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Essiac® and Flor-Essence® herbal tonics stimulate the in vitro growth of human breast cancer cells

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Abstract

Background: People diagnosed with cancer often self-administer complementary and alternative medicines (CAMs) to supplement their conventional treatments, improve health, or prevent recurrence. Flor-Essence® and Essiac® Herbal Tonics are commercially available complex mixtures of herbal extracts sold as dietary supplements and used by cancer patients based on anecdotal evidence that they can treat or prevent disease. In this study, we evaluated Flor-Essence® and Essiac® for their effects on the growth of human tumor cells in culture.

Methods: The effect of Flor-Essence® and Essiac® herbal tonics on cell proliferation was tested in MCF-7, MDA-MB-436, MDA-MB-231, and T47D cancer cells isolated from human breast tumors. Estrogen receptor (ER) dependent activation of a luciferase reporter construct was tested in MCF-7 cells. Specific binding to the ER was tested using an ICI 182,780 competition assay.

Results: Flor-Essence® and Essiac® herbal tonics at 1%, 2%, 4% and 8% stimulated cell proliferation relative to untreated controls and activated ER dependent luciferase activity in MCF-7 cells. A 10^{-7} M concentration of ICI 870,780 inhibited the induction of ER dependent luciferase activity by Flor-Essence® and Essiac®, but did not affect cell proliferation.

Conclusions: Flor-Essence® and Essiac® Herbal Tonics can stimulate the growth of human breast cancer cells through ER mediated as well as ER independent mechanisms of action.

Cancer patients and health care providers can use this information to make informed decisions about the use of these CAMs.

Introduction

There is a pressing scientific need to understand the biologic effects of alternative therapies [1, 2]. Complementary and alternative medicine (CAM) is used by a reported 50% of the general public in North America [3-5] and among some patient populations CAM use is as high as 85% [4, 6, 7]. The use of CAM reflects the growing trend in the United States of turning to alternative medicines to replace or supplement conventional therapies [8]. The increased use of CAM is driven by many factors including increases in prices of prescription drugs, media reports of adverse effects of prescription drugs, managed health care- mandated restricted access to physician care, and importantly, free access to product information from the internet [9, 10]. Patients combine CAM and conventional approaches with a hope to improve quality of life, counter side effects, and achieve a sense of control over their therapy [11].

Essiac herbal extract formulations are commercially available from many sources and used as a CAM by some cancer patients and survivors. The two most widely used commercially available essiac herbal tonics are Flor-Essence® and Essiac®. Both formulations contain the same four basic herbs: burdock root (*Arctium lappa*), sheep sorrel (*Rumex acetosella*), slippery elm bark (*Ulus rubra)*and Turkish rhubarb (*Rheum palmatum*). In addition, Flor-Essence® contains red clover (*Trifolum pratense*), blessed thistle (*Carduus benedictus*), kelp (*Laminaria digitata*), and watercress (*Nasturt officinale*). These herbal tonics are advertised to improve health and boost the immune system. Their use by cancer patients is largely fueled by anecdotal evidence that they can treat or prevent disease [7]. In a survey of 318 cancer patients treated by the Royal Mardsen Hospital in London, 6.0% reported using Essiac, one of the 5 most popular alternative remedies cited [11]. The particular popularity of Flor-Essence® and Essiac® as complementary therapies among breast cancer survivors and patients may be related to the first described use of

this herbal tonic as a cure for a breast lump [12-14]. In a recent field study, designed to determine which natural products were recommended by health food store employees to individuals seeking treatment for breast cancer, Flor-Essence® and Essiac ® were the two most frequently recommended products [15].

Flor-Essence® and Essiac® herbal tonics are marketed as dietary supplements and so not regulated by the FDA in the same way as are prescription and over the counter drugs [8, 9]. The available literature focusing on Flor-Essence® and Essiac® is widely disseminated in the lay press [13, 14, 16] and on numerous web sites and is rarely backed by scientific information [1, 2, 14]. In spite of these approaches to capture the interest of consumers, patients are beginning to demand science-based information about herbal products with which to make informed decisions about their use [17]. Advising patients on the use of unconventional therapies is difficult when rigorous scientific data and documentation about their safety, efficacy, and potential herbal-drug interactions are unavailable [8, 9, 17].

Although the effects of the tonics have not been thoroughly studied, the effects of the individual herbs used in the Flor-Essence® and Essiac® have been investigated because of their important dietary and medicinal roles [18]. Characteristics of these herbs include estrogenic, cytotoxic, anti-estrogenic, anti-tumor, anti-mutagenic, anti-oxidant, and anti-inflammatory properties, among others. The lay literature suggests that the components act synergistically to convey beneficial properties [13, 14]; however, this has not been scientifically demonstrated [18].

We were interested in determining if Flor-Essence® and Essiac® tonics effect tumor cell growth either by estrogen-dependent or –independent mechanisms. Estrogenic activity was hypothesized since several of the herbal tonic components (burdock root, sheep sorrel, and red clover) contain phytoestrogens such as the isoflavones genistein, daidzein, formononetin, and

biochanin-A [18]. Genistein levels in red clover are ten-fold greater than in soy [18]. Administration of red clover to ovariectomized rats causes a dose-dependent increase in uterine wet weight, a biologic measure of estrogenic activity [19].

Several model systems can be used to evaluate complex mixtures for functional and biologic relevance to human exposure including *in vitro* cell proliferation and reporter assays, and *in vivo* uterotropic assays in rodents [20-29]. We report here our successful use of cell proliferation and estrogen receptor (ER) dependent luciferase reporter assay systems to evaluate the effect of Flor-Essence® and Essiac® herbal tonics on cancer cell growth. Using these assays, we show that these tonics stimulate ER activation and dramatically increase breast cancer cell proliferation.

Materials and Methods:

Human Breast Cancer Cells

Estrogen receptor positive (ER+) MCF-7, and T47D, and estrogen receptor negative (ER-) MDA-MB-231 and MDA-MB-436 human breast cancer cell lines, obtained from American Type Culture Collection (ATCC, Manassas, VA), were used for the described studies. MCF-7 cells were grown in DMEM with 5% fetal bovine serum (FBS), 1% non-essential amino acids, 10 µg/ml insulin, 2 mM L-glutamine, and 1% penicillin/streptomycin. MDA-MB-436 cells were grown in Leibovitz'z L-15 medium supplemented with 10 µg/ml insulin, 16 µg/ml glutathione, 2mM L-glutamine, 1% penicillin/streptomycin, and 10% FBS. T47D cells were grown in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate and supplemented with 0.2 units/ml bovine insulin, 1% penicillin/streptomycin and 10% FBS. MDA-MB-231 cells were grown in

RPMI 1640 medium with 2 mM L-glutamine, 1% penicillin/streptomycin and 10% FBS. All cells were maintained at 37°C with 5% CO_2

For cell proliferation assays and estrogen responsive reporter assays, Opti-MEM phenol red-free medium (Gibco) was used with 5% charcoal stripped fetal bovine serum (Clonetech) to minimize estrogen-like activity contributed by the phenol red and serum [29, 30].

Herbal Tonic preparation

Flor-Essence® (Flora Manufacturing) was obtained as a sterile pre-packaged and prepared product at a local health food store. It was used directly out of the bottle for the described tissue culture applications, which is how an individual who had purchased the product for self-administration would consume it. Similarly, Essiac® (Reserpine Corporation) was obtained as a dry formulation, and was prepared in the laboratory as recommended by the manufacturer. Both tonics were kept refrigerated and not exposed to extremes in temperature in accordance with manufacturer's instructions.

Recommendations from the available lay literature on Essiac formulations suggest daily doses ranging from 1 to 6 oz [12, 13, 16]: However, dose recommendations are not based on weight and although the majority of people who self-administer Flor-Essence® herbal tonic self-report following the recommended doses, some admit to consuming more [7]. Because the final concentration of tonic at the level of the target cells is unknown, making it difficult to mimic a human exposure, we chose a range of Flor-Essence® and Essiac® tonic concentrations (1% to 32%) to evaluate a dose response.

Cell proliferation assay

For cell proliferation assays, 3 x 10^3 cells were seeded into each well of a 96-well tissue culture plate. Twenty-four hours after plating, cells were treated with Opti-MEM medium supplemented with 1%, 2%, 4%, or 8% Flor-Essence® or Essiac® herbal tonic, 10^{-7} to 10^{-10} M 17-β-estradiol (E2) (Sigma), or left untreated. The effect of tonic exposure on cell growth was evaluated 24 and 72 hours after the initiation of treatment. For incubations longer than 24 hours, cell medium was renewed at the 48 hours time point. Flor-Essence® concentrations up to 32% were tested in the MCF-7 cell line. For the ICI 182,780 experiments, cells were pre-treated with 10^{-7} M ICI 182,780 (Tocris Cookson, Inc., Ellisville MO) for 30 minutes, followed by 10^{-9} M E2, increasing concentrations of Flor-Essence® or Essiac®, or no treatment.

Cell growth was quantified using an Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison WI) with absorbance of the wells measured in a standard multi-well plate reader at 490nm. Briefly, this colormetric assay measures the bioreduction of a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) to a formazan product. The conversion of MTS is directly proportional to the number of living cells in culture. Each experiment was performed at least three individual times with 4 – 6 replicates per experiment. Effect of treatment was determined by comparing cell growth to the appropriate vehicle-treated cells (sterile water) and untreated cells and is presented as percent of control. Error is presented as standard error of the mean. Statistical significance was assessed using a standard Student's T-test.

Estrogen Responsive Reporter Assay

Herbal tonic concentrations ranging from 1% to 8% were directly tested for estrogenic and anti-estrogenic activity using a standard estrogen responsive reporter plasmid containing three

vitellogenin estrogen responsive elements (EREs) upstream of the luciferase reporter gene (EREx3-Luc). EREx3-Luc was co-transfected with a commercially available control renilla reporter plasmid (pRL-TK, Promega) to control for transfection efficiency and for the normalization of the results. The reporter plasmids were transfected into MCF-7 cells [31-34], which have well-characterized responses to estrogenic stimuli. The ability of the herbal tonics to induce luciferase reporter gene activity was compared to 10^{-9} M E2 and appropriate negative controls, as previously described [35, 36].

MCF-7 cells were plated in 96-well plates, designed specifically for use in a luminometer (Perkin Elmer, Wellesley, MA) at a density of $1X10^5$ cells/well in Opti-MEM medium. The cells were left untransfected or were co-transfected with 200 ng each of luciferase reporter and renilla control plasmids using lipofectAMINE™ 2000 (Invitrogen) optimized for 96-well plate experiments according to directions. After a 5-hour transfection, the untransfected cells were incubated with culture medium made from Opti-MEM and 5% charcoal stripped FBS to quantify background levels. The transfected cells were incubated with the same culture media alone or supplemented with 1%, 2%, 4%, or 8% Essiac® or Flor-Essence® herbal tonics.

The cells were incubated at 37°C for 18 hours and then assayed for luciferase and renilla activity using a dual luciferase/renilla reporter assay kit (Promega) according to directions. Each experimental condition was duplicated six to eight times in the 96-welled plate and each plate was read in the luminometer twice. Luciferase activity was normalized to the renilla controls.

Co-incubating the cells with 10^{-9} M E2 and increasing concentrations of both tonics was used to measure potential anti-estrogenic activity. To indirectly measure specific binding to the ER, the anti-estrogen ICI 182,780 was added to the cells and the change in Flor-Essence® and Essiac®-mediated induction of luciferase activity was measured. Transfected cells were exposed

to 10^{-7} M ICI 182,780 for 30 minutes before addition of 10^{-9} M E2, 4% Flor-Essence®, or 4% Essiac®.

All experiments were repeated more than 4 times (with 5-6 replicates per experiment) and all data were normalized to E2 (10^{-9}M) activation. Error is presented as standard error of the mean. Statistical significance was determined using a standard Student's T-test.

Results

Flor-Essence® and Essiac® Herbal Tonic induce human breast cancer cell proliferation at low concentrations.

Flor-Essence® treatment was associated with a statistically significant increase in cell proliferation that ranged from 1.5-fold to 2.1-fold ($p < 0.01$) (Figure 1A) at 24 hours after treatment. The increase in cell growth was similar for all cell lines tested, regardless of ER status. In addition, these concentrations resulted in a greater induction of cell growth than estrogen alone (10^{-10} M or 10^{-9} M), which was 1.1-fold greater than control in the MCF-7 cell line (data not shown). Concentrations of Flor-Essence® up to 32% were tested in the MCF-7 cell line; treatment with 16% Flor-Essence® resulted in growth that was not significantly different from controls and 32% was cytotoxic to the cells (data not shown). These concentrations were not used in subsequent experiments.

The 1%, 2%, 4%, and 8% Essiac® treatments induced proliferation 1.2-fold to 2.2-fold above untreated cells (Figure 1B) 24 hours after exposure and was statistically significant ($p < 0.01$, with the exception of MCF-7 cells treated with 8% Essiac®, which was $p < 0.05$). The ER+ MCF-7 cells were less stimulated by Essiac® treatment relative to other cell lines tested ($p<0.01$). However the T47D ER+ cell line, showed proliferation that was not significantly

different than the ER- cell lines. All Essiac® concentrations tested induced proliferation to a greater degree than 10^{-10} M or 10^{-9} M E2 in any cell line at 24 hours after treatment (data not shown).

Comparing the response of the 4 cell lines to the two tonics demonstrated that Flor-Essence® and Essiac® produced a generally similar increase in cell proliferation in the T47D, MDA-MB-436 and the MDA-MB-231 cell lines. In the MCF-7 cells, Flor-Essence® produced a statistically significant increased proliferation at all concentrations tested ($p<0.01$), compared to Essiac®. In addition, low concentrations of Flor-Essence® (1%) produced a significant increase in proliferation in the ER- cell lines, when compared to Essiac®.

To determine if continuous exposure would result in an acute increase in cell proliferation followed by acclimation and a return to basal levels or a sustained increase in cell proliferation we treated the cell lines for extended periods of time with the two tonics at multiple concentrations. Cells were treated with 4% Flor-Essence® (Figure 2A) or 4% Essiac® (Figure 2B) for up to 72 hours. Induced proliferation was significantly different than control ($p <0.01$, with the exception of 72 hours treatment of the T47D cells, which was $p < 0.05$) although the effect of increasing time of tonic treatment was not the same for all cell lines tested. For T47D, MDA-MB-436, and Essiac®-treated MDA-MB-231, the increased cell proliferation demonstrated in the 24 hour time point was not sustained at 72 hours tonic treatment. Flor-Essence®- treated MDA-MB-231 cells and Essiac®-treated MCF-7 cells showed a time-dependent increase in cell proliferation (72 hrs > 24 hrs, $p<0.01$) and Flor-Essence®-treated MCF-7 cells showed no difference in the 24 and 72 hour time points.

The time-dependent effects of two tonics were not significantly different for the T47D or MDA-MB-436 cells lines. However, 24 hour incubation with Flor-Essence® caused a significant

increase in MCF-7 cell proliferation compared to Essiac® (also demonstrated in Figure 1). Similarly, 72 hours incubation with Flor-Essence® produced a statistically significant increase in cell proliferation in the MDA-MB-231 cells, compared to Essiac® ($p<0.01$).

Flor-Essence® and Essiac® induce estrogen-receptor dependent luciferase reporter activity

Both the Essiac® and Flor-Essence® herbal tonics induced luciferase expression from an ER dependent reporter construct transfected into MCF-7 cells. Flor-Essence® administered at 1%, 2%, 4%, and 8% induced luciferase activity 3.8-, 4.9-, 5.0-, and 3.3-fold over negative controls and was statistically significant ($p < 0.0001$; Figure 3). Lower concentrations of Flor-Essence® (1%, 2%, and 4%) produced luciferase induction that was not statistically different from the 4.5-fold increase observed with 10^{-9} M E2 relative to the negative control. MCF-7 cells exposed to 1%, 2%, 4% and 8% Essiac® induced luciferase activity 2.8-, 3.1-, 3.4-, and 3.9-fold above untreated controls ($p < 0.0001$). However, the induced luciferase activity was significantly less than cells treated with 10^{-9} M E2 ($p < 0.01$) or low concentrations of Flor-Essence®. One, 2 and 4% Flor-Essence® caused significantly more luciferase induction compared to the same concentrations of Essiac®. The effect of 8% Flor-Essence® treatment was significantly less than the effect of 10^{-9} M E2 and not significantly different from the same concentration of Essiac® ($p < 0.01$).

Genistein, a known phytoestrogen, was also tested in the luciferase assay and induced estrogen receptor dependent luciferace expression. Genistein concentrations of 0.1 ug/ml and 1 ug/ml elicited a 5.1- and 5.0-fold greater luciferase expression than the negative control that was statistically significant ($p < 0.01$) (Figure 3). This response is similar to that observed for 2% and 4% Flor-Essence® and not significantly different from E2 exposure. A low genistein

concentration (0.01 ug/ml) did not induce luciferase activity above control. Similarly, a relatively high genistein concentration of 10 ug/ml was not efficient at inducing luciferase activity.

To test for potential anti-estrogenic activity, 1%, 2%, 4% and 8% Flor-Essence® and 1%, 2%, 4% and 8% Essiac® were co-incubated with 10^{-9} M E2 in the luciferase assay. Combining the tonic exposure with E2 did not significantly affect E2 induction of luciferase expression (data not shown).

Effect of ICI 182,780 on Flor-Essence® and Essiac® induced luciferase activity and cell proliferation

ICI 182,780 is an anti-estrogen that specifically antagonizes the binding of the estrogen-receptor binding to its natural ligand at the estradiol binding site [37, 38]. If a compound is acting through an estrogen-receptor dependent mechanism of action, ICI 182,780 will inhibit mediation of the effect. ICI 182,780 pre-treatment followed by 10^{-10} M E2 caused a 7.2-fold reduction in luciferase activity compared to 10^{-10} M E2 alone. Pre-treatment with ICI 182,780 before exposure to 4% Flor-Essence® and 4% Essiac® caused a 4.6- and 3.3-fold decrease in luciferase activity, respectively (Figure 4A).

To determine if specific binding of ICI 182,780 to the ER produced a parallel inhibition of cell proliferation, cells were treated with ICI 182,780 for 30 minutes prior to treatment with 2% and 4% Flor-Essence® or Essiac®. Cell proliferation was measured after 24 hours incubation. Figure 4B shows that incubation with ICI produced no significant reduction in cell proliferation, compared to tonic treatment alone. However, pre-treatment with ICI 182,780 prevented E2-mediated increases in cell proliferation. Longer incubation times (48 hours) or repeating the experiment in the MDA-MB-436 cell line did not change the results (data not shown).

Discussion

In this paper we demonstrate for the first time that both Essiac® and Flor-Essence® herbal tonics stimulate cell growth in ER+ and ER- breast cancer cell lines and can activate ER dependent transcription. The results of the transcriptional activation studies provide mechanistic information to suggest that specific components within the tonic activate the ER. Based on our knowledge of the ingredients in these two tonics, it is likely that phytoestrogens such as genestein, daidzein, formononetin, and biochanin-A are responsible for this activity. However, because the tonics are equally able to stimulate growth in ER- cell lines and in the presence of the anti-estrogen ICI 182,780, our results also suggest that the tonics are able to affect growth through other, ER independent pathways. In fact, the various components of these complex mixtures may be acting together with the phytoestrogens to increase cell proliferation through ER dependent mechanisms, ER independent mechanisms, or receptor cross-talk with subsequent estrogen responsive element (ERE) binding.

The effect of the tonics on cell proliferation was tested using a modified E-SCREEN assay [39]. This assay was developed to take advantage of the ability of estrogen-responsive MCF-7 cells to proliferate in the presence of compounds that mimic estrogen. Although a standard assay for estrogenicity screening, the proliferative response of the MCF-7 cells varies depending on the MCF-7 sub-line employed. In these studies we used ATCC wild-type MCF-7 cells, which have been shown to vary in their response to estrogen incubation [39]. Using this assay, we demonstrated that Essiac® and Flor-Essence® herbal tonics stimulate cell proliferation to a greater degree than estradiol treatment in the MCF-7 cells.

Further modification of the E-SCREEN assay to accommodate different media conditions enabled us to test additional ER+ and ER- human breast cancer cell lines. Using this approach, we demonstrated that Flor-Essence® and Essiac® herbal tonics induced the proliferation of T47D, MDA-MB-231 and MDA-MB-436 breast cell lines at concentrations ranging from 1 to 8%, regardless of the presence of the ER. Mechanistically, this suggests that the ER is not necessary for the proliferative effect of Essiac® and Flor-Essence®. Evaluating cell proliferation after the co-administration of the anti-estrogen ICI 182, 780 and the tonics further supports the conclusion that the ER is not essential for the cell growth effect. In these experiments we demonstrate that specifically blocking the estradiol binding site on the ER did not significantly affect the ability of the tonics to stimulate cell proliferation.

We investigated the effect of chronic exposure to the tonic by incubating the cell lines with Flor-Essence® and Essiac® for up to 72 hours. Prolonged exposure to the two tonics resulted in an increase in cell proliferation that continued with exposure. The cells did not acclimate to the exposure by returning to the baseline or control growth rate during the 3 day exposure time course. This may indicate that a long duration of exposure to the tonics (as would be expected, based on the recommended dosing regime in the product literature) could result in continued stimulation of cell proliferation of human breast cells.

Both tonics are able to activate ER-dependent transcription in MCF-7 cells transfected with a standard estrogen responsive luciferase reporter plasmid, indicating that the tonics can interact with the endogenous ER present in MCF-7 cells to effect transcription. Tonic-induced ER activation suggests that although these tonics are complex mixtures of herbal extracts, estrogenic activity contributed by constituent components are detectable and can be correlated with effects on cell growth. Sheep sorrel and burdock root are present in Flor-Essence® and Essiac® and red

clover is a component of Flor-Essence® all there extracts are reported to contain phytoestrogens, which may be directly interacting with the ER.

ICI 182,780 is an anti-estrogen that specifically binds the ER and blocks estrogen binding. This compound was used to investigate the interaction of components of Flor-Essence® and Essiac® at the level of the ER in the transcription assay. Pre-incubation with ICI 182,780 inhibited tonic-stimulated increased luciferase activity, suggesting that the tonics effect transcription through direct interaction with ER at the estradiol binding site. ER activity was reduced, but not completely abolished, by the addition of ICI 182,780 suggesting that the tonics may activate ER transcription via mechanisms that do not rely on direct binding to the estradiol binding site. In addition, pre-incubation with ICI 182, 780 in cell proliferation studies had no effect on the ability of the tonics to increase cell growth, further suggesting that the tonics can stimulate cell proliferation by mechanisms that do not involve direct interaction with the estradiol site on the ER.

Although there was little difference in the effects of the two tonics on cell proliferation, Flor-Essence® tended to produce a greater response in the ER+ MCF-7 cell line. This observation, in combination with the luciferase assay, suggests that the additional components present in Flor-Essence®, relative to Essiac®, including red clover, blessed thistle, kelp, and watercress, may be enhancing the response. Red clover has high genistine levels and the inclusion of this plant extract may explain the increased estrogenic activity of Flor-Essence® herbal tonic compared to Essiac®.

Genistein was included in these studies in order to compare the tonics to a known phytoestrogen, which has been well investigated in both *in vitro* and *in vivo* models for breast cancer as well as in epidemiologic studies [40-46]. In the study presented here, 2% and 4% Flor-

Essence® induce luciferase activity at levels similar to 0.1 ug/ml and 1.0 ug/ml genistein. As expected, 10ug/ml genestein demonstrated a reduced ability to stimulate estrogen receptor activation, possibly by down regulating ER- alpha expression [47]. This trend is similar to the results we obtained with increasing concentrations of tonic exposure.

Two other studies have been recently published, demonstrating the effect of Essiac® and/or Flor-Essence® on human cell lines [48, 49]. Ottenweller et.al. demonstrated decreased cell proliferation of Chinese Hamster Ovary (CHO) cells and the human prostate cancer cell line, LNCaP. This study also demonstrated an augmentation of T-cell proliferation at low doses of Essiac® [48]. The study by Tai, et. al. reported a slight increase in cell proliferation in ATCC MCF-7 cells at 1 and 2 % tonic concentrations. The results of the Tai study also show a weak antiproliferative effect of both Flor-Essence® and Essiac® on 3 cancer cell lines at doses spanning 1-10% tonic at 96 h incubation time and a differentiation inducing effect on HL60 cells at high concentrations [49].

It is difficult to directly compare our results to those presented by Otenweller et al and Tai et al. because of the difference in cell lines used as well as the different approaches to study design. We took precautions to ensure that our assay systems were depleted of estrogenic components. We chose to use Opti-MEM phenol red free medium, which is often used when testing compounds in cell systems to determine if they are estrogenic [29, 30]). In addition, although we used normal fetal bovine serum in our medium for the routine propagation of our cell lines, we used charcoal stripped fetal bovine serum for the cell proliferation and estrogen responsive reporter assays. These conditions are designed to minimize the contributions of estrogenic activity from the cell medium in order to fully detect those present in the herbal tonics.

Bi-phasic bioactivity (causing activation at low concentrations and inhibition at high concentrations) has been reported for at least two naturally-occurring estrogenic compounds [50, 51]. By not depleting the culture conditions of inherent estrogens, and then adding the tonics, it is possible that the total concentration of estrogenic compounds in the assay system was never low enough in the Tai et al study to determine an activation effect. We also found that high concentrations of Flor-Essence® (16-32%) did not cause a stimulatory effect or inhibited cell proliferation.

Another factor complicating cross-study comparisons across is product consistency. Herbal tonics are sold as dietary supplements, are not regulated, are not standardized and may demonstrate lot-to-lot variability. The Tai et al study used HPLC to evaluate the lots used for their study and found no significant differences among lots. We have also evaluated lot variation of Flor-Essence® and Essiac® using HPLC and proliferation assays (data not shown). Our HPLC results found some variation in the peak height of the various components of the herbal tonics in different lots, but the proliferation assays consistently showed a cell growth stimulatory effect.

In this report, we have demonstrated that Flor-Essence® and Essiac® can promote the growth of cancer cells and that ER- dependent and independent mechanisms are responsible for mediating the effect. In a previous study, we showed that Flor-Essence® promoted DMBA-initiated mammary tumor formation in Sprague-Dawley rats [52]. The present study provides the rational for a mechanistic basis for the increase in tumor multiplicity seen in the rat study. DMBA-induced rat mammary tumors are hormone sensitive and it is likely that ER mediated growth effects are playing a role in tumor growth. However, due to the complex nature of the herbal mixtures there are likely to be an array of components that affect cell growth through alternative mechanisms.

Cancer patients are desperately seeking information about CAM and frequently seek that information from the internet. Highly credible cancer websites exist that provide reliable information about CAM for cancer treatment and prevention with links to information on CAM. However others websites issue misleading information including, but not limited to, claims for CAM efficacy that are not supported by sound scientific evidence or advice for patients that counsels against conventional therapy [53]. Essiac is frequently mentioned as a "curative" CAM [53] and over 90% of the websites that mentioned FlorEssence® were considered questionable due to vague, inaccurate, anecdotal and nonscientific information [54]. It has been estimated that 50% of internet users use the World Wide Web to gather medical information and that this information can influence a patient's choice of treatments [53, 55, 56]. Thus, there is a strong need for both accurate information and peer-reviewed studies in order that informed decisions can be made when one is considering self-administering CAM. Individuals and health care providers can use the information presented in this report to begin evaluating the risks and benefits of self-administration of Essiac® and Flor-Essence® herbal tonics in the context of individual health needs and concerns. For women and men who have been diagnosed with cancer, these results may be significant and can be used to make informed decisions about herbal tonic use.

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Figure Legends:

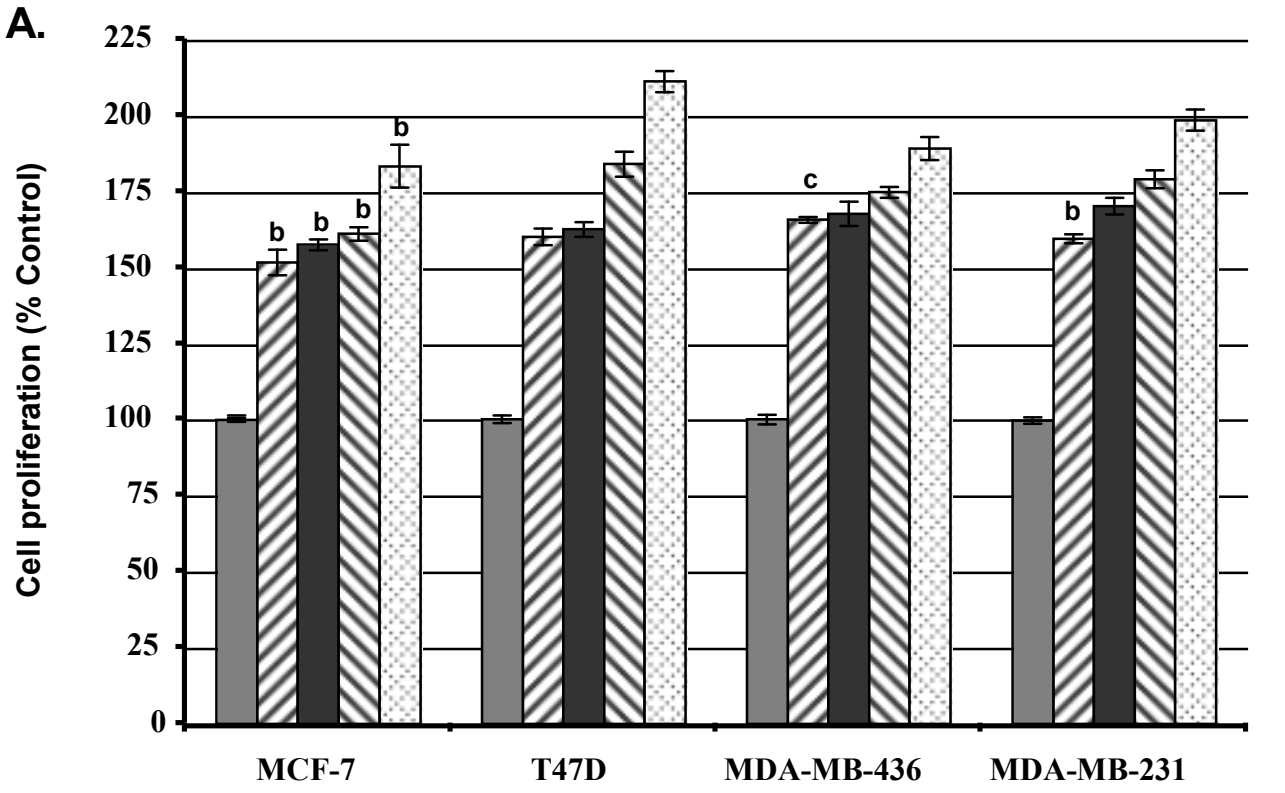
Figure 1: Effect of herbal tonic dose on ER+ and ER- human breast cancer cell proliferation. Cells were treated for 24 hours with increasing concentrations of A: Flor-Essence® and B: Essiac®. All tonic concentrations stimulated proliferation significantly above control ($p < 0.01$), with the exception of a, which is $p< 0.05$. b= Flor-Essence® significantly greater than the same concentration of Essiac®, $p<0.01$. c= Flor-Essence® significantly greater than the same concentration of Essiac®, $p<0.05$.

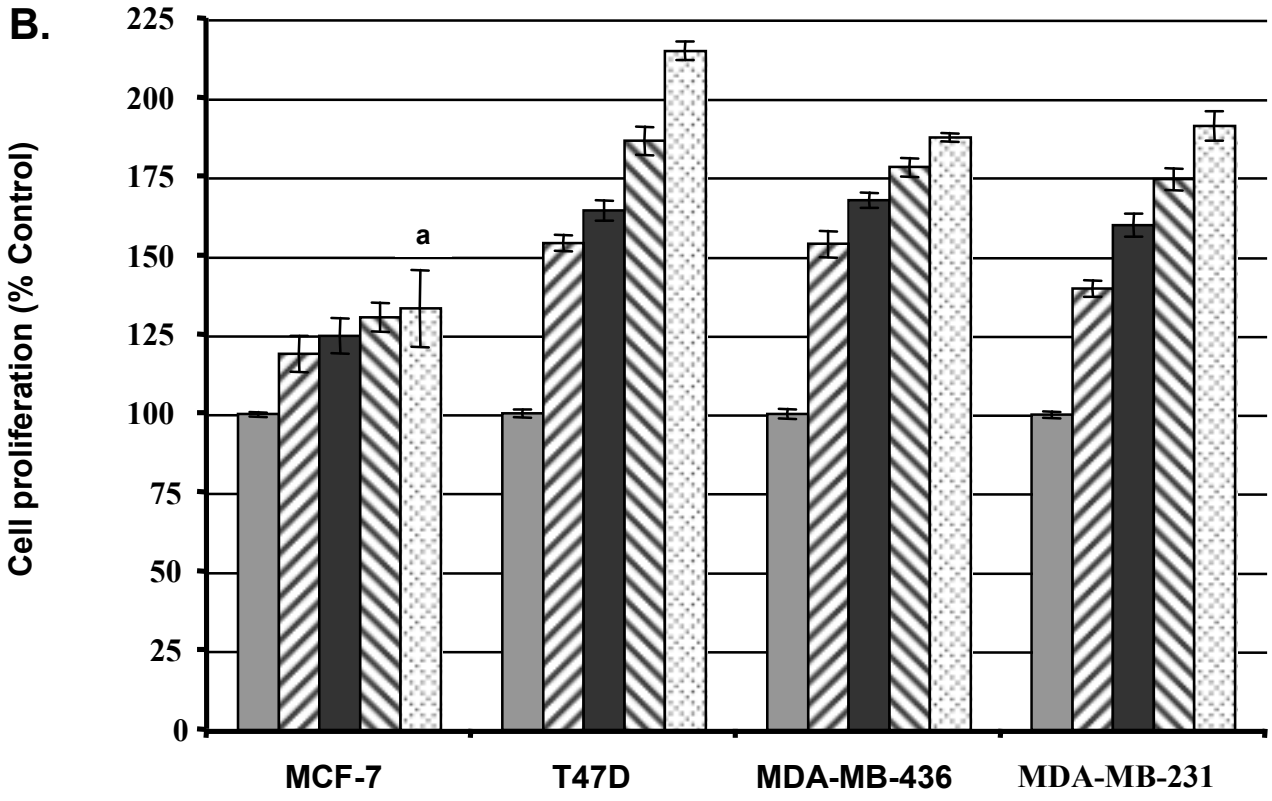
Figure 2: Effect of herbal tonic time of incubation on ER+ and ER- human breast cancer cell proliferation. Cells were treated with 4% Flor-Essence® (A) or 4% Essiac® (B) for up to 72 hours. All times are significantly greater than control, $p<0.01$, with the exception of (a) which is greater than control, $p<0.05$. b= Flor-Essence® significantly greater than Essiac®, $p<0.01$.

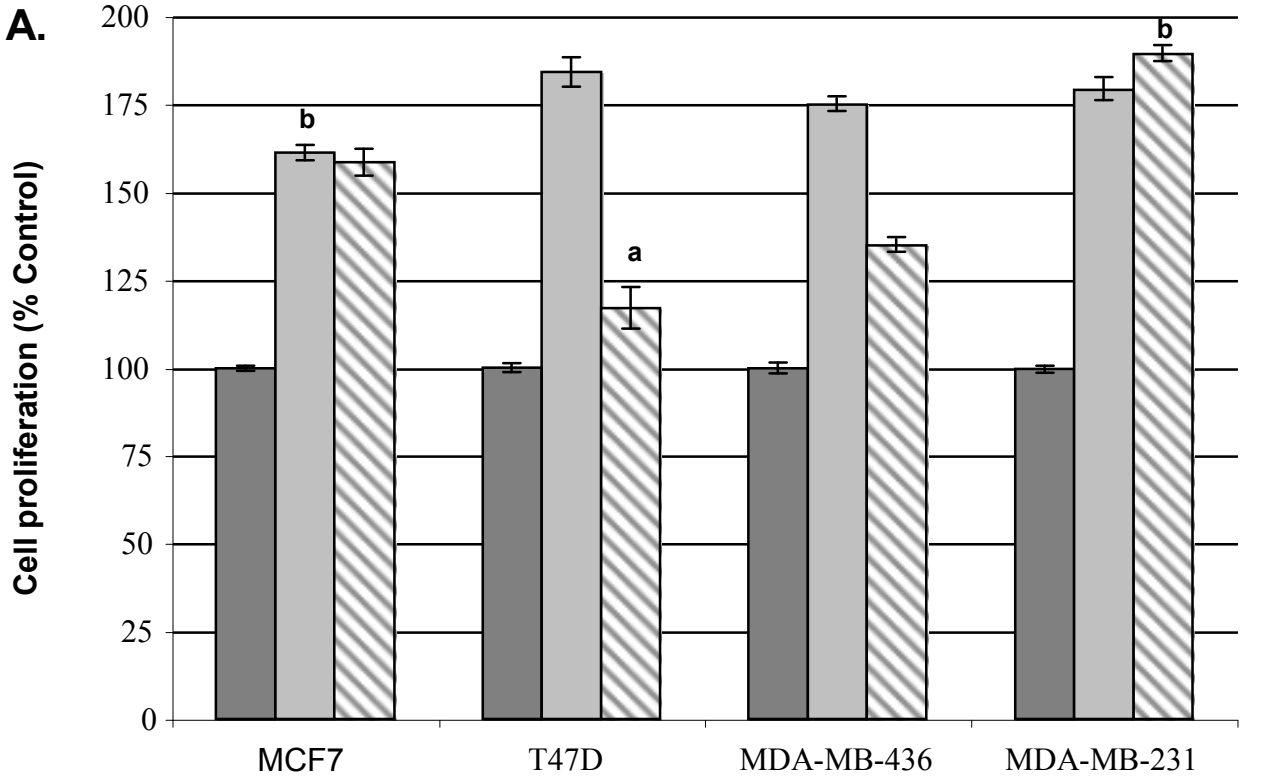
Figure 3. Effect of herbal tonics and genistein on ER activation. MCF-7 cells were left untreated (Ctrl) or treated with 10^{-10}M estradiol (E2), or increasing concentrations of Flor-Essence®, Essiac®, or genistein. Genistein concentrations are µg/ml. Data are normalized to estrogen. a= significantly different from control, $p<0.01$. b= significantly different from estradiol, $p<0.01$. c= significantly different than the same concentration of Flor-Essence®, $p<0.01$.

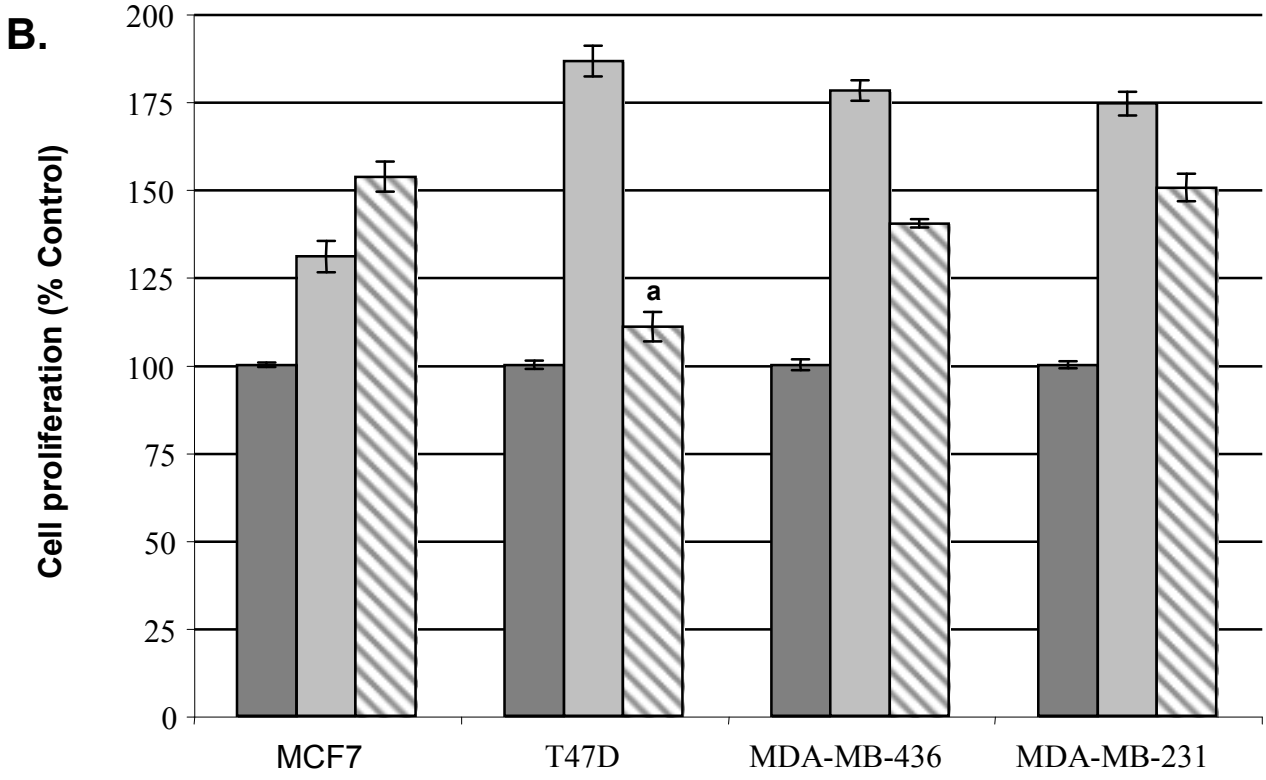
Figure 4. Effect of ICI 182,780 on Flor-Essence®- and Essiac®- induced luciferase gene expression and cell proliferation in MCF-7 cells. A. Cells were treated with estradiol (10^{-10}M),

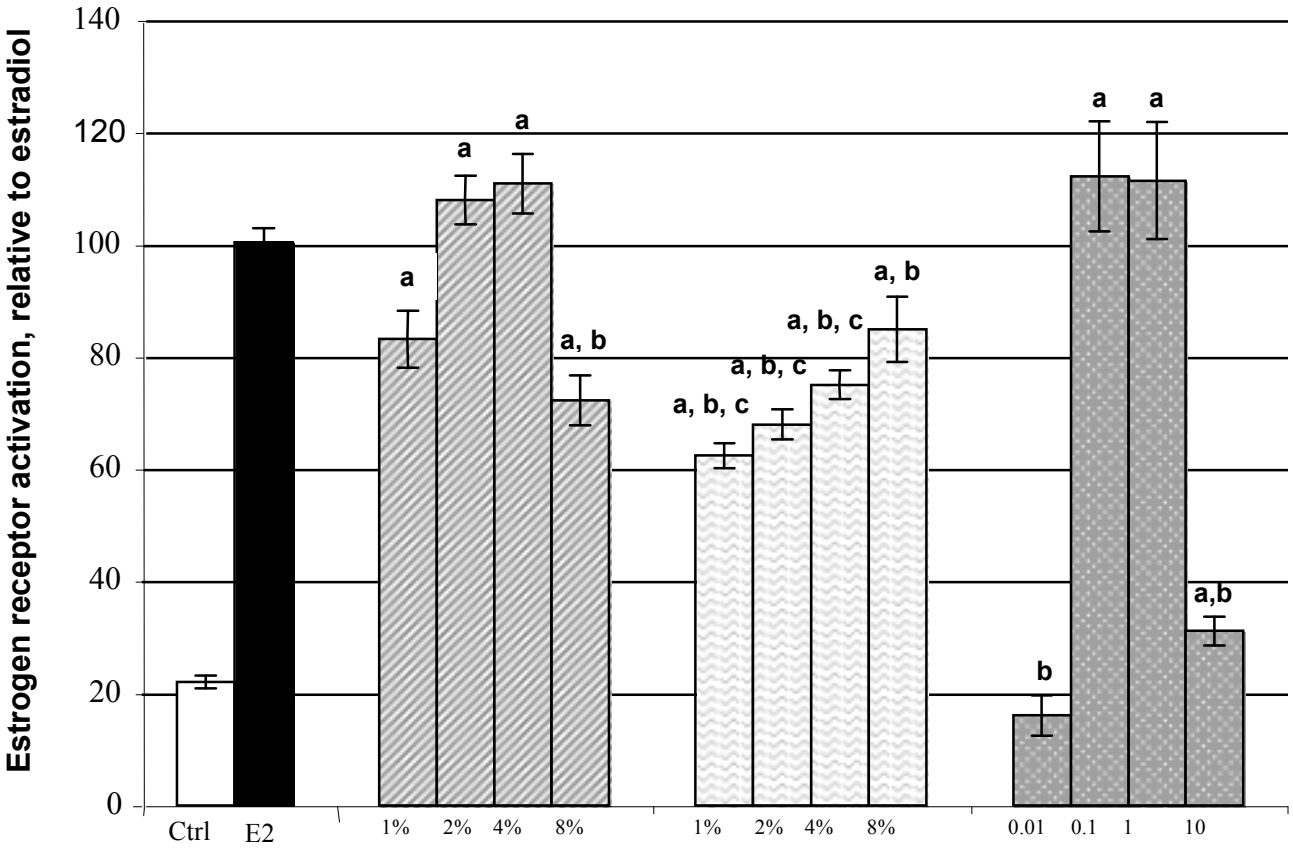
4% Flor-Essence® (F-E®) and 4% Essiac® (Es®) with and without ICI 182,780 (ICI) (10^{-7}M). Data are normalized to estrogen. a = significantly different from treatment without ICI 182,780, $p<0.01$. B. Cells were treated with 2 and 4% Flor-Essence® (F-E®) and 2 and 4% Essiac® (Es®) with and without ICI 182,780 (ICI)(10^{-7}M) for 24 hours. Data are normalized to control. All treatments (except for E2) were significantly different than control, $p<0.01$. E2 w/ ICI was significantly different than either control or E2 alone, $p<0.01$. There is no statistical difference between the cells treated with just tonic and cells treated with tonic and ICI. Other incubation times were tested with no change in results.


A.
Cell proliferation (% Control)
225
200
175
150
125
100
75
50
25
0
b
b
b
b
c
b
MCF-7
T47D
MDA-MB-436
MDA-MB-231

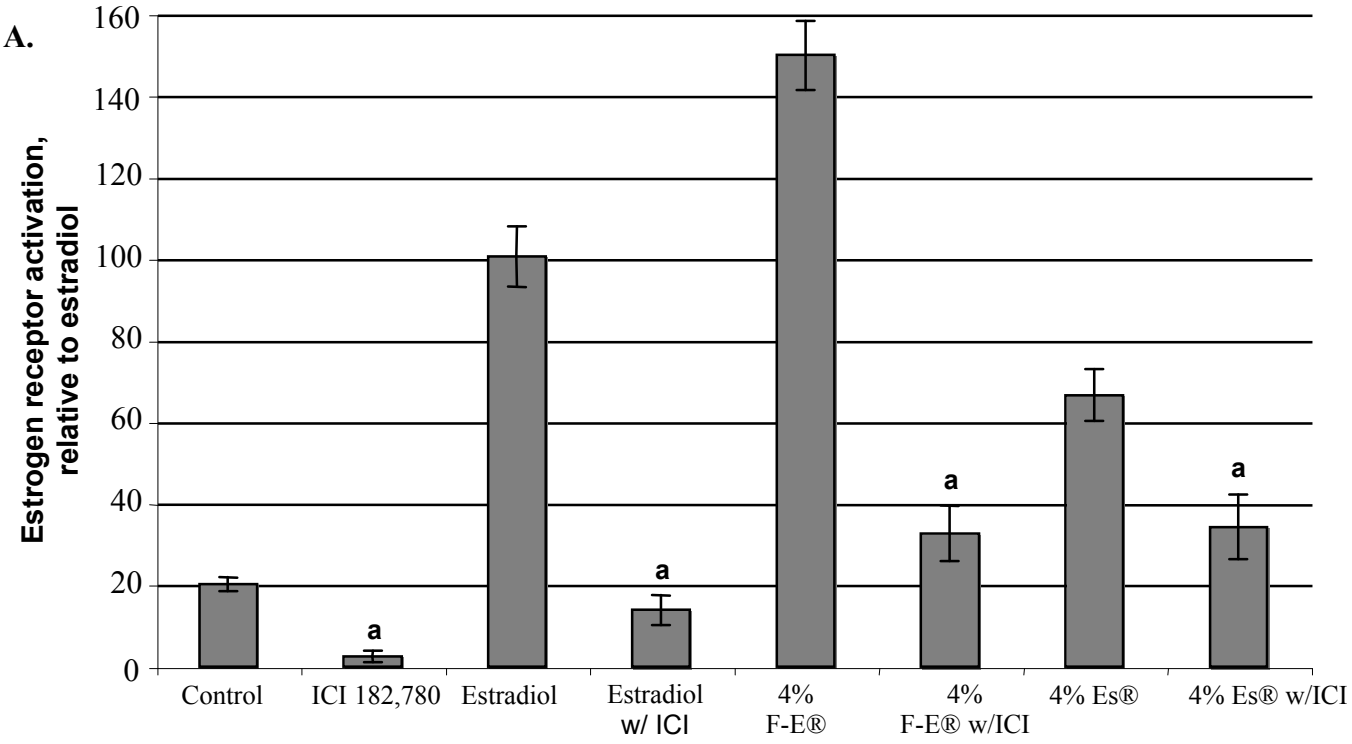

B.
Cell proliferation (% Control)
225
200
175
150
125
100
75
50
25
0
a
MCF-7
T47D
MDA-MB-436
MDA-MB-231


A.
Cell proliferation (% Control)
200
175
150
125
100
75
50
25
0
b
a
b
MCF7
T47D
MDA-MB-436
MDA-MB-231

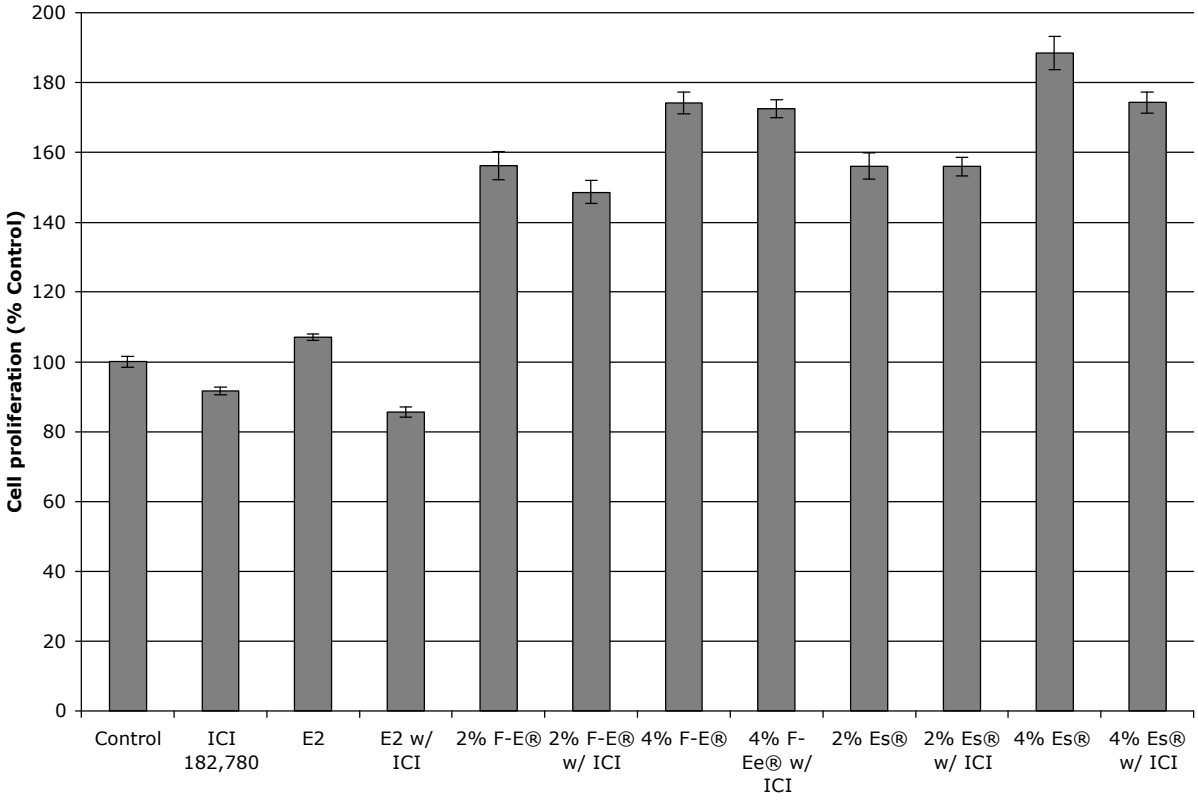

B.
Cell proliferation (% Control)
200
175
150
125
100
75
50
25
0
a
MCF7
T47D
MDA-MB-436
MDA-MB-231


Estrogen receptor activation, relative to estradiol
140
120
100
80
60
40
20
0
Ctrl
E2
a
a
a
a, b
a, b, c
a, b, c
a, b
b
a
a
a,b
1%
2%
4%
8%
1%
2%
4%
8%
0.01
0.1
1
10

A.


Estrogen receptor activation, relative to estradiol
160
140
120
100
80
60
40
20
0
a
a
a
Control
ICI 182,780
Estradiol
Estradiol w/ ICI
4% F-E®
4% F-E® w/ICI
4% Es®
4% Es® w/ICI

B.


Cell proliferation (% Control)
200
180
160
140
120
100
80
60
40
20
0
Control
ICI 182,780
E2
E2 w/ ICI
2% F-E®
2% F-E® w/ ICI
4% F-E®
4% F-Ee® w/ ICI
2% Es®
2% Es® w/ ICI
4% Es®
4% Es® w/ ICI