

Isolation and Characterization of an Antiproliferative Compound in the
Herbal Anticancer Remedy Essiac

An undergraduate research project

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ABSTRACT

Essiac is a popular herbal tea with purported anticancer effects. Despite these claims, previous studies on Essiac have yielded contradictory results, with some reports showing increased proliferation and others showing decreased proliferation of cancerous and transformed cells in the presence of Essiac. We report that whole Essiac tea inhibits the *in vitro* proliferation of transformed Chinese hamster ovary (CHO) cells, as measured by a 5-carboxyfluorescein diacetate acetoxymethyl ester cell viability assay. The antiproliferative effect is lessened when the CHO cells are engineered to overexpress the Myc oncoprotein, suggesting that Myc does not sensitize cells to Essiac-induced death. Upon liquid-liquid extraction with chloroform, acidic aqueous Essiac fractions displayed antiproliferative effects, while neutral, basic, and chloroform fractions showed either no effect or mild proliferative effects. We propose that contradictory findings from previous studies resulted from differential sensitivity in the tested cell lines to the various proliferative and antiproliferative compounds within Essiac, which cannot be deduced when whole Essiac is applied. Furthermore, our investigations suggest that Essiac contains an antiproliferative compound that becomes charged and protonated between pH 5 and 4, suggesting that it may be a large, aromatic amine. Future work in isolating this compound could provide a possible anticancer agent and help explain the paradoxical effects of Essiac on cancer cells.

INTRODUCTION

The use of complementary and alternative medicines (CAMs) to treat cancer is rising in North America (Markman, 2002). Despite this increased popularity, evidence for the effectiveness of many of these CAMs remains largely anecdotal. One CAM, the herbal anticancer tea Essiac, is of particular interest due to its recent emergence, its immense popularity, and conflicting reports regarding its effectiveness (Kulp *et al.*, 2006; Ottenweller *et al.*, 2004; Smith and Boon, 1999).

Essiac is composed of four herbs: burdock root (*Arctium lappa*), slippery elm bark (*Ulmus fulva*), wild sheep sorrel (*Rumex acetosella*), and Turkish rhubarb root (*Rheum palmatum*), most of which have some purported value as folk medicines (Glum, 1988). These herbs have been investigated individually and found to have antioxidant, immunomodulatory, estrogenic, cytotoxic, and antiproliferative properties (Tamayo *et al.*, 2000). Supporters of Essiac maintain that its effectiveness in treating cancer is due to the synergy of these properties and that the individual herbs are far less effective than the combination (Tamayo *et al.*, 2000). As such, most recent work has evaluated whole Essiac rather than its constituents.

Some of these studies have investigated Essiac's immunomodulatory, anti-inflammatory, and antioxidant properties (Cheung *et al.*, 2005; Leonard *et al.*, 2006); however, most have investigated Essiac's antiproliferative effects on cancerous and transformed cells. The results of these antiproliferation studies have been contradictory. Ottenweller *et al.* (2004) found that Essiac decreased the proliferation of prostate cancer cells and transformed, non-cancerous cells significantly, while non-transformed cells proliferated normally. Further studies with leukemia and breast cancer cell lines also showed antiproliferative effects on cancer cells (Tai *et al.*, 2004). However, a more recent study by Kulp *et al.* (2006) has shown the opposite effect: that Essiac

increases the proliferation of breast cancer cells *in vitro*. This was first thought to be due to Essiac's estrogenic properties; however, on subsequent investigation, both estrogenic and non-estrogenic mechanisms for proliferation were found to occur. It remains unclear why some cells proliferate and other cells die in the presence of Essiac.

These conflicting results may be due to the fact that Essiac is a complex mixture with a variety of bioactive compounds. These compounds will affect different cell lines to varying degrees. For example, Essiac contains several isoflavanoids, which act as phytoestrogens (Tamayo *et al.*, 2000). Cells with estrogen receptors (e.g., breast cancer cells) will be significantly affected by these phytoestrogens and undergo proliferation, whereas cells lacking these receptors will be resistant to this effect (Brooks and Skafar, 2004). Similarly, overexpression of a single gene, *MYC*, sensitized cells to Essiac-induced death (Phillips, 2003). Thus, antiproliferative or proliferative compounds in Essiac may have differential effects on different cell lines. This suggests that although Essiac may be benign or even beneficial for the treatment of cancers arising from certain cells, it may cause undesirable effects in cancers arising from other cell types.

We have chosen to investigate the growth-modulating effects of Essiac on two transformed Chinese hamster ovary (CHO) cell lines. Although not cancerous, these cells possess many qualities typical of cancer cells, including immortality and rapid growth. Additionally, one of the lines has been engineered to overexpress the *MYC* oncogene (Gibson *et al.*, 1995). *Myc* overexpression has been shown to cause cells to assume a cancer-like morphology, increase susceptibility to apoptosis when the cell cycle is blocked (an action common to many anticancer drugs), and to increase Essiac-induced death in other cells (Phillips, 2003). By using this engineered cell line, and the original cells as a negative control, we can

investigate the role of the Myc oncogene in Essiac-induced cell death. Furthermore, Ottenweller (2004) has demonstrated that Essiac inhibits proliferation of the non-engineered CHO cells, which will allow us to determine Essiac-induced death regardless of Myc overexpression. Also, because these cells express functional estrogen receptors, they should be sensitive to any phytoestrogens contained in Essiac (Thomas *et al.*, 2003), which will allow us to investigate the paradoxical Essiac-induced growth in breast cancer cells (Kulp *et al.*, 2006). However, although these cells will likely be sensitive to phytoestrogens, and thus exhibit some Essiac-induced proliferation, Ottenweller's work has shown that these particular cells are inhibited by an antiproliferative element in Essiac. This suggests that these CHO cells will be sensitive to both proliferative and antiproliferative compounds in Essiac.

The proliferative and antiproliferative compounds within Essiac may be better understood if these compounds can be separated and characterized by fractionation. Although one antiproliferative compound specific for cancer cells, the polyphenol emodin, has been isolated from one of Essiac's component herbs (Ottenweller *et al.*, 2004), other antiproliferative compounds contributing to Essiac's purported cancer-specific cytotoxicity have not been described. Additionally, by isolating the antiproliferative compounds from Essiac, we can separate out the proliferative compounds. This should reveal if the potentially growth-promoting phytoestrogens do indeed have an effect. By exposing our CHO cells to various Essiac fractions and counting the cells with a cell viability assay, information can be gained about the properties of the proliferative and antiproliferative components in Essiac. Furthermore we can ascertain whether or not the cytotoxicity is specific, or sensitized by the Myc oncoprotein, by using the *MYC*-overexpressing, engineered cell line.

MATERIALS AND METHODS

1. Cell lines and culture conditions

CHO-SVMYC cells, engineered to constitutively overexpress human Myc protein, were gifts from Allan Gibson (Pai *et al.*, 1992). CHO-DHFR cells are engineered the same way, except without the *MYC* gene. Both cell types were cultured in RPMI 1640 media containing 10% fetal bovine serum, 110 units/ml penicillin, and 110 µg/ml streptomycin (Gibco). Cultures were kept in a humidified incubator at 37°C and 5% CO₂.

2. Western blotting

Protein separation and electroblotting were performed according to standard procedures (Gibson, 2005). After electroblotting, the blot was probed with 1-9e10 mouse anti-human myc primary antibody, followed by alkaline phosphatase-conjugated goat anti-mouse antibody. Secondary-antibody controls were performed with alkaline phosphatase-conjugated goat anti-mouse antibody only.

3. Preparation of Essiac

Essiac was prepared according to supplier's instruction (Essiac, 2004). In brief, Essiac powder (Essiac Canada International Incorporated, Ottawa, Canada) was added to distilled water at 2% w/v, stirred, and boiled for 10 minutes. The resulting solution was removed from heat and allowed to sit for 4 hours before being boiled again for 5 minutes. The tea was then stored at 4°C until use (maximum 2 months).

4. Liquid-Liquid extractions

4.a Aqueous extractions of acidic, basic, and neutral Essiac

Essiac (pH 5) was left untreated or adjusted to pH 1 or pH 14 by titration with hydrochloric acid or sodium hydroxide respectively. Solutions were then mixed with equal amounts of chloroform, vortexed, and the phases were allowed to separate. The aqueous phase was diluted 2X with phosphate buffer (pH 7, 100 mM), and adjusted to pH 7 with sodium hydroxide or hydrochloric acid, as needed. Extraction-controls were prepared the same way, with distilled water substituted for Essiac.

4.b Aqueous extractions of citric-acid buffered Essiac

Essiac was adjusted to pH 3, 4, 5, or 6 with citric acid buffer (10 mM), followed by extraction with chloroform. The aqueous phase was diluted 2X with phosphate buffer (pH 7, 100 mM) for a final pH of 7. Extraction-controls were prepared as in 4.a.

4.c Chloroform extractions of acidic, basic, and neutral Essiac

The chloroform phase (4.a) was evaporated to dryness. The remaining solutes were redissolved in phosphate buffer (pH 7, 50 mM), with gentle heating to promote dissolution.

5. Cell growth inhibition assay

Cell numbers were determined using a 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA,AM) assay. Cells were harvested with trypsin-EDTA, then mixed with media and test solution (Essiac, Essiac-extractions, or controls) at roughly a 1:2:1 ratio. These cultures were incubated for 24 hours, whereupon they were incubated with CFDA,AM (final concentration of 0.01 mg/ml) for 30 minutes before being read with a fluorescent plate reader (excitation/emission, 485/528 nm). Unless otherwise indicated, CHO-DHFR cells were used.

6. Statistical treatment of data

Essiac and control treatments were tested for differences by a two-tailed t-test assuming unequal standard deviations. If the p-value was less than 0.05, the results were considered statistically significant.

RESULTS

1. c-Myc expression in CHO and control cells

To ensure that differences in Essiac-induced mortality of Myc overexpressing cells compared to controls was due to Myc expression, we first performed a Western blot on CHO-SVMYC (overexpressors), CHO-DHFR (control), and COLO320 (cancer cells known to express high levels of myc) cells. Both COLO320 and CHO-SVMYC cells expressed detectable levels of human c-Myc, while CHO-DHFR cells did not. Distinct bands at approximately 62 kDa, corresponding to human Myc, were seen in both the COLO320 and CHO SV-MYC lanes (Figure 1). These bands were not seen in the CHO-DHFR negative control or the secondary-antibody only controls (data not shown).

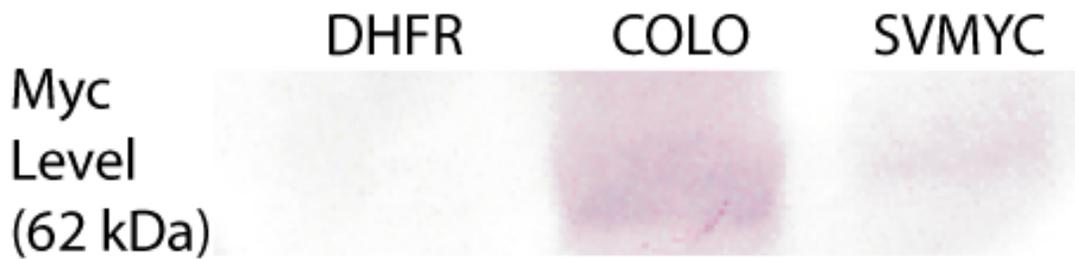


Figure 1. Myc expression in regular (DHFR) and Myc overexpressing (SVMYC) CHO cells as determined with an anti-human myc Western blot. COLO cells are known to express high levels of Myc and serve as a positive control.

2. Cell number assay

Because fluorescence saturation can occur with fluorescent cell viability assays, two-fold dilutions of CHO-DHFR and CHO-SVMYC cells were seeded into a 96-well tray and measured with the CFDA,AM fluorescence assay to find the linear range. CFDA,AM is cleaved by living cells to produce a fluorescent product, which is then measured with a plate reader. The fluorescence was correlated with cell number and found to be linear between cell concentrations

of 5×10^3 and 5×10^4 cells/well for both cell types (data not shown), and subsequent cell measurements were conducted within this range.

3. Growth inhibition of Myc overexpressing cells by whole Essiac

To determine whether the Myc oncoprotein sensitizes CHO cells to Essiac-induced cell death, we harvested CHO-DHFR and CHO-SVMYC cells and incubated them with Essiac for 24 hours. This simple exposure experiment has been found to be sufficient for Essiac to modulate the growth of a variety of cells types (Phillips, 2003), and we did indeed see an effect on cell number over controls in which distilled water was used instead of Essiac. Although Essiac inhibited the growth of both cell types, CHO-DHFR cells were inhibited to a greater extent (Figure 2). This suggests that Essiac is cytotoxic towards CHO cells, but that Myc expression does not increase the susceptibility of CHO cells to Essiac's antiproliferative effects.

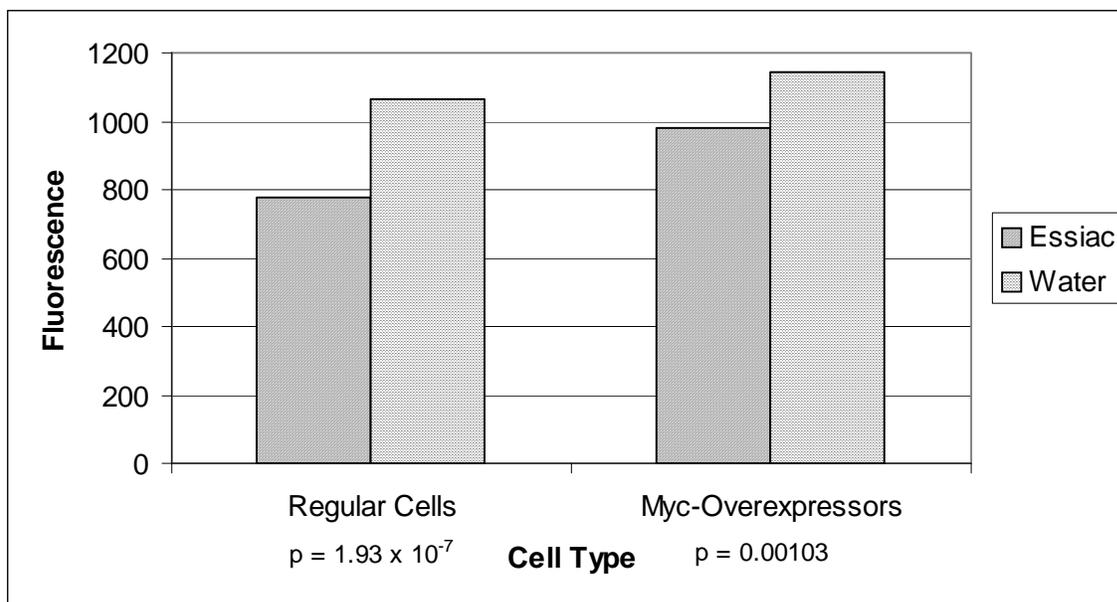


Figure 2. Cytotoxicity of Essiac towards CHO-DHFR (regular) and CHO-SVMYC (Myc-overexpressing) cells. Equal numbers of both cell types were incubated with Essiac for 24-hours before incubation with CFDA,AM. Higher fluorescence indicates more living cells. P-values are for a two-tailed t-test, with $n = 8$.

4. Growth inhibition of CHO cells by aqueous extractions of acidic, basic, and neutral Essiac

To begin an analysis of the active compounds in Essiac, we fractionated whole Essiac with a simple pH-adjusted liquid-liquid extraction and assayed the fractions with the cytotoxicity assay described above. Because CHO-DHFR cells were affected more than CHO-SVMYC in the Myc-sensitization experiment, CHO-DHFR cells were used for all remaining experiments. Neither the neutral nor basic Essiac fractions exhibited any effect on cell number, whereas the acidic fraction showed marked cytotoxicity when compared to control (Figure 3). These results indicate that Essiac contains a cytotoxic compound that is uncharged and hydrophobic at neutral and basic pHs, and positively charged at acidic pHs.

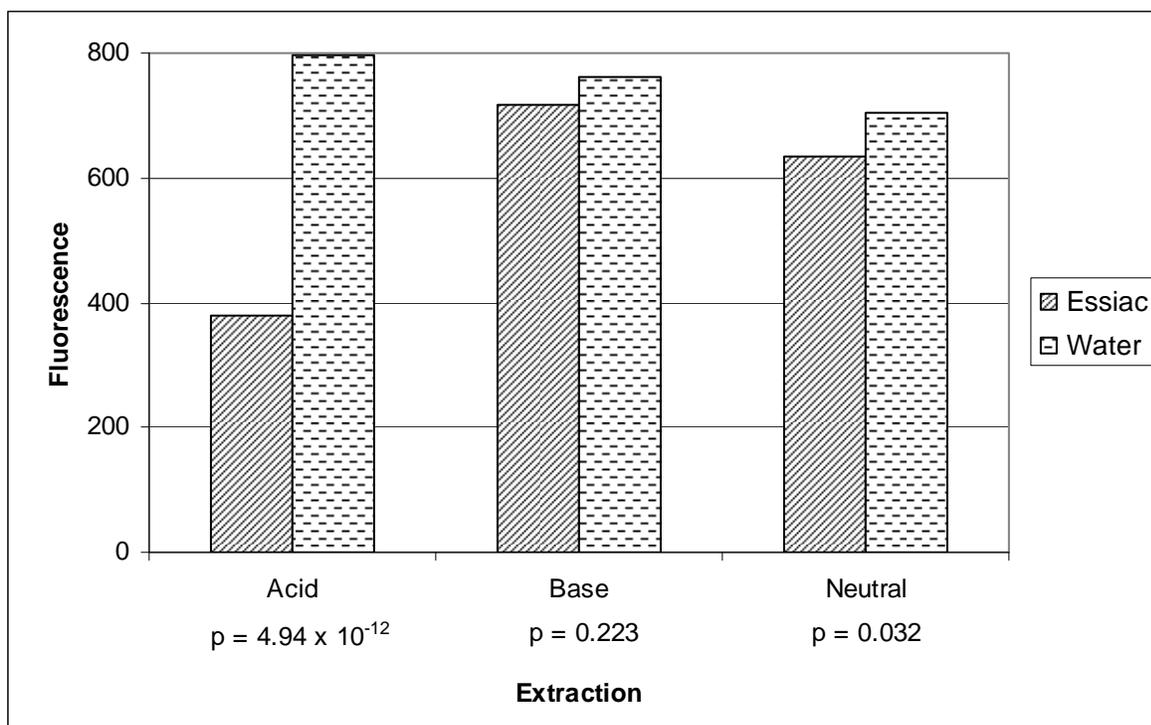


Figure 3. Cytotoxicity of aqueous Essiac extractions. Essiac was pH-adjusted and mixed with chloroform. The aqueous phase was collected, buffered, and returned to neutral pH before being assayed for cytotoxicity. P-values are for a two-tailed t-test, with $n = 8$.

5. Growth inhibition of CHO cells by chloroform extractions of acidic, basic, and neutral Essiac

Although we found a cytotoxic compound in the aqueous, acidic Essiac fraction, this does not preclude cytotoxic or proliferative compounds from being present in the chloroform fractions. Furthermore, the phytoestrogens, which are large and hydrophobic, are expected to be found in these fractions. Neither the acidic nor basic chloroform extractions of Essiac showed any effect on the CHO-DHFR cells (Figure 4). However, the neutral chloroform extraction showed statistically significant proliferative effects, suggesting that phytoestrogens or other growth-promoting compounds may be present in this fraction.

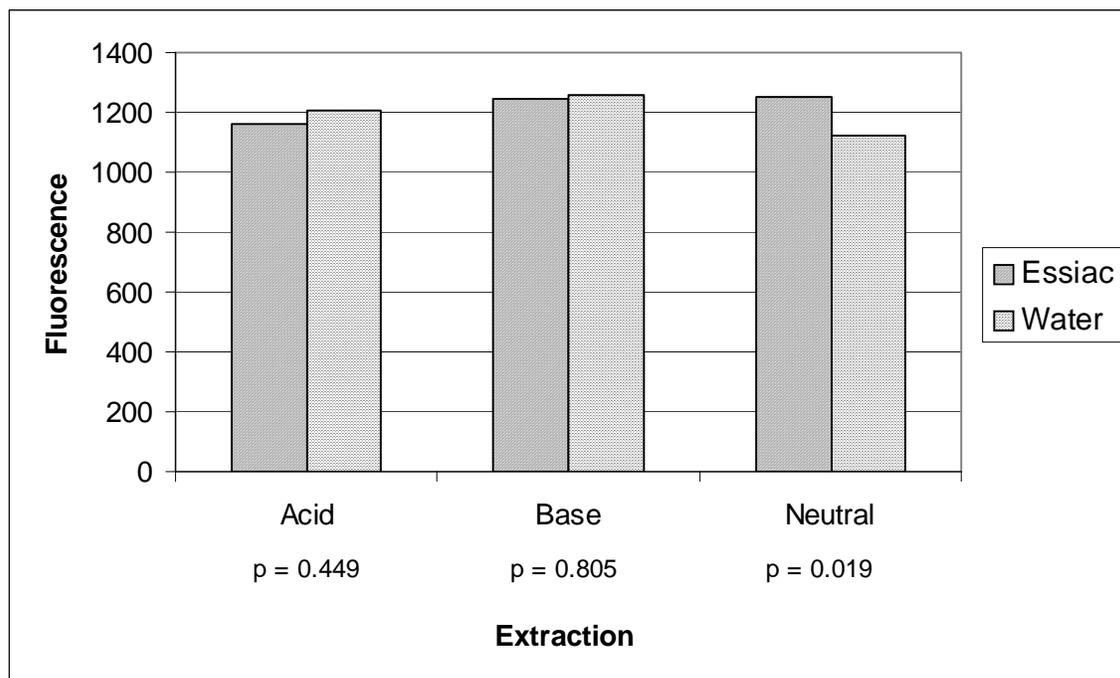


Figure 4. Cytotoxicity of chloroform Essiac extractions. Essiac was pH-adjusted and mixed with chloroform. Chloroform was then collected and evaporated. The remaining solutes were redissolved in buffer and distilled water before being assayed for cytotoxicity. P-values are for a two-tailed t-test, with $n = 8$.

6. Growth inhibition of CHO cells by buffered extractions of acidic Essiac

In order to determine the pH-sensitivity of the cytotoxic compound found previously in the acidic, aqueous phase of the chloroform liquid-liquid extraction, we performed several additional extractions, each buffered at a different pH. This range of pHs will extract compounds according to their pK_a 's and provides information on the cytotoxic compound's pH sensitivity. All aqueous buffered extractions had a significant effect on CHO-DHFR proliferation (Figure 5). Both the pH 3 and 4 extractions showed decreased proliferation, while pH 5 and 6 showed increased proliferation, albeit to a minor extent. This suggests that the cytotoxic compound becomes protonated, and consequently charged, between pH 5 and 4.

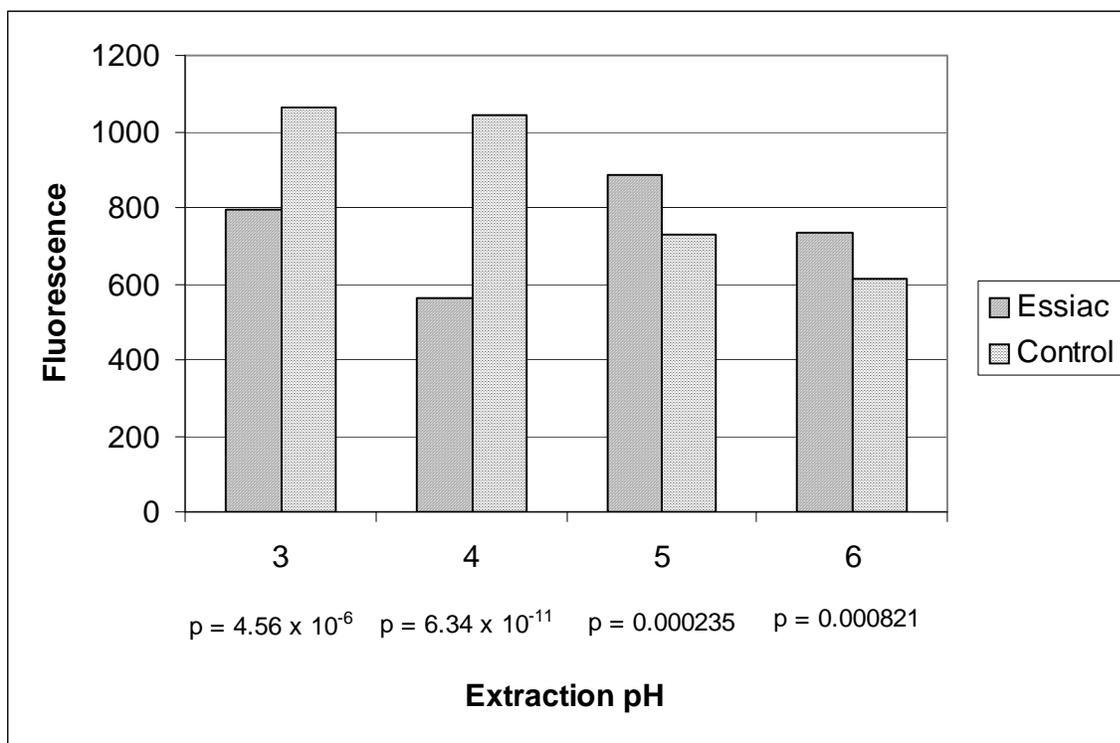


Figure 5. Cytotoxicity of citric acid buffered Essiac extractions. Essiac was pH-adjusted and mixed with chloroform. The aqueous phase was collected, buffered, and returned to neutral pH before being assayed for cytotoxicity. P-values are for a two-tailed t-test, with $n = 8$.

DISCUSSION

Essiac-induced cell death in myc-overexpressing and control CHO cells.

Our results showed that Essiac inhibits proliferation of both the Myc-overexpressing CHO-SVMYC cells and the control CHO-DHFR cells, but inhibits CHO-DHFR cell growth to a greater extent. Earlier studies suggest that Myc overexpression increases susceptibility to apoptosis when progression through the cell cycle is blocked (Evan *et al.*, 1992); the fact that CHO-SVMYC cells were not killed preferentially suggests that Essiac's cytotoxicity may not be due to interactions with the Myc oncoprotein and that Essiac may not prevent passage through the cell cycle, an action common to many anticancer drugs.

These results contrast with previous findings where Myc overexpression increased Essiac-induced death in an NIH 3T3 cell line where Myc overexpression was controlled by an inducible promoter (Phillips, 2003). Although Myc overexpression increased the death of these cells in the presence of Essiac, it was not linked to increased apoptosis, but rather some other mechanism of cell death (Phillips, 2003). These results, in conjunction with our work, indicate that Essiac-induced cell death may not occur through apoptosis. As such, the increased cell death observed in the NIH 3T3 cells is likely due to an indirect effect of Myc overexpression, and not Myc-sensitization to apoptosis.

Myc overexpression causes cells to take on a cancer-like morphology and behaviour (Fest *et al.*, 2002). It is possible that Essiac targets cells with this behaviour independently of Myc expression. If Essiac targets cells that express a cancer-like behaviour, then this may explain our observations with the CHO cell lines. CHO cells, although non-cancerous, are transformed and exhibit several traits characteristic of cancer cells (immortality, lack of differentiation, rapid

growth). Myc overexpression did not appear to affect these traits in the CHO cells significantly, which may explain why the CHO-SVMYC cells were not killed to a greater extent. However, Myc overexpression in the NIH 3T3 cells investigated by Phillips (2003) caused the cells to take on a cancer-like behaviour, and this may be why they were targeted preferentially by Essiac compared to our cells.

Essiac contains a hydrophobic, basic compound that inhibits proliferation of CHO-DHFR cells

Although our pH-adjusted, liquid-liquid extractions did not result in complete isolation of a cytotoxic compound, they do allow us to make inferences about the structure of the compound. Each fraction will contain different compounds, based on their chemical structure and properties (Table 1). Given that cytotoxicity was displayed only by acidic, aqueous fractions (Figures 3 and 5), the cytotoxic compound must be hydrophilic, but only when protonated; if it were hydrophilic under all conditions, it would be present in the aqueous phase of the neutral and basic extractions as well. Furthermore, since there was no cytotoxicity observed at pHs 5 or 6 (Figure 5), but marked cytotoxicity at pH 4, protonation must occur at this point between pH 4 and 5. This is most consistent with an aromatic amine. These compounds have pK_a 's around 4 or 5, below which they are protonated, positively charged, and hydrophilic (McMurry, 2003). Above their pK_a , these aromatic amines are uncharged and would partition into the chloroform.

Table 1. Expected compounds in extractions of whole Essiac. Different compounds will partition into either the aqueous or chloroform phase based on their structural properties, and each extraction will contain certain representative classes (McMurry, 2003).

Solvent	pH	Properties	Representative Compounds
Water	Acidic	hydrophilic/charged, basic	alcohols, amines
	Basic	hydrophilic/charged, acidic	alcohols, carboxylic acids
	Neutral	hydrophilic/charged	Alcohols
Chloroform	Acidic	hydrophobic/uncharged, acidic	polyaromatics, protonated carboxylic acids
	Basic	hydrophobic/uncharged, basic	polyaromatics, deprotonated amines
	Neutral	hydrophobic/uncharged	Polyaromatics

The results described above suggest that the cytotoxic compound is an aromatic amine that is hydrophobic in its neutral state. As such, the cytotoxicity should also have been displayed by the non-acidic chloroform fractions. This was not observed (Figure 4). A likely explanation for this is that there are hydrophobic compounds present in Essiac that offset the cytotoxicity of the other compounds, which may explain the Essiac-induced proliferation of breast cancer cells observed by Kulp *et. al* (2006). Essiac is known to contain a number of phytoestrogens that stimulate the *in vitro* growth of certain cancer cells (Tamayo *et al.*, 2000). These phytoestrogens are all large, polyaromatic compounds and will be present in the chloroform phase of all the extractions. Since these compounds promote cell proliferation in some cell types (Kulp *et al.*, 2006), it is possible that they counteract the effects of the cytotoxic elements when concentrated in the chloroform phase. Certain cell types (i.e., those sensitive to estrogens) will be affected more strongly than others by certain classes of compounds (i.e. phytoestrogens), which may contribute to the contradictory cell proliferation and death found previously (Kulp *et al.*, 2006, Ottenweller *et al.*, 2004).

This confounding cell proliferation may also explain the inability to isolate the cytotoxic compound emodin with our pH-adjusted liquid-liquid extraction. Emodin is a polyaromatic phenol and should be present in the chloroform phase in all extractions, regardless of pH (Ottenweller *et al.*, 2004). Since phytoestrogens will also partition into the chloroform phase, the cytotoxicity of emodin may not be observable with the fractionation scheme that we used.

There is another possible explanation for the anomalous results displayed by the chloroform fractions. The chloroform extractions, which will contain hydrophobic compounds, must be evaporated and redissolved before they can be applied to cells, since chloroform is itself

cytotoxic. As such, the hydrophobic compounds may not have redissolved in the aqueous buffer used. This would prevent observable changes in cell number.

Despite this, our work demonstrates that the growth-modulating effects of Essiac depend on which fraction is applied, suggesting that Essiac contains some compounds that promote growth and others that inhibit growth. Although testing whole Essiac has the advantage of being representative of what consumers are actually ingesting, the problem of confounding growth-modulating compounds makes *in vitro* study of whole Essiac problematic. Isolation of the cytotoxic elements within Essiac would facilitate their study and allow us to determine whether or not they show promise as anticancer agents. Furthermore, our work with whole Essiac on Myc overexpressing cells shows that changes in the expression of a single gene alter sensitivity of cells to Essiac-induced death, supporting the previous finding that Essiac's effects are cell-line dependent.

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