

Effect of the standardized *Cimicifuga foetida* extract on Hsp 27 expression in the MCF-7 cell line

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ABSTRACT

Cimicifuga foetida, an Asian *Cimicifuga* species, has been employed as a cooling and detoxification agent in traditional Chinese medicine since ancient times. For this herb, two cycloartane triterpene glycosides isolated from the rhizomes have demonstrated cytotoxicity on rat tumor and human cancer cell lines. Since human Hsp27 is increased in various human cancers and exhibits cytoprotective activity that affects tumorigenesis and the susceptibility of tumours to cancer treatment, the purpose of this research was to study the expression of this protein in MCF-7 breast cancer cells. To accomplish this aim, MCF-7 cells were exposed to different concentrations of *Cimicifuga foetida* extract showing a reduction in cell number measured by the sulforhodamine assay. In addition, the expression of Hsp-27 mRNA detected by RT-PCR and Hsp-27 protein detected by immunofluorescence was present in all conditions, except when using the highest concentration of *Cimicifuga foetida* extract (2,000 µg /L). We conclude that Hsp 27 expression at 2,000 µg /L *Cimicifuga foetida* extract is diminished. This is the first report showing the Hsp-27 expression after exposure to *Cimicifuga foetida* extract in MCF-7 cells.

Key terms: *Cimicifuga foetida*, cytotoxicity, Hsp-27, Immunofluorescence, MCF-7 cells, RT-PCR.

INTRODUCTION

There is a great need to improve cancer therapies through the search for new medicines with anticancer properties. The herbs used in traditional medicine for cancer treatment are promising candidates.

The genus *Cimicifuga* (Ranunculaceae) consists of more than 18 species whose roots and rhizomes have been widely used in traditional medicine worldwide (Tian et al., 2007). *Cimicifuga racemosa*, (syn. *Actea racemosa*) a famous North American species has shown remarkable antitumor activities in diverse studies. Extracts from black cohosh (*C. racemosa*) have been traditionally used by Native Americans for the treatment of rheumatism, dyspepsia, epilepsy, kidney ailments, dysmenorrhoea and the relief of pain during menses and childbirth (Borrelli and Ernst, 2002). The roots and rhizomes of these plants contain two major classes of compounds, triterpene glycosides and phenylpropanoids (Einbond et al., 2008). For this perennial herb, it has been demonstrated antiproliferative effects, induction of cell cycle arrest and apoptosis in the breast cancer cell line, MCF-7 (Hostanska et al., 2004a, b). Several kinds of extracts from rhizomes of *C. racemosa* were demonstrated the capabilities of killing estrogen receptor-positive (MCF-7), estrogen receptor-negative (MDA-MB231 and MDA-MB-453) human breast carcinoma and androgen-sensitive LNCaP human prostate cancer-derived cell lines (Einbond et al., 2004; Hostanska et al., 2004a, 2005; Jarry et al., 2005; Seidlova-Wuttke et al., 2006).

C. foetida, an Asian *Cimicifuga* species, has been employed as a cooling and detoxification agent in traditional Chinese medicine since ancient times (Tian et al., 2007). Recently, two cycloartane triterpene glycosides isolated from the rhizomes of *C. foetida*, have demonstrated cytotoxicity on the Ehrlich ascites carcinoma (EAC) rat tumor cell line and on SGC7901 and MDA-MB-A231 human cancer cell lines (Sun et al., 2007).

Heat shock proteins (Hsps), which are molecular chaperones, appeared overexpressed and implicated in tumor cell proliferation, metastasis and death. Hsp 27 is a family member of the small heat shock proteins (sHsps), which represent an abundant and ubiquitous family of stress proteins with a monomeric mass ranging between 15 and 30 kDa (Parcellier et al., 2005). This protein has been associated with α estrogen receptors (Er α) in female breast carcinomas and endometrial carcinomas, but not associated with Er α in male breast carcinomas, cervical uterine carcinomas, hepatocellular carcinomas and meningiomas, tissues that may express Er α (Ciocca and Calderwood, 2005).

Since human Hsp27 is increased in various human cancers and exhibits cytoprotective activity that affects tumorigenesis and the susceptibility of tumors to cancer treatment (Fortin et al., 2000), it is interesting to study the expression of this protein in MCF-7 breast cancer cells.

Hsp27 protein and mRNA levels are induced by heat shock, β -estradiol and antagonists of the estrogen receptor such as ICI 164,384, tamoxifen and hidroxi-tamoxifen in estrogen

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receptor positive MCF-7 cells, while its expression is lower when exposed to dioxins as 2,3,7,8-tetrachlorodibenzo-p-dioxin (Edwards et al., 1981; Porter et al., 2001).

Considering that *C. foetida* contains similar triterpenoids and also the reported antitumor activity of *C. racemosa* (Tian et al., 2007), we performed experiments to detect the expression of Hsp-27 in MCF-7 cells treated with different concentrations of *C. foetida* extract (CFE) and report the results herein.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals were reagent or molecular biology grade. β -estradiol stock was 0.2 mM in ethanol, tamoxifen stock was 1 mg/mL in ethanol and *C. foetida* extract (CFE) stock was 0.1 g/L in phosphate buffer saline (PBS). All stock solutions were kept at -20°C.

Plant materials

Commercial dried powder of *C. foetida* was obtained from Stryka Botanics (lot BC031021) and the species qualification was performed using high performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD) for fingerprint analysis.

Cell culture

The human breast adenocarcinoma cell line MCF-7 (American type culture collection, Rockville, MD, USA) was grown in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, PAA Laboratories GmbH, Linz, Austria), 2mM glutamine, 10U/L penicillin and 100 μ g/mL streptomycin. The cells were cultured in a humidified incubator with a 5% CO₂ atmosphere.

Phenol red free media with 10% activated charcoal-adsorbed FBS to remove steroids, was used in all experiments to expose the MCF-7 cells to β -estradiol, tamoxifen and CFE. This media was also used as the control condition in all the experiments.

Sulforhodamide assay

MCF-7 cells were trypsinized and 5,000 cells/well seeded in 96-well plates. Selected dilutions of CFE, tamoxifen and β -estradiol, were then added 24 hours after cell seeding and cells were incubated for an additional two days. Each treatment was performed in triplicate. After this, the treatment-containing media was removed and fresh media was added to allow the remaining viable cells to proliferate. One day later, the cells were fixed by precipitation with 60% trichloroacetic acid for one hour. After extensive washing with water, the cells were stained with 0.4% sulforhodamine in 1% acetic acid, for 10 minutes and extensively washed with 1% acetic acid, then the plates were air-dried and the dye solubilized in 10mM Trizma Base. The optical density was read in a Merckscan (Anthos Labtec Instruments, Salzburg, Austria) plate reader at 540 nm. Survival was calculated by subtraction of the optical densities of the control condition from the experimental condition (Skehan et al., 1990). Negative values mean death or proliferation inhibition.

RNA Isolation

Total cellular RNA was isolated utilizing the Trizol (Invitrogen) method. The cells were directly lysed with 1ml TRIZOL reagent per 1cm² of cell culture surface, following the manufacturer instructions. The RNA pellets were air dried and resuspended in 50-100 μ L of diethyl pyrocarbonate treated ultrapure water (0.01% DEPC, Sigma). The yield and purity of the isolated RNAs were determined in a NanoDrop spectrophotometer. The RNA was kept at -70°C until used.

Primers for the RT-PCR reactions

The primers for the β_2 microglobulin RT-PCR reactions were those published by Laffon et al (2001). The sequences were 5'CCA GCA GAG AAT GGA AAG TC3' for sense and 5'GAT GCT GCT TAC ATG TCT CC3' for antisense primers.

The primers for the Hsp-27 RT-PCR reactions were those published by Cubano and Lewis (2001). The sequences were 5'TGT CCC TGG ATG TCA ACC ACT TC3' for sense and 5'AAA AGA ACA CAC AGG TGG CGG3' for antisense primers.

Reverse transcriptase reaction

The reaction was performed using 0.5 μ g of primer for 1.0 μ g RNA and following the directions of the manufacturer for AMV reverse transcriptase (Promega).

Polymerase Chain Reaction

In the same reaction tube used for the cDNA synthesis, the following reaction mixture was added: 4.6 μ L MgCl₂ (25 mM), 4 μ L PCR buffer 10X (Promega), 6.4 μ L dNTPs mixture (1.25 mM each), 4 μ L of sense and 4 μ L anti-sense primers (10 μ M, each), 0.5 μ L Taq DNA polymerase (2.5 U. Promega) and ultrapure/DEPC water to complete 40 μ L. For Hsp-27 its specific primers were used and the following program in a Thermo PXE 0.5 Thermocycler: an initial 3 minutes step at 95°C, 30 cycles of 1.5 minutes at 95°C, 1 minute at 56°C, 2 minutes at 72°C and a final step of 5 minutes at 72°C. For β_2 microglobulin, the same reaction mixture was used with its specific primers and the following program in a Thermo PXE 0.5 Thermocycler: an initial 5 minute step at 95°C, 30 cycles of 45 seconds at 95°C, 35 seconds at 56°C, 1 minute at 72°C and a final step of 1minute at 72°C.

Detection of Hsp-27 by Immunofluorescence

MCF-7 cells were grown on silanized slides immersed in cell culture medium inside Petri plates. Before reaching confluence, the cells were exposed for 24 hours to phenol red free media with 10% activated charcoal adsorbed FBS. Then, the cells were grown for 48 hours in cell culture media containing either CFE (2,000, 200, 20 or 2 μ g/L), or β estradiol (325 pM), or tamoxifen (240 ng/mL) or phenol red free media 10% adsorbed FBS. To fix the cells on the slides, 4% p-formaldehyde in PBS was used for twenty minutes at 4°C.

Anti-Hsp-27 c-20 (sc-1048 goat polyclonal antibody raised against Hsp-27 of human origin, Santa Cruz Biotechnology, Inc.) in a 1:50 dilution in PBS was utilized as the primary antibody.

As secondary antibody, donkey anti-goat IgG-FITC (sc 2024, Santa Cruz Biotechnology, Inc) was used in a 1:100 dilution in PBS. The mounting medium was Vectashield-Dapi (4,6-diamidino-2-phenylindole) to stain the nuclei (UltraCruz™ Mounting Medium: sc 24941). All samples were visualized with an Olympus BX 51 Fluorescence microscope provided with a U-MWU2 Olympus filter. Most of the images were taken at a magnification of 40X, except for the control and CFE 200 $\mu\text{g}/\text{L}$, to privilege a better image of the immunofluorescence.

Western blot Analysis

Pre-confluent cells were lysed in lysis buffer (20 mM Tris, pH 7.5; 5 mM EDTA, 1% Triton X-100) containing Halt[®] Protease Inhibitor Cocktail Kit (Pierce, Rockford, IL, USA). The lysate equivalent to 20 μg (Hsp27) or 40 μg (actin) of protein was electrophoresed in a 10% polyacrilamide gel. The proteins were electrotransferred to a PVDF membrane, using an electroblot semi-dry apparatus (BIO RAD). The membranes were blocked overnight with blocking solution (2% BSA in TBST: 25 mM Tris, pH 8.0; 125 mM NaCl, 0.1% Tween 20). The membranes were then incubated for 2 hours with the primary antibody, anti-Hsp27 c-20 (sc-1048 goat polyclonal antibody raised against Hsp-27 of human origin, Santa Cruz Biotechnology, Inc.) (Fig. 5A) or anti-actin c-11 (sc-1615 goat polyclonal IgG, Santa Cruz Biotechnology, Inc.) (Fig. 5A), diluted 1:5,000 in blocking solution and after three five-minute washes with TBST, they were incubated for 2 hours in secondary antibody donkey anti-

goat IgG-AP (sc-2022 linked to alkaline phosphatase, Santa Cruz Biotechnology, Inc.) (1:10,000 in blocking solution). After three five-minute washes with TBST, an alkaline phosphatase reaction was performed utilizing the Western Blue[®] Stabilized substrate for Alkaline Phosphatase (Promega). The blots were scanned and subjected to densitometry analysis. The signals for Hsp27 were normalized against the actin signal for each condition.

RESULTS

Fingerprinting of the *Cimicifuga foetida* extract

The extract was analyzed by HPLC with ELSD detector. The chromatogram obtained shows a typical rhizomes and roots of *C. foetida* profile (Fig. 1). The analysis confirmed by LC/MS detected cimifugin and cimifugin glycoside, which are marks of Asian *Cimicifuga* species (He et al., 2006).

Cell growth

Using the sulforhodamine assay, the effect of the CFE (Fig. 2A) on cell growth was assessed and compared to the known effect of the SERM tamoxifen (Fig. 2B). The results of this assay are presented as dot graphics, in which each dot represents the difference between the OD of the cells grown under the experimental condition and cells grown in control cell culture. Every dot under the zero line represents cell death. The cell number progressively diminishes at 24, 48 and 72 hours of

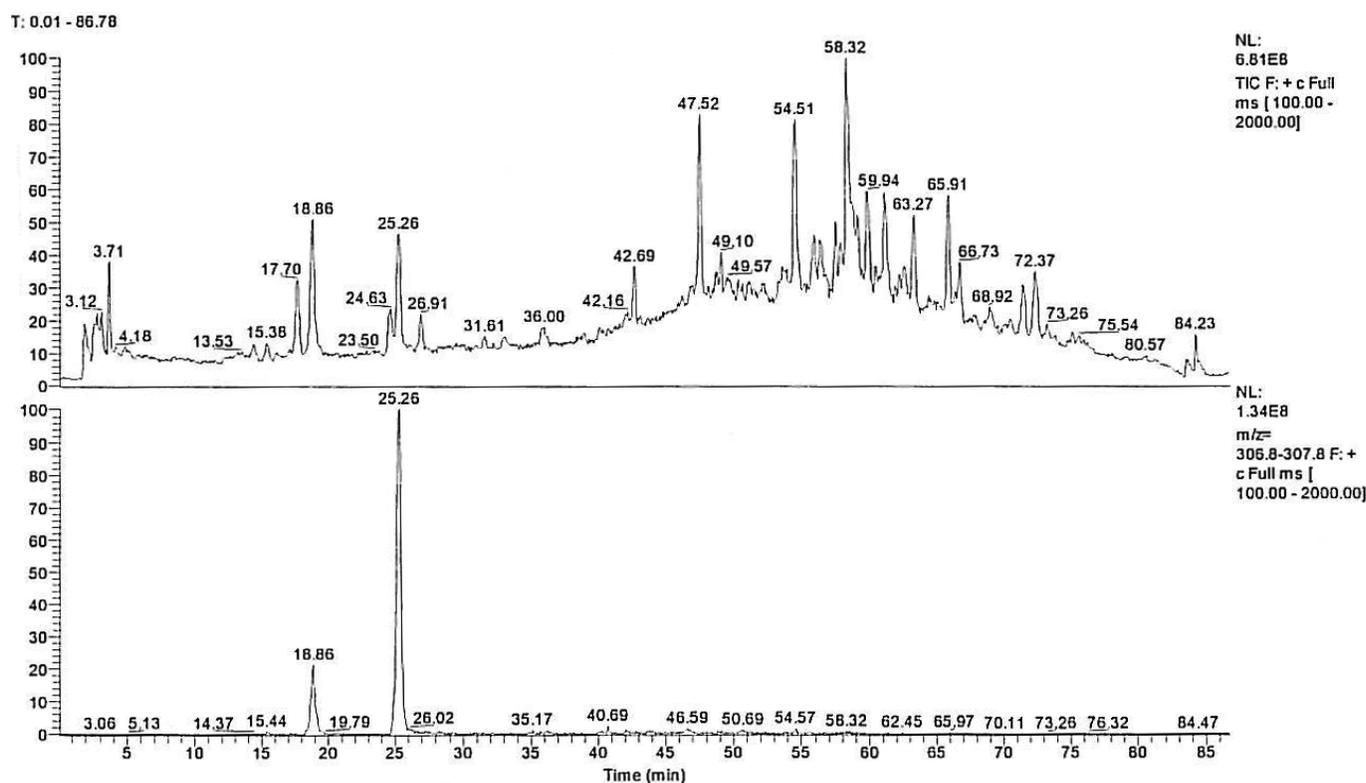


Figure 1: Fingerprinting of the *Cimicifuga foetida* extract. HPLC-ELSD fingerprint chromatogram of CFE showing a typical *C foetida* profile (top). LC-MS analysis confirms this Asian species by detection of cimifugin and its glucoside (bottom).

culture in the presence of 2,000 $\mu\text{g/L}$ of the CFE (Fig. 2A). This is comparable to the effect observed for tamoxifen (480ng/mL) on the same experimental setting (Fig. 2B).

Hsp 27

Hsp 27 was detected through RT-PCR for mRNA and by immunofluorescence and Western blotting for protein detection. RT-PCR analysis revealed that the message for Hsp27 did not change when MCF-7 cells were grown in either control media (Fig. 3A, lane 1), CFE 2, 20, 200 $\mu\text{g/L}$ (Fig. 3A, lanes 2, 3 and 4), tamoxifen (Fig. 3A, lane 7), or β -estradiol (Fig. 3A, lane 8). However, when MCF-7 cells were grown in the presence of 2,000 $\mu\text{g/L}$ of CFE, the message for Hsp 27 was not detectable (Fig. 3A, lane 6). The RT-PCR analysis for the constitutive gene β_2 microglobulin for each cell culture condition is shown in Figure 3B.

Interestingly, the immunofluorescence analysis showed that the expression of Hsp27 at the protein level was undetectable only when the cells were grown on CFE 2,000 $\mu\text{g/L}$ (Fig. 4). Western blot analysis showed protein expression for cells cultivated in the presence of all concentrations of CFE (Fig. 5A). Densitometry analysis showed a modest reduction in Hsp27 expression as compared to control or β -estradiol grown cells (Fig. 5B).

DISCUSSION

We first evaluated the activity of the *C. foetida* extract (CFE) using a proliferation assay and the result showed a reduction in MCF-7 cell number (Fig. 2). This result is in agreement with a previous report using black cohosh extracts that displayed growth inhibitory activity in MCF-7 (Gaubert et al 2007) and in MDA-MB-453 human breast cancer cells (Einbond et al 2008).

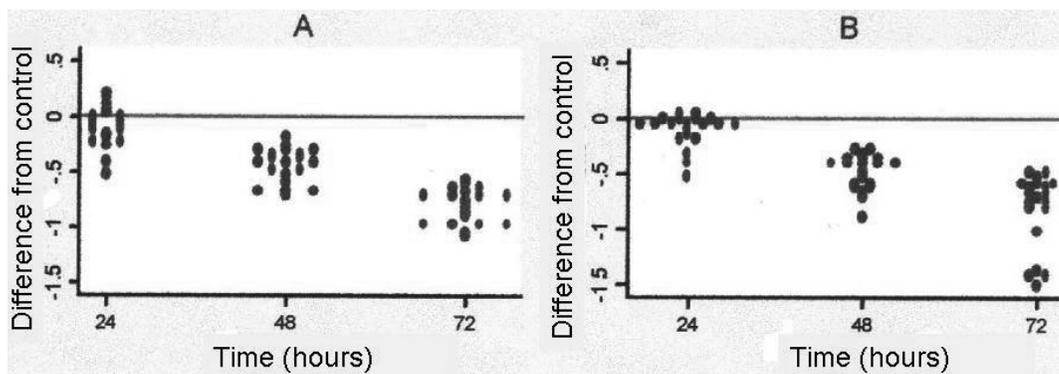


Figure 2: *Cimicifuga foetida* cytotoxicity on MCF-7 cells. Sulforhodamine assay for MCF-7 cells grown in cell culture media containing tamoxifen (Panel A) or *Cimicifuga foetida* extract (Panel B) for different time periods. Each dot represents the difference between the absorbance registered for cells grown under experimental conditions and control conditions for an individual replicate.

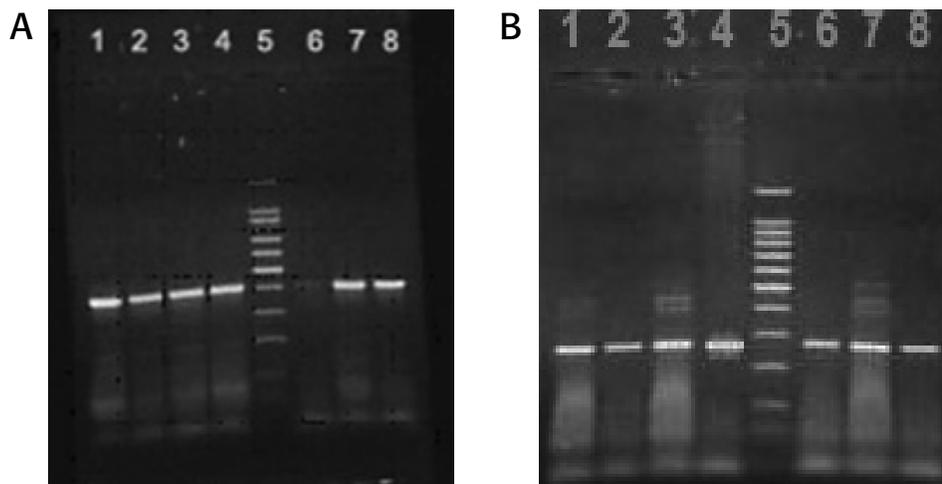


Figure 3: RT-PCR for Hsp-27 and β_2 -microglobulin on MCF-7 cells. RT-PCR in MCF-7 cells grown on the following conditions. Channel 1: Control, Channel 2: CFE 2 $\mu\text{g/L}$, Channel 3: CFE 20 $\mu\text{g/L}$, Channel 4: CFE 200 $\mu\text{g/L}$, Channel 5: DNA ladder (100 bp), Channel 6: CFE 2000 $\mu\text{g/L}$, Channel 7: Tamoxifen 480 ng/mL, Channel 8: β Estradiol 315 pM. Panel A: amplicons for Hsp27 (400 bp) and Panel B: amplicons for β_2 -microglobulin (267 bp), used as constitutive control.

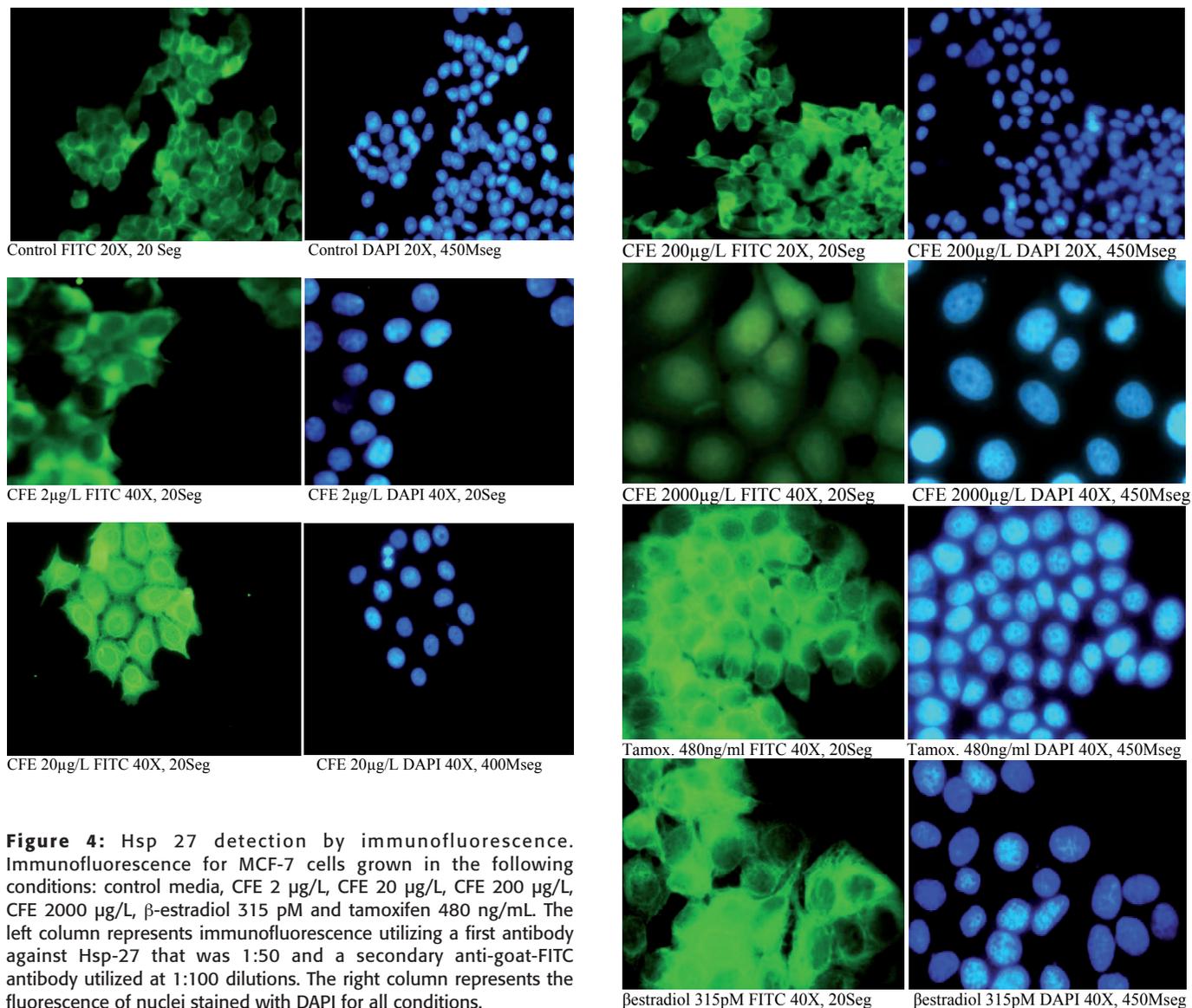


Figure 4: Hsp 27 detection by immunofluorescence. Immunofluorescence for MCF-7 cells grown in the following conditions: control media, CFE 2 µg/L, CFE 20 µg/L, CFE 200 µg/L, CFE 2000 µg/L, β-estradiol 315 pM and tamoxifen 480 ng/mL. The left column represents immunofluorescence utilizing a first antibody against Hsp-27 that was 1:50 and a secondary anti-goat-FITC antibody utilized at 1:100 dilutions. The right column represents the fluorescence of nuclei stained with DAPI for all conditions.

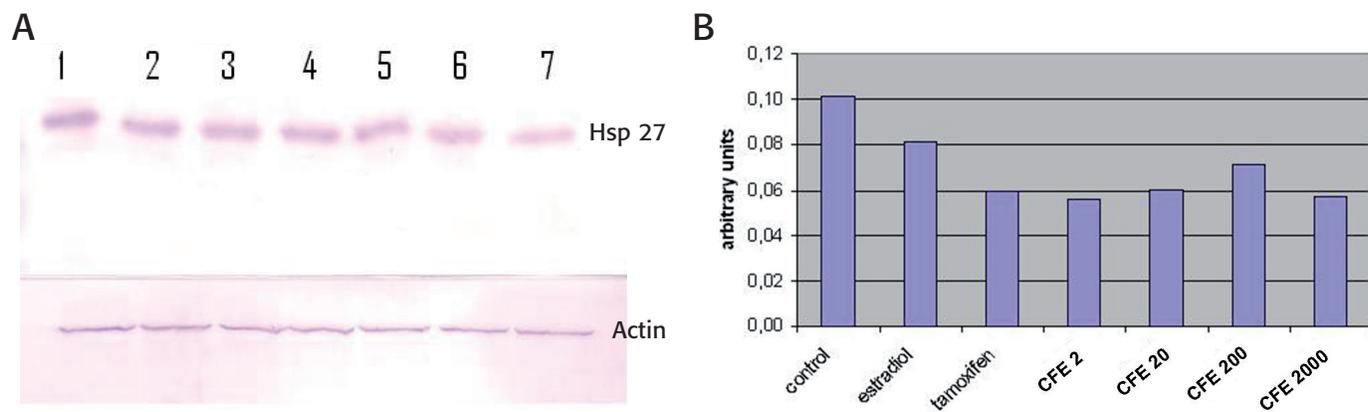


Figure 5: Hsp 27 detection by immunoblot. Panel A. Immunoblot for MCF-7 cells grown in the following conditions: channel 1: control media, channel 2: β-estradiol 315 pM, channel 3: tamoxifen 480 ng/mL, channel 4: CFE 2 µg/L, channel 5: CFE 20 µg/L, channel 6: CFE 200 µg/L, channel 7: CFE 2000 µg/L. Anti-hsp-27 antibody was 1: 5,000 and anti-actin antibody was 1:5,000. In both cases the secondary antibody was donkey anti goat-AP 1: 10,000. Panel B. Histogram for the densitometry analysis of the electrophoretic bands. Results are shown as the rate of Hsp27 over actin signals.

In addition, we report for the first time the study of Hsp-27 expression after MCF-7 cells were exposed to CFE. The stressful conditions characteristic of the tumors' microenvironment facilitate the expression of heat shock proteins. Increased expression of Hsp-27, proportional to the levels of estrogen receptors, has been detected in breast and ovarian cancer cell lines and primary tumors (Langdon et al., 1995, Porter et al., 1996). Moreover, Hsp-27 has been strongly associated with tumor aggressiveness due to several effects, such as apoptosis inhibition, immunosuppressant activity and drug resistance (Rane et al., 2003, Uozaki et al., 1997, Lee et al., 2007). In this study, MCF-7 cells were exposed to different concentrations of CFE and to other stimuli including β -estradiol and tamoxifen. When MCF-7 cells were stimulated with β -estradiol at 315 pM, which is within the physiological range for a premenopausal woman, the expressions of Hsp-27 mRNA and protein were detected by RT-PCR (Fig. 3, lane 8) and protein immunofluorescence (Fig. 4), as well as Western blot (Fig. 5A, lane 2), respectively. These results are consistent and expected according to the literature (Edwards et al., 1981, Porter et al., 1996).

It has been reported that exposure of MCF-7 cells to tamoxifen resulted in decreasing cell proliferation and cell cycle arrest in G₀/G₁ and G₂ phases (Ichikawa et al., 2008). Our results showed that it did not inhibit the Hsp-27 mRNA (Fig. 3, channel 7) or protein expression (Fig. 4, Fig. 5A) after treating MCF-7 cells with tamoxifen. This result was not expected, since tamoxifen is a known estrogen receptor antagonist in breast tissue. However, it is in agreement with previous research showing that certain antagonists of the estrogen receptor had transcriptional activity on the Hsp-27 gene (Porter et al., 2001). Some control experiments were performed with hydroxy-tamoxifen, a known *in vivo* metabolite of tamoxifen and showed no differences on Hsp-27 expression (result not shown).

Our results, upon stimulation using low concentrations of CFE (2, 20, 200 μ g / L), showed Hsp-27 expression in both Hsp-27 mRNA (Fig. 3A, channels 2, 3, 4) and protein (Fig. 4, Fig. 5A). It is suggested that the cytoplasmic and perinuclear immunofluorescence signal in these cells could be explained by phosphorylated Hsp27 binding to denatured F-actin (Pivovarov et al., 2007) in these parts of the cell upon the stress induced by CFE. Similar results were obtained by Einbond et al (2008), but using actein. Actein has been reported producing stress and also inducing apoptosis in MCF-7 and MDA-MB45 cells. The mechanism of action used by the Hsp27 to protect the cytoskeleton, and therefore tumor cell survival, is still not sufficiently understood (Pivovarov et al., 2007).

With a higher concentration of CFE (2,000 μ g / L), similar to that used in MCF-7 cells stimulated with *C. racemosa* (Zierau et al., 2002), no Hsp-27 mRNA or protein expression was detected by RT-PCR (Fig. 3A, channel 6), or protein immunofluorescence (Fig. 4). We speculate that at 2,000 μ g/L of CFE, Hsp-27 expression might be reduced, as well as its cytoprotective effect on MCF-7 cells. However, Western blot analysis using whole cell lysates showed protein expression at this CFE concentration (Fig. 5A). Previous publications have described the possibility that Hsp-27 translocates to the nucleus in some extension, which is in agreement with reports of Ciocca et al (1998) in clinical samples. More experiments will be needed to elucidate these interesting results, especially because the Hsp

family and their transcription factors have been considered as a new gateway for cancer therapy since they are required for cell survival during tumor progression and metastasis (Volloch and Sherman 1999, Hoang et al 2000, Nylandsted et al 2000a,b, Jones et al 2004). Hsp 27 can be the target of antisense oligonucleotide therapies, which has resulted in increased apoptosis in human prostate cancer cells (So et al 2005) and also enhanced apoptosis and delayed progression of prostate tumors (Rocchi et al 2004). This is a promising result considering the association between tumor aggressiveness and Hsp-27 expression, which underscores the potential of the CFE as a new source of antineoplastic molecules. For this reason, it is also necessary to determine which CFE components are mediating its effect on Hsp-27. These might be the ones that are exclusive for *Cimicifuga foetida*, such as cimifugoside H-1, cimicifugin or cimicifugin glucoside (He et al., 2006). We support this statement in unpublished results using *Cimicifuga racemosa*, which showed a different dose response effect on Hsp-27 protein expression.

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