

Effect of *Echinacea angustifolia* extract on cell viability and differentiation in mammary epithelial cells

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Abstract

Echinacea spp. are popularly used as an herbal medicine or food supplement for enhancing the immune system and activating biological property in different tissues. In this study we show the biological effect of *Echinacea angustifolia* extract on cell viability and cell differentiation in mammary epithelial cell lines. These effects have been observed in two different cell line derived from mouse (HC11) and bovine (BME-UV). *Echinacea* extract enhanced cell liability from 100 to 1000 ng/ml in association with growth factors, epidermal growth factor (EGF) or insulin, but also without EGF ($p < 0.05$) up to 37% vs. control. This effect may be modulated by MAPK and Akt activation that *Echinacea* extract treatment increased and/or by a reduction of caspase 3 activity, showed a dose–response decrease after *Echinacea* treatment. Finally *Echinacea* extract was able to increase ($p < 0.05$) at 100 ng/ml β -casein expression in association with PRL (5 μ g/ml). These data demonstrate that *Echinacea angustifolia* extract can stimulate mammary epithelial cell physiology and may be considered a candidate to support mammary gland activity during a mammogenic and lactogenic state.

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Introduction

Echinacea, also known as purple coneflower, has become a top-selling medicinal herb and food supplement in Europe and the United States (Ernst, 2002). Three major species, *Echinacea purpurea*, *Echinacea angustifolia* and *Echinacea pallida*, have been studied for their possible pharmacological and immunological effects (Nieri et al., 2003; Rininger et al., 2000). They represent one of the most common traditional American herbal medicines that is commonly believed to act as an immunostimulant and marketed commercially as a cold and flu remedy. There are three predominant species of

Echinacea used for such purposes: *E. angustifolia*, *E. purpurea* and *E. pallida*. However, until recently, little evidence-based research into a series of claimed effects was performed, and as a result, clear demonstrations of pharmacological efficacy were often not available. Laboratory studies have shown that *E. purpurea* herb and purified polysaccharides from *E. purpurea* cell cultures possessed immunostimulatory activity to murine and human macrophages and mononuclear cells (Coegniet and Elek, 1987; Roesler et al., 1991a, b; See et al., 1997; Stimpel et al., 1984; Wagner et al., 1986). *Echinacea*-activated macrophages and natural killer (NK) cells displayed cytokine [tumor necrosis factor α (TNF- α), interleukin (IL)-1, IL-6] production, enhanced phagocytic activity, cellular proliferation and the capacity to kill tumor cells as well as effectively eliminate bacterial and fungal pathogens *in vitro* (Coegniet and

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Elek, 1987; Luettig et al., 1989). The purified polysaccharides were subsequently shown to protect immunocompromised mice from both fungal and bacterial infection (Roesler et al., 1991a,b; Steinmuller et al., 1993). Testing of the *Echinacea*-derived polysaccharides in human subjects also demonstrated activation of phagocytic cells similar to that seen in mice (Luettig et al., 1989; See et al., 1997).

Furthermore, *Echinacea* extracts have been prescribed as adjunct therapy for cancer. Evidence of this self-medication can be seen in the fact that 16% of patients use *Echinacea* while undergoing cancer chemotherapy (Bernstein and Grasso, 2001). Sparreboom et al. (2004) reviewed the effect of herbal medicines with anticancer drugs and identified that *Echinacea* can interact with anticancer drugs oxidized by the 3A4 isoform of cytochrome P450 and they also discussed substrates of cytochrome P450 enzymes. *E. purpurea* extracts have shown anti-apoptotic activity through upregulation of bcl-2 and downregulation of Fas (Di Carlo et al., 2003). Since *Echinacea* extracts may prevent apoptosis of normal cells, they may prevent apoptosis of cancer cells as well. In the present study we report for the first time the effect of *E. angustifolia* extract on cell viability and cell differentiation in mammary epithelial cells. We show a specific activation of intracellular pathway involved in cell viability and proliferation and a prevention in caspase 3 accumulation that indicates an anti-apoptotic effect of *Echinacea* extract.

Materials and methods

Materials

E. angustifolia extract¹ (Polinacea™, cod. 9045000; lot. 28226/M1; titer HPLC echinacoside: 4.8% p/p; titer HPLC polysaccharide of *Echinacea*: 5.2% p/p, ethanol 0.07 p/p) was kindly provided by Indena Spa (Milan, IT); RPMI 1640, Insulin, ovine recombinant prolactin, epidermal growth factor (EGF) and dexamethasone were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Master mix for real-time PCR was purchased from BioRad Laboratories Inc. (Hercules, CA, USA); fetal calf serum (FCS), glutamine and penicillin were purchased from EuroClone Ltd (West York, UK).

Cell culture

HC11 cell line was derived from midpregnant BALB/c mouse mammary tissue and is considered to retain

important characteristics of normal mammary epithelial cells such as the ability to produce milk protein in response to lactogenic hormones without cultivation on exogenous extracellular matrix or cocultivation with adipocytes or fibroblasts (Marte et al., 1995). These cells were maintained in RPMI 1640 medium containing 10% FCS, 5 µg/ml insulin, 10 ng/ml EGF, 2 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin (growth medium). To induce differentiation, cells were grown to confluence and cultured for an additional day in growth medium. Subsequently, the cells were incubated for up to 4 days in differentiation medium (RPMI 1640 medium containing 3% FCS and the lactogenic hormones dexamethasone (1 µM), insulin (5 µg/ml) and prolactin (5 µg/ml). Differentiation was monitored by measuring expression of the milk protein β -casein (Baratta et al., 2000).

The BME-UV bovine mammary epithelial cell line was kindly provided by Dr. Politis (Agricultural University of Athens, Athens, Greece). BME-UV cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum.

Cell viability

Cell viability was evaluated using WST-1 assay (Roche Diagnostic, Penzberg, Germany). The intensity of the colored compound formed (formazan dye) was quantified with an ELISA microplate reader (Biorad 680). Briefly, 300 cells per well were seeded in a 96-wells microplate. After 12 h of culture, control cells ($n = 6$) were quantified by WST-1 reagent (day 0) to normalize the successive measurements of treated and non-treated groups. When WST-1 reagent was added the wells were subjected to further incubation for a period of 60 min to facilitate the reaction between mitochondrial dehydrogenase released from viable cells and tetrazolium salt of WST-1 reagent. The absorbance was measured at 450 nm, with the reference at 620 nm. Cell viability was routinely evaluated after 24, 48 and 72 h of treatments.

Western blotting

Cells were lysed with EB buffer (10 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100) supplemented with Protease Inhibitor Cocktail (Sigma), 1 mM PMSF and 1 mM sodium orthovanadate. The protein concentration was determined using the BioRad Dc Protein Assay. Twenty micrograms of total protein were run on 10% SDS-PAGE and transferred to Hybond-C extra membranes (Amersham Pharmacia Biotech). Bound antibodies were detected with horseradish peroxidase-conjugated secondary antibody and Supersignal ECL (Pierce) and recorded by X-ray film. Antibodies were obtained as follows:

¹Detailed informations on the analysis and composition of the *Echinacea* extract can be obtained by Internet: <http://www.indena.com/pdf/polinacea.pdf>. See also publication Cozzolino et al. Carbohydrate Polymers, 65 (2006) 263–272: www.sciencedirect.com.

phospho STAT-5 (UpState); STAT-5 (Santa Cruz); cleaved caspase 3 (Cell Signaling.); α -tubulin (Sigma); anti-phospho-MAPK mouse monoclonal antibodies were from Sigma-Aldrich (St. Louis, MO, USA); anti-MAPK rabbit polyclonal, anti-phospho-Akt mouse monoclonal and anti-Akt rabbit polyclonal antibodies were from Cell Signaling Technologies (Danvers, MA, USA).

β -casein gene expression measurement by real-time PCR

Cells were lysed and total RNA was extracted with Macherey-Nagel NucleoSpin® RNA II, (Düren, Germany) as described by the manufacturer. One microgram of total RNA was reverse transcribed with ready-to-go first strand beads (Amersham Pharmacia Biotech Uppsala, Sweden) as described by the manufacturer. Diluted cDNAs (1:5 and 1:50) were used for real-time PCR amplification. Primers for mouse β -casein were: forward 5'-TCACTCCAGCATCCAGTCACA-3', reverse 5'-GGCCCAAGAGATGGCACCA-3'; primers for 18S (reference gene) were: forward 5'-CGTTTG-TGTGGGGAGTGAATGGTG-3', reverse 5'-GCGT-GGGGGTTGGCGGAAAGAGAA-3'. real-time PCR parameters were: cycle 1, 95 °C for 90 s; cycle 2, 95 °C 60 s, 59 °C 10 s for 40 cycles. The 2- $\Delta\Delta$ CT method was used to analyze the data as described by Livak and Schmittgen (2001).

Quantitative analysis of caspase III activity

This procedure has been published in Hermisson et al. (2000). Briefly, the cells were seeded in 96-well plates (10,000 cells per well) and allowed to attach for 24 h. The cells were treated with curcuma extract for 48 h and lysed in lysis buffer containing 25 mM Tris-HCl (pH 8.0), 60 mM NaCl, 2.5 mM EDTA and 0.25% NP40 for 10 min. The Caspase 3 Substrate II, Fluorogenic (Ac-DEVD-amc (12.5 μ M, Merck Chemicals Ltd., Beeston Nottingham UK), diluted in PBS, was added and incubated at 37 °C for 10 min. Caspase activity was measured for 1 h using a CytoFluor 2350 Millipore fluorimeter at 360 nm excitation and 480 nm emission wavelengths. Data are expressed as relative light units (RLU) and are the mean of 10 replicates for each treatment.

Statistical analysis

Gene expression analysis and western blot were repeated three times independently. Cell proliferation test was performed three times and each treatment had six replicates. In proliferation analyses, data collected from HC11 cells were expressed in percentage relative to

the internal control (optical density detected after the first 12 h of culture, day 0). Experimental data are presented as mean \pm SD. Statistical differences between treatments and interactions were calculated with multifactorial ANOVA using the Statgraphics package (STSC Inc., Rockville, MD, USA). When significant differences were found, means were compared by Scheffè's *F*-test.

Results

Effect of Echinacea extract on cell viability

In HC11 cell line EGF increase cell viability and/or proliferation up to 30% after 48 h of incubation. Echinacea extract (1000 ng/ml) significantly ($p < 0.05$) enhanced cell viability when cells were treated without EGF. Further, cells viability increase ($p < 0.05$) when Echinacea extract was added at 100 and 1000 ng/ml (up to 21% and 37% respectively, Fig. 1a). Also in BME-UV cell line, Echinacea extract was able to increase cell viability but only in association with insulin (1 μ g/ml), used as positive control for cell viability in this cell line (Fig. 1b).

Effects of Echinacea extract on MAPK and Akt activation

EGF is known to activate downstream signaling via phosphorylation and activation of MAPK and Akt proteins. Therefore, we monitored the phosphorylation state of MAPK and Akt in mammary cells stimulated for 15 min with EGF and Echinacea extract, alone or in combination (Fig. 3). As expected, EGF-induced MAPK and Akt phosphorylation. Echinacea extract did not significantly modify MAPK activation but significantly enhanced Akt phosphorylation in association with EGF in HC11 cell line (Fig. 2).

Effect of Echinacea extract on cleaved caspase 3

Loss of viability induced by EGF deprivation may be a consequence of an induction of apoptosis. Apoptosis can be brought about by initiation of either the intrinsic or extrinsic pathways, both of which involve caspases (cysteine proteases) as key effectors. We measured if Echinacea extract treatment restored the loss of cell viability with the initiation of an apoptotic event. As shown in Fig. 3 EGF-deprived HC11 cells increased significantly ($p < 0.01$) cleaved caspase 3 signal compared to EGF-treated cells (lanes 1 vs. 3). Echinacea extract showed a slight but significant reduction in caspase III accumulation. In order to determine if this difference could be related to a specific effect of

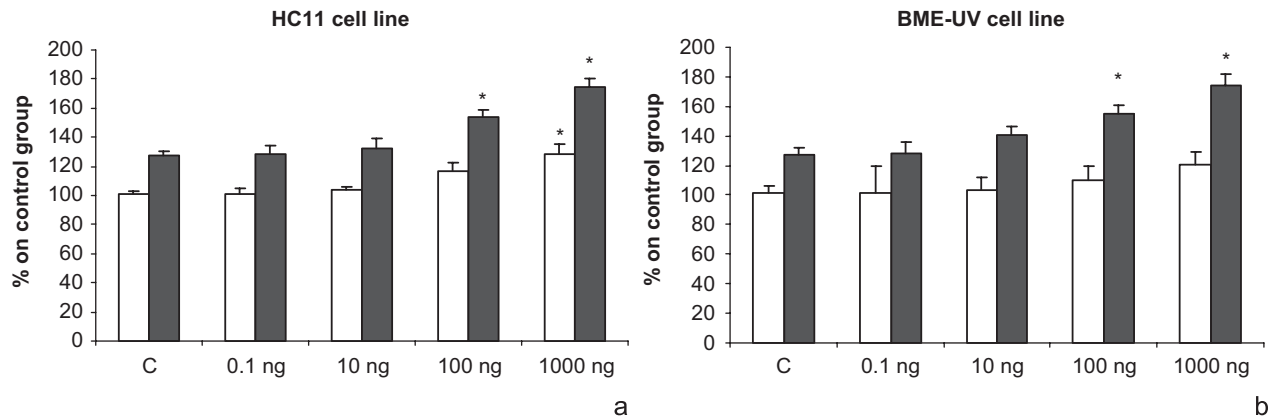


Fig. 1. Effect of Echinacea extract on cell viability in HC11 (a) and BME-UV (b) cell lines. Cells were treated with Echinacea extract (0.1–1000 ng/ml) alone (open bar) or in association with EGF (black bar) for 48 h. Viability was measured by WST-1 test. Data are expressed as percentage on internal control for each cell type measured after 12 h after cell seeding (time 0) and are mean \pm SEM of eight replicated repeated in three independent experiments. *Mean $p < 0.05$.

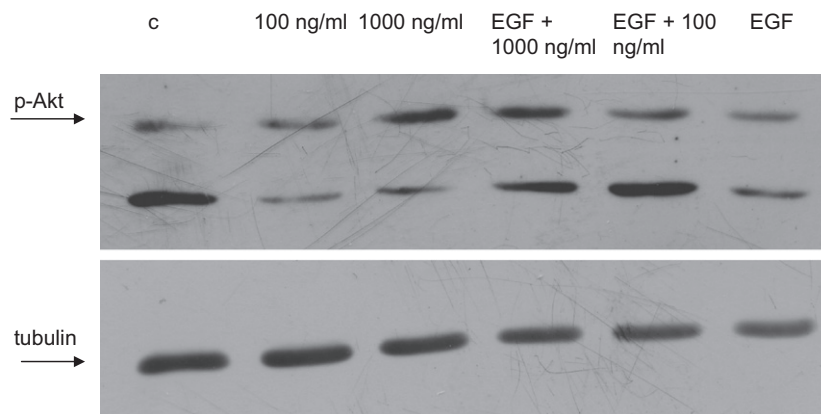


Fig. 2. MAPK (left) and Akt (right) phosphorylation induced by EGF and/or Echinacea extract in HC11 cell line assessed by Western blot. Cells are starved for 12 h in Medium 1640 without serum and growth factors. Echinacea extract increased activation of p-Akt without EGF (line 1 vs. line 2, 100 ng/ml and line 3, 1000 ng/ml) and with EGF (line 6 vs. line 5, 100 ng/ml and line 4, 1000 ng/ml).

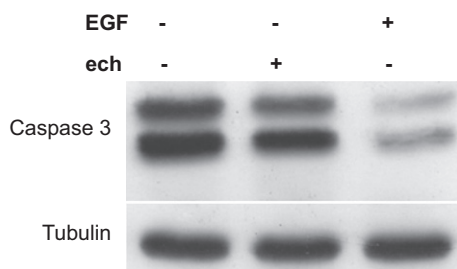


Fig. 3. A representative experiment of cleaved caspase 3 assessed by Western blot: cells were incubated in medium RPMI 1640 3% FCS containing 10 ng/ml insulin, without EGF (lanes 1 and 2) or with EGF (10 ng/ml, lane 3) and with Echinacea extract (100 ng/ml, lane 2).

Echinacea, we measured the quantitative effect of Echinacea extract on caspase 3 enzymatic activity of accumulated caspase 3. In HC11 cell line, Echinacea

extract showed a dose-dependent effect on caspase 3 activity since at very low doses (ranged between 10 and 1000 ng/ml) without the anti-apoptotic role of EGF (Fig. 4a). Also in presence of EGF a positive effect of Echinacea was observed but no dose–response curve was obtained (Fig. 4b). The same effect was observed in BME-UV cell line: Echinacea extract reduced caspase 3 activity in starved BME cells in dose-dependent manner while no dose–response was observed in association with 1 μ g/ml insulin (Fig. 5b).

Effect of Echinacea extract on β -casein gene expression in differentiating mammary epithelial cells

PRL is known to activate downstream signaling via phosphorylation and activation of STAT-5 protein.

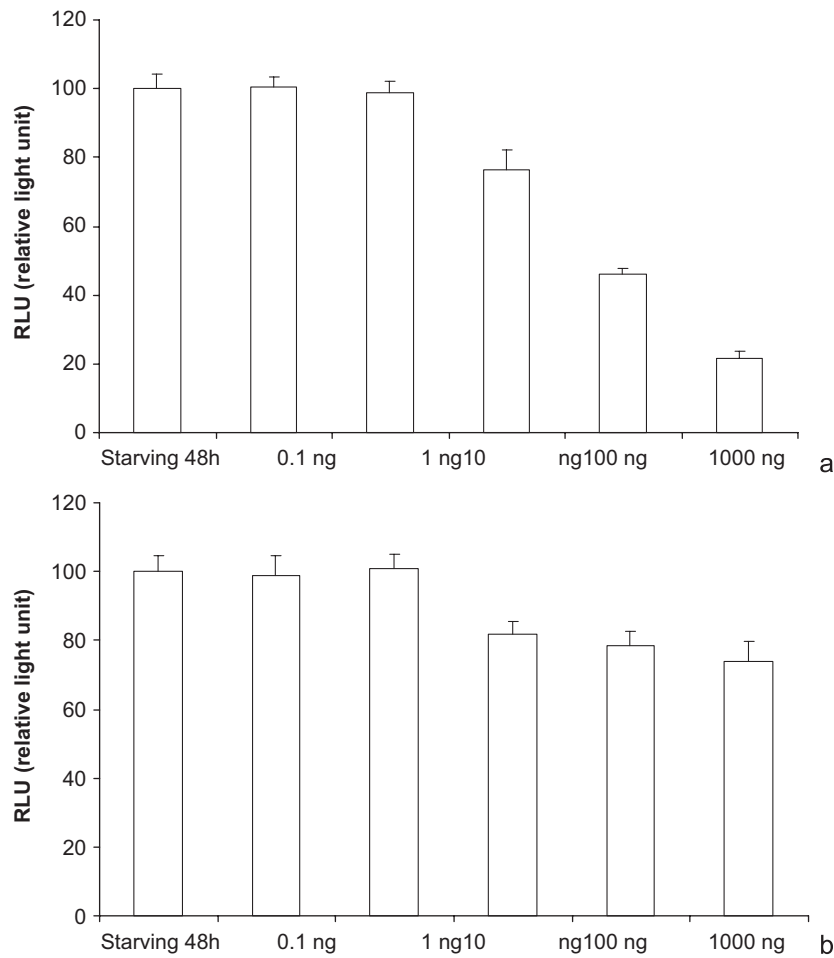


Fig. 4. Echinacea extract on caspase activity measured by caspase 3 substrate II fluorogenic reaction in HC11 cell line. Apoptosis was induced by starvation and data were normalized on the control (starved cell). A significant dose-dependent effect was observed from 10 to 1000 ng/ml (a). In (b) cells were treated with EGF (10 ng/ml), no dose-response curve was obtained but a significant reduction in caspase 3 accumulation from 10 to 1000 ng/ml. Data are expressed as mean \pm SD of 10 replicates, repeated three times.

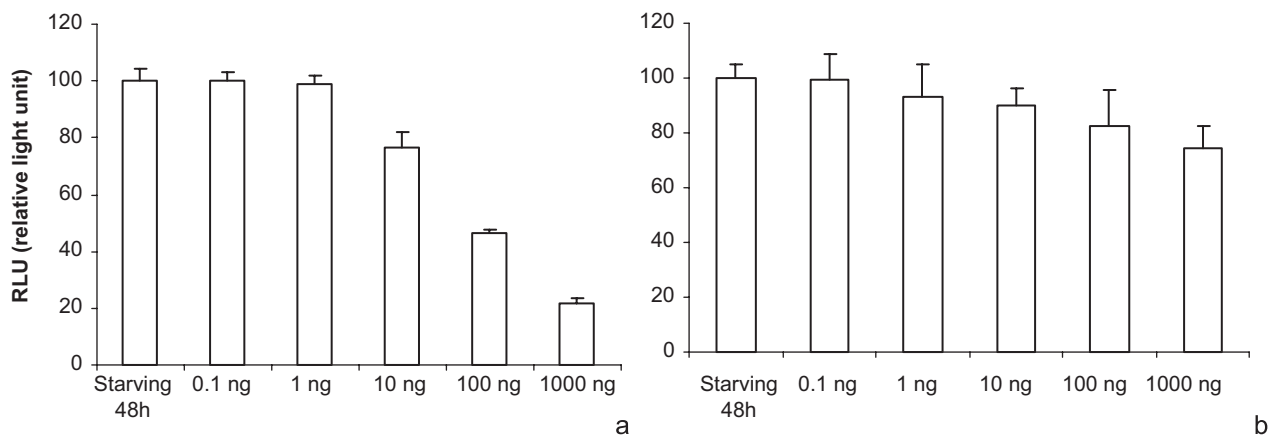


Fig. 5. Echinacea extract on caspase activity measured by caspase 3 substrate II fluorogenic reaction in BME-UV cell line. Apoptosis was induced by starvation and data were normalized on the control (starved cell). A significant dose-dependent effect was observed from 10 to 1000 ng/ml (a). In (b) cells were treated with insulin (1 μ g/ml). Data are expressed as mean \pm SD of 10 replicates, repeated three times.

Therefore, we monitored the phosphorylation state of STAT-5 in HC11 cells stimulated for 10 min with Echinacea extract. As expected, prolactin induced STAT-5 activation, while no effect was observed modulated by Echinacea extract alone or in combination with PRL (5 µg/ml) (Fig. 6).

Mammary epithelial cell line HC11 was able to differentiate and induce expression of the β -casein gene when stimulated with lactogenic hormones. As previously reported (Baratta et al., 2000) treatment of confluent cultures with differentiation medium containing the lactogenic hormones dexamethasone, insulin and prolactin led to a dose-dependent induction of β -casein mRNA expression, while proliferating cells or HC11 cultured in differentiation medium lacking prolactin expressed very low levels of β -casein mRNA. In confluent HC11 cells we treated with Echinacea extract at different concentration (10 and 100 ng/ml): we observed a significant difference in fold induction of β -casein mRNA levels analyzed by real-time PCR (Table 1).

Discussion

Various tissue extracts of Echinacea species have been reported to have immunostimulatory activity on a number of human immune cells, such as macrophages and peripheral blood mononuclear cells (Rininger et al., 2000; Bauer, 2002). *Echinacea* is one of the most popular treatments for the common cold. The glycoproteins, polysaccharides, caffeic acid derivatives and alkylamides have all been reported to have immunostimulating

activity (Bauer et al., 1988). However, a recent study has reported that Echinacea extract stimulated the release of pro-inflammatory cytokines and chemokines released from a cultured line of human bronchial epithelial cells (Sharma et al., 2006a).

Few studies are available on the specific effect of the compounds extracted by this plant on biological property of other cell type. It has been reported the anti-apoptotic activity of *E. purpurea* extracts on non-cancerous cells (Di Carlo et al., 2003). Recently, *E. purpurea* extracts can modulate dendritic cells differentiation and expression of specific immune-related genes (Wang et al., 2006). Furthermore, rhinovirus infection of epithelial cells, and treatment with Echinacea extracts, led to profound effects on numerous transcription factors, which could explain the previously observed modulation of secreted cytokines and chemokines, as well as other signaling pathways (Sharma et al., 2006b). No studies are available to determine if Echinacea extracts may exert their biological function directly throughout specific intracellular pathway. In our study we report in two different mammary epithelial cell lines some effects of Echinacea extract to enhance MAPK e Akt activation. MAPKs are among the central elements that transduce extracellular signaling into cellular responses, and are believed to play a pivotal roles in cell growth and survival (Seger and Krebs, 1995). Akt signaling pathway is also associated with cell motility and invasion (Zhou and Wong, 2006). These pathways are activated by growth factors such as EGF and insulin that, in our models, and are known as essential as mitogenic and survival factors. Further-

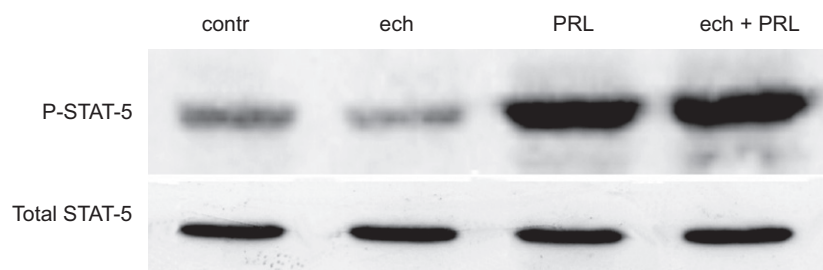


Fig. 6. Echinacea extract does not activate STAT-5 in HC11 cells neither alone (line 2) or in combination with PRL (line 4). Cells were stimulated for 10 min in differentiation medium containing 5 µg/ml prolactin and/or 100 ng/ml Echinacea extract.

Table 1. Echinacea extract effect on the expression of β -casein mRNA

Control	PRL 0.5 µg/ml	PRL 0.5 µg/ml + ech 10 ng/ml	PRL 0.5 µg/ml + ech 100 ng/ml
1 ± 0.2	11.21 ± 0.6	11.21 ± 0.6	13.42 ± 0.7 ^a

HC11 cells were maintained under proliferative conditions, in the presence of 10 ng/ml EGF and 5 µg/ml insulin to reach confluence and then incubated for 72 h in differentiation medium containing 1 µM dexamethasone and 10 ng/ml insulin, 5 µg/ml prolactin with or without Echinacea extract (10 and 100 ng/ml). Levels of β -casein mRNA were monitored by real-time PCR and data are expressed as fold induction compare to undifferentiated cells. Data are mean ± SD of three independent experiments.

^aMeans $p < 0.05$.

more, this extract was able to significantly influence the amount of caspase 3 activity. Caspases are a family of proteins which play a key role in apoptosis induced by various stimuli (Cohen, 1997) and the isoforms 3 and 7 are the main members involved in the induction and execution of the programmed cell death process (Estrov et al., 2003; Luschen et al., 2004). A recent study has reported that the lipophilic root extracts from all the three medicinal *Echinacea* species exert an anti-proliferative activity on human cancer cell lines. The more pronounced concentration- and time-dependent anticancer effect is for *E. pallida* that may be due to its different phytochemical profile as compared to the other two species *E. purpurea* and *E. angustifolia* (Chicca et al., 2007). In that case, it is possible that the different plant composition and preparation may result in a opposite effect, in fact the main class of compounds found in *E. pallida* is polyacetylenes, with only very low traces of alkylamides which represent instead the major components in *E. purpurea* and *E. angustifolia* root hexanic extracts (Barnes et al., 2005).

In our study, it is not possible to identify the molecule that is able to enhance or reduce the functional and biochemical effects observed in these two cells lines. However, it is extremely interesting that the overall response to this preparation lead to a anti-apoptotic effect of *Echinacea* in a model that it is routinely used for functional studies of mammary epithelial cells. Furthermore, *Echinacea* extract is able to exert a positive effect also in cell differentiation. In fact, at least at the higher used concentration, it exerts a positive effect on β -casein expression. It is intriguing to hypothesize that this extract has analog property to prolactin that exerts a significant mitogenetic and lactogenetic effect on mammary epithelium. The effect on β -casein gene expression was not correlated with STAT-5 pathway activation, usually strictly related to PRL effect. A reason of this discrepancy may be a limit of detection in immunoblot analysis or a time-dependent effect of plant extract on cell differentiation. It has been reported that *E. purpurea* stimulates PRL secretion in rat (Di Carlo et al., 2005) but, to our knowledge, no study has correlated *Echinacea* extracts with prolactin function. It has been recently reported that PRL plays an important role in immune system regulation, but in our model PRL exerts a pivotal role in mammary gland biology. Further study should be carried out to examine the possibility that *Echinacea* preparations may be considered an integrator to stimulate mammary physiology during pregnancy and lactation.

Acknowledgments

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