposite effects. More importantly, our results indicate the interaction potential of Leuzea with steroidal anti-inflammatory drugs.

Keywords 20-hydroxyecdysterone; cytotoxicity; dexamethasone; *Leuzea carthamoides*; NF-κB

Abbreviations: 20E, 20-hydroxyecdysterone; AC150, activating concentration 150%; CAF, caffeic acid derivatives (qualified according to UV/VIS spectrum from HPLC fingerprint); ECD, ecdysteroids (qualified according to UV/VIS spectrum); FLA, flavonoids (qualified according to UV/VIS spectrum); MNEC, maximum non-effect concentration.

Introduction

Phytoecdysteroids are plant-occurring hydroxylated steroids, many of them involved with insect moulting hormone activity. 20-hydroxyecdysterone (20E, Figure 1) is the main representative of this chemical group and is widespread across the plant kingdom. Phytoecdysteroids have attracted – since their discovery in the 1960s – great scientific interest due to their insecticidal activity, ecological role and potential applications in plant protection. More recently, the use of phytoecdysteroids in animals and humans has been proposed based on their supposed interference with steroidal metabolic pathways of mammals.[1] While the main focus remains on estrogenic and anabolic effects, little has been published on these compounds' immunomodulatory and anti-inflammatory potential.[2–5] Furthermore, contradictory results have been obtained depending on the test models used. Trenin and Volodin[6] described 20E as a human lymphocyte and neutrophil modulator, while Tanaguchi *et al*.[7] and Harmatha *et al*.[8] found no anti-inflammatory

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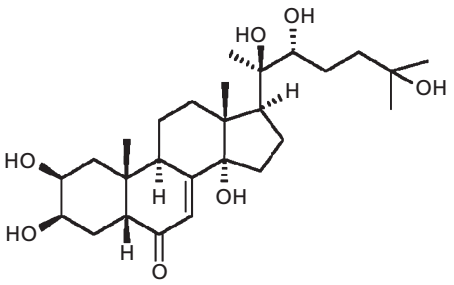


Figure 1 20-hydroxyecdysone (20E).

effect of 20E in rats and isolated mice macrophages, respectively.

20E is the major phytoecdysteroid in *Leuzea carthamoides* (Willd.) DC. (syn. *Rhaponticum carthamoides* (Willd.) Iljin, *Asteraceae*). Based on Leuzea's traditional use, scientific evidence has been built up since the beginning of the 1960s in the Soviet Union, followed in the 1980s by Eastern European countries, before subsequently attracting interest worldwide.[9–11] As a result, a variety of effects have been described for *L. carthamoides*, including anabolic,[12,13] cytotoxic, anticancerogenic, antimetastatic,[14,15] tonic/adaptogenic[16–18] and immunomodulating properties.[19,20] Its medicinal applications have been superseded in favour of its use as anabolic 'functional' fodder and dietary supplement. Products are usually based either on the dry, powdered root or its hydroethanolic extracts, and are offered via the internet as natural alternative to the controversial use of steroids in livestock, but also for human consumption with anabolic claims.[5] Many of these products, which are claimed to have immune-strengthening properties together with anabolic action, do not disclose the species, part of the plant or type of extraction used. This may pose risks, for instance, to patients suffering from chronic immune conditions who may be tempted to combine such products with their ongoing steroid-based therapies without medical supervision. Interestingly, it is stated that whole plant preparations, mainly the traditional hydroethanolic root extract but also preparations from the aerial parts, are preferred to purified 20E formulations, which are also available.[21]

Nuclear factor kappa B (NF-κB) has a central role in cellular immune response, inflammation and cell fate, and its activation is influenced by glucocorticoids.[22] Ecdysteroids such as muristerone A have been shown to interact with the NF-κB system showing comparable effects to dexamethasone and retinoic acid, resulting in apoptotic signalling in murine lymphoma WEHI-231 cells.[23] Ergostane-type ecdysteroids from *Polyporus umbellatus* exhibited potent anti-inflammatory activity in a test of TPA-induced inflammation in mice.[24] These data encouraged us to investigate the effects of 20E and Leuzea extracts on the in-vitro activation of NF-κB, alone or in combination with dexamethasone and other known NF-κB inhibitors. To our knowledge, this is the first time that the influence of 20E and also Leuzea extracts on NF-κB activation has been studied and further qualified by comparison and combination with other NF-κB modulators. For this, a well-established luminometric test system, using a reporter gene in stably transfected HeLa cells, was chosen. NF-κB regulates the expression of many genes that play a role in immunity, including the one coding interleukin-6, a pleiotropic cytokine. Liberman and Baltimore discovered that the promoter region of the interleukin-6 (IL-6) gene has a putative NF-κcB-binding site after finding that a fragment of the IL-6 promoter containing the site specifically binds highly purified NF-κB protein and the NF-cB protein in nuclear extracts of phorbol ester-induced Jurkat cells.[25] The same phorbol-12-myristate-13-acetate (PMA)-inducible nuclear factor binds to the IL-6 promoter and the immunoglobulin κB site, thus making the HeLa luc (Hela-IL-6) model a popular tool in immunological studies. Cytotoxicity and proliferative effects were determined by the MTT assay and an HPLC-DAD fingerprinting methodology, which includes 20E determination, was developed to map chemical patterns in Leuzea-derived products and identify links between composition and bioactivity.

Material and Methods

Reference substances, plant material and extraction

20-hydroxyecdysone (20E), parthenolide, dexamethasone, genistein, xanthohumol, chlorogenic acid and caffeic acid (all Sigma-Aldrich, UK) served as reference standards. Withaferin A and WF-1 (5.6-epoxy-1-oxowitha-2, 14, 24-trienolide isolated from *Withania frutescens* (L.) Pauquy) were kindly provided by Dr Nadya Jacobo (School of Pharmacy, London).

Dry herb and root material from 3-year-old *Leuzea carthamoides*, originating from Research Station for Ecological Agriculture Mikkeli (Finland) and sold as the brand *Lujza*, were cultivated in the Rohrbach district of Upper Austria and were provided by Mr Hubert Falkinger (Putzleinsdorf, Austria). A voucher specimen (No. 658.291) was deposited at the herbarium of the Biologiezentrum der Oberösterreichischen Landesmuseen (Linz, Austria). Plants were cultivated between 2002 and 2005, harvested in June 2005, and dried at room temperature. Before extraction, the dried root material was cut into maximum 10-mm-long pieces. From the overground parts – here further referred to as herb – all major stalks (>10 mm diameter) were removed before cutting down to 10-mm-long pieces.

Root and herb were macerated twice with methanol or ethanol 60% for 2 days, respectively. The crude methanolic extracts were filtered and reduced under vacuum to dryness (**R-Me**, **H-Me**). The herb methanolic extract was fractionated by gradient liquid/liquid separation between water and organic solvents in the order hexane, dichloromethane and ethyl acetate in three steps each. After combination, rotary evaporation and final drying under nitrogen to a constant weight, these gave three fractions (**H-Me-hex**, **H-Me-diclo**, **H-Me-etac**). The ethanol 60% extracts were reduced under vacuum until the majority of the ethanol was evaporated. Ethyl acetate was added to the remaining water layer. This process was repeated until the ethyl acetate fraction remained clear, after which the unified fractions were dried under vacuum (**R-Et60-etac, H-Et60-etac**). In addition, the remaining water fraction of the root (**R-Et60-wa**) was freeze-dried and incorporated into the investigation.

Cell culture

The HeLa-IL-6 cell line was kindly provided by Dr M. L. Lienhard Schmitz, University of Giessen, Germany). It contains a plasmid, constructed as described by Plaisance *et al*., upstream from a luciferase gene, together with the selection plasmid pMEP4. This construct includes the recombination signal sequence binding protein Jkappa, which is constitutively bound to the NF-kappaB site of the interleukin-6 promoter and acts as a negative regulatory factor.[26]

Cells were maintained in DMEM (Invitrogen, UK) supplemented with 10% fetal bovine serum and antibiotics (complete media) at 37°C in a 5% CO_2 humidified atmosphere and split when confluent. Cells were allowed to grow in media to 60–80% confluence before harvesting for the MTT and the NF-κB assays. The cell density was adjusted to 7.5×10^4 cells/ml in media. Using 96 (24) well plates, 200 µl (500 µl) of resuspended cell suspension per well was incubated for 18–24 h at 37°C with 5% CO_2, 95% humidity.

Cell viability and proliferation assays

For the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann in 1983,[27] samples primarily dissolved in DMSO or methanol were diluted in media for a starting concentration of 50 µg/ml for extracts and 100 µM for single compounds. After removal of old media, 100 µl media containing maximum 0.5% solvent was added to each test well, omitting columns 2 (200 µl media as control), 3 (blank, 200 µl media without MTT) and 4 (200 µl test solution as starting concentration). Then 100 µl from column 4 was taken and doubling dilutions were performed from column 5 to column 11. After incubation for 6 (12, 24) hours, removal of old media and washing with PBS (Invitrogen, UK), 150 µl MTT solution (SIGMA, 0.5 mg/ml in PBS) was added and the plates were incubated for another 90 min. The MTT solution was replaced by 200 µl of a DMSO (10%)/isopropanol (90%) mixture, and the plates were wrapped in aluminium foil and left for 10 min before resuspension and measurement using the photometric mode of the Anthos Lucy 1 luminometer at 570 nm (reference filter at 620 nm, ASYS, Eugendorf, Austria). All readings were adjusted by subtracting the blank absorbance. Corrected absorbance values were converted into percentage growth values in comparison to the non-treated control. Viability less than 85% of the control was defined as toxic, viability higher than 115% as proliferative. Toxic effects were expressed in two ways: as the maximum non-toxic concentration (MNTC = 85% of respective control) and as an IC50 value. Samples were tested in triplicate.

The proliferation assay was performed similarly with increased cell numbers using 24-well plates instead of 96-well plates.

NF-κB IL-6 reporter assay

Hela-IL-6 cells were grown to confluence in 24-well plates, media were replaced by serum-free media and with a maximum solvent portion of 2% (0.5% in the case of DMSO) added to the samples. After 40 min incubation the stimulant was added (PMA, 50 ng/ml, TNF-α 10 ng/ml) and the cells were further incubated (37°C) for 6 h or 24 h. After lysis with 70 ml lysis reagent (Promega), 30 µl from each well were added in duplicate to a white 96-well plate. Fifty microlitres of freshly prepared luciferase reagent (Promega) was added automatically using the Anthos Lucy 1 luminometer system. After a reaction time of 10 s the resulting luminometric reading was recorded. Positive (stimulated cells with solvent only) and negative (cells without stimulation) controls were included on each plate to calculate the difference between negative control and sample (or positive control) as a percentage of the positive control. Means were calculated from two readings per sample, two samples per plate, three plates per experiment and two independent experiments. In addition to the IC50 values the MNEC values were calculated as 85% of the positive control. This facilitated comparison of the range of activity taking also into account that inhibition did sometimes not reach 50% of the control or show first cytotoxic effects. For comparison of samples that did not inhibit but further activated NF-κB, the concentrations that caused 50% increased NF-κB activation compared to the positive control (AC150) were calculated in analogy to the IC50 approach. The threshold for MNEC was set at 115% of the positive control.

Combination experiments

To explore the possible role of 20E to the overall effect of Leuzea extracts, 20E was combined with the sesquiterpenlactone parthenolide, and chlorogenic and caffeic acid (PMA activation). Extracts were also tested in combination with different concentrations of 20E (PMA and TNF-α activation).

The interaction potential of 20E was investigated by measuring the combined effect of 20E and the standard glucocorticoid dexamethasone after PMA and TNF-α activation. Accordingly, selected Leuzea extracts were combined with dexamethasone.

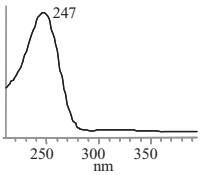
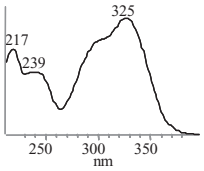
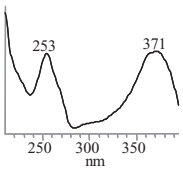
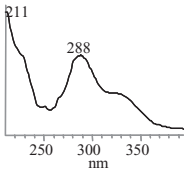
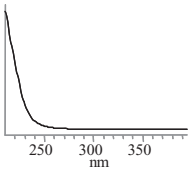
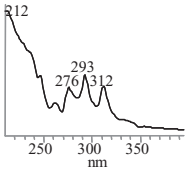
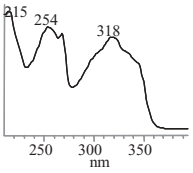
Statistical analysis

When appropriate, pairwise comparisons of specific treatments to the control treatment according to the experimental design were performed as indicated in the figure captions. Relevant control treatments were either the vehicle control or in case of combination experiments, the mono-treatment with one of the combination partners at the respective concentration. All results were statistically evaluated for significance with GraphPad® (InStat 3) statistics software by one-way ANOVA followed by Bonferroni's post-test. Significant differences from selected control or treatment are shown as $*P < 0.05$ or $*P < 0.05$ and $\hat{}P < 0.05$ when referring to two different mono-treatments.

HPLC analysis

Using a HPLC Waters™ system 900, with a Waters™ 996 PDA detector and a Waters™ 717plus autosampler device controlled by EmPower™ software, a 80 min gradient method was applied using solvent mixtures of water (TFA 0.1%), a water–acetonitrile mixture (65 : 35, TFA 0.1%) and acetonitrile with a flow of 0.9 ml/min at 25°C. The system was equipped with an Agilent Zorbax RX-C18 column (5 µm 4.6×250 mm Highchrom, UK) and a Nova-Pak® C8 Guard Column 3.9×20 mm (Waters UK Elstree, UK). Samples (starting concentration of extract 10 mg/ml, standard

Table 1 Main compound groups in the fingerprint of Leuzea extracts according to UV spectra

	ECD	CAF	FLA	Oth
				
Description:	one maximum between 230 and 250 nm	main maximum at around 330 nm	two maxima at around 254 and 370 nm	other UV spectra
Identification:	includes main cyclohexenone containing ecdysteroids	caffeic acid derivatives	flavonoids	other secondary metabolites
Quantification:	total ecdysteroids (ECD) calculated as 20E	total caffeic acid derivatives (CAF) calculated as chlorogenic acid	total flavonoids (FLA) calculated as quercetin	–
	A	**B**	**C**	**D**
				
Description:	one maximum between 270–290 nm sometimes with a second minor maximum at 330–340 nm	no absorption beyond 200 nm	three maxima at around 270, 300, 320 nm	two maxima at around 250 and 316 nm
Identification:	unidentified secondary metabolites including minor ecdysteroids	unidentified secondary metabolites without chromophore	unidentified secondary metabolites	unidentified secondary metabolites

references 1 mg/ml) were diluted in methanol or methanol/water mixtures, occasionally using some drops of DMSO to achieve complete dissolution. Prior to use, all samples were filtered through a 0.45 mm Acrodisc® syringe filter (Fisher Scientific, Loughborough, UK). Every sample solution was injected in triplicate (30 µl).

From the fingerprint, all main peaks were recorded, i.e. those representing more than 1% of the total peak area when integrated at 220 or 256 nm and qualitative, semi-quantitative and quantitative data were derived. Qualitative profile: Peaks were classified according to their UV-spectra (210–400 nm). Beside the typical spectra for caffeic acid derivatives (CAF), flavonoids (FLA) and ecdysteroids occurring in Leuzea, including the characteristic cyclohexenone chromophore (ECD), we found four other dominating types of spectra which were classified into groups A to D. Finally all other compounds with spectra not corresponding to any of the previous classes were included in group 'Other' (Table 1).

Semiquantitative profile: For comparison of the fingerprints, the ratios between the areas under the curve (AUC) were recorded at 220 nm in order to estimate the portion of compound classes in the extract/fraction.

Quantification: 20E was used as standard to calculate the total content of phytoecdysteroids (total ECD), chlorogenic acid to calculate the total content of caffeic acid derivatives (total CAF), and quercetin was used for determination of the total content of flavonoids (total FLA) (Table 1).

Results

Effect of pure 20E on the cell viability and NF-κB activation

20E was compared with the synthetic steroids dexamethasone and mifepristone (RU286), natural compounds with steroidal activities (genistein, xanthohumol, withaferin A, WF-1), the known NF-κB-inhibiting sesquiterpene parthenolide, as well as caffeic and chlorogenic acids. The effect of test compounds alone (without stimulation) on the cell viability (MTT) of HeLa-IL-6 cells was measured after 6 and 24 h (Table 2). 20E had an IC50 value lower than dexamethasone but higher than mifepristone. Clearly more cytotoxic were the two withaferins, parthenolide and xanthohumol, while genistein and caffeic acids had no major effect on the cell viability. There was no measurable proliferative effect on HeLa cells under 20E treatment in the standard MTT procedure using 96-well plates.

Considering the MTT results, 20E was tested at five concentrations (2, 5, 10, 25 and 50 µM) on the modification of PMA triggered NF-κB activation after 6 h. Again, other synthetic and natural steroidal compounds as well as parthenolid, caffeic acid and chlorogenic acid were tested as

Table 2 Effect of 20E, dexamethasone and reference standards on the cell viability after 6 and 24 h incubation (MTT assay, test range 0.4–100 μM) and on PMA-activated NF-κB (6 h after stimulation) determined in HeLa-IL-6 cells

	Cell viability (MTT)				NF-κB inhibition	
	6 h		24 h		6 h	
	MNTC[a]	IC50	MNTC[a]	IC50	MNEC[b]	IC50
20E	34.2 ± 4.4	69.9 ± 8.3	29.2 ± 3.0	59.4 ± 7.7	6.6 ± 2.4	31.8 ± 5.2
Dexamethasone	>100	>100	25.1 ± 3.0	>100	7.1 ± 0.2	35.2 ± 2.7
Mifepristone	6.3 ± 0.7	32.0 ± 6.6	2.7 ± 1.2	12.5 ± 1.4	Further activation	
Genistein	47.6 ± 3.2	>100	15.7 ± 0.9	98.5 ± 5.8	10.4 ± 0.3	43.4 ± 8.5
Xanthohumol	7.4 ± 0.4	24.8 ± 2.9	2.2 ± 0.4	5.8 ± 0.7	2.5 ± 0.5	3.8 ± 1.7
Withaferin A	5.1 ± 1.0	9.6 ± 1.4	1.2 ± 0.3	0.24 ± 0.11	0.25 ± 0.04	1.4 ± 0.3
WF-1	10.1 ± 1.2	18.2 ± 2.0	2.5 ± 0.7	0.35 ± 0.08	0.91 ± 0.07	5.2 ± 1.4
Parthenolide	3.0 ± 0.3	20.7 ± 4.1	2.8 ± 0.5	4.6 ± 1.9	0.8 ± 0.1	3.2 ± 2.2
Caffeic acid	>100	>100	80.0 ± 2.7	>100	48.6 ± 4.5	96.8 ± 9.0
Chlorogenic acid	84.7 ± 7.6	>100	72.6 ± 5.9	>100	37.6 ± 2.7	66.0 ± 11.8

Mean of two determinations in triplicate and standard error of the mean (all in μM)
[a]Sample concentration at 85% of the vehicle control (maximum non-toxic concentration). [b]Sample concentration at 85% of the positive vehicle control (maximum non-effect concentration).

references (Table 2). The strongest NF-κB inhibition was exhibited by parthenolide, xanthohumol and the withanolides (IC50 between 1.4 and 5.2 μM and MNEC of 0.25–2.5 μM), while 20E was comparable to dexamethasone and genistein, exhibiting a moderate but consistent inhibition with IC50 values between 32–43 μM and MNEC of 6.6–10.4 μM. It also had similar concentration–response curves, showing a slight increase in NF-κB activity below 5 μM, and a clear decrease in NF-κB activation at concentrations above 10 μM. However, the dose–response curve usually reached a plateau when approaching 50% inhibition whilst starting to induce toxic effects at around 50 μM. Strikingly, mifepristone gave inconsistent results over seven independent experiments, showing sometimes inhibitory but often activating effects. On the other hand, caffeic and chlorogenic acids only marginally inhibited NF-κB. It became obvious that for strong NF-κB inhibitors the window between effective NF-κB inhibition and toxic effects was very narrow in our cell model. All non-toxic concentrations causing NF-κB inhibition after 6 h turned toxic after 24 h.

Effects of Leuzea root and herb extracts on cell viability and NF-κB activation

Extracts and fractions from Leuzea root and herb were tested for their effect on cell viability using maximum concentrations of 100 μg/ml after 24 h incubation, according to the standard MTT procedure in 96-well plates (Table 3). Only two extracts, H-Me-diclo and H-Et60-etac, affected cell viability negatively with IC50 values of 4.2 and 16 μg/ml, respectively.

In order to test a possible proliferative effect, the MTT assay was repeated with an increased cell number using 24-well plates (with cell density as in the NF-κB assay). After 12 and 24 h there was no significant increase of mitochondria viability, indicating an increased cell number for any of the samples (data not shown).

According to these results, root and herb extracts were then tested for their ability to modify the PMA-induced NF-κB activation at concentrations not affecting cell viability alone (Figure 2). All three root extracts activated NF-κB in comparison to the control (AC150 values: R-Me, 84 μg/ml; R-Et60-etac, 5.2 μg/ml; R-Et60-wa, 125 μg/ml).

As for the herb extracts, H-Me showed a concentration-dependent inhibition of NF-κB (IC50 15.5 μg/ml). Two fractions thereof had no effect (hexane, ethyl acetate), whilst the dichloromethane fraction had a IC50 of 5.9 μg/ml, with a stronger inhibition at higher concentrations than the crude extract but an activating effect at concentrations below 1 μg/ml. H-Et60-etac also inhibited in a concentration-dependent manner (IC50 12.1 μg/ml).

Combined effects of 20E and Leuzea extracts on the NF-κB activation

In order to estimate the contribution of 20E, the main ecdysteroid in Leuzea, to the overall effect of Leuzea extracts, 20E at three concentrations was added (5, 10, 25 μM) both to the NF-κB-activating root extracts and the inhibiting herb extracts. Extract concentrations were close to their IC50 (for the inhibiting extracts) or the AC150 (for the activating extracts) (Figure 3). While 20E alone moderately reduced NF-κB activation, after both PMA and TNFα stimulation, no consistent significant or dose-dependent modification of the extracts' effects could be observed, either for the activating root extracts or the inhibiting herb extracts. Surprisingly, in some cases higher NF-κB levels were found after extract/20E co-incubation than after extract-only incubation.

To ascertain the combined effects of specific classes of compounds present in Leuzea extracts, namely sesquiterpenelactones and phenolcarbonic acids, 20E was combined with the NF-κB inhibitor parthenolide as a model sesquiterpenelactone[28,29] as well as with caffeic and chlorogenic acid because of the characteristic presence of phenolcarbonic acids our Leuzea extracts (Figure 4). The inhibitory effects of parthenolide were not influenced, while 20E addition at concentrations between 5 and 25 μM increased NF-κB moderately in the caffeic and chlorogenic acid treatments 6 h after PMA

Table 3 Extraction yield, effects on cell viability and NF-κB activation and HPLC/DAD-based quantitative profile of extracts from root (R) and herb (H) of *Leuzea carthamoides*

	Yield[a] (%)	Effect on cell viability[b] (MNTC) (μg/ml)	Effect on cell viability[c] (IC50) (μg/ml)	Effect on NF-κB[d] (IC50 or AC150) (μg/ml)	Total CAF[e] (calc. as CLO) (mg/g)	Total FLA[f] (calc. as QUE) (mg/g)	Total ECD[g] (calc. as 20E) (mg/g)	20E (mg/g)
R-Me	9.49	91.1 ± 12.7	>100	Activation 84 ± 2.5	181.2 ± 7.13	2.1 ± 0.42	32.2 ± 2.25	21.1 ± 0.98
R-Et60-etac	4.93	29.1 ± 11.3	84.3 ± 14.2	Activation 5.2 ± 2.0	378.0 ± 13.02	<0.5	46.9 ± 3.25	29.6 ± 5.30
R-Et60-wa	16.54	>100	>100	Activation 125 ± 2.2	66.3 ± 7.63	0.5 ± 0.28	13.0 ± 1.58	12.5 ± 1.25
H-Me	9.94	41.2 ± 5.3	87.5 ± 3.5	Inhibition 15.5 ± 1.4	21.1 ± 1.19	45.5 ± 2.06	11.8 ± 0.52	n.d.[h]
H-Me-hex	1.93	35.1 ± 21.1	76.0 ± 18.4	No effect	4.7 ± 0.52	1.4 ± 0.28	35.4 ± 4.53	<0.5
H-Me-diclo	0.83	1.9 ± 0.2	4.2 ± 0.5	Inhibition 5.9 ± 1.6	2.5 ± 0.19	20.7 ± 1.50	5.7 ± 0.71	<0.5
H-Me-etac	0.85	54.5 ± 9.2	91.1 ± 12.7	No effect	56.6 ± 2.07	289.1 ± 23.78	<0.5	n.d.[h]
H-Et60-etac	3.48	7.3 ± 5.9	16.3 ± 2.8	Inhibition 12.1 ± 0.8	61.6 ± 0.90	416.8 ± 35.3	83.3 ± 6.83	n.d.[h]

[a]Dry mass extract to original dry mass herbal drug. [b]MTT assay HeLa-IL-6, 24h incubation, 85% of the respective vehicle control (maximum non-toxic concentration). [c]MTT assay HeLa-IL-6, 24h incubation, 50% of the respective vehicle control. [d]NF-κB assay HeLa-IL-6, PMA stimulation, 6 h incubation, 50% (150%) of the respective vehicle control. [e]Total caffeic acid derivatives content calculated as chlorogenic acid. [f]Total flavonoid content calculated as quercetin. [g]Total ecdysteroid content calculated as 20E. [h]Not detected.

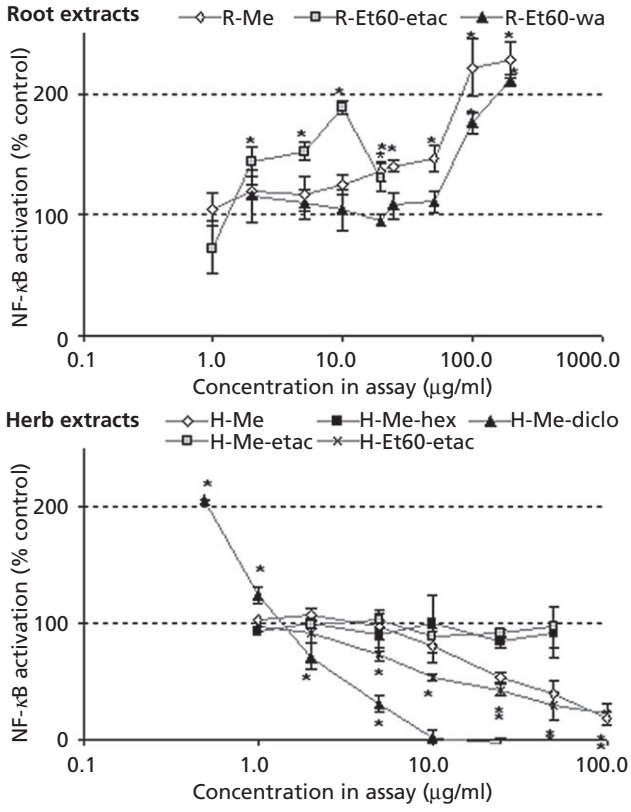


Figure 2 Effect of Leuzea root and herb extracts on the PMA-induced NF-κB inhibition in HeLa-IL-6 cells (percentage relative to vehicle control). Mean of three determinations in triplicate and standard error of the mean. * Indicates significance ($P < 0.05$).

stimulation. No modification was observed when measured 6 and 24 h after TNFα stimulation (data not shown).

In all the combination experiments, the first toxic effects were observed when using 20E at concentrations of 50 μM. Consequently these results were not considered as they may potentially affect luminometric readings.

Combined effects of dexamethasone with 20E or Leuzea extracts on NF-κB

To ascertain possible pharmacodynamic interactions between 20E, Leuzea extracts and steroidal anti-inflammatory drugs, combinations with dexamethasone in different ratios (5 : 1, 2 : 1, 1 : 1, 1 : 2, 1 : 5) were tested in the NF-κB assay. 20E at low concentrations (5 and 10 μM) had no significant influence on the effect of dexamethasone after PMA stimulation. Higher concentrations of 20E (25 μM) enhanced the inhibitory effect of dexamethasone after both PMA or TNFα stimulation (Figure 5).

Dexamethasone (20 min pre-incubation, 20 μM) in combination with active Leuzea extracts was tested in the same cell model (Figure 6). The anti-inflammatory drug alone decreased PMA-induced NF-κB activation dose-dependently and this effect was additive to those of the inhibitory extracts, while the effect of activating extracts appeared reduced (e.g. the water fraction of the 60% ethanol leaf extract). Therefore, the dexamethasone-caused NF-κB inhibition may be enhanced by some Leuzea extracts, whilst others effectively neutralised this effect.

HPLC fingerprint and 20E content in Leuzea extracts

The detection of characteristic compound classes described in Leuzea was achieved by comparison of the UV spectra with pure reference standards of chlorogenic acid, caffeic

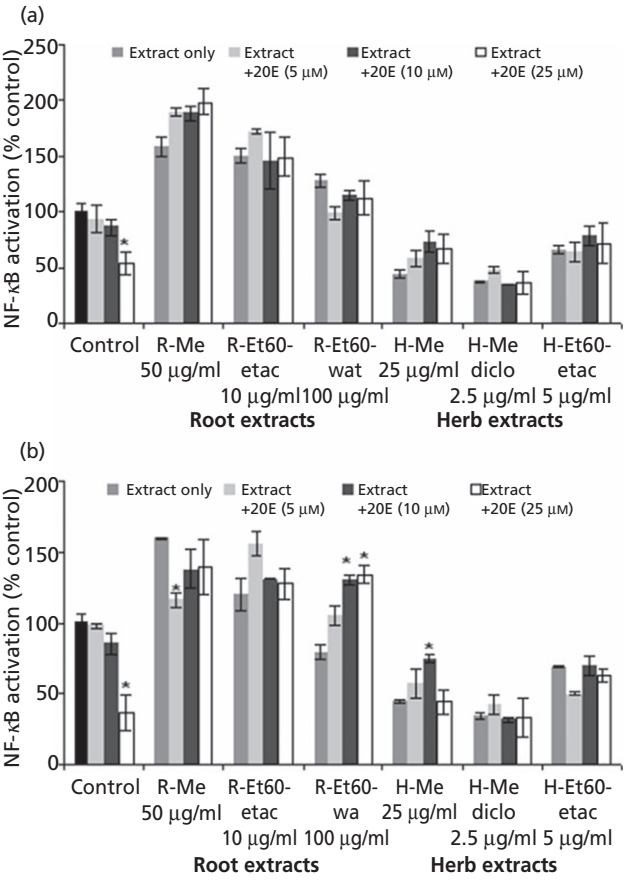


Figure 3 Influence of Leuzea extracts alone and combined with 20E (5, 10 and 25 μM) on the PMA- (a) and TNFα- (b) induced NF-κB activation in HeLa-IL-6 cells. Mean of three determinations in triplicate and standard error of the mean. * Indicates significance ($P < 0.05$) of combination treatment vs extract alone (ANOVA followed by Bonferroni's-test).

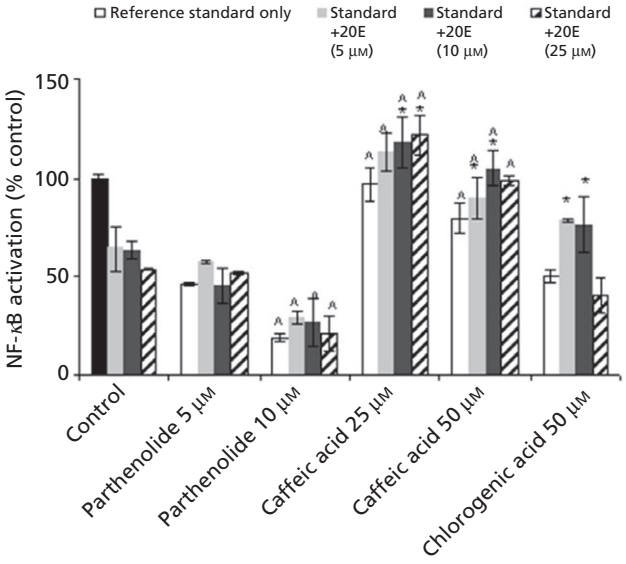


Figure 4 Effect of parthenolide, caffeic acid and chlorogenic acid alone and combined with 20E (5, 10, 25 μM) on the PMA-induced NF-κB activation in HeLa-IL-6 cells. Mean of three determinations in triplicate and standard error of the mean. * Indicates significance ($P < 0.05$) of combination treatment vs reference compounds alone; ^ indicates significance ($P < 0.05$) of combination treatment vs 20E alone (ANOVA followed by Bonferroni's test).

acid, 20E, quercetin and parthenolide (Figure 7). A semi-quantitative method was chosen to compare percentages of the AUC of summarised peaks from those groups (Table 1 and Figure 8). Root extracts (R-Me, R-Et60-etac) contained predominantly phenolcarbonic acids followed by compounds of group D and group ECD (with 20E as the main peak at t_r 22 min), but few flavonoids (Figures 7 and 8). 20E was to a lesser extent also found in the fingerprint of R-Et60-wat. In all three root extracts, however, 20E was not the main peak even at 256 nm, near the absorption maximum of the compound.

The herb-derived extract H-Me, and the H-Me-etac and H-Et60-etac fractions were rich in flavonoids and compounds of group A (Figures 7 and 8). The other two fractions contained no flavonoids but predominantly constituents without chromophores (group B) with minor portions of ECD and group C (H-Me-hex), or mostly presence of type B and C compounds (H-Me-diclo).

The content of 20E as well as total caffeic acid derivatives, total flavonoids and total ecdysteroids, as calculated from external standards (chlorogenic acid, quercetin, 20E, respectively) is shown in Table 3. Only in the root extracts we were able to detect 20E (R-Me 21 mg/g, R-Et60-etac 29 mg/g, R-Et60-wa 12 mg/g). Other compounds with a spectrum similar to 20E, however, were also detected in the herb extracts (maximum 83 mg/g in H-Et60-etac). A reduced presence of 20E in the H-Me, H-Me-etac and H-Et60-etac extracts cannot be excluded, as the fingerprint has shown other peaks (flavonoids and A) co-eluting with this compound. Caffeic acid derivatives ranged between 66 mg/g (R-Et60-wa) and 378 mg/g (R-Et60-etac) in the root extracts and between 2.5 mg/g (H-Me-diclo) and 62 mg/g (H-Et60-etac) in the herb extracts. Higher amounts of flavonoids were only detected in the herb extracts, being particularly prevalent in the ethyl acetate fractions (H-Me-etac 289 mg/g, H-Et60-etac 416 mg/g).

Relationship between classes of metabolites and NF-κB activity of Leuzea extracts

Taken together, the profile of all NF-κB-activating root extracts showed a predominance of CAF together with 20E and other unidentified ecdysteroids. These were accompanied by main peaks for D-type or (water fraction) A- and B-type constituents. The NF-κB-inhibiting herb extracts, in contrast, contained clearly less CAF and ECD but were dominated by other groups, such as B in H-Me-diclo and FLA, and A in H-Me and H-Et60-etac (Figure 7c and 7d). A general pattern of a relationship between compound classes FLA, A or B and the measured NF-κB effect could not be established from the eight extracts analysed here, since the NF-κB-inactive extracts L-Me-hex and L-Me-etac were equally dominated by B or FLA/C constituents, respectively. No simple, straight correlation between the content of 20E, total ecdysteroids, total CAF or total FLA and the pharmacological effects could be found.

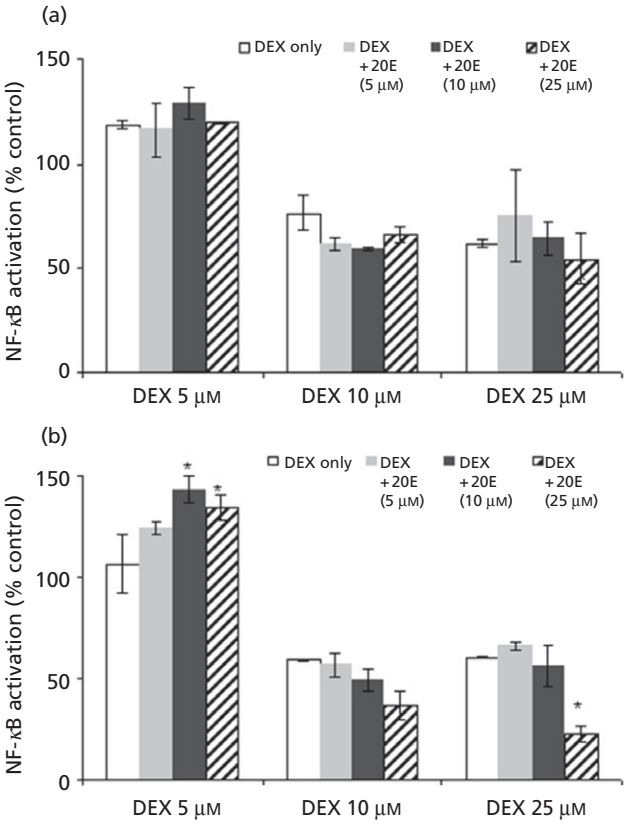


Figure 5 Effect of dexamethasone (DEX 5 μM, 10 μM, 25 μM) on the PMA- (a) and TNFα- (b) induced NF-κB activation in HeLa-IL-6 cells alone and combined with 20E (5, 10, 25 μM). Mean of three determinations in triplicate and standard error of the mean. * Indicates significance ($P < 0.05$) of combination treatment vs dexamethasone alone using ANOVA followed by Bonferroni's test.

Discussion

Effect of pure 20E on cell viability and NF-κB activation

Ecdysteroids have been reported to be of little toxicity, with LD50 values above 10 mg/kg in mice after oral or intraperitoneal administration; in fact, a permanent harmless nutritional exposure of animals and human is assumed.[5] Also in-vitro results for 20E in four different cells types – including HeLa – has indicated low cytoxicity with IC50s ≅ 50 μg/ml.[30] This is in line with our results in HeLa-IL-6 cells, where cytoxicity for 20E was little higher than that of dexamethasone.

The various pharmacological effects of ecdysteroids and ecdysteroid-containing plants encouraged suggestions vis-à-vis a possible interference with mammal steroidal metabolic pathways.[1,5] But ecdysteroids, including 20E, are not supposed to interact with steroidal receptors in mammals and are even widely used in commercial in-vitro test kits for ecdysteroid-inducible transgene expression of mammalian genes. In those systems, highly concentrated ponasterone and muristerone A act as inducers, while other ecdysteroids were found to be inactive.[5] Sometimes, however, it has been questioned whether ecdysteroids really do not influence mammalian cell physiology. Oehme *et al*.[31] for instance reported inhibition of Fas ligand- and TNF- ligand-induced apoptosis by muristerone A at the level of caspase-8. To add to this controversy, it is notable that several plant-derived steroids and non-steroids, including cucurbitacins, withanolides, stilbenoids or lignans, which interact with vertebrate steroidal systems, have been shown to be edysteroid-receptor ligands, i.e. the insect analogues of mammal nuclear hormone receptors, part of the superfamily of transcription regulators.[32–34]

Our data obtained in the HeLa-IL-6 model imply a 20E-caused inhibition of NF-κB comparable to the one induced by dexamethasone or genistein, both known to inhibit this nuclear factor *in vivo* and *in vitro*. Yet the comparison to parthenolide, withanolides and xanthohumol shows that 20E may not reach the potency of more powerful plant-derived NF-κB inhibitors.

It is recognised that the abnormal NF-κB household in cancer cells – one of the mechanisms responsible for evading cell death under toxic conditions for normal cells – gives NF-κB inhibitors the prospect of being able to target cancer cells by reduction of elevated baseline levels, suppressing the 'normal' apoptotic response.[35] Our results show a certain correspondence between potent NF-κB inhibition (6 h) and subsequent cell death (24 h), with comparable IC50 values for all substances. Along these lines, the link between NF-κB inhibition by steroids, including muristerone A and ponasterone, and subsequent apoptosis induction has already been advocated by Donjerković *et al*.,[23] when measuring cell viability at later time points in WEHI-231 cells.

Despite the inhibitory effect of 20E alone at concentrations between 7 and 50 μM, questions remain because of the absence of the expected additive or synergistic inhibition when combined with parthenolide, dexamethasone or Leuzea extracts. Sometimes even opposite effects were found for some of these combinations.

Anti-inflammatory effects for 20E have been reported by some authors (as reviewed by Lafont and Dinan 2003),[5] but there are also reports that could not confirm such effects *in vivo*.[7] Harmatha *et al*.[8] could not detect reduced NO production of peritoneal macrophages upon LPS or interferone stimulation, even after incubation with up to 100 μM 20E. Our experiments are the first report of 20E as a modulator of NF-κB activation, and suggest that further in-vitro and in-vivo tests are required. The inhibitory effect was observed both after PMA and TNFα stimulation, which may suggest independence from the pathway involved; either the classical canonical or the alternative.[36]

Effects of Leuzea root and herb extracts on cell viability and NF-κB activation

The traditional preparations of Leuzea are hydroethanolic extracts of the root, which is reported to contain 0.04–0.81% of 20E.[11] With our methanolic (hydroethanolic) maceration, 20E was obtained at 20 mg/g root (35 mg/g root), which is in the mid-range of previous reports. The total ecdysteroid contents of some herb extracts were comparable to those of the root extracts, suggesting that ecdysteroid-enriched extracts can also be obtained (reported range of 20E in the leaf 0.03–1.22%[11]). The aerial parts may be an economic alternative

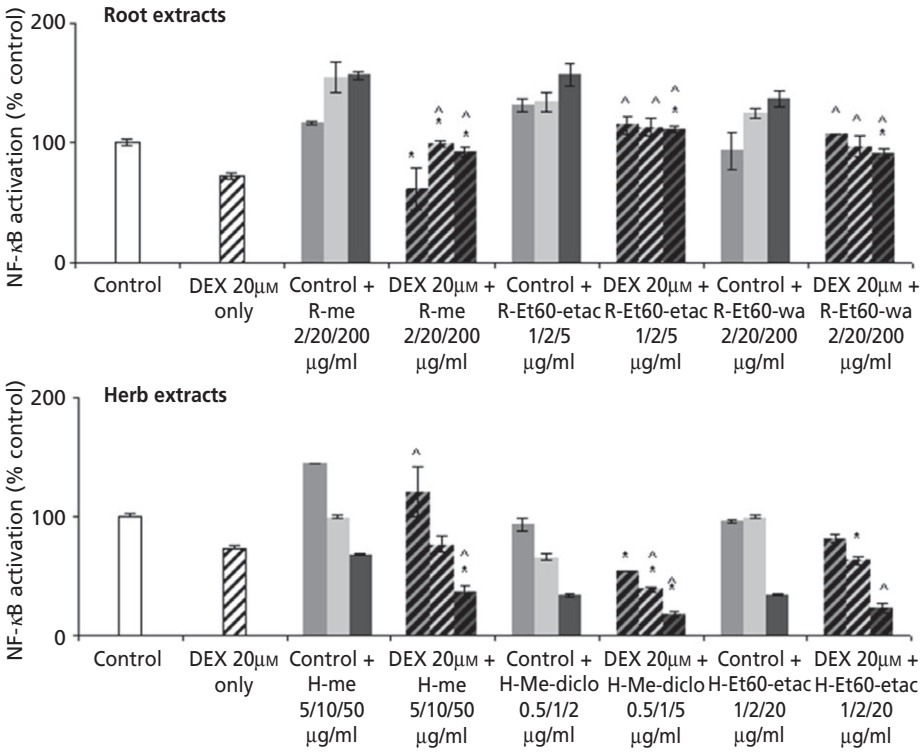


Figure 6 Effect of dexamethasone (DEX 20 μM) alone, Leuzea extracts alone and DEX/extract combinations on the PMA-induced NF-κB inhibition in HeLa-IL-6 cells (percentage relative to vehicle control). Mean of three determinations in triplicate and standard error of the mean. * Indicates significance ($P < 0.05$) of combination treatment vs the extract alone; ^ indicates significance ($P < 0.05$) of combination treatment vs dexamethasone alone using ANOVA followed by Bonferroni's test.

to the root because the perennial plants allow permanent cultivation and repeated harvest.[30]

The tested extracts and fractions show diverse chemical profiles and partly opposite pharmacological effects. The herb-derived extracts were either efficient NF-κB inhibitors or had no effect on NF-κB activation at all. The root extracts caused a moderate further increase of the PMA or TNF-α induced NF-κB activation. This effect was most pronounced for the extract with the highest content in 20E (29.6 mg/g; R-Et60-etac). When considering not only 20E but all ecdysteroid-like compounds, extracts with the highest ecdysteroid content (83; 47; 35 mg/ml) inhibited, activated or had no effect.

Although CAF dominated in root extracts with NF-κB over-activating effects, a decisive role is debatable considering the similar total CAF content in the activating R-Et60-wa (66 mg/g) and the inhibiting H-Et60-etac (62 mg/g) extracts. Likewise concentration of the flavonoids, which were more characteristic for the herb extracts tested here, did not correlate with the effect on NF-κB. Apart from a necessary distinction of single constituents within the groups ECD, CAF and FLA, it may be concluded that compounds other than the typical substances considered here for fingerprint are responsible for the measured activities. Leuzea contains an interesting mix of pharmacologically active classes to which the traditionally described and proven effects may be assigned. Beside the ecdysteroids 20E, makisterone C, polypodin B and ajugasterone C[37,38] and flavonoids,[39,40] more attention may be given to sesquiterpene lactones,[28,29] tiophene polyacetylenes,[41,42] and stilbenes,[43] which are known for their cytotoxic and anti-inflammatory properties in different models involving NF-κB activity modulation (see also review by Kokoska and Janovska [11]). N-feruloylserotonins have also already been suggested.[8] The diverse activities of constituent groups on single physiological targets may cause unpredictable experimental effects when the whole mixtures with distinctive proportions of those groups are tested. On the other hand, the combination of NF-κB-inhibitory and activating principles in Leuzea may play a role in its immunomodulatory and adaptogenic applications in view of the previously supposed bipolar, moderate effects responsible for conditioning of the cellular response towards an attenuated stress reaction.[44]

Contribution of 20E to the in-vitro effect on NF-κB activation of Leuzea extracts

Doubts over the contribution of 20E to the anti-inflammatory effects of Leuzea extracts were expressed by Harmata *et al.* (2008).[8] Although 20E alone was an NF-κB inhibitor in our test model, similar uncertainties arise over the contribution of 20E to the NF-κB inhibition caused by Leuzea extracts for three reasons. Firstly, the chemical pattern of activating, inhibiting and inactive extracts: the fingerprint suggests that the strongest inhibitors were extracts with less than 0.5 mg 20E/g plant material or clearly low 20E content when compared to other constituents. In extracts containing more 20E

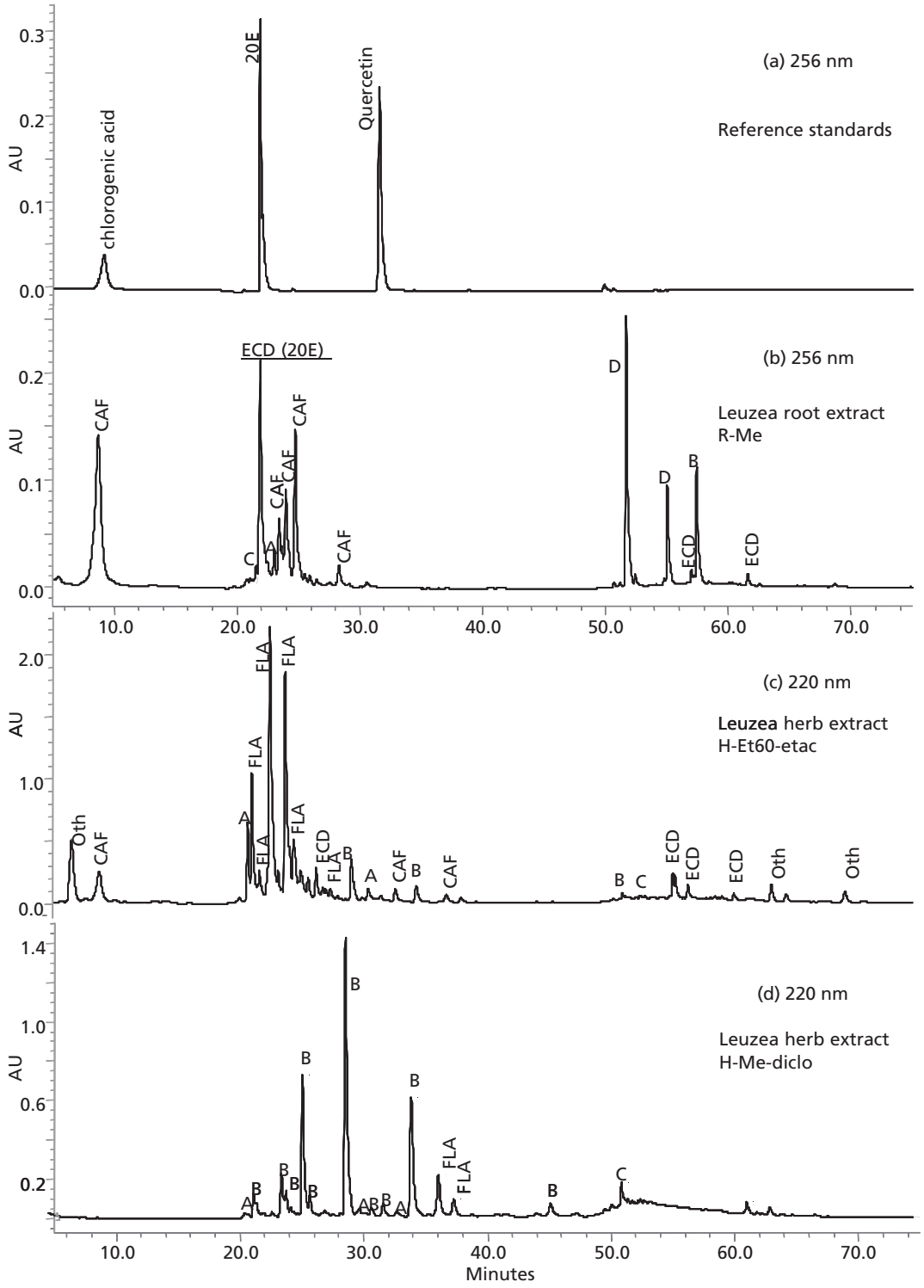


Figure 7 HPLC profiles at 256 nm of (a) reference standards and (b) an extract from Leuzea root (R-Me) and HPLC profiles at 220 nm of two extracts from Leuzea herb: (c) H-Et60-etac and (d) H-Me-diclo. Peaks other than 20E are classified according to UV-spectra (see Table 1).

activated NF-κB there is a much lower 20E concentration in the cell medium for the activity range for extracts compared to tests with 20E alone.

The second reason is the minimal impact of 20E addition to extracts: during our spiking experiments we increased the natural 20E content of the tested extracts (maximum 29 mg/g) by addition of pure 20E. Using extract concentrations at around IC50 levels (maximum 100 mg/ml), the natural 20E content of the extract in the medium of maximum 1.3 ng/ml was artificially increased with 2.28 μg/ml to 11.4 μg/ml pure

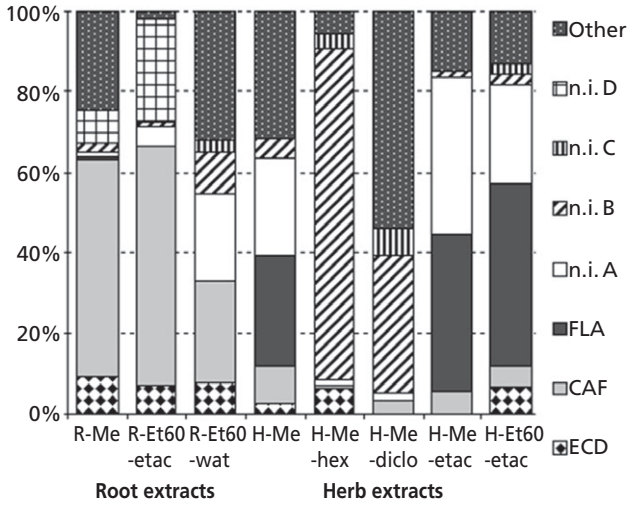


Figure 8 Semiquantitative profile of Leuzea extracts according to main groups identified by UV/VIS spectra 200–400 nm. Percentage of the summarised AUC for each compound group as detected at 220 nm (see Table 1).

20E. Yet the extract activity was not significantly changed compared to the extract alone.

The last reason is the minimal impact when 20E was combined with other NF-κB inhibitors: addition of 20E to parthenolide (ratios 1 : 10–2 : 1), caffeic acid (1 : 2–10 : 1) and chlorogenic acid (1 : 1–10 : 1) either did not significantly change the effect obtained with other compounds alone or even increased NF-κB activity. Also combinations with dexamethasone (1 : 10–5 : 1) did not show any significant effect.

In summary, neither 20E nor ecdysteroid-like compounds seem to be responsible for the NF-κB modulating effects of Leuzea extracts. Although Leuzea extracts may be endowed with an interesting immunomodulatory potential, 20E does not explain the NF-κB modulation. In addition to its doubtful contribution to anti-inflammatory effects, the suitability of 20E as analytical marker when using standard HPLC-UV methods is limited. Doubtless a characteristic quality-indicating component, difficulties in the UV/VIS detection vis-à-vis more prominent compounds and distinction of structurally very similar ecdysteroids have led in the past to a certain reluctance to detect 20E via standard HPLC methods in mixtures.[45]

Although 20E could not always be detected by our fingerprint method, we show that HPLC analysis can deliver useful information of the extract composition for qualitative control. This may be further developed to a standard quantification of 20E and even more importantly to other constituent groups (sesquiterpenlactones, stilbenes). Remarkably, many of the Leuzea products offered for sale on the internet have no specification of the plant part used or the type of extract, and declare only the 20E content. Evidence to establish a relationship between 20E concentration and the pharmacological effect of Leuzea preparations is mostly missing. Our results confirm previous doubts and suggest that more elaborate quality standards for Leuzea extracts are necessary.

Effects of dexamethasone combined with 20E or Leuzea extracts on NF-κB

The ability of glucocorticoids to suppress immune function is closely related to the ability of the glucocorticoid receptor (GR) to inhibit NF-κB transcriptional activity. This is done through direct physical association together with an additional mechanism of antagonism involving competition for common transcriptional cofactor mechanisms of NF-κB-GR antagonism.[46] The points of intersection between these two physiological mediators of inflammation is a popular topic of research.[47] Dexamethasone prevents NF-κB activation by various extranuclear and intranuclear mechanisms. For instance, upregulation of IκB-a synthesis, which depends on the early and transient activation of NF-κB, ligand binding to the C-terminal of glucocorticoid receptors promoting the nuclear translocation of the receptor, and also binding to NF-κB through the glucocorticoid receptor DNA binding domain, have been suggested.[48–50] The NF-κB suppressing effect of dexamethasone in HeLa cells, as detected in our experiments, has previously been described.[51] In contrast to other techniques detecting NF-κB modulation more upstream, luminometric detection of IL-6 expression allows measurement of the overall, final outcome of the glucocorticoid–NF-κB cross-talk at a transcriptional level and independent from the actual upstream mechanisms involved.

The results after addition of 20E to cells treated with dexamethasone were not conclusive. In contrast, a significant modification of the dexamethasone effect on NF-κB activation was detected for both inhibiting and activating Leuzea extracts. Consequently our results imply that 20E may not interfere significantly with other steroid treatments in terms of NF-κB-mediated immunoresponsiveness at a cellular level, while Leuzea extracts do. If confirmed *in vivo*, this potential may open opportunities to lower steroid concentrations when NF-κB inhibition is sought, as in some anti-inflammatory treatments or co-medication in cancer therapies. On the other hand, our results warrant further scrutiny of the widespread use of Leuzea preparations for anabolic purposes, where the combination with other steroids is to be expected. Possible opposite effects, depending on the extract composition, advocate for a better standardisation and pharmacovigilance.

Conclusions

In summary, 20E is here reported for the first time as an in-vitro inhibitor of NF-κB activation, but this compound is unlikely to be a major player in the NF-κB-inhibitory effects displayed by some Leuzea extracts. From our results, caution should prevail towards marketed Leuzea extracts that are non-standardised or standardised on 20E only, since natural variation in the chemistry of the starting material and its extracts may cause divergent effects. More importantly, our results indicate the interaction potential of Leuzea with steroidal anti-inflammatory drugs. This must be further scrutinised *in vivo* to rule out risks in off-label use. It may, however, also provide opportunities for when dose reduction of standard steroidal treatments is desired.

Declarations

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Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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