Effects of *Leuzea carthamoides* on Human Breast Adenocarcinoma MCF-7 Cells Determined by Gene Expression Profiling and Functional Assays

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Key words
- *Leuzea carthamoides*
- Asteraceae
- phytoecdysteroids
- expression profiling
- CYP1A1
- aryl hydrocarbon receptor

Abstract

Products derived from roots of *Leuzea carthamoides* (Maral root) are being promoted as dietary supplements with anti-aging, adaptogenic and anabolic activity, without much scientific evidence. We investigated the effects of a lipophilic *Leuzea* root extract and the major phytoecdysteroid, 20-hydroxyecdysone, in human breast adenocarcinoma MCF-7 cells. Cell proliferation was inhibited by the extract (IC_{50} = 30 μg/mL) but not by 20-hydroxyecdysone. Genome-wide expression profiling using Affymetrix HG U133 Plus 2.0 microarrays was carried out to analyse effects at the transcriptional level. 241 genes appeared to be differentially expressed after *Leuzea* treatment, more than after treatment with either 17β-estradiol or tamoxifen. Transcripts linked to cell cycle regulation and DNA replication were highly over-represented and regulated in an anti-proliferative manner. Genes involved in apoptosis were regulated in a pro-apoptotic manner. Expression levels of several oxidoreductase transcripts were strongly induced, most prominent CYP1A1, known to be regulated via the aryl hydrocarbon receptor pathway. An XRE-dependent reporter gene assay confirmed the AhR-agonistic activity of the *Leuzea* root extract, whereas 20-hydroxyecdysone was not active. *Leuzea* extract also inhibited 5α-reductase, type II. While the extract significantly modulates cellular activities, the phytoecdysteroids, are most likely not the active principles of *L. carthamoides*.

Abbreviations

AhR:	aryl hydrocarbon receptor
AKR:	aldo-keto reductase
CYP1A1:	cytochrome P450, family 1, subfamily A, polypeptide 1
E2:	17β-estratiol
ER:	estrogen receptor
20-HE:	20-hydroxyecdysone

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

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Introduction

Leuzea carthamoides DC. (syn. *Rhaponticum carthamoides* [Willd.] Iljin; "maral root"), is a perennial Asteraceae plant from the Siberian mountain region. Due to its adaptability to rough climates the plant is suited for cultivation as a medicinal plant and fodder crop in Northern and Eastern Europe. The roots contain up to 0.6% phytoecdysteroids [1], mainly 20-hydroxyecdysone (20-HE) (**Fig. 1**), which are believed to be the active principles. Additionally, thiophene polyines [2], [3], serotonin derivatives [4], sterols [5] and other minor components have been identified.

Dietary supplements derived from *Leuzea* root extracts, and ecdysteroids, are promoted as anti-aging and adaptogenic (list of products in [6]). In the 1980 s, Russian athletes reportedly used such preparations as "legal doping" due to their putative anabolic action (the use of these products was dubbed as "Russian secret") [7]. Many other beneficial effects such as anti-inflammatory, blood glucose and cholesterol lowering, as well as antitumor activities have been reported in minor publications [6], [8], [9].

Sound pharmacological data about *Leuzea* root extracts are rare, and little is known about their modes of action. Membrane effects (integration in membrane bilayer and interaction with membrane proteins), or binding affinity to various "orphan" nuclear receptors have been put forward to explain ecdysteroid action in mammals [6], [9].

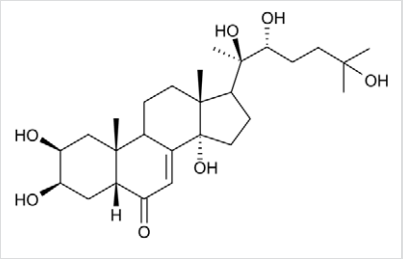

Fig. 1 Chemical structure of 20-hydroxyecdysone.

In arthropods ecdysteroids are known to regulate moulting and reproduction by binding to nuclear receptors, therefore regulating the expression of target genes.

With the emergence of microarrays as a mature technology, the analysis of global drug effects at the gene expression level has become possible. Given that ecdysteroids and *Leuzea* extract might act via transciptionally active nuclear receptors, we conducted genome-wide gene expression profiling to gain a better understanding of the putative pharmacological actions of *L. carthamoides* in mammalian cells. We used the human breast cancer cell line MCF-7, a well-known *in vitro* model for estrogen-responsive mammary cancer that expresses a large number of nuclear receptors, classical (estrogen, androgen, progestin and glucocorticoid receptors) as well as "orphan" nuclear receptors, including the aryl hydrocarbon receptor (AhR), retinoic acid receptors (RARα, RARγ), retinoid X receptor (RXR) and vitamin D receptor (VDR) [10], [11]. Given that nuclear receptor ligands are lipophilic molecules we used a dichloromethane *Leuzea* root extract. Parallel experiments were carried out with 17β-estradiol (E2) and the estrogen receptor antagonist tamoxifen to compare expression profiles with these drugs.

Materials and Methods

▼

Extract

Roots of *Leuzea carthamoides* DC., obtained from the Research Institute for Medicinal Plants, Budakalász, Hungary, were provided by Prof. Eva Szöke, Department of Pharmacognosy, Semmelweis University, Budapest, Hungary. A voucher specimen (specimen number 891 – 1) is deposited in the Institute of Pharmaceutical Biology, University of Basel. Powdered roots (0.5 mm particle size, 10 g) were subjected to pressurized liquid extraction using an ASE® 200 Accelerated Solvent Extractor (Dionex) at 120 bar and 70 °C. After a preheating step (5 min), the sample was defatted with petroleum ether (5 min), followed by extraction with dichloromethane for 10 min. Solutions were collected in different vials. The petroleum ether solution was discarded. After evaporation of the solvent, about 100 mg of dry dichloromethane extract were obtained.

Phytochemical profiling of the extract

Experimental details on the phytochemical profiling of the extract, HPLC chromatograms recorded with PDA, ELSD, and ESIMS detectors, and peak identification are provided as Supporting Information (**Fig. 1S**, **Table 1S**).

Compounds

20-Hydroxyecdysone (≥ 95.0 %) was purchased from Alexis Biocemicals, 17β-Estradiol (≥ 98.0 %) from Sigma-Aldrich and tamoxifen (≥ 99.0 %) from MP Biomedicals.

For subsequent experiments, extract and compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with appropriate assay media. The final concentration of DMSO did not exceed 0.1 %. This DMSO concentration was also used for control experiments.

Cell culture

The human breast adenocarcinoma cell line MCF-7 (obtained from ATCC) was routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) and penicillin/streptomycin (100 U/100 μg/mL) (Biochrom) in a humidified incubator at 37 °C and 5 % CO_2. Before reaching confluence, cells were splitted every 3 – 4 days in a 1 : 4- to 1 : 6-ratio. All experiments were performed with cells at passage numbers 25 – 33. Hormone-free charcoal-dextran stripped serum (CSS) was prepared from FBS by agitating with 0.5 % charcoal (Norit A) (Serva Feinbiochemica) and 0.05 % Dextran-T70 (Pharmacia) at 37 °C for 60 min. After centrifugation at 3500 rpm, CSS was filter-sterilized (0.22 μm) twice and stored at –20 °C.

Proliferation assay

MCF-7 cells were treated with extract and compounds for 120 h. Cell number equivalents were determined using the MTT dye reduction assay [12], [13]. For details, see Supporting Information.

Cell treatment for gene expression profiling

Array and RT-PCR experiments were both conducted in duplicate. MCF-7 cells were seeded in 75 cm^2 culture flasks (Greiner Bio-One) at a density of 20,000 cells/cm^2 under culture conditions and incubated at 37 °C and 5 % CO_2. After 20 hours medium was removed and cells were washed with PBS. Phenol red-free DMEM containing 10 % CSS and 0.1 % DMSO, 30 μg/mL *Leuzea* root extract, 1 nM 17β-estradiol or 10 μM tamoxifen, respectively, were added. Cells were incubated for 24 hours at 37 °C and 5 % CO_2. RNA isolation is described in the Supporting Information.

Microarray experiment

Total RNA from each sample was labelled and hybridized to an Affymetrix GeneChip® Human Genome U133 Plus 2.0 array. Array hybridization as well as primary data analysis [14], [15], [16] are described in the Supporting Information.

Real-time RT-PCR

Expression levels of 13 selected genes were determined by a two step real-time RT-PCR using the LightCycler® system (Roche). Details of experimental setup and primer sequences are given in the Supporting Information.

Western Blot

Protein expression of estrogen receptor α was determined by Western blot using monoclonal antibodies anti-ERα (Cell Signaling). For details, see Supporting Information.

AhR activity

The XRE-driven luciferase assay was conducted in rat hepatoma H4IIE cells, cotransfected with the *Renilla* luciferase gene and a 485 bp fragment of the rat CYP1A1 gene including two xenobiotic response elements (XREs), as previously described [17]. Cells were treated with *Leuzea* root extract or 20-HE for 48 h.

5α-Reductase inhibitory activity

The inhibitory activity of *Leuzea* root extract and 20-hydroxyecdysone towards 5α-reductase, type II was determined using

HEK-II cell homogenates and substrate 1β[^{3}H]-androstenedione (505 nM) as well as the well known 5α-reductase inhibitor finasteride as positive control (IC_{50} = 25 nM) as previously described [18], [19].

Statistics

Statistical analysis was performed using MS Excel or SigmaStat 3.01, respectively. If not otherwise stated, data are given as mean value ± standard deviation (SD) and were analysd by Student's test. Statistically significant difference between two groups is represented as * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

Supporting information

Details on phytochemical characterization of the extract, protocols for proliferation assays, microarray and RT-PCR experiments, a complete list of regulated genes, and RT-PCR data are provided as Supporting Information.

Results

MTT-based proliferation assays were performed to explore the influence of *Leuzea* root extract and the major phytoecdysteroid, 20-HE, on growth of MCF-7 cells over a period of 120 h. 17β-Estradiol, the positive control, significantly stimulated cell proliferation, reaching a plateau of maximal stimulation at a concentration of 1 nM (184.2 ± 28.1 % *vs.* DMSO control; $p < 0.001$). The magnitude was comparable to effects observed in other studies using MCF-7 (ATCC) cells [20], [21]. As expected, the antiestrogen control, tamoxifen (10 μM), reduced cell proliferation to 45.7 ± 6.8 % ($p < 0.001$ *vs*. DMSO control). Treatment with *Leuzea* root extract inhibited MCF-7 cell proliferation in a dose-dependent manner (IC_{50} = 29.7 ± 1.9 μg/mL) (**Fig. 2**). Based on these results we used a concentration of 30 μg/mL for the gene expression studies. 20-HE did not influence cell growth at concentrations up to 100 μM. Therefore, the compound was not included in the gene expression profiling.

MCF-7 cells were treated in parallel with 30 μg/mL *Leuzea* root extract, 1 nM 17β-estradiol, 10 μM tamoxifen and solvent control (0.1 % DMSO) for 24 h in the presence of 10 % charcoal stripped serum. This set of experiments was repeated to obtain independent duplicates of data. After RNA extraction, gene expression profiles were recorded using Affymetrix HG U133 Plus 2.0 GeneChip Arrays as described. For the different treatments regulated (= differentially expressed) genes were identified using the following selection criteria: minimal signal intensity > median and fold change *vs.* DMSO control > 1.5 in both independent experiments. With these criteria we identified 306 probe sets (~0.6 % from the more than 54,00 probe sets on the HG U133 Plus 2.0 Array) representing 241 genes regulated by the *Leuzea* extract. The false positive rate with these criteria was less than 10 % based on random permutation analysis of all gene expression results. From the differentially expressed genes, 91 transcripts (38 %) were up-regulated and 150 transcripts (62 %) were down-regulated. The genes were grouped into functional categories according to Gene Ontology terms and gene description at the NetAffx™ Analysis Center (http://www.affymetrix.com/analysis/index.affx) and, in addition, literature search. Most of the differentially expressed genes could be assigned to five larger groups of functionally related genes (subgroups in brackets): apoptosis, proliferation (cell cycle, DNA replication), general growth (RNA processing, protein processing, transcription, cell structure and organization), signaling and transport (signal transduction, transport) and metabolism (oxidoreductases, biosynthesis and catabolism). Genes that could not be assigned to any of these groups were summarized as other. About one third of all differentially expressed genes could be classified to the proliferation category. As shown in **Fig. 3**, a majority of genes in this group, especially transcripts belonging to the subgroup DNA replication, appeared to be down-regulated. In contrast, almost all genes assigned to oxidoreductases were up-regulated. Transcripts of these categories are statistically highly over-represented among all *Leuzea* regulated genes ($p << 0.001$, two-sided p value Fisher exact test). Expression values of selected genes are presented in **Table 1** (complete list in the Supporting Information, **Table 3S**).

The array data discussed in this publication have also been deposited in NCBIs Gene Expression Omnibus (GEO, http://

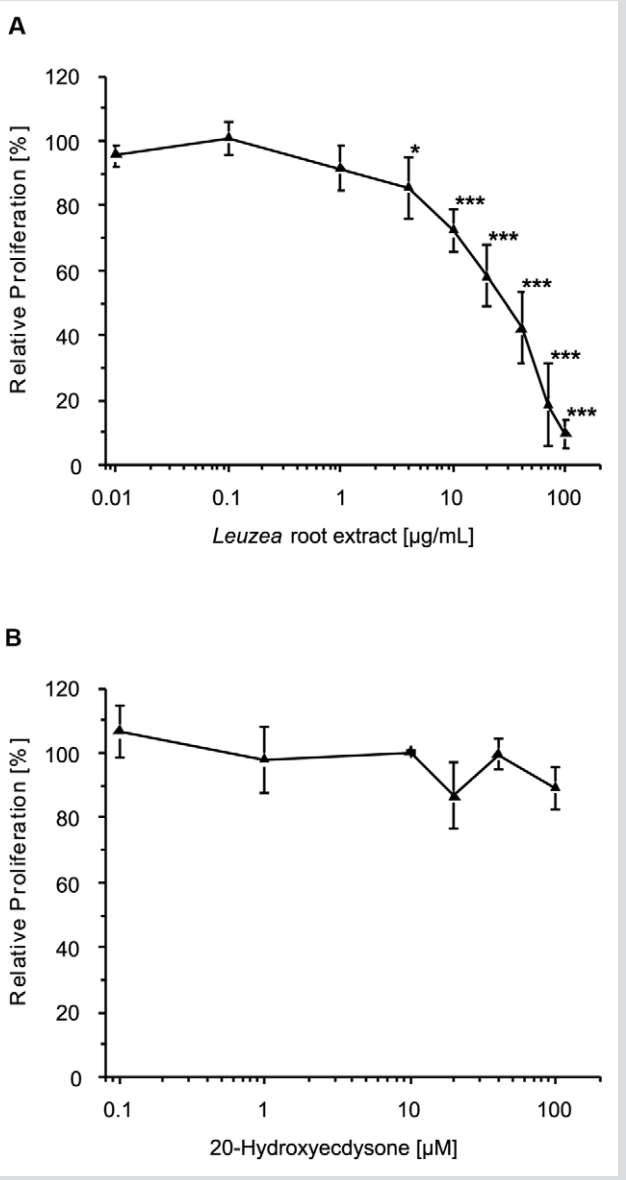

Fig. 2 Effects on the growth rate of MCF-7 cells after 120 h treatment with *Leuzea* root extract (**A**) and 20-hydroxyecdysone (**B**). Cell number equivalents were measured by MTT assay and relative proliferation data *vs.* solvent control (0.1 % DMSO) are presented as means ± SD (n = 3 – 6).

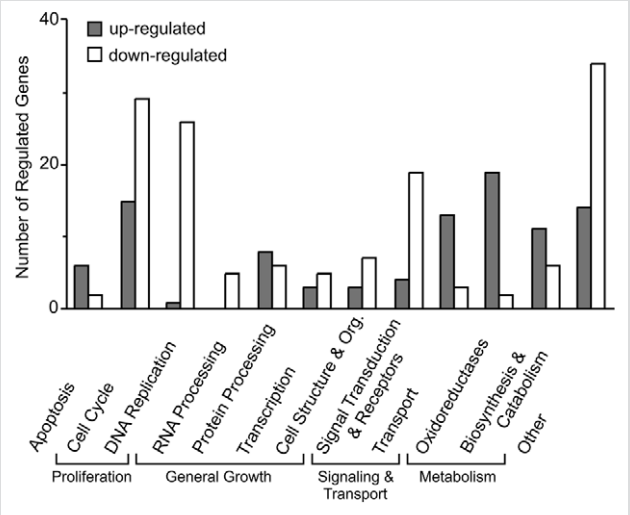

Fig. 3 Functional categorization of 241 transcripts differentially expressed after 24 h treatment of MCF-7 cells with *Leuzea* root extract. Each bar represents the number of genes that were up- (dark) or down-regulated (white) in the respective group/subgroup.

www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE6803.

For verification of the microarray results we determined by real-time RT-PCR the expression levels of 12 genes representing all functional categories as well as the major cellular effects observed after treatment with *Leuzea* extract. All PCR expression data were qualitatively and quantitatively comparable to the microarray results (see **Fig. 5**, **Fig. 6** and Supporting Information, **Fig. 2S**). In most cases PCR analysis showed a higher fold change. This good correlation supports the stringency of the microarray filter settings and, therefore, the significance of the accumulation of genes found in the functional categories described above.

The major transcriptional effects exerted by the *Leuzea* extract are summarized in **Fig. 4** and are discussed in the following.

Gene regulation was observed in an anti-proliferative manner corroborating the results of the proliferation assays. Down-regulation of CCNE2 (cyclin E2), FOXM1 (forkhead box M1), GTSE1 (G-2 and S-phase expressed 1) and PCNA (proliferating cell nuclear antigen) and induced expression of CCNG2 (cyclin G2), GADD45A (growth arrest and DNA-damage-inducible, alpha) and TP53INP1 (tumor protein p53 inducible nuclear protein 1) pointed to cell cyle arrest at the G1/S-transition checkpoint. In addition, some 30 genes linked to DNA replication and synthesis appeared to be down-regulated, suggesting a reduced replication rate and further corroborating an arrest at the G1/S-transition checkpoint.

The regulation of various transcripts was in a pro-apoptotic or apoptosis-sensitizing manner. Transcripts of apoptosis-inducing products such as GADD45A, TP53INP1, GDF15 (growth differentiation factor 15) and DDIT4 (DNA-damage inducible transcript 4) were up-regulated. However, expression levels of GTSE1, BIRC5 (baculoviral IAP repeat-containing 5) and HELLS (lymphoid-specific helicase), coding for proteins with anti-apoptotic functions, appeared to be decreased.

Various transcripts coding for enzymes with oxidoreductase activity, such as AKR1C1 and AKR1C3 (aldo-keto reductase family 1, member C1/3) were strongly upregulated. Expression levels of CYP1A1 (cytochrome P450, family 1, subfamily A, polypeptide 1) were highly induced (48.9 and 43.6 fold) – the most prominent increase in the array experiments. Real-time RT-PCR investigation gave an even higher induction (**Fig. 5**). Other transcripts, which are known to be linked to the aryl hydrocarbon receptor pathway [22], [23], [24], were also up-regulated including CYP1B1, ALDH3A1 (aldehyde dehydrogenase 3 family, member A1), ME1 [malic enzyme 1, NADP(+)-dependent, cytosolic], NQO1 [NAD(P)H dehydrogenase, quinone 1] and TIPARP [TCDD-inducible poly(ADP-ribose) polymerase].

Expression levels of various transcripts known to be involved in tumor initiation, progression and suppression appeared to be affected by the extract. Here, the gene expression changes appeared to be balanced in a tumor-supporting as well as a tumor-suppressing manner (Supporting Information, **Table 4S**).

Parallel to the experiments with the *Leuzea* extract, expression profiles with 17β-estradiol and the estrogen receptor-antagonist tamoxifen were recorded to compare the respective patterns. Intersections of the three treatments are illustrated in **Fig. 7**. After treatment with 1 nM estradiol, 146 transcripts met our selection criteria. Among these were well known estrogen-regulated genes such as GREB1 (GREB1 protein), which was up-regulated 27-fold (Supporting Information, **Fig. 2S**). With 10 μM tamoxifen, 49 genes were significantly regulated. A comparison of the expression patterns of *Leuzea* extract and tamoxifen treatment revealed a correlated regulation of 23 transcripts, meaning that the genes are regulated in the same direction with both treatments. Since *Leuzea* extract and tamoxifen both inhibited cell proliferation (see above), it was not surprising that almost all genes of this intersection were related to cell cycle regulation and apoptosis. Among these genes, the two cell cycle inhibitory transcripts CCNG2 (**Fig. 6**) and TP53INP1 were of particular interest, because they were also regulated by estradiol, but in the opposite direction compared to *Leuzea* and tamoxifen. The repression of these genes by estradiol was in accordance with the stimulatory activity observed in the proliferation assay (see above) and corroborated the reliability of our test system. Nevertheless, comparing *Leuzea* and estradiol treatment, the expression of 24 transcripts appeared to be correlated. Among these were well-known estrogen-sensitive genes such as ESR1 (estrogen receptor 1) (**Fig. 6**), EFEMP1 (EGF-containing fibulin-like extracellular matrix protein 1), HSPB8 (heat shock 22kDa protein 8; syn. estradiol-induced gene 1, E2IG1), LRRC54 (leucine rich repeat containing 54; syn. estradiol-induced gene 4, E2IG4) and CSTA (cystatin A) suggesting some degree of estradiol-like activity of the *Leuzea* root extract.

The expression of ERα, whose transcript was down-regulated by *Leuzea* root extract and estradiol treatment in the array experiment, was evaluated at the protein level. ERα protein was significantly reduced with both treatments (**Fig. 8**). We observed a more than additive reduction when cells were simultaneously treated with *Leuzea* extract and estradiol.

Given that CYP1A1 and other genes linked to the aryl hydrocarbon receptor were strongly up-regulated in the array experiments, a possible AhR activity of the *Leuzea* extract and 20-HE was investigated in a luciferase-based, XRE-driven reporter gene assay in transfected H4IIE rat hepatoma cells. A dose-dependent induction of luciferase activity was observed with *Leuzea* extract (**Fig. 9**). At a concentration (28 μg/mL) similar to the previous experiments in MCF-7 cells, the magnitude of induction was comparable to the AhR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (1 nM). 20-HE, in contrast, did not induce luciferase activity at concentrations up to 50 μM.

Table 1 List of selected genes regulated by *Leuzea* root extract in MCF-7 cells. Genes are listed with symbol, GenBank accession number and fold changes *vs.* DMSO control of two independent microarray experiments.

Gene Title	Gene Symbol	Accession No.	Fold Change	
			#1	#2
CELL CYCLE/PROLIFERATION				
Pregnancy-induced growth inhibitor	OKL38	NM_013370	2.7	2.4
Growth arrest and DNA-damage-inducible, alpha	GADD45A	NM_001924	2.1	2.2
Cyclin G2	CCNG2	AW134535	1.5	1.5
Forkhead box M1 (syn. FKHL16, HFH11, MPP2)	FOXM1	NM_021953	–1.6	–1.7
G-2 and S-phase expressed 1	GTSE1	NM_016426	–1.7	–1.6
Proliferation-related Ki-67 antigen	MKI67	AU147044	–1.7	–1.9
Cyclin E2	CCNE2	AF112857	–1.8	–2.0
Kinesin family member 11 (Eg5)	KIF11	NM_004523	–2.7	–1.9
DNA REPLICATION, SYNTHESIS, REPAIR				
CDC6 cell division cycle 6 homolog (*S. cerevisiae*)	CDC6	U77949	–1.6	–1.6
Flap structure-specific endonuclease 1	FEN1	BC000323	–1.8	–1.8
MCM5 minichromosome maintenance deficient 5 (*S. cerevisiae*)	MCM5	AA807529	–2.1	–1.6
DNA replication complex GINS protein PSF1	PSF1	NM_021067	–2.1	–1.5
MCM2 minichromosome maintenance deficient 2, mitotin (*S. cerevisiae*)	MCM2	NM_004526	–2.2	–1.7
Dihydrofolate reductase	DHFR	NM_000791	–2.2	–1.7
MCM4 minichromosome maintenance deficient 4 (*S. cerevisiae*)	MCM4	AI936566	–2.2	–1.9
Thymidylate synthetase	TYMS	NM_001071	–2.4	–1.6
Proliferating cell nuclear antigen	PCNA	NM_002592	–2.4	–1.5
Thymidine kinase 1, soluble	TK1	BC007986	–2.5	–1.7
APOPTOSIS				
Growth differentiation factor 15	GDF15	AF003934	5.1	5.7
DNA-damage-inducible transcript 4	DDIT4	NM_019058	3.4	3.3
Tumor protein p53 inducible nuclear protein 1	TP53INP1	AW341649	2.0	2.1
Immediate early response 3 (immediate early gene X-1)	IER3	NM_003897	1.9	1.6
Helicase, lymphoid-specific (PASG)	HELLS	NM_018063	–2.0	–2.2
Baculoviral IAP repeat-containing 5 (survivin)	BIRC5	AA648913	–2.3	–1.9
PROTEIN PROCESSING				
TCDD-inducible poly(ADP-ribose) polymerase (syn. PARP7, XRN2)	TIPARP	AL556438	4.5	1.8
Heat shock 22kDa protein 8 (syn. E2IG1, HSP22)	HSPB8	AF133207	2.4	1.7
Cystatin A (stefin A)	CSTA	NM_005213	–1.8	–1.9
EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	AI826799	–4.8	–3.1
SIGNALING and TRANSPORT				
Solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	SLC7A11	AA488687	5.0	3.9
Ferritin, light polypeptide	FTL	BG538564	3.4	3.1
Ferritin, heavy polypeptide 1	FTH1	AA083483	3.1	3.5
Leucine rich repeat containing 54 (syn. E2IG4, TSK)	LRRC54	NM_015516	2.7	2.0
Insulin-like growth factor binding protein 5	IGFBP5	AW007532	–2.3	–2.8
Growth factor receptor-bound protein 14	GRB14	NM_004490	–2.5	–2.9
Estrogen receptor 1	ESR1	NM_000125	–3.2	–3.6
OXIDOREDUCTASES				
Cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	NM_000499	48.9	43.6
Aldo-keto reductase family 1, member C1	AKR1C1	M33376	12.7	11.9
Heme oxygenase (decycling) 1	HMOX1	NM_002133	11.4	10.8
Aldehyde dehydrogenase 3 family, memberA1	ALDH3A1	NM_000691	7.4	4.3
Cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	AU154504	7.1	4.7
Aldo-keto reductase family 1, member C3	AKR1C3	AB018580	4.7	3.7
Cytochrome P450, family 4, subfamily F, polypeptide 11	CYP4F11	NM_021187	3.7	3.9
NAD(P)H dehydrogenase, quinone 1	NQO1	AI039874	2.7	2.4
Glutathione peroxidase 2	GPX2	NM_002083	2.3	1.5
Glutaredoxin (thioltransferase)	GLRX	AF162769	2.0	1.8
Malic enzyme 1, NADP(+)-dependent, cytosolic	ME1	AL049699	1.9	1.6

HEK II cells expressing 5α-reductase, type II, one of the key enzymes in steroid metabolism, were used to examine potential inhibition of this enzyme by the *Leuzea* extract and 20-HE. While the ecdysteroid had no effect, the extract inhibited the activity in a dose-dependent manner (IC_{50} = 38.1 μg/mL).

Discussion

Genome-wide expression profiling is a powerful tool for the identification of unknown pharmacological targets for herbal extracts and their putative active principles [25], [26]. In the

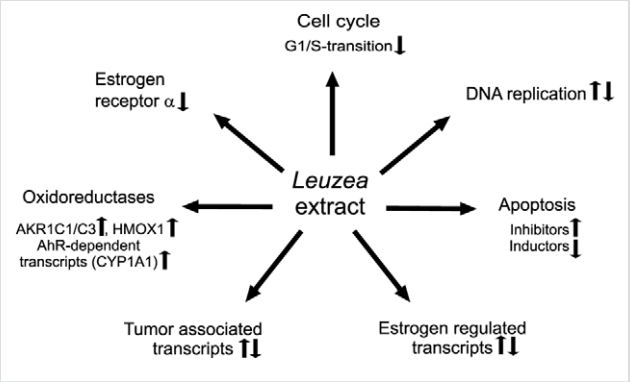

Fig. 4 Summary of effects of *Leuzea* root extract in MCF-7 cells at mRNA level observed with the microarray experiment. ↓ represents inhibition, ↑ represents stimulation.

case of *Leuzea*, a key issue was whether claims of anabolic and anti-aging properties of extracts and the major ecdysteroid, 20-HE, could be substantiated by the patterns of regulated genes. MCF-7 cells were selected as a model because this cell line expresses a wide range of nuclear receptors which could be involved in anabolic stimulation, anti-aging activity, and possibly be a target for the claimed active principle 20-HE, for which no mammalian target has been identified so far.

A first important observation was that *Leuzea* extract inhibited cell proliferation, whereas 20-HE had neither inhibitory nor stimulatory effects on cell growth even at 100 μM. On the other hand, the effects of estradiol and the ER antagonist tamoxifen, on cell proliferation were pronounced, and compounds were used as controls for the gene expression studies.

Comparing the expression patterns of *Leuzea* extract and estradiol, we surprisingly observed correlated regulation of 24 genes, among these many well-known estrogen-sensitive transcripts. On the other hand 23 transcripts were correlated between *Leuzea* and tamoxifen treatments. Most of these genes are involved in cell proliferation, DNA replication and apoptosis (such as CCNG2 and TP53N1) and were regulated in an antiproliferative and pro-apoptotic manner. Overall about one third of the genes regulated by *Leuzea* extract are coding for products involved in the regulation of cell proliferation and DNA replication. The vast majority of these transcripts appeared to be down-regulated, consistent with the results of the proliferation assays. Another salient feature was that transcripts coding for enzymes with oxidoreductase activity were highly over-represented among the *Leuzea* regulated genes. Some genes were strongly up-regulated, such as those of CYP1A1, and, to a lesser extent, CYP1B1. The two oxidoreductases are involved in xenobiotic metabolism, mediate toxic and tumorigenic effects of several chemicals, but are also involved in the metabolism of 17β-estradiol (E2) [27]. CYP1A1 metabolizes E2 to non-carcinogenic 2-hydroxy-E2 whereas CYP1B1 is responsible for the formation of carcinogenic 4-hydroxy-E2. The two enzymes are not necessarily expressed at the same level in tissues. An increased production of 2-hydroxy-E2 relative to 4-hydroxy-E2, due to a preferential expression of CYP1A1 over CYP1B1, has been suggested to contribute to the antitumor activity of natural compounds such as indole-3-carbinol and is, therefore, of clinical importance [27].

CYP1A1, which was up-regulated up to 130-fold after *Leuzea* extract treatment, and a handful of other regulated transcripts (CYP1B1, ALDH3A1, ME1, NQO1, TIPARP) are known target genes of the aryl hydrocarbon receptor (AhR), a member of the superfamily of nuclear receptors. The AhR, upon binding of a ligand, forms a heterodimeric complex with ARNT (aryl hydrocarbon receptor nuclear translocator) which induces transactivation of the CYPs and other target genes via binding to xenobiotic response elements (XREs) in their promoter regions [28]. In a XRE-driven luciferase assay in rat hepatoma H4IIE cells *Leuzea* root extract showed AhR activation comparable to TCDD, a well-

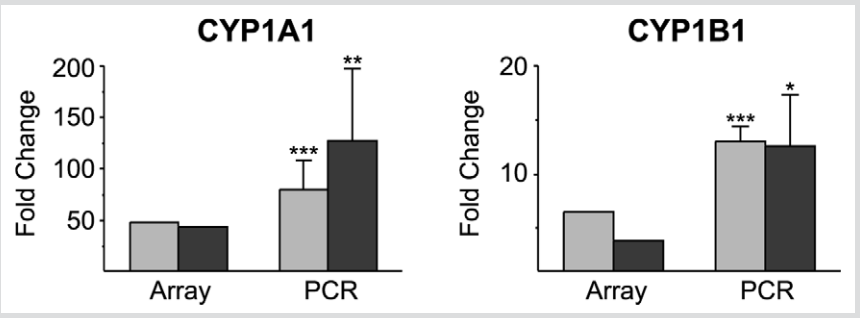

Fig. 5 Gene expression levels of CYP1A1 and 1B1 (cytochrome P450, family 1, subfamily A/B, polypeptide 1) in MCF-7 cells after 24 h treatment with *Leuzea* root extract. Expression data obtained with microarrays (**Array**) and real-time RT-PCR (**PCR**) are shown as fold changes calculated *vs.* DMSO control. RT-PCR measurements were done at least in triplicate, means ± SD are presented.

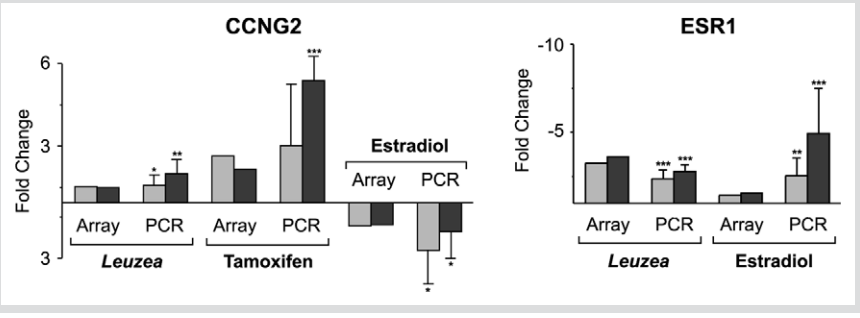

Fig. 6 Gene expression levels of CCNG2 (cyclin G2) and ESR1 (estradiol receptor α) in MCF-7 cells after 24 h treatment with *Leuzea* root extract, estradiol or tamoxifen. Expression data obtained with microarrays (**Array**) and real-time RT-PCR (**PCR**) are shown as fold changes calculated *vs.* DMSO control. RT-PCR measurements were done at least in triplicate, means ± SD are presented.

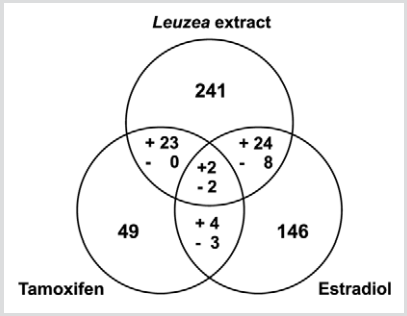

Fig. 7 Intersection diagram of *Leuzea* root extract, estradiol and tamoxifen expression profiles. Numbers within the intersections represent genes regulated with both of the respective treatments or – in the middle – all 3 different treatments. For every intersection genes regulated in the same direction up or down (correlated) are marked with (+). Genes regulated in opposite directions (anti-correlated) are marked with (–).

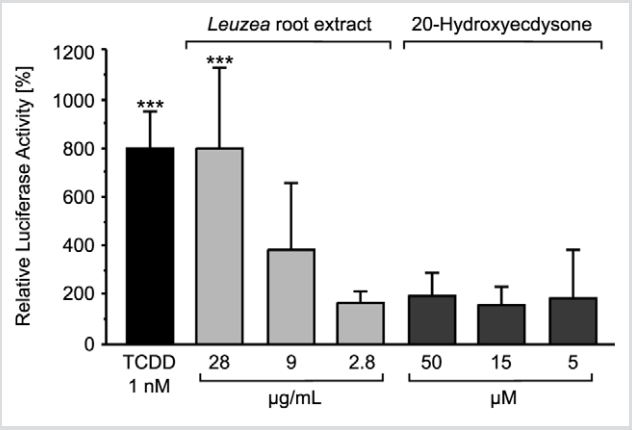

Fig. 9 AhR activity of *Leuzea* root extract but not 20-hydroxyecdysone detected by XRE-dependent luciferase assay in H4IIE cells. 1 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was used as reference compound. Bars represent relative luciferase activity *vs.* solvent control. Data are presented as means ± SD (n = 3)

known AhR ligand. 20-HE, in contrast, did not induce luciferase activity. Given that classical AhR ligands are hydrophobic and planar or coplanar molecules of polycyclic structure, this negative result for 20-HE is not surprising. The AhR ligands in *Leuzea* roots are yet to be identified, as there are no obvious candidates among the currently known secondary metabolites of *Leuzea* (HPLC profiles and a list of identified compounds are given as Supporting Information, **Fig. 1S** and **Table 1S**).

Via AhR activity of the *Leuzea* extract the estrogen-like gene expression as well as a decrease of ERα transcript and protein level might be explained. Western blot analysis showed a marked decrease of ERα protein in cells treated with *Leuzea* extract or E2. The more than additive effect obtained by a simultaneous treatment with extract and E2 suggested a synergistic activity. A decrease of ERα protein content could be either due to down-regulation of transcription or enhanced degradation, as previously described for estradiol treatment [29], [30]. In the presence of E2 a negative cross-talk between the AhR/ARNT complex and ligand-bound ERα has been reported, leading to enhanced proteasome-dependent degradation of both receptors [31]. In the absence of E2, however, ligand-activated AhR/ARNT heterodimers can activate estrogen receptors and, therefore, induce expression of ER target genes [32]. This may explain the regulation of estrogen-sensitive transcripts by *Leuzea* extract.

Within the group of oxidoreductases, transcripts coding for AKR1C1 and AKR1C3 (aldo-keto reductase family 1, member C1/3) were up-regulated. The AKR enzymes are known to be involved in progesterone metabolism in breast cells. Progesterone is converted by AKRs to 4-pregnene derivatives, which exhibit anti-cancer activities [33]. However, the 4-pregnenes and progesterone can be reduced by 5α-reductase to tumor-promoting 5α-pregnanes [33], [34]. Reduced activity and mRNA levels of aldo-keto reductases (AKRs) and increased 5α-reductase activity have been found in breast cancer tissue samples and cell lines, resulting in a high ratio of 5α-pregnanes:4-pregnenes [34], [35], [36]. Treatments counteracting this disproportion, for example, by increased AKR activity and simultaneously decreased 5α-reductase activity, as observed in our studies with *Leuzea* root extract *in vitro*, have been proposed as a new approach in cancer prevention and therapy [34]. Furthermore, an increase of 4-pregnene levels following *Leuzea* treatment might also contribute to the ERα decrease described above [37].

In conclusion, several putative pharmacological targets of a lipophilic *Leuzea* root extract could be identified. The potential anti-cancer properties resulting from AhR activity, and effects on ERα, AKR and 5α-reductase levels/activity have to be further substantiated, given that about 30 transcripts associated with tumor progression and suppression were up- or down-regulated in a manner that did not reveal a consistent overall pattern. This inconsistency is not really unexpected considering that the expression profile was obtained with a multi-component mixture, in which several individual phytochemicals contribute to the observed expression pattern. The phytoecdysteroid 20-HE, however, did neither affect cell proliferation, nor ERα protein and 5α-reductase activity. There is no evidence so far that the compound does play a role in the activity of *Leuzea* extracts.

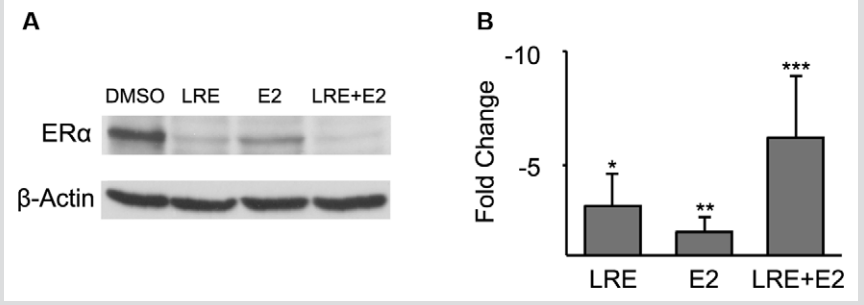

Fig. 8 Western blot analysis of ERα expression in MCF-7 cells after 24 h treatment with *Leuzea* root extract (**LRE**), estradiol (**E2**), and both (**LRE+E2**) (**A**). The diagram (**B**) represents means ± SD of protein expression levels of 4 experiments calculated as fold changes *vs.* DMSO control.

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