Comparative Efficacy of Extracts from Lycium Barbarum Bark and Fruit on Estrogen Receptor Positive Human Mammary Carcinoma MCF-7 Cells

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INTRODUCTION

Chemo-endocrine therapy for estrogen receptor positive (ER+) breast cancer exhibits acquired tumor resistance. Herbal medicines provide integrative support for breast cancer patients. Present study compared the efficacy of aqueous extracts from Lycium barbarum bark (LBB) and Lycium barbarum fruit (LBF) on ER+ MCF-7 cells. Cellular growth and 17β-estradiol (E2) metabolism quantified the efficacy. MCF-7 cells maintained in serum depleted medium + E2 exhibited increased anchorage-dependent and anchorage-independent growth. LBB exhibited greater potency than LBF (95% reduction in IC50). LBB produced a 6.8-fold increase, 40% decrease, and a 3.7-fold increase in 2-hydroxyestrone (2-OHE1), 16α-hydroxyestrone (16α-OHE1), and estriol (E3) formation. The corresponding values for LBF were 3.9, 33, and 10.5. LBB produced a16.3-fold and a twofold increase in 2-OHE1:16α-OHE1 and E3:16α-OHE1 ratios, whereas LBF produced a sixfold and a 2.9-fold increase. The efficacy of LBB is due to increased 2-OHE1 formation, whereas that of LBF is due to accelerated conversion of 16α-OHE1 to E3. Specific growth inhibitory profiles of LBB and LBF may be due to their distinct chemical composition and their complementary actions on E2 metabolism. This study validates a mechanistic approach to identify efficacious herbal extracts for clinical ER+ breast cancer.

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herbal medicines remains to be systematically documented and, therefore, is currently equivocal.

Herbal medicinal products have been extensively used as individual components in complex herbal mixtures for the management of estrogen related health issues (5,7,8). For example, the herbal medicine Lycium barbarum has been reported to sensitize tumor cells against radiation therapy and enhance clinical efficacy of LAK/IL-12 based immuno-therapy in cancer patients (9,10).

Human mammary carcinoma derived ER+ MCF-7 cell line represents a well-established preclinical model for hormone responsive clinical breast cancer. This model has been extensively used both as cell culture approach, as well as in vivo xenotransplant approach for preclinical efficacy studies on synthetic estrogen receptor modulators and inhibitors of estrogen biosynthesis (11). In addition, our published studies on the MCF-7 model have demonstrated that nonfractionated aqueous extracts from several herbal preparations, operating via distinct mechanisms, exhibit cytostatic growth arrest and reduce carcinogenic risk (12–16).

The present study has used the MCF-7 cell culture model to compare the cellular and endocrine effects of nonfractionated aqueous extracts prepared from Lycium barbarum bark (LBB) and Lycium barbarum fruit (LBF). The data generated from this study demonstrate that these two extracts differ in their potency to induce cytostatic growth arrest in the present model. At the mechanistic level, these extracts alter the cellular metabolism of 17β-estradiol (E2) via distinct metabolic pathways. Thus, these data taken together validate the present cell culture model as a rapid mechanism-based approach to screen herbal medicines for their therapeutic efficacy and prioritize promising lead agents for subsequent animal studies and clinical trials for breast cancer therapy.

MATERIALS AND METHODS

Cell Line

The ER+ human breast carcinoma MCF-7 cell line was originally obtained from the Michigan Cancer Foundation (Detroit, MI). These cells were cultured in DME/F12 medium supplemented with 7% heat-inactivated fetal calf serum and recommended additives (17).

For the present experiments, MCF-7 cells were adapted for growth in serum-depleted medium by maintaining the cultures in the medium supplemented with 0.7% serum for at least 5 passages. These stock cultures were routinely maintained in DME/F12 medium supplemented with 0.7% serum in an humidified atmosphere of 95% air: 5% CO2 at 37°C and were subcultured at 1:4 split at about 80% confluency (12,16).

Growth Parameters

The population doubling time was determined during the exponential growth phase by obtaining the viable cell counts from triplicate flasks at 24, 48, 72, and 96 h postseeding of 1.0 × 10⁵ cells per flask, and the mean values from four time points were used to determine the population doubling times. Saturation density was determined from the viable cell number at Day 7 postseeding of 1.0 × 10⁵ cells, and data were expressed as mean ± SD, n = 3 per treatment group.

Anchorage Independent Colony Formation

This assay was performed according to previously published protocol (17,18). Briefly, 1000 MCF-7 cells per 2 ml were suspended in the culture medium containing 0.33% agar. This cell suspension was overlaid on a basement of 0.6% agar in each well of 6-well cluster plates. The cultures were maintained at 37°C in a humidified atmosphere of 95% air: 5% CO2 for 21 days. The cultures with developed colonies were then fixed in Carnoy’s fluid and the colony count in each well was determined microscopically at 10× magnification.

Aqueous Extracts of LBB and LBF

To prepare the aqueous extracts, 20 g of LBB or of LBF were boiled in 200 ml of deionized water until the volume was reduced to 100 ml, and the supernatants were collected (aqueous extracts # 1). The resultant residues were further boiled in 100 ml of water until the volume was reduced to 50 ml (aqueous extracts # 2). The 2 supernatants from individual herbs (extract #1, 100 ml; extract #2, 50 ml) were combined and concentrated by boiling until the volume was reduced to 25 ml. These combined extracts were centrifuged at 5000 rpm at room temperature for 10 min. The resultant supernatants (20 ml) were collected and stored as stock solution in individual aliquots of 5 ml at −20°C. These stock solutions were appropriately diluted in DME/F12 medium to obtain the working concentrations.

Dose Response of LBB and LBF

For the dose-response experiments MCF-7 cells were seeded at the initial density of 1.0 × 10⁴ cells/25 cm² in T-25 flasks. Treatment with LBB or LBF was initiated at Day 1 postseeding and continued up to Day 7 postseeding with a medium change at every 48 h. At the end of the treatment schedule, the cultures were trypsinized and trypan blue excluding viable cell counts were obtained. The data from the viable cell counts were used to determine minimum effective and maximum cytostatic doses of LBB and LBF. These primary data were used to extrapolate inhibitory concentrations (IC₅₀) individually for LBB and LBF.

Sample Preparation for Cellular Metabolism of E₂

The MCF-7 cells at Day 1 postseeding were treated with appropriate concentrations of LBB or LBF in the presence of 20 nM E₂ for 48 h and the medium was analyzed for E₂ metabolites following published methods (19–21). Briefly, a 5-ml aliquot of the medium was diluted 1:1 with sodium acetate buffer (pH 4.65) and 20 μl of β-glucuronidase (110,200 units/ml, Sigma-Aldrich Co., Inc., St. Louis, MO). This solution was incubated at 37°C for 24 h to deconjugate the steroids.
Table 1: Endocrine responsiveness of MCF-7 cells in serum depleted medium

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Serum Concentration</th>
<th>Population Doubling (h)</th>
<th>Saturation Density ×10^5</th>
<th>Anchorage Independent Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.7%</td>
<td>34.4</td>
<td>10.9 ± 0.9^c</td>
<td>17.2 ± 3.4^e</td>
</tr>
<tr>
<td>E₂</td>
<td>20 nM</td>
<td>27.2</td>
<td>18.3 ± 0.3^d</td>
<td>36.7 ± 2.1^f</td>
</tr>
</tbody>
</table>

^a Means ± SD, n = 6 per treatment group. ^b Means ± SD, n = 12 per treatment group. ^c,d P = 0.04. ^d,f P = 0.03.

After the addition of deuterated E₂ as an internal standard (19,20), each sample was thoroughly vortexed. Two volumes of chloroform were added to the samples, and the resulting mixture was vortexed and centrifuged. The chloroform layer was removed and reduced to dryness using a vacuum equipped centrifuge (Labconco, Inc., St. Louis, MO). Each sample was derivitized by adding 10 μl of dry pyridine and 40 μl of bis (trimethylsilyl) trifluoroacetamide (BSTFA), vortexed, and allowed to react at room temperature overnight. One μl of each sample was injected into the GC-MS apparatus without further treatment.

GC-MS Conditions for Analysis of E₂ Metabolites

Select E₂ metabolites were analyzed on an Agilent 6980N gas chromatograph equipped with an Agilent 5973 mass selection detector, an Agilent 7683 injector, and an HP G1701CA MSD Chemstation. The injection port was equipped with a split/splitless capillary inlet system and a silanized glass insert. The temperature of the injection port was maintained at 300°C. The GC-MS interface was maintained at 270°C and the ion source was maintained at 280°C. The ionization energy was 70 eV. The carrier gas was helium at a flow rate of 1 ml/min. Separations of metabolites were carried out using a Hewlett-Packard Ultra 2 capillary column with cross-linked 5% phenyl-methyl silicone (25 m x 0.2 mm x 0.33 μm film thickness). The oven temperature was increased from 60°C to 260°C at 40°C/min then at 1°C/min to 280°C (19–21).

Under selected ion monitoring, the following mass ions and GC elution times of trimethylsilylated estrogens were routinely monitored: E₁ m/z 342, 15.90 min; E₂ m/z 416, 16.40 min; deuterated E₂ m/z 420, 16.40 min; 2-OHE₁ m/z 430, 18.47 min; 4-OHE₁ m/z 430, 18.92 min; 16α-OHE₁ m/z 286 and 430, 19.37 min; and E₃ m/z 504 and 345, 20.76 min. The other E₂ metabolites 2-OHE₂ and 4-OHE₂ were monitored using the mass and base ions m/z 504 and m/z 373 at 19.06 min and 20.15 min, respectively, in a second run using the same parameters as above. The internal standard [2, 4, 16α, 16β-H₄] deuterated estradiol (d₄ E₂) to be used in the present study was synthesized in our laboratory, according to the method of Dehennin et al. (20). The individual metabolites were quantified using a 6-point calibration curve (range: 1 to 50 mg). The data were expressed as ng metabolite per 10⁶ cells.

Modulation of E₂ Metabolism

Because E₁ functions as a common precursor for the formation of 2-OHE₁ and 16α-OHE₁, and because 16α-OHE₁ is converted to the proximate metabolite E₂ (12,14–16), the ratios of 2-OHE₁:16α-OHE₁ and E₂:16α-OHE₁ were also considered for an accurate evaluation of experimental modulation in E₂ metabolism. The 2-OHE₁:16α-OHE₁ ratio was determined by dividing the values of 2-OHE₁ by those of 16α-OHE₁. To determine the portion of total 16α-OHE₁ that was converted to E₂, the E₂:16α-OHE₁ ratio was determined by dividing the values of E₂ by those of 16α-OHE₁+E₂ (12,14–16,18).

Statistical Analysis

Experiments to determine population doubling and saturation density were performed in duplicate with 3 flasks per treatment group per experiment. Experiments for the anchorage independent (AI) growth assay were performed in duplicate with 2 six-well plates per treatment group per experiment. The dose-response experiments for LBB and LBF were performed in duplicate with 3 flasks per treatment group per experiment. The experiments for E₂ metabolism were performed with 3 flasks per treatment group per experiment.

The significance of differences between the control and experimental data points for individual experiment was analyzed by 2-sample t-test using the Prism 4.0 software (Graph Pad Software, Inc., San Diego, CA). P values of <0.05 were considered significant.

Results

Characterization of MCF-7 Cells

The data presented in Table 1 demonstrate that MCF-7 cells maintained in a serum depleted culture medium (0.7% serum, <1 nM E₂) retain their responsiveness to the physiological levels of E₂. Thus, in the presence of E₂ the MCF-7 cells exhibited 20.9% decrease in the population doubling, a 67.9% increase in the saturation density (P = 0.04) and a 113.4% increase (P = 0.03) in the number of AI colonies.

Dose Response of LBB and LBF

The LBB extract inhibited E₂ promoted growth of MCF-7 cells in a dose dependent manner (Fig. 1A). Treatment of MCF-7 cells with LBB at 0.005%, 0.025%, 0.05%, and 0.1% inhibited the E₂ promoted growth by 30.8%, 63.2%, 84.9%, and...
98.4%, respectively. This dose-response experiment identified the IC$_{50}$ concentration as 0.02%, and the maximum cytostatic concentration as 0.05%. LBB treatment at 0.1% induced a cytotoxic response as evidenced by the viable cell number that was less than the initial seeding density of $1.0 \times 10^5$. The LBF extract also inhibited the E$_2$ promoted growth of MCF-7 cells in a dose-dependent manner (Fig. 1B). LBF treatment at the concentrations of 0.1%, 0.5%, 1.0%, and 2.0% resulted in the growth inhibition of 35.8%, 65.3%, 84.4%, and 97.1%, respectively. This dose-response experiment identified the IC$_{50}$ concentration as 0.38%, and the maximum cytostatic concentration as 1%. The LBF concentration of 2% induced a cytotoxic response as evaluated by the viable cell number being lower than the initial seeding density.

**Inhibition of AI Growth by LBB and LBF**

The data for the effect of LBB and LBF on the AI growth of MCF-7 cells is presented in Fig. 2. At their respective maximum cytostatic concentrations of 0.05% LBB and 1.0% LBF, the number of AI colonies was inhibited by 93.2% and 89.2%, respectively, relative to E$_2$ treated controls.

**Effect of LBB and LBF on the Cellular Metabolism of E$_2$**

The effect of LBB on E$_2$ metabolism is presented in Table 2. LBB at its maximum cytostatic concentration produced a 6.8-fold increase in 2-OHE$_1$ formation ($P=0.02$), a 40% decrease in 16α-OHE$_1$ formation ($P=0.04$) and a 3.7-fold increase ($P=0.02$) in E$_3$ formation. The effect of LBF on E$_2$ metabolism is presented in Table 3. Treatment of MCF-7 cells with maximum cytostatic concentration of 1% LBF resulted in a 3.9-fold increase ($P=0.04$) in 2-OHE$_1$ formation, a 33% decrease in 16α-OHE$_1$ formation and a 10.5-fold increase ($P=0.01$) in E$_3$ formation.

**Modulation of E$_2$ Metabolite Ratio**

The effect of LBB and LBF on 2-OHE$_1$:16α-OHE$_1$ ratio is presented in Fig. 3A. LBB treatment resulted in a 16.3-fold increase in this ratio, whereas LBF treatment exhibited only a sixfold increase. The effect of these extracts on the E$_3$:16α-OHE$_1$ ratio is presented in Fig. 3B. LBB and LBF treatment produced a twofold and a 2.9-fold increase, respectively, in this ratio.

**TABLE 2**

Effect of Lycium barbarum bark (LBB) extract on metabolism of 17β-estradiol (E$_2$) in MCF-7 cells

<table>
<thead>
<tr>
<th>E$_2$ metabolite (ng/10^6 cells)</th>
<th>E$_2$</th>
<th>2-OHE$_1$</th>
<th>16α-OHE$_1$</th>
<th>E$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>E$_2$ + LBB</td>
<td>20 nM</td>
<td>2.8 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>E$_2$ + LBB</td>
<td>0.05%</td>
<td>15.1 ± 6.8</td>
<td>3.1 ± 0.2</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

*One-day-old cultures treated with E$_2$ alone or E$_2$ + LBB. E$_2$ metabolites determined after a 48-h treatment. *Means ± SD, n = 3 per treatment group. **$P=0.004$, ***$P=0.02$, ****$P=0.04$. 

**FIG. 1.** A: Dose response of Lycium barbarum bark (LBB) extract on MCF-7 cells. Viable cell number determined at Day 7 post-seeding of $1.0 \times 10^5$ cells. Means ± SD, n = 6 per treatment group. IC$_{50}$: 0.02%. B: Dose response of Lycium barbarum fruit (LBF) extract on MCF-7 cells. Viable cell number determined at Day 7 post-seeding of $1.0 \times 10^5$ cells. Means ± SD, n = 6 per treatment group. IC$_{50}$: 0.38%.

**FIG. 2.** Inhibition of anchorage independent (AI) growth by Lycium barbarum bark (LBB) and Lycium barbarum fruit (LBF) extracts in MCF-7 cells. AI colonies counted at Day 21 postseeding of 1000 cells per well. Means ± SD, n = 12 per treatment group. Serum vs. E$_2$ $P=0.02$, E$_2$ vs. LBB, E$_2$ vs. LBF $P=0.01$. 
TABLE 3
Effect of Lycium barbarum Fruit (LBF) extract on metabolism of 17β-estradiol (E2) in MCF-7 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>E2 Concentration (nm)</th>
<th>E1</th>
<th>2-OHE1</th>
<th>16α-OHE1</th>
<th>E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>20 nM</td>
<td>2.6 ± 0.1a</td>
<td>1.1 ± 0.6a</td>
<td>2.7 ± 0.8</td>
<td>0.4 ± 0.1b</td>
</tr>
<tr>
<td>E2 + LBF</td>
<td>10%</td>
<td>8.3 ± 0.4c</td>
<td>5.4 ± 0.5f</td>
<td>1.8 ± 0.2</td>
<td>4.6 ± 0.3h</td>
</tr>
</tbody>
</table>

*a One-day-old cultures treated with E2 alone or E2 + LBF. E2 metabolites determined after a 48-h treatment. b Means ± SD, n = 3 per treatment group. cd,e,fP = 0.04, ghP = 0.01.

DISCUSSION

The hormone responsive ER⁺ clinical breast cancer is traditionally treated with selective estrogen receptor modulators, aromatase inhibitors, and/or cytotoxic chemotherapeutic drugs (4,22–24). These treatment options are frequently associated with acquired tumor resistance and adverse systemic toxicity, compromising long-term patient compliance. Herbal medicinal products with potential to enhance therapeutic efficacy and reduce toxicity represent an attractive treatment option in complementary and alternative medicine (5–8,10). The long-term safety, efficacy and lack of adverse reactions of herbal medicines with conventional chemo-endocrine therapy, is however, largely unknown.

Human carcinoma derived cell culture models such as the ER⁺ MCF-7 cell line provides a rapid mechanistic approach to obtain clinically translatable data (11). Global gene expression profiling of clinical breast cancers have categorized the subtypes Luminal A, Luminal B, HER-2 enriched, basal-like, and triple negative classes depending on the status of hormone and growth factor receptors and functional gene interactions (2,3). Because of the presence of estrogen and progesterone receptor and absence of HER-2 amplification, in the MCF-7 cells, this human tissue derived preclinical cell culture model resembles the Luminal A subtype.

MCF-7 cells adapted for growth in chemically defined serum depleted (0.7% serum, <1 nM E2) exhibited a positive growth regulatory effect of E2 at physiologically relevant levels, as evidenced by decrease in the population doubling, and increase in the saturation density. AI growth represents a specific and sensitive in vitro surrogate end point biomarker for tumorigenic cell lines (14–18). In accord, it is noteworthy that E2 treatment resulted in increased number of AI colonies. These data taken together provide evidence for persistent endocrine responsiveness of the model.

The dose-response experiments with LBB and LBF to identify effective IC₅₀ concentrations revealed about a 19-fold greater potency of LBB, relative to that of LBF for cyto-static growth arrest in MCF-7 cells. In addition, LBB and LBF at their respective maximum cytostatic concentrations produced a 93.2% and 89.2% reduction in the number of E2 promoted AI colonies. The data generated from these comparative experiments taken together indicate a greater potency of LBB for efficacious growth inhibition of MCF-7 cells. The data generated with LBB and LBF for the induction of cyto-static growth arrest and reduction of carcinogenic risk at relatively low doses identify valuable phenomenological leads for the efficacy of these extracts. The maximally effective low doses may result in acceptable toxicity profiles in future animal experiments and clinical trials. In this context, it is noteworthy that the present MCF-7 model has demonstrated growth inhibitory efficacy of nonfractionated aqueous extracts from several herbal preparations, such as Lycium barbarum, Tabebuia avellanda, and Cornus officinalis, also known as Fructus cornii. These nonfractionated aqueous extracts appear to operate via several distinct mechanisms, including modulation of E2 metabolism, as well as altered expression of several target genes involved in estrogen receptor synthesis, cell cycle progression, cellular apoptosis, and xenobiologic metabolism (12–16). Furthermore, our recent comparative study conducted on isogenic MCF-7 cells selected for functional and nonfunctional ER phenotypes have provided leads to evaluate preferential efficacy of nonfractionated extracts from select Chinese nutritional herbs on the two phenotypes (18). However, it needs to be emphasized that

FIG. 3. A: Modulation of 2-OHE1:16α-OHE1 ratio by Lycium barbarum bark (LBB) and Lycium barbarum fruit (LBF) in MCF-7 cells. Means ± SD, n = 3 per treatment group. E2 vs. LBB P = 0.004, E2 vs. LBF P = 0.003, LBB vs. LBF P = 0.04. B: Modulation of E2:16α-OHE1 ratio by LBB and LBF in MCF-7 cells. Means ± SD, n = 3 per treatment group. E2 vs. LBB P = 0.04, E2 vs. LBF P = 0.04, LBB vs. LBF P = 0.04.
the use of nonfractionated aqueous extracts simulates the route of administration followed in traditional herbal medicine, and therefore, may represent the patient intake of herbal products. It is also conceivable that these nonfractionated extracts may contain a mixture of bio-active components affecting selected cellular functions, responsible for their clinical efficacy.

The cellular metabolism of E2 includes Cyp450-mediated oxidative metabolism. Initially, the mitogenic hormone E2 is converted via C17-oxidation to E1. This intermediate metabolite functions as a common precursor either for the formation of 2-OHE1 via C2-hydroxylation, or for the formation of 16α-OHE1 via C16α-hydroxylation pathways, respectively (25–28). These metabolites have documented growth modulating effects on MCF-7, or other E2 responsive cell lines. Thus, 2-OHE1 has potent antiproliferative effects, whereas 16α-OHE1 functions as a promitogenic metabolite (29–32). The data generated from the experiment, measuring the cellular metabolism of E2, clearly demonstrated that LBB treatment predominantly affected the C2-hydroxylation pathway, resulting in increased formation of antiproliferative 2-OHE1. In contrast, LBF treatment essentially accelerated the reduction of promitogenic 16α-OHE1 to relatively inert E3. The data generated from these experiments essentially extend and confirm our previous report that Lycium barbarum used as a single agent, inhibits the growth of ER+ MCF-7 cells by accelerating the conversion of 16α-OHE1 to E3 (12,15).

Because E1 functions as a bifunctional intermediate metabolite for C2- or C16α-hydroxylation pathways, and because 16α-OHE1 is converted to the proximate metabolite E3 (24,25), the status of 2-OHE1:16α-OHE1 and E3:16α-OHE1 ratios provides a better means to investigate experimental modulation of cellular metabolism of E2 and thereby, may provide novel endocrine biomarkers (12,14–16,18). The primary data from E2 metabolism experiments expressed as 2-OHE1:16α-OHE1 ratio revealed that LBB treatment induced a 16.3-fold increase in the ratio, whereas LBF treatment produced only a sixfold increase. The promitogenic 16α-OHE1 is reduced to inert E3 (12,14–16,18,25–27). In accord, the E3:16α-OHE1 ratio in response to treatment with LBB or LBF exhibited a twofold and a 2.9-fold increase, respectively. These data taken together suggest that LBB and LBF affect distinct pathways of E2 metabolism to exert their growth inhibitory effects on MCF-7 cells.

The data generated from the present study demonstrating growth inhibitory efficacy of LBB and LBF at relatively low concentrations and operating via modulation of E2 metabolism taken together, offer a proof of concept for a strategy to combine LBB and LBF extracts for a superior preventive regimen against hormone responsive clinical breast cancer. This strategy may identify a potential clinical prevention trial to assess efficacy and establish relevant dose schedule.

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This study is dedicated to the memory of Laurie Mezzalingua (1968–2009). During that period, Laurie selflessly and generously devoted herself to helping many others suffering from breast cancer.

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