The Cytotoxic Effect of Oshá on Human Breast Cancer Cells, Normal Human Blood Cells, and Embryonic Mouse Cells

Ashley Jackson
Dr. Mary McCarthy-Hintz, Faculty Mentor

Abstract
Oshá or oshala is the traditional name of plants in the genera Lomatium and Ligusticum that are used medicinally by indigenous peoples in North and Central America. Oshá root has been used medicinally in women’s medicine as a natural contraceptive, an abortifacient, an emmenagogue, and an oxytocic agent. Given its traditional use in women’s medicine, previous researchers hypothesized that it would have an effect on estrogen receptor positive breast cancer cells. In a previous study in this laboratory, ethanolic extracts of certain oshá root extracts showed cytotoxic effects on the breast cancer cell lines MCF-7, BT-474, and MDA-MB-231. Aqueous extracts had no cytotoxic effect on any of the assayed cell lines. The current study investigated the effects these same extracts have on normal human peripheral blood mononuclear cells (PBMC) and mouse embryonic cells (3T3). This study produced the EC50 values of 80 ± 3, >100, and >100 mg/ml for Ligusticum porteri, Ligusticum grayi, and Lomatium californicum extracts, respectively, on PBMC, and 40 ± 1 and 55 ± 1 mg/ml for L. grayi and L. californicum extracts, respectively, on 3T3. These findings indicate that the traditional use of oshá as an abortifacient may be due to its cytotoxicity towards embryonic cells. Further investigation of these extracts and their cytotoxic effects on other types of cells is warranted.

Introduction
Scientists are taught from the very beginning to consider all aspects of biological phenomena, to question every conclusion, to research every angle of a problem, and to dig for knowledge on given topics. However, scientists today often restrict themselves to a Western way of thinking and, therefore, alienate an untapped resource of knowledge that Kimmerer (2002) describes as Traditional Ecological Knowledge (TEK). TEK as described by Berkes, Folke, and Gadgil (1995) is the holistic belief that with the privilege of such knowledge obtained from the earth and the practice thereof comes a responsibility to the earth. Thus, taking advantage of resources that aid in the healing of peoples must be met with the responsibility of not despoiling the environment, the source of aforementioned resources.

As progress is made in the sciences, researchers in fields ranging from pharmaceutical laboratories to agriculture are beginning to tap into the TEK provided by indigenous peoples. These peoples have a vast array of untapped knowledge that includes medicine and pharmacology. Researchers follow a similar line of thinking parallel to that of TEK, Scientific Ecological Knowledge (SEK; Kimmerer 2002). TEK and SEK are derived from the same source but, due to western influences, SEK has become more of a monopolized relationship in which we take from the environment but give nothing back. Both systems yield valuable information that progresses human life, but there is a definite need for integration of TEK and SEK.

As a scientist and a student, this researcher has been trained to examine all the evidence and to discuss alternative pathways for biological happenings. However, in biology and chemistry curricula, scientists are unknowingly ignoring an entire body of knowledge that lies with native peoples and that has the potential to make significant headway in finding cures. Researchers in pharmaceutical laboratories and agriculture are beginning to acknowledge the culture and practices of indigenous peoples in scientific research (Kimmerer 2002). In the past few decades, there has been a movement in biological research to explore this untapped knowledge.

Literature Review
Throughout history, peoples all over the world have been using medicinal plants to cure illnesses. A common example is willow bark, which has been used by peoples in Europe, Africa, and the Americas as a medicinal plant to alleviate a number of symptoms, including fever due to the common cold and painful menstruations (Jochle 1974; Moerman 1986). With the advancement of medicinal chemistry, the active compound in willow bark has been chemically synthesized and modified into the commonly known medicine, aspirin. It was through this example that scientists saw the significant roles that plants can serve as effective alternative treatments for many illnesses.

The study of medicinal plants in different cultures is called ethnopharmacology. Despite the increase in research on flora, only a small percentage of plant species have been chemically and pharmacologically investigated, leaving a vast potential of virtually untapped ethnopharmaceuticals to be studied. There is still a continuous search for new cytotoxic agents from natural sources worldwide (Cragg and Newman 1999). For instance, traditional doctors of Southern Thailand were interviewed and were cited to have employed numerous plants in therapies for cancer treatments (Ihtarat, Singchangchai, and Ratanasawan 1998; Ihtarat et al. 2004). Nature has provided many effective anticancer agents that are currently used. Some have been derived from microorganisms, such as
dactinomycin and doxorubicin, and others from plants, such as vinblastine, taxanes, etc. (Ruffa et al. 2002). Unregulated cell growth is the universal property of tumor cells, and many research projects have investigated the cellular growth control mechanisms; this knowledge has contributed to the identification of compounds with specific antitumoral activity (Kang, Jim, and Jang 2000). With this basis, research labs are able to explore the scientific basis of the effects of many ethnopharmacological agents on cancer (Cordel, Beecher, and Pezzuto 1991; Popoca et al. 1998).

Historically speaking, women’s health has been dealt with through natural means in many cultures around the world. Countless plant extracts have been used as anti-fertility agents in traditional medicinal practices for millennia, with great success. For instance, the targets of traditional remedies concerning women’s health of the Chumash tribe include dysmenorrhea, premenstrual syndrome, feminine hygiene, heavy menstruation, urinary tract infections, parturition, lactation, infant care, menopause, sexually transmitted diseases, fertility, contraception, and abortions (Adams and Garcia 2005). In a recent study, these herbal remedies were found to be used as natural contraceptives (to prevent ovulation or fertilization), abortifacients (to prevent implantation), emmenagogues (to stimulate uterine flow) or oxytocic agents (to stimulate uterine contractions, particularly to promote labor (Conway and Slocumb 1979; Kumar 2011; Ritchie 2001). Researchers today have found many synthetic agents for regulating such things as fertility; however, these often cause many adverse side effects, including hormonal imbalance, hypertension, increased risk of cancer, and weight gain (McNamara 1996). This highlights the need to bridge western knowledge and indigenous knowledge in the field of women’s medicine.

Umbelliferous plants play an important role in various traditional medical systems including Traditional Chinese Medicine and the Traditional Native American Medicine (Li et al. 2003). For instance, the roots of Angelica sinensis (Oliv.) Diels, of the family Umbelliferae, is one of the most commonly used traditional drugs in China, indicated against anemia, menstrual disorders, amenorrhea, and rheumatism (The Pharmacopoeia Commission of the People’s Republic of China 1992). The three plants that were investigated in the current study, Lomatium californicum, Ligusticum grayi, and Ligusticum porteri, are all Umbelliferous plants used in the Americas.

In the western part of North America, Lomatium is the largest genus of the family Umbelliferae (Hardig and Soltis 1999), with numerous species being used as therapeutics, by Native Americans of California (French 1971; Strike, Beck, and Roeder 1994). The roots of L. californicum have been found to contain Z-ligustilide, among other bioactive constituents (Asuming et al. 2005; Chou et al. 2006; Zschocke et al. 1998). A second report on L. californicum described the phytotoxic and antifungal activity of Z-ligustilide (Meepagala et al. 2005), which showed significant cytotoxic activity. A closely related plant species, Lomatium dissectum, was used to treat pulmonary complaints such as colds, coughs, pneumonia, tuberculosis, lung hemorrhage, and asthma (Turner, Bouchard, and Kennedy 1980; Turner et al. 1990). In 1986, Moerman cited numerous native peoples who also used this plant medicinally, and it was purported that many potential antiviral applications could be found. From this launched a study that reported L. dissectum to have antimicrobial activity (Cardellina and Van Wagenen 1985; McCutcheon et al. 1995; Van Wagenen, Huddleston, and Cardellina 1988). The genus Ligusticum, also of the family Umbelliferae, includes 60 species. Numerous studies have been published on the chemical composition of organic extracts or essential oil of plants in the genus Ligusticum, but only a few have reported on their biological activities. Scientific knowledge regarding biological activity and active components of L. porteri, also known as oshá, is sparse (Medina et al. 2005). Like the roots of L. californicum, Ligusticum porteri roots contain Z-ligustilide (Zschocke et al. 1998). In another study, aqueous and ethanol extracts of oshá root showed no significant cytotoxic effects on the human breast cancer cell line MCF7/AZ at a concentration of 50 μg/ml. It was noted, however, that an increased concentration might prove useful (Daniels et al. 2006). Another study done by Cole et al. (2007) showed that the essential oil from the leaf of L. porteri showed considerable cytotoxic activity against three human tumor cell lines, including MCF7. In the current study, the effects of L. porteri root extracts were investigated on MCF7 and other cell lines.

<table>
<thead>
<tr>
<th>Scientific Name</th>
<th>Common Names</th>
<th>Affiliated Tribe(s)</th>
<th>Medicinal Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ligusticum grayi</td>
<td>oshá, oshála, Gray’s licorice root, gray’s lovage</td>
<td>Atsugewi</td>
<td>Cold remedy, cough medicine, gastrointestinal, panacea</td>
</tr>
<tr>
<td>Ligusticum porteri</td>
<td>oshá, chuchupate, porter’s lovage, bear root, mountain ginseng, nipo, Indian root, mountain carrot, Colorado cough root, bear medicine, wild lovage, wild parsley plant, chuchupate</td>
<td>Zuni, Apache, Chiricahua, Mescalero, Chumash, Yuki</td>
<td>Cough, bronchial conditions, painful menstruation, retained placenta, fevers, digestive disorders, toothache, panacea</td>
</tr>
<tr>
<td>Lomatium californicum</td>
<td>oshá, California rock parsnip, celery weed, California lomatium</td>
<td>Karok, Yuki, Kawaiisu, Yuki, Yurok, Poliklah</td>
<td>Dietary aid, cold remedy, gastrointestinal aid, bronchial conditions, panacea</td>
</tr>
</tbody>
</table>

Oshá is a highly palatable forage plant, and peoples from several Native American tribes have long been using the roots of oshá for its medicinal properties. Oshá

Table 1

Ethnobotanical data for the oshá roots investigated. Affiliated Tribes are the Native American tribes known to utilize these plants for medicinal uses. The list is thorough but may not be inclusive.
root prepared as medicine is known as chuchupate, and, before modern medicines were available, it was considered a panacea or cure-all, and its uses are still continued today. It is becoming recognized in the modern western world for its all-purpose medicinal qualities (Dodson and Dunmire 2007). Today, herbalists are beginning to say that it is a whole drug store within itself, where its effects are cleansing and purifying to the entire system (Bowen 1895). The use of chuchupate as a panacea led a previous researcher in this laboratory to hypothesize that it would be useful in cancer treatment. The use of chuchupate as an emmenagogue, which is a substance that has the ability to provoke menstruation, and an abortifacient, to terminate difficult pregnancies, further piqued interest in how this plant would affect breast cancer cells, possibly providing an alternative therapy for healing cancer.

Cytotoxicity screenings provide important preliminary data to help select plant extracts and natural products with anticancer potential for future work (Cardellina et al. 1999). In a previous study, chuchupate was investigated for its ability to inhibit the growth of breast cancer cells (Bautista 2005). The roots of three species of oshá: *L. porteri*, *L. grayi*, and *L. californicum*, were used in the previous study. The anti-proliferative effects (ability to inhibit growth), and/or cytotoxic effects (ability to kill cells) were tested on three human breast cancer cell lines: BT-474 and MCF7, which are both estrogen-receptor positive (proliferate in the presence of estrogen), and MDA-MB 231, which is estrogen-receptor negative (estrogen independent; Bautista 2005). In a subsequent study, it was concluded that all three of the oshá root extracts did indeed have anti-proliferative effects on the BT-474 breast cancer cell line. Even more impressive was *L. porteri* which showed significant anti-proliferative or cytotoxic effects towards all three breast cancer cell lines, indiscriminate of cell type, giving EC50 values (the concentration that kills 50% of the cells) of 32 to 80 mg/ml.

Although it was observed that *L. grayi* and *L. porteri* had a profound cytotoxic effect on breast cancer cells, there was a need to analyze the effects that these same extracts had on normal cells to determine if further research on this medicinal plant was warranted. Specifically, in the current study the EC50 values were determined on normal human peripheral mononuclear blood cells (PMBC) and on mouse embryonic cells (3T3) and compared to the EC50 values found for breast cancer cells.

### Methodology

#### Plant Collection

All three plant samples were collected for a previous research student, Marisa Bautista (Bautista 2005). Dried *L. californicum* was collected near Chuchupate Campground in Los Padres National Forest, California, and was a gift from J. Timbrook of the Santa Barbara Museum of Natural History (California), where a voucher specimen is stored. Dried *L. grayi* was collected at Gold Lake, California, and was a gift from Christopher Hobbs. Dried *L. porteri* was purchased from Garden of Grace Botanicals (Cornville, Arizona).

#### Extraction Procedure

An extraction procedure was prepared during the previous study performed by Marisa Bautista (Bautista 2005). To prepare aqueous extracts, dried oshá root (10.0 g) was chopped finely, added to 250 ml of boiling deionized water, boiled for 20 minutes and steeped for a 24-hour period. After cooling, the pH was adjusted to 7.2 by addition of 0.5 M NaOH. Ethanolic extracts were prepared by grinding one gram of fresh or dried oshá root to a fine meal using a mortar and pestle, and then extracting with 25 ml of 95% ethanol for 20 minutes. All extracts were vacuum-filtered through 3 mm filter paper. Filtrates were centrifuged at 2000 Xg for 5 minutes. Supernatants were sterilized by filtration through a 0.20 mm filter. One milliliter of each extract was dried under vacuum and the mass was determined. Sterile extracts were stored at -20°C. Dilutions were prepared by adding sterile-filtered water or 95% ethanol to aqueous or ethanolic oshá root extracts, respectively. Test media was prepared by adding 1% (v/v) of the various dilutions to growth media. Control media contained 1% (v/v) sterile water or 95% ethanol.

#### Cytotoxicity Assay on Breast Cancer and Embryonic Cell Lines

Three human breast adenocarcinoma cell lines (MDA-MB-231, BT-474, MCF7) were used, as well as the mouse embryonic fibroblast 3T3 cell line. All cell lines were grown in an incubator at 5% CO2 and 37 °C in growth medium (Improved Minimal Essential Media, Richter’s modification [Corning Cellgro] containing 10% fetal bovine serum [Axenia Biologix], 1000 U penicillin [Fisher Bioreagents], and 0.1 mg/ml streptomycin [Fisher Bioreagents]).

For the assay, cells were washed with growth medium. The medium was decanted, and cells were detached with 1.5 ml 0.025% trypsin-EDTA (Sigma), and then medium was added to a volume of 5 ml. The cell pellet, obtained by centrifugation (1200 x g, 5 min), was resuspended in 5 ml of medium to make a cell suspension. Viable cell density was determined by the trypan blue exclusion assay in a hemacytometer, and then cell suspension was diluted with medium to a density of 50,000 cells per ml. One hundred µl of these cell suspensions were seeded into each of ten wells in a 96-well microtiter plate, which was then incubated at 37 °C for 48 hours to allow for cell attachment.

#### Preparation and Cytotoxicity Assay on PBMC

Thirty milliliters of whole blood were collected by venipuncture into evacuated collection tubes containing sodium heparin as anticoagulant on ice, following a
protocol approved by the California State University, Sacramento, Institutional Review Board for use of human subjects. Prior to the PBMC procedure, the collection tubes were inverted to mix the blood.

Fico/Lite™- LymphoH was supplied from Atlanta Biologicals (Atlanta, Georgia) with a density of 1.077 ± 0.001 g/ml @ 20°C and an osmolality of 290 ± 10 mOs/kg. Fifteen ml of the Fico/Lite solution was pipetted into a 50 ml sterile centrifuge tube. Twenty ml of phosphate-buffered saline (PBS; I.9 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, 154 mM NaCl, pH 7.2) was mixed with 20 ml of anti-coagulant-treated whole blood. The blood/PBS mixture was carefully layered onto the Fico/Lite, making sure a distinct boundary existed between the diluted blood and the Fico/Lite solution (Boyum 1968; Fuss et al. 2009; Hokland and Heron 1980). Centrifugation at 1800 rpm for an hour resulted in the separation of PBMCs at the blood-Fico/Lite interface. Other white blood cells and red blood cells, which are unusable, pass through the interface and collect in the bottom layer. Above the PBMC layer, which is in the middle, is the upper layer that contains plasma and platelets. This layer was carefully aspirated without disturbing the mononuclear cell layer at the interface. With a clean pipet, the mononuclear cell layer was carefully collected at the interface, minimizing the amount of Fico/Lite (bottom layer) withdrawn with the sample. The PBMC layer was then washed with sterile PBS to remove any contaminating separation medium. The number of viable cells was assessed with the Trypan Blue exclusion assay (Mishell and Shiigi 1980). After collection and prior to inoculation, cells were resuspended at a density of 1 x 10⁶ cells per ml in media. Then 50 μl of cell suspension was seeded into each well of a 96-well microtiter plate. The cells were then immediately inoculated with 50 μl of a media-extract series (ten wells per concentration). Plates were then incubated for 48 hours, and the numbers of viable cells (relative to the control cells containing only vehicle) were assessed with the Cell Titer 96 Aqueous One Solution Assay (Promega Corporation; Cory et al. 1991). The absorbance of the formazan product was measured at 490 nm with a microtiter plate reader (BioRad). All assays were performed in ten wells. The EC₅₀ value, the concentration of the plant extract that reduced the absorbance at 490 nm to half the control value, was determined using the ED50Plus V1.0 software.

Results and Discussion

The criteria established by the US National Cancer Institute to consider a medicinal plant worthy of further investigation is an EC₅₀ less than 30 μg/ml in the preliminary assay of a crude extract (Suffness and Pezzuto 1990). The range of doses assayed in this study was 20-100 μg/ml. The aqueous extracts exhibited no activity towards any of the tested cell lines (data not shown). EC₅₀ values for ethanolic extracts are displayed in Table 2. The EC₅₀ values differed from those obtained by a previous researcher in the laboratory (Bautista 2005); in most cases, the EC₅₀ values obtained in this study were less than those in the previous study. This may be due to degradation of the extract components over time. As in the previous study, the L. porteri extract was the most potent. Notably, it showed significantly more cytotoxicity against all three breast cancer cell lines than against PBMC. This may indicate that the extract preferentially targets fast-growing cells, since unstimulated PBMC grow very slowly, if at all. The EC₅₀ value of the L. porteri extract against 3T3, another fast-growing cell line, were also well below the EC₅₀ values against PBMC, supporting this hypothesis. Additionally, the cytotoxicity of L. porteri and L. californicum against these embryonic cells offers scientific corroboration of the use of oshá as an abortifacient.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EC50 Value (μg/ml)</th>
<th>L. californica</th>
<th>L. grayi</th>
<th>L. porteri</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT – 474</td>
<td>60 ± 3</td>
<td>81 ± 2</td>
<td>32 ± 1</td>
<td></td>
</tr>
<tr>
<td>MDA – MB – 231</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>60 ± 1</td>
<td></td>
</tr>
<tr>
<td>MCF – 7</td>
<td>&gt; 100</td>
<td>97 ± 5</td>
<td>53 ± 1</td>
<td></td>
</tr>
<tr>
<td>PBMC</td>
<td>&gt; 100</td>
<td>&gt; 100</td>
<td>80 ± 3</td>
<td></td>
</tr>
<tr>
<td>3T3</td>
<td>55 ± 1</td>
<td>N/A</td>
<td>40 ± 1</td>
<td></td>
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</tbody>
</table>

Although all of the EC₅₀ values are above the established NCI value, it is likely that fresh extracts will give different results. Therefore, further testing with fresh extracts, while taking care to decrease error and refine the EC₅₀ values, will be the next step in this study. The results of this study have helped not only to shed light on the scientific richness that can be found in traditional medicine, but also to find supporting evidence for the ethnomedicines that have been utilized by peoples for millenia.
References


