ABSTRACT

Darin N. Sujjavanich. THE EFFECTS OF SIX HERBAL SUPPLEMENTS COMMONLY USED BY WOMEN ON EXPRESSION OF AROMATASE (CYP19) AND THE ACTIVATION OF THE ESTROGEN RECEPTOR ALPHA (HER α) IN HEPG2, H295R, AND CHO-K1-BH4 CELLS. (Under the direction of Dr. James E. Gibson) Department of Biology, April 2007.

Breast cancer patients often consider taking herbal supplements concurrent with prophylactic chemotherapy under the impression that "natural" products will not interfere with the pharmaceutical treatment. The possibility exists that herbal supplements could even counteract chemotherapeutic agents by acting against or antagonizing the effects of selective estrogen receptor modifiers (SERMs) or aromatase inhibitors. The hypothesis is that ginseng, chaste tree berry, flaxseed, garlic, wild yam root, and soybean affect relative potencies, induce aromatase expression and demonstrate estrogenicity when administered at different concentrations to HepG2, H295R, and CHO-K1-BH4 cells. Cells from each of the three lines were treated with varying concentrations of aqueous extract for 72 hours; and IC_{50} values for each extract were determined by comparing cell survival in the treatment group versus the untreated group. This information was used to choose appropriate concentrations of extract for treating the H295R cells and determining whether or not aromatase mRNA levels were increased when compared to the levels in untreated cells. Chaste tree berry, flaxseed, garlic, ginseng, soybean, and wild yam root, did indeed demonstrate varying relative potencies in the three cell lines tested. The extracts in order of decreasing relative potency are garlic, wild yam root, ginseng, and chaste tree berry; flaxseed and soybean had surprisingly little effect on cell survival even at the highest extract concentration tested. Effect of these extracts on aromatase

expression could not be determined based upon qRT-PCR results. A review of the current literature confirms that various extract types and components of all of these herbs, save for garlic, have been shown to bind to or affect activation of human estrogen receptor α. Given the results cited in the contemporary literature, it would seem prudent to advise patients undergoing treatment with SERMs to avoid the use of herbal supplements.

THE EFFECTS OF SIX HERBAL SUPPLEMENTS COMMONLY USED BY WOMEN ON EXPRESSION OF AROMATASE (CYP19) AND THE ACTIVATION OF THE ESTROGEN RECEPTOR ALPHA (HER α) IN HEPG2, H295R, AND CHO-K1-BH4 CELLS

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Darin N. Sujjavanich

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THE EFFECTS OF SIX HERBAL SUPPLEMENTS COMMONLY USED BY WOMEN ON EXPRESSION OF AROMATASE (CYP19) AND THE ACTIVATION OF THE ESTROGEN RECEPTOR ALPHA (HER α) IN HEPG2, H295R, AND CHO-K1-BH4 CELLS

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DEDICATION

To my Mother and Father,

who have always given unwavering

and unconditional love and support.

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Many people, in their acknowledgement sections for their theses, say something to the effect of, "This thesis would not have been possible had it not been for…." There are several people for whom that recognition would certainly be appropriate; however, I feel that those words could never be sufficient to express my sentiments. At best, I can only say that my sincerest thanks go to:

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

I. Herbal supplements

The Dietary Supplement Health and Education Act (DSHEA) of 1994 was a landmark act from Congress that laid out the definitions and guidelines for the sale and distribution of dietary supplements in the United States (US). The DSHEA formal definition of a dietary supplement was also presented. The simplified version of this definition was given in a 1995 review of the act done by the US Food and Drug Administration (FDA); in it, the FDA avers that a dietary supplement:

- is a product (other than tobacco) that is intended to supplement the diet that bears or contains one or more of the following dietary ingredients: a vitamin, a mineral, a herb or other botanical, an amino acid, a dietary substance for use by man to supplement the diet by increasing the total daily intake, or a concentrate, metabolite, constituent, extract, or combinations of these ingredients.
- is intended for ingestion in pill, capsule, tablet, or liquid form.
- is not represented for use as a conventional food or as the sole item of a meal or diet.
- is labeled as a "dietary supplement."
- includes products such as an approved new drug, certified antibiotic, or licensed biologic that was marketed as a dietary supplement or food before approval, certification, or license (unless the Secretary of Health and Human Services waives this provision).

http://www.cfsan.fda.gov/~dms/dietsupp.html.

The DSHEA also formally recognized that millions of Americans are taking herbal supplements and that the industry was a growing one with profits in the billions of dollars. The responsibility for ensuring safety of the dietary supplements fell to the FDA after the supplements were on the market (United States Congress, 1994; United States Food and Drug Administration, 2004a).

While there are provisions for "New Dietary Ingredients" (NDIs), or dietary ingredients introduced to the market after October 15, 1994, there are no legal requirements for efficacy or toxicity testing of dietary supplements by the manufacturer (United States Congress, 1994; United States Food and Drug Administration, 2004a; United States Food and Drug Administration, 2004b). There are, however, many ongoing efforts in the US and in other countries to research and compile information about herbal supplements; most notably in the US. These include the National Center for Complementary and Alternative Medicine (NCCAM), a division of the National Institutes of Health (NIH), an agency focused upon funding and supporting research on both the pharmacology and the clinical effects of dietary supplements.

The absence of any requirement for the supplement manufacturers to provide pharmacological and toxicological data supporting the safety of their supplements is a serious concern and must be addressed in the future (Gibson and Taylor, 2005).

The introduction to Hobbs' 1998 Herbal Remedies for Dummies illustrates the attitude of people in the US towards herbal supplements and pharmaceutical products. In it, it is said:

> "As a primary health care provider, I see many patients who aren't well-served by today's modern health care system – people who are often encouraged to depend on drugs and medical procedures to fix symptoms and conditions without any mention of the personal power they possess to create and maintain health. My experience shows me that people are likely to be healthier, happier, and more successful when they direct their own health care programs. This book gives you safe and effective herbal remedies and other natural means to ease your symptoms and prevent disease." (5)

The use of herbal supplements in the United States has increased dramatically over the past several decades. Survey data confirm that a significant percentage of the population uses herbal supplements to treat acute and chronic medical illness as well as to enhance general health. The average consumer uses herbal supplements to reduce the effects of aging, memory loss, dulling of mental acuity, and menopause. The average consumer may also use herbal supplements to combat insomnia, to increase cardiovascular health, or to prevent the common cold. Many patients, such as those with cancer or AIDS, turn to herbal supplements when their condition becomes terminal. Inexpensive herbal supplements, which are often thought to be safe due to their "natural" origins, are becoming increasingly attractive to a population faced with increasing pharmaceutical expenses and increased media coverage of the negative side effects of those pharmaceuticals (Bent and Ko, 2004).

II. Breast cancer

Sixty percent of all breast cancers diagnosed are classified as Estrogen Receptor Positive. These cancers are characterized by alpha-type estrogen receptors (ERα) on the cancer cells that trigger growth and division cascades when activated. There are two dominant types of chemotherapies for the Estrogen Reeceptor Positive breast cancers: the selective estrogen receptor modifiers (SERMs) and the aromatase inhibitors.

The most widespread therapy for these cancers is Tamoxifen (92-[4-(1,2-diphenylbut-1-enyl) phenoxy]-N,N-dimethyl-ethanamine) which is classified as a SERM. Tamoxifen and other SERMS are competitive antagonists with estrogen for binding on the ERα,

preventing estrogen from activating the receptor and triggering cell growth (Harwood, 2004). In 2002, Young H. Ju et al. found that the soy isoflavone genistein negated the beneficial antagonistic effects of Tamoxifen. Although genistein has a lower affinity for the ERα than does Tamoxifen, its action as a weak agonist at concentrations comparable to those for normal consumption is enough to activate the ERα and cause subsequent upregulation of growth-related gene expression. This discovery raises grave concerns about not only dietary supplement consumption but also normal dietary intake of soy products during conventional chemotherapy (Ju et al., 2002).

Research concerning phytoestrogens, or plant-derived chemicals resembling estrogen, is increasing rapidly. Products like soy, a well known source of phytoestrogens, are promoted extensively in newspapers and magazines as an alternative to hormone therapies. Despite the increasing public interest in the use of phytoestrogens, the bioactivities of most phytoestrogens are still unknown, and it is very clear that these compounds do not necessarily act as estrogen does. The sites of action or lack thereof remain a mystery; furthermore whether or not a SERM and a phytoestrogen, either weak or strong, might react together has yet to be explained.

The other predominant category of chemotherapies used for estrogen responsive positive breast cancers are the aromatase inhibitors. Aromatase is the cytochrome P450 enzyme (CYP19) that catalyzes the end reaction during estrogen synthesis by converting androstenedione into estrogen. Use of an aromatase inhibitor is thought to be an effective chemotherapy as it decreases estrogen levels while demonstrating no partial agonist

effects on the estrogen receptors. The most popular aromatase inhibitor currently being used is anastrozole (2-[3-(1-cyano-1-methyl-ethyl)-5-(1H-1,2,4-triazol-1-ylmethyl) phenyl]-2-methyl- propanenitrile) (Simpson et al., 2002; Harwood, 2004).

These drugs are so efficacious in treatment of breast cancer that one clinical trial ended early in order to allow the placebo patients to receive the aromatase inhibitor treatment as well (Twombly, 2003). It has been found that the chloro-*s*-triazene herbicides and their metabolites will induce aromatase activity (J. T. Sanderson et al., 2001; J. T. Sanderson et al., 2004). Flavones, isoflavones, and phytoestrogens also have been shown to have varied effects on aromatase expression, ranging from induction to inhibition.

Do any other dietary supplements share this ability to upregulate or otherwise alter activity of aromatase? Whether synthetic or botanical in origin, any chemical that upregulates aromatase could potentially negate the action of the aromatase inhibitors. There are many known drugs which alter expression of the cytochrome enzymes and cause changes in prescription drug metabolism, and it is becoming more and more apparent that several dietary constituents alter cytochrome P450 enzyme activity, including bergamottin, a furanocoumarin found in grapefruit juice and isothiocyanates, found in cabbage and cauliflower (Zhou et al., 2004). While the effects of dietary supplements on aromatase activity and the effects of herbal active ingredients on aromatase expression are being researched, the research concerning the ability of dietary supplements themselves to affect aromatase expression is just beginning.

III. Purpose

The purpose of the present research was to investigate selected toxic endpoints for six herbal supplements commonly used by women with breast cancer: *Panax ginseng* (Ginseng), *Vitex agnus-castus* L. (Chaste Tree Berry), *Linum usitatissimum* (Flaxseed), *Allium sativum* L. (Garlic), *Discorea villosa* (Wild Yam), and *Glycine max* (Soy). These herbs were tested in three cell lines, HepG2, H295R, and CHO-K1-BH4 cells to analyze their relative potencies and their effect on aromatase induction. Information from the existing literature was used to evaluate the estrogenicity of each herb as well.

The hypothesis was that chaste tree berry, flaxseed, garlic, ginseng, soy, or wild yam root affect relative potencies, induce aromatase and demonstrate estrogenicity when administered at different concentrations to CHO-K1-BH4, HepG2, and H295R cells. The null hypothesis was that chaste tree berry, flaxseed, garlic, ginseng, soy, or wild yam root would not elicit these effects when administered at different concentrations to the CHO-K1-BH4, HepG2, and H295R cell lines.

The results of this research will be useful in further testing of these extracts' effects on these cell lines, and the demonstrated concentrations inhibiting 50 percent of cell survival (cytotoxic IC_{50}s) are directly translatable into treatment levels of these extracts in these cell lines in future studies.

These studies planned to focus upon two human cell lines, the HepG2 (Human hepatocarcinoma) line and the H295R (Human adrenocortical carcinoma) line and one rodent cell line, the CHO-K1-BH4 cell (Chinese Hamster Ovary Cells).

The HepG2 line is one of the most widely used cell types for estrogenicity assays. These cells are easily transfected with estrogen and steroid receptors, and they are employed by many labs for this purpose (Gaido et al., 2000).

The H295R line is often used for aromatase expression and activity assays. In 2004, Heneweer et al. studied these cells to gauge their suitability for *in vitro* testing of different agents on aromatase; they found that H295R cells had the appropriate aromatase promoter regions that could be utilized for these assays.

The CHO-K1-BH4 line is a preeminent line for doing initial toxicity screens. The CHO-K1-BH4 line consists of epithelial cells with twin female chromosomes and requires proline in the maintenance media due to a lack of endogenous production. This line is also commonly employed in genetic studies (Kao and Puck, 1967).

Herbs are usually sold as either the raw plant or extracts of portions of the plant. Extracted herbs are boiled in water, alcohol, or other solvents to free the supposed biologically active constituents of the herb. This liquid may be further boiled or dried to produce more concentrated substances. Because herbal supplements are considered to be dietary supplements, the industry is largely unregulated, and purity, quality, and concentrations of the ingredients are left to the manufacturer (Bent and Ko, 2004; Gibson and Taylor, 2005).

One major obstacle to understanding the benefits and risks of herbs is that herbal products are very complex by nature. The raw herb may contain complicated mixtures of

organic chemicals, including terpenes, sterols, alkaloids, saponins, glycosides, tannins, fatty acids, and flavonoids. It is very difficult to determine which of these chemicals, if any, is biologically active in humans. It is also often unknown whether or not the effect of the supplement is due to one active constituent or a combination of many constituents at different concentrations. Varying the growth conditions to which these plants are exposed, such as soil type, humidity, daylight, rainfall, dew, and frost, may also affect concentrations of constituents even among batches of herb grown at one location (Kruger and Mann, 2003; Bent and Ko, 2004).

MATERIALS AND METHODS

I. Herbal supplement extract preparation

The protocol for the extraction of each herb tested was based upon that used by Charles et al. (2002) with minor modifications. Dried but otherwise unprocessed herbs (wildcrafted) were obtained from Blessed Herbs, Inc. in Oakham, MA. Dried soybeans, without pods, were obtained from an Asian market. These soybeans, grown in the US, were commercially packaged by the K.L.Y. Trading Company, Inc. in San Francisco, CA.

Upon receipt of the various herbs from Blessed Herbs, they were immediately stored in the 4 °C refrigerator. The soybeans were also stored at 4 °C.

Each of these dried herbs was ground into a fine powder (particles no larger than 1 mm diameter) using a mechanical mill. The herbs, at a concentration of 50 mg/mL, were placed in Biologix conical-bottom 50 mL centrifuge tubes containing 30 mL of HepG2, H295R or CHO maintenance media and were positioned horizontally in a Dubnoff metabolic shaker/incubator (GCA/Precision Scientific) at shaker speed 5 for 24 hours.

Garlic juice was collected from unpeeled, fresh garlic cloves obtained from a local market and diluted 1:6 into the appropriate cell media. The supplements and media were spun at 5 x 10^3 rpm in a Centra CL2 centrifuge with a fixed-angle (801) 12.1 cm radius rotor (International Equipment Company) for 15 minutes. The resulting supernatant was decanted into a 500 mL bottle top filter (0.22 μm polyethersulfone) and filtered into a

sterile 125 mL bottle. The contents of the bottle were placed back into the centrifuge, spun again for 15 minutes, and the supernatant was filter-sterilized into new sterile bottles. Extracts were stored at 4 °C.

The dried herbs were each very different in texture and hardness. The dried soybeans, flaxseed, and chaste tree berry were easily ground to a fine powder in a mechanical mill.

The wild yam root and the ginseng, however, were too large to put directly into the mechanical mill. They were cut down to a usable size and put into a mill for grinding. The ginseng ground easily into a usable powder. A mortar and pestle was used to crush a small amount of Yam root before milling the product into a usable form.

After 24 hours of shaking 50 mL of media and 2.5 grams of dried herbal powder in the lab shaker, the extracts were all thick, slurry, and opaque. The cell media was no longer pink but took on a color consistent with the nature and color of the herbal product. After centrifuging in the 50 mL conical-bottom tubes, the resulting opaque supernatants were pipetted from the top of the tubes into clean tubes. The flaxseed suspension, even after centrifuging for the same amount of time and at the same force as the other suspensions were processed, maintained the properties of a suspension with no identifiable layer to decant. Thus, the flaxseed was used with all of the solid flax suspended in the liquid.

Due to size and amount of debris secreted by the cells during the course of treatment the HepG2 and H295R cells were unable to be counted by the Beckman-Coulter counter and were counted manually by hemocytometer.

II. Cell culture

H295R cells (adrenocarcinoma cells) and HepG2 cells (hepatocarcinoma cells) were obtained from the American Type Culture Collection (ATCC). H295R cells were maintained in 1:1 (v/v) Dulbecco's Modified Eagle's Medium/ Ham's F-12 nutrient mixture with L-glutamine, HEPES, and sodium bicarbonate (GibcoBRL). Added to this solution were 25 µL/mL NuSerum™ (BD Biosciences), 10 µL/mL ITS+™ premix (BD Biosciences), 10 µL/mL 100 U/L penicillin/100 µg/L streptomycin (GibcoBRL), and 55 µg/mL sodium pyruvate.

The HepG2 cells were grown in Eagle's MEM with Earle's salts and nonessential amino acids, 10 µL/mL 200 mM L-glutamine, 110 ng/mL sodium pyruvate, 3µL/mL 10,000U/L nystatin, 10 µL/mL 100 U/L Penicillin/100 µg/L Streptomycin, and 10% fetal bovine serum. The cells were cultured in at 37 °C with 5% CO_2 and 95% humidity as constant conditions. Media was changed twice per week, and the cells were passed once per week.

CHO-K_1-BH_4 cells were originally obtained from Dr. Abraham Hsie, Oak Ridge National Laboratory, Oak Ridge, TN, USA. These cells were maintained and grown in Ham's/F12 nutrient mixture 1 x with 5% Fetal Calf serum supplemented with 100U/mL penicillin-streptomycin, 2.5 µg/mL Fungizone, and 2.5 µg/mL Amphotericin B. The CHO cells were passed every 3 days.

III. Cytotoxicity assay

The protocol was modeled on that used by Charles et al. (2002) with minor modifications. CHO-K1-BH4 cells in logarithmic growth phase were trypsinized and transferred at a density of 4.0 x 10^4 cells/ml into 6-well plates (2 mL/well) approximately 24 hours prior to treatment. At the time of treatment, the culture medium was replaced with dilutions of the test extracts in 2 mL of appropriate medium. Cultures were treated in duplicate wells with the herb extracts for 48 hours. At the end of the treatment, surviving cells were counted with Coulter Counter.

The H295R and HepG2 cells proved to grow very slowly, reaching 80% confluence significantly later than the period of time ATCC quoted in their protocol. Because of this slow turnover rate, the treatment periods for the HepG2 and H295R cell lines were increased from 48 to 72 hours in order to allow for adequate response to treatment. Cell seeding was also increased from the levels used in the CHO cells. HepG2 cells were seeded in 6-well plates at a concentration of 7.5 x 10^5 cells/well, and H295R cells were seeded in 6-well plates at a concentration of 1 x 10^6 cells/well.

Concentrations of supplement extracts ranging from 0-10% were initially tested in both cell lines, and concentrations for subsequent replicates were determined by using previous experimental results. Ethanol was the positive control as in many cytotoxicity studies, and the 0% treatment group served as the negative control. After 72 hours, the cells were trypsinized with 0.25% trypsin/0.02% EDTA and counted manually with hemocytometer. Cytotoxic IC_{50}s were determined based on the numbers of cells present

after the 72 hour treatment period for each concentration of extract as compared to the appropriate 0% treatment groups. Means, standard errors, and coefficients of variation were calculated. The concentration of the herb inhibiting survival of 50% of the cells was determined to be the cytotoxic IC_{50} value.

IV. Aromatase upregulation assay

The expression of aromatase was determined by an assay based on the method of Sanderson et al. (2001) with minor modifications. The H295R cells were cultured, trypsinized, and isolated. Cells were seeded at 1.0×10^6 cells/well in 6-well plates. Each 6-well plate had 1 untreated well and 5 identical treatment wells. Exposure to all treatments was for 72 hours.

Treatment concentrations of extracts for these experiments were:

1. Chaste Tree Berry 1.0%
2. Flaxseed 3.0%
3. Soybean 3.0%
4. Garlic 0.3%
5. Ginseng 0.2%
6. Wild Yam Root 0.1%

These treatment levels were chosen based upon the results of the IC_{50} experiments. In order to have some effect on the H295R cells while allowing for enough of those cells to survive for RNA extraction, the approximate IC_{20} values were used.

RNA was isolated using an AquaPure® RNA Isolation Kit (Bio-Rad®). Cells were harvested by aspirating the media and applying 500 µL 0.25% trypsin/0.02% EDTA to each well and placing in an incubator for 15 minutes. After this time, 1 mL of appropriate cell media was added to stop the trypsinization process. The plates were gently tapped to loosen cells, and a cell scraper was gently brushed across the bottom of each well. Cell suspensions were collected in 15 mL conical-bottom centrifuge tubes, and the tubes were spun for 5 minutes at 3.4 x 10^3 x g in a Centrific Centrifuge (Fisher Sciences). Most of the supernatant was aspirated off, leaving approximately 1 mL of supernatant and the cell pellet. The cells were resuspended and transferred to sterile 1.5 mL microcentrifuge tubes.

The cells were centrifuged at 1.6 x 10^4 x g for 5 seconds in an IEC Microlite RF Refrigerated Microcentrifuge (Thermo Electron Corporation). The supernatant was removed with a 200 µL pipet leaving behind the cell pellet and 20 µL liquid. The microcentrifuge tubes were vortexed for 30 seconds to ensure complete suspension.

The AquaPure® RNA Isolation Kit (Bio-Rad®) was used in order to isolate RNA. 300 µL RNA Lysis solution was added to the cell suspension and pipetted 3 times to lyse cells. 100 µL Protein-DNA Precipitation Solution was added, and the tube was inverted gently 10 times and placed in an ice bath for 5 minutes. The tubes were centrifuged at 1.6 x 10^5 x g for 3 minutes at room temperature. The resulting supernatant was carefully pipetted into a clean microfuge tube with 300 µL 100% molecular-grade isopropanol in order to precipitate the RNA. The resulting product was mixed 30 times by gentle

inversion. The tubes were centrifuged at 1.6 x 10^5 x g for 1 minute. The supernatant was poured off, and the tube was drained briefly on absorbent paper. 300 µL 70% ethanol was added to the tube, and the tube was inverted 5 times to wash the RNA pellet. After centrifugation at 1.6 x 10^5 x g for 1 minute, the ethanol was carefully poured off, the tube was inverted and drained on absorbent paper and allowed to dry for 10 minutes.

50 µL RNA Hydration Solution was added to the tube. RNA was stored at this point in the protocol at -85 °C until needed. Otherwise, the RNA was allowed to rehydrate for 30 minutes on ice. The tubes were vortexed for 5 minutes, pulse spun, and pipetted up and down several times to insure mixing.

RNA purity was checked by characterization of total RNA samples. A NanoDrop 1000 spectrophotometer was used to measure the 260 nm to 280 nm ratio and determine the concentration of RNA in each sample.

qRT-PCR was performed, utilizing iScript™ One-Step RT-PCR Kit with SYBR® Green. Primers for cytochrome P450, family 19, subfamily A, polypeptide 1 (aromatase) and β-actin (housekeeping control gene) were used (SuperArray RT^2 PCR Primer Sets). The assay was performed using the manufacturer's suggested protocol. All components of the kit were thawed at room temperature except for iScript™ Reverse Transcriptase, which remained on ice until used. The reactions were 25 uL per well and were set up on ice as follows:

A. Master Mixes were made for each primer.

1. 12.5 µL Reaction Mix (a 2X reaction buffer with 0.4 mM of each dNTP, Magnesium chloride, iTaq DNA polymerase, 20 nM fluorescein, SYBR ® Green I dye, and stabilizers),
2. 0.3 µL of appropriate primer set, and
3. 0.5 µL reverse transcriptase (Optimized 50X iScript MMLV reverse transcriptase for One-Step RT-PCRs).

13.3 µL of the Master Mix was placed into appropriate wells.

B. Microfuge tubes were set up for each reaction. All mixtures were gently thawed and mixed using side flicking and pipeting small volumes of mixture up and down. All RNA samples were mixed into solution before the samples were taken.

Tube 1: 4273.5 ng of RNA from isolates with DEPC-treated water to volume 1 mL.

Tube 2: 10 µL of Tubes 1 into 90 µL DEPC-treated water.

Tube 3: 10 µL of Tubes 2 into 90 µL DEPC-treated water.

Tube 4: 10 µL of Tubes 3 into 90 µL DEPC-treated water.

11.7 µL of the resulting product samples was placed in each well. Each treatment group had 4 dilutions done in triplicate on 1 plate.

After removing any air bubbles caused by pipeting, the plate was placed into a Bio-Rad® iCycler™. The following protocol was used based upon recommendations by the PCR kit:

cDNA synthesis for 10 minutes at 50 °C

iScript Reverse Transcriptase inactivation for 5 minutes at 95 °C.

PCR cycling and detection, 45 cycles, for 10 seconds at 95 °C and 30 seconds at 57 °C (data collection performed here)

Melt curve analysis was performed as follows:

1 minute at 95 °C

1 minute at 55 °C

10 seconds at 55 °C for 80 cycles, increasing 0.5 °C per cycle.

The plate was kept at 4 °C until it was removed from the thermal cycler. Plates were stored in a -20 °C freezer in case the necessity arose to characterize RT-PCR products. Means, standard errors, and coefficients of variation were calculated. Relative induction of aromatase activity was gauged from the threshold cycle values (Ct values) of the extract at different dilutions versus those of the negative treatment group.

RESULTS

I. Cytotoxic IC_{50} determination

The CHO-K1-BH4 cell experiments were performed first. When the 0-10% extract concentration treatments proved to be too high for Ethanol, Ginseng, and Wild yam root, subsequent plates were made with lower extract concentrations in order to create a graded dose-response curve.

Garlic was not tested due to difficulty with storage of the garlic juice and garlic juice extract.

Figures 1-6 illustrate the results of the CHO cell experiments. All data is presented as a percentage of surviving cells in the treatment group versus number of surviving cells in the negative treatment group. Standard error and coefficients of variation were also calculated, and error bars in the graphs depict standard error. All treatment periods for the CHO cells were 48 hours.

The HepG2 and H295R experiments were performed after the CHO cell results became available. As stated before, the first set of concentrations tested was from 0-10% extract concentration for 72 hours. Later concentrations were adjusted according to the results of initial concentration ranges. Figures 7-11 illustrate the results of the HepG2 experiments, and Figures 12-17 illustrate the results of the H295R experiments. Standard error and coefficients of variation were calculated, and all error bars depict standard errors. Flaxseed extract was not tested in HepG2 cells.

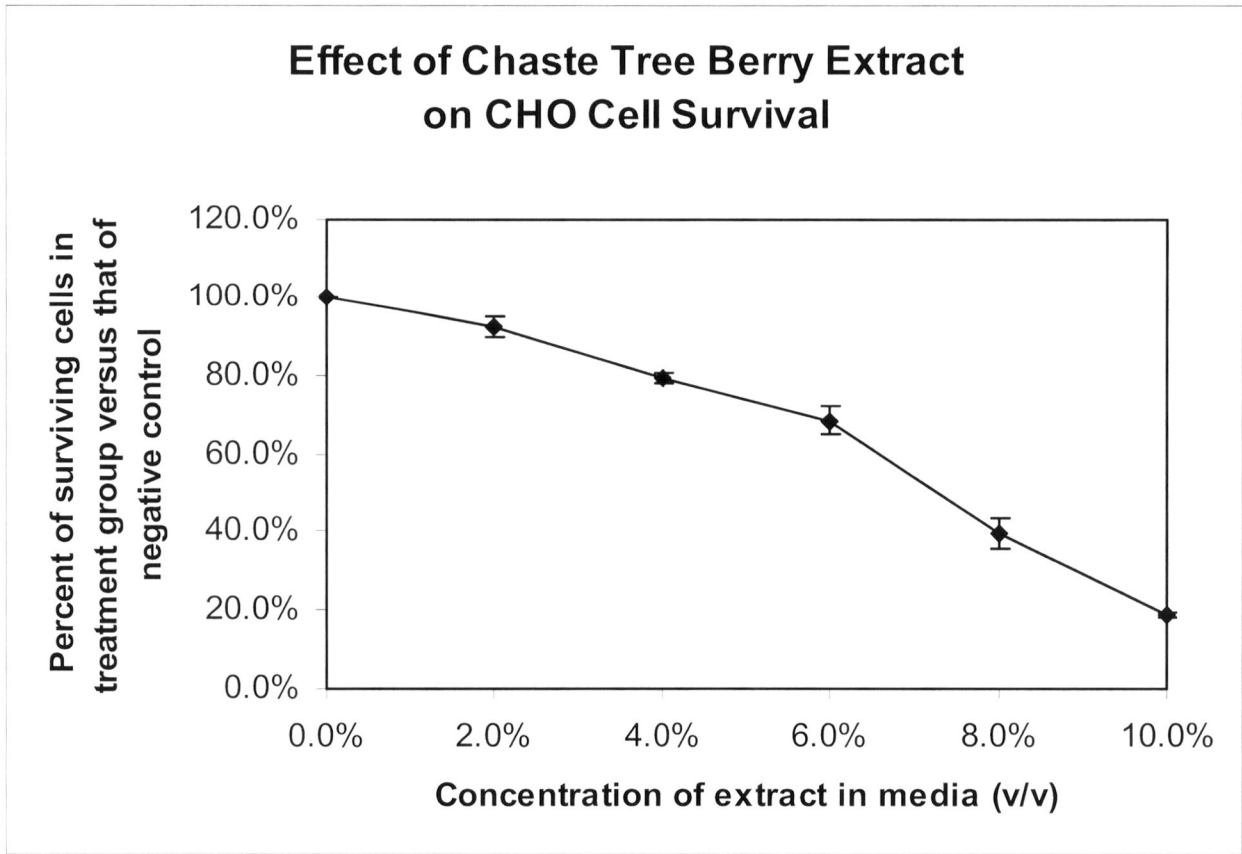

Figure 1. Effect of Chaste Tree Berry on CHO Cell Survival. Treatment period was 48 hours. Error bars represent standard error. n = 4.

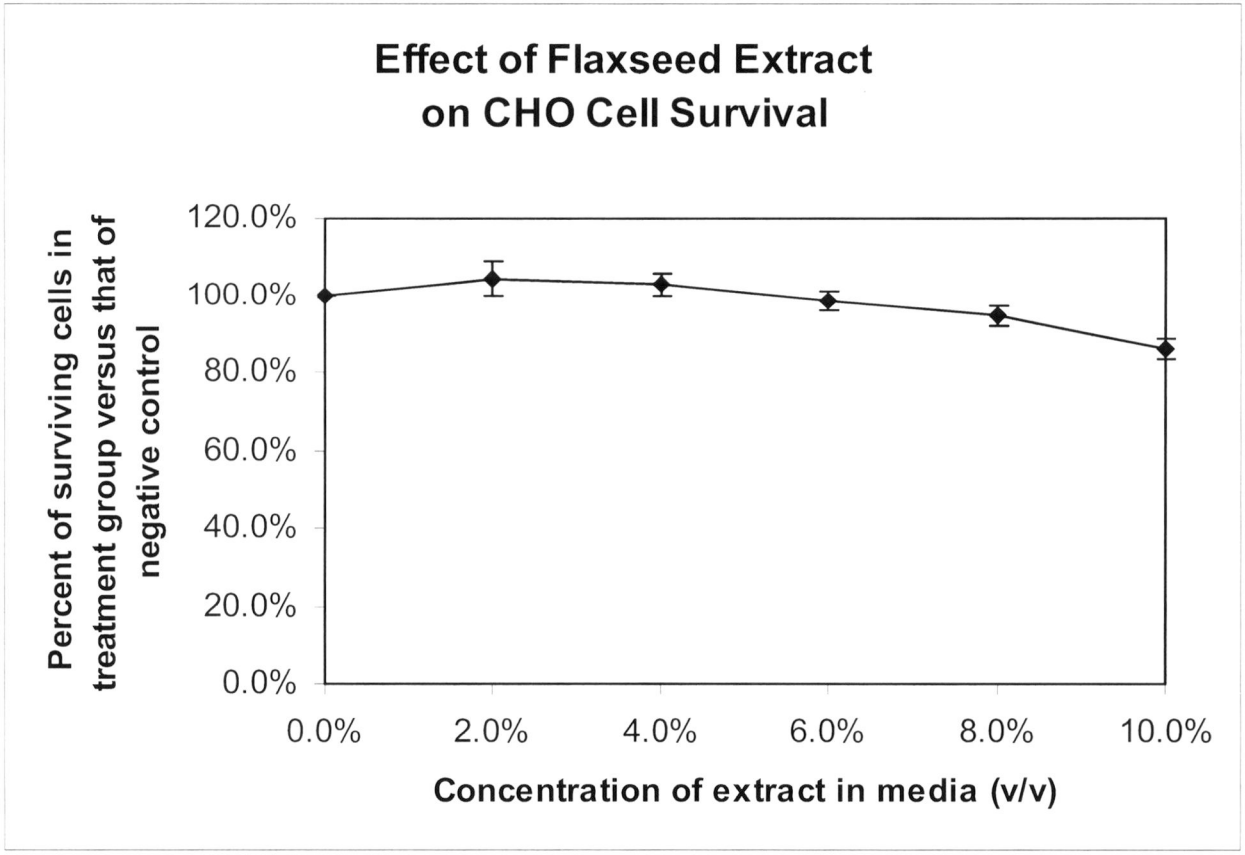

Figure 2. Effect of Flaxseed on CHO Cell Survival. Treatment period was 48 hours. Error bars represent standard error. n = 4.

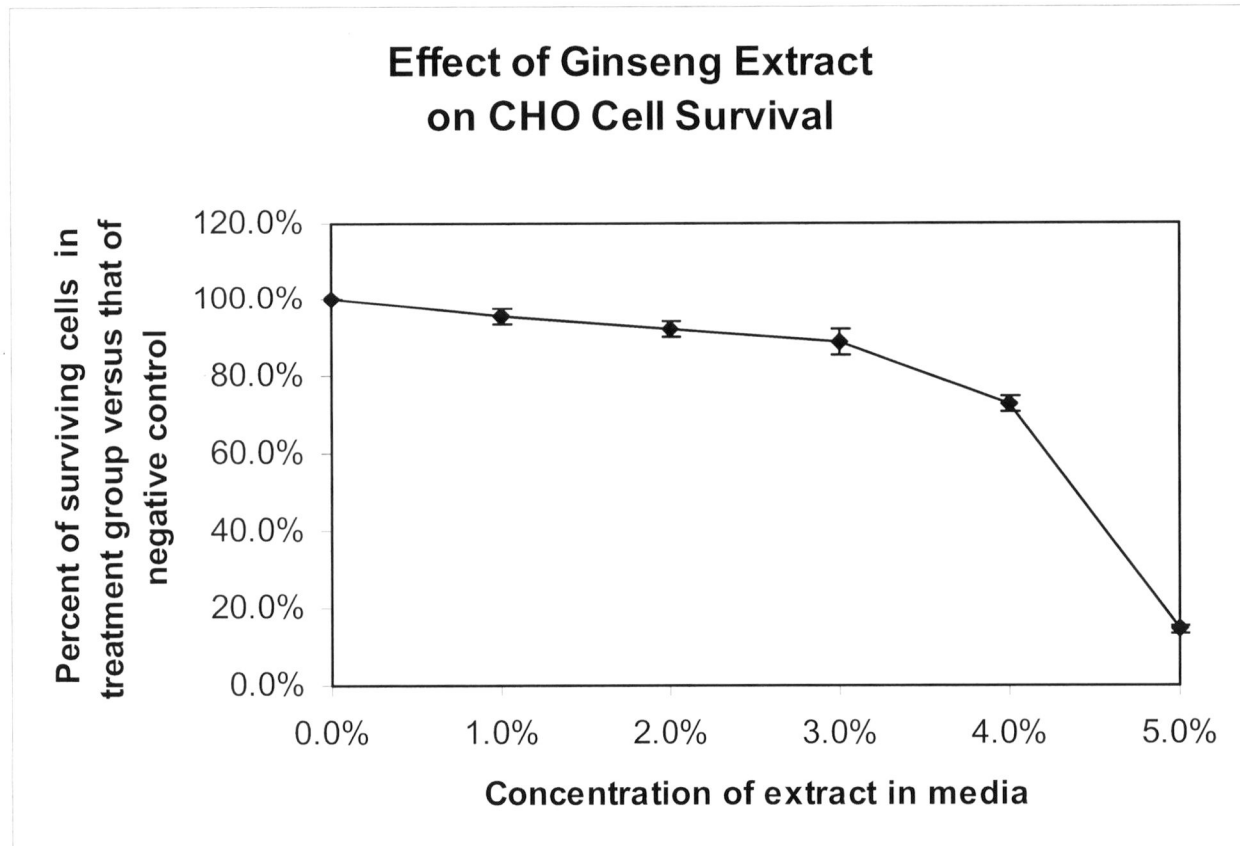

Figure 3. Effect of Ginseng on CHO Cell Survival. Treatment period was 48 hours. Error bars represent standard error. n = 4.

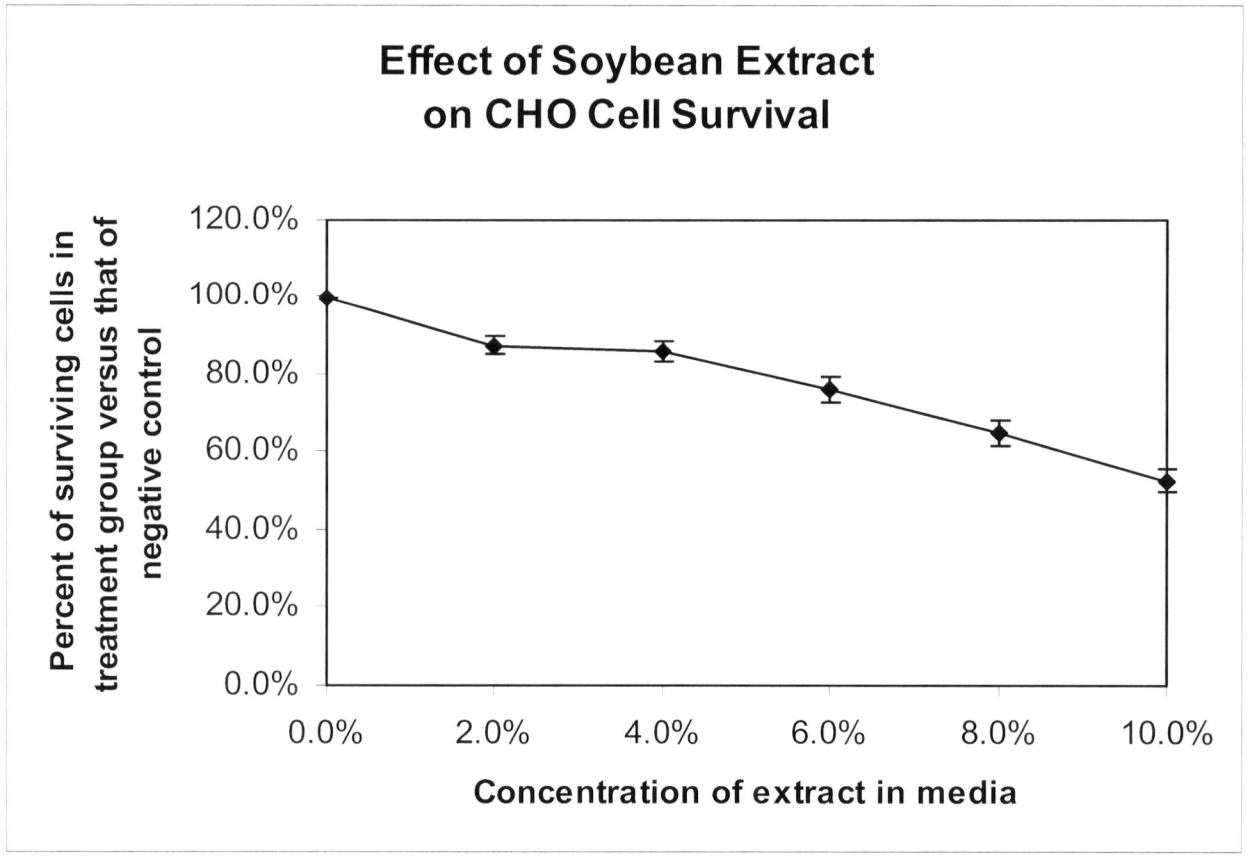

Figure 4. Effect of Soybean on CHO Cell Survival. Treatment period was 48 hours. Error bars represent standard error. n = 4.

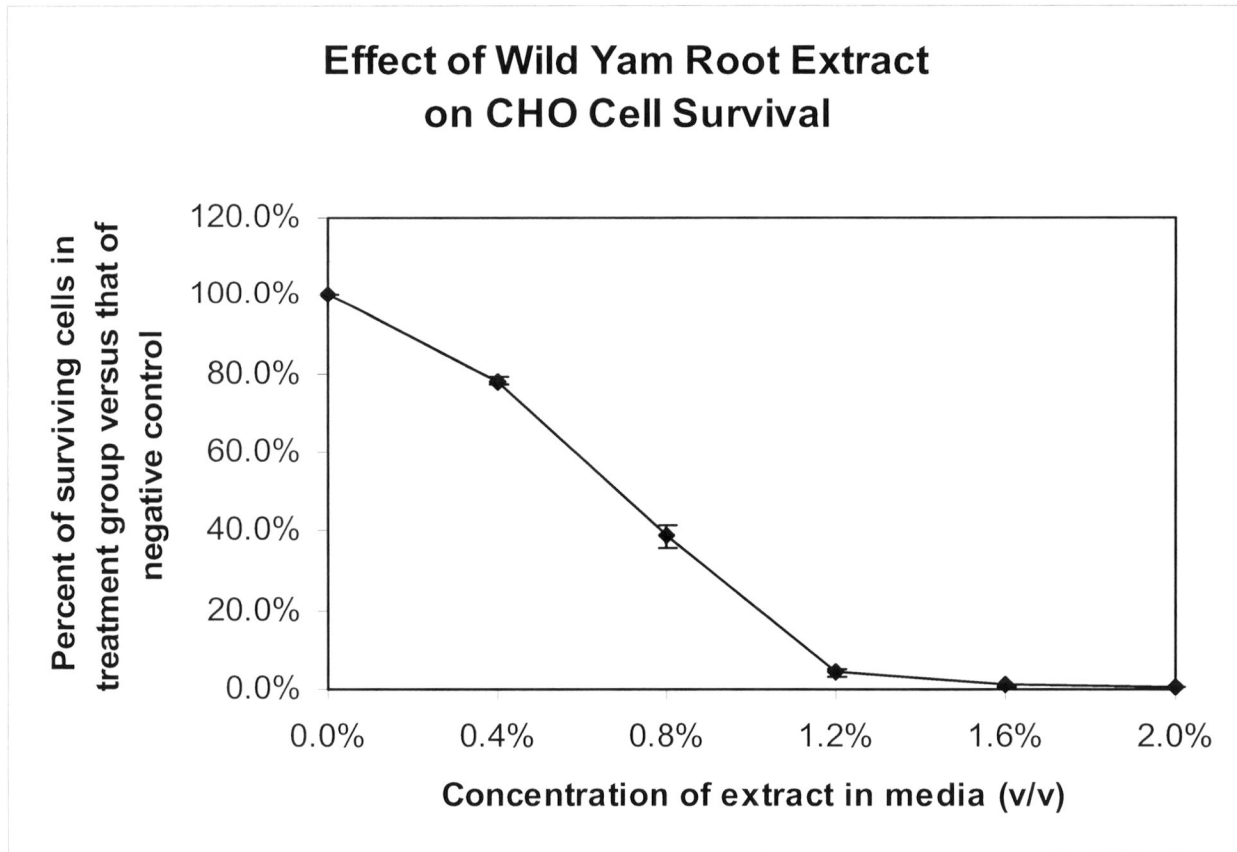

Figure 5. Effect of Wild Yam Root on CHO Cell Survival. Treatment period was 48 hours. Error bars represent standard error. n = 4.

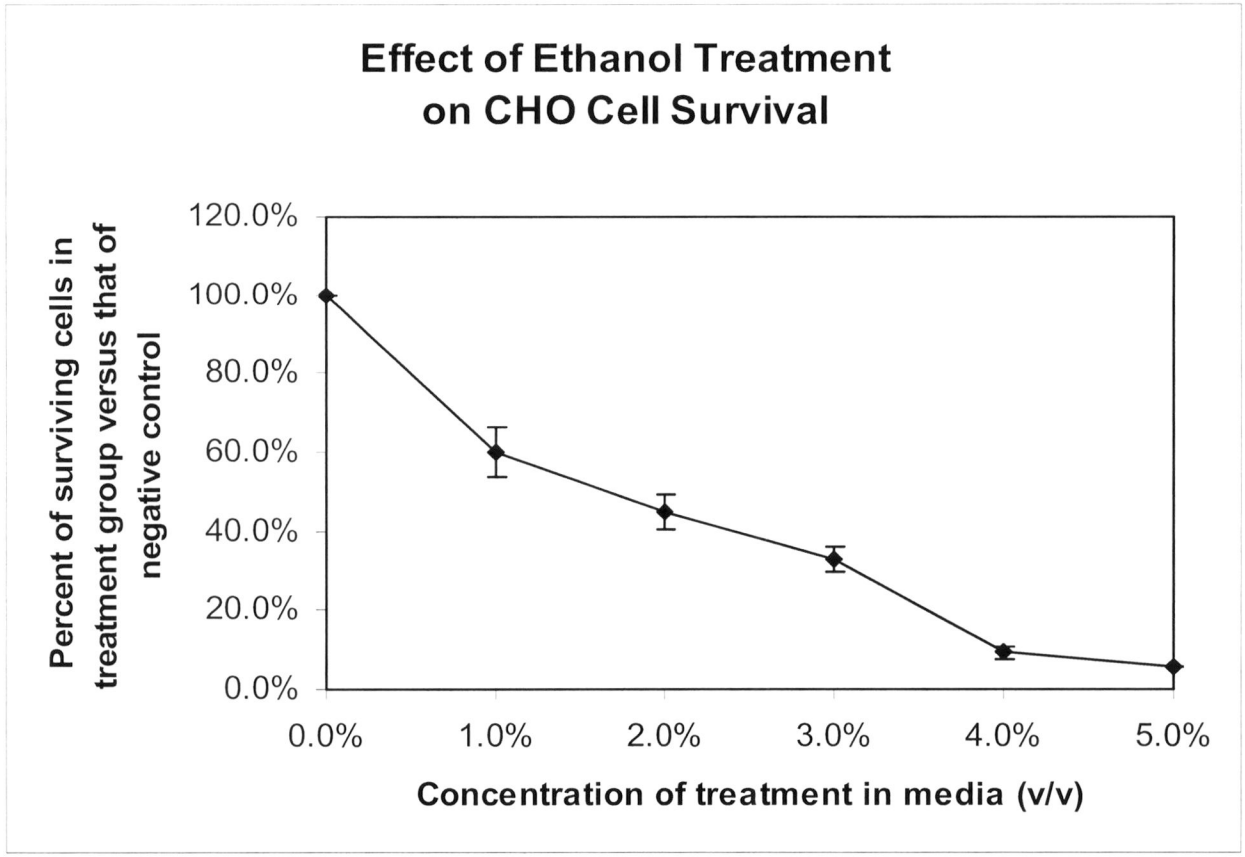

Figure 6. Effect of Ethanol on CHO Cell Survival. Treatment period was 48 hours. Error bars represent standard error. n = 2.

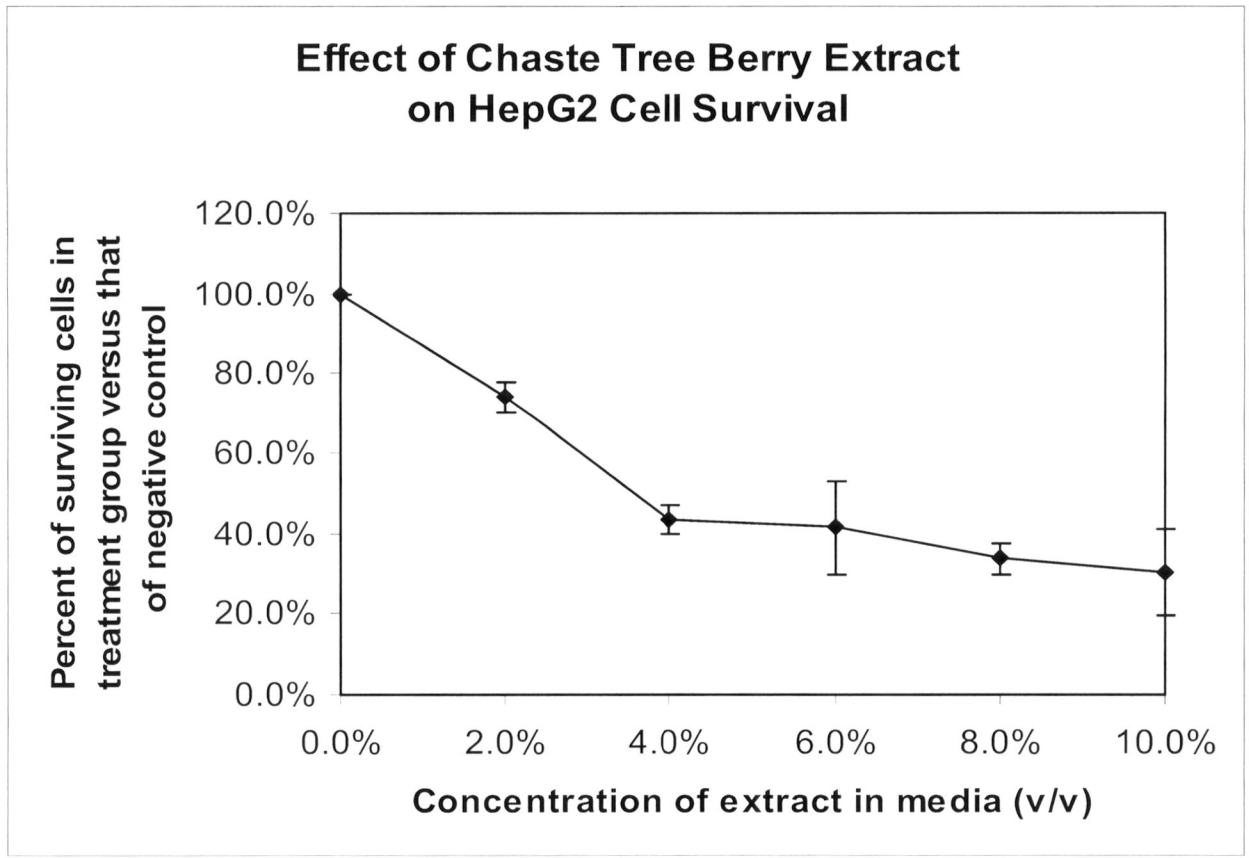

Figure 7. Effect of Chaste Tree Berry on HepG2 Cell Survival. Treatment period was 72 hours. Error bars represent standard error. n = 2.

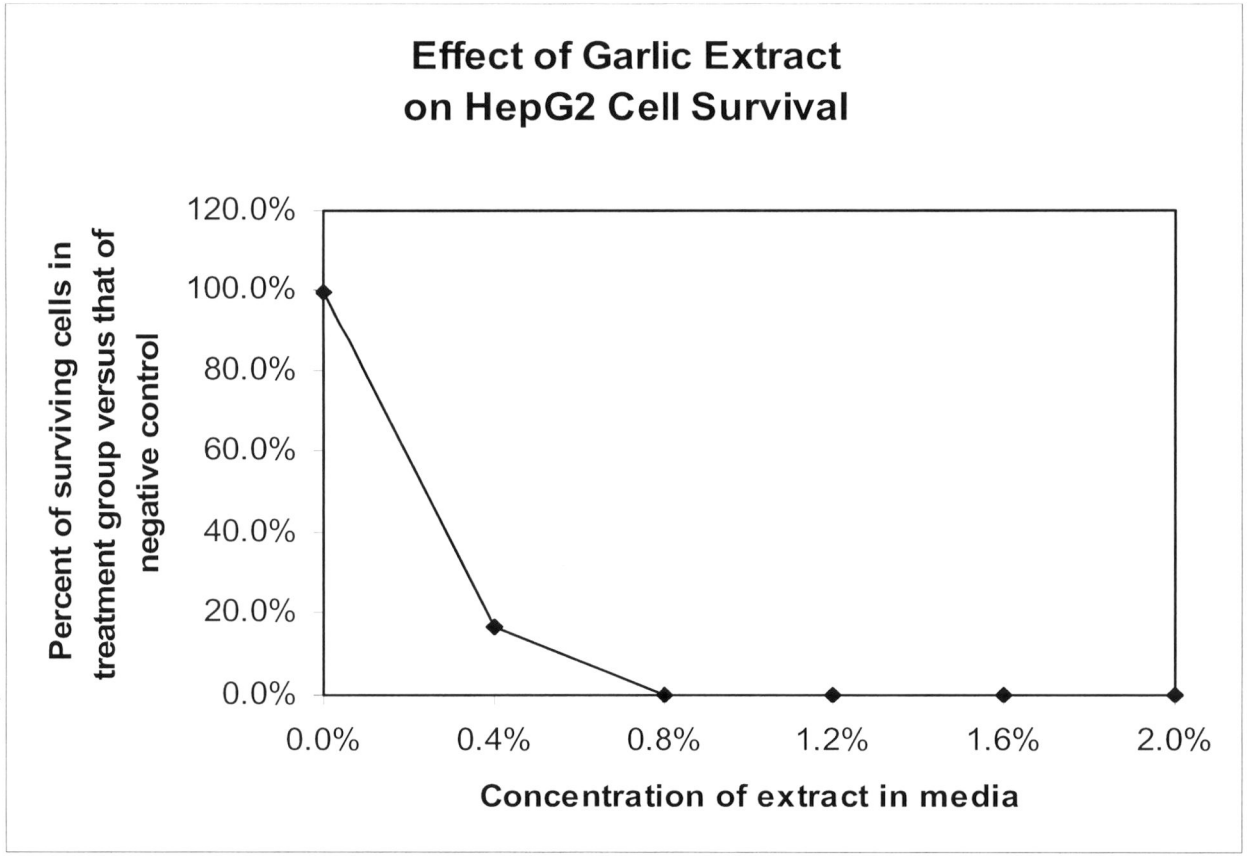

Figure 8a. Effect of Garlic on HepG2 Cell Survival. Treatment period was 72 hours. n = 1.

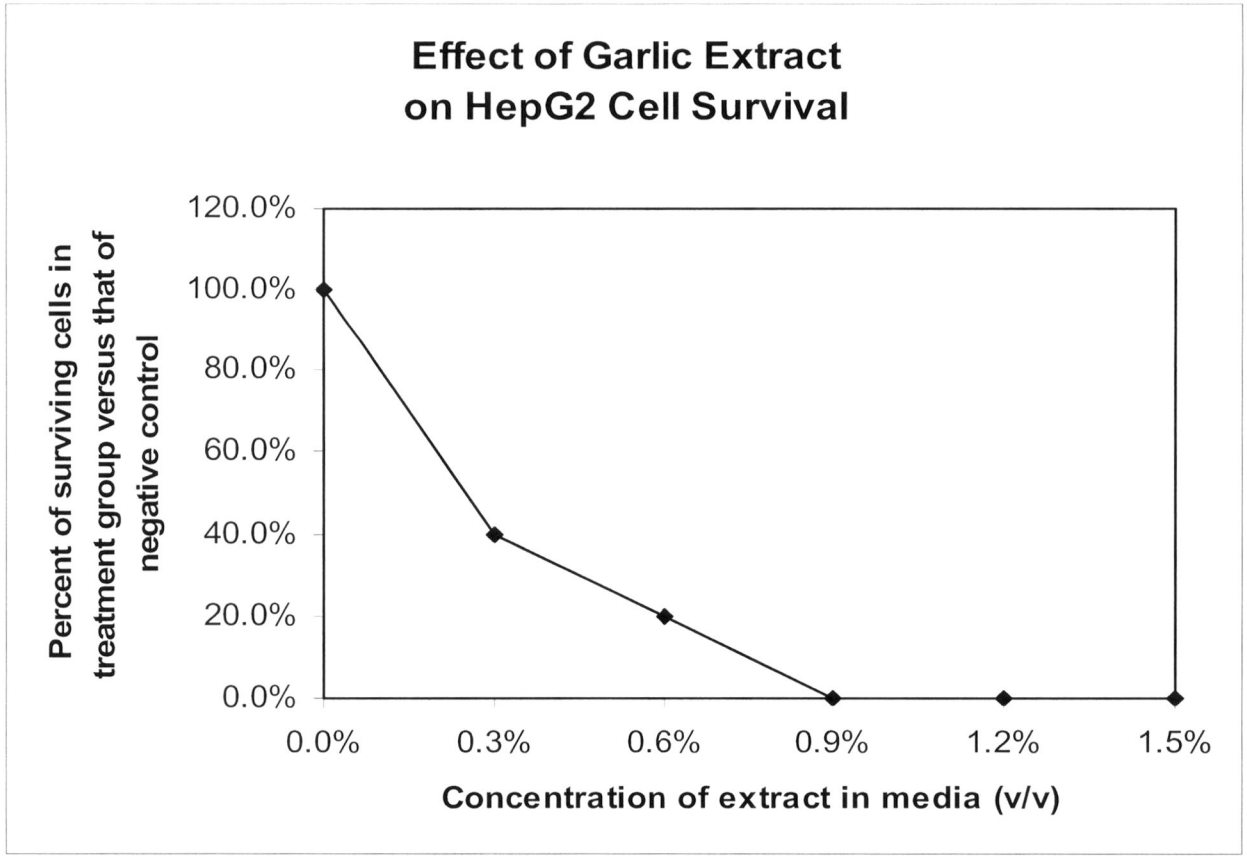

Figure 8b. Effect of Garlic on HepG2 Cell Survival. Treatment period was 72 hours. n = 1.

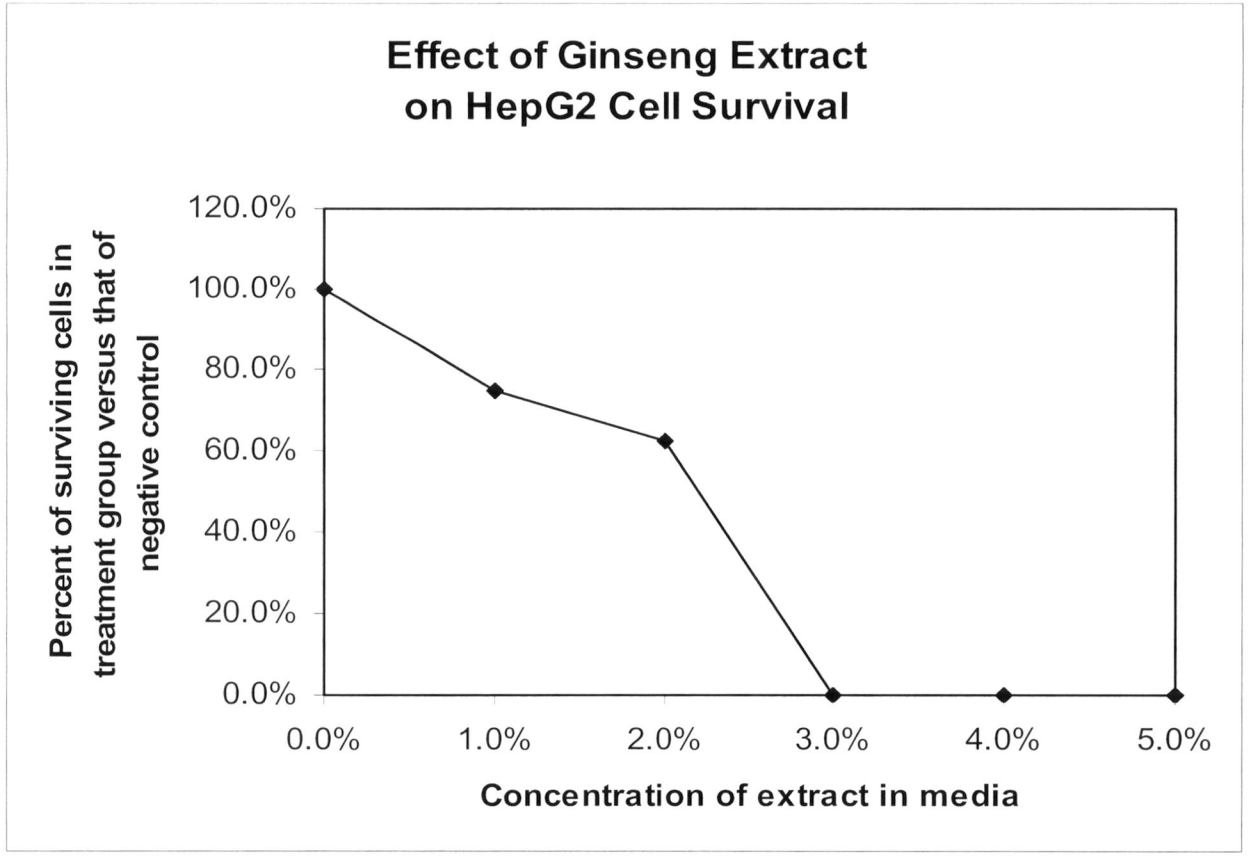

Figure 9. Effect of Ginseng on HepG2 Cell Survival. Treatment period was 72 hours. n = 1.

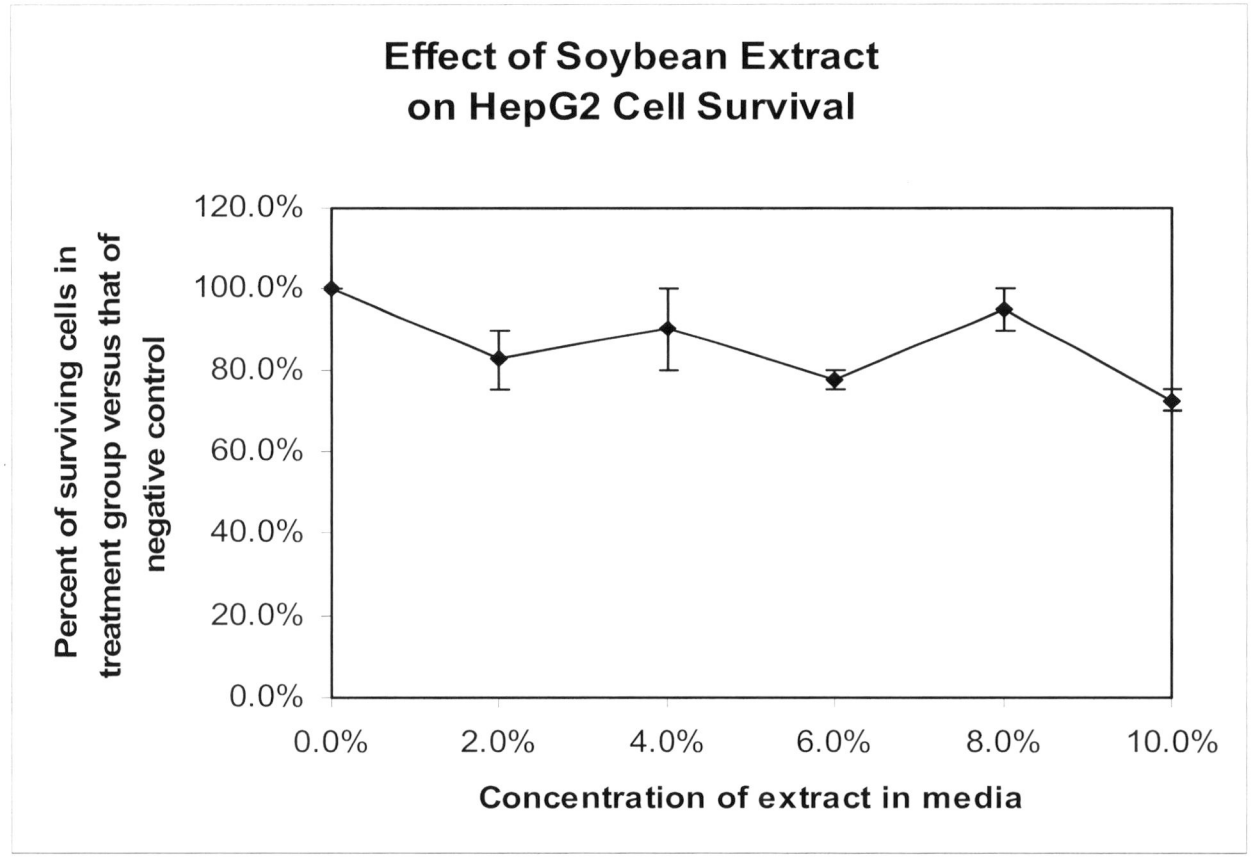

Figure 10. Effect of Soy on HepG2 Cell Survival. Treatment period was 72 hours. Error bars represent standard error. n = 2.

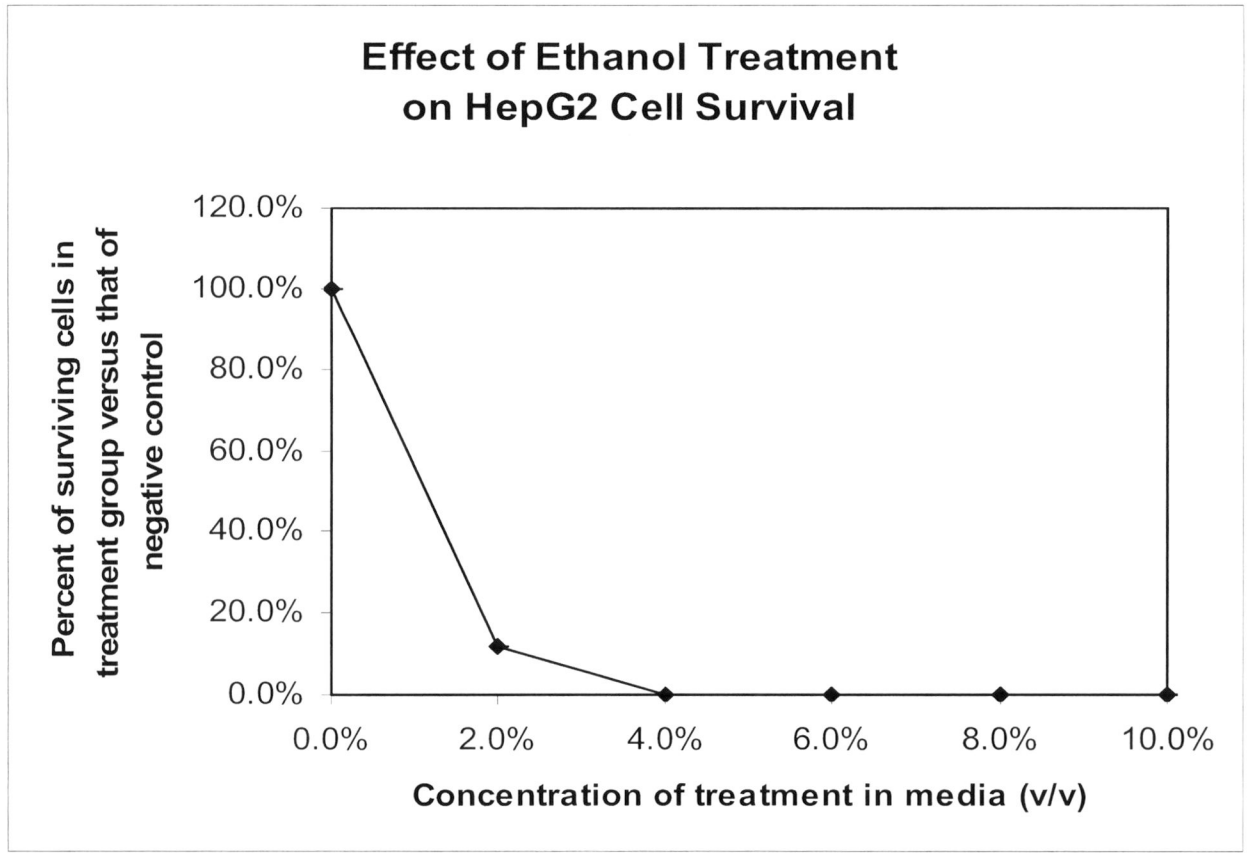

Figure 11. Effect of Ethanol on HepG2 Cell Survival. Treatment period was 72 hours. Standard error were approximately 0. n = 2.

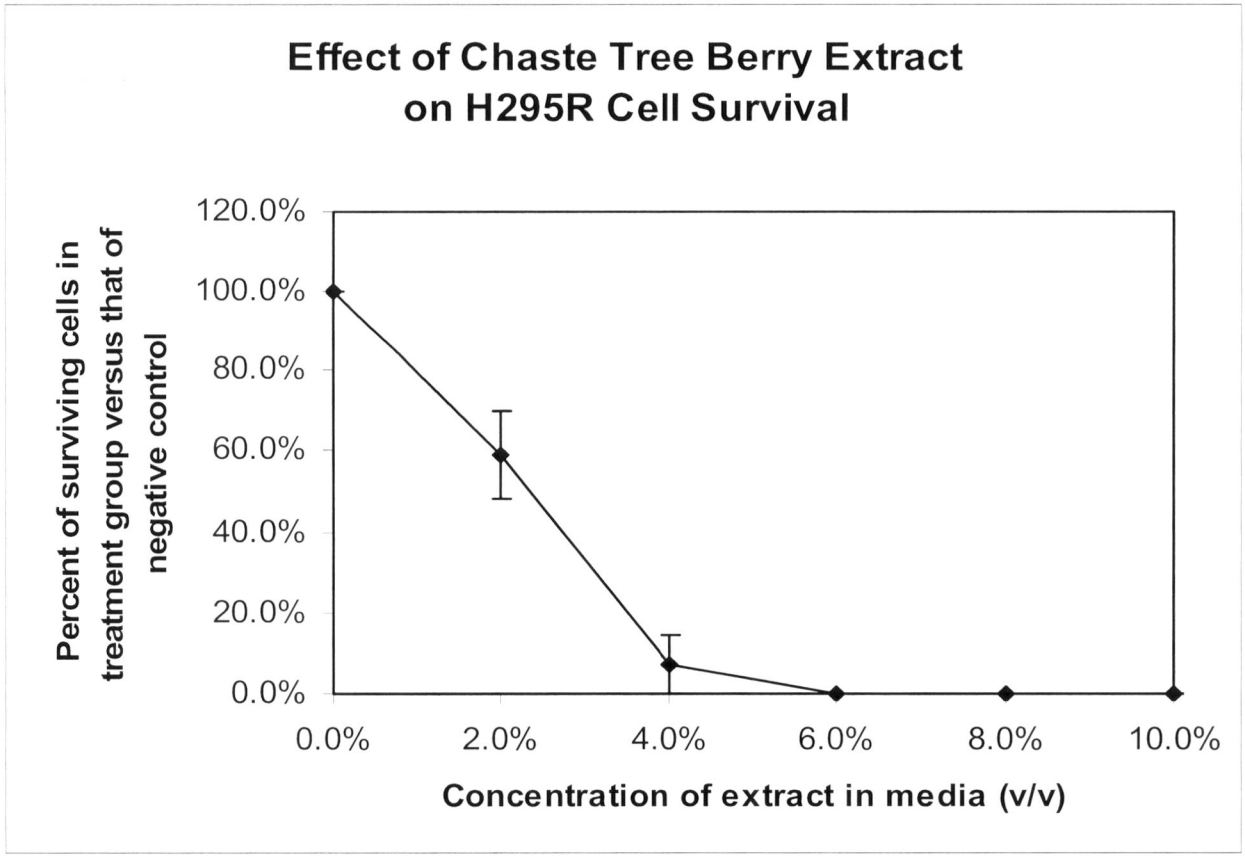

Figure 12a. Effect of Chaste Tree Berry on H295R Cell Survival. Treatment period was 72 hours. Error bars represent standard error. n = 2.

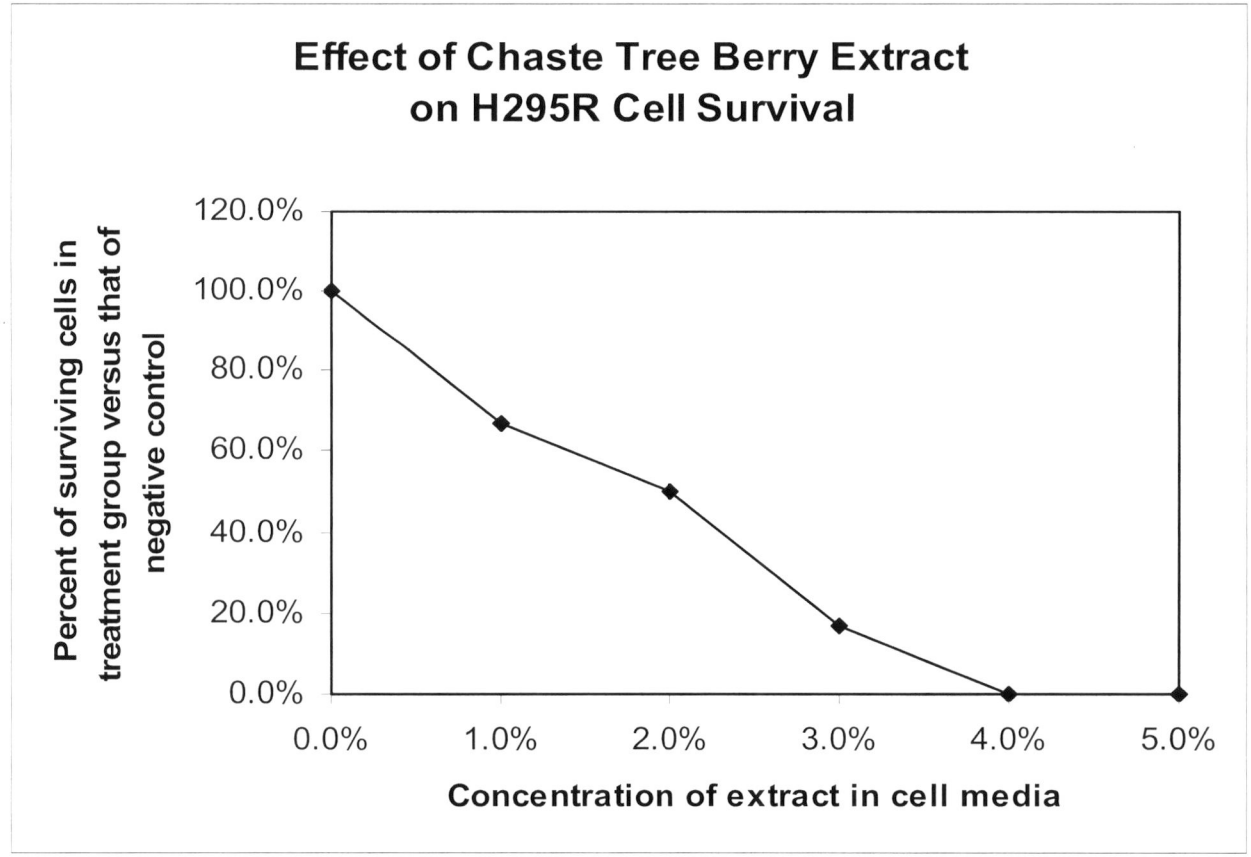

Figure 12b. Effect of Chaste Tree Berry on H295R Cell Survival. Treatment period was 48 hours. n = 1.

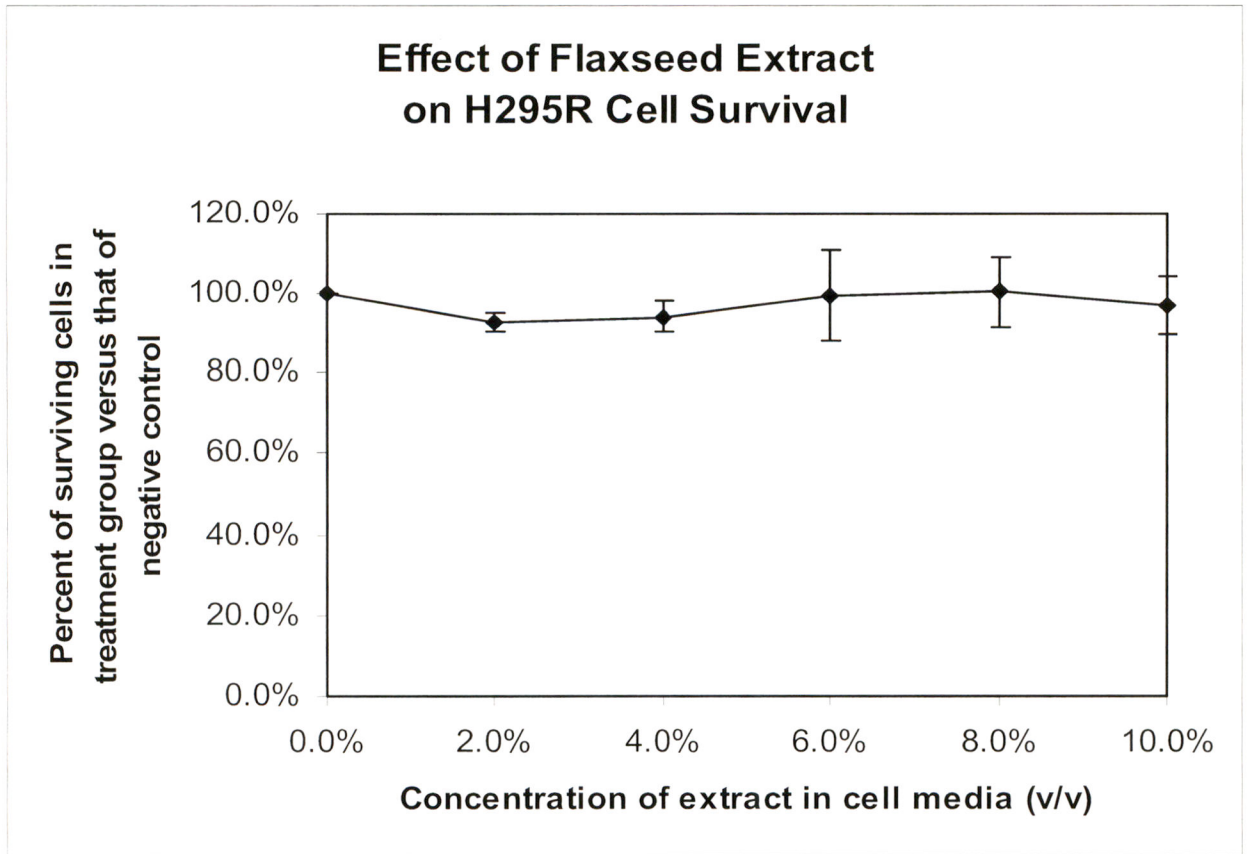

Figure 13. Effect of Flax on H295R Cell Survival. Treatment period was 72 hours. Error bars represent standard error. n = 3.

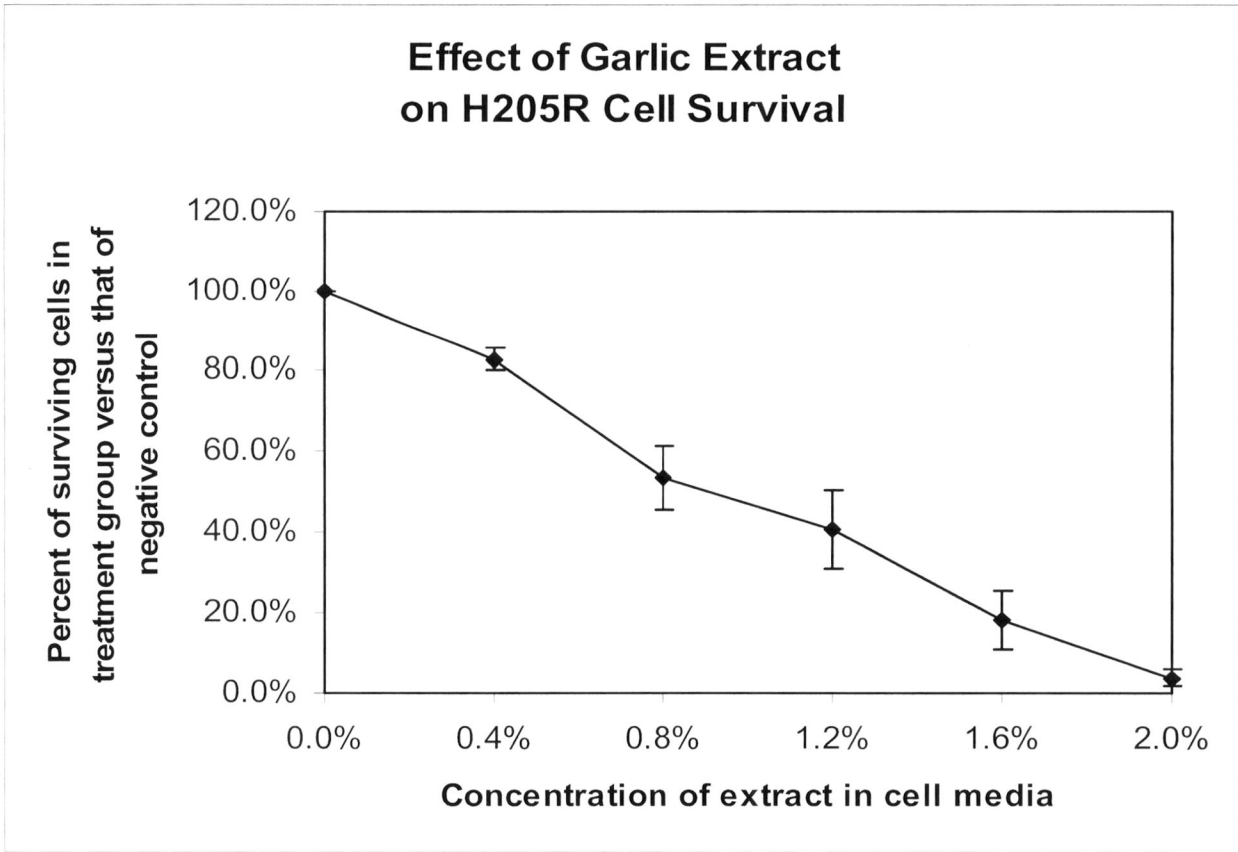

Figure 14. Effect of Garlic on H295R Cell Survival. Treatment period was 72 hours. Error bars represent standard error. n = 4.

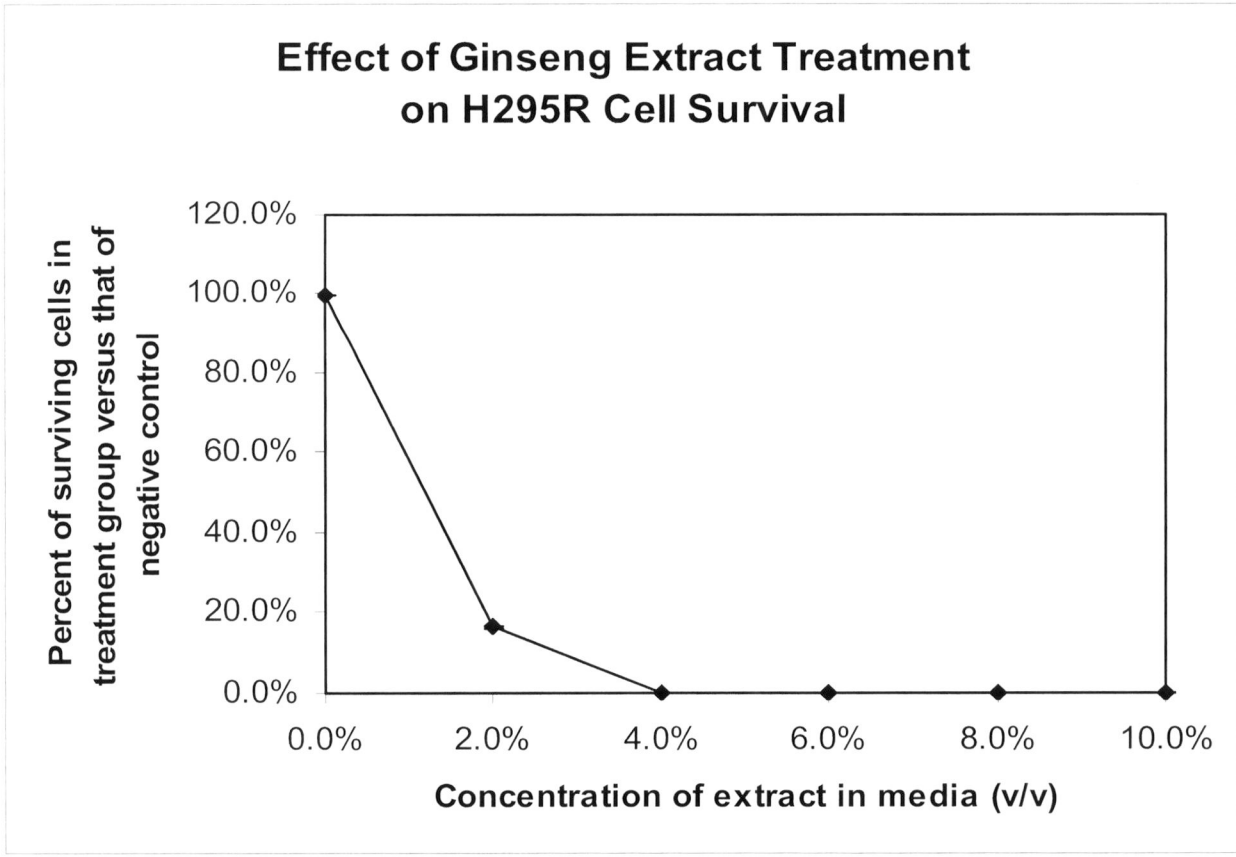

Figure 15. Effect of Ginseng on H295R Cell Survival. Treatment period was 72 hours. All standard errors less than 0.5%. n = 2.

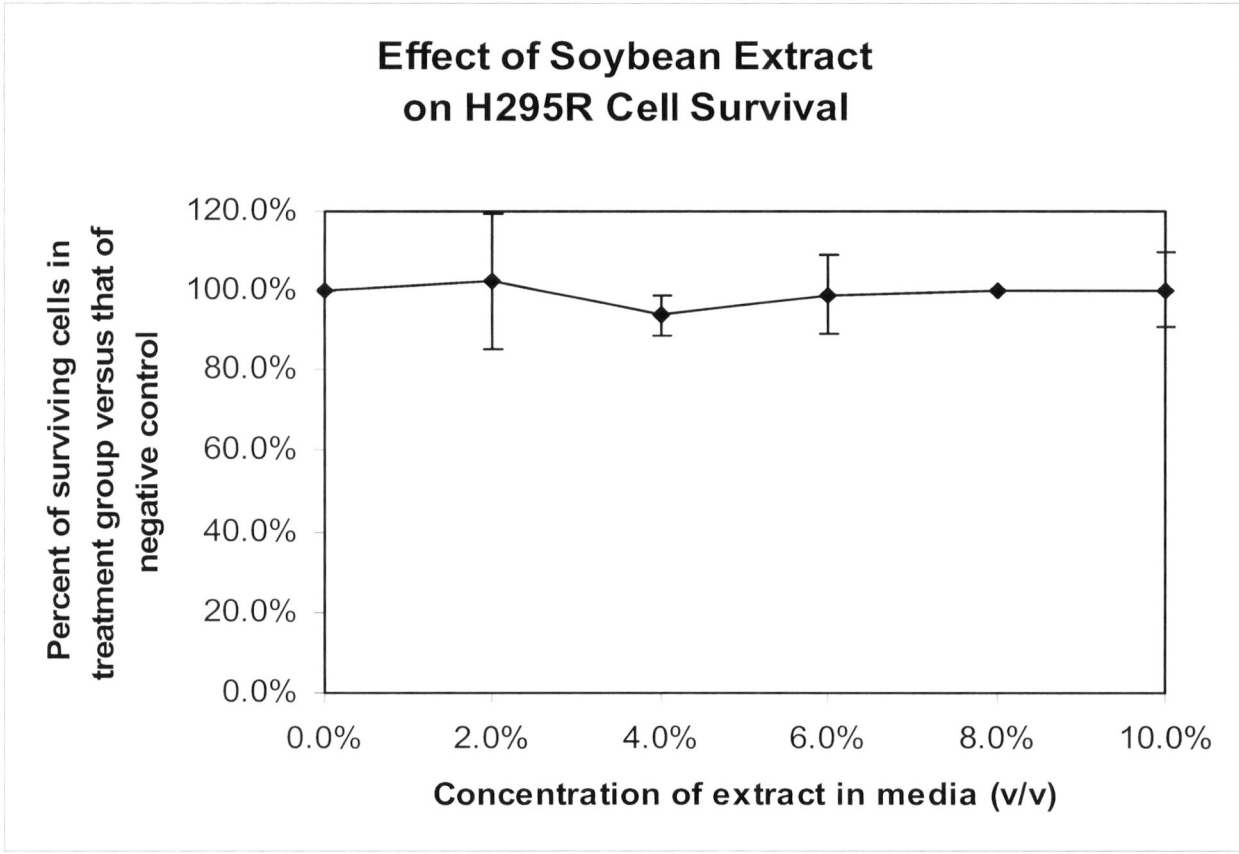

Figure 16. Effect of Soybean on H295R Cell Survival. Treatment was 72 hours. Error bars represent standard error. n = 3.

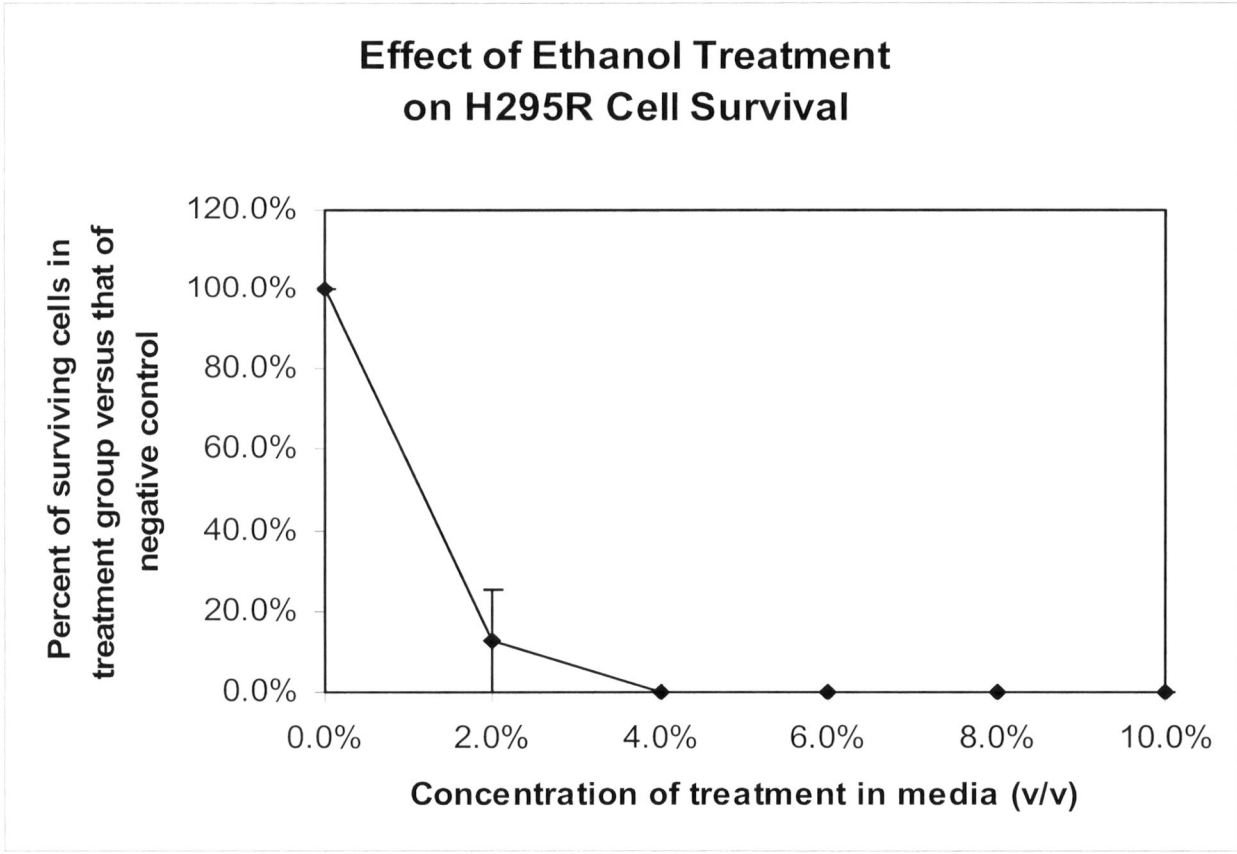

Figure 17a. Effect of Ethanol on H295R Cell Survival. Treatment was 72 hours. Error bars represent standard error. n = 2.

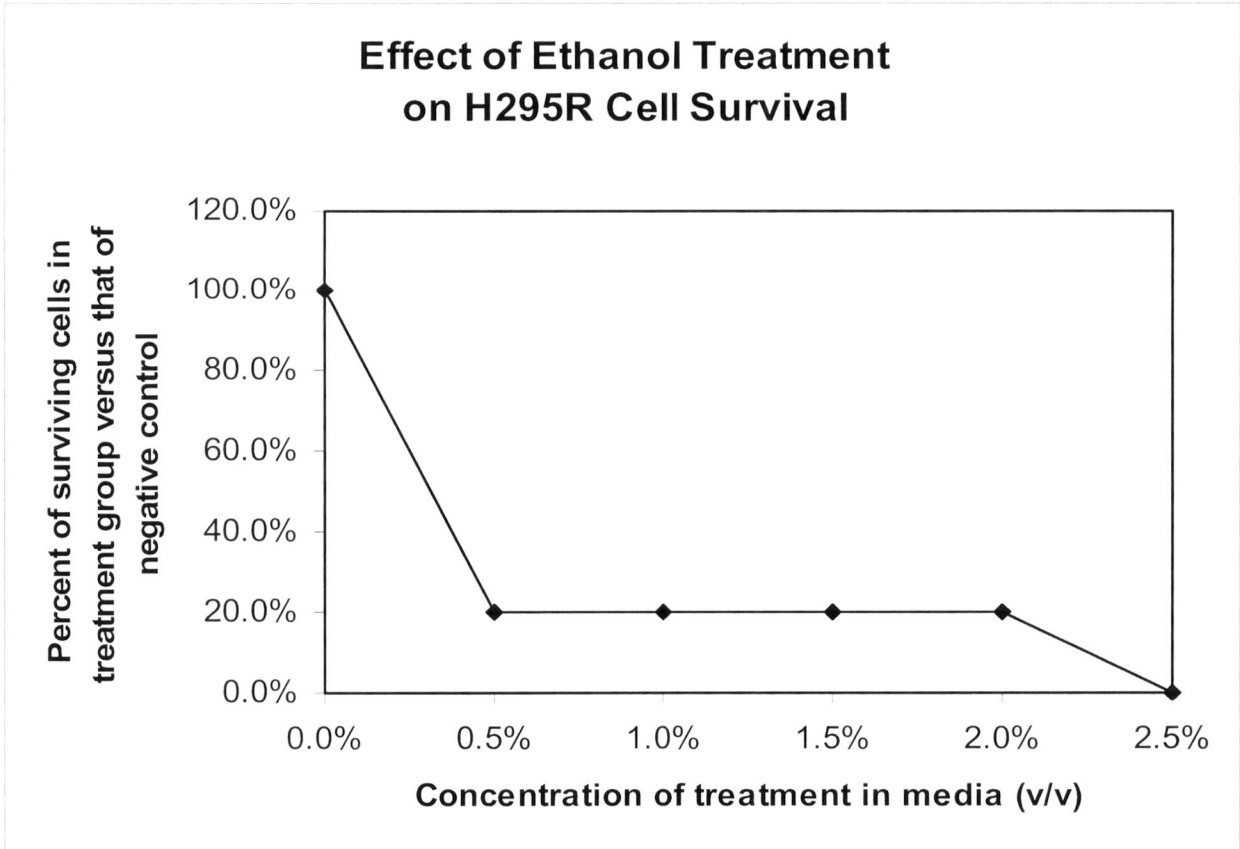

Figure 17b. Effect of Ethanol on H295R Cell Survival. Treatment was 72 hours. n = 1.

III. Aromatase induction assay

Analysis of the qRT-PCR results was done by the Bio-Rad® iCycler™ iQ program, version 3.1.7050. As mentioned before, each treatment group had 4 ten-fold dilutions done in triplicate. The 3 Ct values for each dilution group were averaged and compared to those from the negative treatments. Tables 1-4 each present the results of one 96-well PCR plate, and the average Ct value for each dilution for each treatment was given. Standard error and coefficient of deviation were calculated. Figures 18-21 depict comparisons of these average Ct values by bar graph.

Table 1. Average Aromatase Ct values for Negative and Chaste Tree Berry Treatments.

RNA	Negative			Chaste Tree Berry		
	Ct	SE	CV	Ct	SE	CV
0.5 ng	25.9	0.3	1.9	26.1	0.2	1.5
5.0 ng	25.8	0.1	0.8	25.6	0.2	1.1
50.0 ng	26.1	0.5	3.1	25.8	0.1	0.8
500.0 ng	20.4	1.4	12.3	25.1	1.6	10.8

Chaste tree berry extract was administered to the H295R cells as 1.0% of the media. Each treatment group was done in triplicate. Abbreviations used for all Ct tables: Ct (threshold cycle), SE (standard error), and CV (coefficient of variation).

Table 2. Average Aromatase Ct Values for Negative Control, Flaxseed, and Soybean Treatments.

RNA	Negative			Flaxseed			Soybean		
	Ct	SE	CV	Ct	SE	Cv	Ct	SE	CV
0.5 ng	28.8	0.2	1.0	28.8	0.1	0.7	27.0	0.2	1.1
5.0 ng	27.9	0.2	1.4	28.0	0.3	1.8	27.5	0.2	1.5
50.0 ng	25.0	0.3	2.4	25.6	0.2	1.6	24.6	0.2	1.6
500.0 ng	23.1	0.2	1.7	22.0	0.1	0.9	22.7	0.3	2.6

Both soybean and flaxseed extracts were administered as 3.0% of the media Each treatment group was done in triplicate. Abbreviations used for Ct tables: Ct (threshold cycle), SE (standard error), and CV (coefficient of variation).

Table 3. Average Aromatase Ct values for Negative Control and Garlic Treatments.

RNA	Negative			Garlic		
	Ct	SE	CV	Ct	SE	CV
0.5 ng	30.9	0.4	2.3	30.3	0.5	3.0
5.0 ng	29.9	0.3	2	29.7	0.2	1.3
50.0 ng	28.5	0.3	2.1	27.4	0.2	1.5
500.0 ng	29.0	0.6	3.4	29.1	0.3	1.7

Garlic extract was administered to the H295R cells as 0.3% of the media. Each treatment group was done in triplicate. Abbreviations used for Ct tables: Ct (threshold cycle), SE (standard error), and CV (coefficient of variation).

Table 4. Average Aromatase Ct values for Negative Control, Ginseng, and Wild Yam Root Treatments.

RNA	Negative			Ginseng			Wild Yam Root		
	Ct	SE	CV	Ct	SE	CV	Ct	SE	CV
0.5 ng	24.8	0.4	2.4	34.0	0.9	4.7	21.1	0.2	6.2
5.0 ng	23.6	0.8	5.5	23.8	0.2	1.3	24.8	0.2	5.2
50.0 ng	23.8	0.1	0.8	24.4	0.3	2.5	23.5	0.3	2.6
500.0 ng	22.8	0.1	0.9	22.3	0.5	3.1	23.7	0.6	4.2

Ginseng extract was administered as 0.2% of the media, and wild yam root extract was administered as 0.1% of the media. Each treatment group was done in triplicate. Abbreviations used for Ct tables: Ct (threshold cycle), SE (standard error), and CV (coefficient of variation).

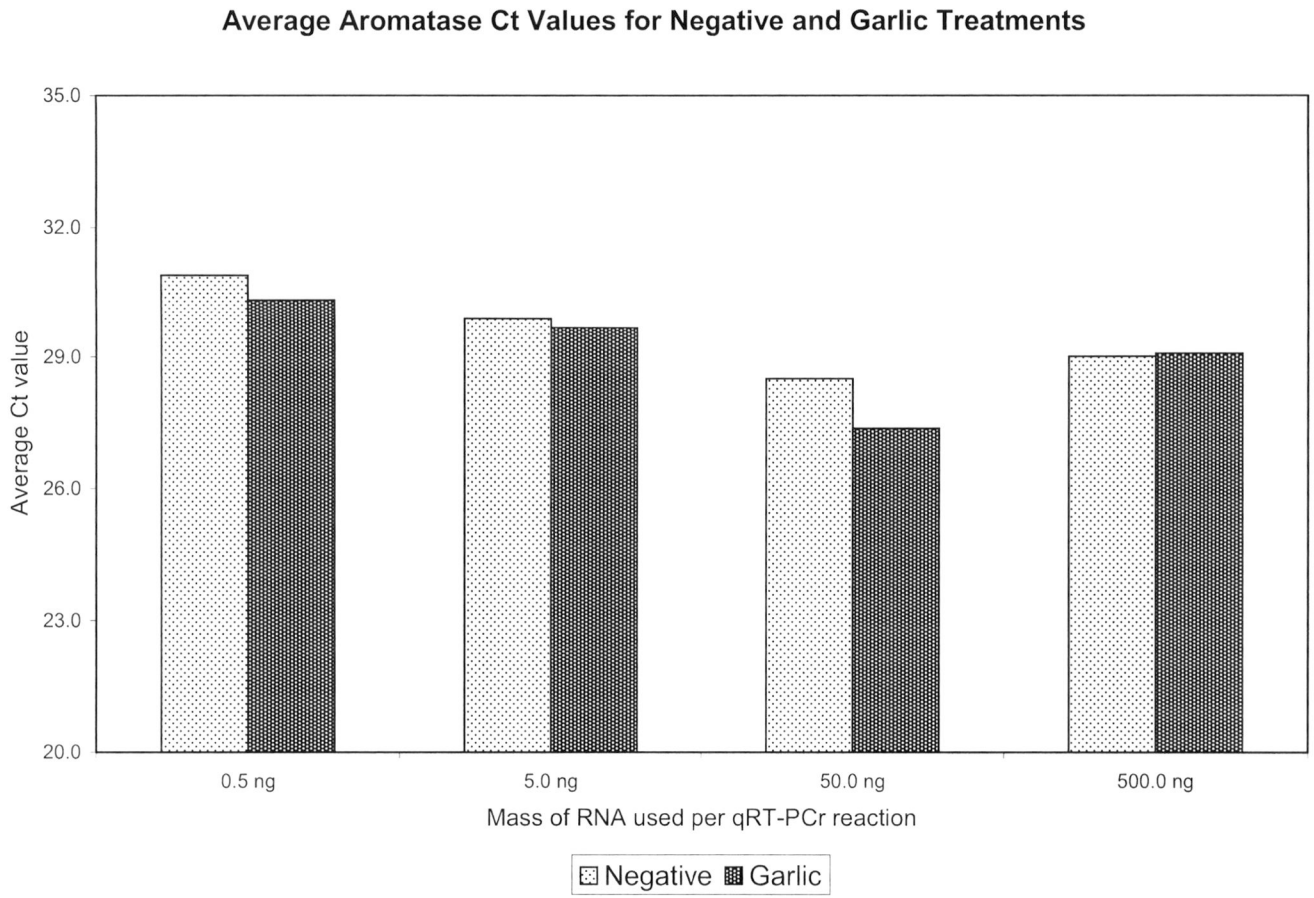

Figure 18. Comparison of Average Aromatase Ct Values for Negative Control and Garlic. Extract concentration 0.3% of cell media. n=1.

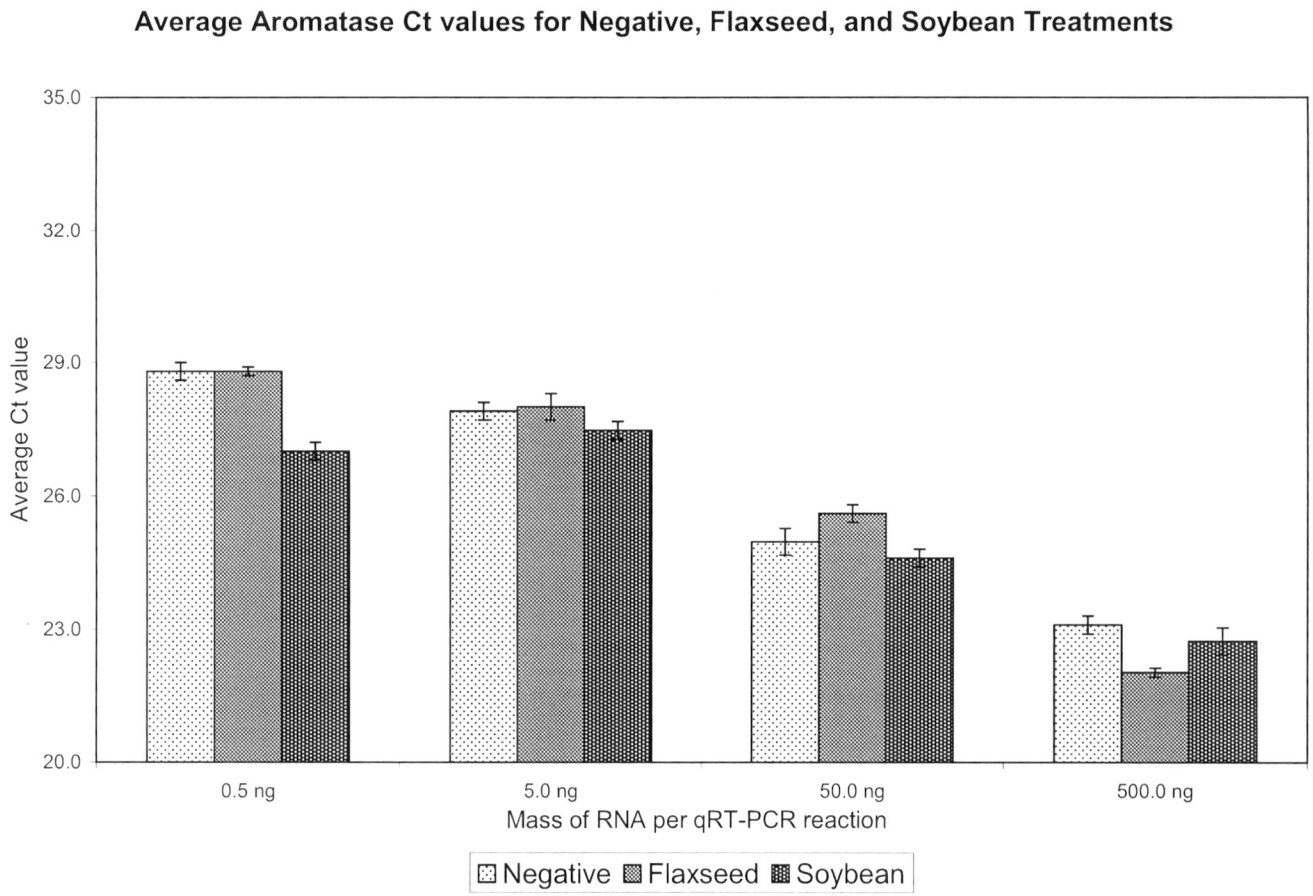

Figure 19. Comparison of Average Aromatase Ct Values for Negative Control, Flaxseed, and Soybean. Extract concentrations for both flaxseed and soybean were 3.0%. n = 1.

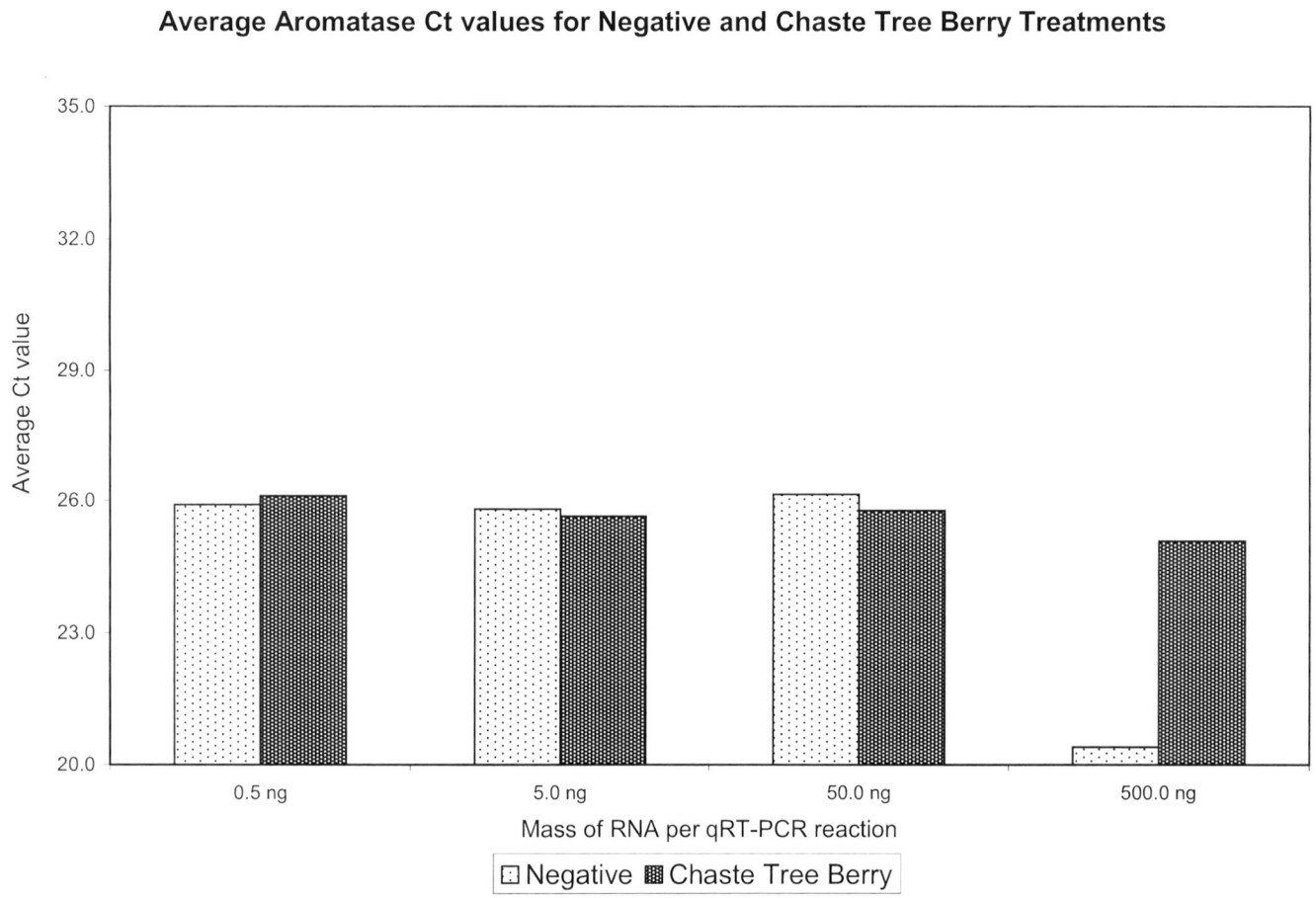

Figure 20. Comparison of Average Aromatase Ct Values for Negative Control and Chaste Tree Berry. Extract concentration for chaste tree berry was 1.0%. n = 1.

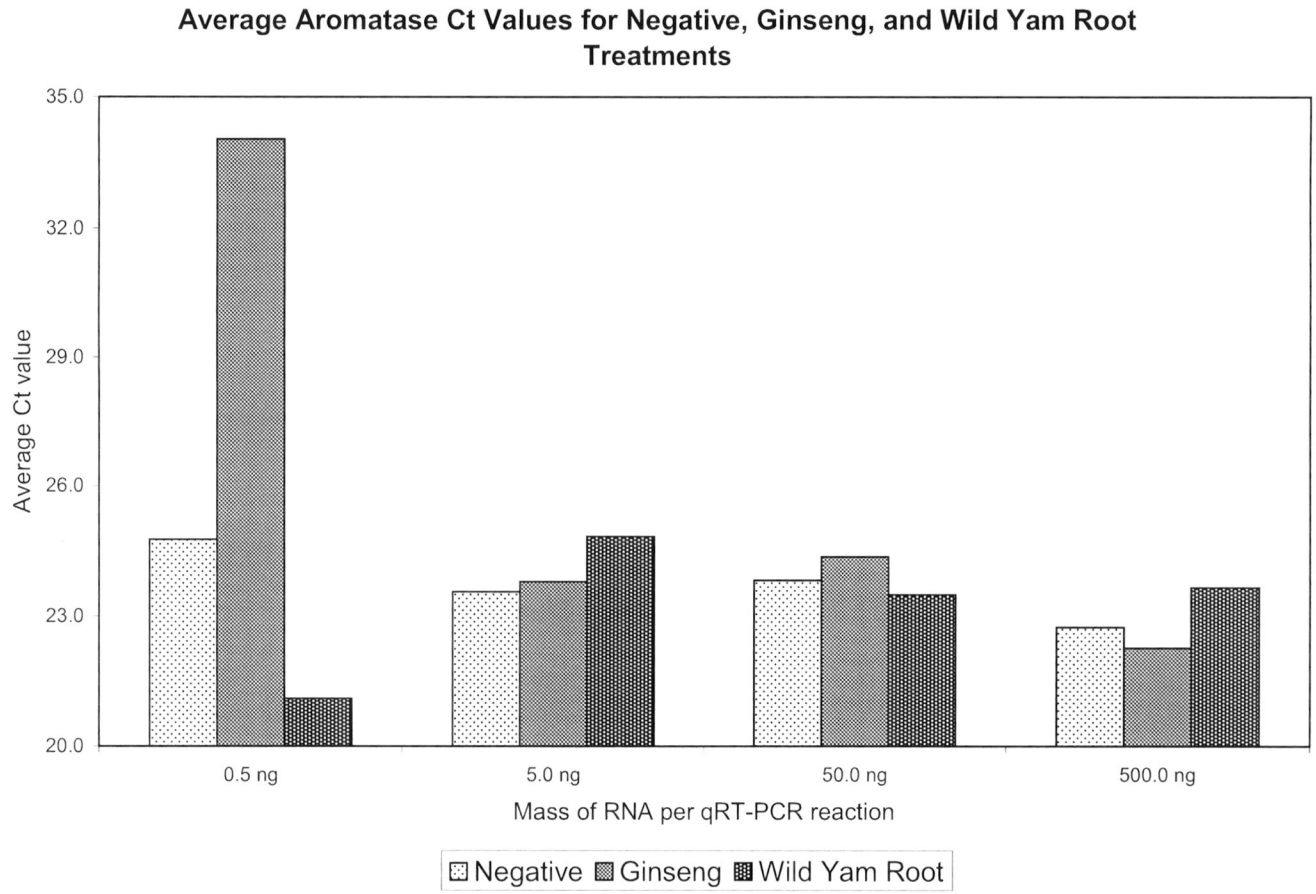

Figure 21. Comparison of Average Aromatase Ct Values for Negative Control, Ginseng, and Wild Yam Root Treatments. Ginseng was tested as 0.2% of the media while wild yam root was tested as 0.1%. n = 1.

DISCUSSION

I. Extract consistency

Constant ratio of mass of ground herb to extraction media was employed in order to quantify treatments applied; this method has been employed in similar studies (Charles et al. 2002). All of the described experiments for the HepG2 and H295R cells were performed using the same extract suspensions in order to be as consistent as possible. The same whole and ground herbs, stored at 4 °C for the duration of the research, were used throughout the experimental period.

II. Cytotoxic IC_{50} determination

The cytotoxic IC_{50} experiments were modeled after that of Charles et al. (2004) where surveying or initial toxicity studies were performed on whole foods. The research was remarkable in that initial toxicity screens much like the ones used for manufactured pharmaceuticals were applied to whole foods. The studies spanned a variety of foods, including garlic, plums, cauliflower, apples, soybeans, white rice, and cumin, that were purchased from local markets. CHO cells were used for these studies.

The Charles lab performed aqueous extractions employing the CHO cell maintenance media as an extraction vehicle, which eliminated any possible influence of other solvents such as water or DMSO on cell survival. A constant ratio of 50 mg of dried, ground material to 1 mL cell media was maintained. Treatments of these extracts were measured by percentage of extract in the cell media, or a volume/volume ratio. Treatments increased from 0% to 10%, as 10% is the generally accepted maximum concentration of

aqueous materials that can be added to cell media without significant osmolality or pH-related toxicity.

The present experiments were carried out in three cell lines, two human carcinoma lines, the HepG2 and the H295R cells, and one rodent line, the CHO-K1-BH4 cells. Each of the cell lines demonstrated different sensitivities to the different extracts. Table 5 depicts the estimated IC_{50} values for each cell line. Figure 23 compares the relative potencies of the extracts in the three cell lines by bar graph.

The positive control used for these experiments was the ethanol treatment. The order of sensitivity of the cell lines to ethanol, demonstrating the most toxic response to the least (or lowest IC_{50} to highest IC_{50}), was H295R, HepG2, then CHO. All IC_{50}s were less than 2.0%.

In the chaste tree berry and ginseng treatment groups, order of sensitivity was the same as in the ethanol treatment group. The H295R cells had a lower IC_{50} value than did the HepG2 cells. The CHO IC_{50} values for these groups were more than double that of either of the other two lines.

The wild yam root treatment demonstrated very low IC_{50} values that were all less than 1.0%. The order of the cell lines displaying the most toxicity to the least was HepG2, then CHO, then H295R.

Table 5. Comparison of IC_{50} values for extracts in HepG2, H295R, and CHO-K1-BH4 cells.

	HepG2	H295R	CHO-K1-BH4
Chaste Tree Berry	3.0%	2.0%	7.0%
Flaxseed	n/a	> 10.0%	> 10.0%
Garlic	0.2%	0.8%	n/a
Ginseng	2.0%	1.0%	4.5%
Soybean	> 10.0%	> 10.0%	10.0%
Wild Yam Root	0.2%	1.0%	0.6%
Ethanol	1.0%	0.3%	1.5%

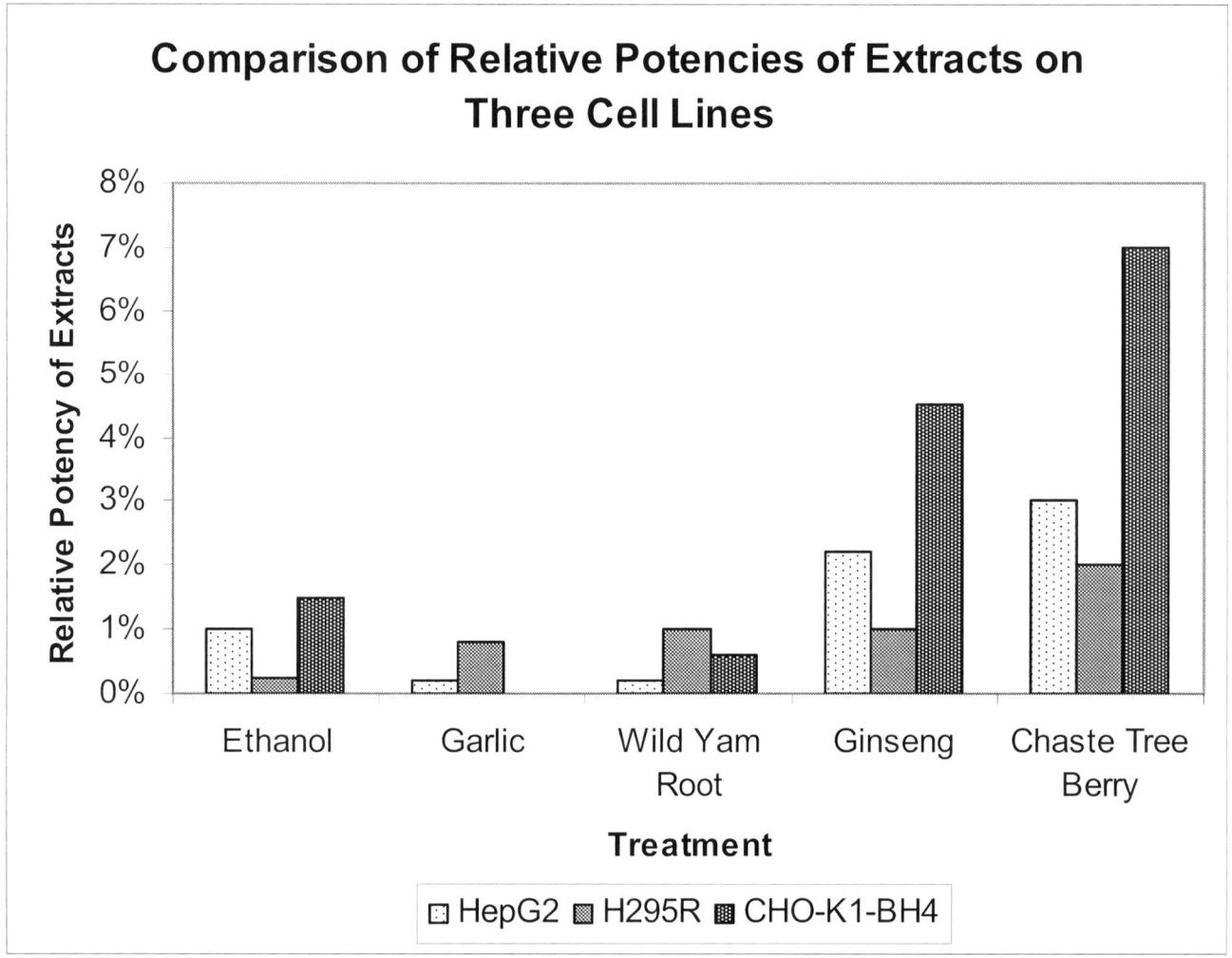

Figure 22. Comparison of relative potencies of the extracts among the three cell lines employed. Relative potency represented by approximate IC_{50} value.

The flaxseed treatments did not cause any remarkable decrease in cell survival for the H295R and CHO cells. Even at 10.0%, the highest concentration tested, the cells surviving in the treatment group numbered significantly more than 50% of the cells surviving in the negative control; hence, an IC_{50} value could not be established. The flaxseed extract was not tested in the HepG2 cells.

Like the wild yam root, the garlic treatment proved to have an IC_{50} value of less than 1.0% in both the HepG2 and H295R cell lines. The HepG2 cells were more sensitive to the treatments than the H295R cells. The garlic extract was not tested in the CHO cells; however, Charles et al. (2004) published an IC_{50} value for garlic on the CHO cells as less than 1.0% under similar conditions.

The soybean treatments in the H295R cells did not appear to negatively affect the cell survival even at 10.0%; the IC_{50} value could only be described as significantly greater than 10%. In the HepG2 cells, increasing treatment did decrease the numbers of cells surviving; however, the number of surviving cells in the 10.0% treatment group was still higher than 50% of those in the negative control. Because of these results, the IC_{50} value for the HepG2 cells can best be described as greater than 10.0% but not to the extent demonstrated by the H295Rs. The CHO cells mirrored the HepG2s in the decline of cell survival in increasing extract concentrations. The surviving cells in the 10.0% group approximately numbered half of those in the negative treatment. Although greater concentrations were not tested to determine whether or not the number of cells surviving

would continue to decline, the trend shown in the results supports 10.0% as an approximate IC_{50} value for the soybean extract in the CHO cell line.

III. Effect of herbal supplements on aromatase induction

One method for measuring expression of small amounts of RNA is Reverse Transcriptase Polymerase Chain Reaction, or qRT-PCR. For this assay, the sample RNA is treated with reverse transcriptase in order to produce cDNA. This resulting cDNA is denatured with heat, treated with a specific set of primers that flank the target sequence and DNA polymerase. Once the polymerase produces a copy of the cDNA, the cycle of heat-renaturing is repeated. These experiments employ SYBR ® green, which binds only to double-stranded DNA. As the amounts of DNA produced increase, the fluorescence increases as well. The cycle number at which the fluorescence, representing the amount of DNA present, begins to increase logarithmically is known as the Ct value. When the sample RNA is diluted, the associated Ct value increases, as it takes longer for less genetic material to grow logarithmically. Along the same lines, if the beginning amount of RNA was greater than the beginning amount of RNA in a negative control, the Ct value would be lower.

The aromatase Ct values in the H295Rs treated with the herbal supplements were compared to those of the negative control and were not found to be significantly different. At the concentrations given, none of the extracts appeared to induce aromatase expression as compared to the negative control.

The garlic, flaxseed, and soybean treated cells did not exhibit different aromatase Ct values from those of the negative controls (Figures 18 and 19). The garlic Ct values did not show a downward trend as expected across the RNA dilutions; this may be attributed to non-optimal qRT-PCR conditions. It should be noted that flaxseed and soybean IC_{50} values were not able to be determined for the H295R cells; 3.0% concentrations were used for both flaxseed and soybean treatments. This may not have been the optimal treatment concentration necessary for an effect on aromatase expression; further studies should test higher concentrations.

The chaste tree berry extract, at the lower 3 RNA dilutions, did not appear to elicit different Ct values than those demonstrated by the negative control (Figure 20). At 500.0 ng, however, there is a remarkable difference between the Ct values of the negative and chaste tree berry treatment. This may be due to the concentration of RNA used; at higher concentrations this trend might have continued to be shown.

The ginseng and wild yam root extracts, at the higher 3 RNA dilutions, did not appear to elicit different Ct values than those demonstrated by the negative control (Figure 21). At the lowest concentration tested, 0.5 ng, the Ct values of the extract groups were markedly different than those demonstrated by the negative controls. This is also most likely due to an optimization problem. Further experimentation would benefit from optimizing levels of primers, RNA, and reagents as well as the cycling protocol.

IV. Estrogenicity of the herbal supplements

Chaste tree berries have long been suspected to be estrogenic due to their historical uses as treatments for premenstrual and menopausal symptoms.

In 2004, Liu et al. studied the binding of a methanol extract of chaste tree berries to the two estrogen receptors, alpha (α) and beta (β). Fresh chaste tree berries were crushed, treated with petroleum ether for defattening, filtered, then extracted twice with methanol; the resulting extract was a combination of the two extractions. The extraction was then excised of methanol, leaving a residue for fractionation on a silica gel flash column. The final product was positively confirmed as linoleic acid. The linoleic acid was dissolved in DMSO and was applied to pure recombinant ERα, supplemented with [^{3}H] estradiol and ER binding buffer. Also tested in this system was the methanol extract. It was shown that both the methanolic extract and the linoleic extract bound to ERα (Liu et al., 2004). This research demonstrated chaste tree berry's estrogenicity to the ERα receptor.

Wild yam root, another herb traditionally used for relief of premenstrual and postmenopausal symptoms, was studied by Rosenberg Zand et al. (2001). Using a commercial extract of wild yam root, the group further diluted the liquid with anhydrous ethanol and treated breast carcinoma cells, BT-474, a cell line positive for estrogen, androgen, and progesterone receptors. For the agonist studies, BT-474 cells were incubated for 7 days with full strength stock dilution and several 10-fold dilutions, after which time the cells were tested for pS2 (protein regulated via estrogen receptor). The wild yam extract did not affect pS2 protein levels, and this extract was determined not to

have significant estrogenic activity. Antagonist activity was also studied extensively by the same treatment as before; however after a 1 hour extract treatment the cells were stimulated with an estrogenic compound and incubated for 7 days. Using antiestrogens such as fulvestrant (7-alpha-[9-(4,4,5,5,5-penta fluoropentylsulphinyl) nonyl]estra-1,3,5-(10)-triene-3,17-beta-diol) and mifepristone (11β-[p-(Dimethylamino)phenyl]-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one) as positive controls, the cells were analyzed for pS2 protein levels, and percentage blocking was quantified by comparing the concentration of pS2 from the extract containing the estrogenic steroid additive to the concentration of pS2 from the estrogenic steroid additive alone. The wild yam root extract was found to have weak antiestrogenic activity (Rosenberg Zand et al., 2001). These findings were confirmed by an earlier study done by Zava et al. in 1998. During the course of these studies it was found that the wild yam products did not bind to the estrogen receptor (Zava et al., 1998).

Flaxseed is a prominent source of lignans, which is one of the major categories of phytoestrogens; however, it has not been found to have estrogenic activity. The lignans in flaxseed do bind to the estrogen receptors but do not stimulate them; lignans act instead to prevent estrogenic compounds from binding and stimulating the estrogen receptor (Smith, 2001). In 2005, the Waldschlager group demonstrated that a crude extract of flaxseed had similar effects as soybean on hormone production and growth of estrogen receptor positive trophoblast tumors; it also inhibited estrogen receptor positive tumor cells in hormone production and in proliferation (Waldschlager et al., 2005).

Ginseng in particular has a great deal of conflicting reports regarding its estrogen receptor binding and activity. Historically used for its anti-fatigue and mood stabilizing effects, the use of ginseng has become more and more popular as a treatment for menopausal symptoms as well as a substitute for hormone replacement therapy (Wylie-Rosett, 2005; King et al., 2006). Extracts of ginseng have been found to increase pS2 (an estrogen-responsive gene) expression (Liu et al., 2001). In direct contrast, other research has found that ginseng extract did not have estrogenic effects (Amato et al., 2002). One explanation for this conflicting data is that the labs were using different extractions – different solvents, herb preparation, and other conditions affecting the herbal extract. In 2006, King et al. validated this point after finding that ginseng extracted with methanol did indeed bind ERα receptors, but the water extraction did not (King et al., 2006).

Soybeans have been the emphasis of the current herbal supplement and complementary medicine craze. Some reports of soybean's estrogenicity speculate that taking this supplement could prevent breast cancer; other reports warn people with high risk for breast cancer to avoid consuming soy products at all. Some products are highly advertised as effective alternatives to hormone replacement therapy in order to decrease the symptoms of menopause; others caution people not to ingest soy products due to possible toxic side effects that can occur in women suffering with breast cancer, whether it is treated with chemotherapeutics or not.

Regardless of the theories propagated by the media and other outlets, soybean extracts do bind estrogen receptors. Ju et al. (2002) discovered that genistein, one of the

predominant isoflavones in Soy, antagonized the effect of the SERM Tamoxifen in estrogen positive breast cancer cells. The pS2 levels were monitored in MCF-7 (breast carcinoma) cells; they were first treated with Tamoxifen, and the pS2 expression decreased as expected. When genistein was added to the treated cells, the pS2 expression increased enough to completely negate the action of the SERM (Ju et al., 2002). Later, the Mueller group published a paper describing their research on the two major soy phytoestrogens, daidzein and genistein. Mueller et al. (2004) performed a competitive binding assay with the phytoestrogens and fluorescein-labeled E2, and it was proven that the phytoestrogens did displace the E2. In addition, the group employed Ishikawa human endometrial cells constitutively expressing estrogen receptors in order to test for increased activity of vitellogenin A, a protein commonly used to assay for agonism or antagonism due to its consensus estrogen responsive element (ERE) which binds an activated estrogen receptor. These studies confirmed the results of the previous binding assay that showed an increase in vitellogenin A activity (Mueller et al., 2004).

Of the six herbs chosen for the present studies, garlic is the herb with the least available information concerning estrogenicity. Used for a wide variety of reasons ranging from antibiotic activity to cardiovascular effects, it is the herb most readily available to the public. There are claims that using garlic may decrease chances of breast cancer, but there are also claims that there is no interaction between the two. This might be explained by varying compositions of extracts used in experiments. Oil extractions were shown to inhibit induced breast cell tumors while water extractions demonstrated little or no influence on them. While it has been shown that garlic compounds will inhibit in vivo

and in vitro growth of estrogen-dependent and estrogen-independent cancer, it has also been shown that these compounds induce apoptosis as well (Nakagawa et al., 2001).

Garlic has not been shown to have any estrogenic properties. There is a great deal of research that studies the effects of garlic extracts upon breast cancer cell models but none concerning the binding or activation of the estrogen receptors. Despite the 2006 research by the Mukherjee group that found garlic to have antiosteoporostic activity similar to that of estrogen, garlic's reputed anti-cancer properties as opposed to actual estrogenic effects are probably responsible for its popularity as a possible alternative therapy for breast cancer patients (Mukherjee et al., 2006).

CONCLUSION

The herbal supplements significantly employed by breast cancer patients, chaste tree berry, flaxseed, garlic, ginseng, soybean, and wild yam root, did indeed have varying effects on relative potencies in the three cell lines HepG2, H295R, and CHO-K1-BH4. The order of relative potency from most toxic to least is garlic, wild yam root, ginseng, and chaste tree berry; flaxseed and soybean did not exhibit adequate toxicity even at the highest levels tested to determine the IC_{50} values. Conclusions about the effects of cell media aqueous extracts in H295R cells on expression of aromatase could not be made. Various extract types and components of all of these herbs, save for garlic, have been shown to bind to the estrogen receptor α. The vast number of possible extraction and formulation techniques complicates the research and the regulation of herbal supplements. Only further study and research in this area, including the identification of the most relevant extracts and compounds to human consumption, can provide needed insight and answers. Given the results cited in the contemporary literature, it would seem prudent to advise patients undergoing treatment with SERMs to avoid the use of supplements containing chaste tree berry, flaxseed, ginseng, soybean and wild yam root.

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