An Evaluation of the Anti-Proliferative Effects of ESSIAC® on \textit{in vitro} and \textit{in vivo} Models of Prostate Cancer

by

Andy Eberding

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Department of **Pharmaceutical Science**

The University of British Columbia  
Vancouver, Canada  

Date  **Aug 22, 2003**
ABSTRACT

Prostate cancer is the most prevalent cancer in men of western nations. Major research advancements in prostate cancer diagnosis and treatment have been made over the past several decades. Unfortunately, there are no curative treatment options available for late stages of prostate cancer. Often patients with these disease states turn to alternative medicines. ESSIAC® is one of these treatments. ESSIAC® is an extract of a blend of Burdock root, Indian Rhubarb root, Slippery Elm bark and Sheep Sorrel.

Purpose: The intention of this study is to provide objective preclinical evidence of ESSIAC’s ability to exert an antiproliferative effect on both in vitro and in vivo prostate cancer cells and the tumors they generate.

Methods: An HPLC method was developed and employed to ensure that successive commercial lots and escalating concentrations of ESSIAC® were similar in content based on the peak area produced. The extract was tested in cell culture by means of both a crystal violet cytotoxicity assay and a cell cycle analysis. The acute toxicity of the extract on healthy athymic nude mice was tested, as well as ESSIAC®’s efficacy in inhibiting subcutaneous PC-3 tumor growth. The treated tumors were analyzed for the expression levels of both Ki-67 and PCNA.

Results: The HPLC analysis indicated that the within-lot variation of the extract was small with <2% RSD for most of the analyzed peaks. The between-lot variation, despite being large when considered on an absolute scale, was small when the peak areas were considered relative to the total respective peak area. No cytotoxicity was recorded with 48 hr ESSIAC® treatments. Similar results were obtained from a cell cycle analysis. Using body weight as an indicator, no toxicity was observed in the athymic nude mice using doses ranging up to 240 mg/kg QD, over 28 days. The orally dosed ESSIAC® treatment groups did not differ significantly from controls
during a 28 day efficacy study. Analysis of proliferation marker expression levels confirmed the results of the efficacy study.

Conclusions: The preclinical evaluations performed in this study suggest ESIAC® aqueous extract has no marked antiproliferative action on the prostate cancer models tested.
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**LIST OF ABBREVIATIONS**

ANOVA ------------ analysis of variance  
AR -------------- androgen receptor  
BID -------------- dosed twice daily  
BPH -------------- benign prostate hyperplasia  
BSA -------------- bovine serum albumin  
CAD -------------- Canadian Dollars  
CAM -------------- complementary and alternative medicine  
CBCRI ------------ Canadian Breast Cancer Research Initiative  
dH₂O -------------- deionized water  
DHT -------------- dihydrotestosterone  
DMEM -------------- Dulbecco’s modified Eagle medium  
DNA -------------- deoxyribonucleic acid  
DRE -------------- digital rectal examination  
EDTA -------------- ethylenediamine tetraacetic acid tetrasodium salt  
FBS -------------- fetal bovine serum  
HPLC -------------- high performance liquid chromatography  
mRNA -------------- messenger ribonucleic acid  
MTT -------------- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
PBS -------------- phosphate buffered saline  
PCNA -------------- proliferating cell nuclear antigen  
PSA -------------- prostate specific antigen  
QD -------------- dosed once daily  
RCF -------------- relative centrifugal force  
RPMI -------------- Roswell Park Memorial Institute  
RSD -------------- relative standard deviation
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DEDICATION

This work is dedicated to...

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Section 1

Introduction
1.1. Background

1.1.1. Cancer

Cancer has been a growing scourge for the last century and is poised to continue as such. It has been predicted that over 136,000 Canadians will be diagnosed with cancer in 2002; in 50% of the males and 46% of the females the disease will be fatal. In men, the most prominently afflicted sites are the prostate, lung, and colorectum. When combined, these sites account for 57% of all new cancer cases. Prostate cancer tops the list of new cancer diagnoses in men; lung cancer, however, has the greatest number of cancer related deaths attributed to it, followed closely by prostate cancer. Prostate cancer’s lower death rate (as compared to lung cancer) is due to a combination of late age, typically, of diagnosis and slow disease progression. Because of the disease’s characteristics, many prostate cancer patients die of unrelated causes before their cancer becomes lethal (1).

1.1.2. Prostate Cancer

1.1.2.1 Epidemiology

Epidemiological studies of prostate cancer have revealed that both genetic and environmental factors contribute to the incidence of the disease. Racial studies have revealed that both incidence and mortality rates are higher within the African-American segment of the US population than it is in the Caucasian segment. In the Chinese, Filipino, Hawaiian, Japanese, and Hispanic segments’ the incidence rates are all considerably lower than the Caucasian incidence rates (2). These data implicates a strong genetic predisposing factor in prostate cancer incidence. In contrast, immigrant Japanese men in America have higher rates of incidence than those men who have remained in Japan (3). This implies that there is a strong environmental factor contributing to incidence.
In western societies, autopsy based reports suggest that as many as 30% of men beyond the age of 50 have undetected prostate cancer (4). It should be noted, however, that this information predates advancements in diagnostic testing for prostate cancer detection, such as the serum prostate specific antigen (PSA) test; therefore, it is quite plausible that the incidence of prostate cancer has decreased markedly since that time. However, prostate cancer can have a long latency period, during which, it is completely asymptomatic.

1.1.2.2 Oncogenes and Tumor Suppressor Genes

The role of oncogenes and tumor suppressor genes in the development of prostate cancer continues to be elusive despite extensive research into the issue. The Ras oncogene, which codes for a signal transduction protein, was initially reported to be found in prostate carcinoma tissue and not benign prostate hyperplastic (BPH) tissue (5). Later studies, however, reputed the conclusiveness of this information (6). Myc, a nuclear phosphoprotein mediating DNA replication, cell cycle regulation and differentiation, was also suggested to have higher expression levels in prostate cancer than in normal and BPH tissues (7), but these findings were also refuted with contrary results (8). Similar conflicting information has been published regarding the oncogene Erb-B2, with some groups finding increased expression in prostate cancer (9), while others report no difference when compared with BPH (10). Definitive evidence supports the finding that the anti-apoptotic Bcl-2 gene has increased expression in hormone independent regrowth and lower levels in earlier stages of progression (11).

The contribution of known tumor suppressor genes to prostate cancer etiology is equally as uncertain. p53 Mutation incidence rates, for example, have been reported ranging from 0-80% (12). It has been indicated that cells develop p53 mutations as the disease progresses (13). The influence
of retinoblastoma, a cell cycle mediator, is also contentious as abnormalities are seen in both prostate cancer and BPH (14). BRCA-1 mutations are implicated in cases where there familial is clustering (15) but its role in the general population has not been elucidated.

1.1.2.3 Androgen Receptor

Androgens are essential for tumor initiation and for the androgen receptor (AR) mediated effects of androgens that lead to prostate cancer progression. Huggins and Hodges (16) first described the effects of altered androgen levels on prostate conditions over 60 years ago. Normal mature prostates require androgen to maintain size and function (17). The ligand with the highest binding affinity to the AR is dihydrotestosterone (DHT). In the prostate, testosterone is converted intracellularly to DHT by 5α-reductase (18). Following dimerization, the DHT/AR complex binds to DNA androgen response elements found in promoter regions of target genes (19).

Developmental studies of the prostate gland have shown how the AR plays a major role in tumor differentiation (20).

Because of the AR’s effects as a transcription factor in prostate cancer cells, the alteration of systemic androgen levels is sometimes used to treat prostate cancer. Unfortunately, the androgen dependent tumor growth inhibition is only temporary; ultimately the tumor growth recurs. When it does, androgen suppressive treatments are an ineffective treatment. There are a variety of contributing factors that allow prostate tumor cells to thrive in the absence of androgen. Firstly, the population of cells prior to treatment is actually heterogeneous, containing both androgen dependent and independent cells (21). Following treatment, the cells have evolved into a more uniformly androgen independent population. During anti-androgen therapy, androgen dependent cells regress. The more scarce population of hormone independent cells eventually form a tumor
mass capable of tumor regrowth and spread. Gene amplification and mutation of the AR are known to occur (22). This alteration allows the cells to proliferate binding to adrenal androgens or other steroidal ligands (23). Cell culture studies have found that these cells can survive without the AR gene, AR mRNA or receptor protein (24). Research continues into the mechanistic basis of this adaptation.

1.1.2.4 Prostate Cancer Stages and Treatments

Prostate cancer treatments are almost as varied as the people who receive them. It is a complex disease and every patient should receive a treatment appropriate for his individual prognosis. There are several aspects that should be considered when deciding upon an appropriate course of treatment including his disease stage, general health, age and level of sexual activity; also, many men find that social and emotional factors have a substantial impact and contribute to their final decision. Despite this fact, clinicians have developed a system for charting stages of the disease (25). When cancer is present but is still sufficiently small so that it remains undetectable during a digital rectal examination (DRE) it is considered a Stage T1a cancer. This group is further classified as Stage T1b if the cancer is in less than 5% of the prostate, and Stage A2 if the cancer exceeds 5% of the prostate. Only 15% of T1a cancers will develop further and those that do require 10-15 years to do so. Of the T1b cancers, only 35% will lead to metastatic cancer. If these patients are old or in poor health no treatment is recommended, however, the younger, healthier patients may receive a radical prostatectomy or radical radiation therapy. Detection at this stage is rare. The earliest stage that prostate cancer is normally detected is T1c (26). The fact that detection is possible through the presence of high serum PSA sets these cancers apart from the ones in the other T1 stages. Stage T1c cancers require pelvic lymph nodes be surgically investigated for indications of metastatic cells. Stage T2a cancers are detectable by DRE with a size of less than 2 cm in diameter, while Stage T2b
cancers are greater than 2 cm in diameter. With aggressive treatments 90% and 50% of patients diagnosed with diseases in Stage T2a and T2b, respectively, can be cured. The treatment for otherwise healthy men might include neo-adjuvant hormone therapy followed by a radical prostatectomy, while older and/or less healthy men might receive hormone therapy only. Stage T3 cancers are present in both sides of the prostate gland and have grown outside the prostate capsule. There is no cure for this disease stage, only treatments, similar to T2b, will act to control the disease. Stages M+ and N+ describe cancers that have metastasized to the lymph and bones, respectively. All treatments at this stage are palliative in nature and might include any of hormone withdrawal, radiation and chemotherapy. Many clinical trials are conducted with new therapeutic agents in this cohort of prostate cancer patients.

1.1.2.5 Androgen Dependence

Prostate cancer may be classified into either androgen dependent or androgen independent disease. This categorization is independent of the staging described above, as all stages may contain both androgen dependent and androgen independent cells. A tumor can be metastatic and still responsive to hormone therapy; alternatively, stage T1 tumors are known to be heterozygous for their androgen responsive nature. Androgen independence progression usually only occurs following androgen ablation; without treatment, androgen dependent cells may eventually metastasize and cause death. Hormone withdrawal therapy of androgen dependent disease simply suppresses tumour growth and thus, prolongs life.

Hormone therapy is the mainstay in prostate cancer treatment. The objective of hormone therapy, therefore, is to decrease or abolish androgen effects. This can be accomplished via surgical or chemical castration (prevention of testicular androgen release by inhibiting the release of stimulating
hormones). This action impedes disease progression, since the prostate cells are dependent on the presence of androgens.

Unfortunately, surgical or chemical castration is only effective temporarily. Some cancerous cells are able to persist and, eventually, flourish in the absence of androgens. This ability defines the hormone independent state of prostate cancer. There is little that can be done to slow hormone independent cancer. There is no accepted ‘gold standard’ treatment for hormone independent prostate cancer (27). Auclerc et al (28) reviewed various chemotherapeutic regimens. This paper states, “...no randomized study has been able to demonstrate the benefit of chemotherapy on survival rate”. However, Auclerc et al recognize doxorubicin, mitoxantrone, taxanes and other chemotherapeutic agents as requiring further study. Most treatments for androgen independent prostate cancer are directed at quality of life. Other treatments that are currently under investigational development include gene targeting therapies (29) and photodynamic therapies (30). It is obvious, therefore, that there is a desperate need for discovery, testing and development of treatments for this disease state.

1.2. Complementary and Alternative Therapies

Complementary and alternative medicine (CAM) use is utilized by a wide cross section of prostate cancer patients; the two groups who employ these treatments the most are often those with the fewest treatment options, individuals with hormone responsive metastatic cancer and individuals with hormone independent prostate cancer progressing to more advanced stages. There are many nutritional and herbal products that claim to be useful for the treatment of prostate cancer, some have begun to be scientifically evaluated while others have not. Below are a few of the most well recognized CAM treatments in use and is by no means a comprehensive list.
1.2.1. Nutrients and Micronutrients

Beta-carotene, a provitamin and carotenoid found in orange and dark green vegetables, has been shown to reduce the growth of prostate cancer cell lines PC-3, DU145 and LNCaP (31, 32). Due to rapid conversion of beta-carotene to Vitamin A in rodents, no studies in animal models are available (33). Beta-carotene has conflicting results in clinical studies (34); interestingly, there appears to be toxicity at higher levels resulting in increased risk of prostate cancer development and tending toward more aggressive disease (35).

Lycopene, another carotenoid found in many red produce items like tomatoes, has been reported to reduce PSA to a statistically insignificant extent in a small study (n=26) of pre-surgical prostate cancer patients (36). Case-control studies have produced conflicting results, however cohort and nested case control studies indicate a benefit is derived from tomatoes, its product and lycopene intake (34). Some conclusions have been ascribed specifically to lycopene, while other micronutrients from the tomato may be contributing to the outcome observed.

Vitamins A, C, D, and E have all been tested for their contributions to prostate cancer development suppression. All four vitamins have shown an ability to decrease proliferation in cultured cells through one mechanism or another (37-40). In a large cohort study, Vitamin A was not able to reduce prostate cancer risk, a five year follow-up identified only a small set of cases were reported (41). Clinical studies regarding the relationship between Vitamin C and prostate cancer development have produced conflicting results (34). Calcitriol, the active form of Vitamin D, causes dangerous serum calcium levels when taken in high doses therefore it is of limited use as a therapeutic in this form. A review of the many studies involving Vitamin E and an individual’s risk of prostate cancer
development finds that there is an inverse relationship and that Vitamin E may have a preventative role (34).

Selenium is a trace element that is a cofactor for the anti-oxidant glutathione peroxidase. High levels of the element reduce prostate cancer risks (42). In patients with high levels of selenium that did develop prostate cancer, the disease was found to be less aggressive than those who had lower levels.

1.2.2. Natural Products

Many natural products are purported to be therapeutic for prostate cancer. Besides those described below the list also includes Pau D’Arco, Mistletoe, Panax Ginseng, Saw Palmetto, Shark Cartilage and Thuja. The prominent CAM treatment for prostate cancer in recent years is PC-SPES. This blend of eight Chinese herbs had gained an unprecedented status for its ability to limit prostate cancer’s growth. An extensive amount of research was performed on the product at all levels (43-53). The results that had been reported were varying in degrees of success at preventing the proliferation of cancer cells. The US Food and Drug Administration halted production of PC-SPES, however, after the synthetic compounds, diethylstilbestrol (DES) (54) and warfarin (55), were detected in the blend. DES is an estrogenic compound that has historically been used to treat prostate cancer (56). It has yet to be determined whether the positive results of earlier studies with PC-SPES were or were not attributable to the adulterants. Green tea, a common drink from China and Japan, contains a component, epigallocatechin-3-gallate, which induces apoptosis in DU145, PC-3 and LNCaP cell lines (57, 58). Garlic has been shown to be successful in inhibiting the proliferation of CRL-1740 a hormone dependent prostate cell line (59).
1.3. ESSIAC®

1.3.1. What is ESSIAC®?

ESSIAC® is a proprietary mixture of four herbs: Burdock root (*Arctium lappa*), Indian Rhubarb root (*Rheum Palmatum*), Slippery Elm ark (*Ulmus fulva* or *Ulmus rubra*) and Sheep Sorrel (*Rumex acetosella*). There are several accounts of the initial conception and the subsequent saga of this blend of herbs, which includes claims for its effectiveness as a cancer therapy and the plight of those who choose to include the treatment in their medical practices (60-62). The history begins in 1922, when a nurse Rene Caisse was working in northern Ontario. A patient, who had professedly been treated for breast cancer exclusively with an Ojibwa herbal tea many years previous, bestowed Caisse with the recipe. Shortly, thereafter, Caisse’s aunt developed untreatable stomach cancer and was given a prognosis of six months to live. Caisse proceeded to tend to her aunt with the formulation she had acquired. Her aunt reportedly lived another two full decades.

Over the next several decades, Caisse spent much time treating terminal cancer patients with the tea (63). She had, by this time, named the formulation Essiac. As well as treating patients, she was struggling with numerous legal challenges arising from the unconventional therapy she was offering without a medical license (63). During this time, she conducted some research, both clinical and animal studies; however, the results were never published in peer-reviewed journals (64). Caisse was reluctant to reveal the recipe to anyone, concerned that the formulation would be exploited for profit (63). This hampered many studies by limiting the amount of the extract available for research purposes. In 1977, shortly before her death Caisse relinquished and sold the formula to Resperin Corporation (62). Eventually, the rights to the blend came to be held by Essiac Products Inc. (63). There are many similar products available from natural health suppliers, by mail order and online.
due to high degree of notoriety the mixture enjoys within the alternative medical community. The most prominent of these replication products is Flor-Essence, however, this product differs substantially from the ESSIAC® in that it contains four additional herbs. Although the producers of Flor-Essence claim to hold a recipe developed by Caisse (65), the origins of the recipe are unclear and it does not trace distinctly back to Caisse. Other products make various claims about the source of their formulation with a wide variation in the persuasiveness of the explanations. This work will only focus on the product manufactured by Essiac Products Inc. and will be referred to as ESSIAC® so as not to be confused with the multitude of 'generic' products available.

1.3.2. Usage

During the early years of ESSIAC® history, usage was limited to the quantity that Caisse was able to concoct, as she alone had the knowledge of how to blend ESSIAC®. Today however, Essiac associated products have quite a substantial sales base. Tamayo (65) reports that Essiac Products Inc. have sold $3.2 million CAD worth of product annually. From the same source, the value for Flor-Essence sales was quoted to be $4.5 million CAD annually. Overall, total sales of Essiac related products were estimated to be above the $8 million CAD mark yearly (65).

A single retail package of the ESSIAC® product sells for $18 to $25 CAD; therefore annual sales represent approximately 130000 retail packages. The manufacturer recommends a course of treatment that requires the use of 8 units per patient. If every user followed this therapeutic regimen, it may be calculated that there are a minimum of 16000 ESSIAC® users annually from this blend alone. Actually, this is likely to be an underestimate of the number of users, as some people take the extract in a preventative capacity, and use less per person, resulting in more individual users.
1.3.3. Current State of Research

1.3.3.1 ESSIAC®

Although it may be true that the product is often used for breast cancer, there has been no research into the disease states that ESSIAC® is most commonly used for nor is the product specifically marketed to any specific group of cancer patients. The Canadian Breast Cancer Research Initiative (CBCRI) compiled the most complete review of the research that has been done on ESSIAC® (64). This review concludes that ESSIAC® shows “Weak evidence of effectiveness. Little evidence of harm. This is a widely used agent which has been incompletely studied.” Health Canada issued an unfavorable statement in 1989 entitled “Essiac: an ineffective cancer treatment”. This review was based on the results of a clinical study that Health Canada gave permission to be conducted in 1978 (66). Other authors, however, counter this statement by charging that the clinical study that was conducted was incomplete. The Government of Canada withdrew its permission to continue the clinical study in 1982; therefore, the data that was used to generate the statement is invalid (60, 62). CBCRI uncovered some preclinical testing that was performed on ESSIAC® by Rene Caisse and Dr. Charles Brusch but the results were never published and no specifics are given. Dr. Brusch is on record as stating that the results of these tests were positive and that there was a synergistic effect in the blend (64). All other information on the blend appears to be incomplete, unpublished or is simply anecdotal.

Despite the fact that Dr. Brusch claimed an important synergistic effect, there are numerous studies available authenticating the presence of anti-cancer compounds within each of the individual herbs and other closely related herbs.
1.3.3.2 Burdock (*Arctium lappa*)

Burdock contains several antioxidants including flavonoids and polyphenols (67). Lin et al (68) reported that Burdock extracts have an antioxidant capacity. The seed of the plant contains polyacetylene and tannin antimicrobials (69-71). An extraction of the plant was reported to reduce the effects of known mutagens in an *in vitro* environment (72). Quercetin, a flavone from Burdock, has been shown to decrease the action of benzo[a]pyrene (73). Yanagihara et al (74) report that several flavones in Burdock, namely genistein, biochanin A, nobiletin, and tangeretin, possess an ability to initiate apoptosis in stomach cancer cells *in vivo*. Two lignans of *Arctium lappa*, Arctegenin 1 and its decanoate ester, have also been shown to slow progression of M1 mouse leukaemia cells (75). Arctiin and arctegenin have been shown to have strong action against HepG2 and sarcoma 180 cells both *in vitro* and *in vivo* (76). Arctiin has displayed mammary, colon and pancreatic chemopreventive capacity (77).

1.3.3.3 Indian Rhubarb root (*Rheum Palmatum*)

*Rheum palmatum* has been shown to contain several anthraquinones; the list includes chrysophenol, emodin, aloe-emodin, rhein, physcionin, citreosein, senosides A, B, C, D, E, and F, napthalins, and stilbenes (78). Emodin is the most highly studied; it is reputed to have anti-inflammatory, antiseptic, antitumor, cytotoxic, and viricidal capacity (78). Emodin displays both cytotoxicity and mutagenicity in breast FM3A and C3H cells (79). Rhubarb extracts used to treat sarcoma 37 cells *in vivo* led to increased tumor necrosis (80, 81). Emodin countered the action of known food mutagens in bacterial cells (82). Aloe-emodin inhibits P-388 lymphocytic leukaemia tumor growth (83). *In vivo* mammary and Ehlich-ascites carcinomas have been inhibited by rhein and emodin (65). Emodin also shows antimutagenic activity (84). Rhubarb administered orally to mice was reported to have immunomodulatory effects (85).
1.3.3.4 Slippery Elm bark (*Ulmus fulva* or *Ulmus rubra*)

Slippery elm contains polysaccharides that forms a mucilage when hydrated (86). The bark also contains several antioxidants such as β-sitosterol and β-carotene (78). Fatty acids found in *ulmus rubra* have been shown to induce cell death of ascitic tumor cells of Ehrlich carcinomas in mice (87). Although not scientifically tested, Slippery elm has traditionally been used to treat carcinomas (88).

1.3.3.5 Sheep Sorrel (*Rumex acetosella*)

The list of compounds found in Sheep Sorrel contains rutin, flavone glycosides, hyperin and the anthraquinones: emodin, aloe-emodin, chrysphanol, rhein and physcion (88, 89). The anthraquinones listed are also found in *Rheum palmatum*. Therefore, research related to these compounds has already been detailed above. Like *Ulbus rubra*, *Rumex acetosella* was widely used by indigenous people as a traditional cancer remedy (90).

1.4. Objective

The comprehensive goal of this work is to determine whether ESSIAC® has effects on prostate cancer cells *in vivo* and *in vitro*.

1.5. Significance of the Research

The overall impact of this work is that it will provide the medical community and prostate cancer patients with a, previously unavailable, pre-clinical evaluation of ESSIAC®'s potential as a treatment for prostate cancer. This work focuses predominately on the effectiveness of the extract on PC-3 cells *in vivo* and *in vitro*. PC-3 cells are generally considered to have the characteristics of late stage
prostate cancer; therefore, the results would be most relevant to patients with a corresponding condition. However, a direct correlation should not be assumed.

1.6. Hypothesis

The need for pre-clinical research with respect to ESSIAC® herbal blend can be justified for a number of reasons. These include anecdotal evidence with respect to the actions of ESSIAC®, the ability of the blend to remain on the market over many decades, widespread use of ESSIAC® as a complementary therapy and the lack of prior rigorous research. In support of the anecdotal evidence, the individual herbs have been shown to contain components with known therapeutic efficacy. Given the above motives for performing pre-clinical research into the actions of ESSIAC®, the research hypothesis is:

ESSIAC® herbal aqueous extract has antiproliferative effects on *in vitro and in vivo* prostate cancer models.
Section 2

Specific Aims and Rationale
2.1. Specific Aims

Aim 1:

To verify the consistency of ESSIAC® aqueous extractions within-lot and between-lot.

Aim 2:

To determine if ESSIAC® extract has an *in vitro* cytotoxic effect on PC-3 and LNCaP prostate cancer cell lines.

Aim 3:

To determine the level at which, if any, ESSIAC® extract is toxic in athymic mice.

Aim 4:

To establish the growth kinetics of the androgen independent tumor (PC-3) in athymic mice.

Aim 5:

To conduct an efficacy study of ESSIAC® extract on an androgen independent prostate tumor model *in vivo* using athymic mice.

Aim 6:

To explore expression of proliferation markers in tumor samples collected in Aim 5 through the use of Tissue Micro Array screening.
2.2. Rationale

2.2.1. Aim 1: To verify the consistency of ESSIAC® aqueous extractions within-lot and between-lot.

The goal in this aim was to verify that components of the product are consistently present in multiple preparations and lots. The objective of the method to be developed was to analyze the homogeneity of the extracts prepared from each lot by comparing extracts prepared from successive lots. Separation of each individual compound was not a goal of this project.

A variation in the chemical make-up of ESSIAC® might be attributed to a number of pre-disposing factors affecting herbal preparations. Firstly, raw plant matter may vary a great deal as a result of the environmental conditions that the plants were grown in, harvesting technique, and plant age (91). Secondly, because individual compounds are not being used as a method of identification there is no way of knowing whether other plants with a similar fingerprint have been substituted into the blend. The manufacturer of ESSIAC® claims to maintain control over raw herb identity purchasing the ingredient herbs from a horticultural supplier that grows the herbs specifically for the manufacturer (Personal correspondence, 2002).

2.2.2. Aim 2: To determine if ESSIAC® extract has an in vitro cytotoxic effect on PC-3 and LNCaP prostate cancer cell lines.

2.2.2.1 Objective

This work was conducted in an effort to observe if ESSIAC® preparation has an ability to slow the proliferation rate of prostate cancer cell lines in an in vitro environment. The in vitro effects of an herbal preparation may be markedly different from the effects in vivo. One reason for this is the
large number of compounds that are found in a crude extraction. Systemic metabolizing enzymes may affect any active agents. If this is true, no effect is likely to be seen in vitro. However, if the compound is active in the absence of metabolizing enzymes, this will indicate that no activation is required. Alternatively, if multiple active agents are responsible, a reduced effect may be seen in the in vitro environment because only some of the component compounds have intrinsic activity.

For an herbal preparation, the applicability of an in vitro system is questionable, however, it may provide insight for future mechanistic studies. The proposed assays are relatively easy to perform and are typically done in pre-clinical evaluations; therefore they have been included in this project.

2.2.2.2 Cell Lines

This Aim uses a system that provides a rapid means of determining if a treatment has cytotoxic effects on an in vitro system. The cell lines to be used here were PC-3 and LNCaP. The PC-3 cell line was derived from a prostate cancer metastatic tumor (92). It has been fully characterized: the cells are androgen independent and do not express the AR or produce PSA. The LNCaP cell line is also very well characterized and used extensively in prostate cancer research. The line originated from a metastatic lesion and displays all the characteristics of a hormone responsive prostate carcinoma (93). It is androgen dependent, expresses PSA and a mutated AR. The natures of these cell lines are characteristic of late androgen independent and transitional androgen responsive for PC-3 and LNCaP, respectively. These cell lines were chosen because they are representative of the later stages of prostate cancer; the stage when patients are the most likely to be using ESSIAC®.
2.2.2.3 Crystal Violet Assay

The method utilized to determine cytotoxicity was the crystal violet assay. Results of the crystal violet assay are based on the overall quantity of membrane protein available for staining in each well (94, 95). Therefore, the absorbance reading for each well reflects the number of cells present, not a functional capacity of the cells. If the treatment is cytotoxic, there will be fewer cells present and, hence, less protein for the stain to bind to, thereby decreasing the absorbance recorded.

Originally, consideration was given to testing the cytotoxic effect of ESSIAC® using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. However, recently published research contraindicates its use with herbal products; phytoestrogens have the potential to reduce MTT to formazan (96, 97). Rather than determine if phytoestrogens in ESSIAC® have such a reductive potential, a decision was made to employ the crystal violet assay.

2.2.2.4 Validation

This portion of the project began with a series of experiments to determine the most favorable conditions in which to carry out the crystal violet assay. The specific variables that were considered included the number of cells required for each cell line and the length of time to allow the cells to proliferate (so absorbance readings can be maximized).

2.2.2.5 Cell Cycle Analysis

Using a DNA binding dye, such as propidium iodide, and a flow cytometer it is possible to determine the amount of DNA present in an individual cell in a high throughput method. This relatively simple technique allows investigators to determine whether or not a treatment influences or causes cell cycle arrest. The classic example of a drug that does this is paclitaxel, which will be
used as a positive control for these experiments. If cell cycle is affected, further experiments may be performed to pinpoint the mechanism by which treatment is producing this effect. This experimental section provides supportive follow up to the crystal violet cytotoxicity assay with more mechanistic detail regarding the confirmation of results; any disruption to the cell cycle should be reflected in the cytotoxicity assay.

2.2.3. Aim 3: To determine the level at which, if any, ESSIAC® extract is toxic in athymic mice.

2.2.3.1 Objective

When novel compounds are being developed to treat any disease, a rigorous pre-clinical evaluation must be conducted in accordance with Health Canada and/or US Food and Drug Administration regulations prior release of the compound to the general public. A Phase I clinical trial is the first step in a series of assessments required to take place and is carried out prior to testing in a diseased human population. The purpose of a Phase I trial is to observe a small number of healthy individuals receiving gradually increasing quantities of the treatment compound (dose escalation) – mainly investigating for effects or toxicities.

Analogously, this aim will serve to perform the same function in a pre-clinical evaluation in mice. Although the test material is a mixture of natural compounds, the treatment could still be toxic. It would be unwise to begin an efficacy study with a large number of animals only to learn that the mice were experiencing toxicity and that the study must be cancelled. Therefore, for the purposes of this objective, healthy athymic mice will be treated with escalating doses of aqueous ESSIAC® and a 20% of greater drop in body weight employed as an endpoint for acute toxicity.
2.2.3.2 Dosages

The dose prepared for the mice will be based upon the manufacturer’s recommendation for humans, converted to an approximate mouse dosage based on the body mass differential. The recommended human dose is 24 mg/kg. This level will be considered a 1X dose. The study will also include 2X, 5X and 10X doses (48, 120, and 240 mg/kg).

A translation from human to mouse dosage to be based solely on body mass is not the only manner in which treatment dosages can be converted. This simple conversion was chosen to represent a minimum value. Other methods of conversion attempt to account for the higher metabolic rate observed for mice when compared to human rates. Ultimately, these conversions result in a requirement for a higher dosage level than the minimum seen here. However, the need to use a more complex method to convert the dosage has been negated, as an escalating series of exposure levels, up to 10-fold of the suggested minimum, are planned. The maximum dose was chosen because of the prohibitive nature of the extraction process. Attempts at extracting ESSIAC® at concentrations higher than 10-fold were unsuccessful, as the raw material has an inherent ability to retain water; this characteristic was attributed to slippery elm bark’s known capability of forming a mucilage. Therefore, the range of concentrations listed includes both high and low extremes without modifying the extraction process or including super-dilutions.

These preparations will be analyzed using the High Performance Liquid Chromatography (HPLC) method developed for Aim 1. The peak areas of the fingerprint will provide an indication of when a saturation point of a component may have been reached. The increase in each peak area should correspond to the increase in concentration e.g. 2X, 5X or 10X. Should one of the peaks not increase the predicted amount it is likely that the representative compound(s) has reached its limit of
solubility. Each extraction will be prepared individually, not through a process of serial dilution; thereby, providing an HPLC fingerprint that truly corresponds to the concentration in an effort to detect such a saturation point.

2.2.4. Aim 4: To establish the growth kinetics of the androgen independent tumor (PC-3) in athymic mice.

2.2.4.1 Objective

Aim 4 has been included for two specific purposes. The first purpose is to establish the growth rate of PC-3 cells in vivo. Although this cell line has been used quite extensively and is reputed have a very high tumor generating ability (by researchers associated with our center), a search of the literature was unable to identify any sources that revealed the specifics of the growth of a PC-3 tumor in a control situation. Our centre published results of a study in which PC-3 tumors were treated with antisense oligonucleotides (98), however, a control group was treated with “mismatch oligonucleotides”; therefore the tumor growth profile of control or no treatment still remains to be determined.

The second purpose is to allow the author the opportunity to develop skills in consistently measuring tumors. Unfortunately, these cells do not express PSA, a biomarker used to follow disease progression in humans. Without the PSA serum concentration as an index of tumor growth, the only method of tracking the tumor growth is by physically measuring the tumor size. Tumors tend to grow in many irregular shapes and sizes. Researchers who are going to use such measurements in their investigations must learn to do so precisely prior to conducting any large-scale studies or the results might be skewed by poor technique.
2.2.5. Aim 5: To conduct an efficacy study of ESSIAC® extract on an androgen independent prostate tumor model \textit{in vivo} using athymic mice.

\textbf{2.2.5.1 Objective}

Aim 5 is designed to test ESSIAC®'s efficacy in the treatment of a human prostate tumorigenic cell line \textit{in vivo}. PC-3 is the cell line to be used. This cell line was chosen because PC-3 cells represent a late stage metastatic prostate cancer cell. This is reflective of the most common period during which ESSIAC® is used by prostate cancer patients.

The dosage groups will be defined by the maximum tolerated dose as determined in the toxicity study results in Aim 3. The proposed number of treatment groups is lower than the number used in Aim 3, however, because more mice are required in each group to provide statistical significance to the acquired efficacy data. Two groups will receive a maximal dosage; one group will be dosed every 12 hrs and the other group will be dosed every 24 hrs. The 12 hr dosing regimen is reflective of the recommended routine for human treatment. The maximal dosing at 24 hr intervals will allow a direct comparison between the efficacy of a 24 hr treatment schedule and an equivalent dose administered on a 12 hr treatment schedule. The third group will replicate the recommended human treatment protocol, in both dosage and schedule. The fourth group will be included as the control; these mice will receive water via oral gavage once per day at the same volume as the ESSIAC® treated groups. The final group will provide a standard or positive control (micellar paclitaxel) to which the results from the other groups can be compared (99). This is not an ideal control as it is delivered intravenously and the study groups will be delivered via oral gavage. Unfortunately, no known positive control treatment for this prostate cancer model is available for oral administration.
and so while micellar paclitaxel is not controlled for with respect to its route of administration, is the closest to an ideal positive control that is currently available.

2.2.6. Aim 6: To explore expression of proliferation markers in tumor samples collected in Aim 5 through the use of Tissue Micro Array screening.

2.2.6.1 Objective

The objective was to compare the expression levels of proliferation markers in ESSIAC® treated tumors with those in control tumors. Tumor tissue harvested from treatment groups in the ESSIAC® in vivo efficacy study of Aim 5 were evaluated. Tissue Micro Arrays involve the placement of multiple small but intact sections together on a single slide and allow tissue samples from numerous sources to be stained simultaneously. This technique ensures that while all samples are situated on a single slide, they receive as near to identical treatments as possible. A Tissue Micro Array is only one possible technique that could be used to determine the potential differential expression levels between the treated and control groups. The benefit of using this technique is the reduction in the amount of time required to obtain results of the analysis. Since multiple slides (easily 50 or more) can easily be produced obvious additional benefits are gained. If the results require further immunohistochemical investigation other slides containing adjacent sections are readily available for analysis with a minimum requirement for preparation.

Proliferating cell nuclear antigen (PCNA) and Ki-67 antigens are the specific proteins that were quantified. The Ki-67 antigen is present in proliferating cells throughout the cell cycle; however, when the cell cycle is arrested the antigen is promptly degraded (100). PCNA proteins are expressed during the G1 and S phases of the cell cycle (101). By quantifying the amount of the markers in each of the treated and control tumor tissue samples, it will be possible to determine if ESSIAC® is
producing an anti-proliferative effect at a molecular level in the tumors. All other work in this project has been geared toward measuring effects at a gross or tissue level. By investigating at a molecular level, it may be possible to identify a response that may not have been quantifiable otherwise. Using a tissue micro array, multiple samples of each tumor can be analyzed for their PCNA and Ki-67 expression levels using immunohistochemistry in an expedient manner.
Section 3

Materials and Methods
3.1. Materials and Reagents

3.1.1. Instrumentation

The chromatographic system consisted of a Hewlett Packard HP 1100 HPLC outfitted with a Zorbax SB-C18 column from Agilent Technologies (Mississauga, ON, Canada) interfaced with Chem-Station for LC 3D (Rev A 08 03 C847) software also from Agilent Technologies (Mississauga, ON, Canada). The crystal violet assay absorbance readings were performed on a Bio-Tek Instruments Power Wave X linked with KC4 Kineticalc for Windows software Version 2.6 Revision 3. The flow cytometry system used was a Beckman Coulter EPIC XL-MCL 2988 Flow Cytometer (Mississauga, ON, Canada) interfaced with Beckman Coulter Expo 32 v1.2 Cytometry List Mode Data Acquisition and Analysis Software. The immunohistochemical staining was performed using an Olympus BX51 microscope coupled with a Diagnostics Instruments RT 2.2.1 color digital camera controlled by SPOT software Version 3.5.5. The extent of positive staining was then analyzed using Image Pro PLUS software version 4.5.1.22. All statistical analysis was performed using Sigma Stat software version 2.03.

3.1.2. Animals

Male athymic Nude mice were purchased at 6-8 weeks of age with a body weight of 20-25 g from Harlan Sprague Dawley Inc (Indianapolis, IN, USA).

3.1.3. Chemicals and Reagents

ESSIAC® herbal powder (Lots 17694, 17903 and 18146) was provided as a donation from Essiac Canada International (Ottawa, ON, Canada). Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) Medium 1640, Fetal Bovine Serum (FBS), 0.25% Trypsin, 1 mM ethylenediamine tetraacetic acid tertasodium salt (EDTA) were purchased from Invitrogen,
Inc (Burlington, ON, Canada). Crystal violet, glutaraldehyde, bovine serum albumin (BSA), dimethylsulfoxide, xylene, Triton, hydrogen peroxide and hydrochloric acid were obtained from Sigma-Aldrich (Oakville, ON, Canada). Trisodium citrate, EDTA, HEPES, sodium phosphate monobasic, sodium phosphate dibasic heptahydrate, sodium bicarbonate and acetonitrile were obtained from Fisher Scientific (Ottawa, ON, Canada). Ethanol was bought from Commercial Alcohols (Brampton, ON, Canada). Micellar paclitaxel used in animal studies was provided by Dr Helen Burt’s Laboratory, Faculty of Pharmaceutical Sciences, UBC, (Vancouver, BC, Canada). Acetic acid, formic acid, phosphoric acid and 10% neutral buffer were purchased from VWR Canlab (Edmonton, AB, Canada). Trifluoroacetic acid was purchased from American Chemicals, (Montreal, PQ, Canada). Ki-67 antibodies, biotinylated secondary antibodies and strepavidin-peroxidase conjugates were obtained from DAKO Cytomation (Mississauga, ON, Canada). Proliferating cell nuclear antigen antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). NovaRED and Hematoxylin were purchased from Vector Laboratories (Burlingame, CA, USA). The paclitaxel used in cell culture experiments was obtained from the BC Cancer Agency Pharmacy (Vancouver, BC, Canada).

3.2. **ESSIAC® Preparation**

ESSIAC® extraction began by adding 1.8 g of the powdered herbal product to 300 ml of filtered water. The mixture was covered and boiled for 10 minutes in a glass beaker. The extract was removed from the heat source and allowed to sit at room temperature for 4 hours. The mixture was subsequently reboiled for 5 minutes. The insoluble material was precipitated for 16 hours at 4° C. The extract was decanted off and 4° C stored at for use within one month of preparation.
<table>
<thead>
<tr>
<th>Designation</th>
<th>ESSIAC® (g)</th>
<th>Water (ml)</th>
<th>Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X</td>
<td>1.8</td>
<td>300</td>
<td>6</td>
</tr>
<tr>
<td>2X</td>
<td>3.6</td>
<td>300</td>
<td>12</td>
</tr>
<tr>
<td>5X</td>
<td>9.0</td>
<td>300</td>
<td>30</td>
</tr>
<tr>
<td>10X</td>
<td>18.0</td>
<td>300</td>
<td>60</td>
</tr>
</tbody>
</table>

**Table 1:** Quantities used to perform extractions of ESSIAC® that were subsequently analyzed for extraction efficiency and eventually used to perform both the toxicity and efficacy studies *in vivo.*

Amendments to this extraction protocol were made when the experimental design required a more concentrated extract be prepared, as was the case for the HPLC analysis, the *in vivo* toxicity study and the *in vivo* efficacy study. The alternative extraction concentrations were all prepared as described above with the exception of the supplementary herbal powder added in each instance. The volume of water used was held constant at 300 ml for each extraction. The four concentrations, denoted 1X, 2X, 5X and 10X, were prepared using 1.8, 3.6, 9.0 and 18.0 g of Essiac powder, respectively (Table 1). The 1X concentration is equivalent to that recommended by the manufacturer for human consumption.

The HPLC analysis required the evaluation of all available commercial lots of ESSIAC®: Lot 17694, Lot 17903 and Lot 18146. Throughout the rest of the study, Lot 17903 was applied exclusively. The use of all three available lots during the *in vivo* portions of the study would have been unethical, as well as being redundant given the results obtained from the HPLC analysis.

### 3.3. HPLC Analysis

Sample aliquots of preparations from each lot analyzed (17694, 17903 and 18146) were taken in triplicate. Each aliquot was centrifuged at 165 RCF for 5 min to remove the particulate matter. Each sample was passed through a 40 μm filter. The HPLC analysis was carried out with a Hewlett Packard HP 1100 series HPLC/DAD using Chem-Station for LC 3D (Rev A 08 03 C847) software.
from Agilent Technologies (Mississauga, ON). The HPLC was fitted with a Zorbax SB-C18 column (4.6×150mm, 5μm) maintained at 25° C. The mobile phase consisted of a 0.1% formic acid in water and acetonitrile gradient system. A flow rate of 0.75 ml/min was

<table>
<thead>
<tr>
<th>Flow Rate</th>
<th>0.75 ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Volume</td>
<td>5 μl</td>
</tr>
<tr>
<td>Solvent A</td>
<td>0.1% Formic Acid in H₂O</td>
</tr>
<tr>
<td>Solvent B</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Column</td>
<td>Zorbax SB-C18, Stable Bond Analytical, 4.6 X 150mm, 5μm</td>
</tr>
<tr>
<td>Column Temperature</td>
<td>25° C</td>
</tr>
<tr>
<td>Wavelength Analyzed</td>
<td>330 nm</td>
</tr>
</tbody>
</table>

**Table 2**: High performance liquid chromatography method specifications developed for analysis of the consistency of ESSIAC® extract. The complete method also included the extraction process, which was completed as outlined by the manufacturer of ESSIAC®, and the solvent gradient as outlined in Table 3.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>79</td>
<td>21</td>
</tr>
<tr>
<td>8.0</td>
<td>79</td>
<td>21</td>
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<tr>
<td>21.0</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>23.0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>25.0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>27.0</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

**Table 3**: Solvent gradient used in HPLC method described in Table 2 to perform the analysis of the consistency of ESSIAC® extract.

used to carry 5 μl of sample solution. The wavelength used for the analysis was 330 nm. The summarized details of the HPLC method are outlined in Table 2 and Table 3.

### 3.4. In vitro Assays

#### 3.4.1. Cytotoxicity Analysis of ESSIAC® with Crystal Violet Assay
Cultured LNCaP and PC-3 cells were plated into 96 well plates using RPMI and DMEM media, respectively, with 5% fetal bovine serum (FBS). To determine the linear range of this assay, a standard curve for both the LNCaP and the PC-3 cells was developed by plating each at a range from 0 to 375000 cells/ml. A hemocytometer was used to count cells; the number of cells contained in four 0.1 μl aliquots of media was averaged and multiplied by 10⁴ to determine the number of cells per ml. Cells were only counted when no aggregation was present. The standard curves were generated using the protocol as described below. The only exception was during the production of standard curves, media with 5% FBS only was added to plates instead of a treatment. Since 4000 cells were determined to be an appropriate absolute number of cells per well and 80 μl was the required volume in each well of a 96-well plate, the stock solution of cells was diluted with the appropriate media (RPMI 1640, 5%FBS – LNCaP; DMEM, 5%FBS – PC-3) to a concentration of 50000 cells/ml. In addition, each plate also contained wells with 80 μl of media only (no cells), to act as a blank control for the experiment. The cells were incubated at 37° C in 5% CO₂ for 24 hr to allow the cells to adhere to the plate. The cells were treated in octuplicate with 20 μl ESSIAC® extract diluted in media with 5% FBS, and 25% deionized water (dH₂O) to concentrations of 30, 300 and 3000 μg/ml. The resulting concentration in each well, after the addition of the treatment to the plating media, was 6, 60 and 600 μg/ml of ESSIAC® and 5% dH₂O. Also, an octuplicate of wells were treated with 20 μl media containing 25% dH₂O, to supply a final concentration of 5% dH₂O; this was included as a control for any cytotoxic effect that the dH₂O in the ESSIAC® extract may have produced. The cells were incubated at 37° C in 5% CO₂ for 48 hr. The addition of 25 μl of 4% glutaraldehyde in dH₂O along with a 5 min incubation at 37° C in 5% CO₂ fixed the cells to the plates. The media was aspirated, the cells were rinsed with 200 μl dH₂O, the dH₂O was
aspirated and the fixed cells were air-dried. The cells in each well were treated with 100 μl of 0.5% crystal violet solution (0.5g crystal violet dissolved 100 ml of 20% ethanol) for 5 min. The stain was aspirated, the cells were rinsed with 200 μl dH2O, the dH2O was aspirated and the stained cells were air-dried. The plates were cleaned manually with cotton swabs dipped in ethanol, removing any stain residue that was not directly bound to the cells. To solubilize the dye, 100 μl of Sorensen’s solution (9 mg trisodium citrate, 195 ml 0.1 N HCl, 500 ml 90% ethanol and 305 ml dH2O) was added to each well and incubated at room temperature for 5 min. The absorbance was recorded using Bio-Tek Instruments Power Wave X interfaced with KC4 Kineticalc for Windows Software Version 2.6 Revision 3. The recorded relative absorbance for each treatment group was normalized by subtracting the average absorbance recorded for the octuplicate of blank wells from each plate. The resulting average relative absorbance for the dH2O treated controls was then used to calculate the percentage of cytotoxicity resulting from each treatment group.

3.4.2. Cell Cycle Analysis

Flow cytometry analysis was initiated when 5x10^5 PC-3 cells were plated onto 100 mm diameter tissue culture plates in DMEM media with 5% FBS. The cells were incubated at 37°C in 5% CO2 for 24 hr to allow the cells to adhere to the plates. The media and non-adherent cells were aspirated from the plates. The cells were treated, in triplicate samples, with 0.6, 0.06, 0.006 mg/ml ESSIAC®, or 0.007 mM Paclitaxel in DMEM with 5% FBS, 5% dH2O. The Paclitaxel treatment media also contained 0.1% dimethylsulfoxide. The cells were incubated for 48 hr at 37°C in 5% CO2. The media was collected from each plate and placed into 15 ml tubes. Each plate had 10 mM EDTA, 20 mM HEPES, 0.1% Bovine Serum Albumin @ pH 7.4 in phosphate buffered saline (PBS) was then added to each plate to lift cells. The cells were incubated for 5 min at 37°C in 5% CO2 and washed.
off the plate by careful and repeated pipetting of the lifting solution with a 1000 µl pipette. The lifting solution was transferred into the 15 ml conical tubes that the corresponding treatment media had been placed into previously. In addition, 35 µl 100 mM CaCl₂, 100 mM MgCl₂ was added to each tube to neutralize the EDTA. The cells were centrifuged at 165 RCF for 5 min. The supernatant was aspirated and each pellet was rinsed with 1 ml PBS, 0.1% glucose. The cells were centrifuged again at 165 RCF for 5 min. The supernatant was aspirated again leaving 200 µl of rinse in the tube. The cells were resuspended by gently vortexing the tube for 10 sec while adding 1 ml 0° C ethanol. The cells were stored for approximately one week at 4° C. The cells were pelleted by centrifugation at 375 RCF for 5 min. The supernatant was aspirated and resuspended in propidium iodide staining solution (10 µl - 10 mg/ml RNase A, 5 µl - 10 mg/ml propidium iodide per 1ml of PBS, 0.1% glucose). After 30 min, the cells were analyzed using a Beckman Coulter EPIC XL-MCL 2988 Flow Cytometer interfaced with Beckman Coulter Expo 32 v1.2 Cytometry List Mode Data Acquisition and Analysis Software.

3.5. Toxicity in Athymic Nude Mice

Eight groups of three male athymic nude mice were administered ESSIAC® at doses of 24, 48, 120, 240 mg/kg daily by oral gavage using the 1X, 2X, 5X, and 10X. Four groups were dosed once daily and four groups were dosed at 12 hr intervals with half the daily dose. The control group was administered water, the vehicle. Groups were organized as shown in Table 4. The mice were dosed for 28 days and observed for signs of acute toxicity such as weight loss, lack of coordination and lethargy. The mice were terminated at the completion of the study by cervical dislocation following asphyxiation with CO₂.
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose</th>
<th>Schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>N/A</td>
<td>24 hr</td>
</tr>
<tr>
<td>2</td>
<td>PO ESSIAC®</td>
<td>24 mg/kg QD</td>
<td>24 hr</td>
</tr>
<tr>
<td>3</td>
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<td>48 mg/kg QD</td>
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</tr>
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<td>4</td>
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<td>24 hr</td>
</tr>
<tr>
<td>6</td>
<td>PO ESSIAC®</td>
<td>12 mg/kg BID</td>
<td>12 hr</td>
</tr>
<tr>
<td>7</td>
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</tr>
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<td>12 hr</td>
</tr>
<tr>
<td>9</td>
<td>PO ESSIAC®</td>
<td>120 mg/kg BID</td>
<td>12 hr</td>
</tr>
</tbody>
</table>

Table 4: Specifications for the treatment groups (n=3) in the 28 day toxicity study of ESSIAC® extract in athymic mice.

3.6. PC-3 Tumor Growth Kinetics in Athymic Nude Mice

Twelve athymic nude mice were subcutaneously inoculated with approximately $2 \times 10^6$ PC-3 cells at a single dorsal position. Once a tumor became palpable, measurements of the developing tumor volume commenced. All twelve mice that were inoculated developed a tumor prior to the initiation of the tumor measurements. The tumors were measured semiweekly until the animals became moribund. The tumors were measured in three dimensions linearly and the volume was calculated using two formulas (102) —

$$\text{Volume} = \left(\frac{1}{6}\pi\right) \times \text{Length} \times \text{Width} \times \text{Depth}$$
$$\text{Volume} = \frac{1}{2} \times \text{Length} \times (\text{Width})^2$$

The tumors were removed at the conclusion of the study and their mass was recorded.

3.7. Effect of ESSIAC® on Tumor Growth in Athymic Nude Mice

Five groups of 11 male athymic nude mice were included in this study. Group One was administered 200 mg/kg ESSIAC®, as a 100 mg/kg dose twice daily. Group Two was administered 200 mg/kg ESSIAC® in a single daily dose. Group Three was administered 20 mg/kg ESSIAC® as a 10 mg/kg dose twice daily. Group Four was administered a bolus of water, equivalent to the volume...
of ESSIAC® received by Group One, in a single daily dose. Groups dosed twice daily received their treatments at 12 hr intervals. All the treatments listed above were administered orally. Group Five was administered intravenous 1.67 mg/kg micellar paclitaxel via a tail vein on Days 1-5 inclusive of a 14 day cycle repeated twice during the study. The treatment groups are outlined in Table 5.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dosage</th>
<th>Schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ESSIAC® po</td>
<td>100 mg/kg</td>
<td>2X/day</td>
</tr>
<tr>
<td>2</td>
<td>ESSIAC® po</td>
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<td>1X/day</td>
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<td>3</td>
<td>ESSIAC® po</td>
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<td>2X/day</td>
</tr>
<tr>
<td>4</td>
<td>Vehicle (Water) po</td>
<td>N/A</td>
<td>1X/day</td>
</tr>
<tr>
<td>5</td>
<td>Micellar Paclitaxel iv</td>
<td>1.67 mg/kg</td>
<td>Day 1, 2, 3, 4, &amp; 5 of a 14 day cycle</td>
</tr>
</tbody>
</table>

Table 5: Specifications for the treatment groups (n=11) in the efficacy study of orally administered ESSIAC® extract in athymic mice. The Paclitaxel treated group was administered a single bolus dose via a tail vein according to the schedule described.

The mice were subcutaneously inoculated with $2 \times 10^6$ PC-3 cells in a single dorsal site and tumors were generated in every mouse inoculated. Mice were stratified according to both tumor size and body mass 20 days following inoculation. For each treatment group, tumors were measured linearly in three dimensions semiweekly using calipers. To provide blinding to the study, technical support was available; this prevented the same person who dosed the mice from also taking the tumor measurements. Mice were terminated by cervical dislocation following asphyxiation with CO₂ and the tumor, liver, small intestine and prostate was collected from each animal. The mass of each tumor was recorded and correlated to the calculated final tumor volume.

Upon harvesting the tumors in Aim 5, the tissue was separated into two approximate half sections. One portion was stored at -80°C for potential future studies, for example, Micro Gene Array or proteomic analyses. The other portion was fixed by immediately immersing the sample in 10% neutral buffer for 24 hr, followed by long-term storage in 80% ethanol for use in Tissue Micro Array analysis in Aim 6.
3.8. Analysis of Tumor Protein Expression

3.8.1. Tissue Micro Array

The tumor samples were enclosed individually in cassettes, placed into a paraformaldehyde bath and sent to VGH Pathology Laboratory (Vancouver, BC, Canada) for paraffin embedding. Seven 0.6 mm diameter core samples from each tumor were transferred from each paraffin embedded tumor sample to a principal recipient paraffin block using a MTA-1 manual tissue arrayer (Beecher Instruments Microarray Technologies, Sun Prairie WI). The principal block contained two 7×11 grids of tissue core samples; one grid containing the 200 mg/kg BID treated and one grid containing the control tumors from the efficacy study. The recipient block was subsequently sent to the Morphological Services Laboratory at UBC Hospital (Vancouver, BC, Canada) where 100 sections at a standard 4 μm thickness were cut and mounted on glass microscope slides.

3.8.2. Ki-67 Immunohistochemical Staining and Analysis

A single tissue micro array section was deparaffinized and rehydrated by incubating in xylene (2× 5 min), 100% ethanol (2× 3 min), 95 % ethanol (2× 3 min), 70% ethanol (3 min) and rinsed in PBS (3× 5 min). The section was washed with 0.2% Triton in PBS (10 min) and washed with PBS (3× 5min). The section was heated in Citrate Buffer for 30 min and gradually cooled to room temperature. Following a PBS wash (3× 5 min) the section was incubated for 30 min with 3% BSA blocking solution and then washed again in PBS (3× 5 min). Prior to staining, 3% H₂O₂ in PBS was applied (10 min) to the section and rinsed in PBS (3× 5 min).

Ki-67 antibodies (DAKO Cytomation, Mississauga ON) were diluted at 1:100 in 1% BSA solution; the tissue micro array section was immersed in the solution and incubated overnight at
room temperature. The section was washed with PBS (3× 5 min) prior to the application of biotinylated secondary antibody (DAKO Cytomation, Mississauga ON) at room temperature for 10 min, rewashed with PBS (3× 5 min), incubated at room temperature for 10 min with strepavidin-peroxidase conjugate (DAKO Cytomation, Mississauga ON) and then washed with PBS (3× 5 min). The section was then stained with NovaRED (Vector Laboratories, Burlingame, CA) followed by washing with dH₂O (3×), counterstained with Hematoxylin (Vector Laboratories, Burlingame, CA) for 3 min and rewashed in dH₂O (3×). The section was rinsed with 2% sodium bicarbonate for 1 min followed by a wash with dH₂O (3×). Sections were dehydrated by dipping 10 times in 95% and 100% ethanol and cleared with xylene.

Each core section of the Ki-67 immunohistochemically stained tissue micro array was digitally photographed using an Olympus BX51 microscope coupled with a Diagnostics Instruments RT2.2.1 colour digital camera controlled by SPOT software Version 3.5.5. The extent of positive staining was then analyzed using Image Pro PLUS software version 4.5.1.22. A threshold level for the intensity of staining was subjectively set for the entire array using the first stained section as a control for the intensity of staining throughout. The number of positively stained cells and the total number of cells were counted in each core section. The result was expressed as the average percentage of positively stained cells per core section.

3.8.3. PCNA Immunohistochemical Staining and Analysis

A single tissue micro array section was deparaffinized and rehydrated by incubating in xylene (2× 5 min), 100% ethanol (2× 3 min), 95 % ethanol (2× 3 min), 70% ethanol (3 min) and rinsed in PBS (3× 5 min). The section was washed with 0.2% Triton in PBS (10 min) with PBS (3× 5min). The section was then heated in Citrate Buffer for 30 min and gradually cooled to room
temperature. Following a PBS wash (3× 5 min) the section was incubated for 30 min with 3% BSA blocking solution to remove background and then washed again in PBS (3× 5 min). Prior to staining, 3% hydrogen peroxide in PBS was applied (10 min) to the sample section and rinsed in PBS (3× 5 min).

PCNA antibodies (Santa Cruz Biotechnology, Santa Cruz CA) were diluted with 1% BSA solution at 1:6400; the tissue micro array was immersed in the solution and incubated overnight at room temperature. The section was washed with PBS (3× 5 min) prior to the application of biotinylated secondary antibody (DAKO Cytomation, Mississauga ON) at room temperature for 10 min, rewashed with PBS (3× 5 min), incubated at room temperature for 10 min with strepavidin-peroxidase conjugate (DAKO Cytomation, Mississauga ON) and then washed with PBS (3× 5 min). The section was then stained with NovaRED (Vector Laboratories, Burlingame, CA) followed by a wash in dH₂O (3×), counterstained with Hematoxylin (Vector Laboratories, Burlingame, CA) for 3 min and rewashed in dH₂O (3×). The section was rinsed with 2% sodium bicarbonate for 1 min followed by washing with dH₂O (3×). Sections were dehydrated by dipping 10 times each in 95% and 100% ethanol and cleared with xylene.

Each core section of the PCNA immunohistochemically stained tissue micro array was digitally photographed using an Olympus BX51 microscope coupled with a Diagnostics Instruments RT 2.2.1 colour digital camera controlled by SPOT software Version 3.5.5. The extent of positive staining was then analyzed using Image Pro PLUS software version 4.5.1.22. A threshold level for the intensity of staining was subjectively set for the entire array using the first stained section as a control for the intensity of staining throughout. The percentage area of positive staining was determined for each core section.
3.9. Statistical Analysis

All statistical analysis carried out throughout this study were performed using Sigma Stat for Windows version 2.03. Cytotoxicity assay and proliferation marker expression levels assessments were assessed using a student t-test with the p-value set at 0.05. The results are expressed as means ± standard deviation. The comparison of the treatment groups in the in vivo efficacy study was made with a Two Way analysis of variance (ANOVA). A post-hoc analysis using a Tukey test was used to indicate which group was significantly different. A One Way ANOVA was performed on the cell cycle data followed by an analysis of the groups using two post-hoc tests, the Tukey test and the Student-Newman-Keuls test. The Tukey test is more conservative; therefore, it less likely to indicate that a specific result is statistically significant than the Student-Newman-Keuls test. The Student-Newman-Keuls test is, alternatively, less likely to give a false negative. By requiring that the results pass both tests before being declared significant produces a more rigorous analysis of the data. The p-value was set at 0.05 for all tests and the results are expressed as means ± standard deviation.
SECTION 4

Results
4.1. ESSIAC® Extract Within-Lot and Between-Lot Variability

The development of the HPLC method as described in Table 2 and 3 was an extensive component of this portion of the study. Several operating conditions were varied or altered during the development of the method including flow rate, column temperature, solvent system and gradient. The mobile phase consisted of a dual solvent system consisting of acetonitrile and water. The polarity of the solvent system required was considered to be adequate, because the sample is an aqueous extract; the expectation is that it will contain primarily polar compounds, therefore, the use of a extremely non-polar solvent should not be necessary. The water was acidified with several different acids including trifluoroacetic, formic and acetic. Once formic acid was determined to be suitable for acidification, the acetonitrile was also acidified; however, no change in the chromatographic resolution was noted. A variety of different solvent profiles were evaluated ranging from an initial isocratic mobile phase to a gradient profile two-minute incremental increase in the concentration of acetonitrile. The optimal gradient profile provides a run length of 23 minutes with a 4 min post run column flushing time. As profiled in Table 3, the initial percentage acetonitrile in the run was 0% and then was rapidly increased to 21% (2.5 min) where it is held constant for 5.5 min. The acetonitrile is then slowly increased to 35% at the 21 min run-time mark and then is gradually increased, reaching 100% by minute 23. To avoid either the excessive run times produced using a flow rate of 0.5 ml/min or the poor resolution brought about by a flow rate of 1.0 ml/min and greater, a compromise was struck between the two. This resulted in the final moderate flow rate of 0.75 ml/min. Column temperatures were varied from 5° to 45° C; the high column temperatures resulted in faster elution and less resolved peaks; Alternatively, the 5° C temperature produced slower elution but also resulted in poor resolution since time was not devoted to adjusting the
Figure 1  A sample HPLC chromatograph of ESSIAC® extract acquired using the method developed specifically for this purpose and outlined in Tables 3A and 3B. The chromatograph has been labeled to identify the specific peaks that were quantified for the HPLC based consistency analysis. The specific chromatograph shown was acquired from a run of Lot 17903 – 60 mg/ml.
gradient for the slower elution rate. The differences between the extreme temperatures did not appear to produce substantially improved chromatographs; consequently the column temperature was controlled at the moderate temperature of 25° C. The final method provided a large number of peaks with sufficient resolution to allow for semi-qualitative analysis. A sample chromatograph can be seen in Figure 1.

Homogeneity of the selected peaks within-lot of Lots 17694, 17903 and 18146 is represented diagrammatically in Figures 2, 3, and 4, while homogeneity of the between-lot analysis of the selected peaks is represented diagrammatically in Figure 5. The variation in peak areas within-lot is predictably small, typically >2% relative standard deviation (RSD), however, one peak (Peak V) has within-lot RSDs of 25 and 35%. The between-lot RSD is high, near 50%, in most instances. Each peak’s relative proportion of the entire area under the curve remains rationally consistent across the three lots, as shown in Figure 6, considering the extracts were taken directly from plant matter.

The objective of the final HPLC analysis was to ensure that an increase in the quantity of material extracted would actually produce a similar increase in the peak areas; in other words, each peak area truly corresponds to the concentration of individual components. This evaluation was performed using ESSIAC® Lot 17694. In Figure 7, the selected absolute peak areas for each concentration prepared is represented. A more meaningful representation of each peak area, with each peak normalized to the size of the corresponding average 1X preparation peak, is displayed in Figure 8. Each peak’s relative proportion of the entire area under the curve is represented diagrammatically in Figure 9. The peak areas in the 2X preparation was determined to be an average of 1.9 times greater than the corresponding 1X preparation peak area. Likewise,
Figure 2 Histogram of HPLC peak areas from multiple extractions of a single commercial lot (17694) and the resulting mean corresponding peak areas ± SD. The specific peaks of interest correlate to those shown in the sample chromatograph in Figure 1.
Figure 3 Histogram of HPLC peak areas from multiple extractions of a single commercial lot (17903) and the resulting mean corresponding peak areas ± SD. The specific peaks of interest correlate to those shown in the sample chromatograph in Figure 1.
Figure 4 Histogram of HPLC peak areas from multiple extractions of a single commercial lot (18146) and the resulting mean corresponding peak areas ± SD. The specific peaks of interest correlate to those shown in the sample chromatograph in Figure 1.
Figure 5 Histogram of average ESSIAC® extract HPLC peak areas from multiple commercial lots and the resulting mean corresponding peak areas ± SD. The specific peaks of interest correlate to those shown in the sample chromatograph in Figure 1.
Figure 6 Histogram of average ESSIAC® extract HPLC peak areas from multiple commercial lots and the resulting mean corresponding peak areas ± SD all expressed as a percentage of overall peak area. The specific peaks of interest correlate to those shown in the sample chromatograph in Figure 1.
Figure 7 Histogram of four concentrations of ESSIAC® extract HPLC peak areas from a single commercial lot (17964) shown with error bars on the 10X bars indicating ± SD (n=3). The concentrations of the raw herbal powder per volume of water used to perform the extraction were 6, 12, 30, and 60 mg/ml denoted 1X, 2X, 5X and 10X, respectively. The specific peaks of interest correlate to those shown in the sample chromatograph in Figure 1.
Figure 8 Histogram of the three concentrations designated 2X, 5X and 10X of ESSIAC® extract HPLC peak areas normalized to the 1X concentration from a single commercial lot (17964). The concentrations of the raw herbal powder per volume of water used to perform the extraction were 6, 12, 30, and 60 mg/ml denoted 1X, 2X, 5X and 10X, respectively. The specific peaks of interest correlate to those shown in the sample chromatograph in Figure 1.
**Figure 9** Histogram of four concentrations of ESSIAC® extract HPLC peak areas expressed as a percentage of overall peak area taken from a single commercial lot (17964) shown with error bars on the 10X bars indicating $\pm$ SD ($n=3$). The concentrations of the raw herbal powder per volume of water used to perform the extraction were 6, 12, 30, and 60 mg/ml denoted 1X, 2X, 5X and 10X, respectively. The specific peaks of interest correlate to those shown in the sample chromatograph in Figure 1.
the peaks in the 5X and 10X preparations were on average 4.2 and 8.3 times greater than the corresponding 1X preparation peak area, respectively. The deviations in these ratios were small across the entire set of peaks analyzed with the exception of Peak V which was substantially lower at 3.0 and 6.1 times the area of the 1X preparation in the 5X and 10X preparations, respectively. The RSD of each peak's relative increase in area compared to the 1X preparation was highly stable remaining at >4% for most peaks, with the notable exception of Peak V.

4.2. The *in vitro* Effects of ESSIAC® on PC-3 and LNCaP Cell Lines

The crystal violet assay was validated to determine its linear range during a 48 hr assay. A standard curve was produced using both LNCaP and PC-3 cells. As represented in Figure 10, the data revealed a highly linear range for LNCaP when applied to a plated cell density of 2000 to 7000 cells per well in 96 well plates performed in octuplicate. Similarly, Figure 11 shows how PC-3 cells display linearity when plated at a cell density of 2000 to 10000 cells per well in a 96 well plate. This is equivalent to 2.5x10⁴ to 8.75 or 12.5x10⁴ cells/ml when initially plated.

The cytotoxicity assay has been performed on both the LNCaP and PC-3 cell lines with ESSIAC®. The effect of 6, 60 and 600 μg/ml ESSIAC® on proliferation in LNCaP cells based on the absorbance of the crystal violet dye was 98.6±6.4%, 87.3±4.6%, and 92.3±6.8%, respectively, of the proliferation seen in cells treated with an equivalent dilution of dH₂O control, as seen in Figure 12. Figure 13 shows the same concentrations applied to PC-3 cells actually produced a marginal, but not statistically significant, enhancement in proliferation of 113.7±6.7%, 126.3±12%, and 113.1±6.1%, respectively, relative to the dH₂O controls. Figure 14 depicts the crystal violet assay results of 48 hr paclitaxel treatment on LNCaP cells. This work was completed in this laboratory by another researcher using the same methodology described for the ESSIAC® crystal violet assay.
Figures 10 & 11 Standard curves produced to determine the linear range of cells plated onto a 96 well cell culture plate at the completion of a 48 hr crystal violet assay when the absorbance reading is recorded. The curves were produced for both PC-3 and LNCaP cell lines. All crystal violet assays performed were initiated by plating 4000 cells per well to assure the experimental results remained in the identified linear range. All values represent mean ± SD (n=8 wells).
Figure 12 & 13 Results of cytotoxic the evaluation of ESSIAC® extract over a 48 hr incubation period using both PC-3 and LNCaP prostate cancer cell lines with an exposure level ranging from 0.006 to 0.6 mg/ml. The results are expressed as a percentage of the controls’ absorbance. All values represent mean ± SD (n=8 wells).

Figure 14 Cytotoxicity of paclitaxel on the LNCaP cell line over a 48 hr incubation period. The data points represent mean ± SD (n=8 wells).
**Figure 15** Cell cycle analysis results of PC-3 cells treated with ESSIAC® extract (0.6, 0.06, and 0.006 mg/ml) and Paclitaxel (0.007 mM) for 48 hrs. Following incubation the cells were fixed with ethanol and the DNA was stained with propidium iodide. Each cell then had its DNA content measured using flow cytometry. Each sample analyzed contained 10000 cells. The histogram shows means ± SD of triplicate samples. A One Way ANOVA was performed on the cell cycle data followed by an analysis of the groups using two post-hoc tests, the Tukey test and the Student-Newman-Keuls test; p<.05 was considered statistically significant.
4.3. Cell Cycle Analysis of ESSIAC® Treated PC-3 Cells

The PC-3 cells that were treated with ESSIAC® had a cell cycle distribution that typically resembled the distribution seen in the control cells. For example, the control cells containing a diploid quantity of DNA, or cells in the G₁/G₀ phase, represented 49.4±0.7% of the all the control cells, while in the 0.6, 0.06, 0.006 mg/ml ESSIAC® treated had 49.1±1.6%, 52.5±1.0%, and 51.7±1.7%, respectively, of G₁/G₀ cells.

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<tr>
<th>Group</th>
<th>Dose</th>
<th>Mean % Sub-G₀</th>
<th>Mean % G₀/G₁</th>
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<th>Mean % G₂/M</th>
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<tr>
<td>Control</td>
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<td>ESSIAC®</td>
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<td>ESSIAC®</td>
<td>0.06 mg/ml</td>
<td>22.8±0.3</td>
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<td>12.5±0.3</td>
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<tr>
<td>ESSIAC®</td>
<td>0.006 mg/ml</td>
<td>23.7±1.9</td>
<td>51.7±1.7</td>
<td>7.3±0.5</td>
<td>12.9±0.6</td>
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<td>18.6±1.1</td>
<td>3.6±0.2</td>
<td>41.3±0.3</td>
</tr>
</tbody>
</table>

Table 6: Cell phase distribution of ESSIAC®, control and paclitaxel treated PC-3 cells as determined with propidium iodide staining and flow cytometry analysis

This similarity was typical across all the cell cycle phases as summarized in Table 6 and illustrated in Figure 15. Alternatively, treatment of PC-3 cells with 7 μM paclitaxel resulted in a dramatically different distribution; for instance, the G₁/G₀ phase contained 18.6±1.1% of the cells. There is no significant difference between that distribution of the control cells and those treated with ESSIAC®.

4.4. Acute Toxicity Testing of ESSIAC® in Athymic Mice

All treatment groups displayed an increase in average body weight over the treatment period ranging from 5.4% to 19.1% of the initial average body weight (Table 7, Figure 16). No animals decreased in body weight, behaved abnormally or exhibited gross indications of toxicity. Overall body weight increase had no relationship to dosage of ESSIAC® administered.
<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dose</th>
<th>Average Size in Relation to Initial Body Weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>N/A</td>
<td>110</td>
</tr>
<tr>
<td>2</td>
<td>PO ESSIAC®</td>
<td>24 mg/kg QD</td>
<td>105</td>
</tr>
<tr>
<td>3</td>
<td>PO ESSIAC®</td>
<td>48 mg/kg QD</td>
<td>109</td>
</tr>
<tr>
<td>4</td>
<td>PO ESSIAC®</td>
<td>120 mg/kg QD</td>
<td>108</td>
</tr>
<tr>
<td>5</td>
<td>PO ESSIAC®</td>
<td>240 mg/kg QD</td>
<td>119</td>
</tr>
<tr>
<td>6</td>
<td>PO ESSIAC®</td>
<td>12 mg/kg BID</td>
<td>115</td>
</tr>
<tr>
<td>7</td>
<td>PO ESSIAC®</td>
<td>24 mg/kg BID</td>
<td>108</td>
</tr>
<tr>
<td>8</td>
<td>PO ESSIAC®</td>
<td>60 mg/kg BID</td>
<td>108</td>
</tr>
<tr>
<td>9</td>
<td>PO ESSIAC®</td>
<td>120 mg/kg BID</td>
<td>115</td>
</tr>
</tbody>
</table>

Table 7: 28 day toxicity study of ESSIAC® extract in vivo treatment of athymic mice final average bodyweights. QD—dosed once daily BID—dosed twice daily (n=3)

4.5. PC-3 Tumor Growth Kinetics in Athymic Nude Mice

PC-3 tumors were initiated on a single dorsal position of twelve athymic nude mice. Once the tumors were palpable linear dimensions were recorded twice weekly. Every mouse that was inoculated with PC-3 cells developed a tumor that was palpable on Day 13. The mice each had a single tumor. The first measurement was always of the longest 'axis', another measurement was taken at the point of the greatest 'depth' and the last was taken at a right angle to the other two. Due to the extent of the tumor growth, the study was halted on Day 57. The only exception was one tumor’s growth rate, which was exceedingly high, such that the mouse was terminated on Day 53.

The mass of each tumor was recorded following termination. This data was correlated with the recorded final tumor volume to verify that calculated volumes were accurate and a valid tool for this study. The volumes calculated using both formulae were correlated.
Figure 16 Average bodyweight chart of the treatment groups in the ESSIAC® extract toxicity study expressed as a percentage of the average initial bodyweight of each group. The control group is shown with error bars representing ± SD. The bodyweight was used as an indicator of the state of the health of the mice. A 20% decrease in bodyweight during the study was considered indicative of acute toxicity. No group or individual mouse had a decrease in bodyweight at the end of the study. No statistical significance was found at critical value set at p<0.05 determined by ANOVA using both Tukey and Student-Newman-Keuls Test.
with the corresponding final tumor masses. Both volume formulas were used and compared with the mass data. The results, which are listed in Table 8 and graphically displayed in Figure 17, establish that $V=xyz(\pi/6)$ is a better representation of the true tumor size than $V=(1/2)xy^2$. The average tumor size and standard deviation is charted in Figure 18, with the calculated tumor volume of each tumor shown in Figure 19.

Using the data collected, the sample size required for the efficacy study was computed and is tabulated in Table 9. After performing a sample size calculation, it was determined that ten mice per group would be a manageable and relevant sample size for an efficacy study. With ten mice per group, the study would have enough power to distinguish with 90% certainty

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Volume $[V=xyz(\pi/6)]$</th>
<th>Volume $[V=(1/2)xy^2]$</th>
<th>Tumor Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1261</td>
<td>1755</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>765</td>
<td>830</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>2691</td>
<td>3728</td>
<td>2.4</td>
</tr>
<tr>
<td>4</td>
<td>4171</td>
<td>4946</td>
<td>3.1</td>
</tr>
<tr>
<td>5</td>
<td>5512</td>
<td>8155</td>
<td>4.6</td>
</tr>
<tr>
<td>6</td>
<td>2139</td>
<td>2234</td>
<td>1.6</td>
</tr>
<tr>
<td>7</td>
<td>548</td>
<td>769</td>
<td>0.3</td>
</tr>
<tr>
<td>8</td>
<td>5480</td>
<td>6266</td>
<td>4.5</td>
</tr>
<tr>
<td>9</td>
<td>4654</td>
<td>5578</td>
<td>4.3</td>
</tr>
<tr>
<td>10</td>
<td>4411</td>
<td>5569</td>
<td>3.4</td>
</tr>
<tr>
<td>11</td>
<td>5889</td>
<td>9634</td>
<td>4.8</td>
</tr>
<tr>
<td>12</td>
<td>4152</td>
<td>6872</td>
<td>3.6</td>
</tr>
</tbody>
</table>

**Table 8**: Final tumor masses and volumes calculated using $V=xyz(\pi/6)$ and $V=(1/2)xy^2$ from growth kinetics data. The correlation between each of the calculated final volumes and the mass were charted in Figure 17 to ensure that the volumes calculated during the experiment were truly representative of the tumor size.
Figure 17 Correlation between each of the calculated final volumes and the mass from growth kinetics data. This correlation was performed to ensure that the volumes calculated during the experiment were truly representative of the tumor size and that the linear tumor measurements had been performed accurately.

<table>
<thead>
<tr>
<th>Percent Decrease in Growth</th>
<th>Absolute Decrease</th>
<th>Z Values</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>91%</td>
<td>80%</td>
</tr>
<tr>
<td>10%</td>
<td></td>
<td>212</td>
<td>160.5</td>
</tr>
<tr>
<td>20%</td>
<td></td>
<td>425</td>
<td>40.1</td>
</tr>
<tr>
<td>30%</td>
<td></td>
<td>637</td>
<td>17.8</td>
</tr>
<tr>
<td>40%</td>
<td></td>
<td>850</td>
<td>6.9</td>
</tr>
<tr>
<td>50%</td>
<td></td>
<td>1062</td>
<td>4.4</td>
</tr>
<tr>
<td>60%</td>
<td></td>
<td>1274</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Table 9: Sample Size Calculation for Efficacy Study Based on the Data Collected During the PC-3 Growth Kinetics Study.

that a 40% decrease in tumor growth was statistically significant. Also, there would be a sufficient number of mice to state with 72% certainty that a 30% decrease in tumor growth was statistically significant. An experimental plan of ten mice per group is both practically and ethically justifiable.
Figure 18 Calculated average tumors volume from mice in PC-3 growth kinetics study. Volume ± SD was calculated by two commonly used formulas $V = xyz(\pi/6)$ and $V = (1/2)xy^2$. $V = xyz(\pi/6)$ was found to have a better correlation to the final tumor masses recorded. The error bars indicate standard deviations.
Figure 19 Calculated volume of tumors from mice in PC-3 growth kinetics study. Volumes were calculated using $V=\frac{xyz}{6}$. Tumors were palpable by Day 13 post-inoculation and the study was terminated after 57 days due to the extreme tumor burden that some mice were carrying. Deviation increased throughout but was extreme following Day 49. The legend numbers are identification numbers for the individual mice in the study.
4.6. Efficacy of ESSIAC® to Inhibit Tumor Growth in Athymic Nude Mice

PC-3 tumors were inoculated in a single dorsal position on 60 athymic nude mice. After 14 days, weight and tumor sizes were recorded. Every mouse that was inoculated with PC-3 cells developed a measurable tumor. At the beginning of the study the mice each had a single well-defined ‘bulbous’ tumor. There was, however, a number of mice who had either extremely elongated or multiple tumors. Five of the mice with the more cumbersome tumors were selected and removed from the study. Four of the mice that had irregular tumors were randomly assigned into groups for the study: two of which had fork shaped tumor masses and two of which had multiple tumors. The volume of the entire tumor was recorded. This required that dimensions were also recorded for the tumor masses that were not part of the main tumor body. Early during the course of treatment, some of the other tumors also developed irregular fork masses that were quantified similarly. By the end of the study, all of the mice had ‘bulbous’ shaped tumors. All tumor volumes specified represent total tumor burden.

The correlation between tumor mass and final calculated volume for all the tumors was calculated similarly to those from the growth kinetics study. The results are displayed in Figure 20. A high degree of correlation was observed using the slope of the best-fit line as an indicator. The body weights and tumor volumes throughout the experiment are shown in Appendix 2. The mean tumor growth for each treatment group is shown in Table 10. The average growth by treatment group is displayed in Figure 21. The tumor volume growth rates for individual mice in the 100 mg/kg BID group, 200 mg/kg QD, 10 mg/kg BID and control group are shown in Figure 22, 23, 24 and 25, respectively. Figure 26 depicts the tumor growth rates for the micellar paclitaxel treated group; because of its completely different growth rate, the scale on this figure is different from those of the
other groups. The only group that showed a significant difference from the control group was the micellar paclitaxel treated group. ESSIAC® treatments did not result in a significant difference in tumor growth at either of the tested concentrations using the dosing regimens applied.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Dosage</th>
<th>Relative Tumor Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PO ESSIAC®</td>
<td>100 mg/kg BID</td>
<td>501±126 %</td>
</tr>
<tr>
<td>2</td>
<td>PO ESSIAC®</td>
<td>200 mg/kg QD</td>
<td>608±234 %</td>
</tr>
<tr>
<td>3</td>
<td>PO ESSIAC®</td>
<td>10 mg/kg BID</td>
<td>490±168 %</td>
</tr>
<tr>
<td>4</td>
<td>PO Vehicle (Water)</td>
<td>N/A</td>
<td>529±160 %</td>
</tr>
<tr>
<td>5</td>
<td>IV Micellar Paclitaxel</td>
<td>1.67 mg/kg†</td>
<td>32±11 %</td>
</tr>
</tbody>
</table>

Table 10: Final tumor growth values following efficacy study with ESSIAC® extract treatments expressed as a percentage of initial tumor size. († - Dosed on Days 1-5 inclusive of a 14 day cycle repeated twice during the study)
Figure 21 Efficacy study average tumor growth chart of all treatment groups expressed in relation to average group tumor size at initiation of treatment. Volumes were calculated using $V=xyz(\pi/6)$. Average of the 100 mg/kg BID treatment group is shown with error bars indicating ± SD. All treatments were administered via oral gavage except micellar paclitaxel, the positive control, which was administered via tail vein injection. For all groups n=11. The comparison of the treatment groups was performed with a Two-Way ANOVA. A post-hoc analysis using a Tukey test was used to indicate which group was significantly different. A p value .05 was considered statistically significant.
Figure 22 Efficacy study tumor growth chart of the 100 mg/kg BID treated group expressed in relation to individual tumor size at initiation of treatment. Volumes were calculated using $V=xyz(\pi/6)$. Mean value ± SD is shown with error bars. The legend numbers are identification numbers for the individual mice in the study.
Figure 23 Efficacy study tumor growth chart of the 200 mg/kg QD treated group expressed in relation to individual tumor size at initiation of treatment. Volumes were calculated using $V=xyz(\pi/6)$. Mean values are shown with error bars indicating ± SD. The legend numbers are identification numbers for the individual mice in the study.
Figure 24 Efficacy study tumor growth chart of the 10 mg/kg BID treated group expressed in relation to individual tumor size at initiation of treatment. Volumes were calculated using $V = xyz(\pi/6)$. Mean values are shown with error bars indicating ± SD. The legend numbers are identification numbers for the individual mice in the study.
Figure 25 Efficacy study tumor growth chart of the control group expressed in relation to individual tumor size at initiation of treatment. Volumes were calculated using $V=xyz(\pi/6)$. Mean values are shown with error bars indicating ± SD. The legend numbers are identification numbers for the individual mice in the study.
Figure 26 Efficacy study tumor growth chart of the micellar paclitaxel treated group expressed in relation to individual tumor size at initiation of treatment. Volumes were calculated using $V=xyz(\pi/6)$. Mean values are shown with error bars indicating ± SD. The legend numbers are identification numbers for the individual mice in the study.
4.7. Tissue Micro Array Analysis

4.7.1. Ki-67 Immunohistochemical Staining

The staining of the tissue micro array slide with Ki-67 proliferation marker revealed that several of the tumors had insufficient positive staining to render a meaningful result. Of the eleven treated tumors, only seven generated sufficient core sections with adequate positive staining to justify incorporation into the analysis; the number of core sections in the samples included ranged from four to seven. Additionally, of the eleven control tumors, only six had sufficient core sections with adequate positive staining to justify incorporation into the analysis; the number of suitable core sections in the samples included also ranged from four to seven.

The average percent of tumor core sections with positive staining ranged from 37.0±10.5% to 59.0±12.8% to produce an overall average of 46.8±12.9%. The average positive staining in the control tumor core sections analyzed ranged from 37.0±7.0% to 54.8±7.0% to produce an overall average of 44.5±10.8%. There is no significant difference between these values. The results are graphically represented in Figure 27 and Figure 28.

4.7.2. PCNA Immunohistochemical Staining

There was sufficient staining in ten of the eleven treated and in eight of the eleven control tumor core sections to include them in the analysis. The number of both the control and treated core section samples included per tumor ranged from four to seven. The average area of positive staining in the treated tumor core sections spanned from 1.5±0.6% to 12.3±3.5%, while the area of positive staining in the control tumor core sections spanned from 2.4±1.2% to 11.5±4.1%. The overall average of the positively stained areas in the treated tumor core sections was
Figures 27 & 28 Percent positive immunohistochemical staining of the ESSIAC® treated and control PC-3 tumors with Ki-67 antibodies. The values shown reflect the percentage of positively stained cells. The core section sample source refers to the identifier of the individual mouse from which the tumor was harvested. Although sections were taken from eleven tumors, many had tissue of insufficient quality insufficient to allow for quantification of the proliferation markers presence. The number of sections used to determine the average staining levels is shown on the individual bars of the histogram. The overall averages of the treated and control sections have no statistically significant difference (p<0.05) determined using a Student t-test.
Figures 28 & 29  Percent positive immunohistochemical staining of the ESSIAC® treated and control PC-3 tumors with PCNA antibodies. The values shown reflect the average percentage area of each equal sized image that was positively stained. The core section sample source refers to the identifier of the individual mouse from which the tumor was harvested. Although sections were taken from eleven tumors, many had tissue of insufficient quality insufficient to allow for quantification of the proliferation markers presence. The number of sections used to determine the average staining levels is shown on the individual bars of the histogram. The overall averages of the treated and control sections have no statistically significant difference (p<0.05) determined using a Student t-test.
8.1±5.2% while the corresponding average for the control tumor core sections was 7.1±4.7%. The results are summarized in Table 11 and graphically represented in Figures 29 and 30. Examples of the stained tumor core sections are shown in Figure 31 and 32.

<table>
<thead>
<tr>
<th></th>
<th>Overall Average Ki-67 Staining</th>
<th>Overall Average PCNA Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treated</td>
<td>46.8±12.9%</td>
<td>8.1±5.2%</td>
</tr>
<tr>
<td>Control</td>
<td>44.5±10.8%</td>
<td>7.1±4.7%</td>
</tr>
</tbody>
</table>

Table 11: Percent positive immunohistochemical staining of the ESSIAC® treated and control PC-3 tumors with PCNA and Ki-67 antibodies. Ki-67 values reflect the percentage of positively stained cells. PCNA values reflect the average percentage area of each equal sized image that was positively stained.

Figure 31 Selected immunohistochemically tumor section images from the Tissue Micro Analysis stained with Ki-67 antibodies. Sections A and B are both samples of control tumors while Sections C and D are both samples of tumors treated with 100 mg/kg ESSIAC® BID. Sections A and C are representative of the sections that were included in the analysis and therefore quantified. Sections B and D are representative of the section that were rejected due to insufficient staining. Blue indicates positive staining for Ki-67.
Figure 32 Selected immunohistochemically tumor section images from the Tissue Micro Analysis stained with PCNA antibodies. Sections A and B are both samples of control tumors while Sections C and D are both samples of tumors treated with 100 mg/kg ESSIAC® BID. Sections A and C are representative of the sections that were included in the analysis and therefore quantified. Sections B and D are representative of the section that were rejected due to insufficient staining. Blue indicates positive staining for PCNA.
SECTION 5

Discussion
5.1. HPLC Analysis of ESSIAC® Extract

There were 23 peaks on the HPLC chromatogram for ESSIAC® that were deemed suitable for inclusion in the comparative analysis. The peaks that were chosen were included because they had both sufficient resolution and were of substantial size. It is not known if any of the selected peaks represent active agents of the extract since this determination was outside the scope of this project. At the time of the completion of this Aim, it was not known if ESSIAC® would display any activity in the planned studies.

No attempt was made to determine the identity of the compounds giving rise to any of the peaks. It would have been illogical to expend the effort to identify the component compounds in an extract that had not been shown to have a substantiated effect. The HPLC analysis was performed predominantly out of a recognized need to execute quality control when dealing with herbal products of unknown integrity. Therefore, performing this HPLC investigation provided assurance of the extract’s uniformity of composition. The technique used is best suited to identifying if the manufacturers had proficiently blended the ingredient herbs.

HPLC chromatography is a poor way to positively identify compounds, but it is an adequate tool to for identifying gross discrepancies in content. The only manufacturing irregularities that might be detected using HPLC techniques are variations in extract concentrations and component proportions. Some of the possible causes of discrepancies include substitutions with taxonomically related (or unrelated) herbs or addition of synthetic adulterants to the product.

Active components can be identified through progressive fractionation. This procedure would be initiated with an assay of the whole extract for biological activity. In this project, the assay employed was used to determine anti-proliferative activity. If the assay indicates that the extract
has the desired activity, the extract could be fractionated through further extraction using other solvents or through column chromatography. The fractions could then be assayed for activity. This process is collectively termed 'bio-activity guided fractionation'. A positive assay indicates which fraction contains the active compound(s). This process can be repeated until the active component(s) has been isolated. The compound can then be identified using a suitable method, such as, mass spectroscopy or nuclear magnetic resonance spectroscopy. This technique is possible for ESSIAC®, the initiation step was performed and is described in Section 5.2.

The within-lot analysis of the extract revealed that the manufacturers preparation protocol could generate a product which, when extracted, produced a HPLC chromatograph that contained typical RSDs of <5% (Figures 2 and 3). The between-lot analysis were less supportive of the protocol however. One lot (18146) contained peak areas that are substantially lower than the equivalent peaks of the other two lots. It is notable that the low RSD values mentioned above were only observed in Lots 17694 and 17903. This result is consistent with, and possibly attributable to, the lower peak areas generated by the extraction of Lot 18146. It appears that Lot 18146 less readily allows for extraction of its components. Despite having only analyzed three lots, it is impossible to state whether Lot 18146 is remarkably different from the others or if the relative similarity between the peak areas of the other two lots is exceptional. To answer this question, it would be necessary to probe further lots. However, because of a sporadic manufacturing schedule new lots have not become available for analysis. Considering the results of the other Aims in this project, the benefit of pursuing further consistency analysis becomes debatable.

Despite the fact that the peak areas are of substantially different size, the relative area of each individual peak with respect to the cumulative peak remained reasonably consistent throughout
the analysis. This may be a reflection of the time of year or the type of growing season in which the raw materials were harvested. It is plausible that a drier growing environment may have generated a more fibrous vegetative starting material. The product, consequently, might have a lower amount of extractable components per net weight. As a result, the manufacturer would have to modify the preparation protocol so that the consumer will dependably extract the desired amount of the components in each extraction. The modified protocol would have to address the higher RSD and correct for it in some manner. It may be that all that is required is an adjustment to the quantity of raw herbal extracted per volume of water and a longer duration at the boiling point, however this is only speculative.

The final component of the HPLC analysis probed the ability of the extraction protocol to yield decoctions that contained components quantitatively related to the amount of raw material processed. Samples were analyzed in which the ESSIAC® powder was increased two, five and ten times greater than the manufacturers specified amount for the equivalent volume of water in the extraction. All other variables of the process were constant. The absolute peak areas had an incremental increase related to the amount of raw material processed (Figure 7); however, the results were not quantitative. The relative peak areas normalized to the baseline (1X) extraction's peak areas were 1.91, 4.23, and 8.27 fold for the 2X, 5X and 10X preparations, respectively. Extractions at higher strength were attempted, however, the powder had an ability to absorb and retain water making it difficult to accumulate large enough volumes of the extract, beyond ten times the concentration suggested by the manufacturer, to be used in analysis.

The values determined are important because two separate Aims of this project require extractions of various concentrations. It is very difficult to accurately express the concentration of an extraction, because this product has not been standardized to a single active component.
Although there are active compounds in the herbs of this product, the analysis performed in this study did not attempt to reveal which HPLC peak might represent them. A standardization of this product is complicated by the fact it contains four distinct herbs, presumably, each contributes different active agents to blend. To standardize the product to a single compound from one herb essentially neglects the importance of the other herbs. However, the current study does not entail an in-depth description of characterization of chemicals found in ESSIAC and, therefore, further consideration is outside the scope of this discussion.

The amount of raw material per volume of water used to perform the extraction is the most tangible way to express the final concentration of the extract. As observed in the analysis of the various concentrations of extract, there is only a relative, not direct, relationship between the concentrations of the final extracts and the quantity of raw product used to produce them. That is to say, if two extractions are performed the one obtained using more herbal powder will result in larger peak areas. There is no obvious quantitative relationship between the mass of raw material processed and the size of the peak area generated. The majority of the raw powder extracted is insoluble and is disposed of following sedimentation. Despite using these concentrations throughout this project to describe the extract, it is evident the associated amount only qualitatively describes the extracts.

5.2. ESSIAC® Extract’s Effects in vitro

5.2.1. Cytotoxicity Assay

The results of the in vitro crystal violet assay of ESSIAC® aqueous extract on LNCaP and PC-3 prostate cancer cell lines indicate that there is no significant anti-proliferative effect on these cell lines. While the quantified UV absorptions from the LNCaP assay produced values that may be indicative of a marginal cytotoxic effect, the UV absorptions from the PC-3 assay are more
indicative of a proliferative effect. However, none of the findings are statistically significant or dose dependent so any assertions made are speculative in nature.

The extract concentration, as stated, is based on the amount of raw herbal powder used to perform the original extraction. The majority of this material is insoluble and disposed of as sediment during preparation. Only a small amount, possibly only 1% or less, of the original biomass is actually extracted. The true concentration of the extract is much smaller than the mass originally extracted, however there is no reference for stating the actual amount. In addition to this, there are numerous compounds in the extract. It is apparent, using HPLC peak areas as a basis for approximation, that no single compound makes up more than roughly 17% of the total composition of the extract. (In reality, it is entirely possible that any of the peaks, do not arise from the elution of a single compound but is the result of multiple undifferentiated compounds.) The extract is diluted extensively (100 fold minimum in the in vitro studies and incalculably in the in vivo study due to pharmacokinetic effects) before the cells receive any exposure to it. All these factors contribute to minimal exposure levels in all the studies.

Nevertheless, these amounts are clinically relevant; paclitaxel, for example, has an IC_{50} value of only 20 nM (103). Using the numbers that are mentioned above and assuming a large molecular weight (paclitaxel's is 854 g/mol), concentrations of 1μM could be expected in vitro.

One assumption that is made when linking the crystal violet assay UV absorption values to cytotoxic effect is that all of the adherent cells in the plate prior to staining are indeed dead. If the mechanism of adhesion is disrupted in such a manner so that unhealthy and dead cells remain attached to the culture plate, the reported results will not truly be indicative of the effect the treatment has on the cells. The recorded absorption will indicate more cells are present and healthy than is the actual case. However, the rate of proliferation of the cell line used in these
experiments should be sufficient to overcome the potential masking effect. If the treatment did indeed have a cytotoxic effect, those cells in the treatment group would not have proliferated at the same rate as the control group. Therefore, the number of cells adhering in a control group after a 48 hr incubation should, logically, still be present in significantly larger numbers than those in a successful treatment group. Using this reasoning and combining the crystal violet assay results with those of the cell cycle analysis, confidence that the results represent the true effect of ESSIAC® is held.

The extract should be assayed against other prostate cancer cell lines, such as, Shionogi and DU-145. These are also well-characterized cell lines used to model prostate cancer. The DU-145 cell line was developed from a brain tumor arising from metastatic prostate cancer (104). The Shionogi cell line is a murine mammary carcinoma, which exhibits androgen dependent characteristics (105). Because prostate cancer is a very heterogeneous disease, the entire disease state is better represented once more cell lines have been included.

5.2.2. Cell Cycle Analysis

The phase distribution of the PC-3 cells treated with ESSIAC® was not significantly different from the control treated cells. The results from the flow cytometry corroborate with the results of the cytotoxicity assay. Had any effects been noted in this study, it almost certainly would have been detected first with the crystal violet assay. The combination of the two assay results provide strong evidence that ESSIAC® produces no detectable effect on PC-3 cells in an in vitro environment. The paclitaxel treatment represents a positive control. It is known that paclitaxel has the ability to enhance assembly of, and stabilize microtubule structures, in turn producing a reduced proliferation rate (106, 107). It is precisely these effects of paclitaxel that would produce the alterations in the cell cycle seen in this study. It was also obvious, through visual
assessment of the cell culture plates, that paclitaxel had an antiproliferative effect on the cultured PC-3 cells and ESSIAC® did not (or if it did it was substantially smaller) when confluence of each was compared with controls.

As previously mentioned, the application of *in vitro* assays of herbal products can easily produce negative results in models of cell types when positive results may be seen clinically. Agent(s) may require activation by metabolizing enzymes to produce the activity. In a culture dish, the tissues required to activate such compounds are not present and the *in vitro* assay results will, therefore, be negative.

There are other reasons that the *in vitro* assays may not be definitive for the testing of herbal treatments. Herbs contain numerous compounds that can act through different mechanisms. Simple cell culture assays designed to study the nature of cancer cells will be inadequate to detect the activity of a compound has angiogenesis inhibitive or immunostimulatory effects, for example.

### 5.3. ESSIAC® *in vivo* Toxicity Study in Athymic Nude Mice

The consistent increase in body weight of every animal in this study indicates that there was no toxic effect at the concentrations evaluated (Figure 15). Although monitored by subjective, visual means, and not in any analytical way, other physiological indicators of toxicity, such as shakiness, lethargy, loss of coordination, or change in body temperature, were not apparent at any stage of the toxicity study. It might be noticed that the two largest dosages evaluated, 240 mg/kg QD and BID, resulted in two of the largest average bodyweight increases recorded. However, it is difficult to make any conjectures based on the results of this toxicity study because each of the groups contained only three animals; therefore, the results are not
statistically significant. The results of this study are validation that the treatment would not induce harm to the treated animals in the subsequent efficacy study of ESSIAC® in athymic mice, since the efficacy study will be of the same length and will use similar dosages.

5.4. Growth Kinetics of Subcutaneous PC-3 Tumors in Athymic Nude Mice

Subcutaneous PC-3 tumors were fully established two weeks following inoculation of $2 \times 10^6$ PC-3 cells. By the 13th day, 100% of the animals in the study group had developed tumors ranging in size from 50 to 163 mm$^3$. The study was continued for 57 days; tumors grew to a size ranging from 548 to 5889 mm$^3$. A single mouse had to be terminated on the 53rd day for ethical reasons as its tumor load had already reached 5512 mm$^3$. This tumor was more than 20% larger than the any of the other tumors in the study. The standard deviation of the tumor sizes on the 13th, 42nd and 57th days was 36, 945 and 1894 mm$^3$, respectively. Based on the data collected, it was decided that it would be best to start treatments in an efficacy study involving in vivo PC-3 tumors at the beginning of the second week when all tumor sizes are quantifiable. The studies should be carried out for four weeks, as the deviations in the tumor sizes tend to increase more quickly beyond this time point making statistical significance more difficult to achieve. This is the timeline that was used for the efficacy study discussed below

The numerical data discussed above were calculated using the formula $V = \frac{\pi xyz}{6}$. This formula showed a higher correlation when the final tumor mass was correlated to the final tumor volume when compared to the volumes calculated using the formula $V = \frac{(xy^2)}{2}$. All tumor volumes, therefore, were calculated and analyzed using $V = \frac{\pi xyz}{6}$. In a study carried out by Tomayko and Reynolds (102) they found that the two equations are “interchangeable” and recommended either one be used above the numerous other tumor volume formulae in the study.
5.5. ESSIAC\textsuperscript{®} \textit{in vivo} Efficacy Study in Athymic Nude Mice

The treatment of the mice in this study with ESSIAC\textsuperscript{®} led to neither tumor growth inhibition nor restrained body weight growth rates. The only group that demonstrated an effect from treatment was the positive control group that was treated with micellar paclitaxel. There was no statistically significant differences in the average tumor growth rates of any groups that were treated with ESSIAC\textsuperscript{®} when compared with the control group. The method of detecting any effect of the ESSIAC\textsuperscript{®} treatment in this study was quite rudimentary. Therefore, the effects on two biochemical proliferation markers in the tumors harvested from this efficacy study were investigated as supporting evidence of the initial findings of this efficacy study.

One of the first aspects that must be considered when acquiring negative results with an orally administered treatment is the bioavailability of the dose. As with any herbal product, bioavailability is a difficult concept to address. This is because of the large number of components included in each dose and the lack of knowledge regarding which component might be active. Each component of the extract potentially has its own bioavailability. To fully answer the question of an herbal's bioavailability, complete knowledge of the identity of the compounds within the extract would have to be obtained and each would have to be tested. This type of evaluation is not practical. In order to avoid such an overwhelming task it would be better to perform a study in which the extract is delivered intravenously, thereby, completely avoiding absorption issues. However relevance to equivalent patient use would be lost and questions arise regarding the usefulness of the resulting data.

The one group that was dosed intravenously with micellar paclitaxel had a statistically significant decrease in tumor growth rate when compared to the control group. This result has
not been previously published in a PC-3 tumor model. It has been reported with a subcutaneous LNCaP tumor model (99). Similar results were reported with mean tumor volumes falling 91% after three cycles of treatment (5 mg/day administered on the first five days of a 21 day cycle). In this study, a 68% decrease was observed after 2 cycles. Angiotech Pharmaceuticals, Inc (Vancouver BC) is currently performing the required research to develop micellar paclitaxel as a cancer therapy (108).

A second pharmacokinetic consideration is distribution. It is known that, in some instances, drugs can be stored in reservoirs such as adipose tissue and bone, as is the case with tetracycline and bisphosphonates (109). It is unlikely that compounds from an aqueous extract would be sequestered into a lipid rich environment such as adipose tissue. The bone, however, represents a more interesting environment. ESSIAC® is alleged to be a successful treatment for late stage cancer. Prostate and breast cancer consistently metastasizes to skeletal sites. If any active compound present in ESSIAC® is sequestered to bone so that it is at higher concentration in this “compartment” than it is systemically, it may be more effective in treating metastases than it is at treating primary tumors. In addition, a longer-term treatment than was employed here might be required to achieve therapeutic levels systemically. However, this is simply speculative as there is no experimental evidence to indicate that this might be occurring.

5.6. Tissue Micro Array Analysis of PC-3 Tumors

Two proliferation markers were quantified in this study, PCNA and Ki-67. Despite both markers being used to indicate proliferative potential of the tissue, each marker is quantified differently due to the nature of the marker. Ki-67 is an absolute marker. It is either present or absent in each cell nucleus depending on the status of the cell cycle, as the Ki-67 protein is rapidly
degraded during Go (110). Therefore, a count of the number of cells that have the marker present
is an accepted method of quantifying proliferation. Alternatively, PCNA is not degraded as
rapidly but dissipates into the cytoplasm (111); because of the loss of nuclear localization, it is
more difficult to establish an accurate cell count using imaging software. The quantification of
this antigen, therefore, was performed by determining the percentage of the area of each
identically sized digital image that was positively immunohistochemically stained above a
threshold level for PCNA. The threshold level was set to represent staining in a proliferating
cell’s nucleus. It is not possible to compare the results of the two marker stains directly to each
other, however, that was not the objective of the work. The intention was to compare the tumors
from the animal treated with ESSIAC® with the tumors from the control animals using
independent proliferation markers.

The overall average percentage of Ki-67 immunohistochemically stained cells per core section
was 46.8±12.9% and 44.5±10.8% for ESSIAC® treated tumors and control tumors, respectively.
The overall average area of PCNA immunohistochemical staining per core section image was
8.1±5.2% and 7.1±4.7% for ESSIAC® treated tumors and control tumors, respectively. In both
instances, ESSIAC® treated tumors were actually shown to have slightly higher amount of
proliferation, however, these results are not statistically significant.

The proliferation marker expression experiments could have been improved in two ways.
Firstly, it would have been useful to have also included a positive control treatment group in the
Tissue Micro Array. Unfortunately, there was only a small amount of micellar paclitaxel treated
tumor tissue available due to the recession in tumor size during the course of the efficacy study.
The tumor tissue harvested, although not histologically examined, probably contained a low
density of striving cancerous cells. This low density would have been reflected in the staining
with a low number of quantifiable sections. Secondly, complete tumor sections should have been processed with hematoxylin-eosin staining to reveal regions of the tumor that were free of necrotic tissue. The selected cores included in the Tissue Micro Array analysis could have been taken from the regions of the tumors that were free of necrosis. This would have prevented the eventual rejection of the numerous tumor sections that were later found to contain necrotic tissue.

5.7. Conclusion

The initial results of this work show that the aqueous extract of the herbal product ESSIAC® despite having a difference in absolute quantities of components between-lot, maintains a stable ratio of its component parts. The in vitro results indicate that 48 hr treatments of PC-3 and LNCaP prostate cancer cells with ESSIAC® at concentrations up to 6 µg/ml have no statistically significant difference from the controls. While no toxicity was registered, as indicated by the body weight of the animals during the four week study of athymic mice dosed with ESSIAC®, neither was any inhibition of tumor growth identified after an in vivo efficacy study of ESSIAC®, of a similar duration, was completed. As a confirmation of the results recorded during the efficacy study, the tumors harvested were probed with the antibodies for Ki-67 and PCNA proliferation markers. The quantification of the immunohistochemical staining verified the previous conclusions.

All the findings in this project have failed to identify any significant difference between ESSIAC® treated and control prostate cancer cells proliferative activity. Throughout the project there has been only marginal difference between the treatment groups. This provides solid evidence of ESSIAC®’s inability to restrict proliferation in a pre-clinical environment. The
benefit of performing further studies in the wake of these findings are debatable unless the experimental design was such that one of the elements that were not controlled for in these experiments, such as bioavailability, was removed as a confounding factor. Despite the high degree of consensus in the results, it is not valid to infer that Essiac® extract is an ineffective treatment for prostate cancer. It is reasonable to state that the results of this study indicate ESSIAC® treatment did not produce a statistically significant antiproliferative effect in the models tested.

5.8. Significance and Limitations of the Results

There are several aspects of this work that bear mentioning as important details that may not have been clearly stated elsewhere. The study of herbal therapies for cancer, or other diseases, always comes with controversy. The tools and techniques used to study pharmaceutical drugs do not always transfer readily when performing studies on herbal products. The difficulty shown in the quantitation of the extract is a good example of this.

Quantification of individual pharmaceuticals is relatively straightforward, purify and measure. However, with herbals, such as ESSIAC®, the act of measuring is quite a difficult task. It is even difficult to determine what exactly it is that is being measured. The HPLC work described in this project shows that the mass of the raw herbal does not necessarily reflect the concentration of the components in the extract. Another option to quality control with herbals is standardization to a known active component. However, there are dangers to using this approach. Firstly, in the situation that was faced at the outset of this study, as an example, the active component was unknown (and following this work even the presence of an “active” remains debatable). Secondly, even if an active component is known the question remains as to whether that single
component is the sole source of activity for the herbal. If two active compounds are present, the validity of standardizing to one or the other is called into question. Thirdly, still other components may be not directly affecting the effect being measured but rather acting in a supportive or chemosensitizing manner, such as an immunostimulants or anti-oxidants. The capacities of these components of the products are completely ignored by the standardization process. However, it is necessary to establish product consistency so that the analysis of the product will have value that could be extrapolated to future lots.

It was stated earlier that the results of these studies would be most relevant to late stage prostate cancer patients because the work focuses mostly around the PC-3 cell line. While true, this statement must be tempered with the knowledge that positive results of cell culture and animal studies do not necessarily imply efficacy in humans, if this was the case Phase II and III clinical trials would be unnecessary. By reversing the logic, negative results in cell culture and animal studies may not preclude efficacy in humans.

As with all experiments, the connotations of the results are limited by the experimental design. The first limitation is that the aggressive growth of the PC-3 tumors combined with the small stature of the carriers (athymic nude mice) only permits a short-term study of approximately one month. With such a short treatment period, it is difficult to suggest that the results of the study are necessarily reflective of a clinical patient who is consuming the extract over a longer period of time to treat a less aggressive tumor than the PC-3 cell line produces.

The second limitation transpires due to the desire to study the effects of human prostate cancer cells in an in vivo model. Nude mice are immunodeficient and were chosen as carriers for this very characteristic. The mice will not reject the inoculated human cell line because of their
inability to mount an immune response. However, for the same reason any immunostimulatory effects ESSIAC® might have will be completely stifled by the experimental design.

The third limitation arises because no active agents were identified or studied exclusively. This is problematic because the metabolic pathway of all (or any) medicinal compounds is unknown. If the compounds must be bio-activated by a metabolic enzyme, no effect will be seen in the in vitro studies. Furthermore, no effect will be seen in the in vivo studies if such an enzyme is present only in humans and not in mice. Alternatively, if a metabolizing enzyme found in mice inactivates the therapeutic agent no efficacy will be seen; if humans have no equivalent enzyme then a potentially positive effect will be disregarded. If any of these scenarios are true, only clinical studies will produce reliable results and that work is beyond the scope of this research.

5.9. Future Studies

The results of this project, despite the consistent lack of efficacy of ESSIAC® extract shown, should be corroborated with further in vivo experiments. The studies should be designed with the focus specifically on three significant factors: ensuring immunocompetence in the carriers, use of a non-mouse animal model, and employment of a tumor model that was less aggressive than the human PC-3 cell line. The Dunning rat is a well-characterized and highly studied rodent model that has been recommended for use therapeutics testing (112). The use of this model will allow in vivo study that has a non-mouse immuno-intact carrier with a slow growing tumor. It should be understood, however, that there would be downsides to every model system and experimental design. The substitution, however, would require that the study be implemented with cancer cells that are not human and express proteins that are atypical for prostate cancer cells. The only way to escape these issues is to do a clinical evaluation. Even with clinical
studies though the control of the experiment is compromised by the confounding factors introduced from the patients' daily lives. This extract does lend itself to clinical evaluation, as it already has wide usage by a population that is in dire need of answers to their healthcare dilemma but the cost of performing such an evaluation on a large scale are prohibitive. A preliminary evaluation, however, could be justified considering this widespread usage combined with a lack of scientific evidence to support the efficacy of ESSIAC® extract. The PC-3 cell line, although being well characterized as an excellent model for hormone refractory prostate cancer, does bring with it some inherent deficiencies. As mentioned above the cell line is highly aggressive. When inoculated into the mice, this high growth rate generates in an experiment that must be relatively short in duration. Furthermore, there are attributes of the PC-3 cell line that, arguably, make it a poor choice to be used as an exclusive representative of hormone refractory prostate cancer. For example, the p53 gene is mutated and nonfunctional in PC-3 cells (113). It would be better to perform studies with multiple models, as all cell lines likely represent only a portion of the cancer type and stage that they are said to characterize.

As well, there are other experiments that could be done to confirm further the results of the in vitro experiments. For example, another experimental option for determining the in vitro antiproliferative effects of ESSIAC® extract is an assay which looks at colony formation of human cell lines in a solid media. Conversely, it is important to understand the mechanisms at work in any in vitro assay applied. For example, an alternative cytotoxicity assay, which might be performed, is the MTT assay. However, it has been shown recently that this assay is incompatible to herbal therapies because components in the extraction have the potential to disrupt the very mechanism that leads to the generation of useful data.
5.10. Synopsis

ESSIAC® extract, despite its reputation within the alternative medicine community in Canada, and abroad, has not revealed an ability to inhibit the proliferation of the prostate cancer models experimentally used in this study. There are numerous factors, put forward in the discourse above, which might have contributed to the negative results observed. It is not possible to control for all the confounding factors, however, the results appear to unwaveringly indicate that ESSIAC® does not have a pre-clinical capacity to negatively affect the growth of prostate cancer. The combined results of both the cytotoxicity assay and the cell cycle analysis are strong evidence that ESSIAC® extract, in an unmetabolized state, cannot produce an inhibition in proliferation of cultured cells. The \textit{in vivo} results, indicated by both the gross measuring of tumor size and by immunohistochemical staining of well recognized proliferation markers, were equally as convincing evidence that there was no difference between the ESSIAC® treatment and a control treatment. The need for further research into ESSIAC®’s® effectiveness pre-clinically as a prostate cancer therapy would have to be supported by some other evidence of efficacy, such as a well designed clinical study, although this is not advocated.
References


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