



Hypericum perforatum Extracts and Hypericin Treatment of a Mouse Mammary Cancer Cell Line Induces Growth Inhibition in a Dose Dependent Manner

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Abstract

Hypericum perforatum, commonly known as St. John's Wort, has been found to exhibit many medicinal, especially anti-depressant, properties. Hypericin is thought to be the main chemical constituent responsible for *H. perforatum*'s medicinal properties. We report here the ability of *H. perforatum* and hypericin to inhibit the growth of mouse mammary cancer CRL2539 cells. *H. perforatum*, at concentrations of 0.4% and 0.8%, significantly ($P < 0.05$) inhibited cell growth in a concentration dependent manner. Hypericin (purity 80-90%) at a concentration of 0.001% also significantly inhibited cell growth but not to the extent which the *H. perforatum* extracts did. In addition, *H. perforatum* at a concentration of 0.8% inhibited cell growth significantly more than *H. perforatum* at a concentration of 0.4%. Our study shows a promising therapeutic strategy in using the whole *H. perforatum* extract as its own form of treatment to effectively slow the growth rate of cancer cells, and potentially overcome the negative side effects associated with current forms of cancer treatment.

Introduction

Hypericum perforatum is a yellow flowering plant naturally found in various locations around the world including West Asia, Europe, and North Africa. *Hypericum perforatum* has been studied for its effects in treating depression and attention deficit hyperactivity disorder¹. Cancer prevention research is one of the new areas that *Hypericum perforatum* and its main chemical constituents are being applied to. Research has shown that the most significant compounds in *Hypericum perforatum* for cancer prevention are hypericin and hyperforin².

Hypericin is used in photodynamic therapy (PDT) as a form of cancer treatment. PDT requires a photosensitizing agent (photosensitizer) and visible light of a wavelength that correlates with the absorption spectrum of the drug. Alone the light and photosensitizer have no therapeutic effect, but when combined produce cytotoxic products which trigger irreversible tumor destruction and cell damage. Out of 36 species of *Hypericum*, 27 held hypericin, the most common being *Hypericum perforatum*³.

The cell type in which hypericin is applied dictates the killing efficacy and the cellular distribution of the drug. In colon carcinoma CAco-2 cells for example, hypericin is found to accumulate in the nuclear and plasma membranes. Hypericin mainly targets cell membranes and can affect critical mitochondrial functions in a photodependant manner. Although hypericin does not gather in mitochondria, hypericin's photodynamic action

primarily targets these cell sites as shown by the impairments in mitochondria bioenergetics when hypericin is present and combined with a visible light wavelength³.

Another form of cancer treatment that involves the use of hypericin is catalytic therapy (CT). CT is a cancer treatment that involves the use of substrate molecules and a catalyst to generate reactive oxygen species (ROS). Hypericin from *Hypericum perforatum* as an active photosensitizer assists in ROS generation in response to light. *Hypericum perforatum* alone has little effect on cell life but when an activation mixture, made up of ascorbic acid, is added, it drastically increases early apoptosis in cells⁴.

These researchers could not find studies delineating the effects of the whole extract as opposed to its component compounds. Although hypericin from *Hypericum perforatum* has had positive effects in cancer treatments so far, when combined with other drugs for in vivo treatment it can cause dangerous side effects such as reduced plasma levels of antiretroviral agents, which increases patients' risk for disease progression². Also, when used as a photosensitizer in photodynamic and catalytic therapy, an increase of dosage of hypericin results in a shift from apoptotic to necrotic death resulting in a harmful inflammatory response in the patient³.

Our study tests the effects of a whole extract of *Hypericum perforatum* and one of its chemical constituents, hypericin, on the growth of a mouse mammary cancer cell line. The first hypothesis is that if *Hypericum perforatum* and hypericin extracts are applied to a mouse mammary cancer cell line, then the cancer cells exposed to the extracts will grow at a slower rate than the control cells. The second hypothesis is that if *Hypericum perforatum* and hypericin extracts are applied to a mouse mammary cancer cell line, they will slow the cell growth at an equal rate. The third hypothesis is that if *Hypericum perforatum* is added in greater concentrations to the mouse mammary cancer cells, then the cells will grow at an increasingly slower rate.

Our study is important is because although hypericin has been used before in cancer such as PDT or CT, there are many negative side effects associated with current treatments such as increased risk for disease progression and induction of an inflammatory response. If we could find the optimal dosage of hypericin and *Hypericum perforatum* extract, it could be used on its own as a separate form of cancer treatment.

Materials and Methods

CRL2539 mouse mammary cancer cells (American Type Culture Collection) were seeded at concentrations of $1.0 \times 10^4/25\text{cm}^2$.



Cancer inhibitory activity of liquid glycerol based *Hypericum perforatum* extract (0.3% hypericin) (Puritan's Pride Incorporated) and hypericin (Planta Analytica) were determined by exposing cells to their various concentrations for 96 – 264 hrs. or until control cells reached confluency. Each set of trials were run with three 25 ml control flasks, three 25 ml 0.4% *Hypericum perforatum* flasks, and three 25 ml 0.8% *Hypericum perforatum* flasks or three 25 ml 0.001% hypericin flasks. Seven mL of media were needed for each flask, so media was made in sets of 21 mL with dilutions as follows. 21 mL of DMEM with 10% FBS were made for the control cells. 0.084 mL of *Hypericum perforatum* and 20.916 mL of DMEM with 10% FBS were made for the 0.4% *Hypericum perforatum* flasks of cells. 0.168 mL of *Hypericum perforatum* and 20.832 mL of DMEM with 10% FBS were made for the 0.8% *Hypericum perforatum* flasks of cells. 0.021g of hypericin was dissolved in 1mL of ethanol for the 0.001% hypericin flasks of cells. This was added to 20mL DMEM. 1mL of ethanol was added to 20mL of DMEM for the ethanol flasks of cells. Cells were cultured in a 5% CO₂, 95% air, fully humidified incubator at 37°C. Control CRL2539 cells were cultured in DMEM containing 10% FBS. Experimental CRL2539 cells were cultured in DMEM containing 10% FBS and 0.4% *Hypericum perforatum*, 0.8% *Hypericum perforatum*, or 0.001% hypericin. Cell growth was estimated at periods of 24 – 48 hrs. using pictures from the inverted microscope. Three pictures were taken at randomly dispersed locations throughout each flask. The cells in each picture were counted and averaged so that the number of cells per mm² was found. This number was multiplied by the surface area of the flask, 1763 mm², to find the average number of cells in each flask at a certain time. At the end of each trial we found the final cells counts in each flask using a hemocytometer. We performed a hemocytometer count per flask three times and average the number of cells counted to ensure an accurate representation. Statistical evaluation of the results was performed with the Student's t-test using the Microsoft Excel software. Probability values equal or less than equal to 0.05 were considered significant. Normal distribution graphs were constructed based t-test values using Fathom Dynamic Data software.

Results

Graphs comparing the control and experimental trials were made to compare the growth rates of the cells over time. Figures 1 and 2 show the average number of cells that were in each of the three control or experimental flasks over time. After 96 hours for the 0.4% *Hypericum perforatum* and after 72 hours for 0.001% hypericin it is clear that the experimental cells are growing slower than the control cells. The difference in the cell populations between the experimental and control flasks increases as time goes on. Figure 3 shows the average number of cells in each of the three 0.4% or 0.8% *Hypericum perforatum* flasks. The figure shows that the cell population of the 0.8% *Hypericum perforatum* concentration is less than the cell population of the 0.4% concentration over time.

To analyze the cell population data, t-tests were run to compare the mean number of cells at various times between the control and experimental flasks. The purpose of this test is to determine if there is a statistically significant difference between the cell populations at the set times, shown in the resulting p-value. As shown in table 1, p-values of less than 0.05 were observed for

Experimental 0.4% *Hypericum perforatum* vs. Control Growth

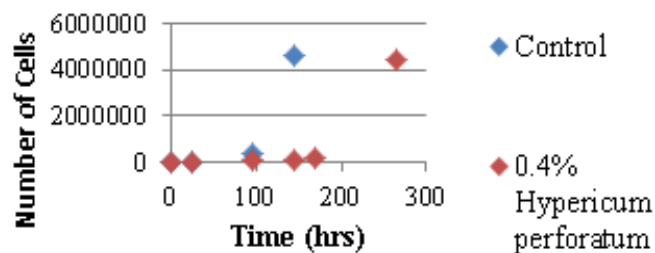


Figure 1: *Hypericum perforatum* at 0.4% inhibited the growth of CRL2539 cells. Cell populations were measured in three control flasks and three experimental flasks until the flasks reached confluence.

Experimental 0.001% Hypericin vs. Control Growth

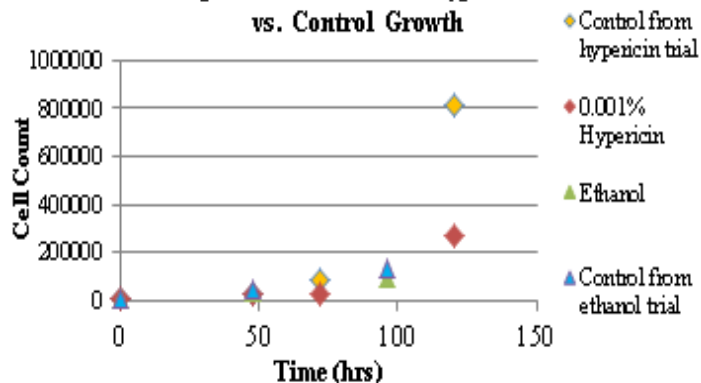


Figure 2. Hypericin at 0.001% inhibited the growth of CRL2539 cells. Cell populations were measured in three control flasks and three experimental flasks until the control cells reached confluence. An ethanol trial was included because the hypericin was dissolved in ethanol before combining it with the DMEM.

Experimental *Hypericum perforatum* 0.4% vs. 0.8% Growth

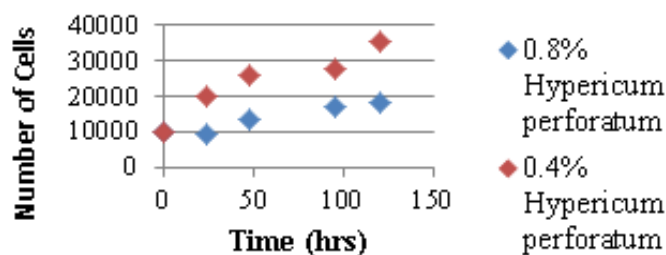


Figure 3. *Hypericum perforatum* 0.8% concentration inhibited the growth of CRL2539 cells further than *Hypericum perforatum* at 0.4% concentration. Cell populations were measured in three experimental flasks for each concentration until 120 hours.



Tables 1 - 4. T-tests on experimental and control samples.

**Table 1. t-Test Two-Sample 24, 96, 144 Hours
0.4% Hypericum perforatum vs. Control**

Time (hrs)	Mean Number of Control Cells	Mean Number of 0.4% <i>Hypericum perforatum</i> Cells	P-value (two tail)
24	25857	23115	0.45
96	338300	63272	0.013
144	4583333	117141	0.0015

**Table 2. t-Test Two-Sample 48, 72, 120 Hours
Control vs. 0.001% Hypericin**

Time (hrs)	Mean Number of Control Cells	Mean Number of 0.001% Hypericin Cells	P-value (two-tail)
48	24682	27229	0.33
72	86975	22919	0.038
120	810000	266667	0.0073

**Table 3. t-Test Two-Sample 48, 96, 120 Hours
0.4% Hypericum perforatum vs. 0.8% Hypericum perforatum**

Time (hrs)	Mean Number of 0.4% <i>Hypericum perforatum</i> Cells	Mean Number of 0.8% <i>Hypericum perforatum</i> Cells	P-value (two-tail)
48	28012	15671	0.041
96	34672	21156	0.032
120	57333	236666	9.4E-05

**Table 4. t-Test Two-Sample 48 Hours
Control vs. Ethanol**

Time (hrs)	Mean Number of Control Cells	Mean Number of Ethanol Cells	P-value (two-tail)
48	43194	33105	0.26

the control compared to the *Hypericum perforatum* (0.4%) at times 96 hours (p-value 0.013) and 144 hours (p-value 0.0015). As shown in table 1, at 24 hours the p-value was not less than 0.05 (p-value 0.45). As shown in table 3, p-values of less than 0.05 were also observed for the control compared to the hypericin (0.001%) at times 72 hours (p-value 0.037) and 120 hours (p-value 0.007). However, at time 48 hours, the p-value for the hypericin trial was not less than 0.05 as shown in table 3. T-tests were also run comparing the cell populations of 0.4% and 0.8% concentrations of *Hypericum perforatum* at certain times. Table 2 shows p-values of less than 0.05 at times 48 hours (p-value 0.04), 96 hours (p-value 0.03), and 120 hours (p-value 9×10^{-5}). In addition, table 4 shows a p-value of more than 0.05 at time 48 hours comparing the ethanol population to the control population. These statistical tests show that the hypericin and *Hypericum perforatum* concentrations significantly slow the rate at which the cancer cells are growing after certain times. Ethanol does not significantly affect the rates at which the cancer cells are growing. From this, it can be concluded that the anti-cancerous properties exhibited by hypericin are not a result of the ethanol the extract was dissolved in.

Figures 4-6 provide a visual demonstration of the statistically significant difference between the experimental and control means. Because none of the graphs had overlapping error bars, the mean values of the control and experimental flasks were all found to be statistically different.

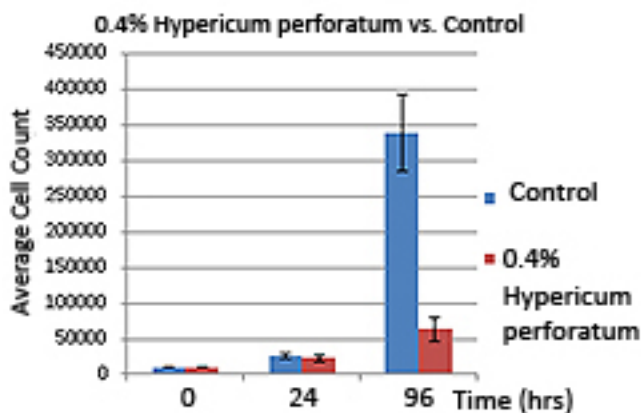


Figure 4: Hypericum perforatum at 0.4% inhibited the growth CRL2539 cells significantly compared to the growth of control cells starting at 96 hours.

Discussion

The first hypothesis, “If *Hypericum perforatum* and hypericin extracts are applied to a mouse mammary cancer cell line, then the cancer cells exposed to the extracts will grow at a slower rate than the control cells” was accepted. It was concluded with confidence that *Hypericum perforatum* and hypericin extracts did significantly slow the growth of the mouse mammary cancer cells after times of 96 hours for the *Hypericum perforatum* extract and 72 hours for the hypericin extract. With time, both extracts decreased cell growth at greater rates as shown in the decreasing p-values over time (0.013 to 0.0015 from times 96hrs to 144hrs for the *Hypericum perforatum* extract and 0.037 to 0.007 from times 72hrs to 129hrs for the hypericin extract). The hypericin did not slow the growth of the cells to as great an extent as the whole *Hypericum perforatum*

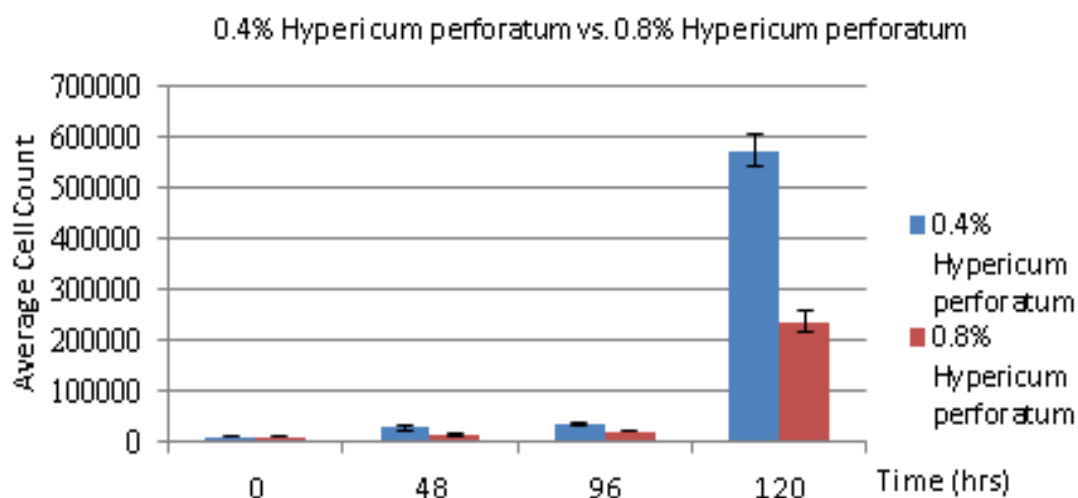


Figure 5: *Hypericum perforatum* at 0.8% inhibited the growth CRL2539 cells significantly compared to the growth of *Hypericum perforatum* at 0.4% cells starting at 48 hours.

extract did shown by the cell populations of the hypericin at the same time as the *Hypericum perforatum* populations which were higher. Therefore, the second hypothesis “if *Hypericum perforatum* and hypericin extracts are applied to a mouse mammary cancer cell line, they will the slow the cell growth at an equal rate” was not accepted. This demonstrates that hypericin is not the only active compound responsible for the anti-cancerous properties of *Hypericum perforatum*. The third hypothesis “if *Hypericum perforatum* is added in greater concentrations to the mouse mammary cancer cells, then the cells will grow at an increasingly slower rate” was accepted because the cells with concentration of 0.8% grew at a significantly slower rate than the cells with concentration 0.4%.

This study demonstrated that *Hypericum perforatum* significantly slows the rate at which mouse mammary cancer cells grow. The results indicate that as concentrations of *Hypericum perforatum* increase, the rate at which the mouse mammary cells are growing decreases. This study did not determine if there is an optimum dosage of *Hypericum perforatum* for the cells. This study also indicated that hypericin significantly decreases the growth rate of the mouse mammary cancer cells. However, the hypericin did not slow the growth rate to as great an extent as the 0.4% *Hypericum perforatum* whole extract did, indicating that hypericin is not the only component of *Hypericum perforatum* responsible for its anti-cancerous properties.

The cell populations of the 0.4% and 0.8% concentrations of *Hypericum perforatum* were also found to be statistically significantly different. The cell populations of the flasks with 0.8% *Hypericum perforatum* concentration were significantly lower than the flasks with the 0.4% concentration. Therefore, it has been demonstrated that with an increase in concentration of *Hypericum perforatum* there is a correlating decrease in the rate at which the mouse mammary cancer cells were growing.

In an unpublished study, *Hypericum perforatum* was found to inhibit or denature the proteins aldolase and enolase in *Drosophila melanogaster* flies. Aldolase and enolase both play a major role in glycolysis. Upregulation of glycolysis has been observed in many cancer studies which suggest that inhibition of the glycolytic pathway may decrease or halt cancer progression. Therefore, we will examine the mechanisms behind the inhibition of cancer progression through *Hypericum perforatum* shown in this study looking specifically for the inhibition or denaturation of aldolase and enolase. Further research could also investigate the mitochondrial membrane to determine if the extracts are interfering with its function thus giving more evidence for the mode of action hypericin uses to inhibit cell growth.

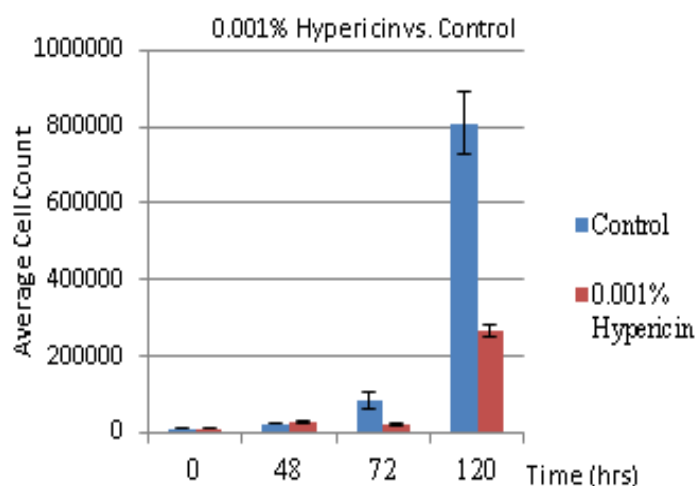


Figure 6: Hypericin at 0.001% inhibited the growth CRL2539 cells significantly compared to the growth of control cells starting at 72 hours.



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