

# Effect of Chinese Herbal Therapy on Breast Cancer Adenocarcinoma Cell Lines

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Despite the widespread use of medicinal herbs to prevent and treat many diseases, including cancer, there are insufficient scientific data on the safety and efficacy of the majority of herbal therapies. The aim of this study was to assess the effect of a unique Chinese herbal therapy (CHT) from controlled manufactured concentrated powders, on an *in vitro* model of breast cancer. Three breast adenocarcinoma cell lines (MDA-231, MDA-453, T47D) were exposed to CHT for 72 h. Cell viability was assessed by XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,

4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulphonate hydrate) assay. Apoptosis and cell cycle stage were determined by fluorescence-activated cell sorting (FACS) analysis. CHT decreased cell survival in a dose-dependent manner in all tested cell lines. FACS analysis of treated and non-treated T47D cells demonstrated that the inhibitory effect of CHT was associated with an increase in apoptosis. A randomized clinical trial is currently underway to investigate CHT as supplementary therapy for breast cancer patients receiving chemotherapy.

KEY WORDS: CHINESE HERBS; BREAST CANCER; MDA-231; MDA-453; T47D; APOPTOSIS; FACS ANALYSIS

## Introduction

Breast cancer is the most prevalent malignancy in women in many countries worldwide; an estimated 1.3 million new cases of invasive breast cancer were expected to occur among women in 2007.<sup>1</sup> Although recent progress in early diagnosis and new therapies has increased the chances of survival among women with breast cancer, it remains a major cause of morbidity and mortality and there is, therefore, an urgent need for the development of new modalities

of treatment.<sup>2</sup>

Some of the chemotherapeutic agents used in the treatment of breast cancer are derivatives of medicinal herbs,<sup>3</sup> such as taxanes, vinblastine and vincristine. The use of botanical medicine is widespread in all regions of the developing world and has been rapidly growing in industrialized countries, especially among patients diagnosed with cancer. A study that assessed the prevalence of complementary and alternative medicine (CAM) used by patients in cancer-centre outpatient clinics at the University of Texas MD Anderson Cancer

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Center (Houston, TX, USA) found that 83.3% had used at least one CAM, of which vitamins and herbs (62.6%) were the most common therapies.<sup>4</sup> Another study on the prevalence of CAM use in cancer patients in a Florida hospital showed that 80% of cancer patients used CAM and that 54% of them used herbal products.<sup>5</sup> A study on the prevalence of the use of CAM among breast cancer patients in 11 European countries found that 44.7% used CAM and that the most common therapies involved herbal medicine (46.4%).<sup>6</sup>

Despite their widespread use, scientific data on the safety and efficacy of the majority of herbal therapies are incomplete.<sup>7</sup> Campbell *et al.*<sup>8</sup> studied the antiproliferative effects of > 70 Chinese herbs on breast cancer cell lines. Remarkably, > 20% of the extracts showed significant growth inhibition on at least four of the five tested cell lines. Shoemaker *et al.*<sup>9</sup> evaluated the antiproliferative activity of 12 Chinese medicinal herbs on eight cancer cell lines: all extracts demonstrated growth inhibitory activity on some or all of the cancer cell lines. In another study, the effect of four extracts of Chinese medicinal herbs was investigated on ovarian cancer cells: these extracts significantly inhibited cancer cell growth and induced apoptosis in all six human ovarian cancer cell lines studied.<sup>10</sup>

The present study was designed to evaluate whether a unique Chinese herbal therapy (CHT) had an inhibitory effect on survival of the breast cancer cell lines tested and whether this effect was associated with simultaneous induction of cell apoptosis.

## Materials and methods

### CELL CULTURE

The human breast adenocarcinoma cell lines MDA-231, MDA-453 and T47D were obtained from the American Type Culture

Collection (ATCC; Manassas, VA, USA). All cell lines were grown and maintained in Dulbecco's modified Eagle's medium (DMEM, Biological Industries, Beit HaEmek, Israel) supplemented with 10% fetal calf serum, 1% penicillin and 1% streptomycin (full medium) at 37 °C, in an atmosphere of 95% oxygen and 5% carbon dioxide.

The unique CHT used (LCS101) is a herbal formulation consisting of a homogeneous mixture of dried Chinese herbal powders containing *Astragalus membranaceus*, *Poria cocos*, *Atractylodes macrocephala*, *Lycium chinense*, *Ligustrum lucidum*, *Paeonia lactiflora*, *Paeonia obovata*, *Citrus reticulata*, *Ophiopogon japonicus*, *Milletia reticulata*, *Oldenlandia diffusa*, *Scutellaria barbata*, *Prunella vulgaris* and *Glehnia littoralis* manufactured under Good Manufacturing Practice conditions. They were imported under license by the Zen Herb Company (Petach Tikva, Israel) in accordance with the regulations of the Israel Ministry of Health.

### CELL VIABILITY ASSAY

To determine the effect of CHT on cell survival, cells ( $2 - 5 \times 10^3$ /well) were seeded in 96-well plastic plates and incubated at 37 °C in full medium for 24 h. CHT was then added in varying concentrations (0 - 10 mg/ml) to each of three replicate wells and incubated for 72 h. A freshly prepared mixture of sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulphonic acid hydrate (XTT) and an activation reagent, *N*-methyl dibenzopyrazine methyl sulphate (PMS) was added to each well (50 µl). Following 2 h of incubation at 37 °C, the plates were placed on the mechanical plate shaker of a computerized automatic microwell plate spectrophotometer and shaken for 30 s, and the optical densities (OD) of the dye read at 450 nm. The measurements were repeated

following 4 and 6 h of incubation. The time point of the assay for OD readings was chosen according to the manufacturer's recommendations. The results for the different time points were normalized and averaged. The OD readings had been previously shown to correlate well ( $r > 0.97 - 0.99$ ) with the number of cells/well.<sup>11-13</sup>

### LIGHT MICROSCOPY

The T47D breast adenocarcinoma cells were plated at a density of  $5 \times 10^6$  cells per 10-cm dish and incubated for 24 h with different concentrations of 0, 0.3 or 0.5 mg/ml of CHT. The cells were then viewed by light microscopy under  $\times 200$  magnification.

### FLOW CYTOMETRY ANALYSIS OF APOPTOSIS

The T47D cells were plated at a density of  $5 \times 10^6$  cells per 10-cm dish and treated with 0, 0.3 or 0.5 mg/ml of CHT. The adherent and non-adherent cells were collected during the exponential growth phase of the cells and counted. A total of  $1 - 2 \times 10^6$  cells were washed in 10 mM phosphate-buffered saline (PBS), pH 7.4, and the pellet fixed in 3 ml ethanol for 1 h at 4°C. Before the analysis, the cells were pelleted and resuspended in 1 ml 10 mM PBS and incubated for 30 min with 0.64 mg/ml RNase at 37°C. They were then stained with 45 µg/ml propidium iodide for at least 1 h before analysis by flow cytometry. Data acquisition was performed on a fluorescence-activated cell sorting (FACS) scanner and analysed by CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). All fluorescence and laser light scatter measurements were made with linear signal processing electronics. Data for at least 15 000 cells were collected for each data file. A standard protocol for cell-cycle distribution and cell size was used. Necrotic cells were

excluded by counting cells following staining with trypan blue before fixation. All experiments were done three times.

### STATISTICAL ANALYSES

The results were calculated as mean  $\pm$  SE. Statistical analyses were carried out using the SPSS® statistical package, version 15.0 (SPSS Inc., Chicago, IL, USA) for Windows®. The difference between the intact and treated cells was evaluated by the one-way Student's *t*-test and significance was established by the *post hoc* Tukey's pairwise comparison. A *P*-value  $< 0.05$  was considered to be statistically significant.

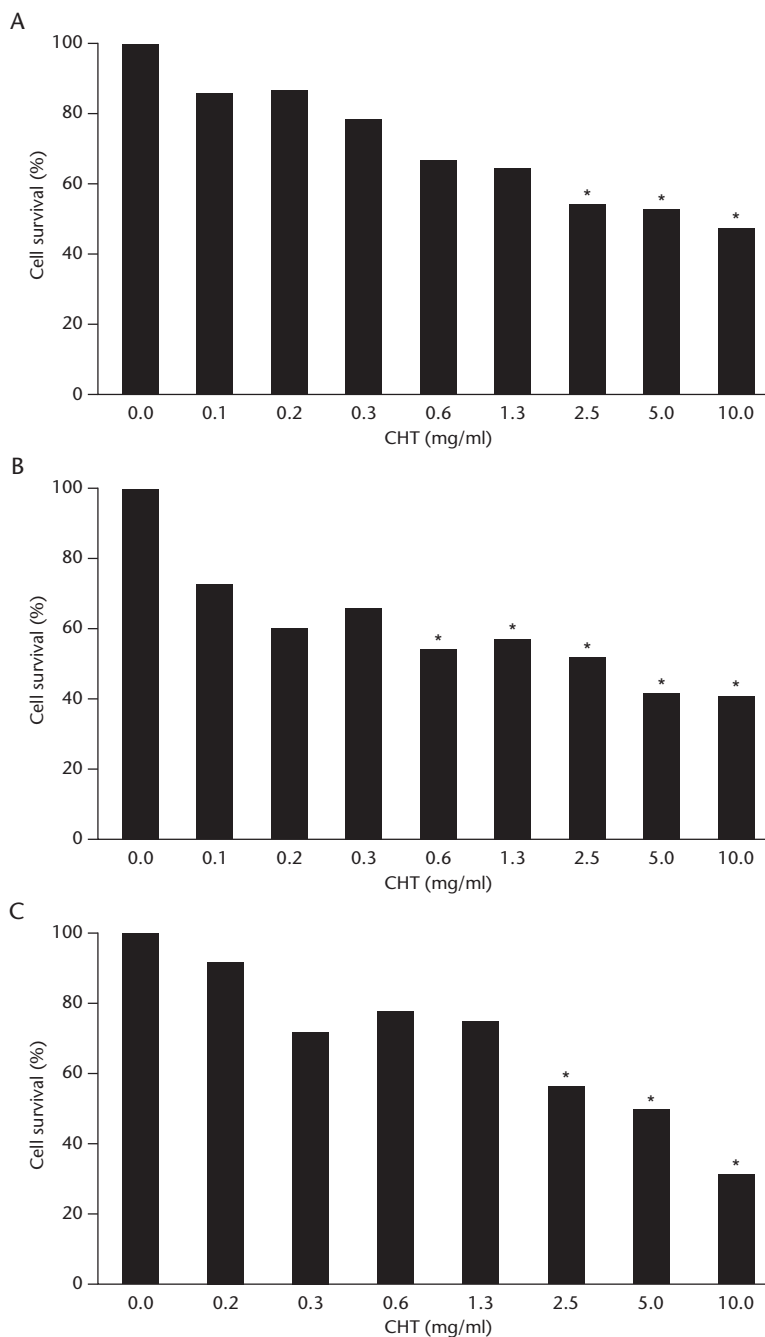
## Results

A dose-dependent inhibitory effect of CHT on cell survival was found in the MDA-231, MDA-453 and T47D human breast carcinoma cell lines ( $P < 0.05$ ; Fig. 1A - 1C). The IC<sub>50</sub> for CHT ranged between 2.5 and 10 mg/ml in the breast carcinoma cell lines tested. Light microscopy on the T47D cell line after 24 h of treatment with CHT demonstrated a dose-dependent inhibition of cell growth (Fig. 2).

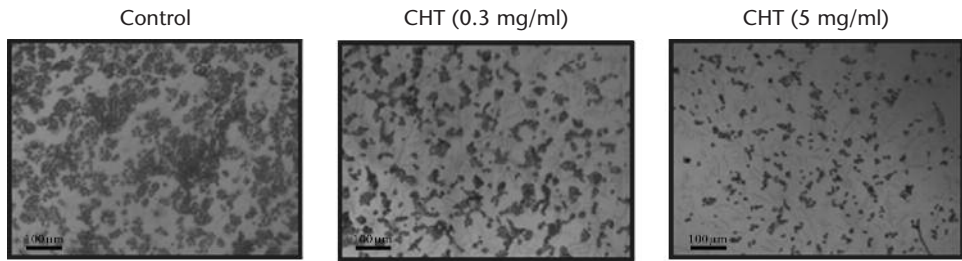
The extent of apoptosis was assessed by flow cytometry analysis following 72 h of exposure of the cells to 0, 0.3 or 5 mg/ml of CHT. CHT increased the percentage of cells with sub-diploid DNA content, the hallmark of apoptosis, in a dose-dependent manner in the T47D carcinoma cell line ( $P < 0.05$ ; Fig. 3).

## Discussion

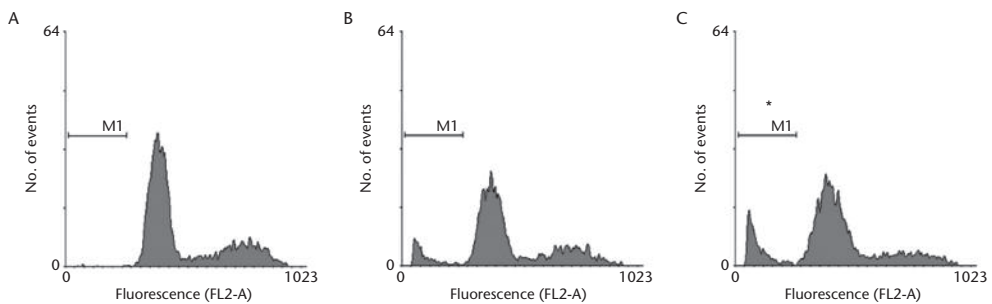
Rather than randomly screening thousands of plants for antibreast cancer activity, the present study focused on plants that have been used for hundreds, perhaps thousands, of years in Chinese medicine. A bioactive formula extracted from controlled manufactured concentrated powders of Chinese botanical herbs was used, and it was



**FIGURE 1:** Effect of 72 h exposure to different concentrations of Chinese herbal therapy (CHT) on survival of three breast adenocarcinoma cell lines: (A) MDA-231; (B) MDA-453; (C) T47D. \* $P < 0.05$  versus control (Student's *t*-test)



**FIGURE 2:** Light microscopy visualization of the effect on cell proliferation of different concentrations of Chinese herbal therapy (CHT) incubated with T47D breast adenocarcinoma cells for 24 h: (A) Control; (B) CHT (0.3 mg/ml); (C) CHT (5 mg/ml)



**FIGURE 3:** Flow cytometry assessment (FL2-A) of the sub-G<sub>1</sub> population of T47D breast adenocarcinoma cells, showing the effect of different concentrations of Chinese herbal therapy (CHT) on the induction of apoptosis. (A) Control; (B) CHT (0.3 mg/ml); (C) CHT (5 mg/ml). \*  $P < 0.05$  versus control (Student's *t*-test)

shown that treatment with CHT inhibited the survival of breast adenocarcinoma cell lines and that this effect was accompanied by an induction of apoptosis.

An increasing number of *in vitro* and *in vivo* studies show the beneficial roles of Chinese herbs in inhibiting proliferation and enhancing apoptosis of cancer cells. *P. cocos* is a mushroom that grows on the roots of pine trees and is widely used as a Chinese herbal drug; it known to have immune-enhancing and antitumour activities. Zhang *et al.*<sup>14</sup> assessed the effect of PCM3-II, a  $\beta$ -glucan derived from *P. cocos*, on human breast carcinoma MCF-7 cells and found a significant growth-inhibitory effect, manifest as cell-cycle arrest and the induction of apoptosis.

Paeonol, the main active compound of *P. lactiflora*, possesses antioxidant, anti-inflammatory and antitumourigenic properties.<sup>15</sup> Nizamutdinova *et al.*<sup>16</sup> showed that the inhibitory effect of paeonol on the expression of intercellular adhesion molecule-1, one of the key molecules in the development of atherosclerosis, is mediated by blocking p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways, and by subsequent inhibition of the activation of nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells (NF- $\kappa$ B). Multiple lines of evidence have shown that NF- $\kappa$ B regulates proliferation, invasion and metastasis of tumour cells.<sup>17</sup>

*Citrus reticulata* has been used

traditionally in Asia to promote liver Qi activity and the function of the digestive system.<sup>18</sup> Several studies have demonstrated its antiproliferative effects on cancer cells, including those of human breast cancer.<sup>19–21</sup>

*Oldenlandia diffusa* extract was shown to inhibit the growth of pancreatic cancer cell lines *in vitro* and to stimulate the induction of apoptosis, but not to affect normal pancreatic cells. In addition, Gupta *et al.*<sup>22</sup> demonstrated a significant inhibition of lung metastases in an *in vivo* model with no noticeable toxicity. Other studies have suggested that *O. diffusa* has immunomodulating activity; for example, Yoshida *et al.*<sup>23</sup> showed that *O. diffusa* stimulated macrophages to produce interleukin-6 and tumour necrosis factor, and Wong *et al.*<sup>24</sup> demonstrated that *O. diffusa* extract augments macrophage oxidative burst and inhibits tumour growth *in vivo*.

*Scutellaria barbata* has been used in traditional Chinese medicine as an anti-inflammatory and antitumour agent.<sup>25,26</sup> Its extract was shown to inhibit growth of several breast cancer cell lines *in vitro* and to induce apoptosis in tumour cells, but not in normal cells.<sup>9</sup>

A recent phase I clinical trial of the herbal extract, BZL101, in patients with advanced

breast cancer showed that the treatment was effective and safe, and the authors suggested that it should be investigated in a phase II clinical trial.<sup>27</sup>

Apoptosis is now recognized as one of the pathways by which cytotoxic agents induce the death of tumour cells. Breast adenocarcinoma cells, especially in advanced stages of the disease, are resistant to apoptotic stimuli as well as being commonly resistant to apoptosis-inducing chemotherapeutic agents.<sup>28,29</sup> It was found in the present study that CHT dose-dependently succeeded in inducing apoptosis in T47D cells. Thus, it could be suggested that the combination of CHT and chemotherapy might be a novel approach for the treatment of resistant breast cancer with higher therapeutic efficacy than the currently available modalities, and that randomized clinical trials are warranted to validate this theory. These results have led to a randomized clinical trial that is currently being conducted in the Tel-Aviv Sourasky Medical Centre to assess further the role of CHT for breast cancer patients.

## Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

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