Proliferative Activity of *Echinacea angustifolia* Root Extracts on Cancer Cells: Interference with Doxorubicin Cytotoxicity

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Doxorubicin is an anticancer drug that causes apoptosis in cells, but cardiotoxicity limits the cumulative dose that can remain in the blood. Echinacea extracts have been prescribed to supplement cancer chemotherapy. In a recent study, it was reported that *Echinacea purpurea* extracts protected noncancerous cells from apoptosis. Our study aimed to determine interference with doxorubicin chemotherapy, and if fractions and compounds from *Echinacea angustifolia* roots protected the cells. Cervical and breast cancer cells were treated with the *Echinacea* samples and doxorubicin. At 0.05 and 0.5 μM doxorubicin concentration, cynarine increased HeLa cell growth by 48–125% and 29–101%, respectively ($p < 0.01$). At 0.05 μM doxorubicin concentration, chicoric acid increased cell growth by 23–100% ($p < 0.01$). When MCF-7 cells were treated with *Echinacea* and doxorubicin, the ethyl acetate fraction increased cell growth by 20–25%, and chicoric acid increased cell growth by 10–15%. Cynarine showed proliferative activity on HeLa cells, but showed antiproliferative activity on MCF-7 cells. Results indicate that phenolic compounds are responsible for proliferative activity. Studies with individual compounds show that chicoric acid and cynarine interfered with cells treated with 0.5 μM doxorubicin. The results of this study show that *Echinacea* herbal medicines affect cell proliferation despite cancer treatment, and that herbal medicines require further study with respect to anticancer drugs.

Introduction. – Doxorubicin is an anthracycline compound that shows cytotoxic activity against several cancer cell lines. It belongs to a class of compounds known as anticancer antibiotics, and several of its analogs have shown anticancer activity. Its mechanism of action has been studied, and has been proposed as its intercalating ability to DNA, or its inhibition of topoisomerase enzymes [1].

Clinical studies on doxorubicin have covered a wide range of applications alone or in combination with other drugs; by the early 1980s, over 5000 clinical papers were published [2]. Toxic side effects included alopecia, stomatitis, myelosuppression, gastroenteritis, and cardiotoxicity. To minimize these effects, an intermittent dose schedule, such as a three-week interval between doses, is recommended [3]. Such a toxicity would limit the cumulative dose that may remain in the body.

Echinacea extracts have been prescribed as adjunct therapy for cancer [4]. Evidence of this self-medication can be seen in the fact that 16% of patients use *Echinacea* while undergoing cancer chemotherapy [5]. *Sparreboom et al.* 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 [6], and

they also discussed substrates of cytochrome P450 enzymes. *Echinacea purpurea* extracts have shown anti-apoptotic activity through upregulation of bcl-2 and downregulation of Fas [7]. Since *Echinacea* extracts may prevent apoptosis of normal cells, they may prevent apoptosis of cancer cells as well. This study was conducted to determine the possible proliferative activity of *Echinacea angustifolia* root extracts on cancer cell lines treated with doxorubicin (*Fig. 1*).

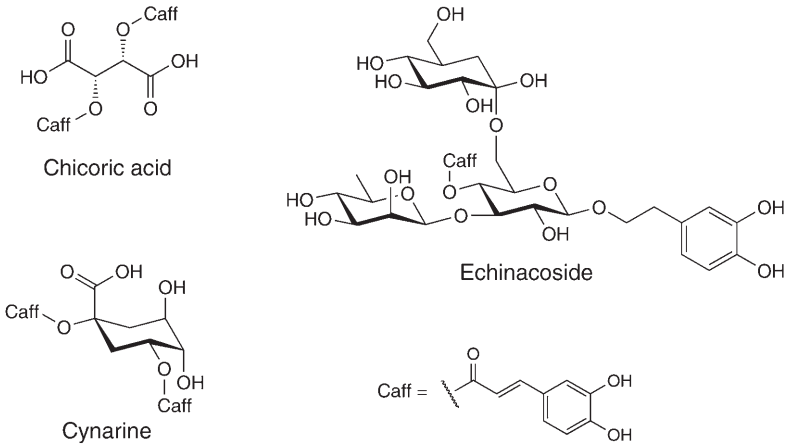

Fig. 1. *Compounds which were studied for their effects on the proliferation of cancer cells treated with doxorubicin and hyaluronidase*

Results. – *Determination of the Interference of* Echinacea *Extracts on HeLa Cells Treated with Doxorubicin*. Doxorubicin showed a concentration-dependent trend ($p \leq 0.01$). Growth of HeLa cells treated with *Echinacea* fractions or commercial extracts is shown in *Fig. 2*. At 0.5 μM doxorubicin, the fingerprinted extract increased cell growth by 35–70% ($p \leq 0.01$). The other fractions showed high cell growths compared to doxorubicin alone, but gave trends that were not dose-dependent as seen in the *Table*.

Determination of the Interference of Echinacea *Compounds on HeLa Cells Treated with Doxorubicin.* When HeLa cells were treated with individual *Echinacea* compounds, they showed higher cell growth compared to doxorubicin alone (*Fig. 3*). At 0.05 and 0.5 μM doxorubicin concentrations, cynarine increased cell growth by 48–125 and 29–101% ($p < 0.01$). At a 0.05 μM doxorubicin concentration, chicoric acid increased cell growth by 23–100% ($p < 0.01$). Echinacoside showed low cell growth compared to doxorubicin and does not show a significant increase with 0.05 μM doxorubicin treatment, but gives higher cell growth at 0.5 μM doxorubicin ($p < 0.01$). This indicates a lack of proliferative activity on HeLa cells treated with doxorubicin.

Determination of the Interference of Echinacea *Extracts on MCF-7 Cells Treated with Doxorubicin.* Doxorubicin showed a cytotoxicity-dependent trend with breast cancer cells ($p \leq 0.01$). Growth of MCF-7 cells treated with *Echinacea* samples and doxorubicin is shown in *Fig. 4*. When MCF-7 cells were treated with 0.5 μM doxorubicin, the AcOEt fraction increased cell growth by 20–25% ($p < 0.01$). Several

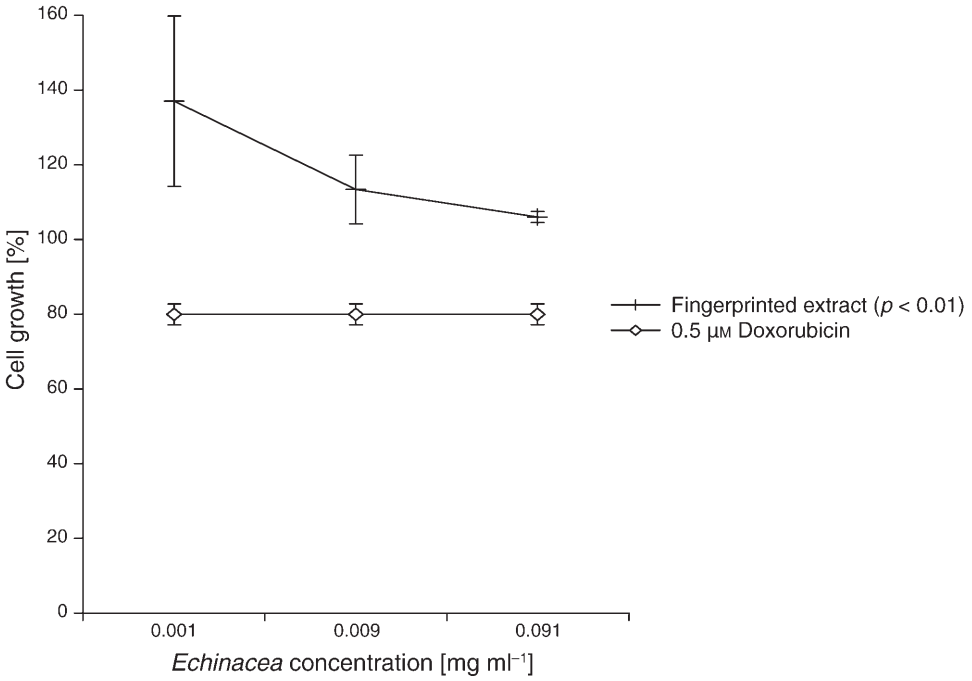

Fig. 2. *Growth of HeLa Cells treated with* Echinacea *fractions, commercial extracts, or chicoric acid, and 0.5 μM doxorubicin.* Only the fingerprinted extract showed a statistically significant response ($p < 0.01$). Samples were checked against cells treated with 0.5 μM doxorubicin alone to determine point of reference.

of the nonpolar fractions showed antiproliferative activity (*Fig. 4*). The remaining fractions and extracts gave statistically insignificant trends (*Table*).

Determination of the Interference of Echinacea *Compounds on MCF-7 Cells Treated with Doxorubicin.* MCF-7 Cells were treated with individual Echinacea compounds and doxorubicin as well (*Fig. 5*) to determine if *Echinacea* interfered with the cell-killing activity of doxorubicin. Chicoric acid only increased cell growth by 10–15% ($p < 0.01$), but at the highest concentration, the cell growth decreased. Cynarine gives a dose-dependent curve with cells treated with 0.5 μM doxorubicin ($p < 0.01$); however, its presence seems to enhance the cytotoxic activity of doxorubicin. Echinacoside did not show significant proliferative activity as seen in the *Table*.

Discussion. – The proliferative activity of Echinacea extracts on cancer cells has not been investigated to a great extent. *Carlo et al.* [7] examined the anti-apoptotic activity of *E. purpurea* extracts on non-cancerous cells. No compounds were identified as the active anti-apoptotic compounds. They investigated the proliferative effect of *E. angustifolia* extracts, fractions, and compounds on cancer cells treated with an anticancer compound. Our study also identified two of the compounds which are responsible for this activity. Cells grew in a proliferative trend despite the presence of doxorubicin.

Table. *Summary of Statistical Significance of All Fractions, Extracts, and Compounds on HeLa and MCF-7 Cells.* Not all samples were included in the figures due to statistical insignificance.

	HeLa[a])	MCF-7[a])
Fractions		
Hexane	0.48	0.03
CH_2Cl_2	0.47	0.02
CH_2Cl_2 (acidic)	0.08	0.02
AcOBu	0.14	0.08
AcOEt	0.13	<0.01
BuOH	0.21	0.13
Extracts		
Fingerprinted extract	<0.01	0.46
Standardized extract	0.76	0.73
Compounds		
Chicoric acid (0.05 μM)	<0.01	<0.01
Echinacoside (0.05 μM)	0.32	0.42
Cynarine (0.05 μM)	<0.01	<0.01
Chicoric acid (0.5 μM)	0.78	0.57
Echinacoside (0.5 μM)	<0.01	0.50
Cynarine (0.5 μM)	<0.01	<0.01

[a]) Unless the doxorubicin concentration is otherwise indicated in parentheses, cells were treated with the *Echinacea* sample and then with 0.05 μM doxorubicin.

Commercial extracts showed higher cell growth on both types of cells. The fingerprinted extract showed a dose-dependent trend ($p<0.01$) at lower concentrations of doxorubicin. Though the standardized extract did not give a dose-dependent curve, it did show higher cell growth than cells growing in medium alone. This suggests that the commercial extracts as a whole are counteracting doxorubicin cytotoxicity. The number of compounds present in the extract may explain the lack of dose dependency.

The fractions, which still consist of several compounds, have fewer compounds than the commercial extracts. The neutral fractions and the acidic CH_2Cl_2 fractions have low proliferative activity. The remaining acidic fractions (AcOBu, AcOEt, and BuOH) show higher cell growth than doxorubicin alone and, at least, similar cell growth compared to cells growing in media alone. Coupled with the lower p values shown for both cell types treated with the acidic fractions (*Table*), this would indicate that phenolic molecules present in the fractions are responsible for counteracting doxorubicin cytotoxicity.

The cellular response observed with the cervical cells could be due to the lower apoptotic rate of the cells, but, since increased cell growth only occurs when cells are treated with *Echinacea* fractions/compounds, the higher cell growth shows that *Echinacea* samples have proliferative/growth-promoting activity. This would mean that *Echinacea* fractions, compounds, and commercial extracts are counteracting the cell-killing activity of doxorubicin on HeLa cells.

The statistical significance of all compounds is shown in the *Table*. MCF-7 Cells treated with echinacoside, and 0.05 and 0.5 μM doxorubicin displayed higher cell growth

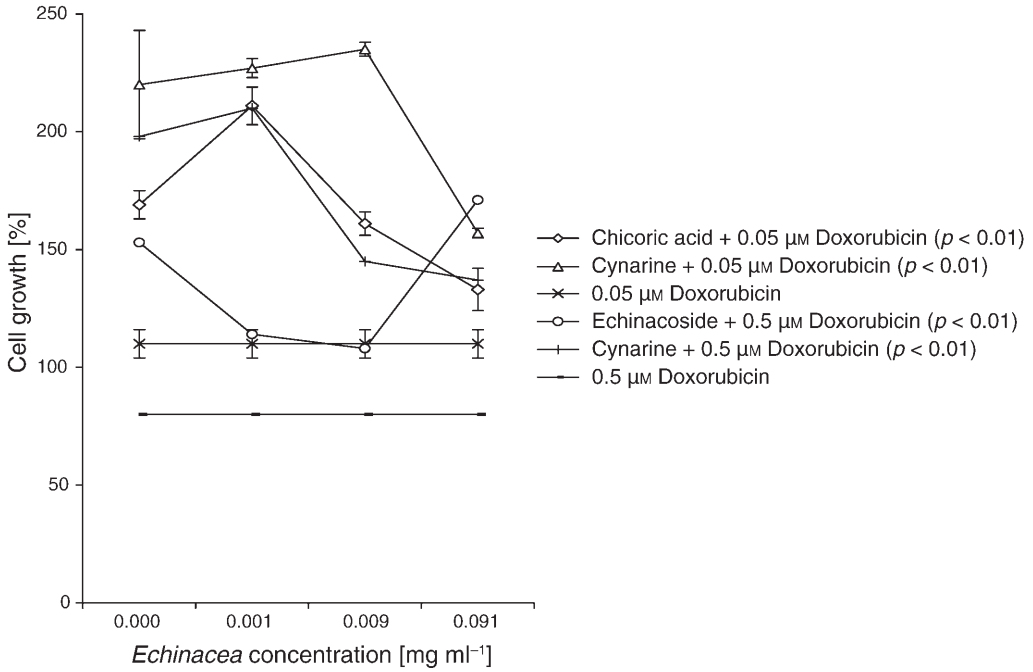

Fig. 3. *HeLa Cells treated with individual* Echinacea *compounds, and 0.05 or 0.5 μM doxorubicin.* Chicoric acid and cynarine showed higher growth when treated with 0.05 μM doxorubicin ($p < 0.01$). Echinacoside shows higher cell growth when treated with 0.5 μM doxorubicin ($p < 0.01$). This can be seen compared to HeLa cells treated with doxorubicin alone.

than both doxorubicin treatments, at p of 0.42 and 0.5, respectively; this indicates proliferative activity which is not dose-dependent. Chicoric acid showed higher cell growths than both doxorubicin treatments, but only gave a significant trend with a 0.05 μM doxorubicin ($p < 0.01$); this indicates proliferative activity, which may work against low-enough concentrations of doxorubicin. Chicoric acid is a major component of *E. purpurea* extracts, and it is present in smaller amounts in *E. angustifolia* extracts [8]. Cynarine and 1,5-*O*-dicaffeoylquinic acid are also genuine compounds found in *Echinacea* root extracts [9]. The compounds occurring in the *E. purpurea* extracts coincide with those found in the *E. angustifolia* root extracts, particularly caffeoyltartaric and caffeoylquinic acids. This would explain the proliferative activity seen in both fingerprinted and standardized extracts. Chicoric acid is the major compound in *E. purpurea* extracts, it is also present in small concentrations in *E. angustifolia* extracts. Cynarine showed proliferative activity and is present in the root extract at a higher concentration than chicoric acid.

The observation that other fractions did not show a statistically significant trend indicates that they do not give a dose-dependent curve. This could be explained by the fact that cells treated with *Echinacea* have higher cell growth compared to cells treated with doxorubicin alone and approximately similar cell growth as cells alone. This means

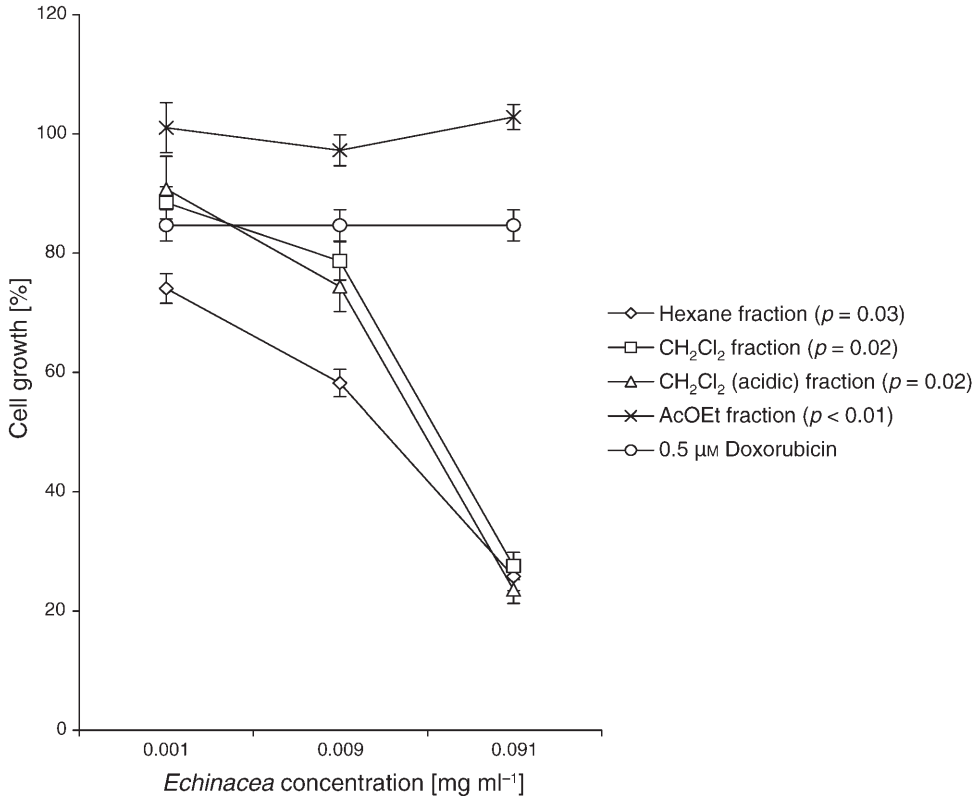

Fig. 4. *MCF-7 Cells treated with* Echinacea *fractions, commercial extracts, or chicoric acid and 0.5 μM doxorubicin.* Of the samples tested, only the hexane, CH_2Cl_2, CH_2Cl_2 (acidic), and AcOEt fractions showed a significant response ($p < 0.05$).

that *Echinacea* fractions and commercial extracts are counteracting the cell-killing activity of doxorubicin on MCF-7.

The results of this study have a number of implications. They show that *Echinacea* extracts may adversely affect cancer chemotherapy, and that cancer patients should not employ self-medication with *Echinacea* during chemotherapy. It was also shown that herbal remedies need closer examination for adverse effects with anticancer agents. The interactions of herbal medicines with anticancer drugs have been discussed by one mechanism of action. The results presented in this paper reinforce the idea that herbal medicines need to be studied more closely for interactions with other anticancer drugs. Finally, they imply that doctors should be more inquisitive as to their patients' use of herbal medicines.

Conclusions. – Several fractions of *E. angustifolia* root extracts, commercial extracts, and individual compounds were examined for possible interference with anticancer drugs. HeLa and MCF-7 cells were chosen for the investigation. The

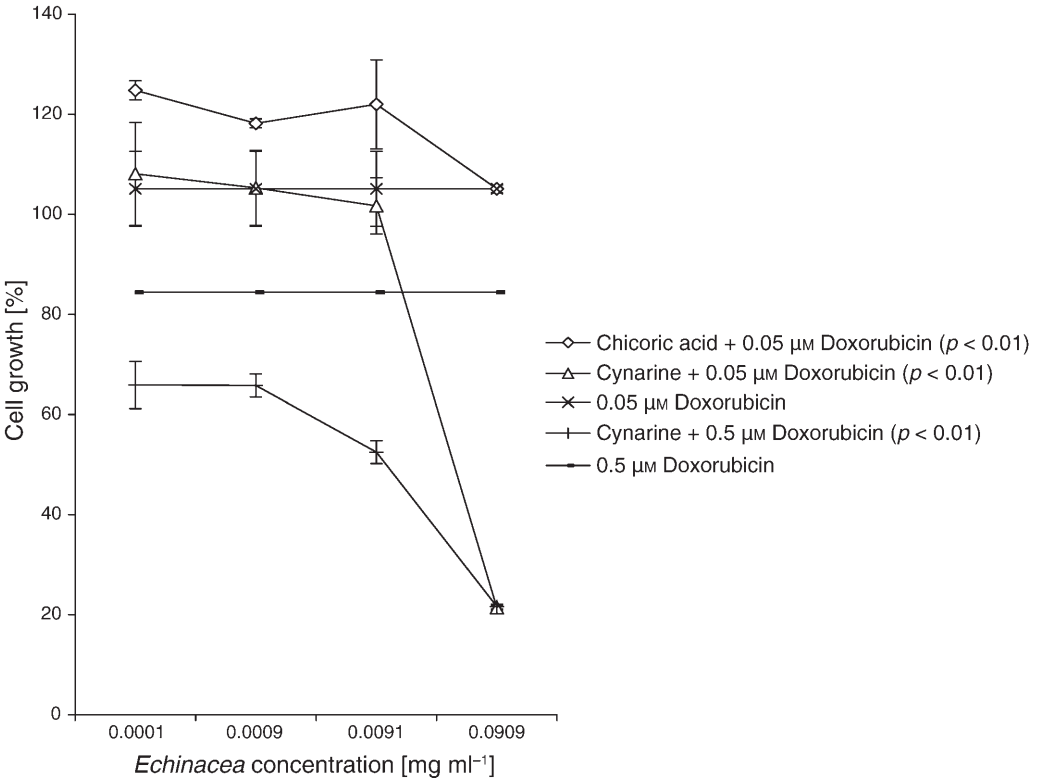

Fig. 5. *MCF-7 Cells treated with individual* Echinacea *compounds, and 0.05 or 0.5 μM doxorubicin.* Chicoric acid and cynarine showed higher growth when treated with 0.05 μM doxorubicin ($p < 0.01$). Echinacoside showed higher cell growth when treated with 0.5 μM doxorubicin although this could be attributed to a random error ($p = 0.05$).

commercial extracts showed higher cell growth/proliferative activity compared to the cells treated with doxorubicin; the lack of statistical significance could be explained by the number of compounds present in the cells. The results from the fractions and individual compounds show that phenolic compounds such as chicoric acid and cynarine are responsible for the increased cell growth. The results of this study demonstrate a greater need to study the *Echinacea* extracts for interference with anticancer drugs.

Experimental Part

Sample Preparation. E. angustifolia seeds were collected from South Dakota and subsequently grown in the botanical garden of South Dakota State University. Plants were harvested and the roots were dried in the shade and ground to a fine powder. The powder was extracted with 70% MeOH/H_2O in a commercial blender. After filtration, the filtrate was concentrated through rotatory evaporation and lyophilization. The freeze-dried extracts were refrigerated until further analysis.

Two commercial *Echinacea* preparations, purchased from *General Nutrition Center* (Watertown, SD), were prepared as well: fingerprinted *Echinacea* root, which contains ground root powder, and

standardized *Echinacea*, which was standardized to contain 4% echinacosides. The liquid herb was diluted accordingly to 10 mg/ml, while the fingerprinted powder was extracted with MeOH and concentrated through rotatory evaporation as indicated. To examine the antihyaluronidase effect further at a molecular level, several caffeic acid derivatives were obtained. Chicoric acid was prepared, and cynarine and echinacoside were purchased from *Apin* (Abingdon, Oxon, UK) and *Phytolab* (Hamburg, Germany), resp. (*Fig. 1*).

The root extract was fractionated by the method of *Facino et al.* [10]. An aq. extract was extracted successively with hexane and CH_2Cl_2. Then the aq. extract was acidified to pH 2 with 6M HCl and extracted with CH_2Cl_2, AcOBu, AcOEt, and BuOH. The six fractions were filtered with anh. Na_2SO_4, concentrated through rotatory evaporation and stored for further analysis.

Determination of the Interference of Echinacea *Extracts on HeLa Cells Treated with Doxorubicin.* All fractions, compounds, and extracts were applied to HeLa and breast cancer cells prior to doxorubicin treatment to determine proliferative activity. All *Echinacea* fractions were prepared at concentrations of 10 mg/ml. The AcOBu, AcOEt, and BuOH fractions were dissolved in H_2O, while the hexane and the two CH_2Cl_2 fractions were dissolved in EtOH. Chicoric acid was prepared in 30% (*v*/*v*) EtOH/H_2O, echinacoside was prepared in H_2O, and cynarine was prepared in EtOH; all compounds were prepared at a concentration of 1.0 mg/ml. The liquid herb and extracted root powder were diluted with H_2O to 10 mg/ml. Doxorubicin was supplied from *Pharmacia* (Italy); stock solns. of 5 and 0.5 μM doxorubicin were prepared.

Cervical cancer cells were grown in a modified *Eagle*'s medium; the medium was fixed to contain 2.4 g/l of Na_2CO_3, 0.32 g/l of glutamine, and 0.11 g/l pyruvic acid, and was supplemented to contain 10% foetal bovin serum (FBS) and standard antibiotics. The media was changed every 3–4 d by pouring off the old media and replacing it with fresh media. Cells were passed by washing with GKN (a soln. of glucose ($2\ g\ l^{-1}$), KCl ($0.4\ g\ l^{-1}$), NaCl ($8\ g\ l^{-1}$), $Na_2HPO_4 \cdot 12\ H_2O$ ($3.56\ g\ l^{-1}$), $NaH_2PO_4 \cdot 2\ H_2O$ ($0.87\ g\ l^{-1}$), and Phenol Red ($0.01\ g\ l^{-1}$)), trypsinizing for 5–10 min until the cells lifted off the flask, and centrifuging the cell suspension at 5° and 1500 rpm for 10 min. The supernatant was poured off, and the cell pellet was resuspended in 10 ml of fresh media. Cells were counted on a hemacytometer, and the cell suspension was diluted accordingly, based on the number of cells needed for the assays.

The proliferation assay is a modification of the method described in [11]. Cells were seeded at 30,000 cells/well and treated with serial dilutions of the *Echinacea* samples and incubated for 24 h. After 24 h, the cells were treated with doxorubicin and incubated for an additional 24 h. Cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium hydrobromide (MTT) [12].

Cytotoxicity was expressed as cell growth, which was calculated by the following equations. The *Eqn. 1* related the sample wells to the controls; the *Eqn. 2* related each absorbance to the control set run at each concentration to factor out solvent effects.

$$CG_a = 100 - \left(\frac{(A_{pc} - A_a - A_{nc})}{(A_{pc} - A_{nc})} \cdot 100\right) \quad (1)$$

$$CG = 100 - \left(\frac{(CG_{sol} - CG_a)}{CG_{sol}} \cdot 100\right) \quad (2)$$

where CG_a, CG_{sol}, and CG are the cell growth values for *Echinacea*, solvent, and overall cell growth of the well, respectively. A_a is the absorbance of the wells treated with *Echinacea* and doxorubicin. A_{pc} and A_{nc} are the absorbance of the wells containing the positive and negative controls, resp.

To determine the effect of *Echinacea* samples on HeLa cells treated with doxorubicin, cells were cultured and seeded as before. Cells were treated with tenfold dilutions of the *Echinacea* samples, incubated for 24 h, and treated with 0.5 μM doxorubicin for another 24 h. Cells were stained with MTT as before.

Once the data was collected and processed, the data sets were tested with ANOVA. Statistical analysis was conducted with STATISTICA [13]. The *p* values are shown in the *Table* to determine statistical significance.

Determination of the Interference of Echinacea *Extracts on MCF-7 Cells Treated with Doxorubicin.* Mammary carcinoma fibroblasts (MCF-7) cells are round cells that grow to 70% confluency. Breast cancer cells were cultured in *RPMI-1640* supplemented with 10% FBS, antibiotic, and antifungal agents. The media was changed every 2–3 d by pouring off the spent media and replacing it with fresh media. The cells were passed by washing off cellular debris with 10 ml of GKN and trypsinizing (10 ml) for 5–10 min until the cells became suspended in the trypsin. A 10-ml volume of growth media was added to the mixture, and the cell suspension was centrifuged at 5° and 3000 rpm for 10 min. The supernatant was poured off and re-suspended in 10 ml of media. Cells were counted on a hemacytometer and diluted accordingly, based on the number of cells needed for the assay.

Cells were added at 30,000 cells/well, but were incubated 24 h. After 24 h, cells were treated with tenfold dilutions of *Echinacea*. The cells were incubated for 24 h, and the cells were treated with 0.5 μM doxorubicin for 24 h. Cells were incubated for 6 d to detect long-term apoptosis.

The fractions, commercial extracts, and pure compounds were measured for proliferative activity on breast cancer cells. Cells were seeded at 30,000 cells/well and grown for 24 h. Cells were incubated for 24 h, followed by doxorubicin (0.05 and 0.5 μM) for 24 h. After the treatment, the cells were grown for 6 d to detect the long-term apoptotic effects on the cells. Cells were stained with MTT and measured on a 96-well plate reader. Absorbance was measured and calculated according to *Eqn. 1*. Controls included doxorubicin, cells alone, and media alone. Solvent controls were provided depending on the solvent used. In the case of EtOH, the cell growth at each concentration was calculated with *Eqn. 1*, and the cell growth values were calculated with *Eqn. 2*. The cell growth was plotted against *Echinacea* concentration, which was graphed against the cell growth in doxorubicin alone.

Once the data was collected and processed, the data sets were tested with ANOVA. Statistical analysis was conducted through STATISTICA [13]. This gave the *p* values, shown in the *Table*, to determine statistical significance.

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